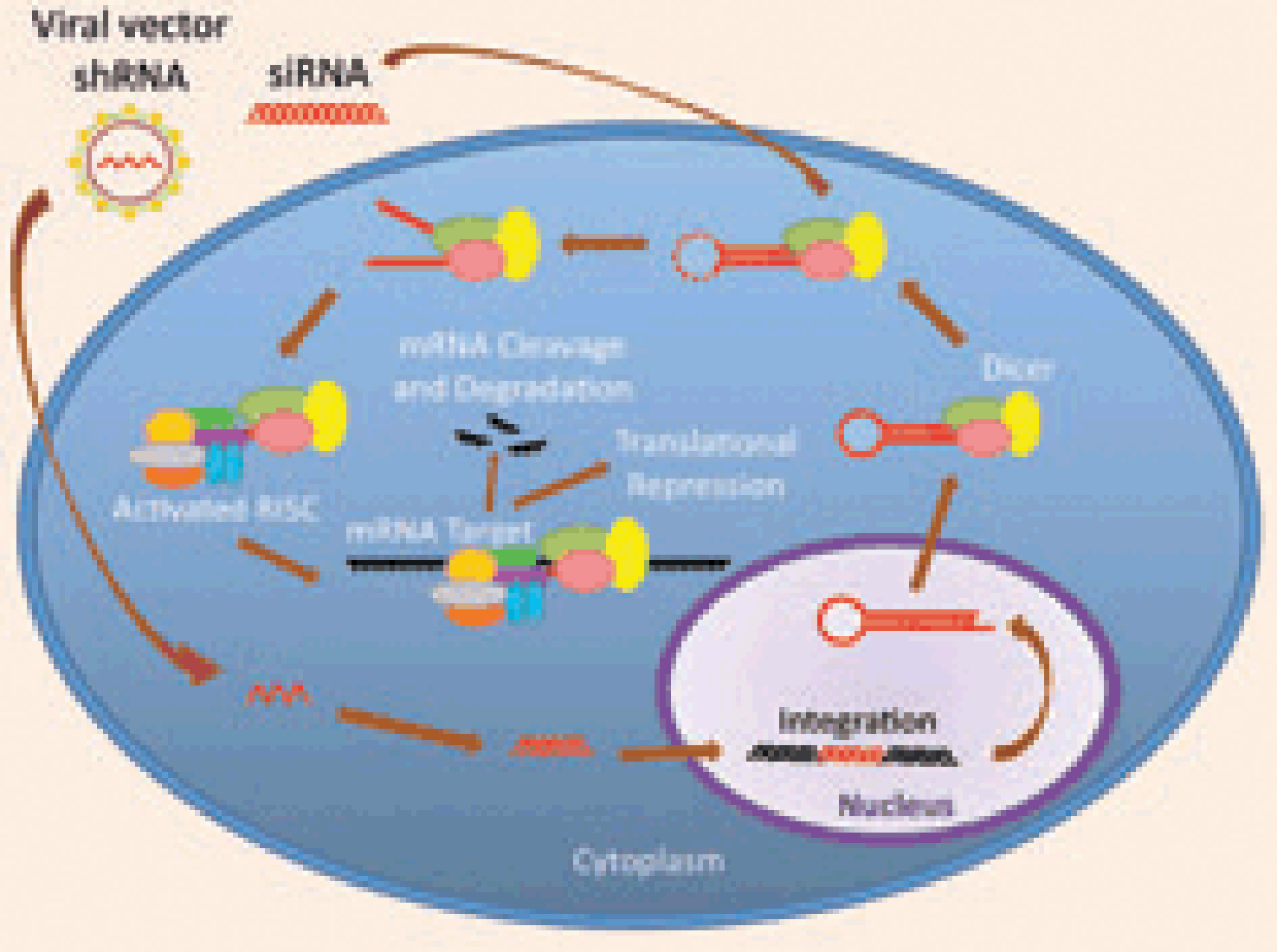
Viral vector
shRNA
siRNA
mRNA Cleavage
and Degradation
Dicer
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Repression
Activated RISC
mRNA Target
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Nucleus
Cytoplasm

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Neurosurg Focus 28 (1):E1, 2010

Introduction: neurogenomics and neuroproteomics

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For this issue of *Neurosurgical Focus*, we are pleased to present a spectrum of interesting articles covering a wide range of contemporary genomic and proteomic topics specifically related to neurosurgical diseases. The authors review a broad range of contemporary population genetic and molecular techniques available for the study of neurological disease. The issue begins with articles on broadly applied population genetic tools used to identify the relative risk of neurosurgical diseases among the kindred of affected individuals. One such example is the Utah population data bank described by Niazi et al. Subsequent articles describe game-changing contemporary molecular techniques used to provide screening for genetic alterations associated with a specific disease or phenotype. Examples are the genome-wide association studies described by Cowperthwaite et al. and the next-generation screening techniques for genetic alteration applied to medulloblastomas by Taylor et al. A review of the role of miRNAs in brain tumor biology is provided by Kalani et al. Chen and colleagues describe the identification of distinct functional genomic alterations among vast genetic changes as a strategy to target tumor cell vulnerabilities with novel therapies. The application of siRNA technology to induce therapeutic functional changes in gene expression in glioblastoma multiforme cells is described in the article by Thaker et al. In the remaining articles the authors discuss genetic and proteomic changes associated with specific diseases, including head trauma (Dardiotis et al.), Parkinson disease (Hadjigeorgiou et al.), and neurofibromatosis Type 1 (Gottfried et al.). Dhandapani et al. discuss their novel proteomic studies in patients with subarachnoid hemorrhage, which provide proof of principle and promise for future development of diagnostic and therapeutic innovations in this emerging and fascinating area of research. Finally, Vannemreddy and colleagues provide data supporting a role for eNOS in the clinical presentation of intraventricular hemorrhage. Collectively, these papers serve as a solid primer in genomics and proteomics for the practicing neurosurgeon and convey some of the most exciting recent molecular science developments relevant to neurosurgical disease.

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Utah Population Database: a tool to study the hereditary element of nonsyndromic neurosurgical diseases

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Understanding the genetic alterations that contribute to the development of different disease states is key to the ability to screen and treat patients afflicted with these diseases and may someday enable us to prevent their development. The high degree of morbidity and mortality associated with neurosurgical diseases makes this understanding particularly important. Mechanisms for early diagnosis, followed by appropriate treatment in these patients, could significantly alter the morbidity and mortality rates in patients with neurosurgical diseases. The Utah Population Database (UPDB) is a unique resource that allows population-based analysis of the familial nature of diseases with the identification of high-risk pedigrees and predisposition genes. The UPDB was initially used to study the hereditary element of cancer, but its application is now expanding to include the neurogenomics of neurosurgical diseases. Specifically, the familial and genetic contribution to intracranial aneurysms and nonsyndromic intracranial malignant diseases has been reported in the literature. The UPDB identifies the familial contribution by examining genealogical records that have been linked with clinical, demographic, and diagnostic data compiled in state records. From this information, one can identify possible genetic targets for future therapies. *(DOI: 10.3171/2009.10.FOCUS09214)*

Key Words • Utah Population Database • glioma • relative risk • intracranial aneurysm • pituitary adenoma • genealogical index of familiality

Understanding the genetic alterations that contribute to the development of different disease states is key to the ability to screen and treat patients afflicted with these diseases and may someday enable us to prevent their development. The high morbidity and mortality rates associated with neurosurgical diseases make this understanding particularly important. Although several heritable disorders that are responsible for neurosurgical diseases, such as Li-Fraumeni syndrome, tuberous sclerosis, multiple endocrine neoplasia, and Ehlers-Danlos syndrome, are well known, these represent only a minority of patients afflicted with neurosurgical disease. The nonsyndromic genetic component of other neurosurgical diseases, such as malignant gliomas, pituitary tumors, and intracranial aneurysms, has been less well studied. Great strides have been made in the ability of researchers to investigate the genetic component of diseases. As new technology and resources become available, our ability to investigate the genetic aspects of neurosurgical conditions has evolved. In this paper, we describe one resource that was originally created to describe the hereditary element of cancer and is now being used to investigate other phenotypes, including neurosurgical conditions.

Abbreviations used in this paper: RR = relative risk; GIF = genealogical index of familiality; UCR = Utah Cancer Registry; UPDB = Utah Population Database.

The Utah Population Database

At the University of Utah, a unique resource has been available for over 3 decades that links phenotypic data with genealogical records dating back multiple generations in a computerized database. It was initially developed with the goal of investigating the genetic contribution to cancer, but over the last 30 years the applications of the UPDB have expanded, and the genetic contributions to disease phenotypes other than cancer have been evaluated.

The UPDB was created by Mark Skolnick[16] in the early 1970s in an effort to link genealogical information representing Utah's pioneers and their descendants with the individual cancer records represented in the UCR. The UCR is mandated by law to collect and store all data on all patients with cancer diagnosed in the state, including primary tumor site, histology, patient age at diagnosis, tumor stage, tumor grade, duration of patient survival, and treatment and follow-up data.[4] The UPDB is also linked to state death certificates dating back to 1904.[7] This database has now become a dynamic database, receiving annual updates from the Utah Department of Health for births, deaths, marriages, and divorces, as well as records from the Utah Driver's License Division, in addition to the information from the UCR (http://www.huntsmancancer.org/groups/ppr/index.html). More recently, the

University of Utah Health Sciences Center Enterprise Data Warehouse, which contains patient data including diagnosis coding, procedure coding, medications, treatment response, and laboratory results, has been linked to the UPDB (http://www.huntsmancancer.org/groups/ppr/index.html).

Because the database includes identifiable health information, an oversight committee representing all data contributors approves all studies. Only data sufficient and necessary for any study are released. For example, for the familial studies described in this review, no names or other identifiers were used. More details of these guidelines have been described by Wylie and Mineau.[20]

The UPDB now includes more than 7 million individuals and has a subset of over 2.5 million individuals with at least 3 generations of genealogical data; some pedigrees now extend to 11 generations, linking back to the initial settlers in Utah. The founding pioneers were primarily of northern European descent and immigrated to the Utah territory in the mid-1800s. They were largely unrelated with low to normal levels of inbreeding, making them an excellent population on which to base a population study.[4,11]

Evaluation of Heritable Contribution

Using three different methods, data from the UPDB have been used to evaluate the genetic contribution to specific phenotypes.[4,16] The first method, which was developed specifically for use with the UPDB, looks at the estimation of the average relatedness among affected individuals who share a specific phenotype, also termed the genealogical index of familiality (GIF).[10] If the average relatedness among individuals in the database with a selected phenotype is significantly greater than that seen for multiple sets of matched controls, there is evidence of excess familiality (Fig. 1). This familiality can represent both genetic and environmental effects, but the UPDB can provide some indication regarding the effect of these components. For example, if excess relatedness of cases is only seen within first-degree relatives (that is, parents, siblings, and children), the effects may be related to environment or genetics or both, whereas if this greater relatedness spans more than just close relatives, then an inherited genetic predisposition is more likely.[4]

The second method of evaluating genetic contribution from the database examines the estimation of the RR in relatives of those individuals with the affected phenotype. The rationale behind this is that phenotypes with a genetic contribution should occur in relatives of those affected with that phenotype at a rate higher than that seen in the control population.[4] Again, as in the GIF, a higher RR in first-degree relatives can be attributable to environment, genes, or both, but an increased RR in distant relatives, such as second- or third-degree relatives, is less likely to be due to environment and is much more likely to have a genetic component.

Finally, the UPDB can be used to identify high-risk pedigrees with specific phenotypes observed in significant excess, and these pedigrees can be studied to identify the predisposition genes responsible for the observed phenotypes. Such studies are complex and involve identification

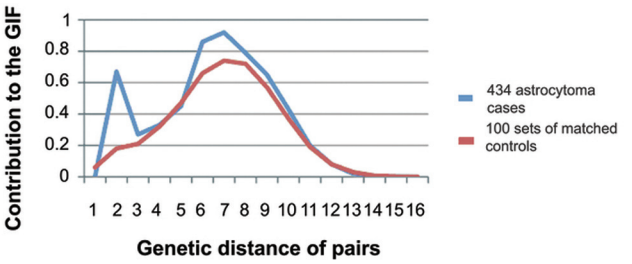

Fig. 1. Line graph illustrating the contribution to the GIF relatedness statistic for 434 astrocytoma cases (based on data reported by Blumenthal and Cannon-Albright in 2008). This figure allows comparison of the distance of genetic relationships contributing to the excess relatedness in cases versus controls.

of informative high-risk pedigrees, appropriate recruitment of pedigree members, validation of phenotypes, collection of blood for DNA, genotyping, and appropriate genetic analysis. Several predisposition genes have been identified using pedigrees identified in the UPDB, including genes for breast cancer (*BRCA1* and *BRCA2*), melanoma (*p16*), familial adenomatous polyposis (*APC*), and Alport syndrome linked to the X chromosome.[5,12,13,18]

Identification of Predisposition Genes or Loci

Once one has identified in the UPDB a phenotype that harbors evidence of a heritable component with associated high-risk pedigrees, predisposition genes can be sought. For example, in 1988, Atkin et al.[2] examined high-risk pedigrees associated with Alport syndrome in an attempt to localize this disease to a specific gene locus. Twenty-three pedigrees whose individuals were at high risk for Alport syndrome were identified, and 3 of the 23 high-risk pedigrees with the highest proportion of affected individuals were studied specifically. Blood samples were obtained from the majority of affected individuals within the 3 selected pedigrees, genomic DNA was isolated, and likelihood analysis was performed to test for linkage, locus order, and heterogeneity. Because of the nature of this disease, with 85% penetrance in female heterozygotes, X-linked inheritance was assumed. Test statistics for linkage were then evaluated, and these are reported as "LOD" scores. The LOD score is defined as the common logarithm of the ratio of the likelihood at a given recombination fraction to the likelihood of free recombination. An LOD score of > 3 is accepted as evidence of linkage, and a negative LOD score is evidence against linkage. The LOD scores for linkage on the X-chromosome for Alport syndrome suggested that the disease locus is located on the long arm of the X chromosome distal to p19–2.

Identified Heritable Contributions

The UPDB has been a vital component in dozens of studies describing the heritable contribution to cancer and other phenotypes and in studies identifying high-risk pedigrees and predisposition genes responsible for disease states. Breast cancer, early melanoma, lymphocytic leukemias, and early lip cancers have all exhibited excess relatedness among identified cases.[6] Additional

conditions that have been evaluated and shown evidence for a genetic contribution to familial clustering have included rotator cuff disease,[17] diabetes,[19] and death from influenza.[1] To date, the UPDB has been used to investigate 3 neurosurgical diseases—intracranial aneurysms,[7] malignant gliomas,[3] and pituitary adenomas (manuscript in submission and not discussed here); all 3 phenotypes were examined because of special interest expressed by clinicians. For all 3 phenotypes, a strong genetic basis within the Utah population has been demonstrated.Additional phenotypes that can be studied only require that sufficient sample sizes are available in the Utah resource and that affected individuals can be identified using some standardized diagnosis or procedure coding or registry.

Aneurysms. A familial nature of aneurysms has been recognized,[9,14,15] but extensive genealogical data are often missing from these studies, which are generally limited to nuclear family members. In 2003, Cannon-Albright et al.[7] used the UPDB to look at the heritable predisposition to aneurysms, examining all 3 specific phenotypes: intracranial, aortic, and other locations. In their analysis of familiality for the 3 different phenotypes, the GIF was statistically significantly higher in all 3 phenotypes compared with the control GIF. In fact, they noted that the GIFs were all higher than GIFs estimated for any previously examined cancer sites (even those for which predisposition genes had already been identified). The RR of each phenotype in first-degree family members and second- and third-degree family members was also evaluated. For intracranial aneurysms, the increased RR was significant in first-degree family members but not in second-degree relatives, and it was also increased in siblings in comparison with the RR between parents and offspring. The authors also used the UPDB to determine whether relatives of affected individuals with one type of aneurysm were at increased risk for another type of aneurysm. They noted no increased risk of intracranial aneurysms in first-degree relatives of patients who died of aortic aneurysms and vice versa, but they did observe an increased RR of other aneurysms.

This study also was able to identify 245 pedigrees in which there were clusters of patients with aneurysms descended from the same ancestor in the UPDB, including one pedigree with 10 individuals with intracranial aneurysms. These high-risk pedigrees are currently the subject of investigation to better elucidate predisposition genes.[8] Identification of predisposition genes would not only offer the opportunity to develop preventative measures or treatments, but identifying and screening those patients at highest risk for ruptured intracranial aneurysms would have significant potential to reduce morbidity and mortality, increase efficiency, and lower cost than general population screening.

Nonsyndromic Brain Cancers. In 2008, using the UPDB, Blumenthal and Cannon-Albright[3] demonstrated that there was evidence for a genetic contribution to the development of predisposition to primary brain tumors that were not syndromic in nature. They examined the relatedness of cases of astrocytoma and glioma, both as a whole and as separate groups. Their findings showed significant excess relatedness using the GIF for astrocytomas and glioblastomas when considered together and for astrocytomas alone, but not for glioblastomas alone. They also found an increased RR for astrocytomas in first- and second-degree relatives of patients with astrocytomas considered separately and an increased RR in first-degree, but not second-degree, relatives of patients with glioblastomas. Similarly, the risk for astrocytoma or glioblastoma in first-degree relatives of patients with either tumor type was elevated. These findings suggest a heritable component to astrocytoma predisposition, and the authors noted that the significantly elevated RR in first-degree relatives of patients with glioblastoma may suggest a heritable component in the subgroup of individuals with secondary glioblastomas (which transform from lower-grade tumors) that is obscured by more common de novo tumors.[3] In addition, 101 high-risk pedigrees that included at least 3 brain tumors and evidence of brain tumors among the descendants of founders were identified from the UPDB data. This study lends further credence to the hypothesis that there are predisposition genes that contribute to nonsyndromic intracranial malignancy.

Conclusions

The UPDB is a dynamic resource whose functionality has continued to expand since its inception in the 1970s as additional genealogical, demographic, and medical information has been linked. The UPDB has been invaluable in the localization and identification of several major cancer predisposing genes (*BRCA1, BRCA2,* and *p16*) through the identification and study of high-risk pedigrees. Evaluation of the degree of familiality of afflicted individuals and the RR of relatives of these individuals has also been able to provide strong support for a genetic contribution to predisposition to selected diseases. The high GIFs observed with intracranial aneurysms and increased RRs for astrocytoma and glioblastoma suggest that these neurosurgical diseases likely have a heritable element. With the high-risk pedigrees for intracranial aneurysms and nonsyndromic intracranial malignancies that have been identified in the UPDB, the identification of predisposition genes is well on its way. In addition to identifying possible targets for future therapies, the ability to identify individuals at increased risk of neurosurgical disease states will offer the opportunity of screening these high-risk individuals for early diagnosis and potentially decrease overall morbidity and mortality rates, even if population-wide screening is unfeasible. For example, in the study of high-risk aneurysm pedigrees, approximately 40 aneurysms were detected in about 500 screenings. Even if the gene is not specifically found, screening individuals at high risk defined by a positive family history could prove beneficial. Finally, these findings hint at the possibility of using the database for developing more personalized medicine. Once genetic variants are identified, the screening can be adjusted accordingly. This will provide the ability to offer earlier diagnosis, leading to prevention or better treatment.

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Genome-wide association studies: a powerful tool for neurogenomics

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As their power and utility increase, genome-wide association (GWA) studies are poised to become an important element of the neurosurgeon's toolkit for diagnosing and treating disease. In this paper, the authors review recent findings and discuss issues associated with gathering and analyzing GWA data for the study of neurological diseases and disorders, including those of neurosurgical importance. Their goal is to provide neurosurgeons and other clinicians with a better understanding of the practical and theoretical issues associated with this line of research. A modern GWA study involves testing hundreds of thousands of genetic markers across an entire genome, often in thousands of individuals, for any significant association with a particular disease. The number of markers assayed in a study presents several practical and theoretical issues that must be considered when planning the study. Genome-wide association studies show great promise in our understanding of the genes underlying common neurological diseases and disorders, as well as in leading to a new generation of genetic tests for clinicians. *(DOI: 10.3171/2010.10.FOCUS09186)*

Key Words • **neurogenomics** • **genetics** • **neurosurgery**

A goal of molecular genetics is to discover the genetic architecture of human phenotypes, especially diseases. The research community has recently made great strides toward associating loci (genes) with phenotypes (diseases), but much work remains.[80] These advances have resulted from significant increases in the scale and power of genetic-linkage tests, which have grown from candidate-gene analyses to GWA studies.

Genome-wide association studies are intended to address some of the shortcomings of traditional candidate-gene linkage tests. Classic linkage studies are typically difficult to conduct, at least in part because they require a priori knowledge about the biology of the disease under study (to select candidate genes) as well as a familiarity with the genetic variants (that is, mutations) in the candidate genes that could alter function or expression.[113] Additionally, there is an inherent bias in the candidate-gene approach stemming from the typically small number of genes that are selected for testing. The low-throughput nature of candidate-gene studies obviously makes them ill suited for testing roughly 30,000 genes and the millions of observed genetic variants in the human genome.

There has been a significant increase in the number of GWA studies being conducted, with ~ 400 published to date.[52,53] In general, these studies have 1) reinforced the importance of the genetic variation that underlies phenotypic variation, 2) illustrated that genetic variation almost always results from multiple Mendelian mutations rather than a single mutation, and 3) demonstrated that genetic variation typically explains only a small fraction of the observed phenotypic variation.[4,80,114] Within the field of neuroscience, recent GWA studies have provided insights into the genetic basis of many common neurological diseases and disorders (Table 1). Such studies have been conducted for conditions including Parkinson disease,[39,67,93,113] malignant gliomas,[23,95,109,143] multiple sclerosis,[7,29,90] Alzheimer disease,[15,18,41,72,73] autism,[3,8,22,45,75,107,133] schizophrenia,[25,119,138] lumbar disc disease,[131] idiopathic scoliosis,[43,140] and restless-leg syndrome.[36,139]

Without doubt, the field of genomics is going to play a central role in the clinical care of the future neurological patient. Physicians will therefore need to have at least a basic understanding of the research tools and concepts routinely used in this field. The purpose of this review is to familiarize the clinician with the fundamentals of GWA studies and to highlight their potential clinical application.

Genome-Wide Association Models

Recent progress toward understanding human genetic variation has advanced genetic-linkage and genet-

Abbreviations used in this paper: GWA = genome-wide association; HWE = Hardy-Weinberg equilibrium; SNP = single-nucleotide polymorphism.

TABLE 1: Genome-wide association studies of common diseases and disorders of the brain, spine, and nervous system*

Condition Studied	Reference
neurooncology	
glioma	109
high-grade glioma	143
neuroblastoma	77
high-risk neuroblastoma	24
cerebrovascular disease	
intracranial aneurysm	20
hemorrhagic stroke	9
ischemic stroke	47, 79, 144
neurological disease	
age-related macular degeneration	62
Alzheimer disease	2, 15, 18, 26, 41, 72, 73, 96, 103, 135, 136
amyotrophic lateral sclerosis	21, 27, 34, 35, 38, 63, 105, 129, 130
Creutzfeldt-Jakob susceptibility	81
multiple sclerosis	6, 7, 12, 28, 29, 57, 59, 90
Parkinson disease	42, 76, 93
progressive supranuclear palsy	82
restless legs syndrome	36, 104, 139
brain function & physiology	
cognition	97, 108
memory	94
pain	60
brain vol	12
sleep	46
attention deficit hyperactivity disorder	5, 64, 65, 68, 83, 87, 117
autism	75, 133
bipolar disorder	13, 40, 51, 106, 115, 116, 137, 145
major depressive disorder	85, 120
panic disorder	91
neuroticism	111, 128
schizophrenia	25, 58, 61, 66, 88, 89, 110, 112, 118, 119, 121, 132, 138
personality dimensions	123
addiction	
alcohol dependence	125
methamphetamine dependence	126
nicotine dependence	17, 19, 37, 124, 127

* Compiled from information contained in the National Human Genome Research Institute GWA study catalog.[52,53]

ic-association studies from candidate-gene analyses to GWA studies.[98] The power of the GWA approach lies in the breadth and number of genetic variants tested during the course of a study. The GWA study also has the advantage of being an unbiased search for the genetic variants associated with a particular disease and therefore offers the possibility of discovering new associations of genes and pathways with diseases.[4]

Genome-Wide Association Theory

A phenotype is an observable trait produced by an underlying genotype. The genetic differences among individuals in the human population are commonly called "mutations" and most frequently are single-nucleotide changes within the DNA sequences of genes.[11,80] Many mutations are expected to be harmful and thus to be removed from the population by natural selection. Fewer mutations are expected to be beneficial or fitness neutral, and such mutations can persist in a population over time while proceeding to fixation (every individual carries it) or loss (lost by the actions of selection and drift); with either fate, any genetic variation is lost and therefore not observable. Prior to being fixed or lost, a mutation is carried by only part of the population and is referred to as a "polymorphism." Single-nucleotide polymorphisms are commonly used as genetic markers in GWA studies and are the focus of this review—although alternative genetic features can be used for GWA studies. These alternatives may be particularly useful for GWA studies of psychiatric disorders, in which genetic features such as gene copy-number variations[14,45,107,119] and gross chromosomal rearrangements[8] appear to be important to the genetic etiology of this class of diseases.

The aim of a typical GWA study is to associate one or more SNPs with a particular disease phenotype (Fig. 1). The tested SNPs are not expected to be the causal genetic factors; rather, they are used to mark ("tag") particular regions of chromosomes that likely contain many genetic variants in high linkage disequilibrium with the tested SNP. Linkage disequilibrium occurs when 2 or more alleles at distinct genetic loci occur together significantly more or less frequently than expected by chance based on the constituent allele frequencies. Single-nucleotide polymorphisms are therefore an efficient way to screen many mutations at once, and thus identify chromosomal locations within which the true causal variants are likely to reside. In fact, the true causal variant may not itself be a nucleotide mutation—it may be an insertion or deletion mutation.

Genome-wide association studies differ in their assumptions about the type of genetic variation underlying the disease of interest. Most such studies operate under the "common disease/common variant" hypothesis, which proposes that phenotypic variation is the result of many common SNPs, each of which contributes only a modest effect.[100] An alternative hypothesis, the "multiple rare variant" hypothesis, proposes that phenotypic variation results from the potentially more modest effects of many rare SNPs.[98] These two hypotheses are not necessarily mutually exclusive—the variation underlying a disease may fall into both categories; rather they are in-

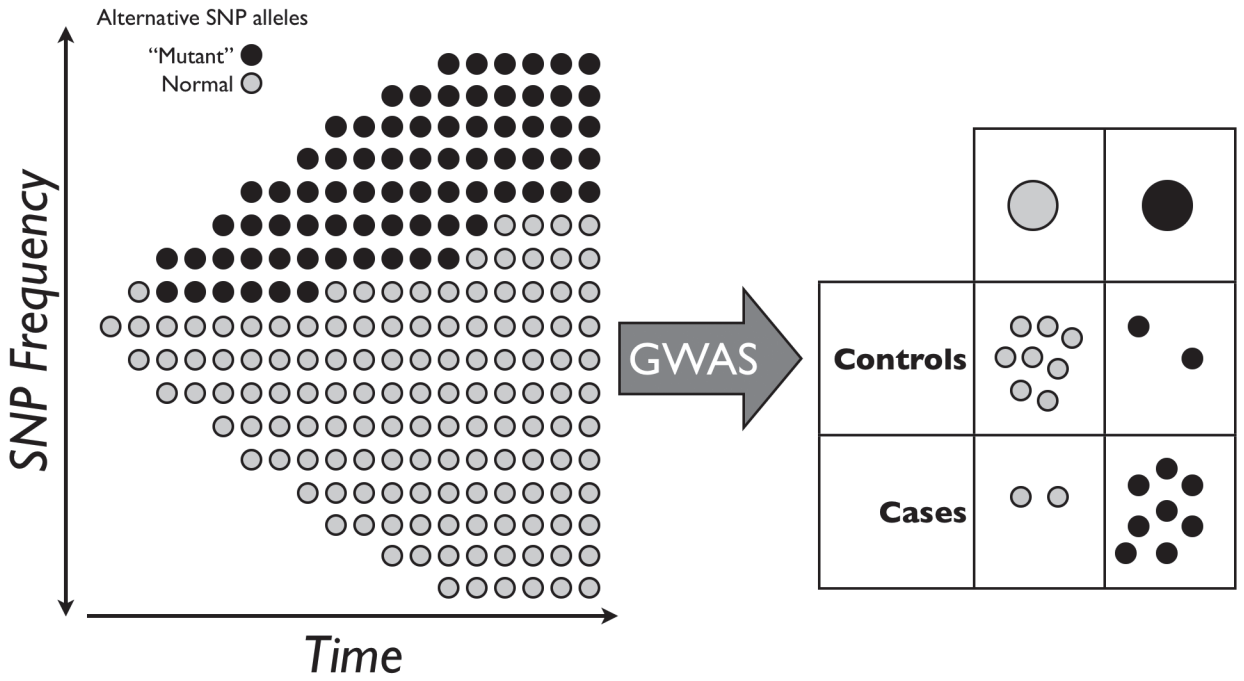

Fig. 1. Schematic representation of a GWA study. ***Left:*** A mutation *(black circles)* entering and spreading through a population over time. ***Right:*** A contingency table in which the mutation is significantly more abundant among the cases than the controls.

tended to guide the design, analysis, and interpretation of GWA studies.

Genetic variation can interact differently to produce the particular disease under study. It can interact additively, in which case alleles confer a mean effect that does not depend on the state of other alleles, or nonadditively, which means that the effect of an allele results from dominance effects and epistatic interactions with other loci. Additive genetic variation is most commonly considered in GWA studies, in which SNPs are typically considered as independent entities.[4] Recently, there has been significant progress toward developing statistical models that assess nonadditive genetic effects, and these models promise to greatly enhance the scope and power of GWA studies.[30,32]

Genome-Wide Association Study Design

The design of a GWA study primarily depends on the specific project goals, but practical factors such as budget and time must be considered as well. The most common design for a GWA study is the case-control format, in which there is a cohort of cases (affected individuals) and a cohort of controls (unaffected individuals). The individuals in the case cohort are assumed to have a greater prevalence of disease-causing alleles than those in the control cohort,[80] a hypothesis that can be assessed by one or more statistical methods discussed below.

Power is the most critical aspect to consider when designing a GWA study. Study power determines the likelihood that a trial will detect significant genetic differences between case and control populations, if any such differences truly exist. Sample size profoundly affects the study power, and, in general, the largest sample that is feasible to genotype should be used. Study power can also be increased by carefully controlling for any population substructure and by cautiously selecting the control population.[4,11,80]

The individuals in the case and control populations are assumed to be "unrelated," which means that their ancestral relationships are distant and therefore unknown.[11] However, the case and control individuals should not be so unrelated that there are distinct subgroups that share a common ancestry (for example, Western European or African heritage). When such subgroups exist, there is said to be population substructure or population stratification, which can be especially problematic when correlated with the case-control delineation. Population stratification can produce false-positive signals because some genetic variants can occur at different frequencies in the two groups as a result of ancestry, even though they are unrelated to the disease under study. Many statistical methods exist to control for population stratification[11,80] and have been implemented in common software packages such as PLINK.[99] Furthermore, recent evidence has suggested that many concerns about population stratification may be overinflated; however, every effort should be made to eliminate its effects.[11,80,137]

Selection of the control population is crucial to the success of a GWA study. Common-control populations, which are genotyped populations that can be used across studies, have been successfully utilized in GWA studies.[137] The main concerns about common controls are the presence of population stratification and the potential loss of power given the presence of latent (undiagnosed) disease

in the control population. A study-specific control population is almost always ideal, but can greatly increase the time and cost of a GWA study (controls must be genotyped) and is subject to selection bias when differences, in addition to the disease under study, exist between cases and controls.[80] Historical controls, which are previously genotyped individuals used in current studies, have the potential to reduce study power when there is significant genetic divergence between extant cases and historical controls. Historical controls may be the best option available, however, and recent evidence has suggested that some of the drawbacks may be overcome by increases in sample size.[137]

An alternative study design is "family-based association" in which genetic tests are performed within families. Family-based association studies offer strong control of background genetic differences at the cost of overall study power. The additional costs associated with studying large numbers of families and recent improvements in statistical methods to control for population stratification make family-based studies attractive for only specialized circumstances.[80]

Genome-Wide Association Study Methods

Genotype Calling

Genotyping is the phase in which an instrument determines ("calls") the genotype, or state of both alleles, at every SNP locus tested. Genotype calling is typically performed in a high-throughput manner by using highly automated instruments, such as those commercially available from Affymetrix and Illumina. Commercial genotyping instruments use high-density microarrays of SNPs ("SNP chips," informally) that have been identified through projects such as the International HapMap Consortium.[56]

The latest generation of commercial SNP genotyping platforms can routinely test ~ 2 million genetic loci in a single assay. The loci include ~ 1 million SNPs and an approximately equal number of copy-number variants; a state-of-the-art instrument with robotic automation can generate ~ 40–50 million genotypes per day. However, investigators in most published studies have used earlier genotyping platforms that tested ~ 300,000–500,000 SNPs. One advantage of commercial genotyping platforms is that the selection of high-quality SNPs to test is no longer a challenge left to individual researchers. The latest genotyping platforms include SNPs that are spaced, on average, 1–2 kilobases apart and cover ~ 95% of the human genome, including sex chromosomes and mitochondrial genomes. Current genotyping instruments poorly sample regions of the human genome with infrequent restriction enzyme sites, which precludes isolating SNPs in these regions. An additional benefit of commercial platforms is that they lead to further standardization of the SNPs tested across studies.

Quality Control of Genotype Data

Genome-wide association studies produce enormous amounts of data, and therefore data quality is of paramount importance. Rigorous quality control measures must be implemented at each stage of the study—from DNA extraction and amplification through to analyzing and interpreting the data. A common source of errors is genotype calling, which must strike a balance between stringency and call rate. If base calling is excessively stringent, then most markers will have a low call rate, which can inflate the false-positive rate.[11] On the other hand, overly relaxed genotype calling will produce significant numbers of miscalled genotypes. Finally, a threshold call rate must be selected, and SNPs whose call rates fall below this threshold should be excluded from consideration. Individuals with low overall call rates should be removed because such rates suggest that their DNA samples may be problematic.

Each marker should be tested in the control population to ensure it is in HWE, which describes the expected genotype frequencies (based on allele frequencies) in a randomly mating population in the absence of selection, mutation, and migration.[50] Extreme deviations from HWE might be symptomatic of genotype calling errors, and such markers can generally be removed with impunity.[80] On the other hand, moderate deviations from HWE may be expected in the cases, and therefore the inclusion threshold should not remove these markers because they may provide additional information when searching for disease-associated SNPs.[11,142] The HWE inclusion threshold must be carefully selected to balance overall inclusiveness with the purging of potentially problematic markers. A flexible and powerful approach is the use of the observed distribution of HWE values for each marker to determine an appropriate threshold for inclusion.[137]

There are a few remaining aspects to consider when implementing quality-control measures. Individuals whose genotype does not agree with their stated ethnicity or sex should not be included. Methods should be implemented to ensure all individuals in the study share a common ancestral background (for example, all of Western European descent) to minimize population substructure. Individuals with evidence of genetic syndromes (for example, fragile-X syndrome or trisomy 21) should also be excluded. In family-based studies, markers with unusually high rates of Mendelian errors—potentially a sign of frequent miscalling of genotypes—should be discarded.[11,80]

Testing for Association

After obtaining a high-quality set of genotypes, a GWA study typically moves into the analysis phase during which SNPs are tested for their association with the phenotype of interest. This phase essentially consists of applying one or more statistical tests of association to each marker tested. There are several statistical models available, although the viable options may be constrained by the study design (for example, phenotypic variable). The statistics underlying these methods can be quite difficult to understand, and therefore only the general features of the most common tests are discussed herein. The interested reader is encouraged to investigate one of many recent reviews for more detailed treatments of GWA study statistics.[11,134]

A significant problem with many tests of association

is the extensive multiple-testing burden;[11,80,134] this problem becomes more significant as the number of SNPs tested increases. Multiple-testing burden refers to the increased false-positive rate that results from performing multiple independent tests on the same data set. Several methods exist to correct the p values for multiple testing and reduce the likelihood of false-positive signals.[10,54,134] The most conservative approach is a Bonferroni correction in which individual p values are each multiplied by *N* (the number of SNPs tested) to maintain the false-positive rate at a desired level. More flexible and relaxed false-discovery rate (FDR) calculations can be used as well.[16] Such corrections yield a greater number of potential SNPs positively associated with the disease under study, but also create a greater risk of including false-positive associations. Simulation models can also be used to empirically determine a threshold p value that appropriately balances overall inclusiveness with false-positive risk. Under any multiple-testing correction approach, the required p value for attaining significance for any particular SNP is exceedingly small because modern GWA studies effectively conduct hundreds of thousands of statistical tests.

The most basic test for a single SNP in a case-control design is that for independence between the 2 rows (cases and controls) and 3 columns (genotypes) of a contingency table.[11] A chi-square or Fisher exact test would be an appropriate statistical test. Alternatively, one could use a Cochran-Armitage test, which is based on the differences in allele counts rather than genotype counts, and may be more powerful for complex traits for which the contribution of individual SNPs is thought to be roughly additive.[80]

More advanced statistical approaches based on regression modeling are routinely used when analyzing GWA data. Logistic regression models are suitable for case-control studies in which the phenotype is binary (for example, presence or absence of a disease), whereas a linear regression or an ANOVA model is suitable for testing continuous phenotypes (for example, degree of spine curvature in a scoliosis study). One advantage of using regression models is that epistatic interactions between SNPs can be readily incorporated into the model, as can other covariates such as age or sex. An important theoretical consideration is that regression-based methods assume that phenotypes are observed prospectively, whereas most GWA studies select individuals based on phenotype and then determine the genotype.[11]

Family-based association studies model SNP flow through pedigrees. Software packages such as MERLIN and LAMP (freely available at http://csg.sph.umich.edu) are widely used suites of programs for the analysis of large pedigree data sets.[1] MERLIN is designed to test quantitative trait association, whereas LAMP[69,70] is intended for discrete trait association. Both software programs use likelihood statistics to assess the relative probabilities of alternative patterns of gene flow through the pedigrees in the data set.

Interpretation of Results

Once a set of associated SNPs has been identified, a search for additional evidence to support the observed association must begin. Ideally, the study would be replicated with a different population of cases and controls to ensure that the same SNPs would be identified in these new individuals. Such replication may not be feasible, however, for reasons such as cost or time.

Vendor-supplied annotation files can be used to determine the physical and genomic location of each SNP within the genome. The annotation for each SNP may also include additional information, such as whether the SNP is located within a gene (intronic/exonic) or an intergenic region. Using this information, one can search databases such as Entrez for additional DNA, RNA, or protein sequence information, or Medline can be queried for other studies corroborating an observed SNP association. For example, genes adjacent to an associated SNP can be checked to determine if any have prior evidence linking them to the disease under study, which could identify genomic locations that might be good targets for more extensive sequencing efforts.

Limitations of GWA Studies

Theoretical Limitations

There is appreciable work to be done on the practical and analytical front for improving the current generation of GWA methods. First and foremost, even the most powerful GWA study can only explain a small percentage of the observed phenotypic variation. This fact partially emphasizes the need for models and methods that explicitly consider the interactions of genes with their environment. Gene-by-environment interactions present a significant practical challenge to GWA studies; it is extremely difficult to determine the relevant times and environmental variables to measure.[31,33] In the future, statistical methods that consider the interactions between multiple genetic variants must be advanced.[32,55] The power to model and detect epistatic interactions among mutations in a genome offers great hope to more completely explain phenotypic variation and uncover a greater number of loci. Loci that are not strongly associated with a trait individually may be very strongly associated when considered in combination with other mutations.

Genome-wide association studies only test for a statistically significant association of a marker with a trait and cannot make a causal statement. The direct test for causality between associated SNPs and their flanking genes would involve the mutation of each candidate gene and the determination of the resulting phenotype. Obviously, this is impossible with humans, but other organisms, such as yeast, mice, and primates, may be good surrogate models for the human disease. One or more of these model organisms might be useful in refining the set of associated SNPs and further understanding the genes and pathways mutated in diseased individuals. It is a long road from a GWA study to determining which genes and pathways are defective in the diseased state; however, GWA analysis can be an important first step in identifying otherwise unknown genes and pathways involved in diseases.

Practical Limitations

At present, the most significant practical limitation for GWA studies is cost. Genotyping is expected to cost ~ $500 per individual, not considering the necessary instrumentation. Current genotyping instruments require a significant initial investment of roughly $300,000. Alternatively, research service providers can be contracted for some or all stages of data production (DNA extraction, genotyping, and so forth). Significant costs may also be associated with obtaining sufficient high-quality data, especially for rare diseases with a low incidence. Moreover, significant time and resources may be required to generate the genetic data (genotyping) for the control population.

The large amounts of data generated during the course of a GWA study must be processed on relatively powerful computers with a large storage capacity. Additionally, computer software may need to be developed in-house to store and analyze the data, or existing software programs may need to be acquired. Commercial software packages are available to analyze the data, and there are freely available options as well.

Clinical Applications of GWA Studies

Genetic Testing

In addition to identifying a set of alleles associated with a particular disease, the GWA approach can be used to identify risk alleles, that is, those that appear to confer an increased risk for developing a disease. Genetic risks have been based on family history or candidate gene testing. Genome-wide association approaches may one day be able to provide a comprehensive picture of an individual's risk for developing any of a wide range of disorders.

Although it has been proposed that risk calculations based on GWA data might ultimately replace those based on family history,[102] genetic tests have gained only limited acceptance in the medical community.[48,49] The clinical utility of genetic testing has been difficult to demonstrate for a variety of reasons, but significant effort is currently being expended to overcome these obstacles. Single-nucleotide polymorphisms implicated in the most powerful GWA studies typically explain only a small fraction of the observed variation for a disease, which partly stems from a combination of methodological and practical limitations. Genome-wide association tests carry significant direct costs. It has been difficult to demonstrate their cost-effectiveness because 1) risk loci have typically low penetrance, 2) the benefits of genetic testing are hard to quantify because treatment for the disease may improve over time or a patient's adherence to preventive measures may decline over time, and 3) there is a potential cost to the patient in terms of stigmatization by society or psychological stress resulting from his or her knowledge about potential future disease.[101] Nonetheless, GWA screens have been conducted for a number of diseases of neurosurgical importance.[114]

Malignant glioma is the most common type of primary brain tumor, and the prognosis remains poor despite surgical and oncological advancements.[43,74] As in other types of cancer, there is great interest in identifying susceptibility loci for these aggressive tumors. Such loci would allow for the estimation of the risk of developing malignant glioma in a particular individual during his or her lifetime. Significantly at-risk individuals could be monitored more closely, with the hope that increased surveillance might lead to earlier detection and better treatment outcomes.

Recently, authors of a large GWA study were able to identify 5 genetic loci that appear to confer a significant risk for the development of malignant gliomas.[109] The SNPs identified potentially support the importance of the cyclin-dependent kinase inhibitor 2A–cyclin-dependent kinase 4 (CDKN2A-CDK4) signaling pathway, as well as the genes involved in genomic stability and telomere preservation. A few of the genes and chromosomal regions have been implicated in other types of cancer. Interestingly, 2 of these loci appear to be associated with a greater risk for the development of high-grade gliomas.[143] One of the chromosomal regions identified is the 9p21 region in which the *CDKN2B* gene resides. This gene participates in the control of cell division and is frequently deleted in high-grade gliomas. The other identified chromosomal region is 20q13 in which *RTEL1* is located. The *RTEL1* gene encodes a DNA helicase that is critical for the maintenance of telomere length.

Idiopathic scoliosis is the most common childhood spine disorder, affecting almost 3% of children globally.[140,141] The disease appears to cluster within families, although the precise mode of inheritance has yet to be determined. A recent GWA study revealed significant linkage of a region of chromosome 8 with idiopathic scoliosis, specifically with the *CHD7* gene.[44]

Lumbar disc disease is a significant source of disability, and one of the most common disorders seen in neurosurgical practices.[92] A recent small-scale GWA study demonstrated an association between a region of chromosome 21 and lumbar disc disease.[131] The results of this study are encouraging, although larger and more densely sampled GWA studies must be conducted to corroborate the data. Furthermore, lumbar disc disease may present special problems relating to phenotype scoring since many individuals carry asymptomatic disc herniations.

Diagnostics, Tumor Grading, and Prognosis

The GWA study framework also holds great promise for molecular diagnostics in medicine. Molecular diagnostics use genetic markers to ascertain the clinical status of a patient's tissue sample. For example, a patient's brain tumor tissue sample is traditionally sent for anatomical pathology analysis. In the future, a patient's specimen may be sent to a genetics laboratory for analysis. The potential advantages of genetics-based diagnostics are that such analyses are typically unambiguous, unbiased, and completely objective. There is still much work to be done for this field to move from potential applications to effective clinical solutions.

Recently, significant progress has been made toward developing molecular diagnostics for brain tumors, in particular malignant gliomas. For example, there is a great deal of interest in developing tumor-grading meth-

ods based on the genetic states of tumors rather than their anatomical appearance.[86,122] These methods would use genetic markers as an adjunct to traditional grading of tumors by a pathology lab. There is also significant interest in developing tumor prognosis classifiers based on genetic markers, with some recent success.[95] Progress has been made in developing artificial-intelligence classifier models that predict survival time based on the genomic expression patterns of select genetic loci.[71,78]

Personalized Medicine and Tumor Drugs

Lastly, we note that GWA studies may one day lead to clinical regimens that are individually tailored to each patient. For instance, genetic testing is routinely being done for oligodendrogliomas, with the 1p/19q screen for chemotherapeutic effectiveness. However, the GWA approach permits screening for more markers in a single sweep and offers much more precision in associating genotypes with clinically important phenotypes. In addition, understanding genes, as opposed to chromosome arms, will allow for a deeper understanding into the molecular genetic mechanisms behind drug function and metabolism, and lead to better therapies in the future.[84,122]

Conclusions

Genome-wide association studies hold great promise for decrypting the complex genetic architecture of many diseases. Furthermore, GWA approaches have the potential to power a new generation of genetic tests, which one day may be used to estimate an individual's risk for a particular disease or to predict which chemotherapeutic agent or biological treatment will be most effective. As the costs decline and the analytical methods become more powerful, genetic testing may become a feasible option for patients seeking to understand the health risks conferred by the mutations residing in their DNA. Given their advancement to date, the current generation of neurosurgeons and neurologists can expect to use patient genetics as part of their clinical decision-making at the bedside.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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The many roles of microRNAs in brain tumor biology

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MicroRNAs (miRNAs) are now recognized as the primary RNAs involved in the purposeful silencing of the cell's own message. In addition to the established role of miRNAs as developmental regulators of normal cellular function, they have recently been shown to be important players in pathological states such as cancer. The authors review the literature on the role of miRNAs in the formation and propagation of gliomas and medulloblastomas, highlighting the potential of these molecules and their inhibitors as therapeutics. *(DOI: 10.3171/2009.10.FOCUS09207)*

Key Words • microRNA • glioma • medulloblastoma • cancer stem cell

MicroRNAs are now recognized as the primary endogenous small RNAs, about 21 nucleotides in length (21–23 nucleotides), involved in the purposeful silencing of the cell's own message in a variety of ways, including translational repression, mRNA cleavage, and deadenylation. They were first described in *Caenorhabditis elegans* with the discovery that *lin-4* coded for an RNA transcript capable of modulating the protein expression of *lin-14*.[42] After their initial discovery in 1993, miRNA research proliferated and a variety of screening efforts have led to the discovery of hundreds of miRNAs. MicoRNAs are now considered fundamental to normal cellular function in eukaryotes, and the alteration of miRNA expression and activity has been implicated in a variety of pathological processes.

Biogenesis

MicroRNAs are stem-loop structures encoded by a cell's own genome. They interact with complementary mRNA leading to the disruption of target protein expression. MicroRNAs are generated by a multistep process (Fig. 1). Each miRNA can be transcribed separately from an individual transcriptional unit, or each transcriptional unit can encode a cluster of distinct miRNAs; miRNAs can also be processed directly from other RNA species, such as introns by Dicer.[2] The primary miRNA transcript (often abbreviated pri-miRNA) is typically transcribed from the genome by RNA polymerase II and is subsequently capped and polyadenylated.[40] The primary miRNA transcript folds into a stem-loop structure, which is essential for the maturation process. In animals, the primary miRNA transcript is then cleaved in the nucleus by Drosha, an RNase III endonuclease, in association with the double-stranded RNA-binding domain protein DGCR8/Pasha in a protein complex referred to as the microprocessor complex.[17] Drosha cleaves both strands of the stem at sites near the base of the primary stem-loop,[43] generating an intermediate known as the miRNA precursor (sometimes abbreviated premiRNA or pre-miRNA). The miRNA precursor is then exported out of the nucleus by Exportin-5 into the cytosol where the RNase III domain–containing nuclease, Dicer, cleaves the terminal loop to generate the ~ 22 nucleotide mature miRNA. Dicer functions with the double-stranded RNA–binding domain proteins TRBP and PACT.[46] Drosha and Dicer cleave with great precision, generating very exact ends, and it is this feature that is likely responsible for the high specificity of miRNA with their target mRNA.[8]

Posttranscriptional Gene Silencing

Immediately after formation of the mature miRNA, the duplex is unwound and loaded onto the RNA-induced silencing complex, which ultimately carries out the silencing of target mRNA. The RISC is a trimeric complex composed of Dicer, TRBP, and a protein of the

Abbreviations used in this paper: miRNA = microRNA; npBAF = neural-progenitor-specific BAF; REST = repressor-element-1-silencing transcription factor; RISC = RNA-induced silencing complex.

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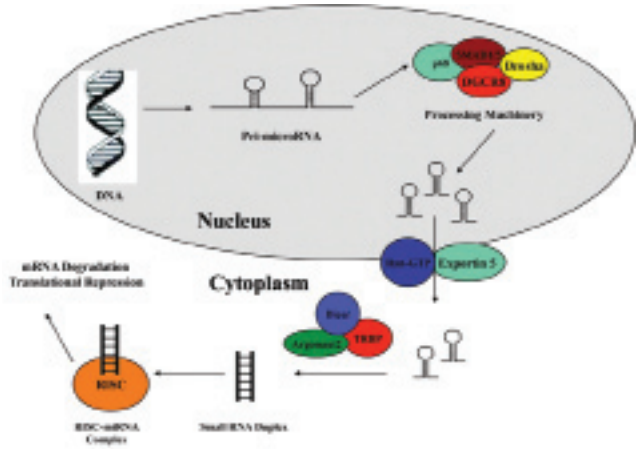

Fig. 1. Long primary miRNAs (pri-microRNAs) are processed in the nucleus into stem-loop precursors by the RNase III endonuclease Drosha and Pasha. The products of the enzymatic reaction are transported into the cytoplasm by exportin 5 and Ran-GTP, and these precursors are further processed into small RNA duplexes of approximately 22 nucleotides by the Dicer RNase III enzyme and Loqacious (Loqs). The microRNA duplex is then loaded onto the RNA-induced silencing complex (RISC). The microRNA guides the RISC to the target mRNA for translational regulation.

Argonaute superfamily (Ago2 in humans).[30] It identifies target mRNA based on complementarity with the associated single-stranded miRNA and results in either mRNA cleavage or translational repression.[2] An estimated one-third of all mRNAs are thought to be susceptible to post-transcriptional gene silencing by miRNAs.[66]

MicroRNA and Neuronal Development

One of the most defining moments in the development of the nervous system is when neuroprogenitors lose multipotency at mitotic exit and begin to develop their terminal cell fate. In the case of neurons, it means they commit to their final topographical position and established stable connections that will persist for the lifetime of the organism. This transition is in large part accompanied by a switch in chromatin-remodeling/regulatory complexes such as in the exchange of the BAF53a and BAF45a subunits within Swi/Snf-like npBAF complexes. The subunits of the npBAF complex are essential for neural-progenitor proliferation. The exchange of the BAF53a and BAF45a in the neuron-specific BAF complex promotes postmitotic neural development and dendritic morphogenesis (Fig. 2). MicroRNA (miR-9* and miR-124) has been implicated in this process by regulating the chromatin architecture and dynamics.[72] Interestingly, miR-9* and miR-124 are repressed by the REST, which mediates cell type- and developmental stage–specific gene repression, gene activation, and long-term gene silencing for protein-coding genes resulting in postmitotic neurons. The expression and function of REST are tightly regulated by context-specific transcriptional and posttranscriptional mechanisms including bidirectional feedback loops with various miRNAs. It is therefore not surprising that deregulation of REST and miRNAs are both implicated in the pathogenesis of various brain tumors.

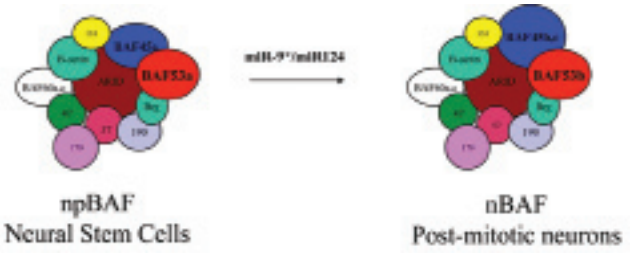

Fig. 2. The subunits of the npBAF complex are essential for neural-progenitor proliferation. The exchange of the BAF53a and BAF45a in the nBAF (neuron-specific BAF) complex promotes postmitotic neural development and dendritic morphogenesis.

MicroRNA Function and Cancer

Following the discovery of miRNAs and other small RNAs, a wealth of data were rapidly generated, which revealed a novel mechanism for the modulation of gene expression. As gene-finding computational models have evolved from simple homology-based searches to more complex multifactorial models, the number of identified miRNAs has continued to grow.[47] Over recent years, there has been a significant push to better understand how they function in both normal and pathological states.

Though miRNAs have been demonstrated to modulate genes involved in a variety of cellular processes, a significant proportion of miRNAs regulate genes associated with cellular fate. It is now well accepted that miRNAs are fundamental to the regulation of proliferation, differentiation, and apoptosis during normal development. It has been shown that miRNAs have a predilection in targeting developmental genes. Genes involved in functions common to all cells, such as in maintenance and general activities, have very few miRNA target sites, and seem to be under selection to avoid targeting by miRNAs.[61] Furthermore, alterations in the expression of miRNAs are seen in a variety of pathological processes, including cancer. Aberrant miRNA expression has been demonstrated in essentially every cancer type studied, including breast[58,64,68,73] and ovarian carcinomas,[48,74] pancreatic cancer,[4,27] non–small cell lung cancer,[41,56] leukemia,[5–7] and brain tumors (Table 1).[52,66] MicroRNA expression can be altered in cancer through a variety of mechanisms including chromosomal changes, epigenetic defects, mutations, and alterations in the machinery involved in miRNA biogenesis.[66]

Beyond mere biomarkers, the altered expression profiles of miRNA implicate them as key regulators of tumorigenesis. The miR-17-92 polycistron, located on chromosome 13q32–33, was the first example of miRNAs acting as mammalian oncogenes.[35] This region of the chromosome is amplified in several types of cancer and can be activated by *c-Myc*, a well-established protooncogene overexpressed in different cancer types.[51,66] The first individual miRNA assigned an oncogenic role was *miR-155*,[18] which when overexpressed in a transgenic mouse model, led to the development of B-cell leukemia and high-grade lymphoma.[15]

There is also evidence accumulating that miRNAs are involved in cell-cycle checkpoint regulation. Using a mutated version of dicer-1, Hatfield and colleagues[33] dem-

TABLE 1: MicroRNAs implicated in glioma and medulloblastoma biology

Tumor	miRNA	Cellular Role
GBM	miR-7	suppresses EGFR expression; independently inhibits Akt pathway
	miR-10b	may promote invasion; found to be increased in invasive high-grade gliomas
	miR-15b	results in cell cycle arrest
	miR-21	anti-apoptosis; suppresses tumor supressors
	miR-26a	targets PTEN; enhances Akt pathway
	miR-124	inhibits proliferation by inducing G0/G1 cell cylce arrest via CDK6 inhibition
	miR-128	expression decreases levels of Bmi-1
	miR-137	inhibits proliferation by inducing G0/G1 cell cylce arrest via CDK6 inhibition
	miR-181a	tumor supressor
	miR-181b	tumor supressor
	miR-221	unknown; found to be increased in invasive high-grade gliomas
	miR-425	upregulated in non-cancer stem cells; promotes differentiation
	miR-451	upregulated in non-cancer stem cells; promotes differentiation
	miR-486	upregulated in non-cancer stem cells; promotes differentiation
MB	miR-let7g	upregulated in anaplastic MB; differentially expressed in desmoplastic MB
	miR-9	regulates proliferation, apoptosis
	miR-19a	over-expressed in hedgehog-dependent MB; upregulated in anaplastic MB
	miR-20	over-expressed in hedgehog-dependent MB
	miR-92	over-expressed in hedgehog-dependent MB
	miR-106b	upregulated in anaplastic MB; differentially expressed in desmoplastic MB
	miR-124	regulates cell cycle via CKD6
	miR-125a	regulates proliferation, apoptosis
	miR-125b	hedgehog dependent proliferation
	miR-191	upregulated in anaplastic MB
	miR-199b-5p	expression correlates with decreased metastatic potential; associated with survival
	miR-324-5p	hedgehog dependent proliferation
	miR-326	hedgehog dependent proliferation

onstrated that miRNAs modulated stem cell division in Drosophila by bypassing the G1/S checkpoint. This work suggested that miRNAs may make stem cells insensitive to environmental stimuli and that a similar mechanism may be implicated in tumorigenesis.[16] More recently, specific miRNAs capable of regulating cellular checkpoints in cancer have been described, including miR-221, miR-222, and miR-27a.[45,49,67]

In addition to their involvement in cancer formation, miRNAs have also been associated with tumor progression and metastatic potential.[3] Although still not well characterized, specific miRNAs—such as miR-10b, miR-21, miR-30a, miR-30e, miR-125b, miR-141, miR-200b, miR-200c, and miR-205—have been suggested to play an important role in tumor invasiveness and metastasis.[1] Some of the targets of these miRNAs have recently been elucidated and include tumor suppressor genes such as *tropomyosin 1* and other targets with metastatic potential such as *PDCD4* and *maspin*.[76]

MicroRNAs and Gliomas

Gliomas are tumors arising from glial cells, the neuroepithelial support cells of the CNS. Gliomas are the most common primary tumor of the CNS comprising over 50% of primary brain tumors.[62] Glioblastoma is the most common and deadliest glioma with approximately 10,000 new cases every year and a median survival of only 14 months even with the most current therapies.[62] Gliomas, like tumors in other parts of the body, develop because of fundamental genetic alterations that cause the formation of a tumor stem cell population that divides without regard to normal physiological biochemical signaling.[11,65] It is no surprise then that the same miRNAs that are involved in stem cell regulation and differentiation are also implicated in tumor stem cell biology of gliomas.

Microarray studies of glioma tissue have implicated a number of miRNAs involved in glioma formation and propagation. In their quantitative reverse transcriptase polymerase chain reaction analysis of high-grade astrocytomas in humans (glioblastomas and anaplastic astrocytomas), Silber et al.[59] found 35 different miRNAs that were significantly deregulated compared with control brain tissue. Those miRNAs that have been shown to play a role in human glioma include miR-7, -10b, -15b, -21, -26a, -124, -128, -137, -181a, -181b, -221, -451, and others. The majority of miRNAs are underexpressed in proliferating glioma cells with the important exception of miR-10b, -21, and -221.

MicroRNAs are key regulators of tumor suppressor genes. The overexpression of miR-21, a recently described

antiapoptotic factor, appears to be vitally important in not only glioma but also cancer arising from other parts of the body.[9] Chen and coworkers[10] used bioinformatics analysis to screen and identify various genes with miR-21 binding sites. Using the glioblastoma cell line T98G, they confirmed that miR-21 binds and silences the tumor suppressor gene *PDCD4*. Corsten et al.[14] transfected human glioma cells with anti-miR-21 oligonucleotides and simultaneously implanted neural precursor cells expressing a secretable variant of the cytotoxic agent TRAIL (S-TRAIL). They found a synergistic effect of knocking down miR-21 levels and adding the apoptotic agent S-TRAIL evidenced by increased caspase activity and decreased cell viability in the human glioma cells in vitro. They then found complete eradication of tumor cells in a murine model transfected with anti-miR-21 and then exposed to S-TRAIL in vivo.

MicroRNAs regulate oncogenes implicated in brain tumor formation. The expression level of miR-128 is inversely correlated with expression level of transcription factor E2F3a, a protein that promotes cell entry into S-phase and implicated in cancer of the bladder and prostate.[75] Godlewski and colleagues[28] found that miR-128 levels were downregulated in glioma cells compared with normal brain tissue. They reported that increasing miR-128 expression leds to a decrease in the expression of the oncogene *Bmi-1*. Overexpression of miR-128 in glioma neurosphere cultures specifically blocks glioma self-renewal consistent with *Bmi-1* downregulation.

Interestingly, other than serving as key regulators of oncogenes and tumor suppressors, microRNAs may also dictate the invasiveness and aggressiveness of tumors. Conti et al.[13] found both miR-21 and miR-221 upregulated in glioma samples; however, they noted that whereas miR-21 was elevated in all gliomas, high levels of miR-221 were only found in high-grade gliomas. MicroRNA-10b has similarly been associated with high-grade tumors. Overexpression of miR-10b may promote glioma invasion via the RhoC and urokinase plasminogen activator receptor (uPAR), which have been implicated in other cancers as prometastatic and proinvasive factors.[55] Huse and colleagues[37] studied the overexpression of miR-26a in a subset of high-grade glioma, most often associated with monoallelic loss of phosphatase and tensin homolog (PTEN). Studying 3 human glioblastoma samples, they found that miR-26a directly targets PTEN and enhances the Akt pathway. By transfecting miR-26a into a murine model, they were able to show that overexpression of miR-26a enhances gliomagenesis. Because miR-26a overexpression appears in only a subset of glioma with monoallelic loss of PTEN, they hypothesized that this overexpression functionally substitutes loss of heterozygosity at the PTEN locus.

MicroRNAs have also been shown to be important regulators of cellular proliferation/differentiation and the cell cycle. Silber and colleagues[59] were the first to discover that miR-124 and miR-137 are downregulated in high-grade gliomas compared with normal controls. These microRNAs are also upregulated during adult neural stem cell differentiation. The upregulation of miR-124 and miR-137 in tumor stem cell populations promotes neuronal differentiation of the tumor stem cells and inhibits proliferation of the tumor cells by inducing G0/G1 cell-cycle arrest. The mechanism of action is via the inhibition of CDK6 expression, a protein essential for cell-cycle progression.[53] Kefas et al.[39] studied miR-7, another miRNA that is downregulated in glioma. They found that miR-7 suppresses EGFR expression and independently inhibits the Akt pathway in glioblastoma. Transfection of glioblastoma with miR-7 decreased viability and invasiveness of primary glioblastoma cell lines and increased the apoptotic fraction of cells. Overexpression of miR-15b, another differentially expressed microRNA, results in cell-cycle arrest while suppression of miR-15b results in more cells in S-phase.[70] Gal and colleagues[23] examined glioblastoma stem (CD133-positive) and nonstem (CD133-negative) cells and found that miR-451, -486, and -425 were significantly upregulated in CD133-negative cells compared with CD133-positive cells. Transfection of glioblastoma cells with these miRNAs inhibited neurosphere formation, and transfection with miR-451 resulted in neurosphere dispersion and inhibited glioblastoma growth. Gal and colleagues found that combining miR-451 transfection with Imatinib mesylate treatment had a cooperative effect in dispersal of glioblastoma neurospheres.

MicroRNAs have recently been shown to function as bona fide tumor suppressors. Shi et al.[57] reported on downregulated miR-181a and miR-181b involved in glioma formation. Their study showed that these miRNAs functioned as tumor suppressors. Transfection of these miRNAs into glioblastoma cells inhibited proliferation in vitro, resulted in loss of anchorage-independent growth, induced apoptosis in glioma cell lines, and depressed the invasion of glioma cells in vitro.

MicroRNAs and Medulloblastoma

Medulloblastoma is the most common malignant brain tumor in children with an incidence of approximately 2 per 100,000. It appears to arise from stem cells and from granule neuron precursors in the external granule layer of the cerebellum[26] or multipotent precursors in the ventricular zone of the cerebellum.[44] About 70% of cases occur before the age of 16 years. Approximately one-third disseminate in the CSF and up to 5% spread systemically. Medulloblastoma treatment most often involves a combination of surgery and radiation therapy. Chemotherapy is usually reserved for children younger than 3 years of age or for recurrent tumors. Five-year survival rates for medulloblastoma have been estimated to range from 35 to 75%.[19]

Evidence for a role of miRNA in medulloblastoma tumorigenesis has only very recently emerged. Pierson and associates[54] were the first to report the involvement of miRNA in medulloblastoma by demonstrating that miR-124 modulates cell-cycle regulation in medulloblastoma cells. They showed that miR-124 expression is significantly decreased in medulloblastoma and that augmentation of miR-124 levels can slow tumor cell growth by targeting CDK6.

Ferretti et al.[21] proposed a role for miRNA in modulating hedgehog signaling, a pathway recently implicated

in tumorigenesis for a subset of medulloblastoma.[29,32,34] Specifically, they showed that miR-125b, miR-326, and miR-324–5p expression was decreased in medulloblastoma and that the altered expression of these miRNAs led to tumor cell proliferation through a hedgehog-dependent mechanism. Expanding on this work, Uziel et al.[63] identified 3 miRNAs overexpressed in hedgehog-active medulloblastomas: miR-92, miR-19a, and miR-20. All 3 miRNAs were encoded by the miR-17/92 cluster, which has been associated with a variety of cancers. Northcutt and colleagues[50] identified a high-level, focal amplification on chromosome 13q31.3, which mapped to the same miRNA cluster. The expression of miR-17/92 was most elevated in medulloblastomas with activated hedgehog signaling and was also associated with elevated c-Myc and n-Myc. These studies suggest that aberrant expression of miRNAs encoded by the miR-17/92 enhance the growth potential of medulloblastoma and that miRNA-mediated modulation of hedgehog signaling may be an important contributing factor to medulloblastoma pathogenesis.

Ferretti et al.[22] used high-throughput screening to examine miRNA expression profiles in 34 patients with medulloblastoma. They identified 78 miRNAs with altered expression in medulloblastoma, compared with normal adult and fetal cerebellar cells. Several of the identified miRNAs have been implicated in other cancer types including glioblastoma.[12,25] The majority of these miRNAs were downregulated in medulloblastoma, supporting a role for miRNAs as tumor suppressors. Additionally, they found increased expression of miR-9 and miR-125a and that increased expression of these miRNAs was capable of decreasing proliferation, augmenting apoptosis, and ultimately promoting arrest of tumor growth. The proapoptotic effect was mediated by miR-9 and miR-125a targeting of the t-TrkC receptor, which was found in this study to be upregulated in medulloblastoma cells. In this study, miRNA expression patterns were also examined in different tumor subsets. The authors found that miR-let7g, miR-19a, miR-106b, and miR-191 were significantly upregulated in anaplastic compared with desmoplastic medulloblastomas; miR-let7g and miR-106b were differentially expressed in desmoplastic compared with classic medulloblastomas; and miR19a was upregulated in anaplastic compared with classic medulloblastomas.

Changes in expression of Her2 (ErbB2) and c-Myc have been demonstrated to impact biological activity and clinical features of medulloblastoma.[26,31,36] Ferretti et al.[22] examined miRNA expression from medulloblastomas overexpressing either Her2 or c-Myc and identified an miRNA signature in each group of medulloblastomas. Expression of miR-10b, miR-135a, miR-135b, miR-125b, miR-153, and miR-199b was altered in Her2-overexpressing tumors, whereas c-Myc overexpressing medulloblastomas had expression changes in miR-181b, miR-128a, and miR-128b. Additionally, the amount of expression change of 2 miRNAs correlated with disease risk. Though miR-31 and miR-153 were downregulated in all medulloblastomas, the group found that the degree of change was directly proportional to disease severity.

It is well established that the Notch signaling pathway regulates the differentiation of granule neuron precursor cells and that Notch2 expression is increased in about 15% of medulloblastomas.[44,60] Expression of the transcriptional repressor HES1, a downstream effector protein of the Notch pathway, normally declines during the process of neuronal differentiation. Conversely, persistent activation of the Notch pathway and HES1 prevents the migration of granule neuron precursor cells out of the ventricular zone and inhibits neuronal differentiation.[38] Based on its role in differentiation, it is not surprising that dysfunction of the Notch pathway has been associated with a subset of medulloblastoma with stem cell–like properties.[20,69,71,77] Garzia et al.[24] examined the role of miRNAs in the regulation of Notch/HES1 signaling in medulloblastoma. They found that miR-199b-5p targeted HES1 and that miR-199b-5p-mediated downregulation of HES1 attenuated cellular proliferation in medulloblastoma cell lines. In medulloblastoma patients, increased expression of miR-199b-5p appeared to decrease metastatic potential and was associated with increased survival.

Conclusions

Although the investigation of miRNAs in brain tumors is still in its infancy, there is strong evidence mounting that miRNAs are integrally involved in brain tumor development and progression. It is becoming clear that miRNAs are essential regulators of many of the key pathways implicated in tumor pathogenesis. While adding another layer of complexity, the discovery of the role miRNAs in brain tumors has also revealed a new category of therapeutic targets. As miRNA research continues to evolve, novel therapeutic targets for the treatment of brain tumors will continue to emerge.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Functional genomic analysis of glioblastoma multiforme through short interfering RNA screening: a paradigm for therapeutic development

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Glioblastoma multiforme (GBM) is a high-grade brain malignancy arising from astrocytes. Despite aggressive surgical approaches, optimized radiation therapy regimens, and the application of cytotoxic chemotherapies, the median survival of patients with GBM from time of diagnosis remains less than 15 months, having changed little in decades. Approaches that target genes and biological pathways responsible for tumorigenesis or potentiate the activity of current therapeutic modalities could improve treatment efficacy. In this regard, several genomic and proteomic strategies promise to impact significantly on the drug discovery process. High-throughput genome-wide screening with short interfering RNA (siRNA) is one strategy for systematically exploring possible therapeutically relevant targets in GBM. Statistical methods and protein-protein interaction network databases can also be applied to the screening data to explore the genes and pathways that underlie the pathological basis and development of GBM. In this study, we highlight several genome-wide siRNA screens and implement these experimental concepts in the T98G GBM cell line to uncover the genes and pathways that regulate GBM cell death and survival. These studies will ultimately influence the development of a new avenue of neurosurgical therapy by placing the drug discovery process in the context of the entire biological system. *(DOI: 10.3171/2009.10.FOCUS09210)*

Key Words • RNAi • glioblastoma multiforme • short interfering RNA • genome-wide screening • protein-protein interaction network

Glioblastoma multiforme is the most common form of primary human brain tumor,[30] and despite major advances in the management and treatment of these tumors, the prognosis remains dismal. Resistance of GBM to conventional chemotherapy and radiation therapy has necessitated a search for more effective therapies, which are beginning to encompass modern molecular biology and drug discovery techniques to identify and target the specific molecular genetic aberrations that underlie the pathogenesis of GBM.[58] However, the results of first-generation clinical trials with molecularly targeted agents have generally been disappointing, owing to tumor heterogeneity and an incomplete understanding of the interconnecting molecular pathways that promote and maintain tumor growth.

Therapeutic strategies that target genes and biological pathways responsible for the development of tumors or potentiation of current therapies could improve patient outcomes. Accordingly, several genomic and proteomic methodologies promise to expand the current drug discovery process. High-throughput screening with siRNA is one strategy for systematically exploring the possible therapeutically relevant targets in cancers, such as GBM. Short interfering RNA are 20–25 nucleotide-long double-stranded RNA molecules that can selectively silence specific genes through sequence-specific mRNA transcript degradation.[55,57] The availability of siRNA libraries and automated liquid handling platforms have spawned an evolution in genome-wide investigations of loss-of-function phenotypes.[25,30,52,75,77] However, this genomics approach has not yet been implemented in neurooncology and will require an analysis of biological pathways as a central reference point to provide a global perspective on the development, function, and pathological basis of neurosurgical disease.

In this study, we describe the drug discovery process, considerations and applications of genome-wide siRNA screening, and the integration of high-content statistical

Abbreviations used in this paper: GBM = glioblastoma multiforme; IPA = Ingenuity Pathways Analysis; KEGG = Kyoto Encyclopedia of Genes and Genomes; MAD = median of the absolute deviation; RISC = RNA-induced silencing complex; RNAi = RNA interference; siRNA = short interfering RNA; TNF = tumor necrosis factor; V-ATPase = vacuolar type H^+-ATPase.

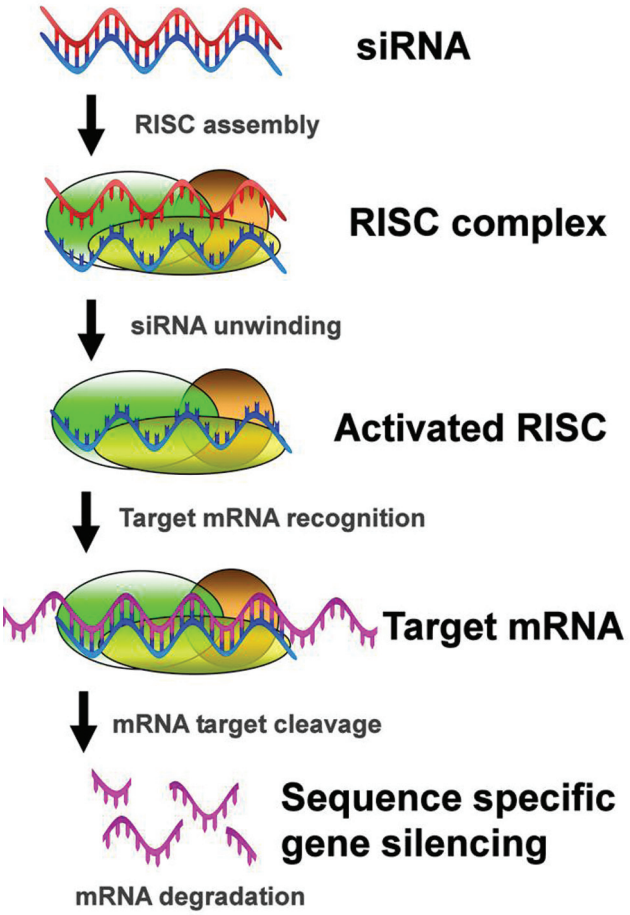

Fig. 1. The mechanism of RNAi. Long double-stranded RNA molecules are cleaved by the RNase-III–like enzyme Dicer into siRNA molecules 20–25 base pairs long with 3′ base pair overhangs. Synthesized siRNA molecules may be directly transfected into cells and do not undergo processing with Dicer. The antisense strand of this siRNA molecule is then incorporated in the RISC complex. This sequence binds to a complementary sequence on an mRNA, and an RNase within the RISC complex cleaves and destroys the mRNA by endonucleolytic cleavage, resulting in silencing of gene expression and reduction of protein levels (Kittler and Buchholz; Martinez et al.).

methods and protein-protein interaction network databases. We then highlight several high-throughput genome-wide siRNA screens in a spectrum of disease models and use these experimental concepts to implement a high-throughput siRNA screen in the T98G GBM cell line to uncover the genes and pathways that modulate GBM cell death and survival.

Drug Discovery and the Druggable Genome

The identification and validation of novel drug targets has remained difficult and costly, despite advances in molecular biology, molecularly targeted therapies, and high-throughput screening technologies. Techniques that target gene expression can facilitate the drug discovery process by replicating the potential effects of pharmacologically blocking a given protein, thereby providing insights for the drug development process. In recent years, siRNA has become a powerful tool for assessing the loss-of-function

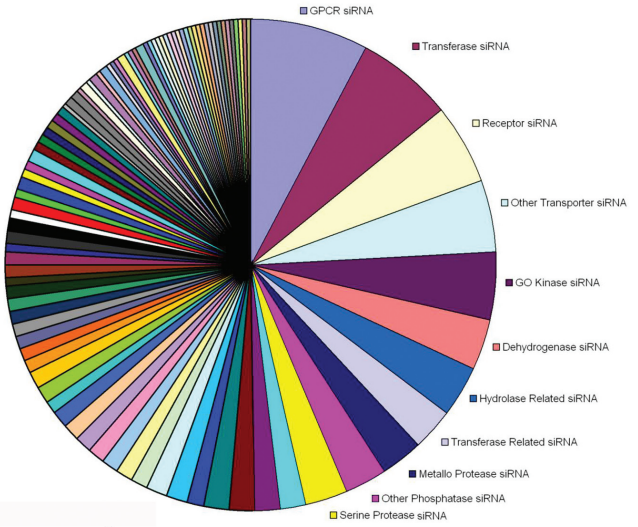

Fig. 2. Commonly targeted gene families of the druggable genome. These gene families represent the genes that are targeted by RNAi screening libraries. This pie chart is approximately based on the Ambion Silencer Druggable Genome siRNA Library version 1.0. GO = gene ontology; GPCR = G-protein coupled receptor.

phenotype associated with protein knockdown within the cell. In the RNAi mechanism, gene expression is silenced through sequence-specific mRNA transcript degradation modulated by sequence complementarity within the RNA-induced silencing complex (RISC)[4] (Fig. 1). Short interfering RNA technology has also facilitated multiple steps of the drug discovery process, which includes target identification, target validation, compound screening, lead optimization, and clinical applications.

Druggable Genome

The drug discovery process may be enhanced through an assessment of the genes and proteins that represent opportunities for therapeutic intervention.[24] Whole-genome sequencing has facilitated the functional annotation of a list of prospective drug targets, and "in silico" experimental molecular techniques have further allowed refinement of the list of molecules that can be targeted with drugs or drug-like molecules.[24,56] Analysis of this so-called "druggable genome" provides a basis for tailoring drug discovery efforts to focus on a high-yield subset of genes, and RNAi libraries have been developed to consist of molecules that specifically target these druggable proteins[48] (Fig. 2). Given the high attrition rate with conventional drug development strategies, this approach promises to rapidly assess and prioritize the most therapeutically promising targets.

Implementing siRNA Screens

Genome-wide screening has significantly contributed to our understanding of biology; these studies have examined signaling pathways, disease-associated genes, and genes involved in viability, secretion, chromosome segregation, neuron development, and neuron outgrowth.[11,18–20,26,36,38,40,42,45,60,65,67,69,70,74,75] Short interfering

TABLE 1: Summary of genome-wide RNAi screens in mammalian model systems

Authors & Year	Model	Summary
Whitehurst et al., 2007	non–small cell lung cancer	paclitaxel chemosensitivity
Giroux et al., 2006	pancreatic adenocarcinoma	spontaneous apoptosis & gemcitabine chemosensitivity
Morgan-Lappe et al., 2006	renal & pancreatic carcinoma	chemosensitivity to Akt inhibition
Tu et al., 2009	adipocytes	insulin signaling pathway constituents that modulate insulin resistance
Ganesan et al., 2008	melanocytes	genes & pathways that modulate melanogenesis
Tai et al., 2009	hepatitis C virus	cellular cofactors of hepatitis C virus replication
Gobeil et al., 2008	melanoma	identification of melanoma metastasis suppressor genes by shRNA screen
Leal et al., 2008	mouse embryonic fibroblast	downregulation of S-adenosylhomocysteine contributes to tumorigenesis
Tang et al., 2008	cervical & colorectal cancer	TCF transcription factors identified in Wnt pathway activation
Sepp et al., 2008	embryonic cerebral cortical neurons	identifying neural outgrowth genes
Loh et al., 2007	neuroblastoma	kinase cluster required for neurite outgrowth & retraction
Hu et al, 2009	mouse embryonic stem cells	transcriptional modules required for self-renewal
Turner et al., 2008	breast & cervical cancer	genes mediating sensitivity to PARP inhibition
MacKeigan et al., 2005	cervical cancer	survival kinases & phosphatases
Collins et al., 2006	ovarian carcinoma	MAP4K4 identified as a promigratory kinase
Westbrook et al., 2005	human mammary epithelial cells & colorectal cancer	identification of a previously unrecognized tumor suppressor REST
Luo et al., 2009	Ras mutant cells	identification of PLK1 & the proteasome as synthetic lethal targets in Ras mutant cells

RNA screening offers an unbiased, systematic strategy for uncovering the biological genes or pathways that underlie disease processes, allowing a deeper understanding of the poorly described molecular mechanisms governing various cellular processes or diseases.

The essential first step in the cancer drug discovery process is the identification of novel drug targets. Recent target identification has relied on characterization of genomic mutational spectra or proteomic expression signatures for correlation of target identification or tumor response to therapies.[1,50] In this context, siRNA screening represents a complementary hypothesis-generating approach by defining the consequences of blocking a given target. The events that underlie tumorigenesis, tumor progression, and tumor response to conventional therapies each represent excellent targets for selective killing of cancer cells versus normal cells that may be delineated on an siRNA-based screen, although the potential applications of these approaches in neurooncology and neurosurgery may have relevance from both a therapeutic and mechanistic perspective.[38,60] Recent siRNA screens in cancer and disease models have focused on: 1) reversing the cancer phenotype, 2) identifying synthetic lethal targets, 3) developing synergistic drug combinations, and 4) clarifying underlying mechanisms of biological processes (Table 1).

Reversing the Cancer Phenotype

The cancer phenotype is associated with several well-defined hallmarks, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, and genome instability.[22,30] Notably, invasion and metastatic spread of cancer are complex biological processes that are directly involved in the pathological process.[11] In an siRNA screen targeting 5234 human genes in an ovarian carcinoma cell line, the authors identified the potential therapeutic utility of targeting mitogen-activated protein kinase pathway in cancer progression.[11] This effect was also reproduced with a small-molecule inhibitor of c-Jun N-terminal kinase (JNK). In another genome-wide RNAi study, the authors described the role of *Growth arrest-specific 1* as a novel tumor suppressor gene that effectively suppressed melanoma metastasis.[20] Furthermore, another RNAi screen identified a novel tumor suppressor gene *REST/NRSF,* which is a transcriptional repressor of neuronal gene expression, in human mammary epithelial cells and observed its associated frequent deletion in colorectal cancer cells.[74] These studies demonstrate the ability of RNAi screening to identify novel targets that may represent cruxes of the cancer phenotype, and the identified proteins may represent nodes of chemosensitivity in various disease models.

Synthetic Lethality

Of utmost importance to clinicians is the development of novel combination therapies that can be swiftly translated into clinical application. Modern drug discovery aims to create novel drug combinations that will selectively kill cancer cells while leaving normal cells unharmed. However, this process has been difficult, owing to exploitation of normal enzyme functionality by oncogenes and the inability to pharmacologically target tumor-suppressor genes that have low or absent activity.[28] Synthetic lethality holds promise to evade some of these difficulties, and RNAi screening can be used to identify these synthetic lethal relationships using high-throughput technologies. Two genes are considered synthetic lethal if mutation of either is compatible with cell viability but

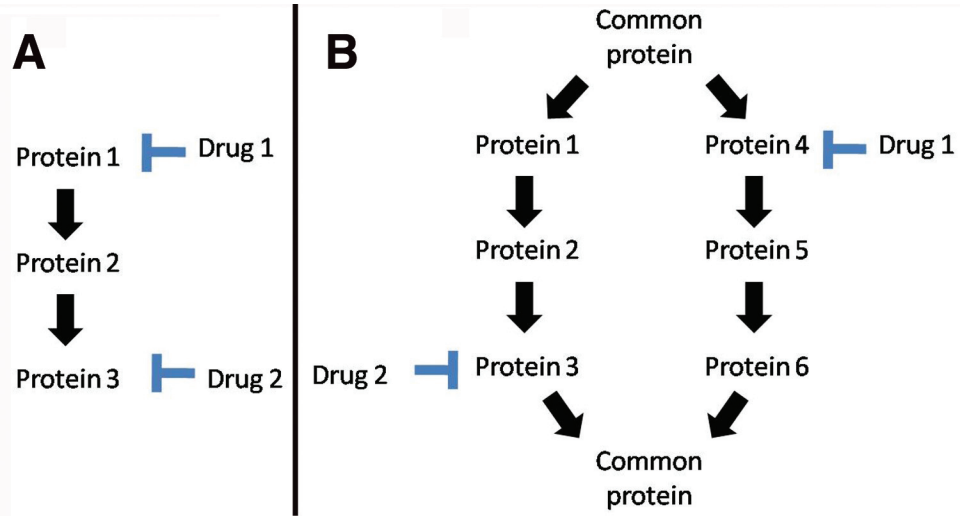

Fig. 3. Schematic diagram depicting abrogation of protein function in single, parallel, converging, and diverging pathways. **A:** Abrogation of enzymatic or protein function in a single, essential pathway can occur upstream or downstream in the pathway. **B:** Abrogation of 2 proteins simultaneously in a parallel pathway, a converging pathway, or a diverging pathway can be assessed with synthetic lethal or chemosensitizer screening assays.

mutation of both leads to cell death.[31] Once this screening tool has identified such genes, anticancer therapies can be developed to target the molecular pathways. For instance, mutation of 2 essential components on 1 linear pathway, such as Proteins 1 and 3 in Fig. 3A, or mutation of 2 components of parallel, converging, or diverging pathways, such as Proteins 3 and 4 in Fig. 3B, may be synthetically lethal.

A well-known synthetic lethal relationship has been demonstrated by the inhibition of poly (ADP-ribose)-polymerase-1 (PARP1) in breast cancer cells deficient in *BRCA1*, *BRCA2*, or other components of the homologous recombination pathway, while normal cells remain unaffected.[8,15,16,59,70] In normal cells, both the homologous recombination and base-excision repair pathways repair damaged DNA; PARP1 is an enzyme required for base-excision repair, which is a pathway that repairs single-strand breaks; *BRCA1* and *BRCA2*, which are tumor-suppressor genes, are required for DNA double-strand break repair by homologous recombination, and mutations in *BRCA1* and *BRCA2* predispose to breast and ovarian carcinomas. Loss of PARP1 increases DNA damage repair through the homologous recombination pathway; thus, abrogation of the homologous recombination pathway concomitant with base-excision repair pathway inhibition could lead to significant cell death. Indeed, recent studies have shown that *BRCA1* or *BRCA2* mutation or absence sensitizes cells to inhibition of the PARP1 enzyme,[15] and patients with hereditary breast or ovarian cancers may be excellent candidates for treatment with PARP1 inhibitors.

This synthetically lethal relationship was deduced by leveraging current understanding of cell biology and the known molecular genetic alterations within cancer. However, additional synthetically lethal combinations may not be readily deduced through a rational mechanistic understanding of cancer cell biology, and an unbiased screening method is needed to systematically detect novel relationships. In a recent genome-wide RNAi study, Turner et al.[70] identified targets that modulated the sensitivity of breast cancer cells to the effects of PARP1 inhibition. Silencing of several kinases strongly sensitized PARP1 inhibition, and these targets included cyclin-dependent kinase 5 (CDK5), MAPK12, PLK3, PNKP, STK22c, and STK36. The presence of CDK5, which is required for DNA-damage checkpoint activation, suggests that normal checkpoint function may be essential for DNA repair when the PARP1 enzyme is inhibited.[28,70] Genome-wide RNAi screens, therefore, offer a unique opportunity to implement a systematic, rapid, and unbiased method to uncover novel synthetic lethal relationships.

Synergistic Drug Combinations

Despite promising in vitro and in vivo data, intrinsic or acquired resistance to conventional therapies has been a major therapeutic obstacle. Tumor heterogeneity, redundancy and parallel processing of intracellular signaling pathways, inactivating metabolism, mutation within a specific targeted pathway, loss of negative inhibition, mutations leading to constitutive activation, and limited drug delivery are the most common resistance mechanisms.[51] Given these therapeutic challenges and the multiple mutations leading to tumorigenesis, tumor cells will need to be targeted with several agents simultaneously to ensure a cure or long-term survival. Combination therapies that target multiple signaling pathways or different constituents in the same pathway (Fig. 3) may overcome resistance mechanisms and widen the therapeutic window, ultimately enhancing the effect on tumor cells without increasing toxicity for normal cells. However, therapeutic combinations are limitless, and a strategy is necessary to select only the most effective and synergistic of combinations.

Investigators have used RNAi screens to identify targets that chemosensitize cancer cells to conventional treatments or molecularly targeted therapies[19,42,45,75] (Table

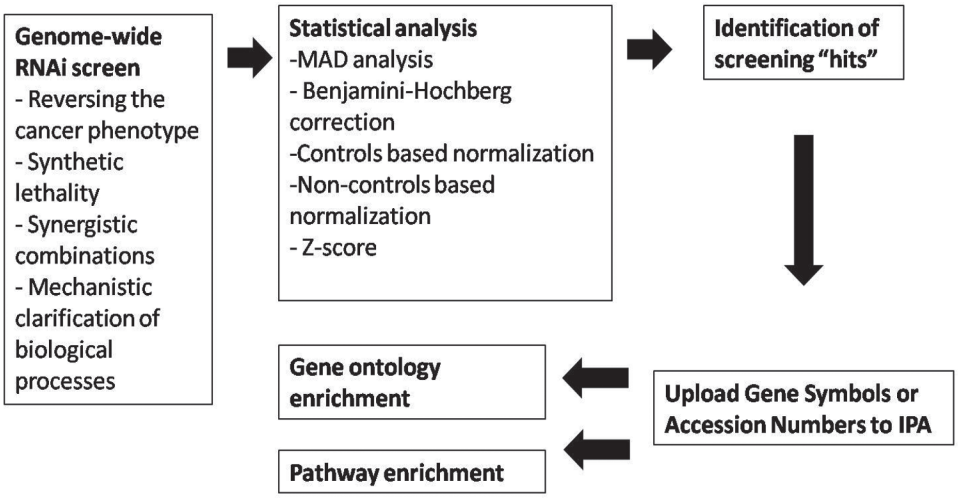

Fig. 4. Functional genomic analysis through genome-wide siRNA screening. This flow diagram depicts the chronology of events in the implementation and functional genomic analysis of a genome-wide siRNA screen. After statistical analysis with one or several methods, screening "hits" are uploaded to the web-based protein-protein interaction network IPA. Gene ontology enrichment is conducted with the Fisher exact test at $\alpha = 0.05$ by comparing the "hits" to a list of ontology categories. Pathway enrichment is similarly conducted by comparing "hits" to the KEGG list of pathways.

1). In the most well-defined of these studies, Whitehurst et al.[75] identified gene loci that chemosensitized non–small cell lung cancer cells to the microtubule stabilizer paclitaxel. As a proof of principle, the authors identified several proteasome components as chemosensitizing targets, and proteasome inhibitors have been shown to enhance paclitaxel-induced apoptosis in several cancers.[41,49] The authors also reported that exposure to the V-ATPase inhibitor salicylihalamide A combined with low concentrations of paclitaxel achieved a synergistic decrease in cell viability. Such proof-of-principle synergism studies are also being applied to neurooncology models, which will be described below.

Mechanistic Clarification of Biological Processes

Systematic phenotyping by means of RNAi and other approaches will also provide novel perspectives on a gene's or pathway's function in the context of the genome. Function and development of the brain require the coordinated action of numerous genes, but we currently understand the functions of only a small fraction of them.[60] The integration of phenotypic information from genomic and proteomic data sets has revealed many important cellular processes at an unprecedented resolution.[17] There are several such studies in neuroscience that have provided insight into the development and function of the nervous system, including neural outgrowth[60] and identification of kinase clusters that are required for neurite outgrowth and retraction[38] (Table 1).

Utilizing an RNAi screening approach, a recent study identified a pathogenic link between the endocytic pathway and neuronal dysfunction in synucleinopathies, such as Parkinson disease.[34] Other RNAi studies have focused on dissecting various cell processes, such as insulin signaling, melanogenesis, Wnt pathway signaling, and self-renewal (Table 1), and this screening strategy holds promise for the mechanistic clarification behind brain development, function, and the pathological basis of diseases, such as GBM.

Analyzing RNAi Screens

With the large amount of data generated from siRNA screening, there is a clear need for data reduction methods, which would allow prioritization of targets and determination of the gene expression products most significantly affected by siRNAs in the disease model.[9] Screening typically relies on sophisticated automation, appropriate controls, and state-of-the-art detection technologies to organize and analyze thousands of test samples.[5,7] Unfortunately, siRNA screening is associated with "off-target" effects, which can affect the analysis and final results. Data output, therefore, requires sophisticated and rigorous analysis methods to reduce the number of high-confidence "hits." Statistical analyses and the use of protein-protein interaction network databases are 2 such methods that can facilitate the data analysis process. An overview of several such data analysis methodologies is depicted in Fig. 4.

Statistical Analysis

Although a comprehensive review of statistical methods for high-throughput screening "hit" selection is beyond the scope of this review, we present the most popular methods with accompanying references for further review. As with any high-throughput methodology, the output varies due to: 1) systematic variation, or 2) unsystematic, random influences. Systematic effects that are not adjusted for can bias the final results of the screen, creating false-positive and false-negative results. The level of random "noise" can also similarly confound the results of a study and therefore also needs to be accounted for in the "hit" selection process.

Most high-throughput screens are conducted in 384-

well plate formats, and most well-designed experiments will include an in-plate positive control (a control that will yield a positive result; for example, cell death in a toxicity assay) and a negative control (a control that will yield a negative result; for example, minimal cell death in a toxicity assay). These controls are used to normalize the wells with targeting siRNAs. Although this methodology is frequently used and is the traditional way that biologists view changes in biological activity, controls-based methods have several potential problems. These include positional variability based on the location of the well on the plate; systematic biases among the controls; variability between control wells; and outliers due to measurement problems.[7,12]

With these issues in mind, some investigators are utilizing non–controls-based normalization, such as normalizing to the median of all values on a plate. The median, unlike the mean, is not affected by outliers. Because it is the outliers on a plate that may be of greatest interest (that is, those wells that had the greatest amount of cancer cell death), this method would help distinguish outliers from the majority of the screening plate. Several popular analysis methods include Z-scores,[76] viability ratios and Benjamini-Hochberg correction,[75] median of the absolute deviation (MAD) method,[10,23,29] other non–controls-based normalization methods,[7,12] and orthogonal analysis methods based on a combination of these statistical methods.[75] We have uniquely developed and applied the MAD method for the detection and removal of outlier data points from high-throughput screening data and further describe our techniques below.

Protein-Protein Interaction Network Analysis

Despite many decades of experiments and many thousands of data points, the cellular and molecular functions of the cancer genome or proteome have not yet been systematized. Because of the complexity of the cellular network, biologists have preferred to consider parts of it by subdividing it into biological pathways that comprise sets of molecules involved in a particular function or process.[2] Pathways can therefore serve as a scaffold for assessing the impact of single molecules on the network of cellular proteins. For instance, proteins that connect to numerous molecules within interaction networks are more likely to produce a cytotoxic effect when deleted (knocked down), whereas proteins that may be part of parallel or redundant biological pathways are less likely to cause lethality when deleted (Fig. 3). Several commercially available protein-protein interaction network databases[13,14,68] have focused on the pathways relevant to human disease, and these interaction analyses can be used in the target identification and validation phases of the drug discovery process.

Protein-protein interaction network analysis can uncover the underlying enriched (over-represented) gene functions and pathways that may not be readily apparent otherwise. Subsequent gene ontology and pathway enrichment analyses are then used to narrow down the list of "hits" to uncover biological functions that are most significantly affected by siRNAs[21] (Fig. 4). To allow functional enrichment according to existing functional annotation systems, the Fisher exact test is adopted to measure gene enrichment of annotation classifications such as Gene Ontology terms[21,63] or pathways from the KEGG database.[32] For each annotation term, the Fisher exact probability describes the probability of sampling without replacement from a finite population consisting of 2 types of elements, and an analogous approach is implemented for the pathway enrichment of siRNA targets. Such an analysis can now be performed using open-access interaction network analyses.[27]

Several studies have implemented this analysis on gene expression data and RNAi screening data.[6,46,69,72] For instance, Bredel et al.[6] identified 3 novel MYC-interacting genes in human gliomas through functional network analysis of gene expression data with Ingenuity Pathways Analysis (IPA); Mori et al.[46] reported that gene profiling and pathway analysis helped to elucidate the molecular mechanisms involved in neuroendocrine transdifferentiation of prostate cancer cells; and Tu et al.[69] used an siRNA screening approach to identify a reliable set of components/modulators of the insulin signaling pathway. Thus, functional genomic analyses that leverage multiple types of information have begun to show promise in uncovering important biology not apparent from standard analysis methods.

Druggable Genome-Wide siRNA Screening in GBM: Working Examples

Cancer cell survival depends on the balance of signaling through survival and apoptotic pathways.[66] An increase in survival signaling, through increased survival factors or decreased apoptotic signaling, could confer a proliferative advantage, which may ultimately enhance chemoresistance. Conversely, uncovering the genes or pathways that are most essential for cancer cell survival may enhance the drug discovery process by identifying promising drug targets. To identify the genes and pathways that represent these chemoresistance and chemosensitivity nodes in GBM, we conducted several druggable genome–wide siRNA screens to identify the gene nodes and pathways that modulate GBM cell death and survival.

Methods

We used a high-throughput siRNA screen with 16,560 siRNAs targeting 5520 unique human genes in the T98G GBM cell line. We selected the T98G cell line because it is a widely available, GBM-derived, human cell line, with a well-characterized radioresistance and chemoresistance profile,[61,62,73] and it provides an in vitro surrogate for the identification and subsequent validation of novel therapeutic drug targets for GBM. We measured cell viability at 96 hours after siRNA transfection with a resazurin fluorescent dye assay and normalized the targeting siRNA wells to in-plate positive and negative controls. Viability ratios were calculated by normalizing cell viabilities to the overall median cell viability of all 5520 genes when averaged over the screening replicates.

For the siRNA-only screen, we selected the viability ratios that were 3 SDs above the median after statistical analysis with the MAD method (see *Statistical Analysis*

TABLE 2: Protective genes in the T98G GBM cell line

Gene Symbol	Gene Accession	Full Gene Name	Viability Ratio
TRPC4AP	NM_199368	transient receptor potential cation channel, subfamily C, member 4 associated protein	1.544873
BTN3A1	NM_194441	butyrophilin, subfamily 3, member A1	1.520764
SSTR2	NM_001050	somatostatin receptor 2	1.514676
RHOV	NM_133639	ras homolog gene family, member V	1.514118
PDPR	NM_017990	PDPR	1.508784
RARRES2	NM_002889	retinoic acid receptor responder (tazarotene induced) 2	1.498987
FLJ10858	NM_018248	nei endonuclease VIII–like 3 (E. coli)	1.497508
NR2F2	NM_021005	nuclear receptor subfamily 2, group F, member 2	1.493387
TAS2R39	NM_176881	taste receptor, type 2, member 39	1.49172
NR4A2	NM_173172	nuclear receptor subfamily 4, group A, member 2	1.491645
COX4I1	NM_001861	cytochrome c oxidase subunit IV isoform 1	1.488063
TPRA40	NM_016372	G protein–coupled receptor 175	1.485445
ADAM18	NM_014237	a disintegrin and metalloproteinase domain 18	1.483975
PGAM1	NM_002629	phosphoglycerate mutase 1 (brain)	1.480993
RHOBTB3	NM_014899	Rho-related BTB domain containing 3	1.477528
GNAL	NM_182978	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	1.469045

above) and classified this set of genes by shared molecular and biological functions using the protein analysis through evolutionary relationships classification system (PANTHER).[44] We then uploaded these lists of "hits" to a web-based application for analysis of biological functions, disease categories, toxicological categories, canonical signaling pathways, drug inhibitors, and pathway and gene ontology enrichment analysis.[68] The Fisher exact test was used with $\alpha = 0.05$ to calculate the probability that each function and pathway classification assigned to the set of survival genes was due to chance. This procedure is detailed in Fig. 4.

Results and Discussion

We identified 16 targeting siRNA reactions that resulted in a significant increase in cell viability (Table 2). Knockdown of these genes appear to enhance T98G GBM cell survival, suggesting that these genes may be functionally associated with cell death pathways. Interestingly, the products of these genes function as nuclear hormone receptors, GTPases, G-protein coupled receptors, oxidases, and mutases, while several genes were unclassified. Utilizing a knowledge-based interaction network, we found that these genes have been implicated in various biological processes including neurological disease, genetic disease, cellular movement, nervous system development and function, and cell signaling. Nine of 16 genes were reportedly overexpressed in primary or secondary glioma,[54] which provided further clinical evidence of the importance of these nodes in glioma cells. The most statistically significant protein-protein interaction network consisted of genes implicated in gene expression, cell death, and endocrine system disorders and was centered around beta-estradiol, TNF, and the NF-κB complex (Fig. 5). Recent studies have described the role of the NF-κB pathway in resistance to TNF-mediated cell death in human glioma, and its role in inflammation, tumor growth, immunity, and an invasive phenotype.[53,64] Thus, our results highlight several cellular factors and complexes that may be implicated in GBM cell death pathways.

In our recent work, we implemented an siRNA screen to uncover the core genes and pathways that are essential for GBM cell survival (that is, where siRNA-induced protein knockdown induced cell death).[68] Interestingly, several identified genes were components of the proteasome complex, suggesting that these components may be essential for cell survival or proteasome structure or function or may have the most rapid protein turnover. Further mechanistic validation has shown that disruption of these components may actually induce instability of the proteasome complex by accumulating intermediate forms, which could contribute to loss of cell viability. Furthermore, using the protein-protein interaction network database, we identified clusters of cellular processes that included protein ubiquitination, purine and pyrimidine metabolism, nucleotide excision repair, and NF-κB signaling, among others. Overall, these findings regarding the significance of the proteasome complex in GBM cell survival represent an unpredicted observation that would not have been obtained without an siRNA-based screening approach.

However, because GBM is a notoriously heterogenous tumor (with respect to cells from different anatomical regions of a patient's tumor as well as between patients), identifying targets in a single cell line, such as the T98G GBM cell line, may not represent drug targets that are effective and consistent outside of this cell line. While our preliminary investigation with this single cell line is proof of principle for this screening approach, we

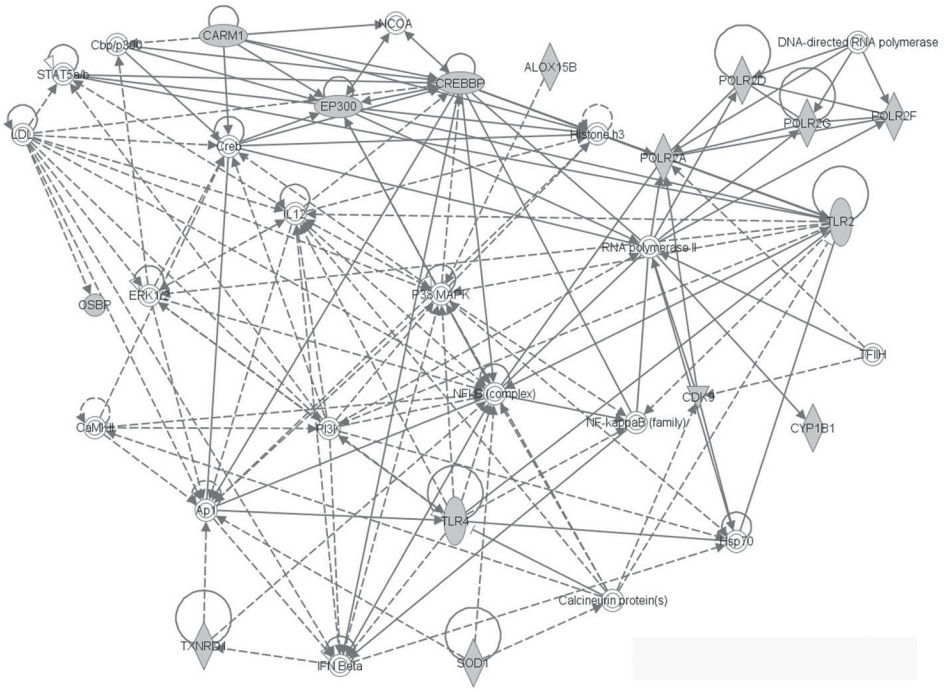

Fig. 5. Mapping of protective genes onto a protein-protein interaction network. Functional analysis of protective genes was performed with IPA. The genes are represented as *nodes*, and *edges* connecting 2 *nodes* represent a biological relationship that is supported by at least 1 published reference or the IPA knowledge base. *Shaded nodes* represent protective genes. This protein-protein interaction network consisted of genes implicated in gene expression, cell death, and endocrine system disorders and was centered around beta-estradiol, TNF, and the NF-κB complex.

additionally applied this genetic tool to a panel of glioma cell lines and nonglioma cancer cell lines to determine if this approach yielded consistent groups of genomic targets within and between cancer cell lines despite the well-characterized molecular heterogeneity. For instance, the glioma cell lines T98G, U373, U87, LN-Z308, LN-Z428, and A172; breast adenocarcinoma cell line MCF7; and lung adenocarcinoma epithelial cell line A549 were transfected with PSMB4 siRNA, and cell viability was measured at 96 hours. The A549 and A172 cell lines were most sensitive, while LN-Z308 and LN-Z428 were most resistant to cell death. Growth inhibition for all cell lines was significantly different from that in control cells ($p < 0.05$).[68] We also reproduced these cytotoxic effects using the small-molecule proteasome inhibitor MG-132.[68] Despite the heterogeneity that exists between and within tumors, screening in a single cell line can yield generalizable results, although they must be ultimately corroborated with a focused secondary screen in other cell lines or primary tumor-derived cell lines. Overall, genome-wide screening in a range of cancer cell lines will continue to provide insight into the similarities and differences in the molecular mechanisms that regulate specific cellular processes.

These studies provide examples of the power and utility of systematic and unbiased functional genomic analysis tools for the identification of novel chemotherapeutic treatment strategies for GBM, and targeting these genes and pathways may provide promising avenues for drug development.

Conclusions and Future Directions

Recent advances in genomics, such as genome-wide sequencing and the discovery of RNAi, have enabled a detailed study of the integrative nature of cellular signaling and protein-protein interactions. Genome-wide siRNA screening can be used to systematically interrogate the loss-of-function phenotypes associated with protein knock-down and can provide insight into previously unknown gene or pathway functions. Until now, this functional genomics approach has not been applied to the study of neurooncological diseases, such as GBM. In this study, we highlighted several genome-wide siRNA screens conducted in various disease models (Table 1) and then implemented a high-throughput screen to test the protective and synergistic effects of the knockdown of 5520 druggable human genes in the T98G GBM cell line. These studies have yielded a global view of the genes that are implicated in GBM cell survival, chemoresistance, and chemosensitization to various chemotherapeutic agents.

Short interfering RNA screening can be used to develop novel avenues of neurosurgical and neurooncological therapies. The aim of proteomics, which can be described as the study of the role of each gene product in its cellular context, in drug discovery is to identify potential novel drug targets and to achieve a comprehensive description of complex molecular mechanisms.[3] Once we have identified an siRNA molecule that confers a phenotype of interest (for example, when protein knockdown of gene X results in cell death), we can focus our efforts on

the development of a small-molecule inhibitor that can phenocopy the effect of the siRNA. Utilizing this new target for lead optimization, we can then streamline the development of novel monotherapies and combination therapies. Small interfering RNA screening can further impact neurosurgical treatment by identifying promising drug targets, uncovering side-effect profiles of novel and old therapies, allowing rapid assessment of promising drug targets, catalyzing swifter movement through the validation phase of the drug discovery process, identifying novel combination therapies through synthetic lethal and synergism screens, and furthering development of a systems biology understanding of the molecular mechanisms underlying neurological diseases and drug action.

Overall, these genetic studies can be directed toward the therapeutic targeting of essentially any cellular process. This screening approach is not only limited to cell viability assays, as have been described in this work, but can also be used to target various facets of tumor biology, such as tumor microenvironment, tumor invasion, factors that enhance growth in hypoxic conditions, and angiogenesis in a wide range of cell types. This screening tool can also be used to determine the effects of multimodal therapies such as chemo- and radiotherapies on cancer survival. As high-throughput screening technology improves, a more complex assay–end point can be used. For instance, instead of measuring cell viability at the end of the assay, we can measure modulation of angiogenesis using cell-culture and animal models,[39] cell invasion using migration assays,[71] and microtubule destabilization using high-content confocal microscopy.[47] Through these studies, this genetic tool will provide further insight into the mechanisms behind cellular processes and gene functions.

On a systems biology level, gene-expression signatures can now be accurately compared, essentially independent of the platform on which they were generated.[35] The future of high-throughput siRNA screening technology will include integration with DNA microarrays, protein-protein interaction data, and tools like the ConnectivityMap[35] to provide molecular clarification of novel loss-of-function phenotypes in various cell-based systems. It is also possible that high-throughput proteomic profiling could be combined with siRNA and small-molecule experiments to further inform drug development. However, current cellular networks are incomplete, since only well-studied proteins and interactions are represented (that is, typical nuclear or cytoplasmic proteins). As cancer and disease models become more sophisticated and comprehensive, it will also become important to define standards for communicating genomic profiles across diverse experimental systems. The RNAi technology is allowing the rapid development and implementation of genome-wide screens for disease processes and functions[37] and will influence the development and understanding of new avenues of neurosurgical therapy.

This genome-wide screening strategy will also have multiple impacts on neurooncological treatments within the clinical setting. For instance, functional genomic profiling of a patient's tumor will enable a more individualized and targeted therapeutic design based on the molecular genetic aberrations unique to the tumor's genome. As RNAi transfection technology in primary cells improves, this screening approach will be directly conducted in patient-derived tumor cells, which will in turn allow selection of single-agent or multiagent molecularly targeted therapies that target the biological weaknesses specific to the patient's tumor.[51] Additionally, this screening strategy will be used to identify the most promising in vitro drug combinations for future clinical trials. By implementing an unbiased, systematic interrogation of the druggable genome, this screening approach will identify novel drug combinations that would not have been identified based on current mechanistic knowledge and will thereby create a list of high-confidence drug combinations for future clinical trials. This screening strategy will also facilitate patient stratification in clinical trials based on the functional significance of specific mutations. Based on these rapidly evolving future screening applications, this screening strategy will significantly impact the clinical approach to individualized genome-based therapies.

Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: NG Thaker, PR McDonald, JS Lazo, IF Pollack. Acquisition of data: NG Thaker, F Zhang. Analysis and interpretation of data: NG Thaker, F Zhang, TY Shun, JS Lazo, IF Pollack. Drafting the article: NG Thaker, IF Pollack. Critically revising the article: NG Thaker, F Zhang, JS Lazo, IF Pollack. Reviewed final version of manuscript and approved it for submission: NG Thaker, F Zhang, JS Lazo. Statistical analysis: TY Shun. Administrative/technical/material support: JS Lazo, IF Pollack. Study supervision: JS Lazo, Ian F Pollack.

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Functional genomics to explore cancer cell vulnerabilities

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Our understanding of glioblastoma multiforme (GBM), the most common form of primary brain cancer, has been significantly advanced by recent efforts to characterize the cancer genome using unbiased high-throughput sequencing analyses. While these studies have documented hundreds of mutations, gene copy alterations, and chromosomal abnormalities, only a subset of these alterations are likely to impact tumor initiation or maintenance. Furthermore, genes that are not altered at the genomic level may play essential roles in tumor initiation and maintenance. Identification of these genes is critical for therapeutic development and investigative methodologies that afford insight into biological function. This requirement has largely been fulfilled with the emergence of RNA interference (RNAi) and high-throughput screening technology. In this article, the authors discuss the application of genome-wide, high-throughput RNAi-based genetic screening as a powerful tool for the rapid and cost-effective identification of genes essential for cancer proliferation and survival. They describe how these technologies have been used to identify genes that are themselves selectively lethal to cancer cells, or synthetically lethal with other oncogenic mutations. The article is intended to provide a platform for how RNAi libraries might contribute to uncovering glioma cell vulnerabilities and provide information that is highly complementary to the structural characterization of the glioblastoma genome. The authors emphasize that unbiased, systems-level structural and functional genetic approaches are complementary efforts that should facilitate the identification of genes involved in the pathogenesis of GBM and permit the identification of novel drug targets. *(DOI: 10.3171/2009.10.FOCUS09212)*

Key Words • functional genomics • RNA interference • short interfering RNA • glioblastoma multiforme • cancer

Glioblastoma multiforme is the most common and lethal of all primary brain tumors. It is diagnosed in approximately 10,000 new patients each year in the US. Despite its comparatively low incidence of about 2–3 new cases per 100,000 people per year (for comparison, the incidence of colon cancer in the US is approximately 1 new case per 1800 people per year), the total number of deaths per year attributable to GBM rivals that of other major cancers. Diffuse invasion into the surrounding brain is a cardinal feature of GBM, essentially preventing surgical cure and complicating the delivery of therapeutic agents.[14,15] Uniformly—if untreated—patients with GBM die within 3 months of their diagnosis. Current therapies, while prolonging overall survival, remain largely palliative, with most patients succumbing to their disease within 1 year of diagnosis. Novel targeted therapeutic approaches are desperately needed.

As with many other types of cancer, a major bottleneck in devising effective targeted therapies for GBM lies in the identification of relevant drug targets. For a given tumor, the choice of targets will depend on the particular genetic network that supports its cancerous phenotype. The identification of genes mutated in human cancer has resulted in novel, pathogenesis-oriented treatment strategies. Gain-of-function mutations in oncogenes represent promising targets for future drug development because many tumors, via a dependency termed "oncogene addiction," rely on these genetic alterations for survival.[32] Substantial efforts have been devoted to the development of cancer therapeutics targeting oncogenes. Notable successes that have resulted from this strategy include the tyrosine kinase inhibitor Gleevec (which blocks the *BCR-Abl* gene fusion product) in the treatment of leukemias, and erlotinib and gefitinib (EGFR inhibitors) in the treatment of non–small cell lung cancers harboring activating *EGFR* mutations.[8] The clinical utility of these drugs has reinforced the notion that a more thorough understanding

Abbreviations used in this paper: GBM = glioblastoma multiforme; MOI = multiplicity of infection; RISC = RNA-induced silencing complex; RNAi = RNA interference; shRNA = RNA short hairpin; siRNA = short interfering RNA.

* Drs. Kahle and Kozono contributed equally to this manuscript.

of the molecular networks governing cancer pathogenesis can be translated into substantial clinical benefits.

However, human cancers harbor hundreds of genetic alterations, and only a subset of these alterations likely drive tumor initiation and maintenance, making it difficult to identify which mutations are the critical drivers of cell transformation, and how multiple mutations within single cells interact to promote tumorigenesis. Furthermore, genes not altered at the genomic level play essential roles in tumor development, in so-called "nononcogene addiction."[29] For instance, cancer cells exhibit increased dependence on the heat shock response, although genes required for this response are not oncogenic.[35]

Given the limitations inherent within the genomic approach, a systematic functional assessment of the contribution of specific genes to cancer phenotypes is an alternative but complementary approach to structural characterizations of the cancer genome. The recent development of genome-wide human RNAi libraries has enabled systematic genetic studies in normal and human breast and colon tumor cells using arrayed and pooled screens.[3–5,12,19,24,27,33] It seems likely that systematic RNAi screens aimed at generating either cancer lethal signatures using a large panel of cancer and normal human cell lines, or synthetic lethal signatures against specific oncogenes or tumor suppressors or cells, should reveal the key glioma cell vulnerabilities that could serve as the basis for the rational design of novel therapeutics.

Mutational Analysis of the GBM Genome

A traditional approach used to discover novel drug targets is to identify genetic lesions in cancer cells and exploit this knowledge for therapeutics. Such an approach is currently used by the Cancer Genome Atlas and other similar efforts that aim to characterize—in a large number of cancer types—genomic alterations, including copy-number variation, transcriptional profiles, epigenetic modifications, and DNA sequence alterations. This approach has the potential to identify common alterations in oncogenes and tumor suppressors for further functional analysis and to uncover oncogene addiction pathways that can be targeted.[7,21] For example, Yan et al.,[36] by sequencing 20,661 protein-coding genes, determining the presence of amplifications and deletions using high-density oligonucleotide arrays, and performing gene expression analyses using next-generation sequencing technologies in 22 human GBM samples, found alterations in a variety of previously known and unknown GBM-associated genes. Most notably, novel mutations in the active site of *isocitrate dehydrogenase 1 (IDH1)*, were demonstrated in 12% of patients with GBM; most of these mutations occurred in a large fraction of young patients and in most patients with secondary GBMs and were associated with an increase in overall survival.

In the Cancer Genome Atlas study, besides recapitulating the mutational events in GBM that have been known for decades on a large scale, the authors found that some genetic lesions—such as *TP53* mutation, *NF1* deletion or mutation, and *ERBB2* amplification—were more common than previously reported.[7] Together, these systems-level studies identified the major genetic events in human GBMs,[7,21] including: 1) dysregulation of growth factor signaling via amplification and mutational activation of receptor tyrosine kinase (RTK) genes; 2) activation of the phosphatidylinositol-3-OH kinase (PI(3)K) pathway; and 3) inactivation of the p53 and retinoblastoma (Rb) tumor suppressor pathways. Recent genome-wide profiling studies have also demonstrated the remarkable genomic heterogeneity among GBMs, along with the existence of different molecular subclasses of GBMs that may allow for treatment stratification.[13,22] However, with rare exceptions, such as *IDH1*, few of the discoveries from these structural studies are anticipated to generate immediately actionable drug targets; for common but currently undruggable oncogenes such as *RAS*, and tumor suppressors, such as *PTEN* and *TP53*, the Cancer Genome Atlas will likely provide little translational insight.

Limitations of Structural Analyses of Cancer Genomes for Drug Discovery

Although informative, structural genetic approaches have limitations with respect to target discovery. First, current sequencing capacity is largely limited to coding regions, though whole-genome sequencing will become feasible with the development of cheaper and more highly parallel sequencing methods. Second, targeting oncogene addiction alone is unlikely to be a solution because: 1) many known oncogenes are challenging therapeutic targets (for example, efforts to develop drugs that inhibit oncogenic *RAS* proteins have been largely unsuccessful, despite the fact that *RAS* is mutated in nearly 30% of human tumors and is essential for their viability); 2) although recent sequencing/mapping efforts have revealed hundreds of potential oncogenic mutations and gene copy alternations, without functional characterization it is unknown which of these mutations make good drug targets; and 3) because tumors rapidly develop resistance to targeted therapy with single agents, multiple agents with different mechanisms of action are necessary to suppress drug resistance.

In addition to oncogene addiction, the stress phenotype of cancer cells makes them more dependent on the function of certain essential genes that are not themselves oncogenes or otherwise mutated; however, many if not all of these proteins can be rate-limiting to their pathways. Elledge and colleagues coined this phenomenon of cancer cells the "non-oncogene addiction."[29] The cancer cells' increased dependence on the heat shock response and their metabolic adaptation for aerobic glycolysis are 2 examples of non-oncogene addiction that have received therapeutic attention.[6,35] Because genes involved in non-oncogene addiction do not themselves harbor genetic alterations, they will be missed by DNA sequencing efforts alone. Therefore, the identification of non-oncogene addiction genes requires functional approaches.[29]

Functional Genomics: An Alternative Method for Exploring Cancer Cell Vulnerabilities

A second approach takes that of functional systems

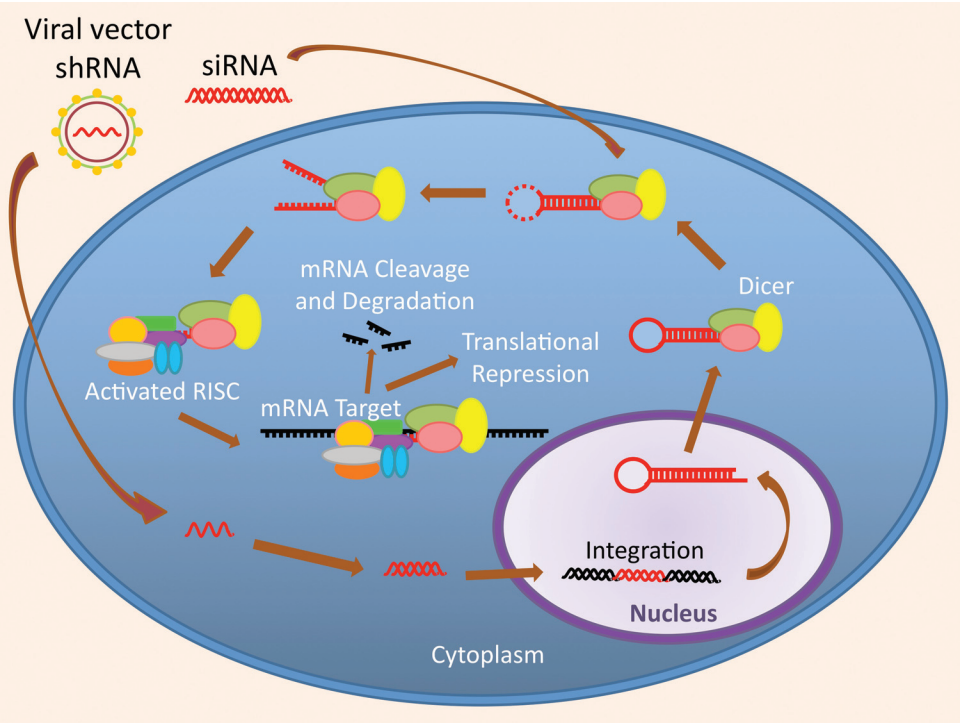

Fig. 1. The RNA interference pathway. Gene knockdown can be readily achieved in mammalian cells by transient transfection with chemically synthesized siRNAs or stable transfection with plasmids or viruses expressing shRNAs. In the latter case, the shRNA sequences integrate into the host genome and are expressed via Pol II– or Pol III–mediated transcription. The shRNAs are then processed by Dicer into double-stranded siRNA. Via either mechanism, the resulting siRNA becomes incorporated into a RISC, which directs target mRNA degradation or translational suppression in a sequence-specific manner, thus resulting in decreased protein expression.

biology to identify cancer cell vulnerabilities.[19,24,27] Advances in RNAi technology in human cancer cell lines have made it possible to systematically interrogate genes for potential roles in tumorigenesis. By silencing or decreasing the expression of specific gene products in cancer-specific assays, these loss-of-function screens can provide insight into their biological function.

A functional genetic approach using genome-wide RNAi offers several advantages in target discovery over structural genomics: 1) RNAi-mediated protein knockdown results in either partial or complete loss of function, oftentimes mimicking the effect of a potential pharmacological inhibitor; 2) RNAi can be applied in an unbiased manner to any annotated gene in the human genome based on sequence information alone without a priori knowledge of the gene's mutational status or biological function, thereby helping uncover novel cancer genes; 3) RNAi can be readily applied in mammalian model systems (including the many different mouse models of cancer), thus providing an opportunity for rapid target validation in vivo without first developing a drug that inhibits the gene product of interest; 4) RNAi screening has the ability to identify genes not mutated in cancers and exhibit features of non-oncogene addiction.

It is possible to induce RNAi-mediated gene knockdown transiently by the introduction of chemically synthesized small interfering RNAs (siRNAs), or stably by plasmids or viruses expressing RNA short hairpins (shRNAs), which are subsequently processed into siRNAs by the protein Dicer. Via either mechanism, the resulting siRNA directs degradation or translational suppression in a sequence-specific manner of the mRNA of the gene of interest by becoming incorporated into the RNA-induced silencing complex (RISC), resulting in decreased protein expression (Fig. 1). While chemically synthesized oligonucleotide siRNAs are effective in inducing gene suppression, such reagents are expensive and can only be used for transient loss-of-function experiments. This is limiting for many assays specific for cancer-related phenotypes, such as anchorage-independent colony formation or tumor xenografts, where long-term gene suppression is required. Vector-based RNAi systems have circumvented this problem by providing stable expression of the RNAi in mammalian cells through incorporation of the construct into the DNA of the host cell's genome. These vector-based systems also have the added benefit of being a renewable resource because of their propagation in *Escherichia coli*. Moreover, vector-based systems can be used to create retroviruses that harbor the RNAi construct, thereby expanding the type of cells into which such constructs can be introduced.[20,23,26] Both siRNA and shRNA libraries have been used successfully in transfection-based arrayed screens investigating phenotypes that develop shortly after gene suppression, such as apoptosis, cell signaling events, or cell cycle distribution.[1,11,34]

The use of RNAi provides a straightforward, func-

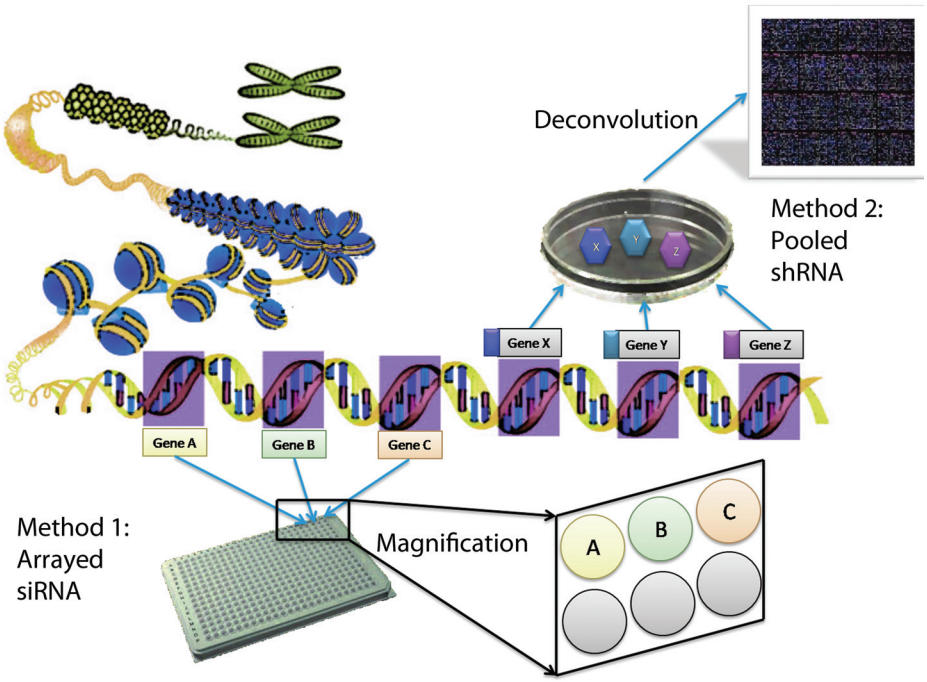

Fig. 2. Whole genome RNAi screening. The effect of gene knockdown of each of the approximately 25,000 genes of the human genome can be achieved using either of 2 general methods. In Method 1, siRNAs are arrayed into 96- or 384-well microtiter plates such that each well contains 1 siRNA directed against a single gene. After a few days, each well can be scored for readouts, such as cell viability, altered morphology, fluorescently tagged proteins, and so forth. In Method 2, thousands of different shRNA constructs that are each tagged with a unique barcode are used to transfect or infect pools of cells. Because these sequences integrate stably into the host genome, the effects of gene knockdown can be deconvoluted by barcode microarrays after several cell doublings.

tional approach to identify new cancer drug targets by identifying genes whose knockdown is lethal to cancer cells but not normal cells. To gain statistical power, one must screen multiple cancer and normal cell lines. Because of the sheer scale of screening that is required, the standard siRNA transfection or shRNA transduction screen in a well-by-well format using multiwell plates is prohibitively expensive and time consuming. Currently, several laboratories are equipped to execute such high-throughput approaches to manipulate gene expression in mammalian cell lines. However, novel technologies are developing that will make it cheaper and easier to execute such large-scale screens. Barcoded shRNA libraries, together with pool-based screening platforms, circumvent these barriers to large-scale screening (Fig. 2). Elledge and colleagues have pioneered the construction of shRNA libraries that target the human genome and have optimized delivery vectors that allow potent shRNA expression and efficient gene knockdown; these efforts have resulted in a barcoded, microRNA-based shRNA library targeting the entire human genome that can be expressed efficiently from either retroviral or lentiviral vectors in a variety of cell types for stable gene knockdown.[20,26,27] The current generation shRNA library consists of 87,283 shRNAs targeting 32,216 human genes, with an average of 3 distinct shRNA sequences per gene.[18]

There are 3 unique advantages of the Elledge-Hannon shRNA library: 1) The shRNA is embedded in the backbone of a naturally occurring microRNA, mir30, enabling efficient expression and processing of the shRNA for incorporation into the RISC (Fig. 3); 2) the shRNA of interest is cloned into a modified retroviral vector permitting sufficient expression so that single proviral integration is sufficient for gene knockdown—an essential characteristic for a pool-based, ultra high–throughput screening platform; and 3) each shRNA is tagged with a unique 60-nucleotide barcode sequence that can be used to track its abundance in a pool by microarray hybridization (Fig. 3).[20,26,27] Elledge and colleagues have also developed an ultra high–throughput platform (see below) to

Fig. 3. Endogenous microRNA-based shRNA retroviral vector. This construct yields the advantage of sufficient gene knockdown following single-copy integration, which is essential for successful pooled screens. LTR = long terminal repeat; ψ = psi retroviral packaging element; miR30 = human endogenous microRNA 30; PGK = phosphoglycerate kinase promoter; Puro = puromycin resistance gene.

screen complex pools of shRNAs using barcodes coupled with microarray deconvolution. Barcodes are critical for pool-based dropout screens that are designed to identify cell-lethal or drug-sensitive shRNAs[19] and provide independent means to track the abundance of shRNAs in a complex pool. Hairpins depleted over time can be identified through the competitive hybridization of barcodes derived from the shRNA population before and after selection to microarrays.[21,27,28] This technology can handle more than 10,000 shRNAs per pool and involves low costs.[27,34]

Cancer Lethal Signatures

The central goal for developing pool-based shRNA screening platforms is to attain the means to rapidly perform dropout screens for the systematic identification of genes required for cancer cell proliferation and survival that could represent new drug targets. A proof-of-principle screen using the Elledge-Hannon shRNA library in human colon and breast cancer cells has recently been published.[24]

In their study, Schlabach et al.[24] used a highly complex pool of 8203 distinct shRNAs targeting 2924 genes consisting of annotated kinases, phosphatases, ubiquitination pathway and cancer-related genes—all central regulators of signaling pathways that should provide a rich source of phenotypic perturbation—to interrogate multiple human colon cancer and breast cancer cell lines, and normal human epithelial cell lines. Two types of cancers with distinct origins were compared with identified common and cancer-specific growth regulatory pathways. Cells were infected with an average representation of 1000 per shRNA and an MOI of 1–2. Initial reference samples were collected 2–3 days postinfection; the remaining cells were puromycin-selected, propagated for several weeks, and collected again as the end samples. Polymerase chain reaction was used to recover the half-hairpin (HH) barcodes from genomic DNA, labeled with Cy5 and Cy3 dyes, respectively, and hybridized to an HH barcode microarray. For each probe, the Cy3/Cy5 signal ratio reports the change in relative abundance of a particular shRNA between the beginning and the end of the experiment. An shRNA that causes growth inhibition or cell death will drop out from the pool over time and show a $\log_2$ Cy5/Cy3 ratio of < 0. Conversely, a shRNA that enhances cell proliferation will enrich in the pool over time and show a $\log_2$ Cy5/Cy3 ratio of > 0. Lastly, an shRNA that has little effect on cell growth will show a $\log_2$ Cy5/Cy3 ratio of ~ 0. Whereas the majority of shRNAs show little changes in their abundance over time ($\log_2$ ratio between –1 and 1), a small fraction of shRNAs dropped out over time ($\log_2$ ratio < -1), reflecting differences between cancer and normal cells. Interestingly, the 2 colon cancer cell lines were more similar to each other than to the breast cancer line, likely reflecting tissue-specific differences and mechanisms of tumorigenesis.

As expected, the screen recovered multiple components of core cellular pathways essential for survival; for example, shRNAs against multiple subunits of the anaphase promoting complex (APC/C) and the eukaryotic translation initiation factor 3 (eIF3) complex were lethal in all cell types. In addition to the common set of shRNAs that impair viability in all cell lines, a number of genes selectively required for proliferation of individual cell lines were identified, including *PPP1R12A*—a regulatory subunit of *protein phosphatase 1*; *PPP1R12A* was selectively required for breast cancer cell viability, but not colon cancer cell viability. Conversely, colon cancer cell lines were more sensitive to knockdown of *PRPS2*, a gene involved in nucleoside metabolism. Importantly, comparison of the lethal signatures between breast cancer cell lines and nontransformed human mammary epithelial cell lines identified a number of genes—including the cell cycle regulator kinase gene *BUB1*—whose knockdown caused greater toxicity to breast cancer cells than normal mammary epithelial cells, suggesting *BUB1* could be further explored as a potential drug target for the treatment of breast cancer.

This study proved the feasibility of ultra high–throughput dropout screens with highly complex pools of shRNAs with barcode microarray deconvolution to identify lethal shRNAs for multiple cancerous and normal epithelial cell lines. These studies resulted in the discovery of genes that are essential for general cell viability and are selectively required for cancer cell viability. Others have described the development and application of genome-scale high-throughput methods using lentiviral RNAi libraries to systematically assess cancer gene function and to integrate structural and functional approaches in the study of cancer.[17]

Synthetic Lethal Signatures

"Synthetic lethality" occurs when alteration of a gene results in cell death only in the presence of another genetic mutation. Cancer cells, due to oncogenic mutations, develop secondary dependencies on genes that are themselves not oncogenes. This fact is exploited in synthetic lethal genetic screens, where perturbation of genes within the same pathway, a parallel pathway, a distant pathway, or in genes with other essential cellular functions, result in oncogene-specific synthetic lethal interactions, thereby defining novel therapeutic targets.[9,10] Synthetic lethal interactions were first described in model organisms like yeast and worms,[2,16] but recent studies have demonstrated the concept of synthetic lethality is relevant for mammalian cells.[28,30] The use of RNAi is also relevant for identifying genetic interdependencies in human cancer; functional genetic screens have identified genes whose knockdown sensitizes cancer cell lines[31,34] or untransformed cells with ectopic oncogene expression to the effects of specific environmental conditions, like a drug.

Recently, in synthetic lethality screens using shRNAs in human cancer cells expressing mutant K-RAS, Gilliland and colleagues and Elledge and colleagues identified 2 different kinases—STK33 (serine/threonine kinase 33) and PLK1 (polo-like kinase 1)—respectively, as potential novel drug targets for RAS-driven cancers.[18,25] In a panel of 8 human cancer cell lines (4 *K-RAS* wild-type and 4 *K-RAS* mutant), using a well-by-well approach in which the biological effects of each hairpin are scored individually, Gilliland and colleagues screened 5000 shRNAs targeting 1000 genes. Annotation of the hits that scored

specifically in the 4 *K-RAS* mutant lines yielded a small list of genes, which included *STK33* at the top of the list. This gene was validated in separate in vitro assays, and in vivo in tumor xenograft models. This large-scale, well-by-well screening approach requires an expensive high-throughput platform that, while powerful, would likely be difficult for most other academic labs to recapitulate. Scaling such technology for genome-wide screens would also be challenging.

Elledge and colleagues use a pooled screening approach with an isogenic cell line pair. In their screen, a library of 75,000 shRNAs targeting 30,000 mRNA transcripts from 12,000 genes was introduced into a *K-RAS* mutant cancer cell line as well as an isogenic wild-type *K-RAS* control.[18] As detected by barcode arrays, shRNAs targeting 400 genes were selectively depleted in *K-RAS* mutant cells after multiple population doublings. These genes were deemed candidate *RAS* synthetic lethal genes. A subset of these were validated in a repeat screen in another *K-RAS* mutant isogenic cell line pair, resulting in a list of 77 validated *RAS* synthetic lethal genes. Due to the larger scale of the screening approach, no single gene was classified as a top hit; however, computational analysis revealed increased dependence of *K-RAS* mutant cells on multiple genes involved in mitotic machinery (including the kinase gene *PLK1*) and the proteasome. Consistent with these results, *K-RAS* mutant cells were preferentially killed by drugs like paclitaxel (which target mitotic spindle function), a preclinical PLK1 inhibitor, and bortezomib (a proteasome inhibitor). These data also bolstered the notion that cancer cells must adapt to avoid stress-induced death associated with their actively proliferating state. Standard chemical approaches should be able to identify inhibitors of these kinases. Ultimately, validation of the synthetic lethal screening strategies outlined will be achieved if patients with *K-RAS* mutant tumors benefit from treatment with STK33 or PLK1 inhibitors.

Future Directions

Within a short time of its discovery, RNAi has emerged as the preferred means for studying gene function. Marriage of RNAi to high-throughput screening technologies has revolutionized biological and biomedical investigations. Applications of genome-wide RNAi screens to GBM will undoubtedly unveil genes critical for tumor initiation and vulnerabilities inherent within the cancer cells. The hybridization of this information with the genomic landscape as characterized by the sequencing efforts will yield novel insights into glioblastoma pathogenesis and shape strategies for therapeutic development.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Genomics of medulloblastoma: from Giemsa-banding to next-generation sequencing in 20 years

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Advances in the field of genomics have recently enabled the unprecedented characterization of the cancer genome, providing novel insight into the molecular mechanisms underlying malignancies in humans. The application of high-resolution microarray platforms to the study of medulloblastoma has revealed new oncogenes and tumor suppressors and has implicated changes in DNA copy number, gene expression, and methylation state in its etiology. Additionally, the integration of medulloblastoma genomics with patient clinical data has confirmed molecular markers of prognostic significance and highlighted the potential utility of molecular disease stratification. The advent of next-generation sequencing technologies promises to greatly transform our understanding of medulloblastoma pathogenesis in the next few years, permitting comprehensive analyses of all aspects of the genome and increasing the likelihood that genomic medicine will become part of the routine diagnosis and treatment of medulloblastoma. *(DOI: 10.3171/2009.10.FOCUS09218)*

Key Words • medulloblastoma • genomics • microarrays • next-generation sequencing

Genomics involves the study of genes and their function, typically in the context of an organism, a tissue, or a particular cell type. Cancer is a genomic disease that accounted for an estimated ~ 640,000 deaths in the US and Canada in 2008.[73,97] The goal of cancer genomics is to develop a comprehensive inventory of the full spectrum of mutations, whether inherited or acquired, that contribute to tumorigenesis. Ultimately, through a better understanding of the cancer genome, targeted treatment options can be developed and implemented so that deaths due to cancer can be reduced in the future.

The human genome consists of ~ 3 billion base pairs of DNA and encodes an estimated ~ 24–25,000 unique protein-coding genes.[71,133] During tumorigenesis, a variety of somatic mutations arise at the level of the genome, collectively providing a selective growth advantage to cells harboring these mutations and promoting the onset of cancer. Some examples of the somatic mutations in the cancer genome include single base pair substitutions, insertions and deletions of DNA segments, structural rearrangements such as duplications, inversions, and translocations, as well as gene amplifications and deletions.[149] Estimates from recent genome-wide sequencing efforts have suggested that a given cancer may contain anywhere from 40 to over 100 somatic mutations.[78,125,173] Since these numbers do not directly account for genes affected by structural changes and copy number aberrations, the actual number of genes targeted for mutation in a given tumor is probably even higher. Beyond the genome, deregulation of the epigenome, including hypermethylation of gene promoters and changes to the histone code, also contributes to cellular transformation.[23,75,76,156] Collectively, these abnormal genomic and epigenomic states in a cancer cell aberrantly impact gene expression, leading to the disruption of normal cellular processes, including cell division. Comprehensive cancer genomics, therefore, include studies at the level of the genome, epigenome, and transcriptome.

Medulloblastoma is an embryonal tumor of the cerebellum and the most common malignant brain tumor

Abbreviations used in this paper: aCGH = array comparative genomic hybridization; CGNP = cerebellar granule neuron precursor; FISH = fluorescence in situ hybridization; G-banding = Giemsa banding; MAPK = mitogen-activated protein kinase; miRNA = microRNA; NGS = next-generation sequencing; PDGFR = platelet-derived growth factor receptor; RT-PCR = reverse transcriptase–polymerase chain reaction; SAGE = serial analysis of gene expression; SHH = sonic hedgehog; siRNA = short interfering RNA; SKY = spectral karyotyping; SNP = single-nucleotide polymorphism; sPNET = supratentorial primitive neuroectodermal tumor; TCGA = The Cancer Genome Atlas; TMA = tissue microarray; 5-aza = 5-aza-2′-deoxycytidine.

in childhood.[53,101] Although 5-year overall survival rates have reached 60–80%, survivors often face a variety of long-term neurological, neuroendocrine, and social sequelae as a result of conventional treatment regimens (surgery, radiotherapy, and chemotherapy).[40,46] It is therefore imperative for us to gain a better understanding of the genes driving medulloblastoma oncogenesis so that future targeted therapies that are more effective and less toxic can be made available.

Much of our current understanding of the molecular basis of medulloblastoma has been derived from insights into hereditary tumor syndromes[151] and candidate gene approaches focused on developmental signaling pathways.[60,96,170] For instance, individuals with Gorlin or Turcot syndrome possess germline mutations in the *PTCH1* and *APC* tumor suppressor genes, respectively, and are predisposed to medulloblastoma, among other cancers.[6,51,62,63] Studies of the *PTCH1* gene in Gorlin syndrome and sporadic medulloblastomas, as well as knockout studies of its mouse homolog, *Ptc*, have helped to establish a role for aberrant SHH signaling in ~ 25–35% of medulloblastomas.[55,96] Similarly, the identification of *APC* mutations in Turcot syndrome and more frequent mutations of *CTNNB1* in sporadic cases have implicated the Wnt signaling cascade in ~ 10–15% of patients with medulloblastomas.[55,96] Furthermore, patients with Li-Fraumeni syndrome have germline *TP53* mutations and can have a broad spectrum of cancer types, including medulloblastoma.[95,148]

Aside from what has been learned from the study of these familial tumor syndromes, the majority of additional oncogenes and tumor suppressor genes implicated in medulloblastoma have been discovered from a priori candidate gene selection. Mutational screening has further implicated additional SHH (*SUFU* and *SMO*) and Wnt *(AXIN1)* pathway genes.[55,96] In addition, the Notch pathway is deregulated in a subset of human medulloblastomas and activated in certain mouse models.[55,96] Furthermore, candidate epigenetic approaches have revealed hypermethylation of the promoter regions of known tumor suppressor genes: *HIC1*, *RASSF1A*, *CASP8,* and others.[90] The relevance of several of these genes has been further validated in mouse models of medulloblastoma.[50,129,134] Although these single-gene and/or candidate-gene studies have shed significant light on our understanding of medulloblastoma pathogenesis, the candidates identified to date very likely represent only a small piece of the genomic puzzle responsible for the onset and progression of this pediatric tumor. Indeed, recent data from whole-genome sequencing projects of multiple tumor types implicate as many as 100 mutated genes per genome.[78,125,173] If such estimates prove applicable to the medulloblastoma genome, many candidates have yet to be identified.

The goal of this review was to detail the progress that has been made in our understanding of the medulloblastoma genome over the last 20+ years, with specific emphasis on global, unbiased genomic profiling. Although what has been learned from gene- and pathway-specific studies of medulloblastoma has been indispensable to our knowledge of this disease, these findings will not be discussed in detail here but have been reviewed elsewhere.[55,96] The summary presented here describes how the technologies available to study the medulloblastoma genome have evolved over the last 2 decades (Fig. 1) and shows how much of the progress in this field has been dictated by both the size of the sample cohorts analyzed and the resolution of the technologies used in their study. A glimpse into what is to come in the near future of medulloblastoma genomics will also be discussed.

Early Cytogenetics and Karyotyping of Medulloblastoma

It has been over 20 years since G-banding was first used to disclose chromosomal abnormalities in medulloblastoma. It is a classic staining technique used to visualize a cell's karyotype, producing an alternating pattern of dark (heterochromatin) and light (euchromatin) bands along metaphase chromosomes.[11,147] Early studies conducted at Duke University Medical Center and The Children's Hospital of Philadelphia provided original and informative descriptions of the medulloblastoma karyotype.[17,19,59] Of significant interest, both of these groups reported an isochromosome of the long arm of chromosome 17 [i(17q)] as the most frequent structural abnormality and, in at least a few cases, the only aberration observed. Isochromosome 17q is the most common isochromosome in human cancer,[105] generating a net loss of 1 copy of the majority of 17p and a net gain of 1 copy of 17q (Fig. 2A). Frequent loss of heterozygosity on chromosome 17p in medulloblastoma was independently confirmed by multiple groups in the early 1990s, typically through deletion mapping by restriction fragment length polymorphism (RFLP) analysis.[29,30,72,135,153] At present, cytogenetic aberrations affecting chromosome 17 remain the most common structural changes noted in medulloblastoma (Table 1);[119,126,138] however, insight into the individual gene or combination of genes on this chromosome that drive tumorigenesis has not significantly improved since these early findings.

The establishment and cytogenetic characterization of permanent medulloblastoma cell lines and xenografts in the late 80s and early 90s also provided initial insight into the prevalence of oncogene amplification in medulloblastoma. Amplification of the *MYC* locus on 8q24, often in the form of double minutes, was reported in multiple cell lines and confirmed in primary tumors by several groups.[8,18,48,130,157] The *MYC* family of protooncogenes (*MYC*, *MYCN*, and *MYCL1*) remain among the most prevalent targets of gene amplification in medulloblastoma (Fig. 2B).[119,126]

The application of CGH to the cytogenetic characterization of medulloblastoma in the late 90s resulted in a much greater appreciation of the degree of genomic imbalance present in this cancer. Using CGH to profile a panel of 27 primary medulloblastomas, Reardon and colleagues[136] described frequent losses on chromosomes 10q, 11, 16q, 17p, and 8p as well as recurrent gains on chromosomes 7 and 17q. Several complementary follow-up studies based on a combination of G-banding, CGH, SKY, and FISH confirmed these now well-recognized regions of genomic instability in medulloblastoma and

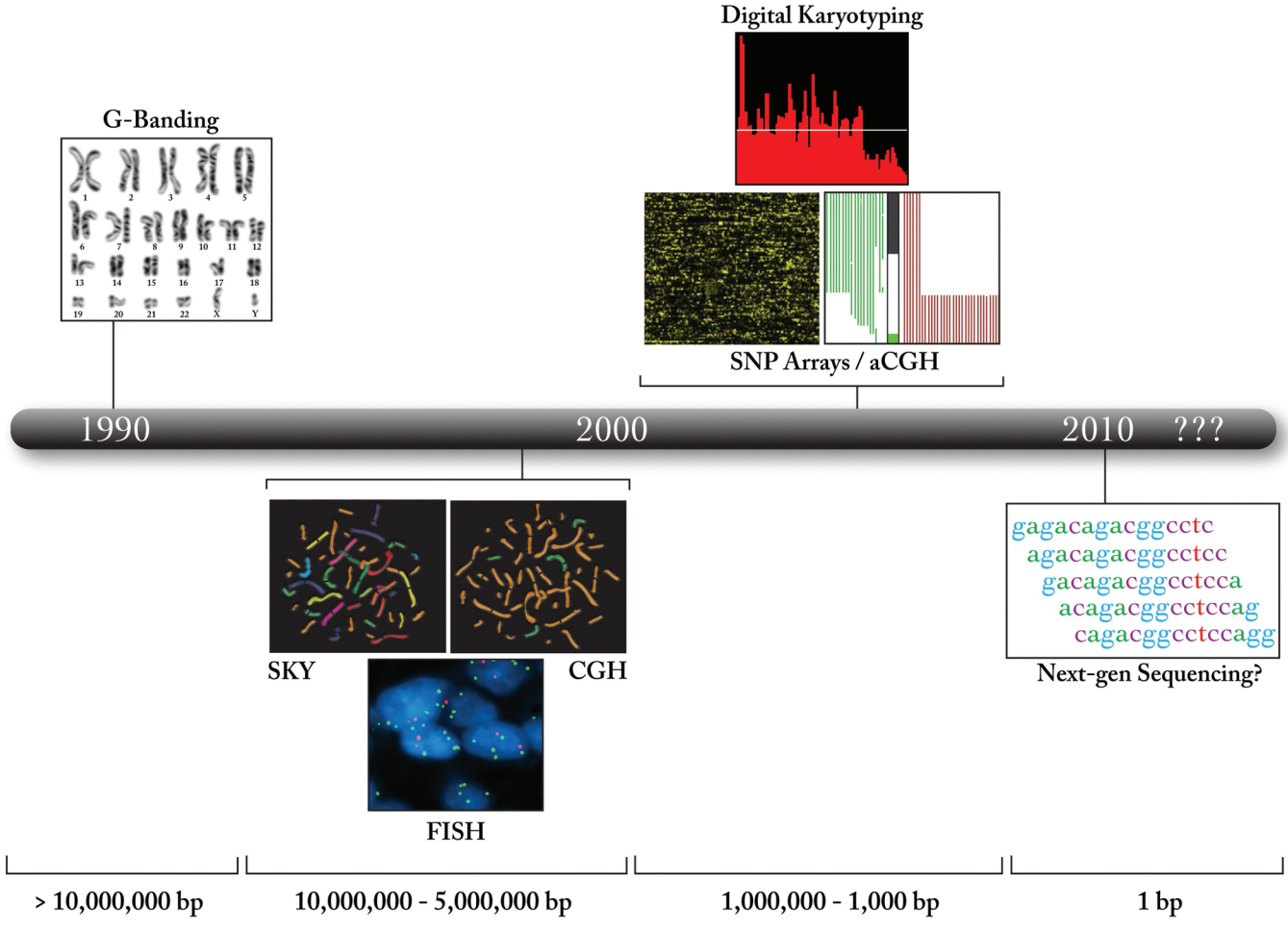

Fig. 1. Timeline showing the evolution of genomic technologies over the last 2 decades and their application to the study of medulloblastoma. Dates reflect the application of referenced technologies to studies of the medulloblastoma genome. Listed below each time period is the approximate resolution (bp) of the techniques shown.

shed light on additional candidate regions.[2,7,12,20,32,38,56,117] An innovative study involving members of our group retrospectively documented a series of 19 primary medulloblastomas (in addition to 5 sPNETs) using classic G-banding, FISH, CGH, and SKY.[12] Spectral karyotyping is a multicolored FISH procedure that permits the identification of structural rearrangements and origins of marker chromosomes in the genome in a single experiment.[13] This "chromosome painting" technique is particularly useful for detecting structural aberrations lacking a net change in copy number, such as balanced translocations. The use of SKY in this study enabled the comprehensive identification of recurrent structural rearrangements in medulloblastoma, including those on chromosomes 7, 17, 3, 14, 10, and 22—something not possible through the use of G-banding or CGH alone.

Although these cumulative efforts provided the pediatric brain tumor community with relatively detailed summaries of the medulloblastoma karyotype, not until the advent of new technologies capable of detecting copy number changes at a much higher resolution could novel candidate oncogenes and tumor suppressors in medulloblastoma be more efficiently identified through the use of genomics. Over the past 5 years, novel, high-resolution (that is, submegabase) genomic technologies have become available.[34,112,147] The applications of some of these technologies in studies of the medulloblastoma genome are discussed in detail below.

High-Resolution Analysis of Medulloblastoma: Digital Karyotyping, aCGH, and SNP Genotyping Arrays

High-resolution genomic profiling of medulloblastoma has recently implicated multiple candidate oncogenes that are recurrently amplified in this malignancy (Table 2). In 2005, 2 very similar but independent studies led by Greg Riggins[21] and Hai Yan[35] used digital karyotyping to identify novel regions of copy number aberrations in the medulloblastoma genome. Digital karyotyping uses short sequence tags derived from specific genomic loci to provide a quantitative and relatively high-resolution profile

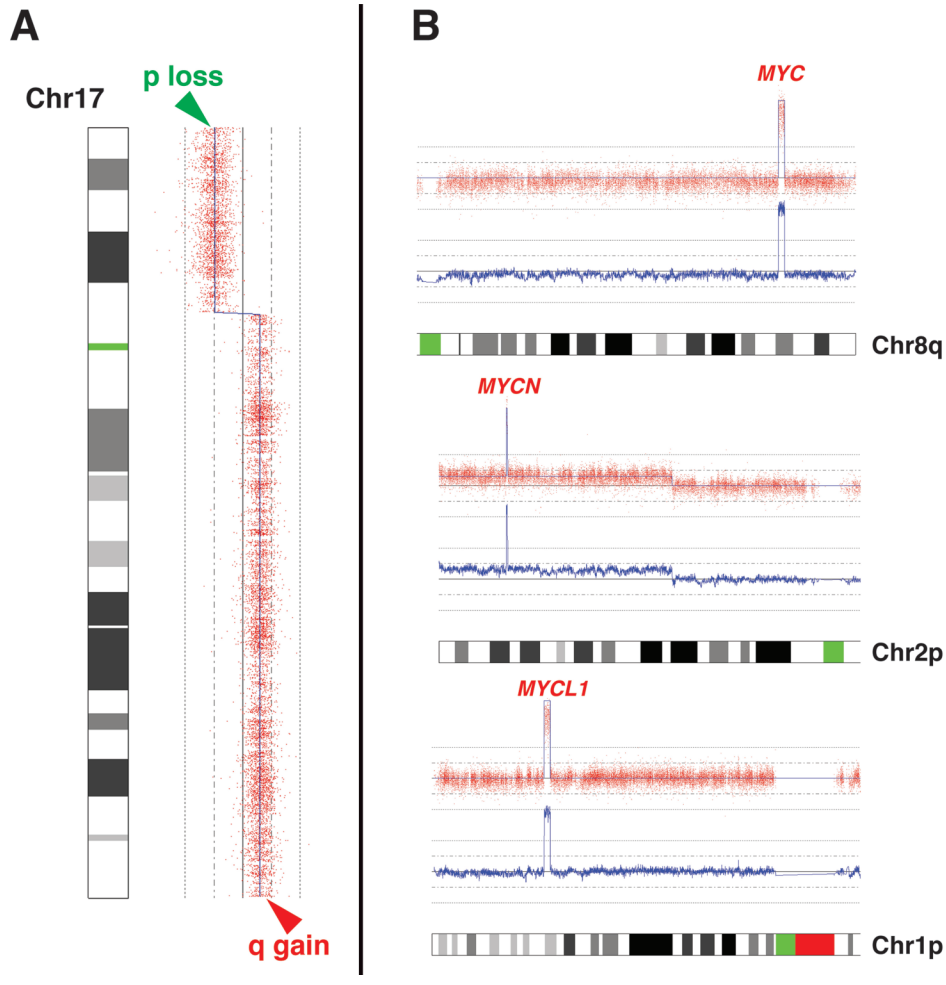

Fig. 2. Prominent genomic aberrations in medulloblastoma. **A:** Isochromosome 17q in medulloblastoma. Single-nucleotide polymorphism array copy number profile for a medulloblastoma patient with a characteristic i(17q) abnormality. Isochromosome 17q is the most common cytogenetic aberration in medulloblastoma, identified in ~ 30–40% of cases. This structural abnormality results in a net loss of 1 copy of chromosome 17p and a net gain in 1 copy of 17q. Chromosome 17p loss and q gain are indicated in the copy number plot with *green* and *red arrowheads*, respectively. **B:** The *MYC* family amplification in medulloblastoma. Single-nucleotide polymorphism array copy number output showing focal, high-level amplification of *MYC* (8q24, *upper portion* of **B**), *MYCN* (2p24, *middle portion* of **B**), and *MYCL1* (1p34, *lower portion* of **B**) in primary medulloblastomas. The *MYC* family protooncogenes are collectively targeted for amplification in ~ 10% of primary medulloblastoma cases, more frequently than any other known oncogenes.

of copy number aberrations throughout the genome.[87,168] Boon et al.[21] karyotyped 5 medulloblastoma cell lines by sequencing ~ 200,000 genomic tags per genome, identifying amplification of the *OTX2* homeobox gene on chromosome 14q22 in the D487Med cell line. Using quantitative PCR, the authors confirmed recurrent amplification of *OTX2* in both medulloblastoma cell lines (D425Med) and primary tumors. In the parallel study published by Di et al.,[35] *OTX2* amplification was revealed in the D458Med cell line, also by digital karyotyping. In addition, by using data from SAGE libraries and quantitative RT-PCR, OTX2 (homeobox protein OTX2) was shown to be specifically overexpressed in medulloblastomas, especially lesions of anaplastic histology, as compared with a wide variety of other malignancies. Furthermore, the inhibition of OTX2 expression by siRNA-mediated knockdown or all-trans retinoic acid (ATRA) repressed medulloblastoma cell growth in vitro, suggesting that OTX2 may represent an attractive target for therapy, particularly in medulloblastomas of the anaplastic subtype.

Among the first authors to apply aCGH to medulloblastomas were Mendrzyk et al.,[104] who profiled 47 primary cases. These authors confirmed typical cytogenetic abnormalities including gains of chromosomes 17q, 7, and 1q as well as losses of 17p, 11p, 10q, and 8. Importantly, they also identified a minimal region of recurrent, high-level amplification targeting the *CDK6* protooncogene on chromosome 7q21.2. They validated *CDK6* copy number aberrations by using FISH and established a negative correlation between moderate-high CDK6 protein expression and overall survival by measuring CDK6 status on a medulloblastoma TMA.

Note that *FOXG1* is another candidate gene implicated in medulloblastoma pathogenesis given its recurrent gain on 14q12 as revealed by aCGH analysis.[1] Adesina et al.[1] have analyzed a small panel of medulloblastomas

TABLE 1: Most prominent cytogenetic aberrations in medulloblastoma

Gains		Losses	
Chromosome	Frequency (%)*	Chromosome	Frequency (%)*
1q	19	8p	12
2p	20	9q	10
7	24	10q	18
17q	44	11p	14
i(17q)	30	16q	12
		17p	36

* Frequency based on results of 500,000 SNP array profilings of 122 primary medulloblastomas.

using a combination of conventional CGH (19 cases) and aCGH (9 cases) and reported a gain of the *FOXG1* locus in 6 of 9 cases in the test set and 55 of 59 cases in a validation series of tumors. Expression of FOXG1 (forkhead box protein G1) correlated with the gene copy number and inversely correlated with p21 protein levels, a relationship that was strengthened in vitro as siRNA-mediated knockdown of FOXG1 in DAOY medulloblastoma cells resulted in increased p21 expression.

Amplifications of *MYCL1*, *PDGFRA*, and *KIT*—all protooncogenes not previously reported to be targeted in medulloblastoma—were also noted using aCGH technology.[100] Amplicons targeting these cancer genes have since been observed in our SNP array studies and by others, suggesting that they are relevant oncogenes in medulloblastoma.[92,119]

An earlier aCGH study of medulloblastoma focused on a series of 16 primary cases and 3 medulloblastoma cell lines.[70] The authors noted a novel region of homozygous deletion on chromosome 6q23 in the DAOY cell line that targeted only 2 previously uncharacterized genes, both of which exhibited reduced expression in a large percentage of primary medulloblastomas analyzed. Our group has since validated this region of homozygous deletion in DAOY and functionally confirmed *L3MBTL3* as a putative medulloblastoma tumor suppressor gene mapping to this locus.[119]

Medulloblastomas can be histologically classified into 5 recognizable subtypes: classic, desmoplastic, anaplastic, large-cell, and medulloblastoma with extensive nodularity.[55] Classic medulloblastoma is by far the most common, followed by the desmoplastic subtype, which makes up ~ 10–20% of cases, and large-cell and anaplastic tumors, which account for ~ 5–10% of cases. Although there is considerable variability in terms of patient outcome between the different histological subtypes and although histological staging has proven to be a less than ideal method of stratification, there is a great deal of interest in defining their molecular basis. To gain an improved understanding of the genomics of desmoplastic medulloblastomas, Ehrbrecht et al.[39] performed conventional CGH on a set of 22 sporadic cases of this subtype, followed by aCGH on a subset. In their analysis, novel regions of amplification were reported on chromosomes 9p and 17q22–24, implicating candidate oncogenes in these regions. Notably, *JMJD2C* was suggested as a putative oncogene mapping to the amplified region found on 9p, and we have since identified it as recurrently amplified and overexpressed in an independent sample cohort and affecting the state of methylation on histone lysines in normal progenitor cells of the developing cerebellum (that is, CGNPs).[119]

TABLE 2 : Candidate oncogenes recurrently amplified in medulloblastoma

Gene	Cytoband	Reference(s)
MYCL1	1p34.2	82, 92, 100, 119
MYCN	2p24.3	82, 119, 126, and many others
PDGFRA	4q12	100, 119
KIT	4q12	100, 119
TERT	5p15.33	38, 136
CDK6	7q21.2	104, 119
MYST3	8p11.21	119
MYC	8q24.21	82, 119, 126, and many others
JMJD2C	9p24.1	39, 119
miR-17/92	13q31.3	118, 119
IRS2	13q34	119
FOXG1	14q12	1
OTX2	14q23.1	21, 35, 119

There have been an impressive number of inquiries into the relationship between developmental signal transduction pathways and their role in medulloblastoma. Mutations in the Wnt, SHH, and Notch pathways have been well described in the medulloblastoma literature.[55,96] Nonetheless, a comprehensive understanding of how specific genomic events contribute to aberrant signaling of these pathways has not been established. An important finding relevant to deregulated Wnt signaling in medulloblastoma was reported in 2006 in 2 independent but related studies.[28,154] Clifford et al.[28] have profiled 19 primary medulloblastomas by aCGH, with the specific intent to genomically describe tumors with Wnt pathway activation (nuclear β-catenin; *CTNNB1* or *APC* mutation). Interestingly, in both the initial cohort (19 cases) and a validation series (32 cases), a single copy deletion of chromosome 6 (monosomy 6) was found exclusively in the Wnt pathway tumors. Identical findings were reported by Thompson et al.,[154] who consistently observed a correlation between the Wnt pathway signature (Wnt pathway expression; *CTNNB1* mutation) and markedly reduced expression of genes mapping to chromosome 6 because of deletion. Monosomy 6 is now widely accepted in the medulloblastoma community as a genomic marker of Wnt pathway tumors that is consistently associated with *CTNNB1* mutation.[43,82,154] From a clinical perspective, monosomy 6/*CTNNB1* mutation is among the most reliable genetic markers in medulloblastoma, correlating with a highly favorable prognosis.[28,43,52,126] Indeed, 100% of patients found to belong to the Wnt immunohistochemical category in the recent St Jude Medulloblastoma-96 clinical trial were event free at 5-years, compared with only 65% of patients in the SHH category.[52]

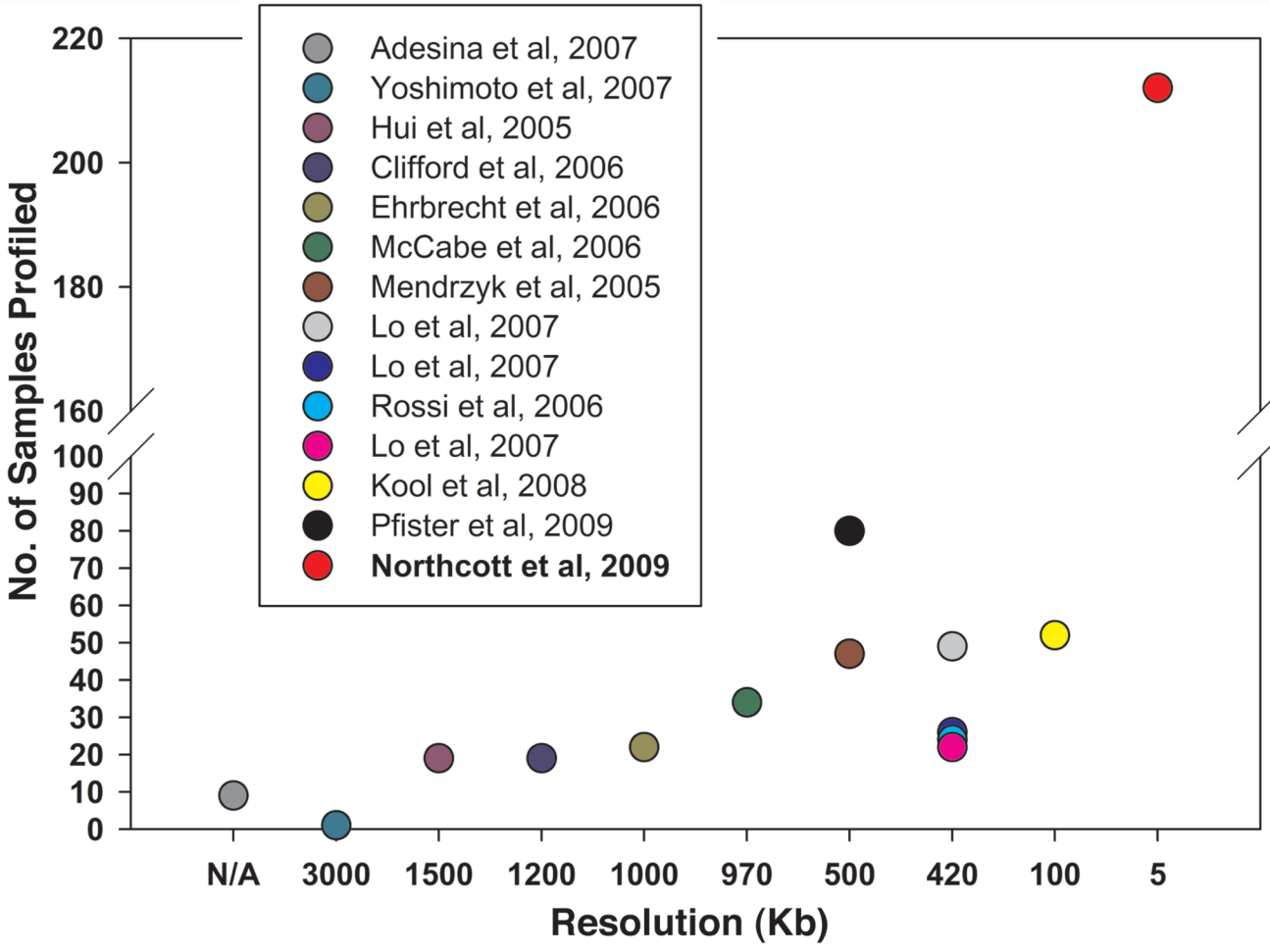

Fig. 3. Scatterplot identifying reports in the literature that have profiled medulloblastoma copy number aberrations using array-based genomic technologies. Each publication is represented as a *colored circle*, with its position along the y axis determined by the number of samples analyzed and its position along the x axis determined by the approximate median resolution of the array platform(s) used in the study. Study referred to in figure is Northcott et al., 2009.[119]

Very recently, Pfister and colleagues[126] proposed a model for molecular risk stratification of pediatric medulloblastoma based on DNA copy number aberrations affecting chromosomes 6q, 17q, and *MYC/MYCN* loci. Using aCGH, the authors initially profiled 80 primary medulloblastomas in an attempt to identify genomic aberrations of prognostic value, and found a gain of chromosome 6q, amplification of *MYC* and *MYCN*, isolated gain of 17q, and i(17q) all to be associated with a poor clinical outcome. In contrast, the loss of chromosome 6q was indicative of an excellent prognosis, consistent with findings in the current literature.[28,43] Validation of these prognostic markers in a nonoverlapping set of 260 primary cases by using interphase FISH on a medulloblastoma TMA, Pfister et al. were able to establish an elegant staging system whereby patient outcome could be predicted based on the genomic status of only 4 markers (arranged from worst to best outcome): *MYC/MYCN* amplification, 6q gain, 17q gain, 6q/17q balanced, and 6q loss.

Although several of the aCGH studies described above have been informative and have enhanced our understanding of the medulloblastoma genome, most have profiled relatively modest sample cohorts (median sample size: ~ 24 cases) using arrays that—although an improvement over classic CGH—are of insufficient density and thus resolution (median resolution ~ 500 kb) to detect very focal genetic events. To address these caveats, we retrospectively collected an unprecedented cohort of 201 fresh-frozen primary medulloblastomas and 11 medulloblastoma cell lines and analyzed their genomes using high-resolution SNP genotyping arrays.[119] These oligonucleotide arrays consisted of 25mer probes designed to detect the genotype (that is, A or B allele) of known SNPs at loci distributed across the genome.[69,85,98,99] The median intermarker distances (that is, resolution) of probes on the 100,000 and 500,000 arrays used in this study were 8.5 and 2.5 kb, respectively, which are at least an order of magnitude higher in terms of resolution than any previous array-based study of the medulloblastoma genome (Fig. 3). In addition to reporting cytogenetic gains and losses at frequencies already known in medulloblastoma (Fig. 4 and Table 1), this strategy of profiling a large number of samples on a high-resolution platform led to the identification of 191 high-level amplifications and 159 homozy-

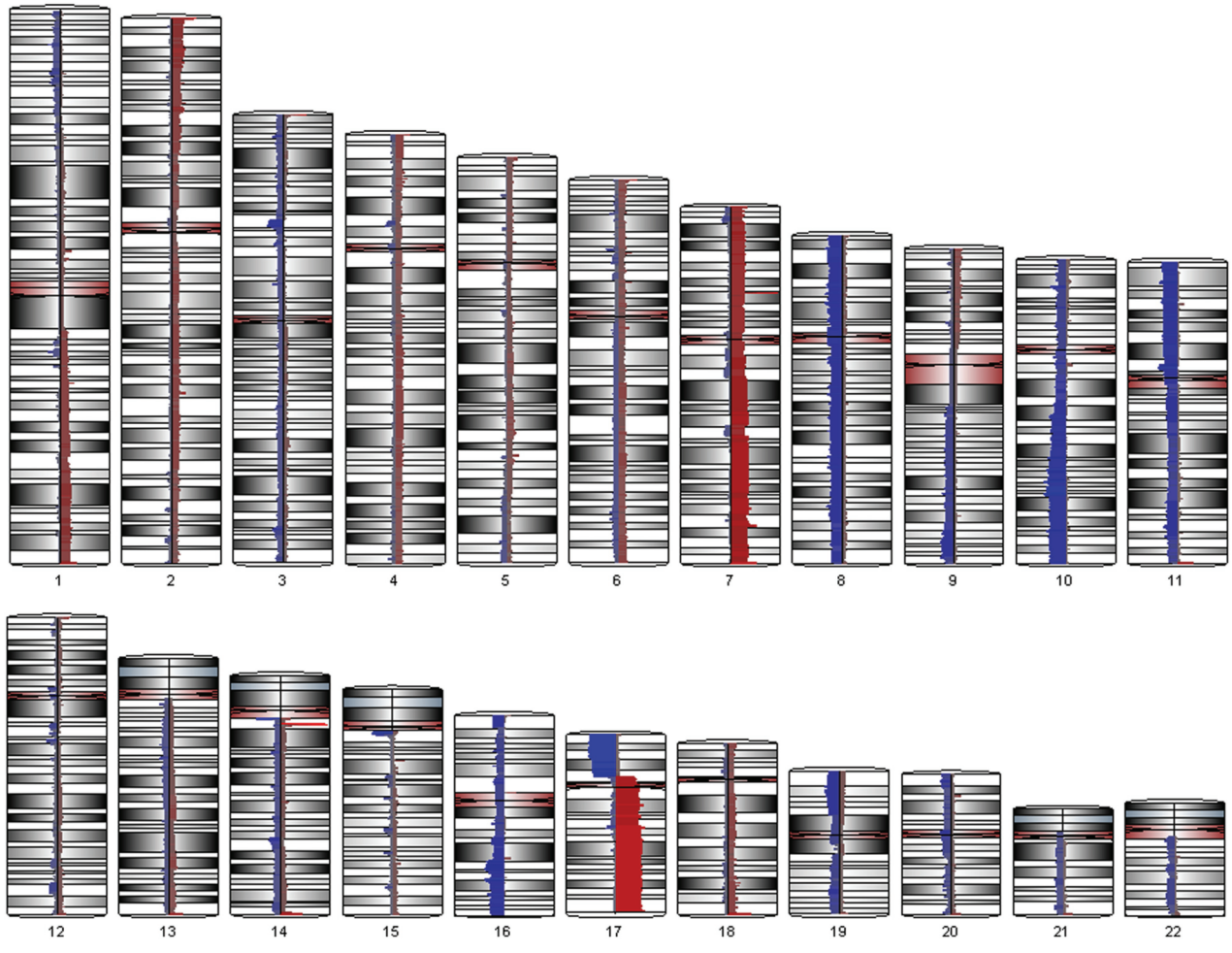

Fig. 4. Visual representation of gains and losses in the genome of medulloblastoma. High-resolution copy number data derived from 122 primary medulloblastomas is represented as a histogram for each of the 22 autosomes, with the relative frequency of gain depicted on the right of each chromosome in *red*, and the frequency of losses shown on the left of each chromosome in *blue*. The most frequent cytogenetic gains in this representative cohort are localized to chromosomes 1q, 7, and 17q, whereas recurrent losses are most notable on chromosomes 8, 9q, 10q, 11, 16q, and 17p.

gous deletions, most of which had not been reported in medulloblastoma. Surprisingly, only 12 recurrent amplifications were identified, and a mere 6 homozygous deletions were found in more than 1 sample. Of the recurrent homozygous deletions, *EHMT1*, a euchromatic histone (H3K9) methyltransferase, was the lone gene mapping to the minimal common region of a deleted region on chromosome 9q34, suggesting that it may represent a novel tumor suppressor gene in medulloblastoma. Note that EHMT1 functions as part of a transcriptional repressor complex that mediates gene silencing by promoting dimethylation of H3K9 ($H3K9me^2$),[121,150] a repressive epigenetic modification,[16,84] in the promoter regions of target genes. The expression of EHMT1 was shown to be significantly downregulated at both the mRNA and protein level, and staining for both EHMT1 and $H3K9me^2$ on a medulloblastoma TMA showed a significant correlation between EHMT1 status and the H3K9 methylation state, consistent with a model in which the loss of EHMT1 leads to H3K9 hypomethylation in medulloblastoma. In addition to *EHMT1*, 7 other genes with a putative role in the regulation of histone lysine methylation were also found to be the target of focal copy number aberrations in the data set, including *SMYD4*, *L3MBTL2*, *L3MBTL3*, *SCML2*, *JMJD2C*, *JMJD2B*, and *MYST3*. Recurrent targeting of genes sharing a common role in the modulation of histone lysine residues in medulloblastoma suggests that deregulation of the histone code, particularly histone lysine methylation, very likely contributes to the pathogenesis of at least some medulloblastomas.

Medulloblastoma Transcriptome Profiling

Typically, strategies aimed at the transcriptional profiling of cancer have involved the comparison of gene expression signatures obtained for normal and neoplastic tissues (Fig. 5). In one of the earliest studies of medulloblastoma gene expression profiling, Michiels et al.[107] used SAGE to compare genes expressed in medulloblastoma

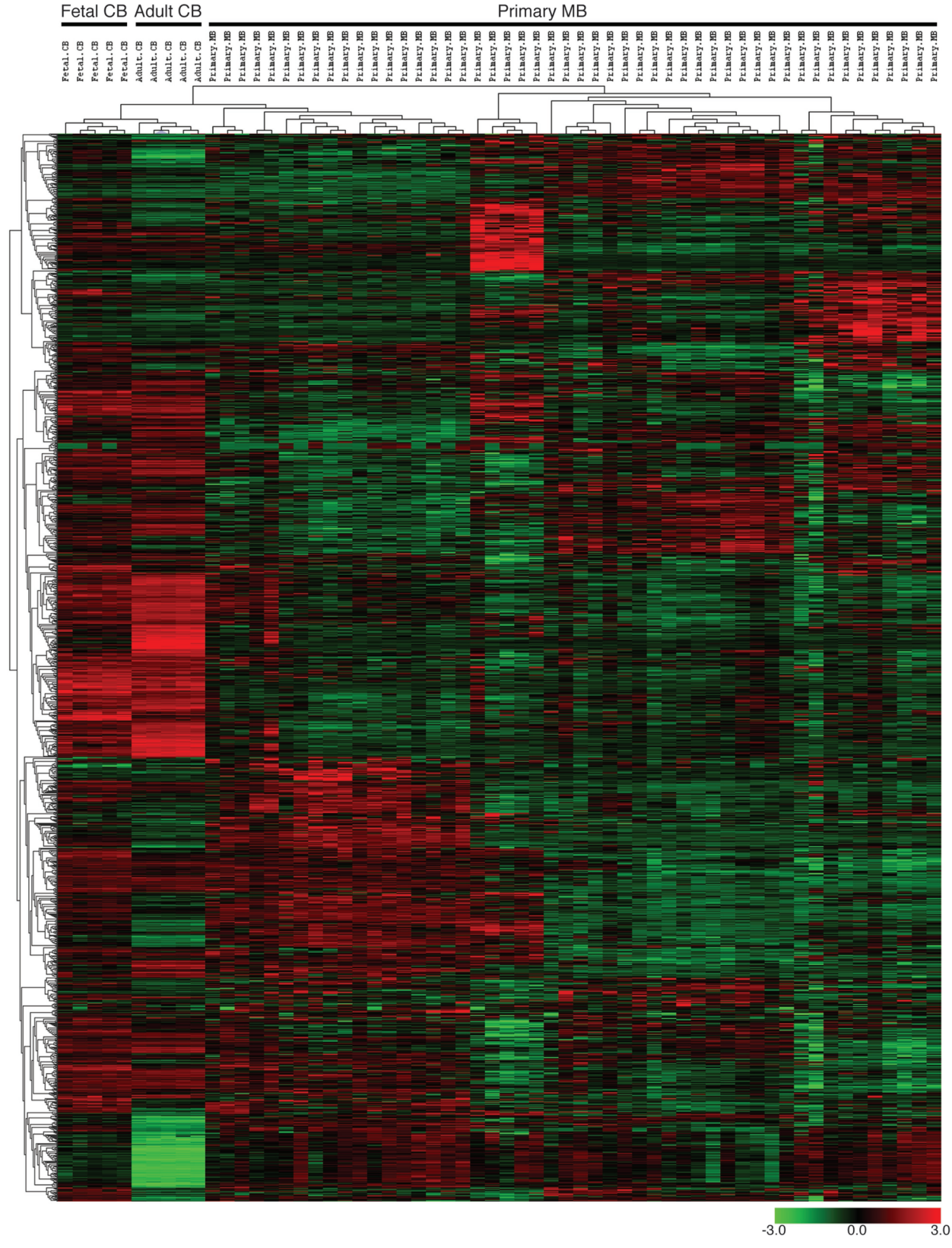

Fig. 5. Heatmap showing gene expression data for 10 normal cerebellum (CB) samples (5 fetal and 5 adult) and 50 primary medulloblastomas analyzed using Affymetrix arrays. Genes exhibiting elevated expression are shown in *red*, whereas genes with reduced expression are depicted in *green*. Unsupervised hierarchical clustering of samples using the most differentially expressed genes results in a clear distinction between normal cerebellar samples and primary tumors.

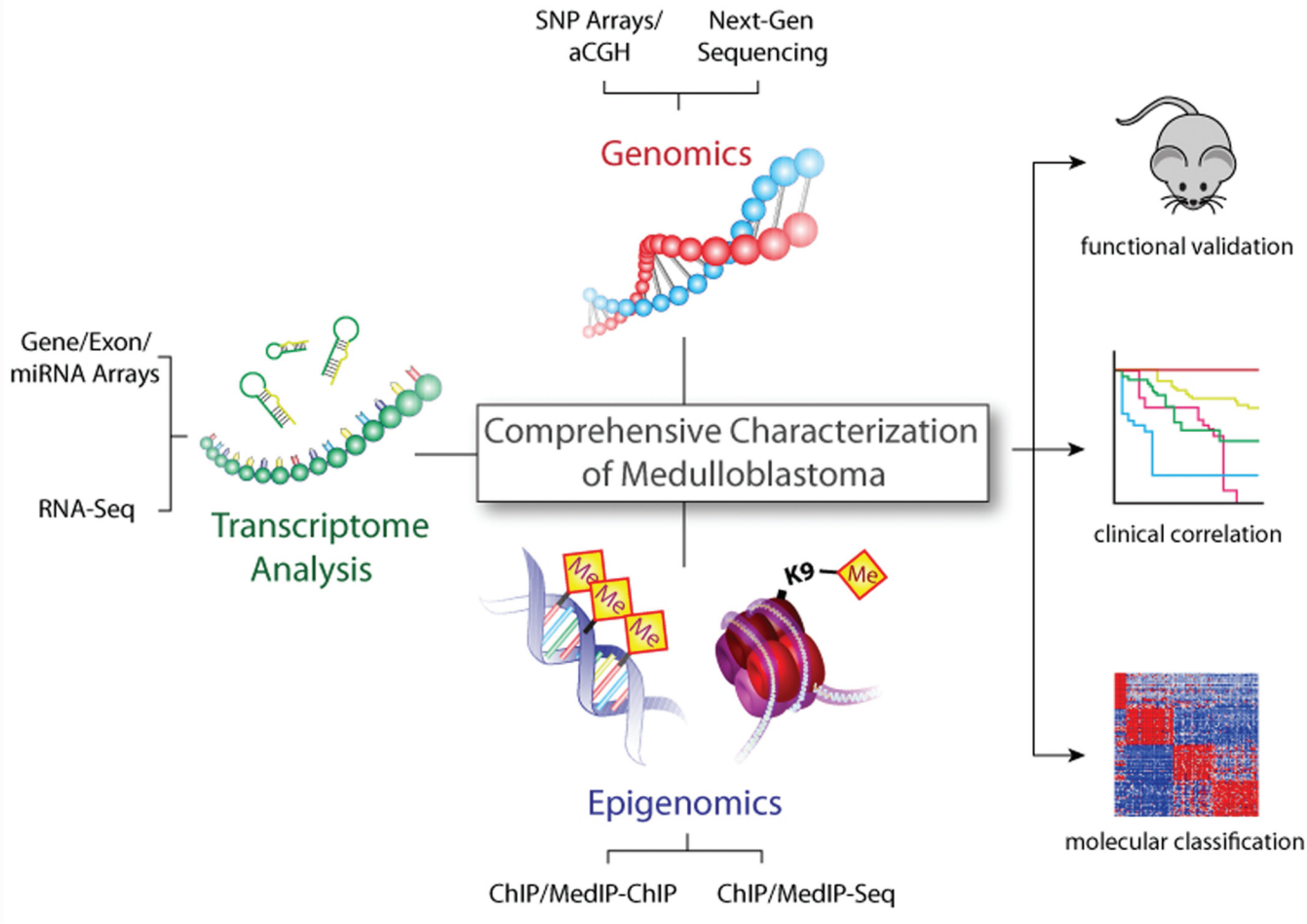

Fig. 6. Schematic illustrating how collaborative approaches that involve integration of genomics, transcriptome analysis, and epigenomics will be applied in the future characterization of medulloblastoma. For each category, some of the leading technologies of the present and future are listed, with applications relying on NGS (Next-Gen Sequencing) present in each. The union of these orthogonal genome/transcriptome/epigenome interrogation strategies will greatly accelerate the identification of novel candidate genes and pathways involved in medulloblastoma genesis and permit follow-up functional validation studies, correlation with clinical variables, and improved molecular classification.

with those in the fetal brain (24.5 weeks). Serial analysis of gene expression uses DNA sequencing technology to digitally quantify mRNA abundance by counting the frequency with which a short sequence tag (that is, transcript) appears in a cDNA library.[161,162] Serial analysis of gene expression technology is very effective in quantifying gene expression and in identifying novel genes/transcripts, as no a priori knowledge of the genome under study is required.[131,144,159,160] In the study by Michiels and colleagues, ~ 10,000 tags were sequenced in both medulloblastoma and fetal brain, with ~ 6000 unique genes identified in each transcriptome. A comparison of the SAGE data revealed 138 genes with significant differential expression between the 2 sources, including ZIC1 (zinc finger protein ZIC 1) and OTX2, both showing significantly elevated expression in medulloblastoma that was confirmed by Northern blotting in multiple independent samples. As these genes are highly expressed in cerebellar germinal zones (that is, the external granule layer and subventricular zone), this study provided early clues into the origins of medulloblastoma.

The presence of disseminated disease at diagnosis is a strong, independent indicator of a poor prognosis for medulloblastoma, occurring in ~ 1 of every 3 cases.[52,83,152,177] Therefore, understanding the molecular basis of metastatic medulloblastoma is extremely important clinically. The first study to specifically compare metastatic (M+) with nonmetastatic (M0) medulloblastoma at a gene expression level was performed by MacDonald and colleagues in 2001.[94] Twenty-three primary medulloblastomas designated as either M+ or M0 were analyzed using Affymetrix G110 cancer arrays, which identified 85 genes as differentially expressed between the 2 classes. Using a supervised class prediction algorithm, this 85-gene signature classified the M+ and M0 tumors with 72% accuracy. Interestingly, PDGFR and members of the Ras/MAPK signaling cascade were reported to be significantly upregulated in metastatic versus nonmetastatic cases. The overexpression of PDGFR in metastatic disease was confirmed by immunohistochemistry in an independent set of tumors. In vitro assays performed in the DAOY medulloblastoma cell line showed that the

PDGF ligand activated the Ras/MAPK pathway and promoted cell migration in this system, whereas neutralizing antibodies against PDGFR attenuated MAPK signaling and prevented ligand-mediated migration. In a follow-up study by Gilbertson and Clifford,[54] the association between PDGFR overexpression and metastatic medulloblastoma was confirmed, further supporting the validity of this pathway as a candidate for targeted therapy.

Undoubtedly, one of the most seminal papers in medulloblastoma genomics was written by Pomeroy and colleagues,[132] who surveyed the expression profiles of a large series of primary brain tumors and made at least 3 findings of clinical significance. Initially, 42 samples consisting of medulloblastomas (10 patients), atypical teratoid rhabdoid tumors (5 patients), renal and extrarenal rhabdoid tumors (5 patients), sPNETs (8 patients), and nonembryonal brain tumors (malignant glioma; 10 patients), and normal human cerebellum (4 patients) were analyzed using Affymetrix HuGeneFl arrays containing ~ 6000 known genes. Based on differentially expressed transcripts, these authors showed a clear distinction between the different tumor types, establishing that histologically similar tumors such as medulloblastomas and sPNETs were molecularly distinct. The molecular distinction between medulloblastomas and sPNETs has important clinical implications; because of their similar histology, these tumors were historically classified under the same broad category of PNET and thus were treated with the same protocols.[141] In recent years, however, it has become evident that medulloblastomas and sPNETs are both molecularly and biologically distinct, with sPNETs typically exhibiting a worse prognosis.[31,89,100,127,137,155]

These same authors also compared the expression profiles in a set of 34 medulloblastomas of either classic (25 cases) or desmoplastic (9 cases) histology, revealing a notable degree of statistically significant differential expression between the 2 subtypes. Genes signifying desmoplastic medulloblastoma included *PTCH*, *GLI*, *MYCN*, and *IGF2*, all of which are now well-described targets of SHH signaling. Although a link between mutations in the SHH pathway and medulloblastoma pathogenesis had already been discovered, this study was among the first to document an association between aberrant SHH signaling and sporadic desmoplastic medulloblastoma.

A third key finding in this study stemmed from the authors' use of gene expression data to predict the outcome of 60 patients with medulloblastoma for whom clinical follow-up data were available. Using a class prediction algorithm, an 8-gene classification model was generated and successfully predicted the survival status for 47 of the 60 patients profiled. Genes correlated with a favorable outcome included markers of cerebellar differentiation (β-*NAP*, *NSCL1*, and *TRKC*) as well as genes encoding components of the extracellular matrix (lysyl hydroxylase [*PLOD*], collagen Type Vαi, and elastin). In contrast, genes associated with a poor outcome included those with a role in cell proliferation and metabolism (*MYBL2*, enolase 1, *LDH*, *HMG1[Y]*, and cytochrome C oxidase) as well as ribosomal protein-encoding genes. Much like the study by MacDonald et al.[94] described earlier, the report by Pomeroy and colleagues[132] demonstrates the utility of correlating gene expression profiles in medulloblastoma with a particular phenotype (that is, favorable vs poor outcome) and provides a rationale for the incorporation of similar molecular profiling strategies in the future diagnosis and treatment of patients with medulloblastoma.

Following the Pomeroy study, a number of independent groups, including our own, have engaged in medulloblastoma transcriptome profiling efforts using a variety of technologies.[22,79,115,123,175] Boon et al.[22] used SAGE to analyze 20 primary medulloblastomas, identifying 30 transcripts exhibiting elevated expression in tumors compared with normal cerebellum and additional regions of the brain. The cancer-testis antigen *PRAME, CD24*, *PRL*, *TOP2A*, *MYCN*, and *BARHL1* were all overexpressed in this study. Data from more recent array-based studies by our group have confirmed the aberrant expression of cancer-testis antigens from the *MAGE* and *GAGE* families in medulloblastoma cell lines and in some primary samples, suggesting that these genes may be important in medulloblastoma.[79]

In an earlier study we used suppression subtractive hybridization to identify genes deregulated in both human and mouse ($Ptc^{+/-}$) medulloblastoma compared with normal, species-matched cerebellum.[175] In suppression subtractive hybridization, double-stranded cDNA libraries are first prepared from tester (that is, medulloblastoma) and driver (that is, normal cerebellum) RNA samples.[164] Heat-denatured tester cDNA is subsequently digested, adapter ligated, and then hybridized with the denatured driver cDNA to generate a subtracted cDNA library that is PCR amplified and cloned into a recipient plasmid for bacterial transformation and sequencing of clones for gene identification. In this effort, over 100 upregulated cDNA fragments were identified in the human library, including *ULIP* (also known as *DPYSL3)*, *SOX4*, *NNAT*, and the previously implicated *BARHL1* and *OTX2* genes. In addition, genes identified as upregulated in medulloblastomas from $Ptc^{+/-}$ mice included *CCND2*, *TMPO*, *Musashi-1*, and others.

Another informative expression profile of medulloblastoma was generated by Neben et al.,[115] who analyzed ~ 4200 genes in 35 primary medulloblastomas in an attempt to identify those associated with patient outcome. Based on mRNA levels, 54 genes were shown to be markers of poor outcome, and a subset of these (9 genes) was further evaluated by immunohistochemistry in a nonoverlapping set of 180 cases on a medulloblastoma TMA. Of these candidate genes, *STK15* positivity was identified as a negative prognostic marker of overall survival, whereas other putative markers implicated in the study (that is, cyclin D1 and stathmin 1) were not.

Molecular Classification of Medulloblastoma

Over the past decade, significant progress has been made in how we study the cancer genome. Indeed, gene expression profiling has proven to be an effective tool for the molecular classification of cancer, including brain tumors.[57,109,120,132] Following the studies of Macdonald and Pomeroy, Thompson et al.[154] were the first to truly

establish the existence of unique molecular subgroups of medulloblastoma using gene expression data. Profiling a series of 46 primary medulloblastomas, these authors performed unsupervised hierarchical clustering with the most informative genes in the data set, identifying 5 molecular subgroups of medulloblastoma. By integrating immunohistochemistry, FISH, and mutational screening data generated from these samples, it was shown that molecular subgroups of medulloblastoma have specific genomic and genetic features. Importantly, this study was the first to demonstrate that Wnt (that is, monosomy 6 and *CTNNB1* mutation) and SHH tumors (that is, *PTCH1* and *SUFU* mutation) are mutually exclusive.

In a more recent study, Kool et al.[82] utilized a similar integrative genomics approach to further characterize molecular subgroups of medulloblastoma. By combining array-based gene expression and copy number profiles for 52 primary cases, Kool and colleagues recapitulated the 5 molecular subgroups described by Thompson et al.[11] and correlated the different subgroups with specific genomic and clinical features. Importantly, the authors furthered our knowledge of non-Wnt/SHH tumors (subgroups A and B), showing that the 3 remaining subgroups (C, D, and E) are closely related and marked by elevated expression of neuronal differentiation (subgroups C and D) and retinal (subgroups D and E) genes. Furthermore, metastatic disease was shown to be more highly associated with subgroups C, D, and E, providing further support for the potential stratification of patients based on molecular subgrouping.

Beyond Protein-Coding Genes: miRNAs in Medulloblastoma

Over the course of the past 5 or so years, there has been a literal explosion in the miRNA field, especially with respect to elucidating their role in human disease—in particular, cancer.[24,41,64] Small, noncoding, single-stranded RNA molecules, miRNAs posttranscriptionally regulate gene expression through their interaction with complementary sequences in the 3′ untranslated regions of target mRNAs.[3,10] Target mRNAs are either degraded or translationally repressed by specific miRNAs, depending on the degree of complementarity between the miRNA and its target. Despite an intense amount of investigation into the involvement of miRNAs in a variety of cancer types, knowledge of their role in medulloblastoma pathogenesis is still in its infancy. The few studies of the entire miRNAome that have been conducted to date are discussed below.

Ferretti and colleagues[45] recently performed a TaqMan quantitative RT-PCR–based profiling of 248 miRNAs in a panel of medulloblastomas (14 cases) and normal cerebellar controls (7 cases), reporting an overwhelming bias toward downregulation of miRNAs in tumors versus controls. A subset of 86 miRNAs previously reported to be expressed in neuronal tissues and/or implicated in cancer were further analyzed in a larger cohort of tumors (34 cases), with the authors selecting miR-9 and miR-125a as 2 neuronal candidates downregulated in medulloblastoma for functional studies. The expression of both miR-9 and miR-125a was induced by retinoic acid treatment of D283 medulloblastoma cells, an agent known to inhibit medulloblastoma cell proliferation. In addition, ectopic overexpression of miR-9 and miR-125a inhibited proliferation, impaired anchorage-independent growth, and promoted apoptosis of D283 cells. Truncated trkC was identified as a target for posttranscriptional repression by both miR-9 and miR-125a in this study, suggesting a possible correlation between the loss of miR-9/miR-125a and the upregulation of the proproliferative truncated trkC in medulloblastoma.

In an effort to discriminate miRNAs deregulated in SHH-driven medulloblastomas from non-SHH cases, the same group of authors[44] used the Gli1 expression status to stratify a panel of 31 medulloblastomas into 2 classes ($Gli1^{high}$ and $Gli1^{low}$) before profiling a set of 250 miRNAs using TaqMan-based quantitative RT-PCR. This approach revealed a set of 34 miRNAs exhibiting significant differential expression between the 2 classes. Three candidates exhibiting reduced expression in $Gli1^{high}$ tumors—miR-125b, miR-324–5p, and miR-326—were chosen for functional analysis based on their predicted capacity to target and repress the SHH family members, Smo and Gli1. Indeed, all 3 candidates were shown to repress Smo mRNA levels when overexpressed in DAOY medulloblastoma cells. Additionally, the expression of these candidate miRNAs correlated with the differentiation state of cultured CGNPs, presumed cells of origin for SHH-driven medulloblastomas,[14,47,55] and their ectopic expression reduced SHH-mediated proliferation and promoted neurite outgrowth in the same cell type.

In 2 distinct but parallel comprehensive analyses of the human and mouse medulloblastoma miRNAomes, the *miR-17/92* polycistron was identified as a putative medulloblastoma oncogene.[118,158] A bona fide oncogene in B-cell lymphoma, *miR-17/92* has been reported to be aberrantly expressed in a variety of human tumors.[66,103] To identify miRNAs deregulated in mouse models of medulloblastoma, Uziel et al.[158] performed unbiased NGS to quantify miRNA abundance in medulloblastoma cells isolated from spontaneous tumors of $Ink4c^{-/-}$; $Ptc^{+/-}$ or $Ink4c^{-/-}$; and $p53^{-/-}$ genotypes as compared with wild-type control cerebellum (1 month old) and CGNPs (6 days old). This strategy revealed 26 miRNAs with elevated expression and 24 with reduced expression in the tumor models. Among upregulated miRNAs in murine medulloblastoma cells, miR-17/92 and related paralogs accounted for 9 of 26. In addition, the authors provided evidence that miR-17/92 might cooperate with SHH signaling in medulloblastoma, showing preferential upregulation of miR-17/92 in the SHH subtype by quantitative RT-PCR profiling of a small panel of human tumors (5 SHH lesions and 5 non-SHH lesions). To evaluate its oncogenic potential in a context relevant to SHH-driven medulloblastoma, miR-17/92 was retrovirally transduced into 6-day-old CGNPs isolated from both $Ink4c^{-/-}$, $Ptc^{+/-}$ and $Ink4c^{-/-}$, $p53^{-/-}$ mice prior to orthotopic transplantation of miR-17/92–expressing CGNPs into immunocompromised mice. Notably, only cells derived from the $Ptc^{+/-}$ background developed medulloblastoma (9 of 9 cases) in this model. Furthermore, tumor cells were sensitive to

the Smo inhibitor cyclopamine, exhibited elevated Math1 and *Gli1* mRNA levels, and lost expression of the wild-type *Ptc* allele—all markers of activated SHH signaling and supportive of a synergistic connection between miR-17/92 and SHH in these tumors.

As detailed earlier, we recently performed high-resolution SNP array profiling on a group of > 200 medulloblastomas.[119] This effort revealed multiple regions of previously unreported copy number aberrations in the medulloblastoma genome, including recurrent, high-level amplification of *miR-17/92* on chromosome 13q31.[118,119] Subsequent interphase FISH performed on a medulloblastoma TMA consisting of a nonoverlapping series of tumors confirmed *miR-17/92* amplification in ~ 6% of cases. To gain further insight into the role of miRNAs in medulloblastoma, we next used miRNA microarrays to globally profile the human medulloblastoma miRNAome in a series of 90 primary medulloblastomas and 10 normal cerebellar controls (5 fetal and 5 adult samples). Remarkably, miR-17/92 and related paralogs (miR-106a/363 and miR-106b/25) were identified as the most highly upregulated miRNAs in medulloblastoma when compared with normal cerebellum in this analysis. The combination of miR-17/92 amplification and consistent overexpression suggested miR-17/92 as a key player in medulloblastoma pathogenesis.

As shown by the aforementioned studies of Thompson[154] and Kool,[82] medulloblastomas can be classified into unique molecular subgroups based on distinct gene expression signatures and specific genomic and genetic features.[82,154] Using Affymetrix exon arrays to comprehensively profile the transcriptome of the same 90 primary medulloblastomas analyzed by miRNA microarray, we described 4 distinct molecular subgroups of medulloblastoma.[118] These subgroups include the well-characterized Wnt and SHH subgroups described earlier, as well as 2 independent subgroups we have designated Groups C and D. Through the integration of genomics (copy number), mRNA, and miRNA expression, we found that miR-17/92 was most highly expressed in SHH-driven medulloblastomas, in agreement with the observations reported by Uziel and colleagues.[158] Additionally, we showed elevated miR-17/92 levels in tumors exhibiting high MYCN (SHH) and MYC (Group C, Wnt) expression, indicative of miR-17/92 transcriptional upregulation by N-Myc and Myc and confirming miR-17/92 aberrancy in a large percentage of human medulloblastomas (~ 60%). Deregulation of miR-17/92 was conserved in well-characterized, SHH-driven mouse models of medulloblastoma—*Ptc*$^{+/-}$ and *SmoA1*—also in concordance with the findings mentioned above. Finally, using CGNPs isolated from wild-type mice, we showed that miR-17/92 is transcriptionally induced by SHH through N-Myc, maintains CGNPs in a proliferative state in the absence of SHH, and synergizes with SHH to enhance CGNP cell growth. Cumulatively, the results of Uziel et al. and our own strongly support miR-17/92 as a legitimate medulloblastoma oncogene that cooperates with SHH signaling to promote and/or enhance CGNP proliferation.

Beyond Genomics: the Medulloblastoma Epigenome

Until recently, the majority of cancer research efforts had focused on describing the genetic basis of cancer, studying everything from large cytogenetic aberrations to SNPs and mutations. Over the past few years, however, there has been an ever-growing volume of literature linking the deregulation of epigenetics to malignancy.[23,42,75,76,156] Epigenetics is defined as "mitotically heritable changes in gene expression that are not accompanied by modifications in primary DNA sequence." Epigenetic modifications include DNA methylation on cytosine residues, most often in the context of CpG dinucleotides, as well as posttranslational modification of histone proteins, such as methylation, acetylation, phosphorylation, and ubiquitination.[16,84] Hypermethylation of CpG islands located at the 5′ end of genes has been reported in most cancers and, either alone or in combination with genetic mechanisms (that is, gene deletion or mutation), can contribute to tumor suppressor gene silencing. Although a handful of known tumor suppressors can be silenced by promoter methylation in medulloblastoma by using candidate gene approaches (that is, *HIC1*, *RASSF1A*, and *CASP8*),[58,61,65,68,91,93,140,166] the application of unbiased, whole-epigenome strategies to identify novel candidates have been scant to date, consisting of only those instances described in the few published reports mentioned below.

Among the earliest studies to implicate aberrant promoter methylation in medulloblastoma on a global scale was an effort led by Frühwald and colleagues[49] who used the technique of restriction landmark genomic scanning to analyze DNA methylation patterns in 17 primary medulloblastomas and 5 medulloblastoma cell lines. Using this method, the authors identified methylation in up to 1% of all CpG islands in primary tumors and up to 6% in medulloblastoma cell lines. In addition, an association between hypermethylated sequences in medulloblastoma and a poor prognosis was implied. Collectively, these findings provided early evidence that epigenetic events are likely to play a role in medulloblastoma pathogenesis.

In a study using microarray-based differential methylation hybridization, Waha et al.[165] identified hypermethylation of the *SCG5* (secretory granule, neuroendocrine protein 1 [7B2 protein] gene) in 16 (~ 70%) of 23 primary medulloblastomas and 7 (~ 87%) of 8 medulloblastoma cell lines. Differential methylation hybridization involves a series of enzymatic digestions with methylation-insensitive followed by methylation-sensitive restriction enzymes, and uncut (methylated) fragments are PCR-amplified before hybridization to microarrays containing probes designed to interrogate CpG islands throughout the genome.[174] The expression of *SCG5* was found to be downregulated in the majority of primary samples and cell lines as compared with normal cerebellar controls, and *SCG5* transcription was restored in cell lines treated with the demethylating agent, 5-aza (5-aza-2′-deoxycytidine). Furthermore, the reexpression of *SCG5* in the D283Med cell line resulted in growth suppression and reduced colony formation, suggesting that *SCG5* may be a putative tumor suppressor gene in medulloblastoma.

Pfister and colleagues[128] developed and applied a technique known as array-based profiling of reference-independent methylation status (aPRIMES) to globally survey DNA methylation patterns in the medulloblastoma genome. This technique compares 2 differentially digested (methylation-sensitive and methylation-specific) aliquots from the same sample genome by competitive hybridization to a CpG island microarray. The advantage of using test-versus-test as opposed to test-versus-control hybridization is the avoidance of both the influences of tissue-specific methylation that may be present in the control sample and the genomic aberrations that may exist in the test sample and not in the control genome. Using this methodology, Pfister et al. showed a striking association between samples classified as either "low methylators" or "high methylators" and patient outcome, with the high-methylator group exhibiting reduced overall survival. In addition, the GLI C2H2-type zinc-finger protein family member *ZIC2* was identified as a hypermethylated candidate using aPRIMES and was subsequently confirmed to be epigenetically silenced in a panel of primary medulloblastomas by using a combination of pyrosequencing and quantitative RT-PCR analysis.

In 2 technically similar yet independent genome-wide methylation studies conducted by Anderton et al.[4] and Kongkham et al.,[81] 5-aza–treated medulloblastoma cell lines were profiled on Affymetrix expression arrays in an effort to uncover novel tumor suppressor genes silenced by aberrant promoter methylation. In the report by Anderton and colleagues, 3 medulloblastoma cell lines (D425Med, D283Med, and MED8A) were either left untreated or exposed to 5-aza, and transcripts showing increased expression by microarray in response to the DNA methyltransferase inhibitor were investigated further by bioinformatically confirming the presence of a 5′ CpG island and assessing methylation status through bisulfite sequencing.[4] This approach combined with gene expression analysis identified *COL1A2* as an epigenetically silenced candidate in medulloblastoma that is preferentially inactivated in nondesmoplastic and noninfant (> 3 years) desmoplastic cases.

Kongkham et al.[81] performed a similar genome-wide 5-aza screen in a larger group of 9 medulloblastoma cell lines but incorporated multiple additional criteria when filtering identified candidate genes as compared with those in the Anderton et al.[4] study. These authors selected for further analysis those genes that demonstrated in at least 2 cell lines a > 2-fold upregulation in expression following 5-aza treatment, contained a predicted CpG island in their promoter region, and were identified as targets of loss of heterozygosity based on SNP genotyping studies.[119] Under these criteria, *SPINT2*, a negative regulator of the HGF/Met signaling pathway, was identified, exhibiting robust reexpression in 6 of the 9 medulloblastoma cell lines profiled. The authors confirmed the downregulation of *SPINT2* in a significant percentage of primary medulloblastomas (> 2-fold in 41 of 56 samples) analyzed by quantitative RT-PCR, and, importantly, that aberrant promoter methylation (assessed by methylation-specific PCR) correlated with the observed reduction in gene expression in most cases. Stable reexpression of *SPINT2* in medulloblastoma cell lines resulted in the attenuation of the malignant phenotype, inhibiting cell proliferation, anchorage-independent growth in soft agar, and cell motility. Furthermore, the orthotopic transplantation of D283 cells stably reexpressing *SPINT2* into recipient nude mice significantly delayed the time to death compared with empty vector control cells in an intracerebellar xenograft model. These data strongly implicate *SPINT2* as a putative tumor suppressor gene in medulloblastoma and shed further light on the apparent role of aberrant HGF/Met signaling in medulloblastoma etiology.

Collectively, these recent studies of the medulloblastoma epigenome have proven informative and have further implicated epigenetic gene silencing as an important mechanism of tumor suppressor gene inactivation in medulloblastoma. The future application of strategies that enrich for epigenetic modifications (that is, methylation-dependent immunoprecipitation [MedIP] or chromatin immunoprecipitation [ChIP]) combined with high-resolution microarrays or NGS technologies will probably lead to an improved appreciation of the role that epigenetics plays in medulloblastoma.

Next-Generation Genomics of Medulloblastoma

Over the past few years, microarray technologies have significantly increased our understanding of the medulloblastoma genome, transcriptome, and, to some extent, epigenome. Moving forward, array platforms will undoubtedly continue to be used in genome-wide profiling of medulloblastoma, especially as the resolution and coverage of these methods continue to improve and the cost of these screens remains affordable. However, recent breakthroughs in DNA sequencing technologies have taken the genomics community by storm, and their application in medulloblastoma research is, without question, imminent.

Since the early 1990s, the capillary-based Sanger method of DNA sequencing has been the mainstay for most applications in molecular biology, even the first drafts of the human genome published in 2000.[86,163] More recently, conventional sequencing has been successfully used in large-scale resequencing efforts, profiling anywhere from a few hundred genes to all known protein-coding genes in a single cancer genome.[26,36,77,78,125,173] Indeed, initial exon resequencing of the colorectal, breast, pancreatic, and glioblastoma multiforme genomes has revealed new genes and pathways involved in the pathogenesis of the respective cancer types.[77,78,125,173] However, these studies relied on the PCR-mediated amplification of literally hundreds of thousands of exons combined with an enormous workload of conventional sequencing, unrealistic tasks for most of the cancer genomics community.

Fortunately, over the last few years a revolution in DNA sequencing technology has occurred and is rapidly changing the field of cancer genomics.[33,143,145,146] Next-generation (also known as "next-gen" or "deep") sequencing biochemistries now permit the parallel acquisition of up to 10s of gigabases (Gb) of DNA sequence of variable "read" length in a single experiment. Multiple NGS options are currently available—454 genome sequencer

(Roche), gene analyzer (Solexa/Illumina), SOLiD system (Applied Biosystems)[5,146]—each with its own strengths and weaknesses. The repertoire and capabilities of these platforms are continually improving. For example, the SOLiD 3 System currently boasts > 20 Gb of DNA sequence per run, compared with ~ 750–1000 bp generated using traditional Sanger sequencing. Although the cost/base ratio is significantly lower for next-generation technologies, the current cost of a single SOLiD 3 run is in the neighborhood of $15,000 (vs ~ $5 for conventional sequencing), making next-generation tools prohibitively expensive for a large percentage of the research community, especially when considering the sequencing of large numbers of patient samples.

The biochemistry and real-time imaging-based data acquisition involved in NGS are what allow these technologies the parallel, high-throughput capacity that is not feasible with conventional Sanger sequencing; detailed reviews on the technical principles of conventional sequencing and NGS have been reported.[5,106,111,146] In the Sanger method, DNA to be sequenced is either randomly fragmented and cloned into a high-copy number plasmid prior to bacterial transformation (shotgun sequencing) or PCR-amplified using target-specific primers (gene-specific sequencing). The amplified template is then subjected to a series of sequencing cycles whereby the template is denatured, primers are annealed, and a new complementary strand is synthesized in the presence of fluorescent-labeled dideoxynucleotide triphosphates ([ddNTPs], 1 unique color for each of the 4). End-labeled DNA fragments are subsequently separated using high-resolution capillary electrophoresis followed by laser excitation of labeled fragment ends, with the emission spectra producing a 4-color chromatogram that can be translated into DNA sequence. Modern Sanger sequencing units can simultaneously run samples in 96- or 384-well format, generating reads of up to 1000 bp in length per sample and providing some degree of throughput.

Despite differences in template amplification and sequencing biochemistry associated with current next-generation platforms, the principles and workflow involved in these technologies are relatively similar. Initially, a template library is prepared through random DNA fragmentation and adapter ligation. Ligated fragments are then bound to micron-sized beads (Roche and Applied Biosystems) or a planar substrate (Solexa/Illumina) and PCR amplified (that is, emulsion PCR or bridge PCR) as clusters or colonies consisting of thousands of clonal "features" to be sequenced. An array or flow-cell can consist of literally millions of clustered features, enabling massively parallel downstream sequencing. So-called sequencing-by-synthesis is then performed with either a polymerase (Roche and Solexa/Illumina) or ligase (Applied Biosystems) that serially extends primed templates. Light (pyrosequencing with Roche) or fluorescence (Solexa/Illumina and Applied Biosystems) emitted following the incorporation of dNTPs (deoxynucleoside triphosphates) or oligonucleotides is then captured by imaging the full array of synthesized features at the end of each cycle. The final result of a full next-generation run currently ranges from up to ~ 1,000,000,000 reads of ~ 400 bp with Roche, ~ 150,000,000 reads of 35 bp with Solexa/Illumina, and ~ 300,000,000 reads of 50 bp with the Applied Biosystems. These figures translate into 10s of Gb of DNA sequence in a single run (1 human genome comprises ~ 3 Gb), allowing for high-coverage, whole-genome sequencing on 1 machine within a few weeks—this in sharp contrast to the sequencing "factories" and several years required for the initial Sanger-based sequencing of the human genome not more than a decade ago.

Multiple "proof-of-principle" studies using next-generation technology have now been performed to analyze the various aspects of both normal and cancer genomes. Published reports have described array-based, targeted capture, and NGS of ~ 200,000 protein-coding exons in the human genome, allowing specific identification of both common and rare sequence variants.[67,116] Unbiased, whole-genome NGS has also been reported for multiple normal human genomes[15,80,167,171] and, recently, the acute myeloid leukemia genome from a single individual.[88] Producing nearly 100 billion bases of sequence, authors of the acute myeloid leukemia study described 10 genes with acquired somatic mutations that were not present in the patient-matched genome from normal skin cells.

Perhaps equally impressive, NGS has not been limited to studies aimed at the identification of sequence variants and mutations.[5,111] Structural aberrations, including inter- and intrachromosomal rearrangements (inversions, inverted/tandem duplications, and translocations) as well as copy number aberrations (amplifications and deletions), have been identified in human cancer cells using NGS, with improved specificity and sensitivity compared with array-based methods.[25,27] Whole-transcriptome profiling (also known as RNA-Seq) has also been described using next-generation approaches, permitting the quantification of transcript abundance (mRNA, miRNA, and so forth) and the identification of novel genes and isoforms in an unbiased manner.[110,113,114,122,169,172] In contrast to the array-based technologies used in gene expression analyses, RNA-Seq requires no a priori knowledge of the transcriptome under investigation—thus enabling full-transcriptome characterization—and eliminates biases associated with array content. Similarly, unbiased NGS has been extended to studies of the mammalian epigenome, including genome-wide analyses of DNA methylomes,[37,102] mapping of histone modifications,[9,108] and detailing of the locations of DNA-binding proteins.[74,139]

Collectively, these emerging NGS-based approaches for studying the cancer genome hold great promise for comprehensive analyses of medulloblastoma. As the cost and bioinformatics involved in next-generation become more mainstream, next-generation–based profiling of the medulloblastoma genome, transcriptome, and epigenome will surely be the priority of several investigative groups (Fig. 6). Undoubtedly, these efforts will lead to a more complete understanding of the genes and pathways involved in the initiation, maintenance, and progression of medulloblastoma. Moreover, as larger patient cohorts are gathered and profiled using these advanced methods, more specific and reliable molecular classification of medulloblastoma will probably be possible. Finally, the correlation of genomic data with patient clinical data,

such as the presence of metastatic disease and overall survival, will undoubtedly be improved.

Conclusions

Twenty years of studying the medulloblastoma genome has facilitated a detailed description of the medulloblastoma karyotype, led to the identification and validation of bona fide oncogenes and tumor suppressors, and implicated key signaling pathways and networks that are recurrently deregulated. Much of the recent progress in this field is owed to improvements in the technologies available for analyzing the genome. To comprehensively appreciate a cancer genome such as medulloblastoma, unbiased, high-resolution, genome-wide investigations must be undertaken, ideally using a combination of complementary (microarrays and NGS) and integrative (genome, transcriptome, and epigenome) technologies. Of course, an adequate sample size is critical to such studies if a full range of both common and rare genomic changes is to be captured. Large, coordinated multiinstitutional consortiums such as The Cancer Genome Atlas (TCGA) are now applying this philosophy to study the genomes of brain (glioblastoma multiforme), lung (squamous carcinoma), and ovarian (serous cystadenocarcinoma) cancers.[26] As part of their pilot project, TCGA is profiling large numbers of tumors of these origins to assess aberrations in DNA sequence (substitutions and indels), genomic copy number (amplifications and deletions), chromosomal composition (rearrangements), DNA methylation (hyper– and hypo–promoter methylation), and gene expression (aberrant expression and splicing). Results from TCGA and other similar large-scale collaborative efforts utilizing this type of broad approach to cancer genomics have recently demonstrated success.[26,36,78,125] To make progress in the fine genomic mapping of medulloblastoma, analogous strategies are warranted.

The generation of large genomic data sets of medulloblastoma using multiple orthogonal technologies will have its challenges. A logistical issue facing potential large-scale genomic projects on medulloblastoma relates to the immensity of the bioinformatics involved, as profiling large sample cohorts inevitably produces large amounts of data of varying complexity. The integration of multiple genomic data sets for maximal extraction of biological information will be a major priority if these new technologies are to be used to their full potential. In addition, the use of NGS technology for cancer genomics studies is still in its infancy, and the cost as well as the bioinformatics and computing challenges related to this promising technology remain a significant hurdle. Before NGS can be efficiently applied to large-scale medulloblastoma projects, financial feasibility and informatics considerations must be addressed.

Discriminating between so-called driver and passenger mutations is also a common yet critical dilemma in cancer genomics studies.[124,149] In other words, not all somatic alterations in a cancer genome actually contribute to cancer development and confer a clonal advantage to a tumor cell. Distinguishing genes that provide a clonal advantage (drivers) in tumorigenesis from those that do not (passengers) requires the integration of multiple data types (copy number, sequencing, and expression) and, ideally, functional validation—neither of which is a simple task when considering possibly hundreds of candidate cancer genes. Thus, the use of genomic studies alone will be insufficient for determining the genes "driving" medulloblastomas, and follow-up functional studies, including mouse models, will be a necessity.

A third issue to consider is the reality of intratumoral heterogeneity and how global profiling strategies are typically performed using nucleic acid extracted from bulk tumor tissue. Methods such as array-based copy number and gene expression profiling essentially produce an average for a genomic region or gene in a given template. Since medulloblastomas are neither histologically nor molecularly monoclonal, genomic events that are present in only a small percentage of cells will be under-called or missed outright when bulk tumor is the source of the template queried. Indeed, the amplification of known medulloblastoma oncogenes, such as *MYC* and *MYCN,* is often found in only 10–20% of cells as determined by FISH (S. Pfister, personal communication, 2009), suggesting that these events are under-called by array profiling. Strategies that involve the analysis of distinct cell populations (that is, laser capture microdissection and cell sorting) or even single cells within a tumor will be required to avoid losing potentially valuable genetic information present only in tumor cell subpopulations.

Despite these relevant technical and logistical considerations, the next few years promise to be an exciting period for the community studying medulloblastoma. Large-scale, collaborative genomics projects will provide a more detailed characterization of this genome than ever before, and, optimistically, many new candidates will be uncovered. These efforts should continue to improve our ability to diagnose, stratify, and treat medulloblastoma, eventually leading to decreased deaths and improved quality of life for patients.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Genetic basis of Parkinson disease

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Over the past few years, considerable progress has been made in understanding the molecular mechanisms of Parkinson disease (PD). Mutations in certain genes are found to cause monogenic forms of the disorder, with autosomal dominant or autosomal recessive inheritance. These genes include *alpha-synuclein, parkin, PINK1, DJ-1, LRRK2,* and *ATP13A2*. The monogenic variants are important tools in identifying cellular pathways that shed light on the pathogenesis of this disease. Certain common genetic variants are also likely to modulate the risk of PD. International collaborative studies and meta-analyses have identified common variants as genetic susceptibility risk/protective factors for sporadic PD. *(DOI: 10.3171/2009.10.FOCUS09220)*

Key Words • Parkinson disease • genetic association study • polymorphism • gene mutation

Parkinson disease is the second most common neurodegenerative disorder after Alzheimer disease. The average age at onset is between 60 and 80 years, and ~ 1% of the general population older than 65 is affected.[12,89]

Although PD appears to be sporadic in most cases, linkage and positional cloning studies were instrumental in the identification of certain genes that cause familial forms of the disease that feature autosomal dominant or autosomal recessive inheritance.[12,89] Approximately 5–10% of patients with the clinical picture of PD carry a mutation in one of the known genes that cause monogenic forms of the disorder.[12,89] There is some evidence that these genes may also play a role in the much more common sporadic form of the disease. The biochemical function of these genes can underlie various pathogenetic pathways, such as oxidative stress, mitochondrial dysfunction, disturbance in protein quality control, and altered kinase activity that may lead to PD.

The vast majority of cases of this disorder are sporadic, resulting from complex interactions among genes, and between genes and environmental factors. Genetic variations may be susceptibility factors for disease that also affect penetrance, age at onset, severity, the appearance of certain clinical features, and also the disease progression.

In this paper, a concise summary of the main genes responsible for monogenic forms of PD (Table 1), and some of the common genetic variants that may play a role in the disease, is provided.

Monogenic Forms of PD

Alpha-synuclein (SNCA-PARK1/PARK4) is central to the pathophysiological mechanisms of familial and sporadic PD. This protein is the major component of Lewy bodies and Lewy neuritis in PD, and also in other synucleinopathies. Three missense mutations (A53T, A30P, E64 K), and duplications or triplications of the locus containing SNCA have been identified previously in families with autosomal dominant PD.[29,42,65,67,76,77,90,92] The phenotype of patients with SNCA point mutations is that of levodopa-responsive parkinsonism in patients with a relatively young age at onset, rapid progression, and high prevalence of dementia, psychiatric, and autonomic disturbances. Patients with duplications resemble those with idiopathic PD,[10] and those with triplications have earlier onset, faster disease progression, severe dementia, and frequent dysautonomia.[23,76] There is consensus that any change in the levels of alpha-synuclein expression or the presence of mutations in alpha-synuclein has a toxic ef-

Abbreviations used in this paper: DBS = deep brain stimulation; PD = Parkinson disease; SNP = single nucleotide polymorphism.

TABLE 1: Genetic loci and genes associated with monogenic forms of PD*

Locus	Gene	Inheritance & Comments	OMIM No.
PARK1	SNCA	AD; 1st PD gene identified	168601
PARK2	PRKN	AR; most common cause of recessive juvenile PD	602544
PARK3	SPR?	AD	602404 & 182125
PARK4	SNCA	AD	168601
PARK5	UCHL1	AD	191342
PARK6	PINK1	AR; 2nd most common cause of recessive juvenile PD	605909
PARK7	DJ-1	AR	606324
PARK8	LRRK2	AD; most common cause of dominant PD	607060
PARK9	ATP13A2	AR; PD plus dementia & spasticity	606693
PARK10	unknown	?	606852
PARK11	GIGYF2?	AD	607688
PARK12	unknown	?	300557
PARK13	Omi/HTRA2	?	610297
PARK14	PLA2G6	AR; PD plus adult-onset dystonia	612953
PARK15	FBXO7	AR; PD plus dementia & spasticity	260300

* AD = autosomal dominant; AR = autosomal recessive; OMIM = Online Mendelian Inheritance in Man; ? = controversial or unidentified.

fect on dopaminergic neurons.[18] Alpha-synuclein monomers interact under certain circumstances to form protofibrils or fibrillar β-pleated sheets.[84,86,89] Toxicity caused by protofibrils may involve the leakage of dopamine from synaptic vesicles because of perforation of the vesicular membranes by these protofibrils.[29] This may account for the selective toxicity of alpha-synuclein in the dopamine-producing neurons of the substantia nigra.[13,56,84]

Parkin (PRKN, PARK2) was the first gene identified for an autosomal recessive form of PD.[1,5,35,40,83] Parkin protein localizes, although not predominantly, to the synapse and associates with membranes. Its main function is as an ubiquitin ligase in the cellular ubiquitination protein degradation pathway. Severe and selective degeneration in the substantia nigra pars compacta but without Lewy bodies has been described, suggesting that the disease may differ in some important ways from typical idiopathic PD.[35,68,83,91] *Parkin* mutations turned out to be a very common cause of parkinsonism. All types of mutations; missense mutations, nonsense mutations, and exonic rearrangements were identified. The vast majority of patients with *parkin* mutations manifest the disease before the age of 40 years, with slow progression, but with levodopa-associated fluctuations and dyskinesias occurring early and frequently. Unusual features such as focal dystonia, early postural instability, and autonomic failure may also be present.

Mutations in the *PINK1* gene *(PARK6)* have also been identified as a cause of autosomal recessive early-onset parkinsonism.[81,82] This gene links PD to mitochondrial dysfunction and oxidative stress as it encodes a primarily mitochondrial protein kinase. This protein may exert a protective effect on the cell.[2,69] No pathological mechanism has yet been reported in patients with *PINK1* mutations. Patients with mutations in this gene are characterized by early onset of parkinsonism (between 32 and 48 years of age), with slow progression and sustained response to levodopa. There are also some indications that they have a higher prevalence of psychiatric disturbances.[3,17]

The third locus for autosomal recessive juvenile parkinsonism was mapped also to chromosome 1p36, and the gene was identified as the oncogene *DJ-1 (PARK7)*.[6] The function of the DJ-1 protein is not entirely clear. The main hypothesis is that it acts as a sensor for oxidative stress, providing neuroprotection in situations of increased demand.[7,93] Parkinson disease causing *DJ-1* mutations is rare and accounts for only ~ 1–2% of early-onset autosomal recessive PD cases. The phenotype closely resembles that found in patients with *parkin* and *PINK1* mutations.[6] The associated pathological characteristics are still unknown, because no autopsies have been reported.

Mutations in *LRRK2 (PARK8)* have been found in a large number of patients with PD.[63,94] More than 40 different variants, almost all missense mutations, have been reported.[4,37] The *G2019S* mutation in particular was detected in 5–6% of autosomal dominant familial PD cases and in 1–2% of sporadic cases.[28,46,62,80] Specific populations such as Ashkenazi Jews and North African Arabs

were found to have an even higher prevalence.[45,62] The LRRK2 is a large protein with multiple protein interactions and catalytic domains that have possible roles in intracellular signaling pathways.[58,85] Some pathogenetic mutations seem to be associated with an increase of kinase activity, which appears to be necessary for neurotoxicity in vitro.[32,39] Although LRRK2 was not found to interact with either alpha-synuclein or tau, the identification of Lewy body or neurofibrillary tangle pathological features in patients with *LRRK2* mutations suggested a possible common role of LRRK2 in the processing of those two proteins.[85] The phenotype of LRRK2 mutation carriers is that of idiopathic PD, although the whole clinical picture is more benign.

Other Genes and Loci

The *ATP13A2* gene is mapped at the PARK9 locus and is responsible for Kufor-Rakeb disease, a recessive, juvenile-onset, atypical Parkinsonism with pyramidal degeneration and cognitive dysfunction.[71] The *ATP13A2* gene encodes a large lysosomal P-type adenosine triphosphatase, which is involved in the lysosomal degradation pathway that clears SNCA aggregates. Lysosomal dysfunction caused by mutations in this gene might contribute to the pathogenesis of parkinsonism.[83]

A heterozygous missense mutation in the *ubiquitin carboxy-terminal hydrolase L1* gene *(UCHL1, PARK5)*, which is located on chromosome 4p, has been identified in a single affected family of German ancestry. However, whether *UCHL1* is really a PD gene is not clear yet.[87]

Mutations in the *glucocerebrosidase* gene *(GBA)* (which has not been assigned a PARK locus yet) are the cause of a recessive lysosomal storage disorder—Gaucher disease. More than 200 mutations have been described in *GBA*. Phenotypes of Gaucher disease and PD do not overlap significantly, but the first indication for a relationship between the two actually came from clinical descriptions. Mutations in *GBA* have a conspicuously high prevalence in patients with PD.[57] Between 2 and 4% of Caucasian patients with PD have been found to have mutations in *GBA*. Those variants are likely to act as risk factors rather than as high-penetrance disease genes.[59] The nature of the association between PD and Gaucher disease remains elusive. However, the pathogenic mechanisms leading to PD in carriers of mutant *GBA* may be related to the faulty processing of toxic proteins, aggravated by the relative decrease in *GBA* activity and accumulation of glucocerebrosidase.[50,61] Moreover, recent findings indicate that Gaucher disease and PD share pathophysiological features.[88]

Other mendelian forms of PD remain to be identified. Either the causal genes at several loci have not yet been identified *(PARK12)*, or the role of the candidate genes at these loci is still controversial *(PARK3, PARK10, PARK13)*. Two novel genes, the *FBXO7 (PARK15)*, a member of the F-box family of proteins active in the ubiquitin-proteasome protein degradation pathway,[14] and the *PLA2G6* gene *(PARK14)* on chromosome 22, have also been identified in families with atypical PD.[33]

Neurosurgical Treatment in Familial PD

The identification of inherited forms of PD has also helped in developing a more appropriate application of neurosurgical treatments of the disease. Deep brain stimulation provides symptomatic benefits to patients with idiopathic PD in terms of both motor activity and quality of life.[72] In hereditary PD, DBS is an efficacious symptomatic treatment for patients with parkinsonism and mutation of the *parkin* and *PINK1* genes.[8,73] Patients with *parkin* mutations are specifically expected to be very good candidates for DBS and to benefit more and longer than other patients because of their younger age at onset, lower daily doses of levodopa, and slower disease progression.[8,51,73] The response to DBS among patients carrying mutations of the *LRRK2* gene is not well established.[46,75] There are some studies that show that the response to DBS was worse among patients with the R1441G mutation in *LRRK2* compared with patients with idiopathic PD.

Based on the aforementioned findings, we conclude that the effectiveness of DBS in different genetic forms of PD has not been studied adequately. The main reason for this is that patient selection is based predominantly on clinical criteria.[9,52] A multidisciplinary approach involving a neurosurgeon, a neurologist, and a neuropsychologist is important to determine the appropriate surgical candidate. The best prognostic indicator of a patient's suitability for DBS surgery is his/her response to levodopa.[9] The patient's age also is another major factor determining how an individual will cope with the surgical procedure and behave postoperatively.[74] Based on these criteria, many patients with genetic forms of the disease who have a sustained response to levodopa[38] or atypical symptoms are excluded from this procedure. As a result, it is impossible, based solely on clinical characteristics of monogenic PD forms, to study the effectiveness of DBS in these patients. Another reason for this is that most patients who have undergone DBS surgery are not screened for mutations in various genes responsible for monogenic forms of the disease.

The detailed evaluation of these patients and the genetic analysis based on criteria such as the family history, phenotype, age at onset, and response to levodopa are of great importance. However, larger series of patients with mutations and longer follow-ups will be needed for evidence of specific genotype-related differences.

Another matter worth discussing is that there are also no studies showing the optimal DBS target based on genetics. Generally the literature demonstrates a trend that the subthalamic nucleus may be more efficient in managing the symptoms of PD, based on institutional experience, surgical and programming management, lower current requirements, and significant reduction in dopaminergic medication.[38,41,66] For these reasons, all of the previous studies in patients with mutations have referred to the effectiveness of DBS of the subthalamic nucleus.[30,51,75] Maybe the genetic forms of the disease with the different neuropathological characteristics will enable deciphering of the mechanisms of DBS on the basis of the function and pathophysiological characteristics of the

cortico-basal-thalamo-cortical loops when different DBS targets are being applied to them.

Genetic Susceptibility Factors in Sporadic PD

Monogenic forms represent < 10% of cases of PD. Common PD, on the other hand, is thought to result from complex interactions involving genetic and environmental risk factors. The discovery that 1-methyl-4-phenyl tetrahydropyridine, a contaminant of a synthetic opiate, can cause parkinsonism through its neurotoxic metabolite, 1-methyl-4-phenylpyridinium, stimulated interest in environmental chemical exposures as risk factors for PD.[44] Many studies have investigated the association between PD and pesticide use, and some, but not all, have found an association.[22,70,95] Use of well water, rural living, and agricultural employment have also been implicated as risk factors, although studies have given conflicting results.[43,95]

On the other hand, the extent of the genetic component remains elusive. Common genetic variations (mainly SNPs) may be either susceptibility factors or disease modifiers, affecting penetrance, age at onset, severity, or disease progression.

Genetic association studies that compare the frequency of putative risk alleles in cohorts of patients and controls are controversial because they have failed to reproduce the initial positive findings most of the time. Almost 800 genetic association studies have been performed so far on more than 500 genes regarding PD (see www.pdgene.org). The vast majority of genetic association studies have focused on candidate genes involved in detoxification of metabolites, dopamine metabolism, mitochondrial function, and familial PD.[78] Some of these findings were exciting at the beginning because the encoded proteins of these genes appear to be closely linked to the pathophysiological mechanisms of PD; however, none of these candidate gene variants have been consistently replicated since then. Thus, theoretically attractive, broad-based meta-analyses yielded no true common genetic risk variant. Potential biases include population stratification, small sample size, misclassification, and inappropriate statistical methods.[36]

Nevertheless, specific polymorphic variants have been validated as genetic susceptibility factors. The Rep 1, a mixed nucleotide repeat in the promoter region of SNCA, has been confirmed as a risk factor.[11,34,53,55,60,64] A polymorphism in microtubule-associated protein tau has also been detected.[26] The combination of risk genotypes in SNCA and microtubule-associated protein tau doubles the risk of PD.[27,31] Two variants in the *LRRK2* gene, *G2385R* and *R1628P,* confer susceptibility to PD in Asian populations.[15,21,24,80] An S18Y variant of the *UCHL1* gene has been demonstrated to be protective against PD in some association studies and meta-analyses.[5,79] The number of polymorphisms that have been studied until now is very large, but so far these are some of the main risk alleles for sporadic PD that seems to be robustly reproducible.

With the completion of the human "HapMap" project and the availability of the SNP databases, there is increasing interest in using the whole-genome association approach to unravel genetic susceptibility factors. Genome-wide association studies of PD, which use haplotype tagging strategies to study variation across the entire human genome, provided little evidence until now about genetic variants that influence the risk for disease. A 2-stage genome-wide association study with a 200-SNP map, and a 1-stage study with more informative markers found no positive associations.[25,54] The most strongly associated SNPs identified in the 2-stage study were not replicated in a subsequent association study with a large number of participants.[16,54] However, the combination of genome-wide databases with meta-analytical techniques can improve the detection of genetic variants with small effect sizes. The GAK-DGKQ region on chromosome 4 has been identified by this strategy, albeit not replicated in a recent report.[19,20] The genomic pathway approach that combines SNPs with axon guidance pathway genes has also been applied to genome-wide association studies, with one positive result,[48] but again without replication.[49] Genome-wide association studies require large sample series and international collaborations, so probably we will have to wait for a few years to identify possible common genetic risk variants and clearly understand their role in the disease.[20,25,36,47,54]

Conclusions

It is hoped that an understanding of the genetic basis of PD will allow us to identify upstream key facts of the pathogenesis and lead to new targeted therapeutic strategies in the future. Large-scale multicenter collaborations, public availability of the International HapMap Project, and genome-wide association PD databases will hopefully arm researchers with information that could be used for modifying the natural course of the disease.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Neurofibromatosis Type 1 and tumorigenesis: molecular mechanisms and therapeutic implications

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Neurofibromatosis Type 1 (NF1) is a common autosomal dominant disease characterized by complex and multicellular neurofibroma tumors, and less frequently by malignant peripheral nerve sheath tumors (MPNSTs) and optic nerve gliomas. Significant advances have been made in elucidating the cellular, genetic, and molecular biology involved in tumor formation in NF1. Neurofibromatosis Type 1 is caused by germline mutations of the *NF1* tumor suppressor gene, which generally result in decreased intracellular neurofibromin protein levels, leading to increased cascade Ras signaling to its downstream effectors. Multiple key pathways are involved with the development of tumors in NF1, including Ras/mitogen-activated protein kinase (MAPK) and Akt/mammalian target of rapamycin (mTOR). Interestingly, recent studies demonstrate that multiple other developmental syndromes (in addition to NF1) share phenotypic features resulting from germline mutations in genes responsible for components of the Ras/MAPK pathway. In general, a somatic loss of the second *NF1* allele, also referred to as loss of heterozygosity, in the progenitor cell, either the Schwann cell or its precursor, combined with haploinsufficiency in multiple supporting cells is required for tumor formation. Importantly, a complex series of interactions with these other cell types in neurofibroma tumorigenesis is mediated by abnormal expression of growth factors and their receptors and modification of gene expression, a key example of which is the process of recruitment and involvement of the $NF1^{+/-}$ heterozygous mast cell. In general, for malignant transformation to occur, there must be accumulation of additional mutations of multiple genes including *INK4A/ARF* and *P53,* with resulting abnormalities of their respective signal cascades. Further, abnormalities of the *NF1* gene and molecular cascade described above have been implicated in the tumorigenesis of NF1 and some sporadically occurring gliomas, and thus, these treatment options may have wider applicability. Finally, increased knowledge of molecular and cellular mechanisms involved with NF1 tumorigenesis has led to multiple preclinical and clinical studies of targeted therapy, including the mTOR inhibitor rapamycin, which is demonstrating promising preclinical results for treatment of MPNSTs and gliomas. *(DOI: 10.3171/2009.11.FOCUS09221)*

Key Words • **optic nerve glioma** • **loss of heterozygosity** • **malignant peripheral nerve sheath tumor** • **neurofibroma** • **plexiform neurofibroma**

Neurofibromatosis Type 1 is a common autosomal dominant disease and is characterized by neural crest-derived tumors.[18,101] The key feature of NF1, neurofibromas, are complex tumors arising from peripheral nerve sheaths.[59,101] Neurofibromas consist primarily of Schwann cells, fibroblasts, and a large amount of extracellular matrix with collagen surrounding an axon, but they also contain many other cell types including perineural cells, mast cells, pericytes, and endothelial cells.[9,59,80,85,101] Malignant tumors can arise in either childhood or adulthood, with MPNSTs being most common.[16] In addition to the lesions associated with the peripheral nervous system, patients with NF1 are also at risk for CNS tumors, specifically gliomas.[2,42] In childhood, these gliomas are primarily located in the optic pathway and less frequently in the hypothalamus and brainstem; adults are more likely to develop higher-grade gliomas.[2]

Our group has previously published an overview of the genetics and pathogenesis of neurofibromas in NF1.[38] In the present paper, we update the relevant information related to the genetic alterations that predispose individuals to this tumor and also discuss the mechanisms involved in the tumorigenesis of MPNSTs and optic nerve gliomas, with attention on implications for targeted therapies (not previously presented).

Abbreviations used in this paper: GAP = GTPase activating protein; GDP = guanosine diphosphate; GRD = GAP-related domain; GTP = guanosine triphosphate; MPAK = mitogen-activated protein kinase; MPNST = malignant peripheral nerve sheath tumor; mTOR = mammalian target of rapamycin; NF1 = neurofibromatosis Type 1.

Genetics

As background information, we include here a brief summary of the genetics of this disease. In this auto-

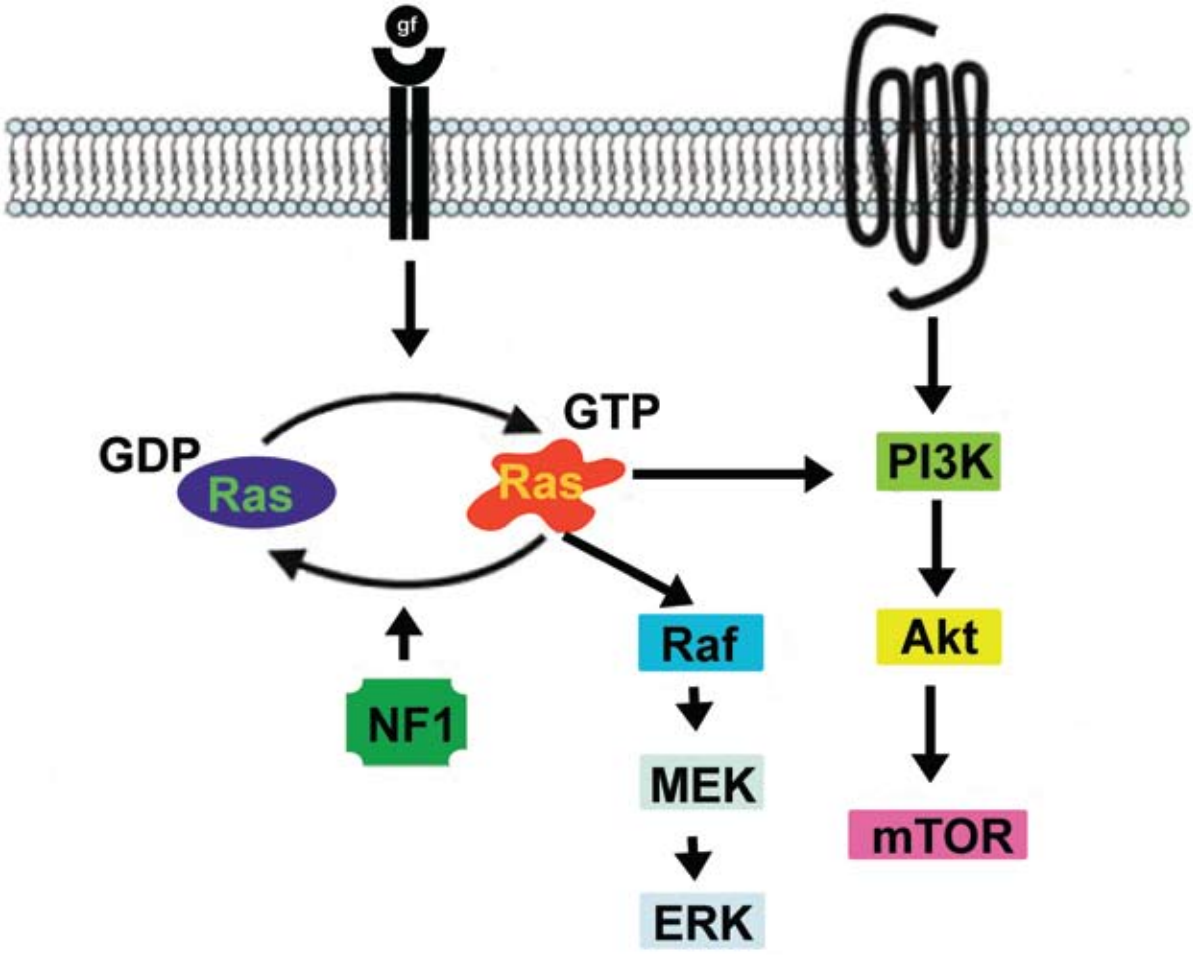

Fig. 1. Illustration depicting the signal transduction pathways involved in NF1 tumorigenesis. Growth factors (gf) interact with receptors at the cell surface, activating guanine nucleotide exchange factors and resulting in activated Ras. Activated Ras sends intracellular signals activating the phosphoinositol 3′ kinase (PI3K), AKT, and mTOR pathway. Activated Ras also activates the Raf-MAK (mitogen-activated kinase)/MEK pathway to stimulate ERK (extracellular signal–related kinase), which can enter the nucleus and promote transcription. Normally, neurofibromin downregulates Ras through its GAP-related domain, and therefore in its absence or at decreased levels in NF1, signaling is increased through all of these pathways resulting in cell proliferation and inhibited apoptosis. Adapted by permission from Macmillan Publishers Ltd: **Oncogene 26**: 4609–4616, copyright 2007.

somal dominant–inherited condition, in which homozygosity is lethal to embryos,[15,46] all affected individuals are heterozygous for an *NF1* mutation.[35] One *NF1* allele carries a genetic alteration in all cells of a patient with NF1, and therefore, a loss of the second *NF1* allele (loss of heterozygosity, LOH) results in complete functional loss of neurofibromin[31,52,86,87] and risk of tumor formation.[31,32,47,86] Point mutations affecting the correct splicing of the *NF1* gene are a common cause of NF1,[4,33,73] responsible for both somatic and germline mutations.[87] Many mutations in the *NF1* gene result in truncation of the protein product, neurofibromin.[96] Approximately half of cases represent new mutations,[35] and a mutation at a given locus may provide a selective and proliferative advantage in a germ-cell precursor.[84]

Positional cloning identified the *NF1* gene at 17q11.2.[19,97,103] It produces an mRNA[9,67] that is expressed in almost all tissues[103] but most highly in brain, spinal cord, and the peripheral nervous system.[24,98] Neurofibromin, the protein product of the *NF1* gene, is found in neurons, oligodendrocytes, and Schwann cells in adults,[24,98] and is also expressed in other cell types, such as keratinocytes, adrenal medulla, and white blood cells.[40,98] Neurofibromin is reduced or absent in neurofibroma cells,[39,55] which are composed principally of Schwann cells without functional neurofibromin.[77]

Signal Transduction Pathways

Although it only occupies a small area of the protein (360 amino acids), the Ras-GTPase activating protein (GAP)-related domain (GRD) is an important functional region of neurofibromin[17,106,107] that stimulates the intrinsic GTPase of p21-Ras-GTP to hydrolyze GTP to GDP, inactivating p21-Ras (Fig. 1).[1,6,14,68,93,106] Inactivation of the active Ras-GTP is the main function of neurofibromin.

The functional domain of neurofibromin, Ras, acts as

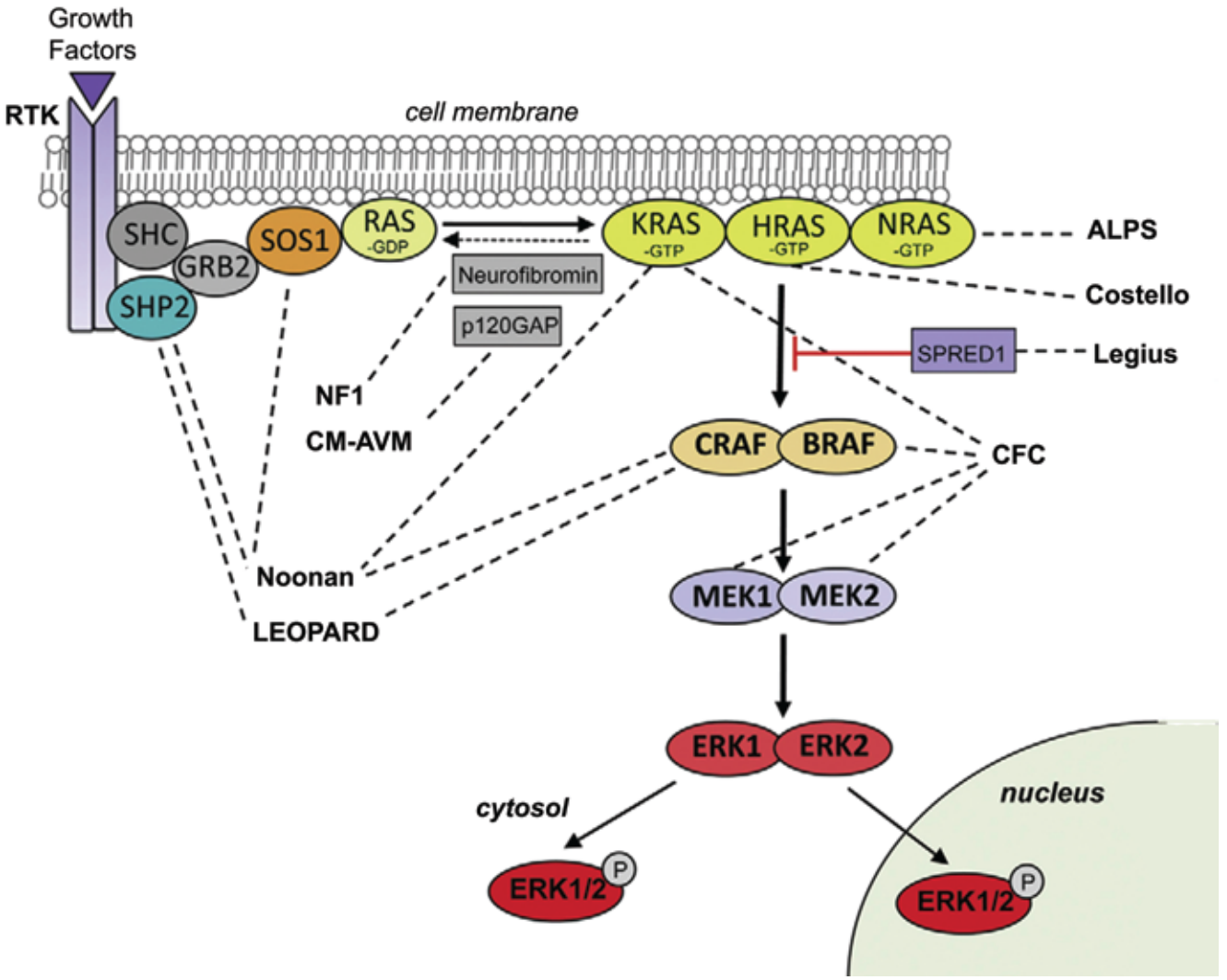

Fig. 2. Drawing demonstrating the common Ras/MAPK pathways responsible for multiple developmental syndromes, including NF1. In addition to similar molecular origins, these Ras-related diseases, including Noonan syndrome, LEOPARD syndrome, hereditary gingival fibromatosis 1, capillary malformation–arteriovenous malformation (CM-AVM) syndrome, Costello syndrome, autoimmune lymphoproliferative syndrome (ALPS), cardio-facio-cutaneous (CFC) syndrome, and Legius syndrome, have considerable phenotypic overlap. Thus, targeted molecular treatment may be effective for multiple diseases. Figure reproduced with permission from Tidyman and Rauen.[92]

part of a signal transduction pathway that is activated by growth factors and their receptors.[8] Increased Ras-GTP leads to increased signaling through Raf kinase, which activates a kinase cascade involving MEK kinase and the Erk1 and Erk2 isoforms of MAPK resulting in cell proliferation.[13,27,91] Increased Ras-GTP also protects cells from apoptosis by activating mTOR.[10,51] Studies confirm that neurofibromin negatively regulates this mTOR pathway with loss of neurofibromin expression in established human neurofibroma cell lines associated with high levels of mTOR activity.[10,51] The mTOR pathway is constitutively activated in both NF1-deficient primary cells and human tumor, is dependent on Ras and PI3K activation, and is mediated by the phosphorylation and inactivation of the TSC2-encoded protein tuberin by AKT.[51] Overall, Ras is a key component of many growth factor signaling pathways, and in the absence of neurofibromin it is constitutively activated, resulting in increased cell proliferation and survival.[98]

The Ras/MAPK pathway is critical to normal development by its regulation of cell proliferation, differentiation, motility, growth, apoptosis, and cell senescence.[92] Interestingly, there are multiple developmental syndromes in addition to NF1 that form tumors by abnormalities in the Ras pathway, and they are referred to as the "RASopathies" or "neuro-cardio-facial-cutaneous syndromes."[26,92] These diseases include Noonan syndrome, LEOPARD syndrome, Costello syndrome, capillary malformation–arteriovenous malformation, and cardio-facio-cutaneous syndrome; these syndromes and their relation to the Ras/MAPK pathway are reviewed in detail in Tidyman and Rauen (Fig. 2).[92] They are related by germline mutations in genes that encode protein components of the Ras/MAPK pathway, and these mutations may result in

increased signal transduction.[92] Each syndrome exhibits unique phenotypic features; however, there are overlapping clinical features including characteristic facial features, cardiac defects, skin abnormalities, developmental delay, and a risk of malignancy.[92] Interestingly, there are several NF1-related pathways that are significant in the pathogenesis of other developmental syndromes, including mTOR, which contributes to tuberous sclerosis.[30,44,74,75] Denayer and colleagues[26] noted that these syndromes with common molecular pathways demonstrated the important roles for evolutionarily conserved pathways not only in oncogenesis, but also in cognition, growth, and development.

Malignant Peripheral Nerve Sheath Tumors

Additional molecular cascades may be involved in tumorigenesis for malignant NF1 tumors.[12] For example, Ral overactivation is a novel cell signaling abnormality in MPNSTs.[12] Of note, overactivation of Ras and many of its downstream effectors occurred in only a fraction of MPNST cell lines, but Ral was overactivated in all MPNST cells and tumor samples.[12] Silencing Ral or inhibiting it with a dominant-negative Ral caused a significant reduction in proliferation, invasiveness, and in vivo tumorigenicity of MPNST cells.[12] Also, neurofibromin is shown to regulate Ral activation, and NF1-GRD treatment caused a significant decrease in proliferation, invasiveness, and cell cycle progression, and increased cell death.[12] The mTOR pathway has also been shown to have an important role in NF1-associated malignancies including MPNST.[50]

Among the genetic abnormalities previously reported to be involved in the transformation to malignancy in NF1,[39] the loss or mutation of the *P53* gene in NF1-related MPNSTs (but never in benign neurofibromas)[11,37,49,58,63,64,66,72,78,82,95,105] is associated with a poor prognosis.[65] The loss of the *P53* gene results in abnormalities in DNA damage–induced cell cycle arrest and apoptosis.[95] Approximately half of MPNSTs in patients with NF1 show homozygous deletions at the *CDKN2A* (also known as *INK4A* or *INK4A/ARF*) gene, which contributes to increasing cellular proliferation.[95] Also, MPNSTs demonstrate a high frequency of microsatellite instability, and thus, additional loci become targets for mutations during the malignant transformation.

Gliomas

There is evidence that the *NF1* gene is involved in the tumorigenesis of both NF1-related and sporadically occurring gliomas.[70,79,112] For example, a genomic analysis of human glioblastoma multiforme identified that the *NF1* gene was mutated in 15% of tumors.[79] As in other NF1 tumors, biallelic *NF1* gene inactivation is observed in NF1-associated gliomas and the heterozygous supporting cell or astrocyte is also important for tumorigenesis.[5] Of note, McGillicuddy et al.[70] found that *NF1* function is inactivated in sporadic gliomas, and it occurs by both proteasomal degradation and genetic loss. For example, neurofibromin protein destabilization is triggered by the hyperactivation of protein kinase C (PKC) and thus, these tumors may be sensitive to PKC inhibitors. They found that complete genetic loss of *NF1* occurs when p53 is inactivated and allows sensitivity to mTOR inhibitors.[70] Yeh et al.[112] found that there is variability in *NF1* gene expression in distinct populations of glia and increased glial cell proliferation in the optic nerve and brainstem but not in other areas after *NF1* inactivation in vitro and in vivo, and thus differences in *NF1* gene expression might contribute to the regional localization of brain tumors. Recent studies also demonstrated that the mTOR pathway is involved in tumorigenesis of gliomas in NF1,[16,42,50,51,70] and its inhibitor rapamycin is effective in decreasing tumor proliferation in an animal model.[42]

Neurofibromas

Neurofibromas are unique among tumors because they exhibit extensive cellular heterogeneity.[85,89] Reciprocal signaling among the many cell types is known to occur in normal peripheral nerve sheath and in neurofibromas,[48,56] and decreased neurofibromin in neurofibromas may result in altered responses. Despite this heterogeneity, the Schwann cell lineage has been recognized as the primary cell type for neurofibroma formation. This cell type is the major one amplified in neurofibromas and is the primary target of growth factors stimulating neurofibroma formation.[81,90,102] In addition, the loss of both *NF1* alleles occurs exclusively in the Schwann cell, whereas a wild-type gene is retained in the other neurofibroma cells, including fibroblasts.[57,88] This Schwann cell, which lacks functional neurofibromin, has a substantial growth advantage[77] and a loss of negative autocrine growth control.[76]

The stage of differentiation of the Schwann cell as the progenitor cell for NF1 tumors is still the subject of some debate.[53,61] In 2009, Le and coworkers[61] proposed that the neural crest stem cell is the cell of origin for cutaneous neurofibromas after identifying a population of stem/progenitor cells residing in the dermis (called skin-derived precursors) that, through loss of *NF1*, form neurofibromas. Interestingly, in another study, Joseph et al.[53] showed that stem cells did not persist postnatally in the location of future neurofibromas or MPNSTs and proposed that the cell of origin is a more differentiated glial cell. Finally, Zheng and coworkers[113] noted that plexiform neurofibroma results from *NF1* deficiency in progenitor cells of peripheral nerves. These cells did not cause hyperproliferation or tumorigenesis in the early postnatal period and peripheral nerve development appeared largely normal except for abnormal Remak bundles, the nonmyelinated axon-Schwann cell unit, identified in postnatal mutant nerves. Subsequent degeneration of abnormal Remak bundles occurred concurrently with expansion of nonmyelinating Schwann cells. These findings led the authors to suggest that abnormally differentiated Remak bundles might be the cell of origin for plexiform neurofibroma.

Haploinsufficiency of the *NF1* gene in the microenvironment of neurofibromas contributes to its tumorigenicity.[71] The single active *NF1* allele in the heterozygous supporting cell does not generate enough functional protein

to achieve an appropriate biological response,[29] and this confers a growth advantage that contributes to tumorigenesis.[45] The neurofibromin-deficient NF1$^{+/-}$ Schwann cell and other heterozygous supporting cells are absolutely necessary for tumor formation and are critically involved in the pathogenesis of neurofibromas.[77,114] Also, inflammatory cells, including mast cells, are important to tumor initiation, progression, and angiogenesis.[22,23,41] Neurofibromin-deficient Schwann cells secrete increased kit ligand, which serves as a chemoattractant for mast cells expressing c-kit receptor. Also, heterozygous inactivation of NF1$^{+/-}$ promotes migration of mast cells on α4β1 integrins (mast cell surface proteins), in response to the kit ligand. Overall, the loss of *NF1* in Schwann cells results in an increase in growth factor production that initiates a paracrine loop, and it is important for tumor initiation and progression.[109]

Normally, growth factors cooperate to suppress cell death in Schwann cell precursors,[36] but growth factor dysregulation is thought to be involved in tumorigenesis.[83] Changes in growth factor expression may be the direct result of *NF1* gene loss or from secondary genetic events.[69] Abnormal growth factor receptor expression also has a role in tumorigenesis, progression, and malignant transformation.

Preclinical Models

The development of animal models for preclinical testing in NF1 is key to identifying novel targets for future therapies.[43] Early xenograft models for studying neurofibroma formation produced tumors by injecting human neurofibroma tissue or Schwann cell preparations into the sciatic nerve of immunodeficient mice.[3,62,89] Subsequently, *Nf1* transgene mouse models were developed based on a mutation identified in patients with NF1.[15,46] It was hoped that transgene mouse models would prove to be a suitable model for human NF1, but mice heterozygous for the *Nf1* mutation (*Nf1*$^{+}$/*Nf1*^{n31}) do not develop the classic phenotype of NF1 disease including neurofibromas,[46] and mice that are homozygous for the *Nf1* mutation die in utero.[15,46] Later, chimeric mice (*Nf1*$^{-/-}$:*Nf1*$^{+/+}$) were developed by injection of *Nf1*$^{-/-}$ homozygous mutant embryonic stem cells at an early developmental stage (blastocysts) to overcome the lethality of the germline homozygous genotype with some success.[20] Although the mice developed multiple neurofibromas resembling human plexiform neurofibromas, the cell type in which the *Nf1* was deleted could not be controlled.[71] Next, a conditional mouse model was developed that resulted in somatic inactivation of *Nf1* and ablation of neurofibromin function specifically in Schwann cells.[114] This animal model, *Nf1*$^{flox/-}$;Krox20-cre, provided evidence for the necessity for haploinsufficient supporting cells in neurofibroma formation,[114] and all mice developed plexiform neurofibromas with all of the typical supporting cells (*Nf1*$^{+/-}$).

A mouse model was developed for MPNSTs by generating mice with mutations in both *Nf1* and *p53* genes.[20,100] These mice (*Nf1*$^{+/-}$:*p53*$^{+/-}$) developed soft-tissue sarcomas and MPNSTs in neural crest–derived tissue.[20,100] This model demonstrates that a mutation in the *p53* gene in addition to that in *Nf1* is required for malignant transformation of cells of neural crest origin.[20,100]

There are also *NF1*-deficient animal models of NF1 that produce gliomas.[5,42] In contrast to astrocyte-restricted *NF1* conditional knockout mice, heterozygous mice lacking *NF1* in astrocytes develop optic nerve gliomas. Thus, NF1 glioma formation requires additional cellular or genetic conditions. This mouse model demonstrates that *NF1*$^{+/-}$ cells contribute to the pathogenesis of gliomas in NF1 and provides a tool for the preclinical evaluation of potential therapeutic interventions.

Targeted Treatment

Targeted therapy will have a great impact for patients with numerous neurofibromas, very massive plexiform neurofibromas, or MPNSTs for which surgery and radiation have not been widely successful,[54,94] and as an adjunct or primary treatment of gliomas. Currently, the principal mode of therapy for spinal neurofibromas or plexiform neurofibromas is surgery. Although isolated single–nerve root neurofibromas can be resected without significant morbidity, patients with multiple spinal neurofibromas often require multiple surgeries during their lifetime. Also, patients with large plexiform neurofibromas represent a surgical challenge; have a high recurrence rate if tumors are subtotally resected, intense adherence, and invasion into local tissue; and have a risk of malignant transformation.[54,94] Many preclinical studies, as well as a few clinical trials using biological-based therapeutic approaches to target specific genetic or molecular events involved in NF1 tumorigenesis are in progress. In the future, these medical therapies may be useful for treatment of residual tumors, or as a measure to reduce tumor size prior to surgery, or even to decrease the development of tumors. It may also be possible to apply new therapeutic agents to the local tumor environment to reduce the incidence of recurrences.

Preclinical or clinical studies have targeted various aspects of mast cell functioning, the Ras signaling pathways, rapamycin, and growth factors and receptors, but future therapies may be targeted at almost any molecule involved in tumorigenesis (Fig. 1). A number of strategies were discussed in our previous review. Proposed treatments that reduce the mast cell activity, migration, or numbers within neurofibromas and mast cell stabilizers have been considered as possible therapeutic strategies.[109,110] Options for treatment include pharmacological inhibition of PI3K, kit activity, or α4β1 adhesion.[109,110] The Ras signal transduction pathways have also been targeted for treatments.[104,108] Anti-Ras therapies are an ideal target, because *RAS* is one of the most common oncogenes mutated in human malignancies,[104] and Ras-GTP is elevated for both benign and malignant NF1 neurofibromas.[39] Treatment options include the delivery of gene therapy vectors with functional NF1 GRD proteins or with dominant negative Ras, which blocks Ras activity.[60,98] Other options include the injection of neutralizing Ras antibodies, which inhibits cell proliferation,[7,25] or viruses that target and infect cells with activated Ras signaling.[21] For example, the human reovirus requires an activated Ras

signaling pathway for infection of cultured cells and has been shown to cause tumor regression in mouse models.[21] The downstream effectors of the Ras signaling pathway,[104] including agents that inhibit MEK[28,34] and PI3K,[99,111] are promising targets for future neurofibroma treatment.

Newer studies are focusing on the mTOR pathway and its inhibitor rapamycin.[10,16,42,50,51,70] Importantly, tumor cell lines derived from patients with NF1, and a genetically engineered cell system that requires *Nf1*-deficiency for transformation, are highly sensitive to rapamycin. For example, Bhola et al.[10] developed and characterized a human NF1-MPNST explant grown subcutaneously in mice to evaluate the effect of rapamycin. Rapamycin significantly inhibited human NF1-MPNST mTOR pathway activation and growth without systemic toxicities. It was effective at reducing NF1-MPNST proliferation and angiogenesis, but did not increase apoptosis. Rapamycin effectively decreased activation downstream of mTOR, but there was increased AKT activation. This study therefore demonstrated the therapeutic potential and limits of rapamycin in NF1-associated and possibly sporadic MPNST. Johannessen et al.[50] demonstrated that rapamycin suppresses the growth of aggressive NF1-associated malignancies in a murine model. Interestingly, they demonstrated that rapamycin does not function via mechanisms generally assumed to mediate tumor suppression, including inhibition of HIF-1α and indirect suppression of AKT, but does suppress the mTOR target Cyclin D1. Finally, in a genetically engineered mouse model of low-grade optic glioma resulting from inactivation of the *NF1* gene, pharmacological mTOR inhibition in vivo led to decreased tumor cell proliferation in a dose-dependent fashion associated with a decrease in tumor volume.[42] It is important to realize that rapamycin is an effective treatment in other developmental syndromes with abnormalities of the mTOR pathway, including tuberous sclerosis, where it may be efficacious in the management of cognitive or developmental disorders and epilepsy and in preventing or limiting tumor development.[30,44,74,75]

Overall, these medical therapies targeting specific genetic or molecular events involved in NF1 tumorigenesis may be useful for treatment of residual tumors, to reduce tumor size prior to surgery, to decrease the incidence of tumor formation, or to apply to the local tumor environment at the time of resection.

Conclusions

Remarkable progress has been made toward understanding the pathogenesis of neurofibromas since the cloning of the *NF1* gene in 1990. Neurofibromatosis Type 1 is caused by germline mutations of the *NF1* tumor suppressor gene, which generally results in decreased intracellular neurofibromin protein, leading to increased cascade Ras signaling to its downstream effectors, including Ras/MAPK and Akt/mTOR. In general, a somatic loss of the second *NF1* allele in the progenitor cell, either the Schwann cell or its precursor, combined with haploinsufficiency in multiple supporting cells is required for tumor formation. Importantly, there is a complex series of interactions with these other cell types, such as $NF1^{+/-}$ heterozygous mast cells, in neurofibroma tumorigenesis. In general, for malignant transformation to occur there must be accumulation of additional mutations of multiple genes including *INK4A/ARF* and *P53*. Increased knowledge of molecular and cellular mechanisms involved with NF1 tumorigenesis has led to more accurate animal models of NF1 tumors to evaluate new agents for targeted molecular therapy, including the mTOR inhibitor rapamycin, which are demonstrating promising preclinical results for treatment of MPNSTs and gliomas.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: WT Couldwell, ON Gottfried, DH Viskochil. Drafting the article: ON Gottfried. Critically revising the article: WT Couldwell, ON Gottfried, DH Viskochil. Reviewed final version of the manuscript and approved it for submission: WT Couldwell, ON Gottfried. Study supervision: WT Couldwell, DH Viskochil.

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Genetic association studies in patients with traumatic brain injury

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Traumatic brain injury (TBI) constitutes a major cause of mortality and disability worldwide, especially among young individuals. It is estimated that despite all the recent advances in the management of TBI, approximately half of the patients suffering head injuries still have unfavorable outcomes, which represents a substantial health care, social, and economic burden to societies.

Considerable variability exists in the clinical outcome after TBI, which is only partially explained by known factors. Accumulating evidence has implicated various genetic elements in the pathophysiology of brain trauma. The extent of brain injury after TBI seems to be modulated to some degree by genetic variants.

The authors' current review focuses on the up-to-date state of knowledge regarding genetic association studies in patients sustaining TBI, with particular emphasis on the mechanisms underlying the implication of genes in the pathophysiology of TBI. *(DOI: 10.3171/2009.10.FOCUS09215)*

Key Words • genetic association study • polymorphism • genes • traumatic brain injury

Pathophysiology of TBI

Clinical and experimental data have demonstrated that several complex and multifactorial pathophysiological cascades are initiated in the brain after a traumatic event. Primary brain damage is induced by direct impact to the brain parenchyma leading to focal or diffuse tissue distortion, destruction, tearing, and/or hemorrhage. After initial trauma, cerebral edema, increased intracranial pressure, tissue hypoxia-ischemia, and disruption of the blood-brain barrier[33] may occur. Secondary brain changes, which appear either immediately after TBI or in the following hours or days, include cellular, neurochemical, and molecular responses to TBI, such as neuronal cell death, apoptosis, excitotoxicity, inflammatory infiltration, Aβ-peptide deposition, disruption of calcium homeostasis, oxidative stress, and cytoskeletal and mitochondrial dysfunction.[34,35] Expression studies have shown that several genes are implicated in the pathophysiology of secondary brain damage.[67,73] Secondary processes were found to dramatically worsen primary damage, leading to the activation of a cascade of neuronal and axonal pathologies, which in turn determine the patient's overall clinical outcome.

Genetic Association Studies

Recent evidence from genetic association studies supports the view that genetic factors play an important role in the outcome of various CNS disorders including TBI.[55,136] Genetic association studies are useful tools in investigating possible relationships between gene polymorphisms and disease outcome. Recent advances in genotyping technologies have greatly expanded the number of studies that can test possible associations between gene polymorphisms and certain phenotypes.

Genetic variations include insertions, deletions, duplications, or SNPs. Genetic polymorphisms may affect the clinical phenotype by altering the function of the encoded protein, either by changing the structure of this protein or by modifying the expression of a gene. Increased frequency of an allele in a phenotype (favorable

Abbreviations used in this paper: ACE = angiotensin converting enzyme; ApoE = apolipoprotein E; COMT = catechol-O-methyltransferase; CPP = cerebral perfusion pressure; GOS = Glasgow Outcome Scale; IL = interleukin; SNP = single nucleotide polymorphism; TBI = traumatic brain injury.

outcome) compared with another phenotype (unfavorable outcome) constitutes a strong evidence of genetic association. However, genetic association does not necessarily mean gene identification. It may, for example, be the result of stratification bias, linkage disequilibrium with the causative variant, or other inherent limitations of association studies.[14] A genetic association study requires biological evidence and functional significance that the risk variant is implicated in the pathogenesis of the disease. Susceptibility genes are expected to have weak effects and account for only a small or modest increase in risk (OR 1.2–1.4). Moreover, gene functions can be modified by other genes (gene-gene interactions), proteins (protein-protein interactions), or environmental factors (gene-environment interactions), and these modifications render the detection of their effects more difficult.

In general, there are 2 approaches to perform genetic association studies: the candidate-gene approach (also known as the hypothesis-driven approach) and the genome-wide approach (also known as the agnostic approach). In the candidate-gene approach, a few SNPs are genotyped on a gene of interest, which is chosen based on a biological hypothesis for the disease. On the other hand, genome-wide association studies attempt to survey the entire genome at the same time, in a hypothesis-free manner, using hundreds of thousands of SNPs.[94] Candidate-gene association studies are generally small in sample size and presumably have the tendency to lack the statistical power to detect a significant association. For example, to achieve a greater than 80% statistical power to identify a modest genetic effect (OR 1.2) of a polymorphism present in 10% of individuals, a sample size of 10,000 patients or more is necessary.[140] Therefore, the sample sizes required to predict associations have to be far beyond what is currently available, and no single institution is able to provide a statistically powerful association study. Strategies to overcome this problem and to increase the large sample size limitation include meta-analyses of multiple studies,[140] or collaborative, multiinstitutional, larger-scale studies and consortia, which allow pooling of data.[31,77] Another approach to increase the power of genetic association studies is by implementing haplotype analysis, which subdivides the studied group into more genetically distinct subcategories. In addition, the statistical power of a study can be increased using well-defined intermediate phenotypes, which may be closer to the TBI pathophysiology and the pathogenic genotype, instead of the clinical phenotype, which is complex and multifactorial. Intermediate phenotypes used in TBI association studies include, for example, the Aβ-peptide deposits,[100] the hematoma volume,[69] or the presence of brain hemorrhage.[37]

The *ApoE* Gene

Apolipoprotein E is a plasma lipoprotein implicated mainly in transporting cholesterol and lipids throughout tissues including the CNS. In the brain, ApoE is synthesized primarily by the astrocytes and the microglia and plays a vital role in the maintenance of neuronal membranes, neuronal tissue repair, remodeling, and synaptogenesis. Apolipoprotein E–deficient mice showed increased ischemic neuronal damage compared with wild-type mice.[45] It has been demonstrated that intraventricular infusion of ApoE reduces neuronal damage.[46] After CNS insults, ApoE is locally upregulated and released by the astrocytes into the extracellular space, and is subsequently absorbed by the neurons.[44]

In humans, there are 3 major isoforms of ApoE (ε2, ε3, and ε4), which differ in amino acid sequence at positions 112 and 158. In the ApoE4 isoform, the amino acid substitution predisposes the protein to reduced stability, and this mediates an interaction between domains, which subsequently results in a more compact structure.[76] This conformation variation is thought to account for the adverse pathological functions of the ApoE4 isoform.

The ApoE4 isoform was found to bind Aβ peptides with a higher avidity,[124] and to promote more rapid aggregation into amyloid fibrils.[123] In a previous experimental head-injury study in mice, fatality was found to be increased in the ε4 isoform group among animals with different isoforms of ApoE.[114] The ApoE isoforms also show different interactions with tau protein, a microtubule-associated protein. Apolipoprotein E3 forms a stable complex with tau, preventing it from phosphorylation, aggregation, and formation of neurofibrillary tangles. In addition, it has been demonstrated that ApoE3 increases neurite outgrowth in cultures of dorsal root ganglion neurons compared with ApoE4.[97] These effects of ApoE3 on sprouting and synaptic remodeling are probably mediated by its effect on microtubule stability.

The ApoE4 isoform has also been associated with neurotoxicity and neurodegeneration, inflammation, mitochondrial dysfunction, impairment of the antioxidative defense system, increased intracellular calcium, disruption of cholinergic transmission, dysregulation of the neuronal signaling pathways, and apoptosis.[55,76]

Evidence from epidemiological and pathological studies has linked TBI to Alzheimer disease.[93] Deposition of Aβ was detected in approximately 30% of individuals dying shortly after severe TBI,[111] and this may imply a genetic predisposition to Aβ accumulation. In head-injured individuals, Aβ deposition was shown to be determined, though in part, by the presence of the *ApoE4* allele.[100] Furthermore, immunostaining of amyloid deposits, which appear early after head injury, was positive for ApoE, whereas the number of immunoreactive plaques was associated with the *ApoE4* allele in a dose-dependent manner.[43]

Several association studies have investigated the role of the *ApoE* gene polymorphism in patients sustaining TBI.[55] The design and results of each study are summarized in Table 1. Despite all the possible limitations, these studies provide valuable information concerning the implication of the *ApoE* genotype in the pathophysiology of TBI.

Most genetic studies in TBI have investigated possible association between *ApoE* polymorphism and functional outcome after TBI measured by the GOS.[2,12,15,17,25,40,69,87,98,130,131,135] In a recent meta-analysis of 14 cohort studies (of 23 relevant studies identified from the literature) and 2427 participants, it was found that the *ApoE4*

TABLE 1: Previously published association studies of patients who had suffered TBI*

Authors & Year	Gene	Polymorphism	Methodology	Phenotype	Results	Comments
Nicoll et al., 1995	*ApoE*	ε2/ε3/ε4	90 autopsy TBI cases (23 Aβ+)	Aβ deposition	pos	*ApoE4* allele, p<0.00001
Sorbi et al., 1995	*ApoE*	ε2/ε3/ε4	16 young pts w/ TBI	posttraumatic unawareness	pos	*ApoE4* allele, p<0.01
Mayeux et al., 1995	*ApoE*	ε2/ε3/ε4	236 community-dwelling elderly persons w/ Hx of TBI	risk of AD	pos	10-fold increase for AD in presence of *ApoE4* allele & Hx of TBI
Katzman et al., 1996	*ApoE*	ε2/ε3/ε4	160 pts w/ AD, 69 controls w/ Hx of TBI	risk of AD	pos	*ApoE4* allele & TBI increased the risk of AD, OR 13.5 (95% CI 2.63–69.12), p=0.0018
Teasdale et al., 1997	*ApoE*	ε2/ε3/ε4	89 pts w/ TBI	poor GOS score at 6 mos	pos	*ApoE4* allele, OR 0.23 (95% CI 0.06–0.82), p=0.024
Jordan et al., 1997	*ApoE*	ε2/ε3/ε4	30 boxers	neurological impairment (CBI scale)	pos	high-exposure boxers w/ *ApoE4* allele had increased severity of neurological deficits p<0.01
Friedman et al., 1999	*ApoE*	ε2/ε3/ε4	69 pts w/ TBI	poor clinical outcome	pos	*ApoE4* allele
Mehta et al., 1999	*ApoE*	ε2/ε3/ε4	797 participants of a population-based cohort w/ Hx of TBI	risk of dementia & AD after mean 2.1-yr follow-up	neg	—
Plassman et al., 2000	*ApoE*	ε2/ε3/ε4	46 w/ AD, 356 non-AD military men w/ Hx of TBI	risk of AD	neg	—
Lichtman et al., 2000	*ApoE*	ε2/ε3/ε4	31 TBI pts w/ diffuse axonal injury	functional independence measures at 6 mos	pos	*ApoE4* allele carriers had lower scores in functional independence measures, p=0.05
Guo et al., 2000	*ApoE*	ε2/ε3/ε4	942 probands w/ AD & Hx of TBI, 327 controls	risk of AD	pos	TBI increased risk of AD in the absence of *ApoE4* allele, OR 3.3 (95% CI 2.0–5.5)
Kutner et al., 2000	*ApoE*	ε2/ε3/ε4	53 active football players	neuropsych assessments	pos	older players w/ *ApoE4* allele had lower cognitive test scores, p=0.004
Liaquat et al., 2002	*ApoE*	ε2/ε3/ε4	129 pts w/ TBI	hematoma volume poor GOS at 6 mos	pos pos	E4 associated w/ larger hematomas, p=0.0056 *ApoE4* allele, p=0.015
Crawford et al., 2002	*ApoE*	ε2/ε3/ε4	110 pts w/ TBI	memory performance w/in 6 mos of injury	pos	*ApoE4* allele carriers had worse memory performance
Liberman et al., 2002	*ApoE*	ε2/ε3/ε4	87 pts w/ mild or moderate TBI	neuropsych tests at 3 & 6 wks	pos	*ApoE4* allele carriers had lower test scores at first visit
Chiang et al., 2003	*ApoE*	ε2/ε3/ε4	100 pts w/ TBI	poor GOS score at 6 mos	pos	*ApoE4* allele, OR 3.01 (95% CI 1.02–8.88), p=0.04
Nathoo et al., 2003	*ApoE*	ε2/ε3/ε4	110 black Zulu-speaking pts w/ TBI	poor GOS score at 6 mos	neg	—
Diaz-Arrastia et al., 2003	*ApoE*	ε2/ε3/ε4	106 pts w/ moderate or severe TBI	posttraumatic seizures GOS expanded score at 6 mos	pos neg	increased risk in E4, OR 2.41 (95% CI 1.15–5.07), p=0.03 —
Millar et al., 2003	*ApoE*	ε2/ε3/ε4	396 pts w/ TBI	GOS score at 6 mos, neuropsych outcome of 18 yrs later	neg	—
Kerr et al., 2003	*ApoE*	ε2/ε3/ε4	91 pts w/ severe TBI	concentrations of amino acid neurotransmitters (aspartate, glutamine) & energy metabolites L/P ratio	pos	those w/ *ApoE4* allele had significant increased & sustained levels of aspartate & L/P ratio post-TBI
Chamelian et al., 2004	*ApoE*	ε2/ε3/ε4	90 pts w/ mild to moderate TBI	GOS score, neuropsych outcome	neg	—

(continued)

TABLE 1: Previously published association studies of patients who had suffered TBI* *(continued)*

Authors & Year	Gene	Polymorphism	Methodology	Phenotype	Results	Comments
Sundström et al., 2004	*ApoE*	ε2/ε3/ε4	34 pts w/ mild TBI pre- & postinjury	neuropsych tests	pos	*ApoE4* allele associated w/ decreased postinjury performance
Quinn et al., 2004	*ApoE*	ε2/ε3/ε4	106 autopsy cases (2–19 yrs old)	brain swelling	neg	—
Koponen et al., 2004	*ApoE*	ε2/ε3/ε4	60 pts w/ TBI	dementia psychiatric disorders after ~30 yrs	pos neg	*ApoE4* allele increased risk of dementia, p=0.028 —
Teasdale et al., 2005	*ApoE*	ε2/ε3/ε4	1094 pts w/ TBI	GOS score at 6 mos	neg	*ApoE4* allele associated w/ poor outcome only in pts <15 yrs, OR 3.06 (95% CI 1.22–7.65)
Leclercq et al., 2005	*ApoE*	ε2/ε3/ε4	88 TBI autopsies	cerebral amyloid angiopathy	pos	*ApoE4* allele associated w/ cerebral amyloid angiopathy, p=0.021
Ariza, Pueyo et al., 2006	*ApoE*	ε2/ε3/ε4	77 pts w/ TBI	neuropsych tasks ≥6 mos post-TBI	pos	*ApoE4* allele increased risk of worse performance
Smith et al., 2006	*ApoE*	ε2/ε3/ε4	239 cases of fatal TBI	moderate/severe contusions severe ischemic damage	pos neg	*ApoE4* allele increased risk of contusions, p=0.05 p=0.08
Jiang et al., 2006	*ApoE*	ε2/ε3/ε4	110 pts w/ TBI	clinical deterioration in acute stage (<7 days post-TBI)	pos	*ApoE4* allele increased risk of clinical deterioration, OR 4.84 (95% CI 1.44–16.21), p=0.011
Isoniemi et al., 2006	*ApoE*	ε2/ε3/ε4	58 pts w/ TBI	hippocampal vol, brain atrophy on average 31.3 yrs post-TBI	neg	—
Kerr et al., 2006	*ApoE*	ε2/ε3/ε4	54 pts w/ severe TBI	CBF detected by Xe-CT w/in 24 hrs postinjury	pos	*ApoE4* allele associated w/ higher CBF
Ponsford et al., 2007	*ApoE*	ε2/ε3/ε4	120 pts w/ moderate or severe TBI	cognitive performance at 3, 6, & 12 mos	neg	—
Han et al., 2007	*ApoE*	ε2/ε3/ε4	78 pts w/ mild to moderate TBI	neuropsych measures at 1 mo	pos	*ApoE4* carriers associated w/ better performance in some tests
Alexander et al., 2007	*ApoE*	ε2/ε3/ε4	123 pts w/ severe TBI	GOS score at 3, 6, 12, & 24 mos postinjury	pos	*ApoE4* allele carriers had a slower recovery rate
Hiekkanen et al., 2007	*ApoE*	ε2/ε3/ε4	33 pts w/ nontrivial TBI	brain lesions determined w/ MRI ~1 wk & 1 yr post-TBI	neg	—
Zhou et al., 2008	*ApoE*	ε2/ε3/ε4	meta-analysis: 14 studies, 2427 participants	initial GCS score poor GOS score at 6 mos	neg pos	— *ApoE4* allele, RR 1.36 (95% CI 1.04–1.78)
Willemse-van Son et al., 2008	*ApoE*	ε2/ε3/ε4	79 pts w/ moderate or severe TBI	GOS score at 3, 6, 12, 18, 24, & 36 mos postinjury	pos	*ApoE4* allele pts had better recovery OR 0.26 (95% CI 0.02–0.51), p=0.037
Tanriverdi et al., 2008	*ApoE*	ε2/ε3/ε4	93 pts w/ TBI, 27 healthy controls	risk of pituitary dysfunction post-TBI	pos	lower risk of pituitary dysfunction in *ApoE3/E3* individuals, OR 0.29 (95% CI 0.11–0.78), p=0.01
Brichtová & Kozák, 2008	*ApoE*	ε2/ε3/ε4	70 children w/ TBI	GCS score at admission, GOS score at 1 yr	pos	children w/ *ApoE4* genotype had unfavorable neurological outcome after TBI
Luukinen et al., 2008	*ApoE*	ε2/ε3/ε4	134 pts >70 yrs, 28 w/ head injury w/o explicit TBI	risk of dementia after 9-yr follow-up	pos	increased risk of dementia in pts carrying the *ApoE4* allele, OR 2.70 (95% CI 1.02–7.16)
Ost et al., 2008	*ApoE*	ε2/ε3/ε4	96 pts w/ severe TBI	death 1 yr postinjury	pos	males w/ *ApoE4* had enhanced 1-yr mortality; females did not, p=0.0079

TABLE 1: Previously published association studies of patients who had suffered TBI* *(continued)*

Authors & Year	Gene	Polymorphism	Methodology	Phenotype	Results	Comments
Rapoport et al., 2008	*ApoE*	ε2/ε3/ε4	49 pts w/ mild TBI, 68 controls	neuropsych performance at 1 & 2 yrs postinjury	neg	—
Kristman et al., 2008	*ApoE*	ε2/ε3/ε4	318 student athletes (28 w/ concussion)	risk of concussion	neg	—
Ashman et al., 2008	*ApoE*	ε2/ε3/ε4	54 older adults w/ TBI, 40 controls	neuropsych tests, reexamination 2–5 yrs later	neg	—
Han et al., 2009	*ApoE*	ε2/ε3/ε4	46 military participants w/ mild to moderate TBI	change in job status post-TBI	pos	*ApoE4* allele may affect change in job status after TBI
Müller et al., 2009	*ApoE*	ε2/ε3/ε4	59 pts w/ mild TBI	neuropsych testing before & 6 mos after discharge	pos	*ApoE4* genotype associated w/ impaired cognitive performance, p=0.046
Hiekkanen et al., 2009	*ApoE*	ε2/ε3/ε4	33 pts w/ TBI	injury symptom checklist, GOS extended version at 1 yr postinjury	neg	—
Lo et al., 2009	*ApoE*	ε2/ε3/ε4	65 critically ill children	CPP insult	pos	significantly less CPP insult among *ApoE4* allele carriers w/ poor outcome, p=0.03
Lendon et al., 2003	*ApoE* promoter	−219G/T −491A/T −427C/T	92 pts w/ TBI	GOS at 6 mos	neg neg neg	nonsignificant when adjusted by logistic regression — —
Jiang et al., 2007	*ApoE* promoter	−219G/T −491A/T −427C/T	110 pts w/ TBI	clinical deterioration in acute stage (<7 days after TBI)	neg neg neg	— 491AA genotype act synergistically w/ *ApoE4* allele —
Jiang et al., 2008	*ApoE* promoter	−219G/T −491A/T −427C/T	110 pts w/ TBI	CT worsening in acute stage (<7 days post-TBI)	neg neg neg	— — —
Terrell et al., 2008	*ApoE* *tau* *ApoE* promoter	ε2/ε3/ε4 His47Tyr Ser53Pro −219G/T	195 active male football players & male/female soccer players, 72 w/ Hx of concussions over the previous 8 yrs	risk of concussions	neg neg neg pos	— — — TT genotype increased risk of concussions, OR 2.7 (95% CI 1.1–6.8), p=0.03
Johnson et al., 2009	*neprilysin*	GT repeats in the promoter	81 TBI autopsies	Aβ plaques formation	pos	increased risk if total GT repeat number >41, OR 10.1 (95% CI 3.1–32.5), p=0.0001
Martínez-Lucas et al., 2005	*p53*	Arg72Pro	90 pts w/ severe TBI	poor GOS score at discharge GOS score at 6 mos	pos neg	Arg/Arg, OR 2.9 (95% CI 1.05–8.31), p=0.039 —
Ariza, Matarin, et al., 2006	*ACE*	insertion/deletion	73 pts w/ moderate or severe TBI	neuropsych tests after resolution of posttraumatic amnesia	pos	*D* allele carriers had better performance on certain tests
Uzan et al., 2005	*IL-1β*	+3953 C/T −511 A/G	69 pts w/ TBI	poor GOS score at 6 mos poor GOS score at 6 mos	pos pos	allele 2, OR 0.25 (95% CI 0.12–0.55), p= 0.0004 allele 2, p=0.005
Hadjigeorgiou et al., 2005	*IL-1ra* *IL-1β*	VNTR −511 A/G	151 pts w/ TBI	cerebral hemorrhage	pos neg	allele 2, OR 4.57 (95% CI 1.67–12.96), p = 0.004 —

(continued)

TABLE 1: Previously published association studies of patients who had suffered TBI* *(continued)*

Authors & Year	Gene	Polymorphism	Methodology	Phenotype	Results	Comments
Tanriverdi et al., 2006	*IL-1α*	−889 C/T	71 pts w/ TBI	GOS at 6 mos	neg	—
Dardiotis et al., 2006	*IL-1α*	−889 C/T	215 pts w/ TBI	GOS at 6 mos	neg	—
Miñambres et al., 2003	*IL-6*	−174 C/G	62 pts w/ severe TBI	GOS at 6 mos	neg	—
McAllister et al., 2005	*ANKK1*	rs1800497	39 pts w/ mild TBI, 27 controls	neuropsych tests 38.4 ± 24.4 days post-TBI	pos	allele *T* associated w/ slower response latencies after TBI
McAllister et al., 2008	*ANKK1 NCAM DRD2*	31 polymorphisms	93 pts w/ TBI, 21 controls	neuropsych tests 43.1 ± 15.8 days post-TBI	pos	allele *T* (rs1800497) and a haploblock of 3 SNPs w/in ANKK1 were associated w/ cognitive outcome
Lipsky et al., 2005	*COMT*	Val158Met	113 pts w/ TBI	neuropsych tests	pos	Val homozygotes had worse performance
Chan et al., 2008	*SLC6A4*	5-HTTLPR, rs25531	174 pts w/ TBI	depression	neg	—

* AD = Alzheimer disease; CBF = cerebral blood flow; GCS = Glasgow Coma Scale; Hx = history; L/P = lactate/pyruvate ratio; neg = negative; neuropsych = neuropsychological; pos = positive; pts = patients; VNTR = variable number tandem repeat; — = no significant association.

allele increases the risk of poor clinical outcome, which was evaluated 6 months after the injury.[139]

The *ApoE* polymorphism was also tested in patients who had suffered TBI in relation to several other neuropathological, laboratory, or imaging intermediate phenotypes, such as Aβ deposition,[100] hippocampal volume and brain atrophy,[48] cerebral blood flow,[59] presence of brain lesions,[41] hematoma volume,[69] cerebral contusions, ischemic damage,[61,121,132] brain swelling,[107] cerebral amyloid angiopathy,[65] concentrations of amino acid neurotransmitters,[58] and CPP insult.[74] Other phenotypes included clinical neurological impairment,[32,40,51,56] mortality,[101] functional independence measures,[71] neuropsychological assessments,[7,9,15,20,38,62,70,87,95,106,109,125] changes in work status,[39] and risks of posttraumatic unawareness,[121] Alzheimer disease,[36,57,60,75,80,86,105] pituitary dysfunction,[127] posttraumatic seizures,[25] and psychiatric disorders.[60]

In addition to the aforementioned coding sequence polymorphisms, the *ApoE* gene is also polymorphic in the regulatory region. Promoter −219G/T and −491A/T polymorphisms were found to substantially alter the transcriptional activity of the *ApoE* gene.[8] It has been reported that these polymorphisms confer susceptibility to Alzheimer disease,[10] possibly by facilitating the Aβ deposition.[64] However, in patients who have suffered TBI, the effects of promoter polymorphisms have remained inconclusive.[50,52,68,132]

Neprilysin

The accumulation of β-amyloid peptide in some patients shortly after TBI may be the result of an imbalance between production and clearance of Aβ. Of note, a major therapeutic strategy for Alzheimer disease is the activation of proteases involved in the Aβ degradation process. One such protease is neprilysin, which was found to play the most important role in the degradation of Aβ.[49] Neprilysin levels were decreased in cerebral cortex and CSF in cases of early Alzheimer disease.[79,113] In addition, knockout models for *neprilysin* were shown to have an increased burden of brain Aβ, while overexpression of *neprilysin* was associated with reduction in Aβ levels and retardation of plaque formation.[49,90] However, it seems that neprilysin may not have an effect on the removal of already existing amyloid plaques but only on the prevention of forming new amyloid plaques.[89]

A GT repeat polymorphism in the *neprilysin* gene was shown to be associated with cerebral amyloid angiopathy and increased risk of Alzheimer disease.[115,138] The polymorphism is located in the regulatory region of the *neprilysin* gene, and it may induce conformational changes in the DNA, influence gene expression, and the degree of the Aβ degradation. In a recent study, this polymorphism was tested in 81 fatal cases of TBI with available autopsy data and a 3.45-day mean survival after injury. It was found that TBI cases with longer GT repeats had increased risk of Aβ plaques,[54] suggesting that *neprilysin* polymorphism may make patients who have suffered TBI more vulnerable to plaque formation.

The *p53* Gene

Experimental and clinical studies have provided evidence of widespread local and remote apoptosis that can be detected from hours to days after TBI. These studies have indicated that apoptosis following TBI occurs in neurons and glia and may contribute to neurological dysfunction.[108,120] Apoptotic cell death after TBI has been associated with decreased expression of survival-promoting proteins, such as Bcl-2 and extracellular signal-regulated kinase and increased expression of death-inducing proteins, such as Bax, c-Jun N-terminal kinase, p53, calpain, and caspases.[99,108] Interestingly, minocycline, a tetracycline derivative currently being tested in spinal cord injury, has been reported to exert neuroprotective and antiapoptotic effects in TBI via inhibition of cytochrome c release, inhibition of caspase-1 and -3, and expression and suppression of microglial activation.[116] The p53 tumor suppressor factor is a key regulator of DNA repair, cell cycle progression, apoptosis, and neuronal damage;[21] p53 is induced shortly after TBI, while its inhibition is assumed to offer neuroprotection.[96,137] A functional polymorphism of the *p53* gene in codon 72, which alters the properties of the produced protein,[27] was studied in a group of 90 severely head-injured patients who were admitted to an intensive care unit.[78] Patients with the *Arg/Arg* genotype were associated with unfavorable outcome at the time of discharge. However, 6 months later, no significant difference was found between the groups of patients with and without the *Arg/Arg* genotype, which may suggest a limited role of p53 in affecting the long-term clinical outcome of patients who have sustained TBI.

The *ACE* Gene

Angiotensin converting enzyme plays an important role in regulating both the production of angiotensin II and the degradation of bradykinin at the endothelial surface. Angiotensin II, which is the main active product of the renin-angiotensin system, has been linked to vascular remodeling, inflammation, and endothelial dysfunction.[28] The *ACE* insertion/deletion polymorphism has been extensively studied in various diseases. It has been associated, among others, with atherosclerosis[118] and Alzheimer disease.[66] The *ACE* insertion/deletion polymorphism appears to be of particular clinical significance as it alters the ACE plasma levels[110] and the local tissue production.[19,23] However, the polymorphism only partially (47% in the study group) determines the variation in plasma ACE levels,[110] and it is uncertain if it represents a functional polymorphism. Actually, despite considerable efforts, the precise location of the *ACE* gene functional polymorphism among the several polymorphic sites that have been described remains unknown.[119] Ariza et al.[6] studied 73 patients who suffered moderate or severe TBI and reported worse neuropsychological performance in the *D* allele carriers of the *ACE* insertion/deletion polymorphism. The authors attributed this association to changes in the blood flow and cerebrovascular tone mediated by the local production of angiotensin II. Angiotensin II can also induce neuronal damage because it was found to have proinflammatory properties and to be implicated in the generation of reactive oxygen species.[112,126]

The *IL* Genes

Brain injury induces a complex sequence of inflammatory processes, which are believed to contribute to the pathogenesis of TBI.[85] The level of these processes determines the patient's clinical presentation and outcome.[3,91,92] Interleukins are induced in response to brain injury and have multiple actions and targets, and often overlapping biological effects. Interleukin-1α and -1β are proinflammatory cytokines with pleiotropic activities, including growth and differentiation of T and B cells, and induction of other interleukins, adhesion molecules, histamine, and thromboxane. Interleukin-1 receptor antagonist (IL-1ra) is a naturally occurring competitive inhibitor of IL-1α and IL-1β and, as such, plays an important role in regulating the inflammatory process.[5,26] In experimental TBI models, rapid induction of IL-1β was reported[42] after brain trauma (increased mRNA concentration occurred 15 minutes after the injury, while increased concentration of the involved protein occurred 6 hours after the injury). Similarly, IL-1ra was upregulated after head injury in the same experimental study (increased concentration of mRNA was observed 6 hours after the injury). Furthermore, administration of exogenous IL-1β markedly exacerbates brain injury,[102] whereas injection or overexpression of IL-1ra significantly inhibits neuronal damage.[133] Additionally, increased IL-1 expression is also detected in CSF in patients with head injury.[84] A randomized phase II study of IL-1ra in acute stroke patients has reported promising results.[30] The *IL-1* gene cluster contains genes encoding IL-1α, IL-1β, and IL-1ra and lies on 2q13 within the 430-kb region in humans. A variable number tandem repeat (VNTR) polymorphism in intron 2 of the *IL-1ra* gene appears to be of particular clinical significance, as it has been associated with a variety of inflammatory diseases.[129] Carriers of the 2-repeat allele *(IL-1RN*2)* have increased IL-1ra plasma levels, enhanced IL-1β production, and decreased local production of IL-1ra in various tissues.[117] Two polymorphisms of the *IL-1β* gene have been studied extensively in the +3953 and –511 positions. The minor alleles of both polymorphisms are considered to be high producers of IL-1β protein.[29,105]

Uzan et al.[134] first provided evidence of an association between *IL-1β* +3953 and –511 polymorphisms and unfavorable prognosis in patients who had sustained TBI. In another study, the *IL-1RN*2* allele carriers were associated with an increased risk of posttraumatic hemorrhagic events, which were used as an intermediate phenotype.[37] Surprisingly, the *IL-1RN*2* allele was associated with more severe initial clinical presentation ($p = 0.045$) and better clinical outcome ($p = 0.02$). These results may suggest that the increased inflammatory processes in *IL-1RN*2* allele carriers may be deleterious in the acute postinjury period, but may participate in neuronal survival and repair in the intermediate and chronic postinjury period, possibly reflecting the dual role of cytokines in neurodegeneration and neuroprotection.[3,91,92]

Regarding the *IL-1α* gene, a polymorphism in the

promoter region (–889) has been studied in various diseases. Although the *IL-1α* (–889) allele 2 was associated with elevated IL-1α and IL-1β protein levels, the functional role of this polymorphism has been questioned.[24] Two studies have investigated the role of this polymorphism in patients who have sustained a TBI, but these studies were not able to establish any positive association.[24,128]

In another study that included patients who had suffered severe TBI and provided autopsy data, Johnson et al.[53] examined a possible association between *IL-1α* and *IL-1β* polymorphisms and the extent of programmed cell death. Interestingly, the amount of TUNEL positivity did not differ between genotypes.

Another cytokine that is implicated in TBI pathophysiology is IL-6. Interleukin-6 has regulatory, antiinflammatory, and neurotrophic effects and is associated with neuronal protection and survival.[92] It has been demonstrated that IL-6–deficient mice have increased numbers of apoptotic neurons after brain injury.[103] However, administration of IL-6 can either enhance or inhibit neuronal injury, probably depending on the time course and extent of expression.[4] In patients who have suffered TBI, the concentrations of IL-6 in CSF were elevated, and this upregulation after injury was associated with better neurological outcome.[18] However, a polymorphism in the promoter of *IL-6* was not found to have any effect on the outcome of patients with TBI.[88]

Other Association Studies

Clinical studies in patients who have sustained TBI have investigated the role of genetic variants in genes that modulate neurotransmitters such as dopamine[81] or serotonin.[16] Traumatic brain injury induces excessive neurotransmitter release, and this may affect motor function, behavior, mood, and cognition.

Dopamine and noradrenaline are key neurotransmitters that regulate cognitive function and affective behavioral processes. Dopamine and noradrenaline interact with specific receptors or are inactivated by COMT. Catechol-O-methyltransferase Val158Met polymorphism is associated with variation in COMT activity. The *158Met* allele, which decreases the activity of the enzyme, is associated with higher dopamine concentrations in the prefrontal cortex[63] and possibly with better cognitive performance. In patients who have suffered TBI, the *COMT* genotype was associated with higher postinjury performance on tests of executive function.[72] In another study, a polymorphism in the *ANKK1* gene, which regulates the adjacent *dopamine D2 receptor* gene, causing a reduction in the expression of D2 receptors, was found to be associated with slower postinjury performance on response latency.[82,83]

Depression is among the most common postinjury sequelae and may contribute to posttraumatic disability. Depression after TBI may be the result of a disturbance in serotonergic neurotransmission.[22] However, a polymorphism in serotonin transporter protein (SLC6A4 solute carrier family 6 member 4) was not associated with increased frequency of depression after TBI.[16]

Conclusions

Over the past years, there has been extensive research in elucidating the pathophysiology of TBI and the determinants of patient outcome, whereas it remains to be seen if this knowledge will lead to the induction of new treatment strategies and improved clinical outcome. Several association studies have revealed a number of genetic variants that confer susceptibility to poor outcome after TBI. Apart from providing insights into the pathophysiology of TBI, genetic studies may have some useful implications including the development of genetic markers for determination of specific molecular profiles in individuals and assessment of phenotype risk. Identification of *ApoE* alleles may determine which patients with TBI will respond, for example, to ApoE administration or to targeted therapies, such as bapineuzumab, which is currently being tested in patients with Alzheimer disease who have a specific *ApoE* genotype. It is also possible that detection of genetic markers, such as IL high-risk alleles, may help in identifying those patients who have sustained TBI with a specific proinflammatory or antiinflammatory phenotype, who may benefit from an early antiinflammatory treatment. This may, in part, explain the lack of clear clinical efficacy in improving outcome of previous antiinflammatory-based treatment trials in patients suffering TBI.[1]

Furthermore, our better understanding of complex genetic pathways may lead to specific genetic manipulation, for example, by targeting genetic modulators such as promoter regions,[47] in the near future. There is also the possibility of neutralizing pathological mRNA, protein products and secondary messengers.[11] Genetic determination of patients' functional alterations in metabolic enzymes may also have a clear beneficial effect on developing specific treatment strategies. It is possible that this pharmacogenomics approach may have a clear impact in determining the optimal therapeutic doses, minimizing the possibility of any side effects, and improving treatment adherence and efficacy.[13] In the future, all of these approaches may offer the prospect of personalized risk assessment and novel, genomic-based, targeted therapies.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Elucidating novel mechanisms of brain injury following subarachnoid hemorrhage: an emerging role for neuroproteomics

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Subarachnoid hemorrhage (SAH) is a devastating neurological injury associated with significant patient morbidity and death. Since the first demonstration of cerebral vasospasm nearly 60 years ago, the preponderance of research has focused on strategies to limit arterial narrowing and delayed cerebral ischemia following SAH. However, recent clinical and preclinical data indicate a functional dissociation between cerebral vasospasm and neurological outcome, signaling the need for a paradigm shift in the study of brain injury following SAH. Early brain injury may contribute to poor outcome and early death following SAH. However, elucidation of the complex cellular mechanisms underlying early brain injury remains a major challenge. The advent of modern neuroproteomics has rapidly advanced scientific discovery by allowing proteome-wide screening in an objective, nonbiased manner, providing novel mechanisms of brain physiology and injury. In the context of neurosurgery, proteomic analysis of patient-derived CSF will permit the identification of biomarkers and/or novel drug targets that may not be intuitively linked with any particular disease. In the present report, the authors discuss the utility of neuroproteomics with a focus on the roles for this technology in understanding SAH. The authors also provide data from our laboratory that identifies high-mobility group box protein-1 as a potential biomarker of neurological outcome following SAH in humans. *(DOI: 10.3171/2009.10.FOCUS09223)*

Key Words • **early brain injury** • **hemorrhagic stroke** • **cerebral aneurysm** • **inflammation** • **cerebrospinal fluid** • **biomarker**

Neurological injuries are associated with long-term disability and significant patient death. Aside from a significant emotional toll, brain injuries place a massive economic burden on society each year. Despite decades of intense investigation, clinical treatment options remain limited, in part, due to the poorly defined sequelae underlying injury progression. The cellular pathways culminating in neurological demise are likely activated within the first minutes to hours following the injury, suggesting that early diagnosis and intervention may be paramount to improving patient prognosis.

The identification of clinically viable therapeutics to limit brain injury remains a subject of intense research focus. Over the past several decades, preclinical research proceeded in a logical, hypothesis-driven manner in which the involvement of a single gene or protein was tested for a given biological function based on existing reports in the literature. Unfortunately, despite great promise in preclinical trials, species differences, drug delivery issues, and poor brain penetration contributed to the inability of most experimental drugs to significantly improve patient outcomes following brain injury. Thus, the incorporation of innovative research strategies may be required to further elucidate the mechanisms of brain injury at the molecular and cellular levels and provide novel targets for improved drug design.

In contrast to the systematic and laborious task of investigating the role of 1 established gene at a time, the advent of genomic and proteomic approaches allows the simultaneous, large-scale screening of all gene/proteins in a biological sample. These advanced screening techniques also allow objective, nonbiased data collection, permitting the identification of biomarkers and/or novel drug targets that may not be intuitively linked with any disease process. These findings may then be exploited in further preclinical and clinical testing. Although these technologies are often criticized as nonhypothesis driven, genomic and proteomic screening methods have significantly increased the mechanistic understanding of

Abbreviations used in this paper: BBB = blood-brain barrier; HMGB = high-mobility group box protein; IL = interleukin; NPH = normal-pressure hydrocephalus; NVU = neurovascular unit; SAH = subarachnoid hemorrhage; TLR = toll-like receptor.

numerous physiological and pathological processes and aided in the identification of disease biomarkers.

We believe that the field of neurosurgery stands to greatly benefit from these rapidly evolving technologies, both in the diagnosis (biomarker discovery) and therapeutic intervention (target discovery, validation, and novel drug design) of complex neurovascular pathologies. To maintain focus, the potential applicability of advanced genomics/proteomics will be discussed in the context of SAH, a type of hemorrhagic stroke caused by the spontaneous rupture of a cerebral aneurysm. Original data from our research group, demonstrating the utility of proteomic screening of patient specimens in novel drug target and biomarker identification, will also be provided.

Therapeutic Targeting Following SAH: Many Questions, Few Answers

Subarachnoid hemorrhage remains a major cause of death and disability in the US, with a prevalence of 1 in 10,000 people (approximately 7% of all strokes).[29,61] Although the overall incidence of stroke declined over the past several decades, the frequency of SAH remains stable despite medical advancements.[111] Patients with SAH exhibit a 30-day mortality rate of 30–40% and sustain a loss of productive life years comparable to that of patients with cerebral infarction, due in part to the young age of onset, dearth of viable therapeutic options, and poor clinical prognosis.[29,38,62,64] The estimated lifetime cost per SAH patient is double that of a patient with ischemic stroke, as ~ 50% of patients with SAH experience permanent disability (such as deficits in verbal and nonverbal memory, psychomotor speed, executive function, and visual-spatial function).[21,39,45,56,64] Overall, ~ 70% of patients with SAH die or require long-term assisted care due to neurological impairments.

The dogmatic view of SAH suggests that delayed cerebral vasospasm, a progressive narrowing of the large cerebral arteries ~ 4–10 days postictus, is the primary cause of neurological demise and death following SAH. Based on the clinical correlation between the onset of cerebral vasospasm and neurological deterioration, intense research efforts were focused on limiting large artery constriction with the hope that this would improve patient outcome. Unfortunately, a recent clinical trial showed that clazosentan, an endothelin receptor antagonist, successfully reduced angiographic vasospasm by ~ 65% without a corresponding improvement in neurological function or 3-month patient outcome.[40] Consistent with this finding, preclinical data from our laboratory and others indicated a similar functional dissociation between cerebral vasospasm and neurological outcome.[74,114] Together, these unexpected findings challenge the view that delayed cerebral vasospasm is solely responsible for brain injury following SAH and suggest that a reevaluation of the disease process may be required.[40,72,74,84,114] Although a detailed assessment of the state of the field is beyond the scope of this report, the reader is directed to several excellent commentaries on the current challenges associated with managing SAH.[40,70,84]

Early Brain Injury as a Cause of Subsequent Neurological Demise?

Early brain injury, a group of detrimental neurovascular pathologies that occur in the acute injury phase following SAH, includes cortical spreading depression, neuroinflammation, microvascular injury, BBB opening, and global cerebral edema.[6,16,21,22,25,26,55,58,68,69,97,101,113,119] Although the initiating events in the cause of early brain injury remain unclear, changes within the NVU are implicated in neurological demise following SAH.[82,123–125] The NVU is composed of neurons, glia, and microvessels organized into discreet units, each of which may communicate with and influence the physiology of the other cell types. For example, astrocytes are in juxtaposition to both neurons and endothelial cells and functionally influence the formation and maintenance of the BBB,[1,47,56] regulation of cerebral blood flow in response to neuronal activity,[46,59,103] maintenance of oxidative balance,[56] promotion of synaptogenesis, and neuroprotection.[71,83,108,109,114] Thus, perturbations within the NVU may negatively impact brain function after SAH. Along these lines, a vasoconstrictive response generated within minutes of SAH reduced cerebral blood flow and initiated a damaging cascade of events, including enhanced ischemic brain injury and early death, following experimental and clinical SAH.[9,12,56,89,91,114,118]

Understanding Neurological Injury After SAH: Where Do We Go From Here?

Whereas novel avenues of exploration into the mechanisms underlying neurological demise (beyond cerebral vasospasm) are clearly needed, the lack of a focused research direction presents a major obstacle in the field. Although there is growing appreciation for the role of early brain injury in determining neurological outcome following SAH, elucidating the complex cellular interactions within the NVU presents unique and technically challenging issues. As such, the traditional research model involving the development of a hypothesis centered upon a single gene/protein in disease progression followed by resource (time, labor, and cost)-intensive experimentation may not provide adequate mechanistic information in a timely manner. Thus, new experimental approaches and tools will be needed to fully understand the pathogenesis of brain injury following SAH. In the following sections, the potential utility of neurogenomics and neuroproteomics to identify and define the molecular and cellular changes within the NVU following SAH are discussed.

The Genomic Revolution

The information gained by the sequencing of the human genome permits the integration of gene expression, gene mutations, epigenetic modifications, and gene polymorphisms (single nucleotide polymorphisms) with biological/functional outcomes, opening an exciting new era in translational research.[2,56] Microarray ("gene chip") analyses permit the large-scale, simultaneous comparison of gene expression between 2 or more study populations (such as healthy control patients vs those with disease),

allowing the identification of individual genes or patterns of gene expression, including those not previously linked with a given disease process. This information may provide novel mechanisms of disease phenotype/progression and/or identify correlations between DNA polymorphisms and disease risk, such as the risk of developing a cerebral aneurysm.

Gene expression profiling technology identified 138 differentially expressed genes within the large cerebral arteries following SAH in rats, providing potential mechanisms of delayed cerebral vasospasm and neurological demise.[112] Of these changes, a known function was ascribed to 77 genes using the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/omim/), which houses genetic information on human disorders and diseases, including a large number implicated in processes related to inflammation, metabolism, oxidative stress, and regulation of the extracellular matrix. A similar study reported the upregulation of 18 genes associated with inflammation and cellular injury within vasospastic cerebral arteries following SAH in dogs,[77] suggesting a possible role for inflammatory mediators following the rupture of a cerebral aneurysm.

Inflammation may signal an adaptive response to promote tissue repair,[23,43] but uncontrolled or chronic inflammation, such as that observed during Alzheimer disease, Parkinson disease, and ischemic brain injury, irreversibly damages tissue and promotes oxidative stress.[44,60,66,67,76,80] Mechanistic studies to support the functional validity of the preclinical microarray data remain largely unexplored; however, initial clinical observations and preclinical data from our laboratory and others suggest that antiinflammatory compounds attenuate acute brain injury following SAH.[48,56,73,85,114,116] For example, mice overexpressing the gene for extracellular superoxide dismutase or copper-zinc superoxide dismutase exhibited a reduction in the development of cerebral vasospasm and attenuation of oxidative stress.[49,72,92] Similarly, CSF or serum from patients with SAH exhibited more immune complexes, complement activation, and increased levels of oxidative and inflammatory mediators (such as IL-6, tumor necrosis factor-α, and intercellular adhesion molecule-1) as compared with control patients.[20,32,34,42,50,56,87,90,101] These preliminary findings indicate the utility of microarray analysis for advancing the mechanistic understanding of acute brain injury following SAH and provide potential therapeutic targets for future preclinical and clinical studies.

Genomic studies after SAH in humans remain completely unexplored, in part due to the ethical and technical issues associated with the collection of tissue samples from patients with SAH. Whereas preclinical models of hemorrhagic stroke provide abundant access to mRNA, brain tissue from patients with SAH is not readily available. In contrast, blood is readily obtained from patients during routine clinical care, allowing blood genomic expression profiling. Although unreported following SAH, microarray analysis using peripheral blood mononuclear cells collected from patients with cerebral ischemia revealed an increase in the expression of hypoxia-induced stress genes, vascular repair genes, and neuroprotective genes, as compared with blood collected from control patients. Although these types of studies potentially provide unique clinical insights into the pathophysiology of brain injury, it remains unclear whether blood genomic responses accurately reflect the brain response to injury (for example, whether immune cells exhibit the identical genomic response as neurons following hypoxia-ischemia). Similarly, it is unknown whether the observed changes in peripheral blood cells indicate specific responses to the injury or whether these changes actually reflect other preexisting conditions within individual patients, such as vascular disease, hypertension, and diabetes.[7,28]

Together these findings suggest that the methods of sample collection and data analysis must be carefully considered when interpreting the findings from these types of studies. Additionally, mRNA expression is dynamic and varies from minute to minute, requiring the ability to repeatedly collect living cells over time. Finally, changes in mRNA expression are a relatively poor predictor of protein expression and biological activity.[37] Thus, while genomics provides an important foundation for identifying novel therapeutic targets, these important caveats may diminish the utility of gene expression following SAH in humans. As such, changes in protein expression and/or modification may provide more meaningful information regarding the complex mechanisms underlying neurological injury following SAH. In the following section, we discuss the potential applicability of proteomics in the study of brain injury.

Proteomics: the Next Step on the Journey

The complement of proteins within a cell or tissue (called the proteome) is considerably larger and more complex than the human genome. For example, alternative splicing of a single transcript may result in several different isoforms of a protein, significantly increasing the number and diversity of proteins. Further complicating the analysis of proteins following brain injury, posttranslational protein modifications such as phosphorylation, glycosylation, and myristoylation modulate protein activation states and protein-protein interactions, providing additional complexity. Thus, advanced technologies are needed to identify the key mediators of neurovascular injury. Once identified, preclinical modeling studies, such as those that use transgenic mice that lack or overexpress a molecule of interest, may be used to define whether a given protein is protective, detrimental, or both. In this section, we highlight the potential utility of proteomic analyses following brain injury. This is followed by the presentation of data from our laboratory, demonstrating the use of proteomic screening in patient-derived CSF to identify a possible biomarker/cellular mechanism of brain injury following SAH.

Proteomics, a method of emerging clinical importance, permits a direct comparison of protein expression between 2 or more populations (controls vs patients with disease). A major benefit of proteomic research is that novel proteins may be discovered and linked with a disease process for the first time, without the need for an existing hypothesis or precedent for that particular protein in the given disease process. To accomplish this goal,

samples of interest are resolved by 2D gel electrophoresis, a method that separates complex protein mixtures by charge, then by molecular weight,[18] resulting in the generation of a 2D gel electrophoresis map and a reference map of all proteins in a given sample. By comparing the spot location for an individual protein between 2 populations, changes in protein expression may be determined. Spots that show distinct differences between experimental groups can then be analyzed by techniques such as liquid chromatography-tandem mass spectrometry, which combines 1 or more chromatographic steps with 2 rounds of mass spectrometry.[74] This procedure allows the identification of individual proteins within complex, heterogeneous biological samples, including serum and CSF.

Proteomic Subdivisions

Bayés and Grant[8] classified proteomics into 4 distinct subdivisions. The first subdivision, expression neuroproteomics, involves the qualitative and quantitative profiling of the proteome and is traditionally accomplished using gel electrophoresis.[78] The second subdivision, functional neuroproteomics, studies the functional properties of individual proteins, including posttranslational modifications and organization of proteins into substructures, complexes, and networks. Data obtained using this method has improved the understanding of complex biological systems, such as the molecular organization of postsynaptic density, which would not otherwise be possible with genomic analyses.[94] For example, many proteins involved in the presynaptic apparatus[14,102] and the postsynaptic anchoring and clustering of *N*-methyl-D-aspartate–type glutamate receptors were identified using this method.[27,33,52,59,63,75]

Clinical neuroproteomics, the third subdivision, focuses on drug discovery and on the identification of novel biomarkers and disease mechanisms for neurological, neurodegenerative, and psychiatric diseases.[17,41,105,110,115,117] In particular, CSF provides an increasingly important resource for identifying novel changes within the brain. The final subdivision, neuroproteomic informatics, addresses the computation tools and databases necessary for handling and analyzing complex proteomic data sets. These technologies are important for determining statistically meaningful data and for the establishment of databases and repositories for proteomic data, which may be mined at a later date by other investigators. In the context of neurosurgery, we believe that clinical neuroproteomics may be particularly useful for identifying novel biomarkers of disease (in controls vs patients with disease) and/or providing novel cellular targets for future drug discovery. As such, the remainder of this review will focus on the potential applicability of this subcategory of neuroproteomics following SAH.

Cerebrospinal Fluid: A Gateway to the Brain?

Human brain specimens are not readily obtainable from patients with SAH, but CSF diversion is routinely performed in the neurointensive care unit, providing an easily accessible source of proteins from patients. Cerebrospinal fluid circulates throughout the brain and contains high amounts of protein (~ 15–40 mg/dl),[30] making this the sample of choice for novel biomarker discovery using proteomic methodologies. Proteomic screening of CSF was first performed nearly 4 decades ago to provide novel insights into human brain physiology and disease.[24,31,51] Initial studies performed in 1980 using 2D gel electrophoresis revealed ~ 300 proteins in human CSF, although most of these proteins remained unidentified at this time.[35] Subsequent studies in the early 1990s, using improved technologies, detected ~ 1000 proteins of which 248 were identified.[121] More recent studies in 2007 using liquid chromatography-tandem mass spectrometry revealed the presence of ~ 2500 proteins,[80,81] providing essential information to generate an atlas of the human CSF proteome, a valuable resource that provides a potential source of brain disease biomarkers.[81] Given the availability of patient CSF within an academic medical center, our research group attempted to identify novel biomarkers of neurological injury following SAH, which may aid in the early diagnosis and treatment of these patients. These original data are presented in the following section.

High-Mobility Group Box Protein-1: A Predictive Biomarker of Neurological Outcome Following SAH?

Using proteomic screening technologies, we detected the expression of a 25 kD protein, HMGB1, in the CSF of all 9 of the patients with SAH who we analyzed. In contrast, HMGB1 was below the level of detection in the CSF of all 7 control patients (Fig. 1), suggesting HMGB1 release may be specific to brain injury. Notably, the levels of HMGB1 within the CSF retrospectively correlated ($r = 0.786$) with neurological outcome, as determined by the Hunt and Hess grading scale (Fig. 2A), and were highly correlated ($r = 0.938$) with the degree of disability or dependence at patient follow-up examinations, as assessed by the modified Rankin scale (Fig. 2C). In contrast, the HMGB1 content of CSF was not strongly correlated ($r = 0.334$) with the appearance of SAH on CT scans, as determined by the Fisher grade (Fig. 2B). Together, these novel data implicate HMGB1 as a possible biomarker for neurological injury and as a predictive marker of patient outcome following SAH. These findings also provide a rationale for characterizing the functional role of HMGB1 in promoting brain injury after SAH.

High-mobility group box protein-1, also called HMG or amphoterin, is an evolutionarily conserved, nonhistone DNA binding protein that is constitutively expressed in most cells throughout the body, including the brain.[57,106] Under physiological conditions, HMGB1 localizes to the nucleus to stabilize nucleosomal structure and to facilitate gene transcription.[15] In contrast, HMGB1 functions as a proinflammatory cytokine when translocated into the extracellular space.[3,4,19,36,56,105] Thus, HMGB1 may be classified as an "alarmin," a multifunctional host protein that activates an immune response to warn neighboring cells of injury.[10,99] Consistent with this assertion, HMGB1 is increased in the CSF in patients with meningitis[105] and in the serum of patients with cerebral ischemia,[36] suggesting HMGB1 may represent a marker of neurological injury.

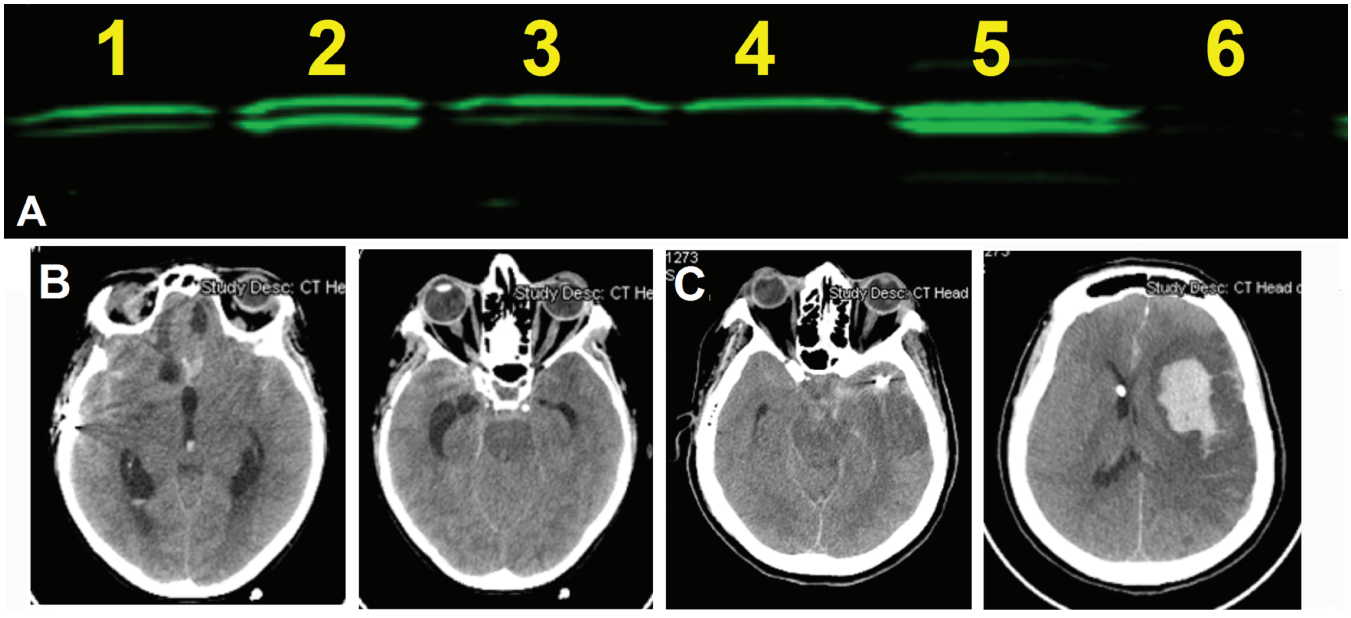

Fig. 1. **A:** Representative Western blot of HMGB1 in CSF samples collected from patients with aneurysmal SAH (lanes 1–5) and a control patient with NPH (lane 6). Blots were imaged using a Li-Cor Odyssey near-infrared imaging system. Patients with SAH consistently presented with an ~ 25 kD band corresponding to HMGB1, whereas patients with NPH did not show a clear band. **B:** Noncontrast axial CT scans of a 45-year-old woman with a Hunt and Hess grade of III and a Fisher grade of 4. The patient was sleepy and confused following a ruptured left posterior communicating artery aneurysm. The patient's HMGB1 sample is depicted in lane 2 (A). **C:** Noncontrast axial CT scans of a 77-year-old woman with a Hunt and Hess grade of IV and a Fisher grade of 4. The patient was stuporous with significant right hemiparesis following a ruptured left middle cerebral aneurysm. The patient's HMGB1 sample is depicted in lane 5 (A).

The function or functions of HMGB1 following SAH remain completely unexplored; however, intracerebroventricular administration of HMGB1 induced the expression of proinflammatory mediators (such as IL-1β, tumor necrosis factor-α, and IL-6) within the rodent brain.[3,76] The mechanisms whereby extracellular HMGB1 increases the expression of neuroinflammatory mediators remain unstudied, but activation of the receptor for advanced glycation end products, TLR2, and/or TLR4 mediate the effects of HMGB1 in the periphery.[5,53,54,56,77,82,100,107,122] Along these same lines, HMGB1 activated the proinflammatory transcription factor nuclear factor–kappa B via a TLR-dependent pathway in mouse macrophages and human kidney cells.[82] Consistent with these reports, the acute expression of TLR4 within astrocytes and vascular endothelium correlated with increased nuclear factor–kappa B activation and neuroinflammation following SAH in rats.[68,69] Because neuroinflammation is an important component of early brain injury and neurological demise after SAH,[82,86,87,96,98,101,104,114,120] HMGB1 may represent a clinically relevant, mechanistic link between acute injury and secondary neurovascular injury following SAH.

In response to inflammatory mediators, such as those that are increased after SAH, macrophages,[11,19] natural killer cells,[95] and myeloid dendritic cells[65] actively secrete HMGB1. In this case, the detection of elevated HMGB1 levels within the CSF may signal an adaptive immune response to clear cellular debris and promote resolution following a cerebral hemorrhage. In contrast, HMGB1 is not actively secreted within the CNS, but it is passively released into the extracellular space and CSF by necrotic neurons following cerebral ischemia.[88] Consistent with these findings, recent data from our laboratory suggests that neuronal HMGB1 is specifically released within the cerebral cortex following hemorrhagic stroke in mice (C.H.A. and K.M.D., unpublished observation, 2009). Thus, neuronally derived HMGB1 may provide an early marker of neurological injury, which if therapeutically targeted, could attenuate early brain injury after SAH. Ongoing work in our laboratory is actively characterizing the contributions of both immune and neuronal cells toward the post-SAH release of HMGB1. This knowledge will undoubtedly improve our understanding of disease pathophysiology and may provide a marker of acute neuronal injury after SAH (and possibly other neurological injuries).

Future Prospects, Challenges, and Direction of the Field

Despite significant advances in neurosurgical approaches, improvements in patient diagnosis, and intense research efforts, the mortality and morbidity following SAH remain unchanged.[93] The notion that neurological demise following SAH is solely caused by the development of delayed cerebral vasospasm was challenged by a number of recent preclinical and clinical reports, in-

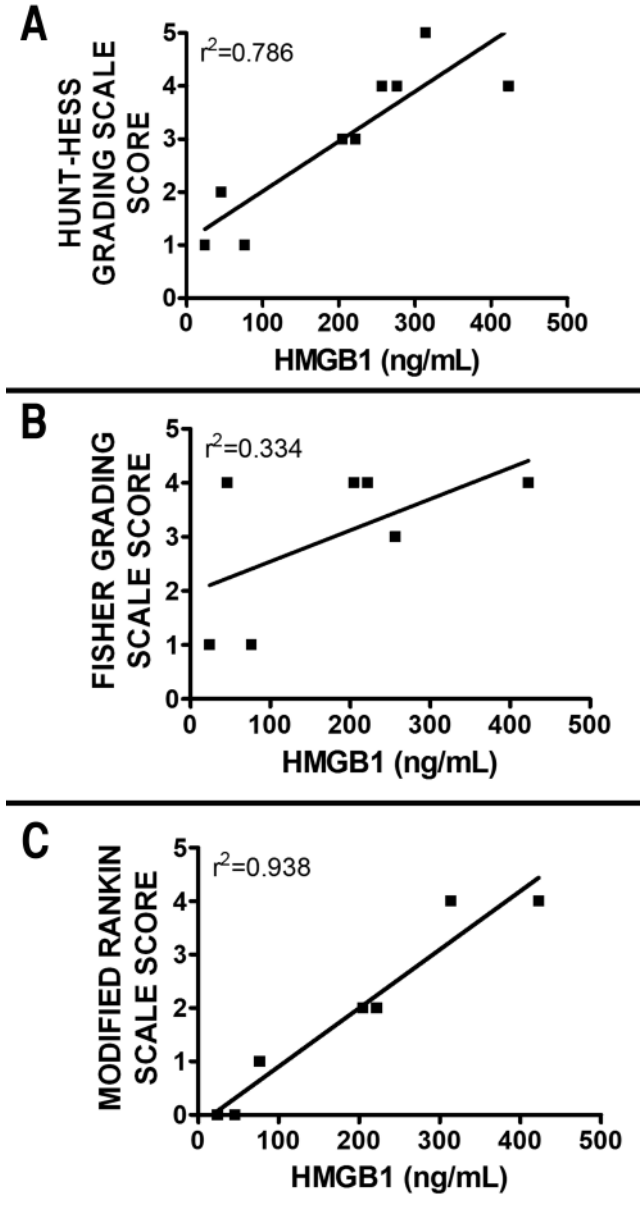

Fig. 2. Graphs of the retrospective correlation analysis of HMGB1 content within the CSF of patients with SAH according to the Hunt and Hess grade **(A)**, Fisher grade **(B)**, and modified Rankin scale score **(C)** at follow-up examination. High-mobility group box protein-1 was quantified by Western blotting. All grades were provided by the attending physician, who was blinded to experimental studies. High-mobility group box protein-1 was not present in the CSF of control patients with NPH.

cluding work by our laboratory,[40,74,114] emphasizing the need for novel therapeutic targets and treatment modalities. Early brain injury is a primary cause of death in patients with SAH[9,13] and emerged as a possible therapeutic target following SAH. Ongoing research suggests early brain injury involves complex neurovascular pathologies, including delayed cerebral ischemia, global edema, BBB disruption, cortical spreading depression, and neuroinflammation;[16,79] however, the signaling pathways and mechanisms involved in the initiation of these events remain largely unknown. Thus, innovative new approaches may be required to understand these complex processes.

Genomic and proteomic analyses are rapidly emerging as important tools for deciphering the complex neurovascular interactions in human physiology and disease. In particular, the incorporation of proteomics into the study of neurological diseases such as SAH may provide exciting new insights into the cellular mechanisms of brain injury. Unlike the retrospective study of individual genes/proteins in postmortem patient specimens, neuroproteomics: 1) allows a sensitive and unbiased method to identify novel proteins associated with a given disease process (biomarkers); 2) can be used to identify novel proteins without the need for a specific hypothesis prior to experimentation; 3) is useful for analyzing patient samples, such as CSF and serum, which are collected in the routine care and treatment of patients on the neurosurgical unit; 4) can aid in deciphering complex cellular interactions, subcellular networks (synaptic function), and posttranslational modifications; 5) can support the development of large databases allowing the comparison of data between multiple medical centers; and 6) can be performed using equipment that is available at most major medical centers. Together, we believe these features of neuroproteomics will allow the widespread incorporation of this technology into clinical research and will provide important new insights regarding the mechanisms underlying SAH and other brain injuries.

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Is an *endothelial nitric oxide synthase* gene mutation a risk factor in the origin of intraventricular hemorrhage?

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Object. Studies have shown decreased levels of nitric oxide (NO), the product of endothelial NO synthase (eNOS) gene activity, in infants with respiratory conditions and intraventricular hemorrhage (IVH). The authors evaluated the association of the *eNOS* gene promoter polymorphism T-786C with the cause of these conditions (respiratory conditions and IVH) in premature infants.

Methods. Blood samples from 124 African American premature infants were studied. The DNA was isolated and microplate polymerase chain reaction–restriction fragment length polymorphism assay was performed. Genotypes were scored as: TT homozygotes with 140 bp and 40 bp; CC homozygotes with 90 bp, 50 bp, and 40 bp; and TC heterozygotes with 140 bp, 90 bp, 50 bp, and 40 bp. Genotypes were stratified according to ethnicity, preterm status, and prematurity conditions.

Results. The mutant allele -786C was present in 15.3% of premature infants with respiratory distress syndrome, bronchopulmonary dysplasia, and IVH, compared with 7.25% in those premature infants without these conditions. A significant 2-fold increase of the mutant allele in patients compared with controls ($p = 0.04$, OR 2.3) reveals that the eNOS -786C allele could be a significant risk factor in the origin of respiratory conditions and IVH in premature infants.

Conclusions. These results suggest that the mutant eNOS -786C allele is a significant risk factor in the origin of respiratory and IVH diseases, probably mediating an insufficient supply of endogenous NO in premature infants. *(DOI: 10.3171/2009.10.FOCUS09143)*

Key Words • hydrocephalus • intraventricular hemorrhage • prematurity • endothelial nitric oxide synthase • African American

Nitric oxide is biosynthesized from L-arginine by the NOS enzyme. There are 3 forms of NOS: eNOS and neuronal NOS, both of which are constitutive forms, and the inducible form. Several single nucleotide polymorphisms in promoter and coding region exon 7, as well as variable number of tandem repeats polymorphisms in intron4 of the *eNOS* gene have been reported. Mutant genotypes of these polymorphisms have been shown to reduce eNOS enzyme activity, resulting in reduced NO levels. Earlier studies have shown that NO is decreased in premature infants with persistent pulmonary hypertension.[13] Our laboratory has previously shown an elevation of mutant endothelial genotypes in premature infants with respiratory distress.[23]

Intraventricular hemorrhage is a condition of preterm neonates that occurs due to rupture of blood vessels within the germinal matrix tissue of the developing brain. The vessels of the germinal matrix are highly susceptible to rupture due to lack of surrounding adventitia in the capillary bed of these vessels and lack of autoregulation in this preterm structure of the brain. Furthermore, the effects of IVH can be devastating, including periventricular scarring, white matter gliosis, and free radical and cytokine generation, all of which produce secondary damage to the early developing brain.[1–5,7] Studies have shown that infants who suffer large IVH are associated with an increased rate of developing major disabilities. As many as 60% of infants with Grade IV IVH will have severe motor and/or cognitive handicaps.[6,18]

Infants who suffer from this condition have been shown to be more likely to develop seizures and hydrocephalus than their gestational age-matched peers without IVH. Many of these infants will subsequently require CSF shunt placement, requiring lifelong maintenance. A previous study conducted by our institution (LSUHSC-Shreveport) found that pediatric shunts placed for post-

Abbreviations used in this paper: ACE = angiotensin converting enzyme; BPD = bronchopulmonary dysplasia; eNOS = endothelial NOS; IL = interleukin; IVH = intraventricular hemorrhage; LSUHSC = Louisiana State University Health Sciences Center; NO = nitric oxide; NOS = NO synthase; PCR = polymerase chain reaction; RDS = respiratory distress syndrome; RFLP = restriction fragment length polymorphism; VLBW = very low birth weight.

hemorrhagic hydrocephalus had significantly shorter survival time when compared with shunts placed for other causes. This study prompted our institution to examine the etiopathology of IVH to further determine if any modifiable factors for this condition of prematurity were present.

Earlier studies have shown that respiratory conditions such as RDS, BPD, or hyaline membrane diseases are closely associated with IVH.[11] Various genetic markers such as factor V Leiden, methylenetetrahydrofolate reductase, and prothrombin II gene polymorphisms have been found to be risk factors for IVH.[20,21] Similarly, we have shown the relationship between several ILs and vascular gene markers with many conditions of prematurity in VLBW premature infants receiving ventilation therapy.[20–22]

In the present investigation, we studied the association of *eNOS* gene promoter polymorphism T-786C with the origin of respiratory and IVH conditions in premature African American infants. The rationale for the present study was that earlier studies from our laboratory have shown significant differences in the genotype frequencies in the *eNOS* gene. For example, the frequency of the -786C mutant genotype in the promoter region of the *eNOS* gene was 2.5-fold higher in Caucasians compared with African Americans. The frequency of the 298Asp mutant genotype in exon 7 of the gene is 3-fold higher in Caucasians compared with that in African Americans. The frequency of variable number of tandem repeats (27 bp repeats) in intron 4 of the gene with 5 repeats (wild-type) is more common among African Americans than Caucasians.[17] Thus, both cases and controls were studied within the African American patient population, and the majority of the patients delivered at our medical center were African Americans.

Methods

Patient Population

Approval to conduct this study was granted by the Institutional Review Board of the LSUHSC. Peripheral blood samples from 124 premature African American infants suffering from both IVH and respiratory conditions of prematurity were collected consecutively from the pediatric neonatal intensive care unit of LSUHSC-Shreveport. The respiratory conditions of prematurity included RDS and BPD. Data from a control group of 124 premature infants without either of these conditions were also collected consecutively. The DNA was subsequently isolated from both patient and control samples using Qiagen DNA isolation kits.

Genotyping Assays

The genotyping assay was modified from that used by Tsukada et al.[16] to suit the large number of samples in our laboratory. Briefly, microplate PCR-RFLP assay was performed in 10 μl with 1 μl DNA, 3.25 mM $MgCl_2$, 0.375 mM deoxyribonucleoside triphosphate mix, 10 × PCR reaction buffer II, 1 unit of Taq Gold polymerase (Applied Biosystems Corp.), and 0.015 μM primer mix with the following sequences: TGG AGA GTG CTG GTG TAC CCC A (forward), and GCC TCC ACC CCC ACC CTG TC (reverse).

TABLE 1: The frequency of eNOS T-786C genotypes in patients and controls

Genotype	No. of Patients	Frequency	No. of Controls	Frequency	p Value (OR, 95% CI)
-786TT	43	0.69	53	0.86	
-786TC	19	0.31	9	0.15	0.03* (2.6, 1.1–6.2)
-786CC	0	0	0	0	
total	62	1.0	62	1.0	

* TC genotype frequency in patients was significantly different from controls.

The following PCR parameters were used: initial denaturation for 10 minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, then 64°C for 30 seconds, and 72°C for 1 minute, with the last extension at 72°C for 5 minutes. Following PCR, the 180-bp product was digested by 2 units of Msp 1 restriction enzyme (New England Biolabs) with 1.2 μl of 10 × Msp 1 buffer at 37°C for 2 hours. The Msp 1 digested PCR fragments were separated by electrophoresis on 2% agarose gel for 3 hours.

Scoring of eNOS T-786C Genotypes

Genotypes were scored by electrophoretic bands using ultraviolet fluorography and ethidium bromide staining as follows: TT homozygotes with 140 bp and 40 bp; CC homozygotes with 90 bp, 50 bp, and 40 bp; and TC heterozygotes with 140 bp, 90 bp, 50 bp, and 40 bp. Genotypes were stratified according to ethnicities, preterm and full-term status, and prematurity conditions.

Results

We analyzed 124 participants with IVH and respiratory conditions of prematurity for the presence of eNOS T-786C mutation by PCR-RFLP analysis. The baseline carrier (-786TC) frequency was 0.15 in the control patients and 0.31 in the study participants, a statistically significant difference (Table 1). The mutant allele -786C was present in 19 (15.3%) of 124 premature infants with RDS, BPD, and IVH conditions compared with 9 (7.25%) of 124 premature control patients (Table 2). Thus, there was a significant 2-fold increase in the mutant allele in the patients compared with the controls ($p < 0.05$, OR > 2.3 [95% CI 1.1–5.2]). Comparison by genotype revealed that the heterozygote mutation was found in 19 (31%) of 62 participants compared with only 9 (15%) of 62 controls, a statistically significant difference ($p = 0.03$). Both the mutated allele and heterozygote eNOS mutation are significant risk factors in the origin of respiratory and IVH conditions in premature infants.

Discussion

The discovery of NO and its vascular effects has led to numerous studies for the treatment and prevention of

TABLE 2: Frequencies of eNOS T-786C alleles in premature infants with and without IVH and respiratory conditions

Allele	No. of Patients	Frequency	No. of Controls	Frequency	p Value (OR, 95% CI)
-786T	105	0.85	115	0.93	
-786C	19	0.15	9	0.07	<0.05* (>2.3, 1.1–5.2)
total	124	1.0	124	1.0	

* C allele frequency in patients was significantly different from controls.

certain diseases. Nitric oxide is produced from the amino acid L-arginine by the enzymatic action of NOS. Nitric oxide synthase occurs in 3 forms: neuronal and endothelial (both constitutive), and inducible.[10,14,17,21,24] Each form is synthesized on a separate gene loci located on 3 different chromosomes. There are 2 endothelial forms of NOS: constitutive NOS (cNOS) and inducible NOS (iNOS).[21] Under basal conditions, NO is continually being produced. The stimulation for the production of NO occurs by 2 different mechanisms. First, increased blood flow produces a force that acts on the vascular endothelium, and the second step involves calcium release and subsequent eNOS activation.

Vascular actions of NO include direct and indirect vasodilatation, antithrombotic effects by inhibiting platelet adhesion to the vascular endothelium, antiinflammatory effects by inhibiting leukocyte adhesion to vascular endothelium, and antiproliferative effects by inhibiting smooth muscle hyperplasia.[5,14,15,20] Thus, impaired production or reduced bioavailability of NO can result in a variety of clinical vascular manifestations. Recent literature has focused on the use of inhaled NO therapy to treat preterm infants with RDS and BPD.[5,11,15] This treatment has been shown to be effective in reducing deaths and the need for extracorporeal membrane oxygenation in full-term and near-term infants and its use is advocated for these groups of infants with hypoxic respiratory failure. In the premature infant, the results are less conclusive. However, it does appear that early, low-dose inhaled NO has a positive effect in acute oxygenation improvement.[5,11] However, these benefits are still under investigation and are not yet considered standard treatment.[5]

Interestingly, inhaled NO does appear to have a secondary beneficial effect in neurological conditions of prematurity. Multiple studies have found that early low-dose NO inhalation therapy decreased the incidence of severe IVH, periventricular leukomalacia, and ventriculomegaly.[1] Neurodevelopment outcomes have also been shown to be improved by the use of inhaled NO in preterm infants specifically, and reduced occurrences of cerebral palsy in those infants treated with inhaled NO. Therefore, eNOS and NO are critically important to both respiratory and cerebral development in the premature infant.

Our laboratory has been intensely involved in the study of genetic markers affecting prematurity such as ILs, CD14, ACE, and NOS. In earlier studies, we have shown that genetic polymorphisms in IL-6, IL-10, and CD14 may alter the risk for blood stream infections and outcomes of sepsis in VLBW infants receiving ventilation therapy. In contrast, we have reported that the ACE insertion/deletion polymorphism does not have a significant effect on the incidence or outcome of sepsis in these VLBW infants.[21] We have also demonstrated that the ACE insertion/deletion polymorphism was not associated with an increased risk of death or BPD in VLBW infants.

In examining respiratory conditions of prematurity, we have reported that the IL10G-1082A polymorphism does not have a major influence on mortality or the development of BPD in VLBW infants receiving ventilation therapy. We have also demonstrated the association of several other genetic markers in VLBW infants receiving ventilation therapy.[21,22] Other genetic polymorphisms have been found at significantly higher rates in preterm infants in general compared with full-term infants. In terms of NOS, however, only iNOS was found to be statistically higher in preterm infants. The eNOS polymorphisms do not appear to be elevated in prematurity alone, but only in specific conditions of prematurity.[8] Most recently, we have found a novel allele of the eNOS polymorphism in asthmatics of the Caucasian population. All of these findings give support to the possibility of genetic mutations as a risk factor for conditions of prematurity, specifically IVH and respiratory conditions.

Earlier studies have shown that thrombotic markers are risk factors in the etiology of IVH in prematurity. In the present study we examined a polymorphism located in the promoter region -786 of the *eNOS* gene as a potential site of alteration, leading to decreased levels of NO. The *eNOS* gene is located on chromosome 7q35–36, with 26 exons spanning 21kb, and producing 1203–1205 residues.[16] Mice that are eNOS deficient have been shown to suffer severe abnormalities in lung morphogenesis, resulting in respiratory distress and death within the first few hours of life.[9] The authors of that study concluded that eNOS plays a significant role in lung development and is a possible contributor to clinical syndromes of respiratory conditions of neonates.

When examining the gene of *eNOS* at chromosome 7, the promoter region -786 has been shown to undergo a base substitution from thymine to cytosine, resulting in the mutated allele with presumed impaired NO production. The mutated C allele of this polymorphism has been shown in numerous studies to be associated with both coronary artery and post-subarachnoid hemorrhage vasospasm.[4,13,16,19] Previous reports have also indicated that the presence of the mutant allele -786C would lead to a decrease in NO levels in the cardiovascular diseases in adults, especially in coronary vasospasm.[4] A recent study by Ko et al.[12] revealed that polymorphism of the eNOS allele led to a statistically significant increase in risk of vasospasm after subarachnoid hemorrhage, specifically the CC genotype of the eNOS -786 polymorphism.[2] We have shown that this C allele is also linked to an increased risk of specific conditions of prematurity.

Due to these significant intracranial vascular effects of NO, we hypothesized that the etiology of IVH must be influenced by NO production, because a genetic mutation of *eNOS* would be a risk factor for IVH. Given earlier

studies in the literature, we chose to examine specifically the eNOS -786 polymorphism. Our study has shown that this mutant allele was present in a statistically significant higher percentage of patients with IVH than those without IVH. This finding suggests that this mutant allele is a significant risk factor in the origin of respiratory conditions and IVH of prematurity. These high-risk patients may benefit from low-dose NO inhalation therapy to help prevent IVH and its potentially devastating consequences.

Conclusions

Our data show that the mutant eNOS -786C allele is a significant risk factor in the origin of respiratory and IVH diseases in premature infants through an insufficient supply of endogenous NO levels in these infants. Those preterm infants who exhibit the -786C mutation have a 2-fold increased risk of developing respiratory disorders and IVH. Inhaled NO therapy may become a pivotal prophylactic therapy for specific high risk infants with this genetic mutation. Further studies are currently being conducted to examine the *eNOS* gene further for additional markers that may be instrumental in the etiology of IVH alone.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Using ex vivo proton magnetic resonance spectroscopy to reveal associations between biochemical and biological features of meningiomas

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Object. The goal in this study was to determine if proton (^{1}H) MR spectroscopy can differentiate meningioma grade and is associated with interpretations of biological behavior; the study was performed using ex vivo high-resolution spectra indicating metabolic characteristics.

Methods. Sixty-eight resected tissue samples of meningiomas were examined using ex vivo ^{1}H MR spectroscopy. Of these meningiomas, 46 were WHO Grade I, 14 were WHO Grade II, and 8 were WHO Grade III. Fifty-nine were primary meningiomas and 9 were recurrences. Invasion of adjacent tissue (dura mater, bone, venous sinus, brain) was found in 32 cases. Thirty-nine meningiomas did not rapidly recur (as defined by expansion on MR imaging within a 5-year follow-up period), whereas rapid recurrence was confirmed in 24 meningiomas, and follow-up status was unknown in 5 cases.

Results. The absolute concentrations of total alanine and creatine were decreased in high-grade compared with low-grade meningiomas, as was the ratio of glycine to alanine (all $p < 0.05$). Additionally, alanine and the glycine/alanine ratio distinguished between primary and recurrent meningiomas (all $p < 0.05$). Finally, the absolute concentrations of alanine and creatine, and the glycine/alanine and choline/glutamate ratios were associated with rapid recurrence ($p < 0.05$).

Conclusions. These data indicate that meningioma tissue can be characterized by metabolic parameters that are not typically identified by histopathological analysis alone. Creatine, glycine, and alanine may be used as markers of meningioma grade, recurrence, and the likelihood of rapid recurrence. These data validate a previous study of a separate group of Grade I meningiomas. *(DOI: 10.3171/2009.11.FOCUS09216)*

Key Words • brain neoplasm • meningioma • tumor metabolism • proton magnetic resonance spectroscopy • creatine • glycine • alanine

Meningiomas, despite categorization as benign lesions, may behave aggressively (that is, at rates as high as 20%), even those of low histological grade.[37,38] In a previous study on Grade I meningioma tumor tissue in which genetic characteristics were correlated with data from ex vivo ^{1}H MR spectroscopy on the same tumor tissue, an identifiable subset of tumor metabolic characteristics was associated with increased aggression, even within only Grade I tumors.[42] Meningiomas may grow quickly, invade adjacent brain, recur rapidly, and ultimately lead to decreased patient survival and quality of life.

Despite the recently revised 2000 WHO grading scheme for meningiomas, in which overall behavior correlates well with grade, aggressive behavior is sometimes difficult to predict.[29] Complicating the follow-up interpretation of prognosis, recurrence, and grade is the fact that resection has an overwhelming influence on outcome, even for high-grade tumors. Given discrepancies between the clinical behavior, histological grading criteria, and biological makeup of these tumors, the need exists both for adjunctive tools for the improved diagnosis and prognostication of outcomes in meningiomas and for a better understanding of the biological pathogenesis of these tumors.

According to current WHO histological grading criteria, intracranial meningiomas are classified as follows: between 85 and 94% are benign (Grade I), with a benign clinical course and a 7–20% recurrence rate. Between 5 and 11% of meningiomas are atypical (Grade II), with a more aggressive clinical course and a 29–40% rate of recurrence. Between 1 and 3% of meningiomas are anaplastic (Grade III), with a very aggressive clinical course, invasion, recurrence, and metastases.[29] Patients with Grade III tumors have a median survival after diagnosis of ~ 1.5

Abbreviations used in this paper: ^{1}H = proton; TSP = sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4.

years, and a 5-year mortality rate of 68%.[37] Usually, clinically aggressive behavior includes arachnoid penetration, bone invasion and destruction, rapid regrowth of a residual tumor, or recurrence of a "totally resected" tumor;[26,37] yet at surgery even fully benign meningiomas may be observed to possess many of these characteristics.

Clinical and pathological findings and resection assessment remain the standard for differentiating between meningioma grades and predicting aggressive tumor behavior, although with a high degree of inaccuracy. Our previous meningioma metabolic studies elaborated on characteristics noted in clinical and pathological studies of meningiomas.[1,18,19,24,29,33,37,38,42,47,53] Variability in recurrence within resection (Simpson) grades suggests that subsets of these tumors exist in which other factors have a greater influence on tumor recurrence than extent of resection.

Various in vitro techniques have been used in the search for prognostic markers in meningiomas. These include immunohistochemical analyses,[3,4,13,34,39] cytogenetic and molecular genetic analyses,[2,5,6,17,20,28,30,41,52,59,60] ^{1}H MR spectroscopy analyses,[22,23,27,36,44] and gene expression analyses.[11,58] Overall, although many of these studies have explored biological differences between meningioma grades and/or survival rates, they have not examined biological parameters against a strictly defined clinical behavior such as rapid recurrence within a specific time period.

Histological analysis alone is no longer sufficient to characterize tumors, and it is now recognized that simply describing genetic variations in tissue or disease may not indicate new avenues for treatment (the ultimate goal). The science of proteomics, or really our understanding of metabolism, may provide the link necessary to exploit genomics. The ^{1}H MR spectroscopy modality has specific diagnostic potential because it can be used to measure the concentrations of major metabolites in brain tumors in vivo,[9,10,12,16,35,43–45,48,54,56] providing a noninvasive quantitative measure of metabolic parameters that can be correlated to clinical parameters. These metabolic features are the proton-containing moieties, which may be part of full-scale proteins or which are proteinogenic, such as the amino acids glycine or alanine.

Such metabolic features can also be generated ex vivo in brain tumor extracts.[7,21,22,25,27,36,55] Analyses of tumor extract metabolite spectra ex vivo have enhanced the ability to interpret in vivo data, not only by allowing extracts to be studied at a higher magnetic field strength, giving greater spectral dispersion than in vivo spectra, but they have also allowed for an improved understanding of how variations in tumor metabolism contribute to variations in phenotype.[7,8,15,56,57] Creatine, glycine, alanine, lactate, choline, glutamine, glutamate, and the glutamine/glutamate complex are the metabolites most often cited as being useful in differentiating meningiomas from other tumors and from normal brain, and they are also the metabolites that are most consistently distinct and well resolved to allow for definitive quantitation.[31,44,45]

We studied the biochemical profiles of a series of 68 clinically and histologically diverse meningiomas. We have previously shown that ^{1}H MR spectroscopy studies can indicate metabolic tumor features associated with clinical aggression or status of recurrence, and with chromosomal profiles, even within a group of so-called benign tumors.[40,42] In this study, we chose to focus on metabolic features based on ^{1}H MR spectroscopy differences between pathological grades of meningiomas with a 5-year follow-up period. In addition, this study did not include the group of tumors from our previous study.[42] Thus, in many respects, this study also provided validation between 2 large groups of samples of meningioma tissue studied for biochemical characteristics, which to date has not been accomplished.

Methods

Patients and Tumor Specimens

Tumor samples were collected in 68 patients (41 women [60%] and 27 men [40%]), whose ages ranged between 29 and 84 years, with a mean of 55 years, who underwent resection of their tumors between 1986 and 2005 at the Barrow Neurological Institute and the Neurosurgical Department of the Donauspital, Sozialmedizinisches Zentrum-Ost. Male patients were somewhat younger than females (mean 57 ± 10 years for men, compared with 59 ± 14 years for women; unless otherwise specified, values are expressed as the mean ± SE). The surgeon recorded the existence of dural, venous sinus, and/or bone invasion as well as brain invasion, and the extent of tumor resection according to the Simpson grading scale.[53] Tumor tissues were immediately snap frozen in liquid nitrogen. The histopathological section of each collected specimen was reviewed to confirm that tissue used for extracts was appropriate. Corresponding paraffin-embedded surgically obtained neuropathology samples were also reviewed to assign grades in accordance with the 2000 WHO criteria.[29] Clinical follow-up ranged from 12 to 108 months, with a mean of 57 ± 15 months. The meningiomas for which an MR imaging–confirmed recurrence was found within 5 years were considered to have rapidly recurred. The meningiomas for which follow-up data were available over a 5-year period and for which no recurrence was found were considered not to have rapidly recurred. None of the tissue samples we examined had been exposed to radiation therapy.

Ex Vivo ^{1}H MR Spectroscopy

Frozen tumor specimens were taken from the same sections in which histological characterization was performed. Preparation of perchloric acid extracts was performed according to the protocol used by Lehnhardt et al.[27] The ^{1}H MR spectroscopy was performed at the Nuclear Magnetic Resonance Facility at Arizona State University. The perchloric acid extracts were redissolved in 0.6 ml deuterium oxide containing 0.05 wt % TSP, cooled to 0°C for 10 minutes, centrifuged to remove any particulates, and transferred into a 5-mm NMR tube. Spectra were acquired at 11.4 T (500 MHz for ^{1}H) with a Varian Inova spectrometer, using 90° single-pulse excitation, 256 transients, a sweep width of 8000 Hz, 1-second weak preirradiation to reduce residual HDO signal, and

TABLE 1: Invasion type in relation to recurrence at follow-up in 63 patients with meningioma

Type of Invasion	Recurrence at Follow-Up		Total
	Yes	No	
none	0	30	30
dural only	2	3	5
bone	5	3	8
sinus	5	3	8
bone & sinus	5	0	5
brain	2	0	2
brain & sinus	2	0	2
brain & bone	3	0	3
total	24*	39	63

* Invasion was associated with rapid recurrence (p = 0.01).

TABLE 2: Resection grade in relation to recurrence at follow-up in 63 patients with meningioma

Resection Grade	Recurrence at Follow-Up		Total
	Yes	No	
1			
invasive	3	3	6
noninvasive	0	21	21
2			
invasive	3	6	9
noninvasive	0	9	9
3			
invasive	18	0	18
noninvasive	0	0	0
total	24*	39	63

* Resection grade (Simpson Grades 1–3) was associated with recurrence (p = 0.001).

a 3.39-second total recycle time, adequate to give full relaxation of all resonances. Spectra were analyzed using commercially available software (MestRe-C NMR Data Processing Package for Windows, Unidade de Resonancia Magnética). Spectra were Fourier transformed, phase corrected and polynomial baseline corrected, and the appropriate peaks were picked by chemical shift and were integrated.[14] The concentration of each metabolite was measured by comparing the intensity of the identified compound with that of the TSP methyl residues. Assignments were confirmed from COSY and HMQC spectra. The spectra of several samples were obtained over the course of 18 hours to control for possible sample degradation, and showed no changes in chemical shifts or integrated intensities.

The ^{1}H MR spectroscopy modality was used to measure the absolute concentrations and ratios of creatine, glycine, alanine, lactate, choline, glutamine, glutamate, and the glutamine/glutamate complex. Metabolites were first examined according to histological grade to look for biochemical alterations that might be correlated with phenotypes as well as specific metabolites that might be used diagnostically in conjunction with standard histological criteria to confirm grade, to distinguish between primary and recurrent tumor, and between invasive or noninvasive behavior.

Data Analysis and Statistical Methods

Summary statistics were completed for several variables for group comparison (ClinMetrics, Inc.). Categorical variables (histological grade, patient sex, primary or recurrent tumor, histopathological subtype, invasion, resection grade, and recurrence on follow-up) summarized by frequencies and percentages were compared using chi-square or Fisher exact tests as appropriate. Continuous variables (patient age, metabolite concentrations, metabolite ratios) were computed using ANOVA. All statistical tests were conducted using a significance level of 0.05.

Results

Clinical Parameters

The most common location was frontotemporal skull base (23 lesions [34%]), followed by convexity (19 [28%]), falx (14 [21%]), and tentorium/posterior fossa (12 [17%]). The extent of resection in 32 tumors (47%) was Simpson Grade 1, in 18 (26%) it was Grade 2, and in 18 (26%) it was Grade 3. According to WHO criteria, tumors were classified as Grade I in 46 cases (68%), Grade II in 14 (21%), and Grade III in 8 (12%). Fifty-nine meningiomas (87%) were primary and 9 (13%) were recurrent tumors. Among 9 histopathological subtypes, 22 transitional (32%), 12 meningothelial (17%), 9 fibrous (13%), 8 atypical (12%), 5 anaplastic (7%), 4 angiomatous (6%), 3 psammomatous (5%), 3 papillary (5%), and 2 fibroplastic (3%) lesions were found. Invasion of adjacent tissue (dura mater, bone, venous sinus, brain) was found in 32 cases (47%). In 4 cases without and in 1 case with bone invasion, clinical follow-up was not available. Invasion by location and by sex was not statistically significant. For greater power of statistical assessment, Grade II tumors were combined with Grade III lesions.

Among the 63 individuals for whom follow-up was available, 7 of the 43 Grade I meningiomas and 17 of the 20 Grades II (9 of 12) and III (8 of 8) meningiomas recurred. This was statistically significant (p = 0.001, Fisher exact test). The relationship between invasion and rapid recurrence was significant (p = 0.01, Fisher exact test; Table 1), as was the association between resection grade and recurrence (p = 0.001, Fisher exact test; Table 2). Only 6 of the 48 patients in whom total tumor resection (Simpson Grade 1 or 2) was achieved experienced recurrence of tumor, compared with all of the 18 patients who had subtotal resection (Simpson Grade 3).

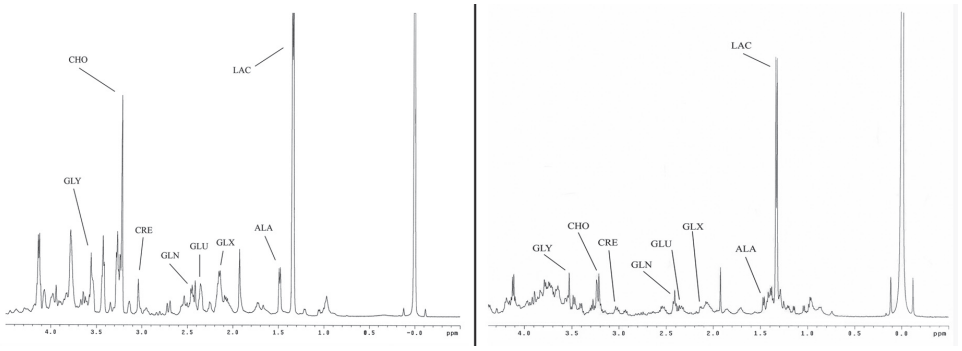

Fig. 1. Left: A ^{1}H MR spectrum from a Grade I meningioma tissue extract shows many metabolites, among which are the prominent choline peak at 3.2 ppm, the alanine peak at 1.4 ppm, and a robust lactate peak at 1.3 ppm. **Right:** In comparison, a spectrum from a recurrent tumor tissue extract shows many of the peaks to be reduced, but there is prominent reduction of choline and alanine. Ala = alanine; Cho = choline; Cre = creatine; Gln = glutamine; Glu = glutamate; Gly = glycine; Lac = lactate.

Ex Vivo ^{1}H MR Spectroscopy

Representative spectra from 2 meningiomas are portrayed in Fig. 1. The mean absolute concentrations of metabolites for Grade I versus Grades II and III meningiomas are shown in Fig. 2. Although several metabolites were selected for analysis and several trends are apparent, only a few metabolite concentrations and ratios were found to correlate significantly with clinical parameters. However, when comparing WHO Grade I (46 lesions) against Grades II and III (22), the mean metabolite values for creatine and alanine are found to be significant between groups ($p < 0.05$). The mean creatine value for Grade I was 183 ± 32 μmol per 100 g wet weight of tissue compared with the other group (Grades II and III; mean 79 ± 21). The mean alanine value was lower for meningiomas categorized as Grades II and III (mean 245 ± 42) compared with Grade I tumors (mean 393 ± 43; $p < 0.05$). In addition, the metabolite ratio of glycine to alanine correlated significantly with tumor grade ($p = 0.002$). The mean glycine/alanine value for Grade I was 0.96 ± 0.31, compared with the mean of 1.8 ± 0.37 for Grades II and III. Hence, alanine and creatine concentrations are lower, whereas glycine/alanine is higher in histologically aggressive meningiomas.

Neither descriptive and demographic variables nor any other metabolite concentrations or ratios correlated significantly with grade. Neither metabolites nor metabolite ratios correlated with histopathological subtype.

Only for alanine were there significant associations between metabolites and primary versus recurrent tumors ($p < 0.05$). Metabolites also may not predict primary/recurrent tumors. There were significant differences observed between primary and recurrent tumors for metabolite ratios of glycine/alanine ($p < 0.001$) and choline/glutamate ($p < 0.05$) (Fig. 3).

Individual metabolites are not associated with presence or absence of invasion. However, several metabolite ratios are associated with invasion: the ratio of lactate to glutamine/glutamate complex showed the highest significance ($p < 0.001$), with glycine/alanine and the ratio of choline to glutamine/glutamate complex following (both $p < 0.05$) (Fig. 4).

Finally, metabolites were examined against the parameter of recurrence within the follow-up period for tumors in which follow-up data were available (63 lesions). Of all metabolite concentrations and ratios studied, creatine and alanine were associated with tumor recurrence. The mean creatine and alanine concentrations were found to be significantly lower in tumors that rapidly recurred

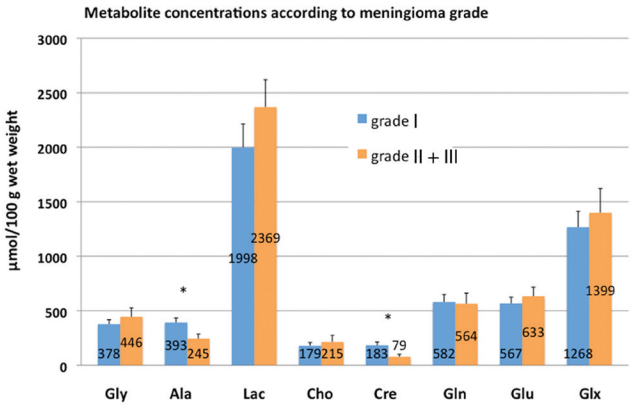

Fig. 2. Bar graph showing values (mean ± SE) for various prominent metabolites in the ^{1}H MR spectrum of meningioma tissue extracts (*$p = 0.002$).

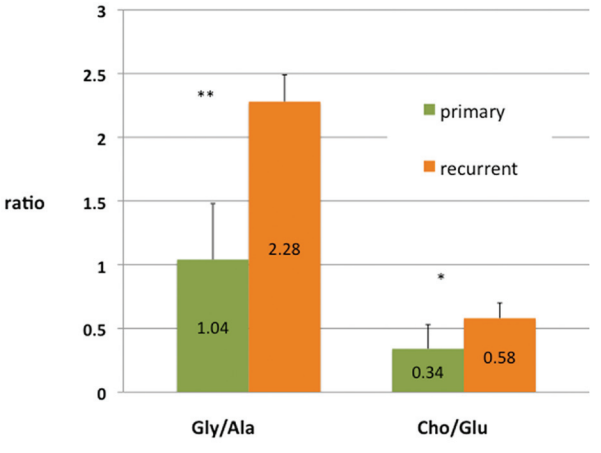

Fig. 3. Bar graph showing selected metabolite ratio values for glycine/alanine and choline/glutamate for 68 primary and recurrent meningiomas (**$p < 0.001$, *$p < 0.05$).

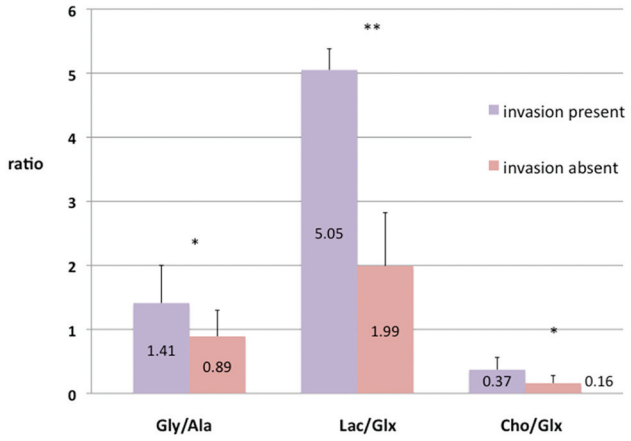

Fig. 4. Bar graph showing metabolite ratio values for 68 meningiomas interpreted as showing invasion (*p < 0.05, **p < 0.001).

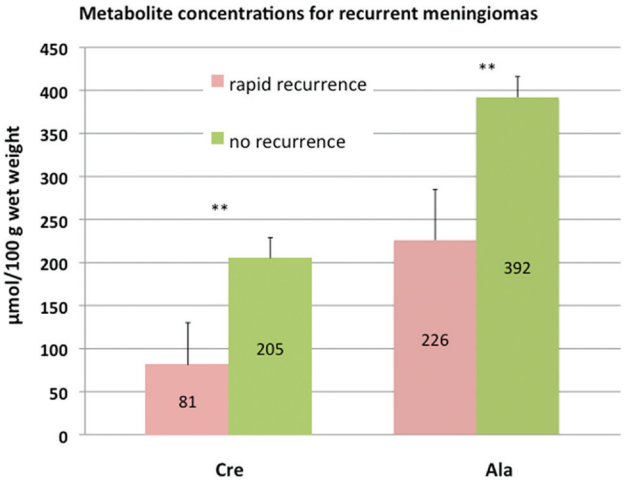

Fig. 5. Bar graph showing mean creatine and alanine values (mean ± SE) with complete follow-up data for 63 meningiomas that rapidly recurred, compared with those that did not recur (**p < 0.001).

compared with those that did not (both $p < 0.001$) (Fig. 5). The glycine/alanine metabolite ratio was also significantly higher in tumors with invasion than in those without ($p = 0.02$). The mean glycine/alanine ratio for patients experiencing recurrent tumors at follow-up was 1.53 ± 0.43, compared with the mean for those who did not have recurrence (1.06 ± 0.14).

Discussion

Accurate diagnosis and prognostication of meningiomas is limited by several factors in the clinic and in the laboratory. From a pathology perspective, these limitations include basing diagnosis on morphological changes downstream of causative molecular events. In the laboratory, studies are often limited by an ambiguous definition of an aggressive meningioma. Often studies either poorly define the phenotypic components of an aggressive meningioma or simply defer to WHO grading. When they base aggression solely on WHO grade, they seek correlations between biological and pathological data instead of correlations between biological and phenotypic data (clinical outcome). We have evaluated the ability of [1]H MR spectroscopy to examine proton-containing proteins and other metabolites to enhance the diagnosis and prognostication of these tumors based on ex vivo examinations of tissue samples. This was accomplished as follows: 1) we have compared [1]H MR spectroscopy to clinical and pathological analysis techniques typically used in the diagnosis and prognostication of meningiomas; and 2) we have evaluated [1]H MR spectroscopy in clearly demarcated clinically aggressive versus clinically benign WHO Grade I meningiomas, narrowly defining clinically aggressive meningiomas in this study as those that recurred on follow-up within 5 years of resection.

Previous work in our laboratory has shown that analyses of chromosomal aberrations and analyses of metabolites yield predictors of clinical aggression that provide vital adjuncts to clinical and pathological analyses within a single grade of meningioma.[40,42] Among all clinical and histopathological findings, resection grade and MIB-1 labeling index have been predictors of recurrence.[40]

Tumors are typically characterized by histologically scanning tissue for the most malignant region. Such interpretations have a significant impact on decisions regarding prognosis and choice of treatment. This examination principle also has merits for a biochemical and molecular approach to tumor behavioral assessment, and is becoming more important in more accurately predicting tumor behavior. Our previous studies of meningiomas show the impact of a regional metabolic and genetic survey of tissue.[40–42] Glycine, alanine, choline, creatine, glutamine, and glutamate were found to play defining roles in the recurring phenotype. In this study we expanded the model we used for examination of "benign" meningiomas into a larger set of lesions that spanned multiple grades in addition to several clinical parameters of aggression. This study was composed of a separate group of tumors from our study of benign meningiomas,[42] although many of the general metabolic characteristics we identified with recurrence, and so on, in our previous study held true in this study. Therefore, this series serves in part as a validation study, which is rare for MR spectroscopy studies of tumors.

Important Metabolites

Creatine. Compared with normal brain, the peak from creatine (creatine + phosphocreatine) is typically nearly absent in meningiomas, especially in comparison with levels seen in more malignant tumors such as medulloblastoma and glioblastoma.[22] In this study, creatine was lowest in rapidly recurring tumors. In our previous work, creatine was also the metabolite whose absolute quantity was closest to approaching a significant association with rapid recurrence, being lower in those meningiomas that rapidly recurred.[42] Creatine is usually used as an indicator of energy metabolism in the cell, although its exact function in many tumors is unknown. However, like high-grade gliomas, which show a lower signal from creatine compared with low-grade gliomas, recurrent meningiomas, which are likely to be more ag-

gressive, show lower signals from creatine compared with Grade I meningiomas.[44–46]

Glycine. This metabolite has been found to be relatively low in normal brain tissue, but is elevated in tumors such as medulloblastoma, ependymoma, and glioblastoma.[22] One study performed by magic angle spinning ^{1}H MR spectroscopy of 6 intact brain tissue specimens showed glycine to be absent in meningiomas or present at low levels.[7] However, similar to our findings, elevated levels of glycine are clearly detected in extracts of meningioma tissue by ^{1}H MR spectroscopy.[22] Neither study differentiated between meningioma types. In our study, glycine appears with high concentrations even in low-grade and clinically benign meningiomas. The high variability in levels of glycine present in some of the tumors may account for the discrepancies in earlier reports, and suggests the existence of more complex subsets within the pathological delineation of Grade I meningiomas.[21–23,36] Glycine appears to be a metabolite worthy of future attention.

Alanine. Alanine has generally been found to be elevated in meningiomas relative to other tumors and normal brain,[32,44,45] and appears in our study at concentrations comparable to literature values. In this study, alanine and glycine/alanine levels correlated with histological grade and with primary/recurrent status of the samples. The alanine concentration was lower in those meningiomas that rapidly recurred and in Grade III tumors. Alanine has been used as a nearly specific marker to distinguish meningiomas from gliomas and metastases by using ^{1}H MR spectroscopy.[44] Why meningiomas display a prominent peak from alanine is unknown, but we have determined that this metabolite is seen in quantity in extracts of dural tissue. Alanine may be produced by meningiomas in relatively larger quantities compared with other tumors, or it may be a by-product and collect within the tissue. Interestingly, alanine appears to be a "normal" part of the meningioma metabolism. As confirmed in this study, Grade I meningiomas show increased resonances for alanine compared with higher-grade meningiomas, and compared with recurrent meningiomas.[42] Thus, as the meningioma becomes more aggressive, it loses its "normal" metabolic process for alanine. Whether alanine collects as a by-product or is specifically produced by the cells, interruption of alanine metabolism may represent a novel, convenient, specific target for developing therapy against meningioma growth.

All of the aforementioned metabolites have been shown to play various roles in cellular metabolism related to oncogenesis and progression; these alterations may be causative or constitutive of the clinically aggressive phenotype. Other metabolites require critical analysis, such as the glutamine/glutamate complex. Beyond identification of metabolites and their patterns, defining their roles and associations to clinical, genetic, and proteomic data is crucial toward a functional understanding of tumors. Few studies have accomplished this goal.[42] Past studies of meningiomas have shown the ability of ^{1}H MR spectroscopy to characterize creatine and alanine levels in vivo. Although glycine has not been as well characterized by ^{1}H MR spectroscopy, our results, for example, suggest that additional effort is warranted to resolve glycine in vivo to allow for further definition of aggressive subtypes of these tumors.[16,44,45]

Recent studies of meningioma progression suggest that complex alterations seen in malignant tumors are already apparent in the early benign stages of Grade I tumors, characteristic of aggressive behavior.[2,42] The presence of certain metabolic aberrations may indicate a more aggressive tumor, but further work needs to be done to define very specific biological subtypes of these tumors, in addition to more thorough examination of separate groups of Grade II and Grade III meningiomas. Standardization of MR spectroscopy analysis techniques will be necessary, yet there will also be influence from sampling bias of the tumor. High-throughput profiling techniques such as gene expression microarray, epigenetic screening, or proteomics assessments may allow for more robust definitions of phenotypic subtypes and correlate with the use of ^{1}H MR spectroscopy for brain tumor classification and therapy planning.[51] Furthermore, it is not known how radiation affects the metabolic behavior of meningiomas. Perhaps somewhat surprisingly, none of the high-grade recurrent tumors had been exposed to radiation therapy.

Conclusions

Because chemical changes precede structural changes for any cell population or tissue, ^{1}H MR spectroscopy may provide a means of biochemical assessment for early detection of more aggressive tumors, or of those that may be in the process of becoming more aggressive. An extrapolation from this work is the use of ex vivo or in vivo MR spectroscopy to monitor brain tumor metabolism under treatment and to observe shifts in tumor activity with progression or regression.[49,50] Meningiomas, however, will be challenging to assess with regard to how and whether such metabolic information will affect treatment planning. These tumors may be aggressive or slow growing, may become quiescent, and are significantly affected by extent of resection and by sampling, and by interpretation of variability and bias with regard to "recurrence" and "invasion" of the arachnoidal membrane. Future work will mandate the application of similar high-resolution MR spectroscopy analyses to a larger number of tumor samples that can guide further exploration of the diagnostic potential of these metabolites in vivo.

Disclosure

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AC Scheck. Reviewed final version of the manuscript and approved it for submission: MC Preul, WK Pfisterer. Statistical analysis: WK Pfisterer, RA Nieman. Administrative/technical/material support: AC Scheck, RF Spetzler. Study supervision: MC Preul.

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