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Review Article

Current status of gene therapy for lung cancer

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CONTENTS

Abstract

Lung cancer is one of the most prevalent cancers and the leading cause of death due to cancer in the world, accounting for more than 28% of all cancer deaths. Despite progress in conventional therapies (surgery, chemotherapy, radiation), the 5-year survival rate remains below 15%. Thus, it is clear that there is a need for novel therapeutic paradigms. Recent advances in our understanding of the biology of lung cancer have led to the development of novel therapies directed at tumor-specific targets. In this regard, gene therapy has emerged as an exciting and promising strategy for the treatment of lung cancer. Several gene-based approaches have been devised to treat lung cancer, by direct control of cancer cell proliferation, by activation of an immune response against the tumor or by inhibition of angiogenesis. Adenoviruses are the vectors used most commonly to deliver genes for lung cancer cells. This review focuses on the known genetic defects that occur in lung cancer, the gene therapy strategies suggested by such defects, and the approaches currently under development for the treatment of this disease.

Introduction

Gene therapy is perhaps the most prominent and well-publicized new approach to the treatment of lung cancer (1). Several gene therapy strategies have been identified. Replacement of tumor suppressor gene function using adenoviruses to transfer wild-type *p53* has particular application in lung cancer therapy. Blockade of dominant oncogene function using antisense technology is showing remarkable promise. Genetic prodrug activation therapy using tumor-selective gene promoters to drive the expression of so-called suicide genes can produce antitumor effects. Immunogene therapy using recombinant DNA constructs to express cytokines and lymphokines and antiangiogenesis gene therapy blocking new vascular growth in the tumor are now entering clinical trials.

Crucial to any strategy that relies on the introduction of foreign genetic material to cells is the ability to deliver genes to the appropriate cell in sufficient numbers to achieve a therapeutic effect (2). Recombinant viruses have generally been highly efficient vectors, and vectors based on human adenoviruses have been used extensively in clinical studies. Nonviral methods of gene delivery are designed to elicit immunogenicity, and toxic effects of viruses and liposomes have already been used frequently to deliver therapeutic genes in preclinical studies in lung cancer. Clinic evaluation of many of these approaches has just begun and initial results provide the basis for a sense of cautious optimism.

Target genes

Evidence is rapidly accumulating that the development of lung cancer is a process that not only involves dysregulation of proliferative factors and activation of oncogenes, but also dysregulation of inhibitory factors and loss of suppressor gene function. In addition to mechanisms of dysregulated proliferation, two separate cellular processes seem to occur to allow progression of the disease: enhanced angiogenesis and evasion of apoptosis. In this section we will discuss the multiple genetic lesions that lead to lung carcinogenesis and focus on the hallmark molecular changes with potential for clinical translation (Table I).

Inactivation of tumor suppressor genes

Tumor suppressor genes encode proteins that play a vital role in the control of normal cell growth and act by providing antigrowth signals to inhibit the process of tumorigenesis. Inactivation of these genes by deletions, mutations or aberrant methylation of normally unmethylated CpG islands in the promoter region of many genes results in the loss of tumor suppressor function. Several tumor suppressor genes that are involved in lung carcinogenesis have been identified.

The tumor suppressor gene *p53*, located on chromosome 17p13.1, is the most frequently altered gene in

Table I: Biological targets in lung cancer.

Marker	Pathway	Aberration	Aberration frequency
p53	Growth regulation	Reduced expression	50%
RB	Growth regulation	Reduced expression	15%
Rb2	Growth regulation	Reduced expression	60%
p16	Growth regulation	Expression	60%
Ras	Growth regulation	Overexpression	30%
myc	Growth regulation	Overexpression	10%
EGFR	Growth regulation	Overexpression	25%
Bcl-2	Apoptosis	Expression	40%
Bax	Apoptosis	Underexpression	30%
Bcl-xL	Apoptosis	Overexpression	Frequent
Fas	Apoptosis	High expression	36-48%
VEGF	Angiogenesis	Overexpression	45-55%
Angiostatin	Angiogenesis	Expression	25%

human cancer (3). It is mutated in approximately 50% of all human cancers and in at least 40% of non-small cell lung cancer (NSCLC) (4-6). It has been designated the guardian of the genome because of its primary role in cell cycle control, DNA repair after radiation damage and apoptosis induction (7). *p53* induces cell cycle arrest through transcription of $p21^{Waf1/Cip1}$, an inhibitor of cyclin-dependent kinases (CDKs), which regulate transition through the cell cycle. p21 induces G1 arrest after DNA damage, thereby preventing cells from entering the S phase. *p53* also transcriptionally activates genes such as *BAX*, which triggers apoptosis or programmed cell death (8). Thus, mutations of *p53* result in an impaired cellular response to various stresses, including DNA damage, growth factor withdrawal and oncogenic transformation, as well as to genomic instability. Moreover, *p53* loss may also abrogate an effective apoptotic response to chemotherapy or radiation treatment.

Another tumor suppressor gene that is frequently inactivated in NSCLC is *RB* (9). The *RB* gene is considered the founder of the *RB* family, because two other genes that are structurally and functionally related, namely *p107* and *Rb2/p130*, have been identified more recently. A relevant biological activity shared by all three members of this family is the ability to negatively control the cell cycle (10). In fact, they negatively modulate the transition between the G1 and S phases, via mechanisms mostly related to inactivation of transcription factors, such as those of the E2F family, that promote cell entry into the S phase. The Rb protein is abnormal in expression level or structure in more than 90% of SCLC and in 20-30% of NSCLC (11). Recent studies of the expression patterns of the Rb family members (pRb/p105, p107 and pRb2/p130) reported mutations in patients with lung tumors, suggesting an independent role for *Rb2/p130* in the development and/or progression of human lung carcinoma (12). Using a tetracycline-regulated gene expression system to control the expression of pRb2/p130 in a JC virus-induced hamster brain tumor cell line, we demonstrated that induced expression of pRb2/p130 reduces the tumor mass in nude mice (13). In another study in nude mice, we showed that ectopic expression of pRb2/p130 suppresses the tumorigenicity of the SK-OV-3 ovarian cancer cell line overexpressing erbB-2 (14). Specifically, in support of the involvement of *Rb2/p130* as a tumor suppressor gene in lung cancer, we showed that *in vivo* retroviral transduction of pRb2/p130 in established tumors, derived from injection of the lung adenocarcinoma cell line NCI-H23 grown in nude mice, reduced the mass 12-fold with respect to the control viruses (12). On the basis of these findings, *Rb2/p130* gene therapy could be a viable therapeutic option for the management of lung cancer (15, 16).

The $p16^{INK4A}$ tumor suppressor gene is the other key component in the Rb pathway and is often inactivated in many solid tumors. Recent genetic and biochemical investigations of the molecular mechanism governing the G to S progression in mammalian cells have demonstrated an important role for *p16*. When activated by cyclin D, CDK4 is able to phosphorylate pRb, leading to the release of associated proteins such as E2F that have the capability to activate genes necessary for cell progression through the G1 phase. *p16* controls cell cycle proliferation during G1, inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate pRb. Homozygous deletion or point mutations of $p16^{INK4}$ are not frequently observed among primary lung cancers but are observed among metastatic and advanced NSCLC (17). An alternative mechanism of $p16^{INK4}$ inactivation is aberrant methylation of the CpG island promoters, and this is common in a number of human cancers. Aberrant methylation of normally unmethylated CpG islands is associated with transcriptional inactivation and loss of expression of tumor suppressor genes in human cancers. Aberrant methylation of the $p16^{INK4}$ gene is observed frequently in NSCLC, *i.e.*, in 36-64% of cell lines (18) and 16-53% of primary tumors (19). Hypermethylation is thought to be the major mechanism through which $p16^{INK4}$ becomes inactivated in primary lung cancers. $p16^{INK4}$ hypermethylation is frequently detected in premalignant lesions (20). However, it is still unknown whether the methylation status of the $p16^{INK4}$ gene changes during the progression of lung carcinoma. Some studies have reported that reintroduction of this gene into NSCLC tumor cells lacking it results in significant tumor growth suppression (21).

Activation of proto-oncogenes

Proto-oncogenes encode proteins that are positive effectors of the transformed phenotype and can be considered positive growth regulators. The activation of these proto-oncogenes via mechanisms that target only one allele (gene amplification, point mutation and constitutive overexpression) causes their functional deregulation, thereby leading to a gain in function or "dominant" effect.

Ras is involved in cell growth and differentiation and the transmission of extracellular stimuli into intracellular signals, a process known as signal transduction (22). Ras proteins are GTP-binding proteins that bind to and enzymatically convert guanosine triphosphate (GTP) to guanosine diphosphate (GDP) once a signal has been transmitted. In the GTP-bound form, the Ras protein is active and serves as a growth-stimulatory signal, whereas in the GDB-bound form, the Ras protein is inactive. When the *Ras* gene is mutated, the resulting protein loses this GTPase activity and is trapped in the active, GTP-bound growth-stimulatory state. Mammalian cells express three types of Ras proteins (H-Ras, N-Ras, K-Ras). Generally, *Ras* oncogenes are activated by point mutations at codons 12, 13 or 61. Mutations in the K-*ras* oncogene occur in 15-20% of NSCLC, especially in adenocarcinomas, but never in SCLC (23). Mutations in K-*ras* account for approximately 90% of Ras mutations in lung adenocarcinomas. The majority of mutations are G-T transversions, which are associated with cigarette smoking. Several studies suggested that patients whose tumors harbor these mutations have a worse prognosis than patients without K-*ras* mutations (24).

Myc is a proto-oncogene that encodes nuclear DNA-binding proteins that are involved in transcriptional regulation of genes that promote cell division (25). Early studies showed that mutations in the L-*myc* oncogene occur frequently in lung cancers, especially in SCLC (26). Generally, *myc* oncogenes are overexpressed by gene amplification or transcriptional dysregulation.

Epidermal growth factor receptor (EGFR, erbB-1) is a member of the erbB family of tyrosine kinase receptor proteins, which also includes erbB-2 (HER2/*neu*), erbB-3 and erbB-4. These receptors play an important role in tumor cell survival and proliferation (27, 28). In lung carcinomas, EGFR is more commonly overexpressed than HER2/*neu* (29). The prognostic association of EGFR overexpression in lung cancer, however, is a controversial issue. Several reports indicated that EGFR was associated with a poor prognosis (30), whereas no prognostic association was indicated by other reports (31).

Enhanced tumor angiogenesis

Tumor angiogenesis is a complicated multistep process that includes the dissolution of the endothelial cell basement membrane and the extracellular matrix in association with the development of new endothelial cells migrating towards an angiogenic stimulus, with the resulting formation of new and functioning capillaries (32). Angiogenesis occurs when there is an imbalance of proangiogenic and antiangiogenic factors. The most important proangiogenic factor is vascular endothelial growth factor (VEGF), the gene for which is located on chromosome 6p21.3. Five VEGF isoforms generated by alternative splicing have been reported, with VEGF-165 being the most abundant isoform in human tissues. VEGF binds to two main high-affinity tyrosine kinase receptors that are expressed primarily on endothelial cells: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Despite the significantly higher VEGF binding affinity for Flt-1, Flk-1 is more important for modulating VEGF mitogenicity. Flt-1 functions primarily as a negative regulator of VEGF and does not participate in endothelial cell migration or proliferation. The binding of VEGF to Flk-1 stimulates endothelial cell proliferation and neovascularization by activation of various pathways, such as Ras, phospholipase C γ (PLCγ) and phosphatidylinositol 3-kinase (PI3-K). VEGF and its receptors are frequently expressed in lung carcinomas, and VEGF expression is significantly associated with new vessel formation and with an adverse outcome in NSCLC patients (33-36). Although VEGF is the primary growth factor involved in the angiogenic process, several other factors also play a role in the development of blood vessels. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and IL-8 are other important angiogenesis regulators and potential therapeutic targets in lung cancer (37-39). As mentioned above, angiogenesis occurs when there is an imbalance of proangiogenic and antiangiogenic factors. In fact, cancer cells are able to produce molecules with antiangiogenic properties such as angiostatin, the role of which as a prognostic factor in lung cancer is under investigation (40).

Evading apoptosis

Apoptosis is essential for carcinogenesis and tumor progression (41-43). Two signaling pathways can lead to apoptosis: one on the cell surface regulated through ligands and receptors (extrinsic pathway) and the other regulated through the mitochondria and cytochrome *c* (intrinsic pathway). These pathways are intimately connected via caspase-8 and Bid. The extrinsic pathway responds to multiple death ligands, such as Fas ligand and Apo2 ligand/TRAIL (TNF-related apoptosis-inducing ligand). These ligands bind their receptors at the plasma membrane, engage the recruitment of a multimeric death-inducing signaling complex and stimulate downstream effector caspases (caspase-3, caspase-7) to realize an organized cell disintegration. In lung cancer cells, death receptors, including Fas and tumor necrosis factor receptor-1 (TNFR-1) and -2 (TNFR-2), are regularly expressed (44, 45). Apoptosis-inducing ligands may be underexpressed in lung cancers. The addition of these death-inducing ligands, such as Apo2 ligand/TRAIL, may increase apoptosis and thereby inhibit the growth of lung cancer cell lines (46, 47). The intrinsic pathway related to mitochondria is regulated by a family of proteins called

Bcl-2 (48, 49). The Bcl-2 family has both proapoptotic (Bax, Bak, Bcl-xs, Bad and Bid) and antiapoptotic factors (Bcl-2, Bcl-xL and Bcl-w). Of these, the antiapoptotic factors Bcl-2 and Bcl-xL can be upregulated in lung cancer. In effect, Bcl-2 expression has been correlated with poor prognosis in a number of malignancies and its presence confers chemo- and radioresistance to cells *in vitro*. Overexpression of the Bcl-2 protein is more common in NSCLC with neuroendocrine features and in SCLC, while mesothelioma and most NSCLC have normal Bcl-2 expression and overactive Bcl-xL (50, 51).

Gene delivery systems

There are a number of strategies designed to introduce genes into cells, including viral and nonviral vectors. Vectors derived from retroviruses, adenoviruses, adeno-associated virus (AAV) and herpes simplex virus (HSV) are used extensively. Nonviral methods of gene delivery include liposomes, cationic polymers and disruption of the cell membrane by physical methods. Nonviral methods of gene delivery are more convenient and have obvious safety advantages over viral methods. However, the majority of current nonviral gene transfer methods result in transient gene expression, and the efficiency of gene transfer is lower compared with most viral methods. Advantages and disadvantages of these systems are summarized in Table II.

Viral vectors

Retroviruses are RNA viruses that are able to integrate DNA within the host cell genome (52-54). The virus enters the cell using envelope glycoproteins to bind to specific receptors on the cell surface. Viral RNA is then reverse-transcribed to DNA by the virally encoded reverse transcriptase. The viral DNA is transported to the nucleus, where it integrates into the host chromosome and directs transcription of the provirus. Viral transcripts are translated by the infected cell to form viral structural proteins. Some of the unspliced viral transcripts are packaged into the newly formed viral particles and are released by budding.

There are several potential limitations of recombinant retroviruses. Retroviruses can transduce only those cells that are actively undergoing mitosis, which limits their utility in certain cell populations, especially hematopoietic stem cells. In addition, the retroviral capacity is limited to about 8 kb. The currently achievable retroviral titers are 1 x 10^7 particles/ml and such titers are not sufficient for the treatment of large tumors. Lastly, they are relatively labile compared with other viruses and cannot be purified without significant loss of infectivity. Advantages include the ability to stably integrate into the host genome, thereby theoretically ensuring prolonged and stable expression of the therapeutic gene, and the absence of viral protein expression.

Adenoviruses are large, complex structures that contain a linear, double-stranded DNA genome approximately 36 kb pairs long (55-57). Their genome can be divided into two main regions —early (E) and late (L)— according to the time at which their genes are expressed during viral replication. Adenoviruses are internalized by receptor-mediated endocytosis and transported to the nucleus, where the immediate early genes *E1A* and *E1B* are expressed. The products of these genes regulate the expression of a variety of host genes and activate the expression of the early delayed genes *E2*, *E3* and *E4*. The *E1* region is essential for viral replication. Therefore, in gene therapy studies, the majority of adenoviral vectors developed have a deletion of this gene. This allows the virus to be able to transfer the gene of interest without replicating. Adenoviral vectors have a number of positive and negative attributes as well. The positive attributes include high frequency of transduction and high levels of transgene expression. Because of the structural stability of the capsid polypeptides of adenovirus, viral particles can be purified and concentrated to very high titers of up to 1 x 10^{13} plaque-forming units (pfu)/ml. This is in contrast to retroviral vectors that achieve much lower titers (1 x 10^7 pfu/ml) because of their envelope instability. Another characteristic of adenoviral vectors is their lack of integration into the human genome. The adenoviral genome remains in the nucleus of the target cells as a nonreplicating extrachromosal entity, thereby avoiding

Table II: Biological properties of commonly used gene delivery systems.

	Retrovirus	Adenovirus	Adeno-associated virus	Liposome
Type	Viral	Viral	Viral	Nonviral
Titer	Low	High	Variable	NA
Efficiency	Moderate	High	Moderate	Low
Duration of expression	Stable	Transient	Stable	Transient
Immunogenicity	Low	Moderate	Low	Low
In vitro toxicity	Low	Low	Low	Low
Repeated dosing	Possible	Not possible	Possible	Possible
Clinical trials	Yes	Yes	Yes	Yes
Advantages	Infects hematopoietic and epithelial cells	Does not need proliferating cells	Stable	Easy to produce; safe
Disadvantages	Unstable; low titer; needs replicating cells	Immunogenic; temporary; does not infect marrow	Small capacity; low titer; requires helper virus	

any potential for mutagenic effects caused by random integration into the host. They have the further advantage of being able to transduce a wide profile of cellular phenotypes, including not only epithelial and carcinoma cells, but also hematopoietic cells. Moreover, adenoviral vectors are trophic for respiratory epithelium and transduce pulmonary cells efficiently. The major disadvantage is their ability to trigger nonspecific inflammatory and specific antiviral immune responses. These problems may be ameliorated by co-administration of immunosuppressant agents, readministering adenoviruses of alternative serotype or using "third-generation", extensively deleted adenoviruses. However, adenoviral DNA remains episomal in the host cell nucleus and the virus does not integrate into cellular DNA, making the infection temporary.

Another viral vector that has been used is AAV, a member of the parvovirus family and a linear single-stranded DNA virus. AAV requires a helper virus, such as adenovirus, for replication (58-60). The AAV integrates at a site-specific area on chromosome 19, and then remains dormant until infection with a helper virus (usually an adenovirus) allows its replication. AAV vectors offer many of the same advantages as adenoviral vectors, including a wide host cell range and relatively high transduction efficiency. Additionally, AAVs are able to raise long-lasting gene expression *in vivo*, even after a single virus injection. Several authors have reported persistence of expression of foreign genes transduced with recombinant AAV (rAAV) from 180 days up to 18 months. Moreover, AAV vectors cause little damage to target cells, unlike adenoviruses which can cause a high degree of cytopathogenicity. Despite these advantages, AAV has not been used clinically to date because it has a small capacity to hold DNA and it has not been produced in high titers. Like all vectors that require promoters that utilize host cell transcriptional machinery, the gene expression of AAV is limited by the ability of a particular promoter to function in a given cell type.

HSV has also been investigated as a potential target for gene delivery (61). It infects a wide range of cell types with prolonged expression and high titers. Unfortunately, virus cytotoxicity and difficulty in manipulating its large genome have limited its clinical use. Because of the natural tropism of HSV for nerve cells, vectors based on HSV have been used mainly to deliver genes to the central nervous system (CNS).

Nonviral vectors

Nonviral methods of gene delivery have employed primarily liposomes. Liposomes are cationic lipids complexed to DNA (62-64). The overall positive surface charge of the cationic liposome interacts with the negative charge of the DNA backbone, forming a stable complex which is internalized into cells due to its electrical charge properties. Cationic lipid formulations have already been used to delivery genes to the lungs *in vivo*. Another method of nonviral gene delivery is to physically deliver the gene into cells, such as by directly injecting the DNA into cells using a microscope and micromanipulator, using a technique named microinjection. Although this technique is useful in the laboratory for the production of transgenic animals, very few cells can be injected manually, and thus this method is not valid for clinical purposes. Another physical method is the "gene gun", which propels gold beads coated with DNA into cells. This method has a potential role in immunization strategies because gene transfer to epithelial cells can be performed *in vivo*.

Gene therapy strategies

A variety of gene transfer techniques and genetic constructs have been evaluated in the laboratory using both *in vitro* and *in vivo* lung cancer models. The information obtained from these studies has been translated into clinical studies investigating the safety and feasibility of delivering genes to patients with advanced or recurrent NSCLC. Published studies are mostly phase I studies that have investigated the efficacy of: 1) reintroduction of tumor suppressor genes; 2) inhibition or downregulation of dominant oncogenes; 3) suicide gene therapy; 4) inhibition of angiogenesis; and 5) enhancing the immune response. Table III lists clinical trials of gene therapy for patients with lung cancer.

Reintroduction of tumor suppressor genes

By far the most popular gene therapy strategy is to introduce tumor suppressor genes into the cancer cells to replace a normal functioning gene. Tumor suppressor genes in cells with a homozygous loss of function could restore normal growth and proliferation pathways. Due to its frequent mutation in lung cancer, *p53* represents an attractive gene therapy target. Adenoviral and retroviral vectors have both been used to successfully deliver *p53* to human lung cancers in athymic mouse models, and antitumor activity after *p53* gene transfer in NSCLC has been demonstrated in a series of preclinical studies *in vitro* and *in vivo* (65, 66). It has been reported that introduction of wild-type *p53* into NSCLC cell lines is able to suppress tumour growth by inducing apoptosis (67). Moreover, recent evidence points to the involvement of antiangiogenic effects in mediating tumor growth suppression (68). After infection with the *p53*-adenoviral vector, a reduced expression of VEGF was reported in transduced compared to nontransduced human lung cancer cells, suggesting the existence of a bystander effect.

In 1996, Roth *et al.* published results of the first clinical trial of *p53* replacement in NSCLC conducted at the M.D. Anderson Cancer Center (69). Wild-type *p53* was administered by direct intratumoral injection of a retrovirus vector carrying a wild-type *p53* cDNA driven by the B-actin promoter. Nine patients entered the protocol. Eight of the 9 patients completed the protocol, and all 8 showed evidence of gene transfer. Three of the 7 patients who were assessable showed evidence of local tumor regression in treated lesions, whereas other untreated

Table III: Clinical trials of gene therapy in lung cancer.

Phase	Drug	Gene strategy	Patients	Results	Ref.
I	Retroviral p53	Replace tumor suppressor genes	9	3 partial responses + 3 stable disease	69
I	Ad-p53 (Sch-58500)	Replace tumor suppressor genes	15	4/6 partial responses	70
I	Ad-p53 (INGN-201)	Replace tumor suppressor genes	28	2 partial responses + 16 stable disease	71
I	Ad-p53 (INGN-201)	Replace tumor suppressor genes	25 BAC	1 partial response + 17 stable disease	72
I	Ad-p53 + cisplatin	Replace tumor suppressor genes	24	2 partial responses + 17 stable disease	77
II	Ad-p53 + carboplatin/ paclitaxel	Replace tumor suppressor genes	25	No benefit	78
II	Ad-p53 + radiotherapy	Replace tumor suppressor genes	19	1 complete response + 11 partial responses + 3 stable disease	79
I	ONYX-015	Replace tumor suppressor genes	2 NSCLC	No response	86
I/II	ISIS-3521 + carboplatin/ paclitaxel	PKC-α antisense	NSCLC	46% response rate; median survival 15.9 months	94
II	Oblimersen + paclitaxel	Bcl-2 antisense	SCLC	No response; 17% stable disease	95
I/II	GVAX®	Tumor cell vaccine	83	3 complete responses in advanced stage	108
I	BEC2	Tumor cell vaccine	15	Good results	110
III	BEC2 maintenance	Tumor cell vaccine	Limited-disease SCLC	No difference in overall survival	111

lesions continued to progress, and 2 patients showed no evidence of viable tumor 4 weeks after treatment. No toxic effects directly attributable to the vectors were observed in the 7 evaluable patients.

As mentioned previously, difficulties in the production of large quantities of retrovirus and poor overall transduction efficiency led to the need to explore the safety of other vectors. Another possible approach for targeting *p53* mutations is a gene replacement strategy using an adenovirus containing the wild-type *p53* gene (Ad-p53 or RPR/INGN-201). Ad-p53 is a vector system in which the wild-type *p53* gene is inserted into a first-generation adenoviral vector (70). Swisher *et al.* conducted a phase I clinical trial of Ad-p53 gene transfer in patients with NSCLC who had progressed on conventional treatments (71). In that study, 28 patients received up to 6 monthly intratumoral injections of Ad-p53. The treatment regimen was well tolerated, the most common adverse effect being transient fever. Other common side effects included headache, pain and edema. No allergic reactions were noted, although pneumothorax was seen in 6 patients, with 2 requiring further intervention. Of the 25 evaluable patients, 2 (8%) exhibited partial responses, 16 (64%) exhibited disease stabilization ranging from 2 to 24 months, and the remaining 7 (28%) exhibited disease progression. Vector DNA was detected in 80% of the evaluable patients, indicating successful gene transfer. Vector-specific *p53* mRNA, as an indicator of gene expression, was detected in 46% of patients. Measurement of apoptosis revealed tumor cells undergoing apoptosis in all but 1 of the group of patients expressing the gene.

An innovative application of Ad-p53 was described by Carbone *et al.* (72). In this study, patients with bronchoalveolar carcinoma (BAC) were treated with Ad-p53 administered by bronchial lavage. The rationale for this study included the fact that BAC is a disease in which tumor cells tend to spread aerogenously in thin layers along distal airways, potentially allowing for disseminated gene transfer by vector administration via the airways. Moreover, it is a disease that involves multiple areas of the lung but no distant metastases. Thus, adequate dissemination of Ad-p53 might be obtained in this locoregional setting and concerns regarding inadequate distant dissemination of this type of agent would be of less importance. In this trial (E6597), a total of 25 patients were treated with up to 14 cycles of therapy. The maximum planned dose of 2×10^{12} viral particles (vp) was tolerated, but 1 of the 4 patients treated at this dose level experienced grade 4 pulmonary toxicity and another died about 1 month after his second cycle. A cohort of 10 patients was therefore treated at the recommended phase II dose of 5×10^{11} vp. At this dose, no greater than grade 3 toxicity was observed. The most frequent toxicity was low-grade fever 24-48 h after bronchoscopy, increased cough after bronchoscopy, and grade 1 or 2 myalgia, arthralgias, headache and fatigue. One month after the start of treatment, 25 patients were evaluated: a partial response

was documented in 1 patient, progressive disease in 7 and stable disease in 17 patients, but disease progression into untreated lobes was found for 6 of the patients in this last group. The overall survival curve showed a median survival of 9 months. One patient showed radiographic responses in the liver and brain, 3 patients had > 20% improvement in the diffusion capacity of carbon monoxide and a number of them showed improvement in symptoms. Results of this study were encouraging and a future study combining airways delivery of Ad-p53 with chemotherapy in BAC has been planned. Alternative approaches to enhance aerosolized gene delivery of *p53* and other genes are also being investigated, including aerosolization of adenoviral vectors incorporated into calcium phosphate precipitates and formulation with cationic polymers such as polyethyleneimine (PEI).

Preclinical studies showed that synergistic growth inhibition could be achieved by combining *p53* gene therapy with cisplatin and irradiation (73-76). On the basis of results of preclinical studies, Nemunaitis *et al.* initiated a phase I trial of *p53* gene transfer in sequence with cisplatin in 24 NSCLC patients with nonfunctional *p53* genes (77). Intravenous cisplatin was administered and 3 days later *p53* was delivered directly into the tumor. A total of up to 6 monthly courses were given. Seventeen patients remained stable for at least 2 months, 2 achieved partial responses and 5 continued with progressive disease. When tumor biopsies were analyzed for apoptosis, 14% demonstrated no change, 7% showed a decrease in apoptosis and 79% demonstrated an increased number of apoptotic cells. Notably, 75% of the patients entered into the trial experienced tumor progression while being treated with cisplatin- or carboplatin-containing regimens.

Schuller *et al.* published results of an interesting international, multicenter, open-label, nonrandomized phase II trial (78). In this study, 25 patients with NSCLC were treated with 3 cycles of regimen A (carboplatin AUC6 on day 1 plus paclitaxel 175 mg/m^2 on day 1) or regimen B (cisplatin 100 mg/m^2 on day 1 plus vinorelbine 25 mg/m^2 on days 1, 8, 15 and 22) in combination with intratumoral injection of 7.5 x 10^{12} particles of Sch-58500 (rAd-p53; on day 1). The primary objective of the study was to compare the response rates of the Sch-58500-injected lesions to the response rates of the noninjected comparator lesions in patients receiving one of the two chemotherapy regimens. This design was chosen to permit detection of any additional local effect of intratumoral wild-type *p53* gene transfer by an intrapatient comparison. The study failed to demonstrate an additional benefit from intratumoral adenoviral *p53* gene therapy in patients receiving an effective first-line chemotherapy for advanced NSCLC. No difference between the response rate of lesions treated with *p53* gene therapy in addition to chemotherapy (52% objective responses) and lesions treated with chemotherapy alone (48% objective responses) was detected. However, median and 1-year survival, as well as the toxicities observed in this trial, compared favorably with those from studies of similar chemotherapy regimens, establishing the safety of multiple intratumoral injections of Sch-58500 in combination with chemotherapy in patients with advanced NSCLC.

A phase II study combining Ad-p53 with radiation therapy was carried out by Swisher *et al.* (79). Patients with locoregionally advanced nonmetastatic NSCLC who could not tolerate chemoradiation because of age or comorbidity and patients with localized disease who were unable to tolerate surgical resection because of poor pulmonary function were eligible. Nineteen patients were treated as outpatients with radiation therapy to 60 Gy over 6 weeks in conjunction with 3 intratumoral injections of Ad-p53 on days 1, 18 and 32. Ad-p53 doses were injected directly into the primary tumor using bronchoscopy or CT guidance. Post-treatment biopsies demonstrated a pathological complete response in 12 of 19 patients who underwent biopsies, suggesting a high pathological control rate at the primary tumor. Indeed, historical data demonstrate only a 20% pathological complete response rate with radiation alone. Radiological evaluation revealed a complete response in 1 of 19 patients (5%), a partial response in 11 patients (58%), stable disease in 3 patients (16%) and progressive disease in 2 patients (11%). The 1-year progression-free survival was 45.5%, with most failures occurring because of metastatic progression rather then local failure, and the survival rate at 1 year was 56%. Treatment was well tolerated and 17 of 19 patients completed all planned radiation and Ad-p53 gene therapy doses as outpatients. Other than injection-related pneumothorax in 13 patients, the most common adverse events were grade 1 or 2 fever and chills. All patients with pneumothorax were managed as outpatients by observation (8 patients) or the use of a percutaneous pleural catheter (5 patients). No treatment-related mortality was observed. This study showed that Ad-p53 can be administered in conjunction with radiation therapy in an oupatient setting in patients with locally advanced NSCLC with low toxicity. The high negative pathological control rate is encouraging, but the continued metastatic failure emphasizes the need to combine Ad-p53 with chemotherapeutic agents to try to address distant disease.

On the basis of these encouraging results, multicenter phase III trials comparing groups of patients with adenoviral *p53* gene therapy plus radiotherapy *versus* radiotherapy alone as a first-line treatment in locoregional nonmetastatic lung cancer are being planned.

Oncolytic virus therapy

ONYX-015 (dl1520) is a replication-selective adenovirus (80). Efficient adenovirus replication is dependent on the expression of proteins that inactivate *p53* (81). The normal *p53* gene product inhibits viral replication. ONYX-015 is an adenovirus that has been modified by deletion of the 55-kD *E1B* DNA fragment. The 55-kD *E1B* gene product inactivates *p53* in complex with E4ORF6 (82). It has been hypothesized that deletion of the 55-kD *E1B* region enables the p53 protein to maintain its function, thereby inhibiting viral replication in cells with normal p53

function; however, in cells that lack normal p53 function, such as malignant cells, the 55-kD E1B gene product may be expendable and the cells should be susceptible to replication and killing after infection. Initial reports suggested that *p53* mutant tumor cells could be lysed in a replication-dependent fashion both *in vitro* and *in vivo* after exposure to ONYX-015 (83). Moreover, the efficacy of ONYX-015 plus chemotherapy (cisplatin, 5-FU) was significantly greater than with either treatment alone (84, 85). Although results of preclinical studies with ONYX-015 are promising, clinical data are not encouraging. A pilot study of *ex vivo* administration of ONYX-015 in patients with cancer metastatic to the lung was performed and included 2 patients with NSCLC (86). In 2 of these patients, intratumoral viral replication without associated replication in surrounding normal lung was documented on a post-treatment biopsy, and increasing viral genome copy number was detected in plasma for at least 7 days, consistent with possible *in vivo* viral replication. Nevertheless, all patients developed anti-adenoviral antibodies and no tumor responses were seen. Because efficacy may be limited by host-mediated immune clearance before the virus can reach its tumor target, direct intratumoral delivery systems may be of greatest benefit in this situation. Further evaluation of ONYX-015 is ongoing.

Antisense therapy

Antisense therapy is a technique designed to ablate expression of dominant oncogenes (87, 88). Antisense oligonucleotides are short (typically 13-20 bases in length) sequences of single-stranded DNA or RNA complementary to expressed genes, chemically modified to protect them from enzymatic breakdown. They are designed to hybridize by Watson-Crick base pairing with mRNA transcripts encoding proteins of interest, and in this way, to silence the expression of that particular gene and, subsequently, its protein product. The earliest attempt to inhibit gene expression using antisense oligonucleotides was reported in 1978. Technology was developed in the early 1980s for automated synthesis of oligonucleotides, with appropriate modifications of the backbones to protect against nuclease digestion and to prolong the duration of effect. With the great economy of large-scale production, gene silencing by synthetic oligonucleotides is becoming an interesting therapeutic gene strategy for cancer. In NSCLC, inhibition of c-*myc* and K-*ras* expression by the antisense technique has also been shown to inhibit cell proliferation *in vitro* (89, 90). Several clinical trials using antisense oligonucleotides delivered by retroviral vectors have been approved for patients with NSCLC.

ISIS-3521 (aprinocarsen sodium, Affinitak™, LY-900003; Isis Pharmaceuticals, Lilly) is an antisense agent that binds to an mRNA sequence specific to protein kinase C-α (PKC-α). PKC is a family of serine/threonine protein kinases that mediate signal transduction (91). Its levels are elevated in various tumors, including lung cancer (92). Phase I studies demonstrated that administration for 3 weeks via continuous infusion followed by a 1-week treatment-free period was safe, with dose-limiting thrombocytopenia and fatigue and a maximum tolerated dose of 2.0 mg/kg/day (93). A phase I/II trial of ISIS-3521 given as a 2-week continuous infusion with a 1-week rest period together with 3-weekly carboplatin and paclitaxel in patients with stage IIIB or IV NSCLC demonstrated a response rate of 46% and a median survival of 15.9 months. The 1-year survival was 54% (94). Based on these results, ISIS-3521 was evaluated in a phase III trial in NSCLC in combination with carboplatin/paclitaxel, but no clinical benefit was seen and the product is no longer under development.

G-3139 (oblimersen sodium, Genasense®; Genta) is an 18-mer phosphorothioate oligonucleotide targeting the first 6 codons of the *BCL2* mRNA open reading frame. G-3139 is currently undergoing phase I evaluation in NSCLC. Initial results of a phase II trial in chemorefractory SCLC with paclitaxel yielded a 17% disease stabilization rate, although objective responses have yet to be seen (95). A phase II/III trial to compare the efficacy of docetaxel with or without G-3139 has completed recruiting patients previously treated patients with NSCLC.

Suicide gene therapy

Suicide gene therapy is a strategy used to transduce cancer cells with a gene construct that is able to convert a prodrug to an active drug that is toxic to target cells (96). The most well-known example of this approach is the herpes simplex thymidine kinase (HSV-tk)/ganciclovir system. Herpes simplex thymidine kinase encodes for an enzyme that converts the normally nontoxic nucleoside analogue ganciclovir to its activated triphosphate form, a toxic compound that leads to cell death. A potential advantage of this technique is the selective uptake of the vector and expression by tumor cells. The process requires a bystander effect to kill cells not infected with vector (97). The bystander effect appears to occur by transfer of phosphorylated ganciclovir from transduced to untransduced cells by intercellular bridges, gap junctions, or by uptake of small vesicles containing activated ganciclovir released by apoptosis. Preclinical studies demonstrated the value of HSV-tk plus ganciclovir in an immunocompetent orthotopic lung cancer model (98, 99). Fukunaga *et al.* reported prolonged survival of mice inoculated with Ad-HSV-tk-transfected tumor cells following treatment with ganciclovir compared with controls. Although this approach has not been assessed in clinical trials in patients with lung cancer, results for other tumor sites have been encouraging. Interesting results have been reported in mesothelioma patients. Two clinical trials utilizing an adenoviral vector to deliver the HSV-tk gene to patients with mesothelioma have been reported (100, 101). Gene transfer was confirmed in more than half of the patients and several partial tumor regressions were noted.

Immunomodulatory gene therapy

Immunogene therapy is used to transduce cancer cells with genes that are able to enhance their immunogenicity. The most common approach involves transducing cancer cells with cytokine genes. The cytokine is produced in high concentrations in the vicinity of the tumor, thereby altering the local immunological environment of the tumor cell so as to either enhance presentation of tumor-specific antigens to antigen-presenting cells (APCs) or enhance the activation of tumor-specific lymphocytes. Many cytokine genes have been introduced into tumor cells with varying effects on both tumorigenicity and immunogenicity. It has not yet been determined which cytokines are optimal for lung cancer, but of particular interest are IL-7, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (102-104). Unfortunately, clinical trials gave discouraging results in lung cancer. Nine patients with NSCLC were treated with intratumoral injection of an adenoviral vector expressing IL-2 without evidence of activity (105). Another study of intratumoral administration of a vaccinia virus expressing IL-2 in patients with chest wall masses associated with pleural mesothelioma has been conducted. Transient tumor-associated expression of IL-2 was detected but immune responses were minimal and no tumor regressions were noted. Neutralizing anti-vaccinia antibody responses were detected in all patients (106).

An alternative approach is to use modified lung cancer cells to deliver a variety of antigens to increase the immune response through concomitant administration of cytokines. This elegant immunogene strategy was used with GM-CSF. GVAX® is an autologous anticancer vaccine in clinical development for various tumor types, including lung cancer. In practice, GM-CSF is introduced into autologous tumor cells. Cells are then reinoculated into the subcutaneous tissues of the patient. The GM-CSF gene produces local GM-CSF that increases the immune response to the autologous tumor. This approach has been evaluated in a phase I trial in patients with advanced NSCLC (107). Thirty-five patients with advanced NSCLC underwent vaccination. Treatment was well tolerated, the most common toxicity being local and mild flu-like symptoms in a minority of patients. The trial revealed that 18 of 25 patients with advanced NSCLC who received the complete course of vaccinations demonstrated enhanced antitumor immunity. However, no significant clinical responses were obtained, although 2 of the patients remain disease-free for more than 3 years after treatment. It was noted that there were limitations in the processing and that the vector was not optimal, so that a novel adenoviral GM-CSF vector provided by Cell Genesys was used in a subsequent study by Neumanitis *et al.* (108). Because immunotherapeutic approaches are more effective in cancer patients with minimal disease, this group explored two cohorts of patients, one with minimal disease and one with advanced-stage disease. Eighty-three patients were entered into the trial (20 with stage IB/IIB and 63 with stage IIIB/IV). Patients underwent surgical harvest of autologous lung tumor tissue and the tissue was processed and transduced with an *E1*-deleted, *E3*-deleted adenoviral GM-CSF gene vector irradiated prior to injection. Patients received a series of 6 vaccinations. Three patients achieved complete response and 7 patients had stable disease with a median duration of at least 6 months. Notably, 2 of the responders had a BAC. Responses in the 2 BAC patients were durable and measured 18 months in 1 patient and exceeded 22 months in the other patient. In addition to the responses in patients with advanced disease, 8 of the 10 patients with early-stage lung cancer who received the GVAX® lung cancer vaccine after surgery were free of disease with a median follow-up of 12 months. A phase II trial (SWOG 0310) of GVAX® in advanced BAC enrolled approximately 100 patients with both untreated and previously treated disease and who received GVAX® with or without cyclophosphamide. The primary endpoint was overall survival and secondary endpoints included progression-free survival and objective response rate. One near-complete response was obtained and about a third of the patients had stable disease. Median progression-free survival was 3.8 months with the vaccine alone and 5.0 months for vaccine + cyclophosphamide, and median overall survival was 5.4 and 9.6 months, respectively (109). Cell Genesys subsequently discontinued development of this vaccine to focus resources on its non-patient-specific GVAX® products.

Another interesting immunogene approach is the construction of vaccines with antigen-specific peptides (110). BEC2 is a murine IgG_{2b} antibody that elicits an anti-idiotypic response to GD3, a glycosphingolipid overexpressed on membranes of SCLC and other tumors derived from the neural crest (111). A pilot study was performed in 15 patients with both limited and advanced disease and long-term survival was reported in patients with limited disease, leading to the initiation of a phase III study in this setting (112). The study, sponsored by the EORTC (the SILVA study), randomized 515 limited-disease SCLC patients in response after chemoradiotherapy to observation or to 5 BEC2 vaccinations with BCG, given on weeks 0, 2, 4, 6 and 10. Treatment was well tolerated but there was no improvement in survival, progression-free survival or quality of life.

Antiangiogenesis gene therapy

Angiogenesis appears to be a fundamental process in all cancers. Several genetic strategies have been employed to block neovascularization. Delivering genes with antiangiogenic properties directly to the tumor vasculature is an emerging and promising therapeutic strategy. Sauter *et al.* published the results of animal studies using adenoviral vectors carrying an endostatin transcription unit (113). These studies showed that the intravenous injection of adenoviral vectors produces constant levels of endostatin and is associated with a reduction in the growth of Lewis lung cancer cells in nude mice. Gene

Table IV: Topics for investigation.

Trials combining different gene therapy strategies targeting multiple signal transduction pathways
Trials combining gene therapy strategies with radiation or chemotherapy
Identification of reliable clinical endpoints to evaluate efficacy of gene therapy
Identification of patients in whom the gene therapy target pathway is the critical signal transduction pathway regulating cancer growth
Designing gene delivery vehicles that provide efficient transfer of exogenous DNA into appropriate cells
Obtaining proper quantitative and qualitative tissue- or cell type-specific expression of transferred genes
Effects of gene therapy as locoregional treatment in nonmetastatic lung cancer

therapy techniques also have been used to directly inhibit VEGF activity. For example, antisense oligonucleotides targeting VEGF are able to decrease tumor growth in lung cancer (114). An alternative approach is a tumor vaccine targeting the VEGFR-2 (also known as Flk-1). An oral DNA vaccine is available and has shown promising results in *in vivo* and *in vitro* studies (115). Currently, these approaches have not yet been examined in clinical programs.

Conclusions and future directions

Significant advances have been made in our understanding of the molecular abnormalities underlying lung cancer and this information is now being translated into the clinic. Phase I studies have shown promising preliminary results, and a number of additional trials are already in progress. Lessons learned from these studies are very important (Table IV).

First, gene therapy has an excellent safety profile and does not appear to enhance the toxicity of chemotherapy or radiation. Thus, if toxicity can be controlled, combinations of gene therapy modalities might work, especially in concert with conventional multimodal therapeutic approaches including surgery, radiation therapy and chemotherapy.

Second, response rates reported are localized to the site of injection of the vector. The use of gene therapy as a co-regional treatment could be another important area of research in lung cancer because, despite improvements in radiation therapy and chemoradiotherapy, locoregional control remains poor in nonmetastatic disease.

Third, the intricate scenario of lung cancer genetics complicates the present situation: lung cancer is the result of cumulative and complex genetic mutations and the restoration or deletion of a single gene function is unlikely to have a real clinical benefit, especially because patients generally have advanced disease. Thus, a combination of different gene therapy modalities may be appropriate.

Furthermore, a number of biological problems remain to be solved, including: the design of gene delivery vehicles that provide efficient delivery of exogenous DNA into cells; targeting gene delivery vehicles to appropriate cells while avoiding the delivery of exogenous genes to inappropriate cells; obtaining proper qualitative tissue- or cell type-specific expression of transferred genes; obtaining proper quantitative expression of transferred genes; obtaining, in some cases, the proper temporal expression of transferred genes; retaining continued expression of transferred gene expression; and avoiding or attenuating immunological responses to foreign gene products. Because the anatomic properties of the respiratory system present unique opportunities for alternate methods of drug delivery that could potentially reduce the toxicity of agents that are administered systemically or for prolonged periods of time, site-directed local therapy, such as aerosolization of gene therapy agents, should be investigated.

Finally, a number of clinical challenges remain. All treatment strategies outlined in this review are obviously cytostatic rather than cytotoxic, and this will require a paradigm shift in the way we approach patients and the design of clinical trials. With regard to patients, a careful selection is necessary because gene therapy can cause tumor regression even in patients who have failed prior chemotherapy combinations, but only a minority of patients will respond to such therapies, probably because they lack the target or because the signal pathway of the target is not essential for growth and survival. It is necessary to understand the molecular and biological abnormalities in an individual tumor and to select therapy according to the tumor's characteristics. With regard to study design, the traditional endpoint in phase I/II trials has been response rate, but it is clear from results of the studies described above that it is difficult to obtain a tumor reduction. Hence, alternative intermediate endpoints must be defined. Preclinical models must serve as a starting point in defining the endpoint, but unfortunately, biological endpoints that confer prognostic information in animal studies are not necessarily applicable in the clinic. Cooperation between basic scientists and clinicians is crucial for the rational design and development of effective therapies that target the specific genetic alterations relevant to malignant lung carcinogenesis.

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