Review

Current Status of Gene Therapy for Lung Cancer and Head and Neck Cancer

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Abstract

Targeting the specific genetic lesions responsible for carcinogenesis and cancer progression is an attractive strategy for developing more effective anticancer therapeutics and reducing treatment-related toxicity. The restoration of defective tumor suppressor gene pathways by replacement of tumor suppressor genes in cancer cells has been studied in lung cancer and head and neck cancer (HNC). The most extensively studied agent is the wild-type p53 tumor suppressor gene delivered by an adenoviral vector. Clinical trials to date in non-small cell lung cancer and HNC have consistently shown evidence of gene transduction and expression, mediation of apoptosis, and clinical responses including pathological complete responses. It is also clear, however, that this approach can be improved further. Promising avenues for investigation include improved gene delivery systems, induction of bystander effects, design of immunogenic and antiangiogenesis gene therapies, and adjuvant use of gene therapy with conventional chemotherapy, radiation therapy, and surgery. These strategies, however, will need further refinement to succeed clinically. This review examines several important issues in cancer gene therapy in general and the most recent achievements in gene therapy for HNC and non-small cell lung cancer.

Introduction

Conventional treatments are not adequate for a majority of lung and HNC2 patients. The failure of conventional therapy occurs because tumors are remarkably resistant to chemotherapeutic or radiation, both of which work by damaging the DNA of the rapidly dividing tumor cells. Attempts to overcome resistance with higher doses of radiation and chemotherapeutics inevitably result in an unacceptable degree of toxicity and bystander damage to normal tissues. Combinations of currently available treatment modalities have been moderately successful, but often these combination therapies cause unacceptably high toxicity without increasing the survival of patients. The major limitation of all these treatments and their combinations is the lack of specificity for the tumor cell and toxicity to the patient.

Recent advances in molecular biology have documented the role of genetic alterations in tumorigenesis and have led to the development of potential new therapeutic approaches designed to target the mutated gene or genes that contributed to initial malignant transformation or that are contributing to tumor progression and metastases. Adult cells are normally maintained in either G0 or G1 stages of the cell cycle, by a balance of signals from the Rb pathway and the p53 pathway. The protein products of several proto-oncogenes and other tumor suppressor genes play critical roles in these pathways, and mutations in some of these genes may lead to cancer by eliminating the tight control of cell proliferation. The Rb protein regulates release from the G1 phase, whereas p53 dictates whether the cell cycle will be arrested in response to stress or DNA damage, or whether the cell will be directed to undergo apoptosis. Major advances have been made in the gene therapy of cancer through replacement of missing or nonfunctional tumor suppressor genes or inactivation of oncogenes. Although several mutations are necessary to achieve transformation, early preclinical and clinical studies have demonstrated that correcting only one of the defective genes may be sufficient to halt tumor progression or eventually even prevent tumor formation. Thus, in this strategy, the gene is used as an anticancer pharmaceutical. The efficacy of this approach is determined by two factors: (a) the potency of the therapeutic gene(s) delivered; and (b) the efficiency of gene delivery to target cells. Therefore, gene therapy will achieve maximum clinical benefit when the sum of the gene potency and the delivery efficiency can be maximized in all target cells (1).

Current studies of cancer gene therapies can generate a

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2 The abbreviations used are: HNC, head and neck cancer; APC, antigen-presenting cell; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; wt, wild-type; HNSCC, head and neck squamous cell carcinoma; ODN, oligodeoxynucleotide; TUNEL, terminal deoxynucleotidyl transferase-mediated biotin UTP nick end labeling; EGF, epidermal growth factor; CEA, carcinoembryonic antigen; HSV-TK, herpes simplex virus thymidine kinase; SP-A, surfactant protein A; GCV, gancyclovir; hTERT, human telomerase reverse transcriptase.
considerable amount of data by which the efficacy of both the genes and the vectors might be assessed (1–5). Any one of a battery of known genes expressed at the right site and at appropriate levels can be very effective at selectively killing cancer cells (6). To overcome the limitations of gene delivery and ensure adequate target cell killing, however, other strategies are being exploited including the induction of a bystander effect, design of immunogene and antiangiogenesis gene therapies, and adjuvant use of gene therapy with conventional cancer therapies [chemotherapy, radiation therapy, and surgery (7–9)]. For cancer gene therapy to achieve optimal clinical benefit, a thorough understanding of the disease being treated and its clinical context is necessary. Only then can cancer gene therapy’s limitations be learned and its full potential exploited.

This article first reviews four important issues in cancer gene therapy: (a) targeting; (b) induction of bystander effect; (c) immunogene therapy; and (d) antiangiogenesis gene therapy. After a review of the molecular biology of lung cancer and HNC, there will be a discussion of recent achievements in the gene therapy for HNC and lung cancer, including some details of preclinical data and results from several clinical trials. Finally, several important clinical issues related to gene therapy in cancer are discussed.

**Four Important Concepts in Cancer Gene Therapy**

**Targeting.** The ability to target delivery systems to tumor cells distributed widely throughout a patient’s body would simultaneously increase efficacy and decrease potential toxicity; thus far, however, no such systemically targeted vectors exist. Injection of vectors into the bloodstream for the treatment of cancer requires not only that the vectors be targeted (to enter only tumor cells) but also that they be protected (from degradation, sequestration, or immune attack) for long periods of time so that they can reach their appropriate sites of action. Moreover, having reached such sites, the vectors must be able to penetrate into the tumor from the bloodstream before carrying out their gene expression.

Progress in vector targeting has been dramatic in the last few years. Surface targeting would be optimal to prevent nonproductive binding and sequestration of vectors before they reach their target cells. It is now possible to activate infection through retroviral envelope binding only in tissues that express, for example, tumor-associated proteases (10), and surface targeting is now also possible for adenoviral vectors (11). The advent of in vivo selection of peptide “addressin” sequences to target tumor cells or vasculature will add greatly to the technology required to target delivery at the level of cell binding (12). The challenge will be to show that such addressin peptides can be efficiently and functionally incorporated into vector systems, such as viral envelopes.

Promoters for transcriptional targeting of tumors need to be active in tumor cells and quiescent in normal cells. In the ideal case, the promoter would be tumor specific. It seems unlikely that such a promoter exists within the human genome. Hence the second best solution is to choose a promoter that is active in tumors and as few nontarget tissues as possible. Transcriptional targeting, as discussed in detail in the lung cancer gene therapy section, has been established as a method of maximizing gene expression in target cells (13). This approach, however, contributes more to decreasing the toxicity of gene therapy than to increasing its efficiency: a transcriptionally targeted vector still has no means of preventing its sequestration by the mass of nontarget cells/tissues that it is likely to encounter before it finds its real target. Thus, despite impressive advances in both promoter design and envelope modification, it remains to be seen whether any of these systems can be used for systemic delivery. On the other hand, a recent study has shown that liposomes have the potential for systemic delivery of genes to distant sites with minimal toxicity (14). Additionally, the liposome delivery system was shown to be restricted by neither gene nor tumor type. Thus, it may be possible to use a liposome-based delivery system in conjunction with the other vector systems to effectively treat human cancers in vivo.

**Induction of a Bystander Effect.** Whatever its mechanism of action, no gene can be a serious contender for therapeutic delivery unless it demonstrates a bystander effect. The requirement for a bystander effect stems directly from the inability of vectors to transduce all tumor cells (6). This bystander effect can be either local (9) or immune mediated (15, 16); a combination of the two is preferable. The proposed mechanisms of bystander killing involve the transfer of toxic metabolites locally between cells, suppression of angiogenesis, and potential death signal delivery by contact with dying cells (16–19). Local bystander killing can certainly be enhanced by coadministration of pharmaceutical or genetic agents that enhance cell-cell communications (20). In addition, immune stimulation through cell killing can also enhance local tumor killing (21) and help to generate systemic immunity to other tumor deposits (22). These effects can be active either over a short period of time by activation of nonspecific immune effector mechanisms (21, 23) or over a longer period by initiation of long-lived, T-cell-mediated immunity to tumor cells (16, 24). Thus, exploitation of mechanisms that combine powerful direct local killing with local and systemic immune activation will be a major priority. Significant advances will come from the discovery of new genes that kill more cells locally and do so with as much immunological aggression as possible (22, 25).

**Antiangiogenic Gene Therapy.** Given the difficulties in generating truly targeted vectors for systemic delivery, the alternative is to target those biological properties of tumors that set them apart from all or most normal tissues. One of the most notable distinguishing features of tumor growth is the absolute requirement for the tumor to provide itself with an expanding blood supply through the process of angiogenesis. There is a wealth of targets at the interface between the malignant population and the supporting stroma that could be exploited by gene therapies (26). For instance, the migration of tumor endothelium can be inhibited by interfering with protease enzyme function (27), and it may even be possible to design molecules with both antiangiogenic activity and tumor-homing properties (28). The identification of naturally circulating factors such as angiotatin and endostatin, which appear to be capable of suppressing angiogenesis, has sparked an explosion in efforts to deliver and express such recombinant molecules (28–31), and more candidates are being reported all of the time.

**Immunogene Therapy.** Many of the first clinical protocols for cancer gene therapy involved the ex vivo modification
of freshly isolated tumor cells with cytokines (32). In the presence of the appropriate in vivo control, however, it became apparent that in many cases, cytokine modification may be little better than more conventional adjuvant-based cancer cell therapies with no gene transfer component (33). In addition, recovering patient tumor cells and maintaining them in culture long enough for transduction with cytokine genes has proved to be laborious, time-consuming, and expensive and may significantly alter the phenotype of the cells and delay urgently needed therapy. Nonetheless, clinical trials have shown encouraging signs that cytokine-modified vaccines can generate significant immune responses in patients while imparting minimal toxicity (34). Given the effort and expense involved in autologous cell gene modification, however, it is unclear whether these sorts of approaches will be practical, and new approaches have been proposed.

Presently, the two areas in which immunogene therapy is likely to make significant progress are in the molecular identification of tumor-associated antigens and exploitation of the central significance of APCs in generating antitumor immune responses (35, 36). One of the most significant advances during the evolution of gene therapy for cancer has been the cloning of tumor-associated antigens from human tumor (usually melanoma) cells that are recognized by either CD8+ T cells (37–39). This development has added molecular credibility to the long-held presumption that tumors can indeed express antigens against which T-cell-mediated responses can be raised. However, because tumors are highly heterogeneous and unlikely to express only one dominant antigen on all of the cells, molecular vaccination with defined antigens will undoubtedly have to use “cocktail” approaches where multiple cDNAs are used in the vaccination protocol. With such antigens in hand, the question remains how to remove tolerance to these antigens, which are often wt self-antigens (40).

Several key studies have shown that tolerance to tumors can be removed as long as tumor antigens, whether clearly defined or not (41, 42), are delivered into suitably activated APCs (35, 41). The magnitude of the resultant antitumor responses will be largely determined by (a) the efficiency of migratory APCs to target tumor sites and return to the lymph nodes, (b) the ability of APCs to internalize and process tumor antigens, and (c) the proficiency of APCs in activating target-specific lymphocytes (43, 44). All three of these APC functions can also be enhanced by immunoadjuvants, such as proinflammatory cytokines (45). Tumor cells that undergo apoptosis in small numbers are unlikely to induce these APC functions significantly. In contrast, cells dying in other scenarios can effectively induce APC function to initiate an immune response. Examples of cell deaths inducing APC functions are death without apoptosis; spilling of cell contents into the stroma; death in the context of foreign antigens, wherein cells succumb to a pathogenic infection (46); or death in the context of widespread apoptosis of cells, wherein the local reticuloendothelial system’s clearance capacity is overwhelmed (47). We now have the ability to identify the key molecules that can serve as targets for immune responses and to isolate and genetically manipulate the central APCs involved in antigen presentation.

One unique potential hazard of immunogene therapy for cancer is autoimmune disease. The induction of autoimmunity to some of these tumor-associated antigens has already been shown and correlates well with the generation of antitumor immune responses (48). However, the same or related antigens may be displayed on other cells or tissues within the body, differing only in relative expression level, and hence may also represent a target for recognition and destruction. Although such side effects can be tolerated for the treatment of tumors where the normal tissue type is not crucial to the patient’s survival (such as melanocytes), the successful induction of autoimmune to tumor antigens could be accompanied by immunological destruction of a wide variety of tissues (41, 42, 48).

### Molecular Biology of Lung Cancer and HNC

When developing gene therapy, it is essential to have very precise information about the genetic etiology of the disease. The information can be very complex in the case of cancer because several mutational hits may have taken place during carcinogenesis (49). Inactivated tumor suppressor genes and activated oncogenes that have been found in lung cancer are presented in Table 1. In SCLC, attention should be focused on the family of myc oncogenes and on the tumor suppressor genes Rb and p53. In NSCLC, high frequencies of alterations are found in the K-ras oncogene and the tumor suppressor genes p16 and p53.

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<thead>
<tr>
<th>Table 1</th>
<th>Oncogenes and tumor suppressor genes altered in lung cancer</th>
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<tr>
<td>Oncogenes</td>
<td>K-ras&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>c-myc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>N-ras&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>L-myc</td>
<td>c-myc&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>N-myc</td>
<td>H-ras</td>
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<td>c-myb</td>
<td>c-raf</td>
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<tr>
<td>c-erbB-1 (EGF-R)</td>
<td>c-far&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>c-fms</td>
<td>c-fes</td>
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<tr>
<td>c-r1f</td>
<td>c-erbB-1 (EGF-R)&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>c-erbB-2 (Her2, neu)</td>
<td>c-sis</td>
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<tr>
<td>c-blc-1</td>
<td>p53&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>c-Rb&lt;sup&gt;e&lt;/sup&gt;</td>
<td>RB</td>
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<sup>e</sup> Most frequently altered genes in tumors or cell lines evaluated. <sup>e</sup> EGF-R, EGF receptor.

The myc oncogenes are often very activated in SCLC (50, 51). Introduction and overexpression of c-myc in SCLC cells contribute to the malignant phenotype by increasing the cellular growth rate in vitro (52). The myc genes encode gene-regulatory proteins that form heterodimers with the Max protein. The Myc-Max complexes bind the core nucleotide sequence CACGTG and activate transcription of target genes that promote cell division (53).

The tumor suppressor gene p53 encodes a M<sub>e</sub> 53,000 transcript factor that activates the transcription of at least six or seven genes known to be involved in the control of the cell cycle and the induction of programmed cell death, apoptosis (54). Normally, the concentration of p53 protein is rather low or even undetectable in the cell because protease activity causes a short protein half-life (about 20 min). When DNA damage occurs in...
the normal cell, the concentration of active p53 protein is increased because of its lengthened half-life and increased expression, together with posttranslational activation of the protein (55). Increased levels of active p53 may induce either G1 cell cycle arrest or apoptosis in the cell. The p53-mediated cell cycle arrest is evidently due to transcriptional activation and increased expression of the tumor suppressor gene p21 (55). Several in vitro studies have shown that introduction of wt p53 in human cancer cells that lack functional p53 induces apoptosis in the cells (56–60). Cell cycle arrest and apoptosis both serve to suppress tumorigenesis. When the cell lacks functional p53, the probability of DNA damage accumulation leading to cell transformation is therefore increased. The fact that p53 contains mutations in more than 50% of all human cancers (61) has caused wt p53 to become the center of most studies in which the therapeutic potential of restoring a tumor suppressor gene in cancer cells has been examined.

In HNCs, 90% of which are squamous cell carcinomas in the United States, the molecular events that occur during carcinogenesis are as well understood as those that occur in lung cancer. The best model to explain HNSCC carcinogenesis is that of “field cancerization,” the repeated exposure of an entire tissue surface to carcinogens (such as tobacco), which increases the tissue’s risk for developing multiple independent premalignant and malignant foci (62). Based on these models, it has been suggested that during the epithelial carcinogenesis of HNSCC, including initiation, promotion, and progression, the dynamic balance between oncogenes and tumor suppressor genes determines the fate of cells exposed to carcinogens (62, 63). Aberrant expression of cellular oncogenes such as myc, ras, neu, bcl, and int is associated with HNSCC carcinogenesis (64, 65). Up to one-third of primary HNSCCs demonstrated either the int-2 or hst-1 gene (both members of the fibroblast growth factor gene family) on chromosome 11q13 (66). Little is known, however, about when these events take place during the multistep carcinogenesis. The most common mutations in HNSCC are in p53, occurring in 40–70% of tumors (67). Moreover, mutations in p53 are present in premalignant areas, including carcinoma in situ or moderate to severe dysplasia, in approximately 20% of cases. Because of such data, most of the gene therapies studied for HNSCC have concentrated on the introduction of wt p53 into tumors for cancer therapy and into premalignant lesions for preventive therapy.

**Gene Therapy in the Management of Lung Cancer and HNC**

Based on the results of many in vitro and in vivo experiments, strategies to maximize gene therapy for lung cancer and HNC have been developed. Although these strategies are commonly used in other types of cancer, we will concentrate mainly on the data from experiments using lung cancer cell lines or lung cancer xenografts. For HNC, because the concepts and methodologies have been similar to those for lung cancer, and, as mentioned above, most of the experiments have been done using wt p53, we will not go over specific details except when discussing the development of the Onyx-015 trials (8). In this section, three subjects (the different genes used for lung cancer and HNC gene therapy, targeted gene delivery, and transcriptional targeting) are described in detail. Finally, clinical data from p53-based gene therapy of lung cancer and HNC are presented.

**Genes Used for Gene Therapy for Lung Cancer and HNSCC**

Although there is still no clear consensus on which gene(s) should be adopted for delivery, genes that directly kill target cells but not normal cells with a wide therapeutic window have significant advantages. Simple suicide genes (9) have been tried, as well as genes for cytokines or immunogenic antigens (10) to induce immunological destruction of tumor cells. Some groups have had success turning the aberrant biology of tumor cells against themselves; by targeting pathways central to the continued survival of the cell, it may be possible to induce tumor killing by triggering apoptotic effector mechanisms indirectly (68) or directly (69).

**Oncogene Inactivation.** Oncogene expression can be blocked by introducing a vector that contains DNA fragments with a sequence complementary to a part of the oncogene. The transcribed DNA fragment will produce antisense RNA that can bind with the oncogene mRNA, resulting in double-stranded RNA that is not translated. Specific gene expression can also be inhibited by introducing short antisense ODNs that can be designed to bind complementary mRNA, blocking the gene expression at the level of translation. Antisense ODNs can furthermore be designed to bind to the major groove of DNA, forming a triple helix structure that inactivates gene expression at the level of transcription (70). Oncogenic inactivation has the main disadvantage that the tumor cells are not always killed by oncogene inactivation, and therefore, the antisense molecules have to be delivered continuously to the cancer cells to maintain the suppression of tumor proliferation. Thus far, however, the data from experiments using cultured cells and xenografts in nude mice (described below) look promising; clinical application of this approach is at an early stage.

In SCLC cells, the antiproliferative effect of introducing L-myc antisense DNA was evaluated in vitro (71). The SCLC cell line NCI-H209, which overexpresses the L-myc gene, was incubated with a modified ODN, a pentadecamer complementary to the sequence covering the translation initiation site in exon 2 of the L-myc mRNA. The number of antisense oligopentadecamer-treated cells decreased slightly after 96 h of incubation, whereas the numbers of cells treated with oligopentadecamer in sense orientation and of untreated cells increased by factors of approximately 4 and 6, respectively. In NSCLC, inhibition of c-myc and K-ras expression by the antisense technique has also been shown to inhibit cell proliferation in vitro (72, 73). When nude mice bearing xenotransplants of large cell lung carcinomas were treated with the antisense insulin-like growth factor I receptor adenovirus vector, prolonged survival was observed (74). Another approach to antisense-mediated oncogene inactivation is the introduction of vectors encoding oncprotein-neutralizing antibody fragments (75). It was also shown that the introduction of an anti-ras single-chain antibody fragment could mediate apoptosis in the ras-transformed lung carcinoma cell line H460 (76). The intratumoral injection of this vector into s.c. tumors in nude mice resulted in tumor regression.
Preclinical Data from Reintroduction of wt p53. The effect of restoring wt p53 expression in tumor cells lacking functional p53 protein has been intensely investigated in numerous cell lines and tissues (60). It was initially shown that introduction of a vector containing wt p53 into NSCLC cell lines that were homozygous for either a deletion or a missense mutation in p53 markedly reduced cell proliferation and tumorigenicity (77). Moderate expression levels of wt p53 were sufficient to suppress tumorigenicity, even though the expression level of wt p53 mRNA was much lower than that of the endogenous mutant p53. Later it was shown that retroviral wt p53 introduction into a NSCLC cell line with homozygously mutated p53 inhibited cell growth by inducing apoptosis (57, 58), as identified by the method of terminal deoxynucleotidyl transferase.

Induction of apoptosis and elimination of the tumor cells is a much more attractive means of suppressing tumor proliferation than the growth inhibition by cell cycle arrest observed in oncogene inactivation. Furthermore, the expression of introduced wt p53 has to be only transient because apoptotic cell death has been observed in NSCLC within 24 h of wt p53 expression in vitro and in vivo (78). Further investigations showed a therapeutic effect of wt p53 introduction in an orthotopic lung cancer model (79). In this model, nude mice were intratracheally inoculated with NSCLC cells homozygous for a mutation in the p53 gene; a retrovirus vector containing wt p53 was intratracheally injected into the tumor cells on days 4, 5, and 6 after tumor cell inoculation. At autopsy, 30 days after inoculation, tumor development was evaluated. In the control groups, endobronchial tumor development was observed in 62% (instillation with a mutant p53 vector, n = 8) to 80% (instillation with vector containing no p53, n = 10) of the animals. In the wt p53 therapy protocol, only 0% (n = 8) to 38% (n = 8) of the mice developed tumors at the site of inoculation.

It is very likely that combination therapy with chemotherapeutic drugs, ionizing radiation, or simultaneous introduction of other tumor suppressor genes can markedly augment the therapeutic response to wt p53 gene therapy. In NSCLC, it was shown that wt p53 transduction of a (p53−/−) H358 cell line induced sensitivity to the DNA-damaging chemotherapeutic drug cisplatin (80). Furthermore, when there is a loss of p53 function in vitro, cellular resistance to a variety of DNA-damaging agents used in cancer therapy is enhanced (81). In s.c. tumor xenografts, the combination of radiation and wt p53 transduction increased the percentage of TUNEL-positive cells and showed synergistic effects in tumor growth suppression.

It was reported recently that complementary tumor suppressor genes, delivered together, can cooperate to induce apoptosis (82). Combinatorial introduction of the tumor suppressor gene p16INK4 and wt p53 demonstrated a synergistic effect on the induction of apoptosis in HuH7 hepatocellular carcinoma cells (mutated p53) and LOVO colon carcinoma cells (very low expression of wt p53) in vitro. p16INK4 (inhibitor of cyclin-dependent kinase 4) indirectly regulates the activity of the tumor suppressor gene Rb (retinoblastoma gene). Overexpression of p16INK4 can induce cell cycle arrest, but only if the cell contains functional Rb protein (83). The mechanisms by which p16INK4 and p53 cooperate to induce apoptosis are not yet fully understood.

Conditionally Replicative Adenoviruses. It has been hypothesized that an adenovirus mutant containing a deletion in the E1B region could only replicate and thereby induce cellular lysis in cells lacking functional p53 because of the putative need for p53 inactivation for adenoviral replication (59). Although this is not gene therapy in the strict sense of delivering a therapeutic gene, it does take advantage of genetic abnormalities in the tumor. When this virus was administered to nude mice transplanted with human tumor xenografts, antitumoral efficacy was observed, especially in combination with chemotherapy (9). One of potential advantages of this viral anticancer therapy is that infected cancer cells will provide neighboring cancer cells with high titers of new virus particles when cellular lysis occurs. This opens the possibility of very high transduction efficiency, which thus far has been a limiting factor of replication-deficient virus vectors administered systemically. Such a reagent, a p53-targeting oncolytic mutant adenovirus, was developed for clinical application by F. McCormick at Onyx Pharmaceuticals. Under the name of Onyx-015, initial Phase I and II clinical trials have been reported since 1998 (84, 85). Several in vitro and in vivo experiments using Onyx-015 showed three important features (86, 87). First, Onyx-015 preferentially destroyed a broad histological spectrum of p53-deficient human tumor cell lines but not the human cell lines with functional p53. Second, the virus was 100-1000 times more toxic to p53-deficient tumor cells than to normal, nontumorigenic cells, indicating a large therapeutic index. It was also shown that cytoxicity arose from viral replication. The virus showed antitumor efficacy (tumor regression and animal survival) in human xenografts in nude mice after intratumoral and systemic administration. Third, the efficacy of Onyx-015 is significantly enhanced by combination with chemotherapy (cisplatin and 5-fluorouracil).

Recent studies, however, have shown that the E1B-deleted adenovirus mutant efficiently replicates in a series of tumor cell lines with wt p53 (88), and wt p53 seemed to be required in the cell lines for the virus-induced cytopathic effect to occur (86). Moreover, Rothman et al. (89) found that Onyx-015 replication is independent of the p53 mutational status in tumor cells and that single-agent Onyx-015 can kill primary cells.

These contradicting observations for the p53 mutational status were partially explained by Ries et al. (90), who discovered that the loss of p14ARF facilitates replication of Onyx-015 in tumor cells, explaining the ability of Onyx-015 to replicate in tumor cells that retain wt p53. This report provides a solid guideline for the therapeutic use of Onyx-015 in tumors with lesions within p53 pathway other than mutation of p53.

Targeted Gene Delivery for Gene Therapy of Lung Cancer and HNSCC

One way to accomplish targeted gene delivery is by coupling receptor-specific ligands to a plasmid containing the therapeutic gene. The ligand-DNA complex will bind only to cells that express the surface receptor specific for the ligand. After binding and receptor-mediated endocytosis, the ligand-DNA complex reaches the endosomal compartment. If the complex does not escape the endosome, it will rapidly be degraded by lysosomal activity. Thus, an agent for endosomal lysis will promote efficient gene expression. Adenovirus particles are capable of endosomal lysis and have been shown to augment in
vitro gene transfer with plasmid vectors without linking the virus to the plasmid (91). Direct linking of replication-defective adenovirus particles to the plasmid is a much more suitable approach for in vivo applications and has been shown to greatly enhance in vivo gene transfer and expression in cotton rat airway epithelium (92). Also, a peptide derived from the NH2 terminus of the influenza virus hemagglutinin subunit HA-2 and a more potent short synthetic peptide have been used as agents for endosomal disruption (93, 94). These endosomal lytic agents are less efficient than adenovirus particles but may prove to be less immunogenic in the host organism.

The EGF receptor is overexpressed in NSCLC tumor cells (95), and EGF and replication-deficient adenovirus (serotype 5) have been attached to a plasmid for gene delivery to NSCLC cells in vitro (96). Overexpression of a target receptor, however, does not seem to be required for efficient gene delivery using ligand-DNA complexes. In SCLC cell lines expressing even low levels of EGF receptor, highly efficient gene delivery through EGF receptor-mediated endocytosis has been accomplished with EGF-DNA complexes (97). Transduction efficiencies of this complex in seven NSCLC cell lines ranged from 16 ± 10.3% to 99 ± 0.6% of the cells. Unfortunately, the direct linkage of the adenovirus particle to the plasmid vector reduced the specificity of complex uptake by means of EGF receptor-mediated endocytosis.

The adenovirus particle used can bind to the cellular Coxsackie adenovirus receptor (98) and thereby promote internalization of the gene delivery system in an EGF receptor-independent manner. This function of the adenovirus as an alternative ligand is obviously very undesirable for a targeting gene delivery system. Adenoviruses bind to their cellular receptors using the 12 fiber spikes protruding from the capsid, and the binding domain for adenovirus serotypes 3 and 5 has been located in the fiber head domain (99). Michael et al. (100) showed that using a monoclonal antibody to the fiber protein of adenovirus serotype 5 resulted in the ablation of virus binding. Furthermore, the antibody-coated adenovirus particles retained their ability to disrupt endosomes when introduced into ligand-DNA complexes.

In addition to EGF, antibodies specific to the EGF receptor and human epidermal growth factor receptor 2 (HER2) have also been used successfully in receptor-mediated gene delivery to cancer cells in vitro (101). Nevertheless, recombinant adenovirus has thus far proven to be the most efficient gene delivery system to different tissues, such as heart and skeletal muscle, after systemic administration in vivo. Therefore, several strategies have been tried to target recombinant adenovirus vectors to specific cell types by manipulating the cell surface binding properties of the viral particle. One approach to adenoviral targeting has involved the use of a fusion protein consisting of an antibody fragment specific to the fiber protein of the virus and a ligand, i.e., EGF (102). This approach is designed to block the normal fiber binding to the Coxsackie adenovirus receptor by means of the bifunctional fusion protein, with concomitant targeting of the virus to the EGF receptor. Using this system, the coated adenovirus particles could specifically target the infection via the EGF receptor in vitro. Addition of the fusion proteins to the adenovirus enhanced the transduction efficiency of the epidermoid carcinoma cell line A431 16-fold at optimal conditions compared with infection with the native adenovirus vector (103). A431 cells greatly overexpress the EGF receptor, and when the cells were preincubated with an excess of free EGF before the modified adenovirus particles were added, transduction was nearly fully ablated. Furthermore, loss of infection was also observed when the virus was incubated with the antibody fragment containing no fused EGF (102). These results indicate that this modification of the adenovirus could both block the native viral tropism and target gene delivery specifically to the EGF receptor.

**Transcriptional Targeting for Gene Therapy of Lung Cancer and HNC**

Promoters for transcriptional targeting of tumors ideally would be active in tumors but have minimal or no activity in nontarget tissues. Promoters that might be used in gene therapy can be divided into the following groups: promoters responsible for tissue-specific or cell type-specific expression of genes; promoters containing cis-acting elements responding to overexpression of transcription factors; and recombinant promoters.

**Cell Type-Specific Promoters.** CEA is thought to function as an adhesion molecule during embryogenesis and tumor development. In healthy adult tissue, CEA is not normally expressed, but it is present in some tumors. Lung cancer patients often display elevated plasma CEA levels (104); therefore, the CEA promoter could be a candidate for targeting CEA-expressing lung cancer cells at a transcriptional level. The cis-acting elements sufficient for cell type-specific expression have been localized to within 424 bp upstream of the translational starting point (105). The activity of this sequence was tested by reporter assays of the cell lines A549 (CEA-producing human lung cancer), CADO-LC9 (non-CEA-producing lung cancer), and HeLa (106). The activity of the CEA promoter in A549 was 26% of that of the SV40 promoter. In non-CEA-producing cells, the activity of the CEA promoter was 9.3% and 3.0% of that of the SV40 promoter. A plasmid containing the CEA promoter upstream of HSV-TK was constructed, and in vitro tests showed that the CEA promoter is active in CEA-producing cell lines.

In addition to the CEA promoter, the hTERT promoter has also been shown to be highly active in tumor cells but repressed in most normal cells. Because this promoter is regulated at the transcriptional level, the hTERT promoter may be used for tumor-specific expression of transgenes. A recent study reported that the induction of Bax gene expression via the hTERT promoter elicited tumor-specific apoptosis while preventing toxicity of the gene in vitro and in vivo (107). It was also shown to suppress tumor growth in nude mice.

**Tissue-Specific Promoters.** SP-A mRNA is found in a high proportion of NSCLC tumor specimens (106). Because the expression of SP-A is limited to the respiratory epithelium, it might be used in targeting NSCLC metastases outside the lung. The transcriptional regulatory sequences of SP-A have been localized within a region extending from −2600 to +178 relative to the transcriptional start (108). Smith et al. (108) showed that in a reporter assay, this DNA fragment promoted reporter gene expression in H441 (a SP-A-producing NSCLC cell line) but not in A549 (a non-SP-A-producing NSCLC cell line). Plasmids containing HSV-TK under transcriptional control of either the SP-A promoter (pSP-I/HSV-TK) or the SV40 pro-
moter (pSV/HSV-TK) were constructed and initially tested for promoter activity. The SV40 promoter showed very high activity in the H441 and A549 cell lines. Transfectants were treated with GCV (48 h after transfection). For A549 transfected with pSV/HSV-TK and pSP-I/HSV-TK, the percentages of cells surviving 24 h after GCV addition were 31.7 ± 1.2% and 104.2 ± 43%, respectively. Therefore, the pSP-I/HSV-TK had no effect in non-SP-A-producing cells. H441 cells transfected with pSV/HSV-TK and pSP-I/HSV-TK showed 24.6 ± 0.9% and 14 ± 0.7% survival, respectively. Despite the significant difference in activity between the SV40 and SP-A promoters in the reporter assay, no appreciable difference was seen in the toxicity experiments. This finding indicates that the amount of HSV-TK produced by pSP-I/HSV-TK is sufficient for obtaining a cytotoxic effect in SP-A-producing cells after GCV administration. Thus, SP-A promoter elements may be useful for specific expression of a therapeutic gene in the treatment of NSCLC tumors or metastases outside the lung.

Recombinant Promoters. In an attempt to target transcription of HSV-TK to Myc-overexpressing lung cancer cells, Kumagai et al. (53) constructed a recombinant promoter. A total of four repeats of the Myc-Max response element (CACGTG) were cloned in front of a part of the HSV-TK promoter. This promoter fragment alone exhibited only 5% activity of the full-length HSV-TK promoter. Cells were stably transfected with a plasmid containing HSV-TK controlled by the synthetic promoter (pTK7) or with appropriate control plasmids, and clones of these transfectants were tested for sensitivity to GCV. Three SCLC cell lines overexpressing myc family oncogenes showed 22-, 33-, and 500-fold increases in GCV sensitivity compared with parental cell lines. The in vivo tests in mice inoculated with parental or transfected cell lines showed an antitumor effect on SCLC cells transfected with pTK7. This study showed that it is possible to use promoters present in the human genome to restrict transcription of a therapeutic gene to specific cell types and that such promoters can produce enough therapeutic gene product for it to perform its function. Each new promoter candidate, however, must be tested for its tissue specificity and efficacy in different gene therapy strategies (109, 110). Removing a promoter from its context in the genome and placing it in a viral vector has sometimes been shown to impair the ability of a putative tissue-specific promoter to restrict transcription to the appropriate tissue (109, 110).

Clinical Trials for p53-Based Gene Therapy

Retroviral Vector for Lung Cancer and Adenoviral Vector for Lung Cancer and HNC. A retrovirus vector carrying wt p53 cDNA driven by the actin promoter was administered to seven lung cancer tumors by direct intratumoral injection (Table 2, Trial 1). The tumors were biopsied before and after wt p53 therapy. In six cases, TUNEL staining was greater in posttreatment biopsies than in pretreatment specimens. In most specimens, the percentage of cells in the posttreatment biopsies that stained positive in the TUNEL assay exceeded the percentage of cells containing vector DNA. This result can be interpreted as a bystander effect (19). In this case, the bystander effect may have been due to phagocytosis of apoptotic bodies by nontransduced tumor cells, inhibition of angiogenesis, or an immunological response against the tumor. Tumor regression was observed in three of the seven patients, and two patients showed no evidence of viable tumor 4 weeks after injection with the retrovirus vector. Thus, for the first time in a clinical setting, the replacement of a tumor suppressor gene has been shown to mediate tumor regression, providing important proof of principle. Importantly, vector-related toxicity was minimal, and a putative mechanism of action, apoptosis, was established.

Adenoviral vectors have an advantage over retrovirus in that they transduce both dividing and nondividing cells, achieve higher expression levels, and can be produced with high titers and in large scale. A replication-deficient type 5 adenovirus (Adp53) in which the viral E1 gene was replaced with a wt p53 expression cassette driven by cytomegalovirus promoter has been evaluated in two Phase I clinical trials in NSCLC patients (111, 112). The adenovirus vector expressing a wt p53 gene was given by intratumoral injection either under computed tomographic guidance or by flexible needle bronchoscopic injection. In a study of 25 patients with treatment-resistant advanced NSCLC (112), the adenoviral construct was found to be safe with little toxicity even after repeated injections (Table 2, Trial 2). From this trial, it was shown that disease stabilization lasted up to 14 months, and >50% tumor regression was seen in two patients. The observed response rate in this heavily pretreated group of patients with progressive disease was encouraging.

Based on results of preclinical studies showing synergistic interactions of wt p53 and cisplatin in mediating apoptosis in human lung cancer cells, Nemunaitis et al. (113) initiated a Phase I trial of p53 gene transfer in sequence with cisplatin in 24 NSCLC patients with nonfunctional p53 genes. Cisplatin was administered i.v., and 3 days later, p53 was delivered by intratumoral injection. Up to a total of six monthly courses were carried out. Seventeen patients remained stable for at least 2 months, two achieved partial responses, and four continued with progressive disease. One patient was un evaluable due to 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. It should be noted that 75% of the patients entered in the trial had tumor progression on cisplatin or carboplatin-containing regimens. However, subsequent multicenter Phase II trials comparing groups of patients with adenoviral p53 gene therapy plus chemotherapy versus chemotherapy alone as a first-line treatment failed to show differences in response rate and survival benefit. From this Phase II study, it was concluded that intratumoral adenoviral p53 gene therapy appears to provide no additional benefit in patients receiving an effective first-line chemotherapy (114).
<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Phase</th>
<th>Vector name</th>
<th>Delivery mode</th>
<th>No. of patients evaluable&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Evidence of gene expression&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. and type of responses</th>
<th>Adverse reactions</th>
<th>Ref. no.</th>
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<tr>
<td>NSCLC</td>
<td>I</td>
<td>LTRp53A (retroviral)</td>
<td>Intratumoral</td>
<td>7</td>
<td>Yes</td>
<td>3 regressed &gt; 50%</td>
<td>Flu-like</td>
<td>Trial 1 (Ref. 2)</td>
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<td></td>
<td></td>
<td></td>
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<td>3 stabilized &gt; 8 weeks</td>
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<td></td>
<td>1 progressive</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 partial response; 16 stable for up to 14 months; 7 progressive</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 partial response; 17 stable for at least 2 months; 4 progressive</td>
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<td>Intratumoral</td>
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<td>Yes</td>
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<td>Fever, pneumothorax</td>
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<td></td>
<td></td>
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<tr>
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<td>Intratumoral plus cisplatin</td>
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<td>Yes</td>
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<td>1 complete response; 11 partial response; 3 stable; 2 progressive</td>
<td>Fever, chill radiation therapy side effect</td>
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<td>Intratumoral</td>
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<td>Intratumoral</td>
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<td>4 partial response; 12 stable; 13 progressive</td>
<td>Flu-like, injection sites pain</td>
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<td>Intratumoral plus cisplatin, 5-fluorouracil</td>
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<td>Sepsis (2 cases), chemotherapy side effect</td>
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<td>Intralesional plus fluorouracil, leucovorin</td>
<td>27</td>
<td>Yes</td>
<td>3 regressed &gt; 50%; 4 minor responses; 9 stable; 11 progressive</td>
<td>Flu-like, hyperbilirubinemia</td>
<td>Trial 9 (Ref. 125)</td>
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</tbody>
</table>

<sup>a</sup> Number of patients evaluable in all studies is smaller than total number of patients entered.

<sup>b</sup> Evidence of transgene gene expression (p53) or viral replication in the injected tumor sites by viral-specific PCR, in situ hybridization, TUNEL assay, and p53 immunocytochemistry. Expression is analyzed for a fraction of the injected patients.

<sup>c</sup> CPR, complete pathological remission.
the interaction of the Adp53 and radiation therapy can potentially improve local tumor control (116). Safety data indicated that this combination had an acceptable safety profile. Thirteen patients underwent 61 computed tomography-guided biopsies or drug administrations. Thirteen (21%) resulted in pneumothoraces, one of which required hospital admission. Six of the 17 patients experienced a grade 3 or 4 adverse event. These results are encouraging and are the basis for a randomized clinical trial in patients with unresectable NSCLC. This trial will compare concurrent chemotherapy and radiation therapy alone to concurrent chemotherapy and radiation therapy with intratumoral injections of adenoviral p53.

An innovative application of Adp53 in patients with bronchoalveolar cell lung cancer was described by Kubba et al. (117). Adp53 was given by bronchoalveolar lavage in 14 patients. Two patients had a pathological response, and 4 of 9 evaluable patients showed a ≧20% improvement in corrected diffusion capacity of carbon monoxide.

In a Phase I study of 33 patients with bulk HNSCC (Table 2, Trial 3), the adenoviral construct was found to be safe with little toxicity, and significant clinical response was observed in 9 of 18 clinically evaluable patients (118). Interestingly, systemic Adp53 DNA was present transiently for <48 h and was demonstrated in blood, urine, and sputum. In Phase II studies, over 200 patients have been enrolled, with approximately 10% of patients with both recurrent or refractory HNC achieving complete or partial responses (119, 120). If patients with prolonged inhibition of tumor growth are included, then 60% of patients showed evidence of antitumor activity. The low toxicity seen with Adp53 administration with less than a 5% incidence of serious adverse events suggests that it can be readily combined with other anticancer treatments without significant increases in treatment-related toxicity (121).

**Onyx-015 for HNC.** An example of therapy mechanistically directed at the p53 pathway, although not truly gene therapy, is the Onyx-015 trial for HNC. As described in detail in the preclinical data sections, a p53-targeting oncolytic mutant adenovirus was used. Early results from several clinical trials with Onyx-015 were encouraging (Table 2, Trials 6–9). Tumor types treated included HNC, gastrointestinal tumors with metastases to the liver, and pancreatic cancers. Intratumoral injection was the preferred route for viral administration. Data from all of the trials showed that Onyx-015 was safe, with no dose-limiting toxicity to normal tissues; its most common side effect was mild flu-like symptoms. All of the patients had neutralizing antibodies to adenoviral proteins after treatment. All of the clinical trials are currently in Phase II evaluation. In HNC trials, of 36 evaluable patients with recurrence/relapse after prior conventional treatment, 4 underwent partial to complete tumor regression, and 13 had stable disease (Table 2, Trial 6). Among 30 evaluable patients with HNC treated with Onyx-015 plus two chemotherapeutic agents, treatment caused tumors to shrink in 25 of the 30 cases. There were 8 complete and 11 partial responses, and by 6 months, none of the responding tumors had progressed, whereas all noninjected tumors treated with chemotherapy alone had progressed (Table 2, Trial 7). The responses of other cancers treated with Onyx-015 plus chemotherapeutic agents have also been evaluated. In pancreatic cancer trials, of 21 evaluable patients with unresectable pancreatic carcinoma who were treated with Onyx-015 plus gemcitabine, 2 had >50% regression, 2 had minor responses, and 6 had stable disease (Table 2, Trial 8). Among 27 evaluable patients with gastrointestinal cancer metastatic to the liver treated with Onyx-015 plus fluorouracil and leucovorin, 3 had >50% regression, 4 had minor responses, and 9 had stable disease (Table 2, Trial 9).

**Conclusion and Future Directions**

Although limitations still exist to the widespread application of gene therapy, the strategy has been shown to be applicable in several clinical situations. Contrary to initial predictions, virus-assisted gene transfer has been shown to be more efficient in cancer cells than in normal tissue cells. Viral vectors appear to spread readily through a tumor and to encourage cell death via apoptosis. Initial concerns that the existence of multiple genetic lesions in cancer cells would prevent the application of gene therapy to cancer appear to be unfounded. Correction of a single genetic lesion has, repeatedly, yielded significant tumor regression.

Clinical trials of p53 gene replacement have provided information that will be useful in the design of future gene therapy strategies. Direct intratumor injection has low toxicity and thus can be readily combined with existing treatments. Postinjection gene expression can be documented and occurs in the presence of an antiadenovirus immune response. Importantly, this treatment can cause tumor regression or prolonged stabilization in patients with recurrent or refractory cancers.

Future research directions will include development of more efficient vectors, use of novel genes, and combined modality approaches. Unresectable tumors are a prominent problem in oncology, with proven therapies such as radiotherapy and chemotherapy controlling, for example, <20% of lung cancers. Based on the preclinical and clinical studies discussed, it now appears that these conventional therapies may provide renewed potential when used in conjunction with the transfer of a functional p53 gene.

Lung cancer and HNC as diseases lend themselves to intratumoral injection gene therapy strategies because, unlike some other organ systems, the tumors can often be accessed relatively noninvasively, and local control remains a problem. Very few complications have arisen from the gene transfer protocol, and a number of patients have undergone treatments on an outpatient basis. Encouraging results in the context of combined modality protocols suggest that this mode of treatment should undergo further study.

A key factor in ensuring the success of gene therapy will be to develop a clear understanding of how it can best play a role in the clinic. For example, immunogene therapies are only ever likely to be effective in clinical situations where patients are at or have been returned to a state of low tumor burden and still have effective, functioning immune systems (68). A consequence of this requirement is that as the early Phase I/II trials move ahead to Phases III and IV, it will take some considerable time and large numbers of patients to demonstrate the true efficacy of the therapies. Moreover, gene therapy is likely to be very effective in combination with pre-existing clinical regimens, such as chemotherapy and radiotherapy. A large number of studies are now showing great potential for combining gene
therapy and pharmaceutical, immunological, and radiotherapeutic approaches to kill cells more effectively and in greater numbers. Development of techniques for systemic delivery of tumor suppressor genes is critical to widening the applicability of gene therapy. This will require new vectors and targeting strategies. There is still a long road ahead before the results gleaned from preclinical and clinical studies of gene therapy strategies approach their full potential, however, these strategies are already proving their worth as the next step in cancer treatment and, because gene therapy targets the etiology of the disease, may eventually have a role in cancer prevention.

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References


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