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Molecular Basis for Heterogeneity in Human Neuroblastomas

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Neuroblastomas demonstrate both clinical and biological heterogeneity. We have proposed that neuroblastomas may be classified in three genetically distinct subtypes, based on cytogenetic and molecular analysis. The first comprises those with hyperdiploid or triploid modal karyotypes (or compatible DNA content by flow cytometry), 1p LOH and *MYCN* amplification are absent, and *TRKA* expression is high. These patients are likely to be infants with low stages of disease (stages 1, 2, or 4S by the International Neuroblastoma Staging System), and they have a very favourable outcome (>90% cure). The second group consists of tumours that generally have a near diploid or tetraploid modal chromosome number or DNA content but lack *MYCN* amplification. They usually have 1p allelic loss, 14q allelic loss or other structural changes, and *TRKA* expression is usually low. These patients are generally older with advanced stages of disease (stages 3 or 4), and they have a slowly progressive course, with a cure rate of 25–50%. The third group is characterised by tumours with *MYCN* amplification. These tumours are generally near diploid or tetraploid, with 1p allelic loss, and low or absent *TRKA* expression. The patients are usually between 1 and 5 years of age with advanced stages of disease, and they have a very poor prognosis (<5%). It remains to be determined if tumours in one group ever evolve into a less unfavourable group, but current evidence suggests that they are distinct genetically. The identification of the oncogenes, suppressor genes and growth factor receptor pathways involved in neuroblastomas has provided great insight into the mechanisms of malignant transformation and progression, and ultimately they may provide the targets for future therapy.

Key words: neuroblastoma, oncogene, suppressor gene, ploidy, *MYCN*, nerve growth factor receptor, differentiation, screening

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INTRODUCTION

NEUROBLASTOMA, a tumour of the peripheral nervous system, is one of the most fascinating but frustrating of childhood neoplasms. In some patients, particularly infants (less than 12 months of age), the tumour may regress spontaneously, whereas in other patients, the tumour may mature into a benign ganglioneuroma. Unfortunately, in approximately half the patients, particularly those over 1 year of age with advanced stages of disease, the tumour will progress despite intensive therapy. Recent genetic and biological studies have led to a better understanding of these seemingly disparate clinical behaviours. What is emerging is that neuroblastoma is a diagnostic term for developmentally related tumours, with different genetic and biological features that are associated with distinct clinical behaviours.

A great deal of progress has been made in the past decade in advancing our understanding of human neuroblastoma at the cellular and molecular level (reviewed in refs [1–11]. Several characteristic genetic rearrangements in the tumours have been identified, including deletion of the distal short arm of chromosome 1 (1p) and amplification of the *MYCN* oncogene. In addition, the importance of the expression and function of neurotrophin receptors in the pathogenesis of neuroblastomas has been elucidated. These and other recent genetic observations have contributed to our understanding of tumour predisposition, malignant transformation, genetic heterogeneity, tumour progression and prognosis.

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GENETICS OF NEUROBLASTOMA

A subset of patients exhibit a predisposition to the development of neuroblastoma, and this predisposition follows an autosomal dominant pattern of inheritance [12–15]. These studies suggest that hereditary factors may be important in the pathogenesis of neuroblastomas, particularly those diagnosed in infants. No one has identified a constitutional genetic syndrome or congenital anomaly that is consistently associated with predisposition to neuroblastoma [16]. However, at least two cases have been described recently in which a constitutional deletion or rearrangement of the distal short arm of chromosome 1 (1p36) was found in children with neuroblastoma [17, 18]. Because this region is characteristically deleted in a subset of tumours (see below), a predisposition gene may reside at this locus.

GENETIC ABNORMALITIES IN NEUROBLASTOMA CELLS

The majority of neuroblastomas studied cytogenetically have modal karyotypes in the diploid range, but a substantial number are hyperdiploid or near triploid [19–23]. Indeed, the hyperdiploid and near triploid tumours in infants frequently have whole

chromosome gains with few if any structural rearrangements, and these patients have a particularly good outcome. In contrast, the near diploid tumours at any age, and the hyperdiploid tumours in older patients, usually have structural rearrangements, such as deletions, translocations, marker chromosomes, or other changes, and their outcome is unfavourable. Flow cytometric analysis of human neuroblastoma cells can also be used to identify cases with substantially increased (or decreased) DNA content [24–28]. Although this approach is much less labour-intensive than cytogenetic analysis, it cannot determine whether or not structural abnormalities are present.

Deletions and loss of putative suppressor genes on 1p and 14q

Deletion of the short arm of chromosome 1 (1p) has emerged as the most characteristic cytogenetic abnormality in neuroblastoma tumour tissue and tumour-derived cell lines [1, 29–33]. We and others have studied pairs of normal and tumour DNAs from patients with neuroblastoma using a panel of restriction fragment length polymorphisms (RFLPs) or polymerase chain reaction (PCR)-based polymorphisms [34–40]. The common region of deletion or loss of heterozygosity (LOH) lies at the distal end of the short arm of chromosome 1 from 1p36.1 to 1p36.3. This suggests that loss or inactivation of a gene (or genes) at this site is critical for the development or progression of neuroblastoma, and so a putative suppressor gene most likely is located within this chromosomal region.

There is recent information that adds complexity to the role of chromosome 1 in neuroblastomas. First, two groups found evidence to suggest that there are two suppressor loci, based on finding two different regions of deletion in subsets of tumours [39–41]. In these studies, one locus was within 1p36, consistent with other studies, and the second locus was possibly proximal to this region. Second, there is evidence that translocations between 1p and 17q occur with increased frequency in neuroblastomas, although the breakpoints on both chromosomes appear to be different [41]. It remains to be determined whether one or multiple genes on 1p are involved in neuroblastomas. However, the current evidence suggests that one gene at 1p36 is deleted in virtually all cases with 1p deletion, and a second proximal locus may be deleted in cases with *MYCN* amplification.

There is recent evidence that LOH for the long arm of chromosome 14 also occurs with increased frequency in neuroblastomas [42–45]. The frequency of 14q allelic loss varies in different studies from 25 to 50%, but it appears to be a consistent finding that probably represents loss of another suppressor gene. Allelic loss has been found involving 11q and 13q in some studies, but not in others [42–45], so the significance of these findings is unclear. Nevertheless, the studies showing allelic loss of 1p, 14q and possibly other chromosomal loci in subsets of tumours provide further evidence for genetic heterogeneity in neuroblastomas.

MYCN *amplification and expression*

For some time there has been cytogenetic evidence of gene amplification in primary neuroblastomas and cell lines, but the nature of the amplified sequences was not known. Recently, Schwab and colleagues identified a novel, *C-MYC*-related oncogene that was amplified in a series of neuroblastoma cell lines [46] and this was confirmed independently by others [47, 48]. The amplified sequence (known as *MYCN*) is found either on extrachromosomal double minutes (DMs), or on the homogeneous staining regions (HSRs) on different chromosomes in neuroblastoma cell lines, and the normal single-copy locus was mapped to the distal short arm of chromosome 2 [47, 49]. Apparently a large region from 2p24 (including the *MYCN* locus) becomes amplified initially as extrachromosomal DMs [50–52]. In a small percentage of primary tumours and approximately half the established neuroblastoma cell lines, the amplified DNA becomes linearly integrated into a chromosome as an HSR.

We studied primary tumours from untreated patients to determine the frequency and significance of *MYCN* amplification. We found amplification (from 3- to 300-fold per haploid genome) in approximately 25% of primary tumours [53, 54]. *MYCN* amplification was strongly associated with advanced stages of disease. However, amplification correlated with rapid tumour progression and a poor outcome, independent of the stage of the tumour or the age of the patient [1, 4, 16, 53–57]. Furthermore, we found a consistent pattern of *MYCN* copy number (either amplified or unamplified) in different tumour samples taken from an individual patient, either simultaneously or consecutively [58]. These results suggest that *MYCN* amplification is an intrinsic biological property of a subset of aggressive neuroblastomas, and tumours without amplification at diagnosis rarely if ever develop this abnormality subsequently.

Interestingly, our studies showed a strong correlation between *MYCN* amplification and 1p LOH [34, 43]. Both *MYCN* amplification and deletion of 1p [20–23, 59] are strongly correlated with a poor outcome and with each other, but it is not yet clear if they are independent prognostic variables. Nevertheless, they appear to characterise a genetically distinct subset of very aggressive neuroblastomas. Since cases with *MYCN* amplification represent a subset of those with 1p deletion, then 1p deletion may precede amplification. Indeed, it may be necessary to delete a gene that regulates *MYCN* expression, or one that mediates programmed cell death in the presence of high *MYCN* gene expression, in order for amplification to occur.

Approximately 25% of neuroblastomas have *MYCN* amplification, and virtually all of these cases have very high *MYCN* expression at the RNA and protein levels [56, 60–63]. Furthermore, there is heterogeneity in the level of expression of *MYCN* in single-copy tumours, but higher expression in non-amplified tumours does not appear to correlate with a worse outcome. It is possible that the level of expression in non-amplified tumours seldom if ever exceeds a certain threshold level necessary to confer an unfavourable outcome, whereas almost all tumours with *MYCN* amplification do exceed this threshold [64]. It is still possible that activation of *MYCN* by mechanisms other than amplification may play an important role [65].

We sought evidence for amplification or overexpression of other oncogenes in a large series of neuroblastomas and tumour-derived cell lines, but found none [1]. Although *NRAS* was first identified as the transforming gene of a human neuroblastoma cell line [66, 67], subsequent studies of primary neuroblastomas by ourselves and others [68–70] indicate that *RAS* activation by mutation of codons 12, 13, 59 or 61 is rare. On the contrary, there is evidence that high expression of *NRAS* in neuroblastomas is associated with a better outcome [71, 72]. Thus, in the subset of the patients lacking *MYCN* amplification, there is no consistent evidence to date for activation of any other oncogene.

Expression of the nerve growth factor receptor, TRKA

Neuroblastoma is derived from the sympathoadrenal lineage of the neural crest. Neurotrophic factors and their receptors have been implicated in the pathogenesis of neuroblastoma, but their role has been obscure. Recently, three tyrosine kinase

receptors for neurotrophic factors of the nerve growth factor (NGF) family have been cloned. The main ligand for the receptors encoded by *TRKA*, *TRKB* and *TRKC* is NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively [73–79].

To evaluate the clinical significance of expression of *TRKA* in neuroblastomas, we studied the relationship between patient survival and their mRNA expression in frozen tissue samples. We studied tumours from 77 children with neuroblastomas and 5 children with ganglioneuromas that had been diagnosed in Japan and the United States from 1982 to 1991 [64]. Patients were treated according to previously described protocols [80], and the median follow-up period after diagnosis was 36 months (range: 8–116).

TRKA expression was detected in 70 of the 77 neuroblastomas (91%), and a high level of expression (≥100 density units) was observed in 63 (82%). All 46 tumours in stages I, II and IVS (according to the Evans staging system) which had no *MYCN* amplification showed a high level of *TRKA* expression. However, 10 of 11 tumours with *MYCN* amplification had an extremely low level of *TRKA* expression. One tumour which had *MYCN* amplification but showed a high level of *TRKA* expression was obtained from an infant patient in stage IVS, and the tumour was regressing at the time of surgery. The expression of *TRKA* correlated strongly with survival: the 5-year cumulative survival rate of the group with a high level of *TRKA* expression was 86%, whereas that of the group with a low level of *TRKA* expression was 14% ($P < 0.001$).

The combination of *TRKA* expression and *MYCN* amplification had a strong influence on cumulative survival. The group with high levels of *TRKA* expression and no *MYCN* amplification showed a cumulative 5-year survival of 87% ($n = 62$). 4 patients' tumours had a normal *MYCN* copy number and a low level of expression of *TRKA*, and their survival was significantly worse than that of the group with a high level of *TRKA* expression ($P = 0.03$). Ten of the eleven patients who had tumours with *MYCN* amplification and low levels of *TRKA* expression died within 2 years. Similar results have been obtained independently by others [81–83], providing further support for the strong correlation between high *TRKA* expression and a favourable outcome. Indeed, the NGF/*TRKA* pathway may be playing an important role in the biological behaviour of some neuroblastomas, namely their propensity to regress or differentiate in selected patients.

Recently, we examined the expression and function of *TRKB* and *TRKC* in neuroblastomas. Both of these neurotrophin receptors can be expressed in a truncated form (lacking the tyrosine kinase) and a full-length form. Interestingly, expression of the full-length *TRKB* is strongly associated with *MYCN* amplified tumours [84]. Since these tumours also express the ligand, BDNF, this may represent an autocrine or paracrine loop providing some survival or growth advantage. Maturing tumours were more likely to express the truncated *TRKB*, whereas the most immature, non-amplified tumours expressed neither [84]. In contrast, the expression of *TRKC* was found predominantly in lower stage tumours (unpublished data), and like *TRKA*, was not expressed in *MYCN* amplified tumours.

Neuroblastoma regression and the biology of tumours identified by screening

Neuroblastomas are known to sometimes undergo spontaneous regression, but the prevalence of this phenomenon has been unclear [85–87]. Nevertheless, insight into the prevalence of clinically detectable tumours has come from mass screening of infants in Japan and elsewhere for neuroblastomas. The prevalence of neuroblastomas in screened populations has doubled from 1 in 8000 live births to approximately 1 in 4000 [88, 89]. This suggests that approximately 1 in 4000 infants develop subclinical neuroblastomas that regress spontaneously. Neuroblastomas identified by mass screening are usually identified in the lower stages, and their cure rate is excellent.

Cytogenetic analysis of these tumours reveals that most are hyperdiploid or near triploid with whole chromosome gains and few if any structural abnormalities [20, 23]. In addition, most have high *TRKA* expression and lack *MYCN* amplification [64, 80], suggesting that they are biologically favourable tumours. Thus, the results of the screening study have suggested either: (1) all neuroblastomas begin as tumours with a more favourable genotype and phenotype, and some evolve into more aggressive tumours with adverse genetic features; or (2) there are at least two different subsets of neuroblastoma, and the more favourable group presents earlier and therefore is the predominant group detected by screening. Our data, combined with those discussed above, are more consistent with the latter explanation [1, 34, 59].

In summary, patterns are emerging, based on cytogenetic, molecular and flow cytometric analysis, which suggest that neuroblastomas may be assigned to three genetically distinct groups (Table 1). The first comprises those with hyperdiploid or triploid modal karyotypes (or compatible DNA content by flow cytometry). *MYCN* amplification is uniformly absent, deletion of 1p or other structural abnormalities is rarely seen, and *TRKA* expression is usually high. These patients are more likely to be infants with low stages of disease (stages 1, 2, or 4S by the International Neuroblastoma Staging System) [90], and they generally have a very favourable prognosis. Most of the infants detected by the neuroblastoma screening studies described above appear to fall into this category. The second group consists of tumours that are generally near diploid or tetraploid modal chromosome number or DNA content, but lack *MYCN* amplification. These tumours frequently have 1p LOH, 14q LOH or other structural abnormalities, and *TRKA* expression is generally low or absent. These patients are more likely to be over 1 year of age and have advanced stages of disease (stages 3 or 4) with an intermediate outcome. The third group of tumours are characterised by *MYCN* amplification. They are generally near diploid or tetraploid, frequently with 1p LOH, and TRK expression is usually low or absent. The patients are also more likely to be over 1 year of age and have advanced stages of disease (stages 3 or 4). In contrast to the second group, they generally respond to treatment only transiently, if at all, and they have rapid progression and die within months to a year or so. It remains to be determined if tumours in one group ever evolve or "progress" into a less unfavourable group, but current evidence would suggest that they are genetically distinct.

The molecular and cytogenetic analysis of human neuroblastomas promises to make available a great deal of information that would be otherwise difficult to obtain. First, these studies may permit the localisation of one or more neuroblastoma predisposition loci. This would enable the identification of hereditary cases in order to provide family counselling, as well as prenatal diagnosis of affected individuals in informative families. Second, genetic markers provide a more objective means of classifying tumours that may appear similar histologically. Third, genetic analysis by karyotype, flow cytometry, determination of *MYCN* copy number and *TRKA* expression

Table 1. Biological/clinical types of neuroblastoma

Feature	Type 1	Type 2	Type 3
MYCN	Normal	Normal	Amplified
DNA ploidy	Hyperdiploid	Near diploid	Near diploid
	Near triploid	Near tetraploid	Near tetraploid
1p LOH	Absent	± Present	Usually present
14q LOH	Absent?	± Present	Usually absent
TRKA expression	High	Variable	Low or absent
Age	Usually <1 year	Usually ≥1 year	Usually 1–5 years
Stage	Usually 1, 2, 4S	Usually 3, 4	Usually 3, 4
3-year survival	95%	25–50%	<5%

provide information that has prognostic significance, and can direct the most appropriate choice of treatment. Hopefully, a better understanding of these and other genetic abnormalities in human neuroblastomas will allow specific proteins and pathways to be identified on which future therapeutic approaches could be focused.

Unfortunately, a number of questions still remain. Is there a common genetic abnormality seen in all neuroblastomas, or do they represent two or three genetically and clinically distinct tumours that resemble each other histologically? Although *MYCN* amplification identifies approximately 25% of patients who have a poor prognosis, what genetic features characterise the remainder of clinically unfavourable tumours? Why is only *MYCN* amplified, and why is it so strongly associated with 1p LOH? How many suppressor genes are involved in the pathogenesis of neuroblastoma, and how many reside on 1p? Where is (or are) the familial predisposition locus (or loci)? What is the role of neurotrophins and their receptors in the pathogenesis of neuroblastomas? These and other questions remain as a challenge to all of us interested in the malignant transformation and progression of this fascinating tumour.

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Regression and Progression in Neuroblastoma. Does Genetics Predict Tumour Behaviour?

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Neuroblastoma (NB) is a heterogeneous disease. The clinical course may range from spontaneous regression and maturation to very aggressive behaviour. Stage 4s is a unique subcategory of NB, generally associated with good prognosis, despite skin and/or liver involvement and the frequent presence of tumour cells in the bone marrow. Another type of NB is the locally invasive tumour without bone and bone marrow involvement which can also have a good prognosis, irrespective of lymph node involvement. Unfortunately, there is only limited biological information on such tumours which have not been treated with cytotoxic therapy despite lymph node involvement, residual tumour mass after surgery and/or bone marrow infiltration. In order to find specific genetic changes common to NBs with a benign clinical course, we studied the genetic abnormalities of these tumours and compared them with highly aggressive tumours. We analysed a series of 54 localised and stage 4s tumours by means of *in situ* hybridisation performed on fresh cells or on paraffin embedded tissues. In addition, we performed classical cytogenetics, Southern blotting and PCR analysis on fresh tumour tissue. The majority of patients had been treated with surgery alone, and in a number of patients tumour resection was incomplete. Deletions at 1p36 and amplifications of the *MYCN* oncogene were absent, and diploidy or tetraploidy were not seen in any case, with residual localised tumours possessing a favourable outcome. Unexpectedly, one patient with a tetraploid 4s tumour without any genetic structural changes not receiving any cytotoxic treatment, did well. Interestingly, this genetic spectrum contrasted with that of progressing tumours, in which most had genetic aberrations, the deletion at 1p36 being the most common event. These data, although limited, suggest that an intact 1p36 (recognised by D1Z2), the absence of *MYCN* amplification and near-triploidy (at least in localised tumours), represent prerequisites for spontaneous regression and/or maturation.

Key words: neuroblastoma, genetics, deletion 1p36, *MYCN*, cytotoxic treatment, *in situ* hybridisation, localised tumours, stage 4s, regression, progression

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