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LUNG CANCER
Prevention, Management, and Emerging Therapies

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Gene-Based Therapies for Lung Cancer

John Nemunaitis and Jack Roth

Abstract Recent advances in genetics, molecular biology, molecular pharmacology, and biomolecular technology have brought targeted therapeutic opportunities to the forefront of clinical development. Physician and patient communities are highly attracted to lung cancer management opportunities that may involve a personalized approach based on utilizing a unique cancer signal with a target-specific therapy. In this chapter, we will review several advanced clinical developments involving gene-based targeted therapies in lung cancer. Discussion will focus on replacement therapies for abnormal p53 function, FUS1 mediated molecular therapy, antisense technologies, and early developments with RNA interference technology.

Keywords Gene • Molecular • Lung • Cancer therapy

Introduction

Non-small cell lung cancer (NSCLC) management over the last 10 years has significantly improved with the successful development of angiogenesis inhibitors and EGFR inhibitors. However, despite these recent additions to our oncology armament, metastatic disease patients receiving frontline treatment with doublet platinum based chemotherapy in combination with angiogenesis inhibition still have a median survival of less than 1 year. Survival of second line patients is approximately 8 months. Survival of small cell lung cancer patients treated with etoposide based chemotherapy regimes is similar to the
survival of patients with advanced NSCLC. In patients with advanced disease, both histologic types of lung cancer have 5-year-survival rates of 1–2% regardless of treatment.

Understanding of the biomolecular basis of cancer has exploded over the last 10 years. Developments in genetics, molecular biology, molecular pharmacology, and biomolecular technology promise to dramatically alter strategies of cancer treatment. Like other cancers, NSCLC and SCLC are driven by a complex adaptive network of dynamic evolving spatial-temporal biomolecular interactions. Six essential alterations in the neoplastic physiome collectively dictate malignant growth. These include self-sufficiency, insensitivity to growth inhibition (including immune “escape”), independence from programmed cell death, unlimited replicative potential, sustained angiogenesis, and local and metastatic invasiveness (1). Although it appears intuitive that disruption of any one of these global physiologic capabilities would provide a therapeutic opportunity, each cancer is a robust system capable of maintaining its functional characteristics following internal or external perturbation (2, 3). Cancer cells are able to buffer the impact of genetic modification by virtue of having redundant functional pathways in which different structural elements have overlapping functions, termed “degeneracy” (4).

Positive and negative feedback controls allow for stochastic robustness by dampening natural noise. Multileveled functional complementation results from self-contained modules at each organizational level (genome → transcriptome → proteome → metabolome) which interrelate in a functional organizational hierarchy (5). Work is now underway to integrate theoretical and experimental programs to map out and model in quantifiable terms topological and dynamic properties of networks which control the behavior of one cell. Development of high throughput data collection techniques (i.e., microarrays) allows for simultaneous interrogation of the status of a cell’s components at any given time. New technology platforms, such as protein chips and yeast two hybrid screens, help define how proteins interact with each other and will enable us to determine various types of interactive networks (protein to protein interaction, metabolic, signaling, and transcription/regulatory networks) (6, 7). Interestingly, the modulation of pathways that produce “robustness” in certain insults are generally associated with enhanced “fragility” of other perturbations (2), thereby exposing an “Achilles heel” of cancer and potentially permitting a reasoned coordinated multitarget lethal attack on the cancer (8, 9). Specifically, technologies have been developed that enable the systematic discovery of the molecular pathways thereby setting the stage for targeted therapeutics which focus on driving reduction in proliferation and tumor growth following transcriptional and translational modulation.

Efforts to improve these statistics recently have centered around a number of innovative approaches involving immune mediated anticancer effect and/or molecular inhibitory approaches. The purpose of this chapter is to summarize key molecular directed approaches in lung cancer, specifically, p53 gene therapy, antisense technology, and RNA interference technology.

Gene Therapy to Replace Genes Including Missing/Defective Tumor Suppressor Genes

Mechanism of p53 Tumor Suppression and Rationale for p53 Gene Therapy

Many studies over the past 20 years have established a genetic basis for lung cancer. Genes that suppress tumors and repair DNA can be damaged by more than 100 carcinogens contained in tobacco smoke (10). Lung cancers show multiple genetic lesions even in histologically normal bronchial mucosa from people with a smoking history. These genetic abnormalities provide an array of targets for therapy. The p53 tumor suppressor gene appears to play a central role in lung cancer development and was the initial focus of gene therapy approaches to lung cancer.

Two tumor suppressor genes, Rb (retinoblastoma gene) and p53, which are both regulated at the protein level by oncogenes and other tumor suppressor genes, regulate cell proliferation. The Rb protein regulates the maintenance of, and release from, the G1 phase. The p53 protein monitors cellular stress and DNA damage, either causing growth arrest to facilitate DNA repair or inducing apoptosis if DNA damage is extensive (11). When a cell is stressed by oncogene activation, hypoxia, or DNA damage, an intact p53 pathway may determine whether the cell will receive a signal to arrest at the G1 stage of the cell cycle, whether DNA repair will be attempted, or whether the cell will self-destruct via apoptosis (programmed cell death). Apoptosis plays a key role in numerous normal cellular mechanisms, from embryogenesis to destruction of cells that have sustained irreparable DNA damage due to random mutations, ionizing radiation, or DNA damaging chemicals including chemotherapeutic agents. The observation that expression of a wild-type p53 gene in a cancer cell triggers apoptosis provided the rationale for gene therapy approaches (12). Previously, it was believed that gene therapy could not replace all the damaged genes in a cancer cell, and thus would not have a significant effect. The fact that restoration of only one of the defective genes is enough to trigger apoptosis suggests that the DNA damage present in a cancer cell may prime it for an apoptotic event that can be provided through a single pathway.

The p53 gene product is a transcription factor that plays a major role in regulating the apoptosis genes (13). p53 also downregulates the prosurvival (or antiapoptotic) genes, including the antiapoptotic genes bcl-2 and bcl-XL, and upregulates the proapoptotic genes bax, bad, bid, puma, and noxa (14). Available transcripts of each of the pro and antiapoptotic genes with bcl2 homology-3 domains interact with one another to form heterodimers, and the relative ratio of proapoptotic to prosurvival proteins in these heterodimers determines the activity of the resulting molecule, thereby determining whether the cell lives or undergoes apoptosis. p53 also targets the death-receptor signaling pathway, including DR5 and Fas/CD95, and the apoptosis machinery, including caspase-6, Apaf-1, and PIDD. It may also directly mediate cytochrome c release.
The p53 pathway is regulated at the protein level by other tumor suppressor genes and by several oncogenes (11). For example, mdm2 normally binds to the N-terminal transactivating domain of p53, prohibiting p53 activation and leading to its rapid degradation. In normal cells, mdm2 is inhibited by expression of p14ARF, a tumor suppressor gene encoded by the same gene locus as p16INK4a but expressed as an alternate reading frame (15). Deletion or mutation of the tumor suppressor gene p14ARF, which has been noted in some cancers, results in increased levels of mdm2 and subsequent inactivation of p53, resulting in an inappropriate progression through the cell cycle. The expression of p14ARF is induced by hyperproliferative signals from oncogenes such as ras and myc, thus indicating an important role for p53 in protecting cells from oncogene activation. Importantly, p53 also plays a central role in mediating cell cycle arrest. This function is significant, as prolonged tumor stability has often been observed in clinical trials of p53 gene replacement, suggesting that this effect may be predominant over apoptosis in some tumors. p53 is involved in regulating cell cycle checkpoints, and p53 expression can promote cell senescence through its control of cell cycle effectors such as p21CIP1/WAF1. Loss of function in the p53 pathway is the most common alteration identified in human cancer to date. About 50% of common epithelial cancers have p53 mutations (16–18). In some cancers, loss of p53 also appears to be linked to resistance to conventional DNA damaging therapies that require functional cellular apoptosis to accomplish cell death.

Preclinical Studies of p53 Gene Replacement

The studies described above suggest that expressing a wild-type p53 gene in cancer cells defective in p53 function could mediate either apoptosis or cell growth arrest, both of which would be of therapeutic benefit to a cancer patient. Initial studies showed that restoration of functional p53 using a retroviral vector suppressed the growth of some, but not all, human lung cancer cell lines (19). Because of limitations inherent in the use of retroviruses, subsequent studies of p53 gene replacement in lung cancer made use of an adenoviral vector (Ad-p53) (20). The original adenoviral vector was a serotype 5 replication-defective vector with a deleted E1 region, which has been used in all p53 clinical trials. The first published study of p53 gene therapy showed suppression of tumor growth in an orthotopic human lung cancer model using a retroviral expression vector (21). This was the first study to show that restoring the function of a single tumor suppressor gene could result in the regression of human cancer cells in vivo.

Ad-p53 also induced apoptosis in cancer cells with nonfunctional p53 without significantly affecting the proliferation of normal cells (22). Subsequent studies with Ad-p53 demonstrated inhibition of tumor growth in a mouse model of human orthotopic lung cancer (23) and induction of apoptosis and suppression of proliferation in various other cancer cell lines and in vivo mouse xenograft tumor models (24–26). Bystander killing (killing of nontransduced cells by transduced cells), now known to be an important phenomenon in the success of gene therapy, appears to involve regulation of angiogenesis (27, 28), immune upregulation (29–31), and secretion of soluble proapoptotic proteins (32).

Clinical Trials of p53 Gene Replacement

The first clinical trial protocol for p53 gene replacement utilized a replication-defective retroviral vector expressing wild-type p53 driven by a beta-actin promoter (33). The gene/vector construct was injected into tumors of nine patients with unresectable NSCLC that had progressed after conventional therapy. Three of the nine patients showed evidence of tumor regression with no vector-related toxicity, demonstrating the feasibility and safety of p53 gene therapy.

Subsequent p53 clinical trials were conducted with the adenovirus p53 vector described above. A phase I trial enrolled 28 NSCLC patients whose cancers had not responded to conventional treatments. Successful gene transfer was demonstrated in 80% of evaluable patients (34). Expression of p53 was detected in 46% of patients, apoptosis was seen in all but one of the patients expressing the gene, and, importantly, no significant toxicity was observed. More than a 50% reduction in tumor size was observed in two patients, with one patient remaining free of tumor more than a year after concluding therapy and another experiencing nearly complete regression of a chemotherapy- and radiation-resistant upper lobe endobronchial tumor. Additional studies in patients with head and neck cancer helped to establish Ad-p53 gene transfer as a clinically feasible strategy resulting in successful gene transfer and gene expression, low toxicity, and strong evidence of tumor regression.

Gene Replacement in Combination with Conventional DNA Damaging Agents in NSCLC

Preclinical studies of p53 gene therapy combined with cisplatin in cultured NSCLC cells and in human xenografts in nude mice showed that sequential administration of cisplatin and p53 gene therapy resulted in enhanced expression of the p53 gene product (35, 36), and similar studies of Ad-p53 gene transfer combined with radiation therapy indicated that delivery of Ad-p53 increases the sensitivity of p53-deficient tumor cells to external beam radiation (26).

Many tumors are resistant to chemotherapy and radiation therapy and, therefore, fail initial therapeutic interventions. P53, often missing or nonfunctional in radiation- and chemotherapy-resistant tumors, is known to play a key role in detecting damage to DNA and either directing repair or inducing apoptosis. Once apoptosis was implicated as a mechanism of cell killing in response to these DNA damaging agents, it followed that a defect in the normal apoptotic pathway might confer resistance to some tumor cells. Due to Ad-p53’s low toxicity (less than a 5% incidence of serious adverse events) in initial trials, therapeutic strategies combining Ad-p53 gene replacement and conventional DNA damaging therapies were logical extensions of earlier studies (37).
Clinical Trials of Tumor Suppressor Gene Replacement Combined with Chemotherapy

Twenty-four NSCLC patients with tumors previously unresponsive to conventional treatment were enrolled in a phase I trial of p53 in sequence with cisplatin (38). Seventy-five percent of the patients had previously experienced tumor progression on cisplatin- or carboplatin-containing regimens. Up to six monthly courses of intravenous cisplatin, each followed 3 days later by intratumoral injection of Ad-p53, resulted in 17 patients remaining stable for at least 2 months, two patients achieving partial responses, four patients continuing to exhibit progressive disease, and one patient unevaluable due to progressive disease. Seventy-nine percent of tumor biopsies showed an increase in the number of apoptotic cells, 7% showed a decrease in apoptosis, and 14% showed no change.

A phase II clinical trial evaluated two comparable metastatic lesions in each NSCLC patient enrolled in the study (39). All patients received chemotherapy, either three cycles of carboplatin plus paclitaxel or three cycles of cisplatin plus vinorelbine, and then Ad-p53 was injected directly into one lesion. Ad-p53 treatment resulted in minimal vector-related toxicity and no overall increase in chemotherapy-related adverse events. Detailed statistical analysis of the data indicated that patients receiving carboplatin plus paclitaxel, the combination of drugs that provided the greatest benefit on its own, did not realize additional benefit from Ad-p53 gene transfer. However, patients treated with the less-successful cisplatin and vinorelbine regimen experienced significantly greater mean local tumor regression, as measured by size, in the Ad-p53-injected lesion than in the control lesion.

Clinical Trials of p53 Gene Replacement Combined with Radiation Therapy

Preclinical studies suggesting that p53 gene replacement might confer radiation sensitivity to some tumors (26, 40–43) led to a phase II clinical trial of p53 gene transfer in conjunction with radiation therapy (44). Patients with a poor performance status who could not undergo surgery and would be at high risk for combined chemotherapy and radiation received 60 Gy over 6 weeks with Ad-p53 injected on days 1, 18, and 32. Nineteen patients with localized NSCLC were treated, resulting in a complete response in one patient (5%), partial response in 11 patients (58%), stable disease in three patients (16%), and progressive disease in two patients (11%). Two patients (11%) were not evaluable due to tumor progression or early death. Three months after the completion of therapy, biopsies revealed no viable tumor in 12 patients (63%) and viable tumor in three (16%). Tumors of four patients (21%) were not biopsied because of tumor progression, early death, or weakness. The 1-year progression-free survival rate was 45.5%. Among 13 evaluable patients after 1 year, five (39%) had a complete response and three (23%) had a partial response or disease stabilization. Most treatment failures were caused by metastatic disease without local progression.

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In that study, biopsies of the tumor were performed before and after treatment so that detailed studies of gene expression were possible. Ad-p53 vector-specific DNA was detected in biopsy specimens from 9 of 12 patients with paired biopsies (days 18 and 19). The ratio of copies of Ad-p53 vector DNA to copies of actin DNA was 0.15 or higher in eight of nine patients (range, 0.05–3.85), with four patients having a ratio >0.5. For 11 patients with adequate samples for both vector DNA and mRNA analysis, eight showed a postinjection increase in mRNA expression associated with detectable vector DNA. Postinjection increases in p53 mRNA were detected in 11 of 12 paired biopsies obtained 24 h after Ad-p53 injection, with 10 of 11 increasing threefold or more. Preinjection biopsy specimens that were shown by immunohistochemistry to be negative for p53 protein expression were stained for p53 protein expression after Ad-p53 injection. Staining results confirmed that the p53 protein was expressed in the posttreatment samples in the nuclei of cancer cells. For p21 (CDKN1A) mRNA, increases of statistical significance were noted 24 h after Ad-p53 injection and during treatment, as compared with the pretreatment biopsy. MDM2 mRNA levels were higher during treatment than before treatment. Levels of FAS mRNA did not change significantly during treatment. BAK mRNA expression increased significantly 24 h after injection of Ad-p53 and thus appeared to be the marker most acutely upregulated by Ad-p53 injection.

The safety profile for intratumoral injection of Ad-p53 has been excellent. The most frequently reported adverse events related to treatment with Ad-p53 injection were fever and chills, asthenia, injection site pain, nausea, and vomiting. The vast majority of these events were mild to moderate.

The maximum tolerated dose for Ad-p53 injection has been established.

Systemic Gene Therapy for Metastases

Local control of cancers is important, but most patients with lung cancer die from systemic metastases. The development of a cancer vaccine to p53 is one approach. Although the p53 protein is expressed by normal cells, it has a short half-life and is thus present at low levels. Mutant p53 is conformationally altered and resists degradation in cancer cells. Thus, it has a prolonged half-life and is expressed at high levels in cancer cells. These differences in expression between normal and cancer cells suggest that p53 could function as a tumor antigen and vaccine target (45–48). Several studies have shown in cultured cells and mouse models induction of anti-p53 cytotoxic lymphocytes that killed cancer cells but not normal cells. A strategy was developed using dendritic cells, which are the most effective antigen-presenting cells, transduced with Ad-p53 (49).

Patients with extensive-stage small-cell lung cancer (SCLC) were entered into a trial. SCLC patients with extensive stage disease have a median survival of 2–4 months untreated or 6–8 months with chemotherapy. In that trial, the patients’ autologous dendritic cells were treated ex vivo with Ad-p53, which activates the cells and results in the expression of high levels of p53 protein. Patients were first treated with conventional chemotherapy. Those who achieved at least stable disease
received the vaccine biweekly for a total of three to six injections. If patients progressed, they were treated with chemotherapy. Of the 29 patients treated, one had a partial response, seven had stable disease, and 21 had progression. Patients having progression then received second-line chemotherapy. Clinical follow-up was completed for 21 patients. Complete or partial responses to the second-line chemotherapy were observed in 61.9% of the 21 patients treated. Eleven of the patients were alive 1 year after the first vaccine treatment. These clinical responses were correlated with induction of immune responses to the vaccine. Published objective response rates for second-line chemotherapy in extensive-stage SCLC patients ranges from 5 to 30%.

Gene delivery to distant sites of cancer is essential for successful cancer gene therapy. Recently, the development of nanoscale synthetic particles that can encapsulate plasmid DNA and deliver it to cells after intravenous injection has been reported. This has been studied in mouse xenograft models of disseminated human lung cancer. In addition to p53, other tumor suppressor genes have been delivered using this technique. Multiple 3p21.3 genes show different degrees of tumor suppression activity in various human cancers in vitro and in preclinical animal models. One of the tumor suppressor genes at this locus is FUS1, which is not expressed in most lung cancers. When wild-type FUS1 is expressed in a lung cancer cell, apoptosis occurs. To translate these findings to clinical applications for molecular cancer therapy, we recently developed a systemic treatment strategy by using a novel FUS1-expressing plasmid vector complexed with DOTAP:cholesterol (DOTAP:Chol) liposome, termed FUS1 nanoparticle, for treating lung cancer and lung metastases (50, 51). In a preclinical trial, we showed that intratumoral administration of FUS1 nanoparticles to subcutaneous NSCLC H1299 and A549 tumor xenografts resulted in significant inhibition of tumor growth. Intravenous injections of FUS1 nanoparticles into mice bearing experimental A549 lung metastasis significantly decreased the number of metastatic tumor nodules. Lung tumor-bearing animals treated with FUS1 nanoparticles survived longer (median survival time: 80 days) than control animals. These results demonstrate the potent tumor suppressive activity of the FUS1 gene, making it a promising therapeutic agent for treatment of primary and disseminated human lung cancer (50, 51). Based on these studies, a phase I clinical trial with FUS1-mediated molecular therapy by systemic administration of FUS1 nanoparticles is now under way in stage IV lung cancer patients at The University of Texas M. D. Anderson Cancer Center in Houston, Texas.

**Summary and Conclusions**

Current therapy such as radiation and chemotherapy controls less than 50% of lung cancers, and overall 5-year survival is only 15%. Combining existing treatments has reached a plateau of efficacy, and the addition of conventional cytotoxic agents is limited because of toxicity. The clinical trials summarized in this article clearly demonstrate that, contrary to initial predictions that gene therapy would not be suitable for cancer, gene replacement therapy targeted to a tumor suppressor gene can cause cancer regression by activation of known pathways with minimal toxicity.

Gene expression has been documented and occurs even in the presence of an antiviral response. Clinical trials have demonstrated that direct intratumoral injection can cause tumor regression or prolonged stabilization of local disease, and the low toxicity associated with gene transfer indicates that tumor suppressor gene replacement can be readily combined with existing and future treatments. Initial concerns that the wide diversity of genetic lesions in cancer cells would prevent the application of gene therapy to cancer appear unfounded; on the contrary, correction of a single genetic lesion has resulted in significant tumor regression.

Studies using the transfer of tumor suppressor genes in combination with conventional DNA damaging treatments indicate that correction of a defect in apoptosis induction can restore sensitivity to radiation and chemotherapy in some resistant tumors, and indications that sensitivity to killing might be enhanced in already sensitive tumors may even lead to reduced toxicity from chemotherapy and radiation therapy. The most recent laboratory data demonstrating damage to tumor suppressor genes in normal tissue and premalignant lesions suggests that these genes could someday be useful in early intervention, diagnosis, and even prevention of cancer. Preclinical studies have shown that systemic delivery for treatment of metastases can be achieved. The ready availability of gene libraries, the ability to administer genes without the extensive reformulation required of small molecules, and their specificity make this an attractive therapeutic approach. Despite the obvious promise evident in the results of these studies, though, it is critical to recognize that there are still gaps in knowledge and technology to address. The major issues for the future development of gene therapy include:

1. Development of more efficient and less toxic gene delivery vectors for systemic gene delivery.
2. Identification of the optimal genes for various tumor types.
3. Optimizing combination therapy.
5. Overcoming resistance pathways.

However, given the rapid progress in the field, it is likely that many of these technological problems will be solved in the near future.

**Antisense Technology in NSCLC**

Antisense oligonucleotides (AS ODNs) are unmodified or chemically modified single-stranded DNA molecules of 13–25 nucleotides in length that are designed to specifically hybridize to corresponding RNA by Watson-Crick binding. They inhibit mRNA function by several mechanisms, one, through inhibition of protein translation by disrupting ribosome assembly, and two, through utilization of endogenous RNase H enzymes that cleave the mRNA strand (52–56). The specificity of
hybridization of an AS ODN to the target mRNA makes the AS strategy attractive for selective modulation of expression of genes involved in the pathogenesis of malignant disease. One AS ODN has been approved for local therapy of cytomegalovirus (CMV) retinitis, and a number of AS ODN's are currently being tested in clinical trials. These include ODN's that target C-Raf kinase, C-Raf 1, H ras, protein kinase A-Type I, protein kinase C-alpha, bcl-2, survivin, and DNA methyltransferase (57, 58).

Most oligonucleotides being clinically tested have a phosphorothioate backbone in which one of the oxygens on the phosphate moiety is replaced with a sulfur. Phosphorothioate oligonucleotides enable mRNA degradation through enzymatic cleavage via activation of RNase H, and they carry a negative charge which has been shown to bind plasma proteins in a manner similar to heparin (59, 60). This characteristic protects them from filtration thereby prolonging product half-life. However, the negative charge has also been correlated with side effects, including thrombocytopenia and activation of the complement cascade (61). Phosphorothioates accumulate predominantly in the liver but also in the kidneys (62–64).

**Protein Kinase C-α: ISIS 3521**

Protein Kinase C (PKC) is a family of phospholipid-dependent cytoplasmic serine threonine kinases which comprises distinct isoenzymes which differ in their biochemical properties, tissue-specific expression, and intracellular localization (65, 66). PKC isoenzymes provide signals that lead to proliferation or differentiation (66, 67). PKC-α activity specifically appears to be involved in signaling (68) malignant transformation and proliferation. Overexpression of the PKC-α gene in breast cancer cells results in increased proliferation, anchorage-independent growth, and enhanced tumorigenicity (69). PKC-α expression is also elevated in human breast cancers (70). Inhibition of PKC-α limits growth of hepatoma (71) and medulloblomma (72). ISIS 3521 (also designated IS 641A) is a 20-mer phosphorothioate oligodeoxynucleotide that hybridizes to the 3'-untranslated region of the human PKC-α mRNA, resulting in a site amenable to degradation by RNase H (72). Phase I testing demonstrated acceptable safety and evidence of clinical activity (two patients with lymphoma had complete response to ISIS 3521) (74).

Early evaluation of ISIS 3521 in NSCLC involved combination with carboplatin and paclitaxel. Forty-eight evaluable patients with advanced stage IIIB or IV NSCLC were entered into trial (75). Minimal toxicity consisting of neutropenia and thrombocytopenia led to treatment delays in less than 15% of patients. Patients received a median of six cycles and achieved a response rate of 48%, with 23% (one patient) obtaining complete response and 66% (22 patients) partial response. The median time to progression and the median survival were 6.3 and 15.9 months, respectively. A second phase II trial tested ISIS 3521 in combination with cisplatin and gemcitabine. Forty-four chemotherapy-naive patients with advanced NSCLC were entered into trial. Toxicity was moderate but included thrombocytopenia, neutropenia, anemia, fatigue, dehydration, sepsis and neutropenic fever (76). In the updated analysis of the trial, the response rate was 37%, including one complete remission and 11 partial remissions.

Based on these phase II data, two large randomized phase III trials were initiated as first-line treatment in patients with NSCLC. The first enrolled 600 patients with stage IV NSCLC using ISIS 3521 in combination with carboplatin and paclitaxel. Results were disappointing. No difference was observed in time to progression or overall survival between treatment and control groups. There were, however, indications of antitumor activity, as a subset of patients who completed the prescribed course of ISIS 3521 (six cycles) had a median survival of 17.4 months when compared with 14.3 months in patients who did not (p = 0.048). Negative results were also obtained in the second phase III trial involving advanced NSCLC patients testing ISIS 3521 in combination with gemcitabine and cisplatin. Therapy was well tolerated, but median survival was roughly 10 months in both groups.

**Clusterin: OGX-011**

Overexpression of clusterin prolongs cell survival and leads to enhanced metastatic potential of cancer cells in vitro (77). AS against clusterin significantly enhanced chemosensitivity in prostate and renal carcinoma cells in vitro (78). A phase I trial using OGX-011 for patients with localized prostate cancer has been published (79). The most frequently reported side effects were mild (grade 1 or 2) and included fevers, rigors, fatigue, and transient elevations of aspartate aminotransferase and alanine aminotransferase. A second phase I study was designed to determine the recommended dose of OGX-011 in combination with docetaxel (Taxotere™) in various solid tumors (80, 81). OGX-011 is currently in phase II development for patients with prostate, breast, and lung cancer.

Combination of OGX-011 and docetaxel in 38 patients with different solid tumors reveals a linear dose-dependent pharmacokinetics of OGX-011, with no apparent interaction with docetaxel. Similar results with OGX-011 were found in combination with cisplatin and gemcitabine. A dose-dependent increase in OGX-011 Cmax and AUC was noted, with no apparent interaction with either chemotherapeutic (82). In another trial, OGX-011 was administered in combination with docetaxel (80). The study enrolled 38 patients with a variety of solid tumors (including NSCLC, and prostate, ovarian, renal cell, and breast cancer). A significant decrease in serum clusterin levels was observed in relation to dose of OGX-011. Of 24 patients with measurable disease, there was one patient with a partial response (PR) and eight patients with stable disease (SD). In a subsequent clinical trial involving ten chemotherapy-naive patients with advanced NSCLC OGX-011 was administered in combination with cisplatin and gemcitabine. Two of nine patients with stable disease to prior therapy achieved a PR to OGX-011, cisplatin and gemcitabine. Toxicity primarily occurred within the first week of therapy and diminished with continued dosing. Hematological adverse effects included grade 1
leukopenia, thrombocytopenia, and anemia. Other self-limiting common adverse events were fever, fatigue and rigors occurring several hours after infusion, and grade 1 and 2 elevations in hepatic transaminase levels. No apparent dose-dependent induction of serum complement was observed (79).

**H-ras: ISIS 2503**

ISIS 2503 is a 20-base phosphorothioate AS ODN that binds to the translation initiation region of human H-ras mRNA (ISIS 2503) and that selectively reduced the expression of H-ras mRNA and protein in cell culture. In a phase I trial, ISIS 2503 administration was not associated with any dose-limiting toxicity. Out of 25 patients, four had stabilization of their disease for six to ten cycles of therapy. No consistent decreases in H-ras mRNA levels were observed in peripheral blood lymphocytes (83). A subsequent multicenter phase II trial analyzed ISIS 2503 in stage IIIB/IV NSCLC. Out of 20 evaluable patients, 7 achieved SD and 13 progressed within the first three cycles. There were no partial or complete responses (84). Given that limited activity was seen and most relevant mutations involving ras oncogene in NSCLC are K-ras rather than H-ras, further development if ISIS 2501 in NSCLC has not been done.

**C-Raf-1: ISIS 5132**

Raf kinases are serine/threonine kinases that regulate mitotic signaling pathway, most notably those involving the mitogen-activated protein kinase pathway signal from ras. This regulation of ras-dependent pathways by raf is potentially important since the ras oncogene is dysregulated or mutated more frequently than any other oncogene studied in human cancer (85, 86). In several tumors, including breast and NSCLC, the presence of a ras mutation is a negative prognostic factor (87). Craf has also been reported to bind to Bcl-2 and to be involved in the regulation of apoptosis. An AS ODN directed to the 3' untranslated region of the c-raf mRNA (ISIS 5132) inhibits growth of human tumor cell lines in vitro and in vivo in association with specific downregulation of target message expression. In a phase I trial, changes in c-raf-1 mRNA expression were analyzed in peripheral blood mononuclear cells (PBMC) collected from patients with advanced cancers treated with ISIS 1532. Significant reductions of c-raf-1 expression from baseline were detected in 13 of 14 patients. Clinical toxicities included fever and fatigue, neither of which were dose limiting. Two patients experienced prolonged disease stabilization for more than 7 months. In both of these cases, this was associated with reduction in c-raf-1 expression in PBMC. Initial results of a phase I trial testing continuous IV infusion of ISIS 5132 in 34 patients with a variety of solid tumors refractory to standard therapy reported one patient at high dose with fever as a dose-limiting toxicity (88), three patients had grade 3 or 4 thrombocytopenia, and one patient had grade 3 leukopenia. One patient with refractory ovarian cancer had a dramatic reduction in her CA-125 level (97%), and two other patients had prolonged disease stabilization for 9 and 10 months, respectively. No objective responses were seen in a phase II trial for 22 patients with progressive lung cancer (18 NSCLC, 4 SCLC) (89). Hematological toxicity did not exceed grade 2. Nonhematological toxicity was mild to moderate. More recently, a different Raf-1 AS ODN has been developed in a new formulation called LERafAON (NeoPharm, Lake Forest, IL) (90). To avoid the need to chemically protect the oligonucleotide from degradation and to improve intracellular delivery, LERafAON has been encapsulated in a cationic liposome (90). LERafAON is undergoing phase I testing in patients with advanced solid tumors (91).

**Bcl-2: Oblimersen**

Oblimersen is an AS ODN which downregulates Bcl-2 protein expression. Animal studies validated mechanism, safety, and clinical opportunity (92–98). Phase I, II, and III studies have been and are being performed testing oblimersen in patients with multiple advanced cancers including lymphoma, melanoma, breast cancer, hormone-refractory prostate cancer, and a small number of lung cancer patients (99, 100).

Phase I and II trial investigation in non Hodgkin's lymphoma (101, 102) demonstrated dose-related safety. Two patients achieved complete remissions. Reduction in Bcl-2 protein as predicted was able to be demonstrated in a subset of patients (102). Fever and transient grade 3 increases in hepatic enzymes were observed.

Phase III investigation involving melanoma (103) did demonstrate limited efficacy; however, it was not sufficient for the Food and Drug Administration (FDA) approval. In melanoma, the overall response rate of the combination of dacarbazine and oblimersen was 12.4% vs. 6.8% for dacarbazine alone (p = 0.0007) (103). The median progression-free survival was 2.4 months vs. 1.6 months (p = 0.0003), but there was only a trend toward improvement in overall median survival (9.0 months vs. 7.8 months, p = 0.077).

Oblimersen has been tested in combination with paclitaxel and in combination with carboplatin and etoposide in advanced small cell lung cancer patients, but limited efficacy has been demonstrated (100).

**Survivin: LY2181308**

Survivin is a member of the IAP gene family, and has an important role in both cell division and apoptosis inhibition (104–106). Survivin is expressed at a high level in a wide range of human cancer types, including lung, colon, pancreas, breast and prostate cancers (105, 107). However, survivin is generally not expressed in normal tissue. Survivin expression levels correlate with lower apoptotic index in tumor
cells and poor prognosis in cancer patients, and serial analysis of gene expression studies have indicated that survivin is the fourth most common gene that is uniformly expressed in cancer cells but not in normal tissues (108). A novel 2’-MOE ASO (called LY2181308) has been constructed. It specifically downregulates survivin expression in a broad range of human cancer cells and has produced potent antitumor activity in human tumor xenograft models (109, 110). Antitumor activity displayed by LY2181308 in these models is oligonucleotide-sequence specific, and is associated with reduced survivin levels in tumor tissue. Clinical development of LY2181308 is moving forward.

RNA Interference

RNA interference (RNAi) is an evolutionarily conserved gene-silencing mechanism which functions during vertebrate embryonic development and is incorporated as an additional layer in the immune defense mechanism (111) whereby small sequences of intrinsic antisense RNA or extrinsic dsRNA (i.e., viral) trigger translational suppression. In cells that endogenously express a gene, introduction of siRNA molecules that target the gene triggers mRNA degradation. The degradation process occurs following interaction of siRNA with ATP dependent helicase and with the ATP dependent Rnase enzyme Dicer through the formation of a “RNA interfering silencing complex” (RISC) (112). Endonucleolytic cleavage of the target mRNA occurs at a single site at the center of the target mRNA-siRNA antisense strand duplex (113) and is mediated by Slicer (Ag02) (114).

The use of synthetic siRNA molecules has gained wide acceptance as a laboratory tool for target validation, but clinical trials in oncology patients have not yet commenced. Nevertheless RNAi has gained greater acceptance in 2 years than traditional antisense oligonucleotides (ASO) and ribozymes (RBZ) achieved in 20 years (115). Unlike single-stranded RNA, duplex RNA is quite stable and does not require chemical modifications to achieve a satisfactory half-life in cell culture media (116, 117). While antisense oligonucleotides have been tested clinically (74, 83, 88), the backbone modifications required for oligonucleotide stability increased the risk of toxicity thereby limiting administration at dose levels sufficient to induce significant tumor response, and siRNA methods could potentially avoid this problem.

Progress in the development of RNAi technology benefited from previous research aimed at optimizing traditional ASO and RBZ nucleotides. For example, cellular uptake was a major obstacle for efficient gene inhibition inside cells and lessons learned from difficulties in transfecting cells with ASO and RBZ’s were applied to RNAi (118). Wide varieties of efficient delivery systems for nucleic acids have now been developed and are commercially available. In addition, researchers using traditional ASOs had already described potential pitfalls and developed criteria for the essential control experiments needed to validate preliminary data (119).

Finally, biodistribution studies of single-stranded ASOs had been performed, providing suggestions about potential target requirements for siRNA (120–122).

Antitumor Effects of RNAi

The exquisite specificity of RNAi has been utilized in multiple studies to exploit phenotypic differences between cancer cells and normal cells. The early work of Martinez and coworkers (123) demonstrated that the p53 mutant-specific RNAi molecule can knockdown the mutant message that differed from wild type by only a single nucleotide, and restored wild type p53 function in heterozygous tumor cells. Similarly, mutant ras silencing by RNAi produced an antitumor effect that nullified the oncogenic phenotype (124). Kawasaki showed that mutant ras decreased by 90% through RNA silencing without altering wild type messages in vitro and in vivo (125, 126). Mutant K-ras knockdown also produced ~70% reduction of cancer cell growth in the human colon carcinoma cell line SW480. Retroviral delivery of an RNAi molecule specifically inhibited the mutant K-ras12 allele in the human pancreatic carcinoma cell line CAPAN-1 without affecting wild type K-ras level, and collaboratively led to a loss of anchor independent growth and tumorigenicity. Similar success has also been attained by targeting the mutant H-ras oncogene (126–128). The targeting of p53 and ras reflect widely different requirements for siRNA reagent design. Insofar as p53 point mutations are located throughout the 11 exon sequences, custom reagents have to be designed for each mutation. By contrast, ras mutations are primarily limited to “hotspots,” thereby allowing a limited number of reagents to cover the most mutated messages. In vitro cancer growth inhibition has been achieved by targeting unique cancer oncogenic messages that are derived from novel gene fusions (e.g., bcr-abl in myelogenous leukemia) (129), virally-expressed genes (HPV E6/E7 in cervical cancer cells) (130), or overexpressed messages (including HER-2/neu in human breast and ovarian cancer cells (131, 132), protein kinase A in pancreatic cancer cells (133), multidrug resistance genes (134), telomerase (135), and the antiapoptotic bcl-2 gene (136). In vivo studies have also led to favorable outcomes by RNAi targeting of critical components for tumor cell growth (124, 137–140), metastasis (141–143), angiogenesis (144, 145), and chemoresistance (146, 147).

As with ASOs and RBZ’s, efficacy of siRNA depends on the cell type as well as the level of expression of the targeted gene (148). Nonetheless, RNAi has repeatedly proven to be more robust in terms of consistency of transcript knockdowns at threshold concentrations that are several orders of magnitude below typically-used ASOs (113, 148–151). Theoretically, approximately 1–3 molecules of duplexed RNA per cell are effective at knocking down gene expression (112), although most studies in mammalian cells require an intracellular concentration at the nanomolar range. At these concentrations, more prolonged knockdown activity has been observed in vivo as compared with ASO and RBZ (152).
RNAi Delivery

Building on the premise that RNAi molecules may have a higher therapeutic index than ASO and RBZ’s, a markedly lower intracellular concentration of RNAi is needed for the desired effect of targeted gene knockdown. Hence the success of RNAi therapy, which requires effective and global delivery of RNAi to target cells, is more likely to be attained. This is particularly applicable to cancer therapy, where multiorgan metastatic foci are largely responsible for the morbidity and mortality of advanced cancer. Murine studies show that RNAi can be administered “hydrodynamically” (153) by rapidly injecting duplex RNAi molecules through the tail vein. This strategy is not feasible clinically and produces severe cardiovascular side effects (152). RNAi molecule delivery by lipid-based technologies (cationic liposomes, liposome-protonated/DNA) (154–156) or viral vectors (157, 158) have also been explored in animal models.

Liposomal-based technologies allow for up to 90% transfection efficiency in vitro, but they are costly, difficult to generate and associated with induction of clinically toxic cytokines (IL6, TNFα) (154). Cell-specific immunoliposomes have been used successfully to deliver chemotherapy drugs to target cells and may serve as a viable alternative for cationic-based RNAi delivery. In a recent study by Zhang et al. (159), weekly intravenous injection of pegylated immunoliposomes effectively delivered epidermal growth factor receptor siRNA to xenografts of intracranial gliomas, resulting in 95% suppression of EGFR function and an 88% increase in survival time.

A number of studies have documented stable transduction of siRNA-expressing constructs with viral vectors. Retroviral delivered siRNA effectively targeted p53 in both cell lines and primary fibroblasts (160). Lentiviral vectors were similarly effective, with a lasting effect of >25 days (112). Lentiviral delivery of antiviral siRNA inhibited HIV production from primary human T cells and macrophages (161, 162) in vitro, and silenced target genes in vivo in transgenic mice (163). However, concern over the risk of insertional mutagenesis with retroviruses precludes their clinical use for cancer therapy at this time. Theoretically, bacterial vectors could also be utilized (164), but to date, most cancer gene transfer trials, whether intratumoral, intravenous or intra-arterial, involve adenoviral delivery vehicles.

Potential Hurdles for siRNA Cancer Therapeutics

siRNA faces unique hurdles as a cancer gene therapeutic in addition to common concerns that it shares with ASO- and RBZ-based therapies. There are concerns regarding the specificity of siRNA gene silencing with respect to interferon (IFN) induction and “off-target” activity. Contrary to the initial observations of Elbashir et al. (113), Sledz et al. identified JAK–STAT pathway activation and global upregulation of IFN-stimulated genes following PKR activation by a 21-bp siRNA molecule (165). However, nonspecific IFN induction or toxicity was not observed in various in vivo studies (112, 166). Other contributing elements of IFN activation include the plasmid vector used for siRNA delivery, which may cause formation of long hairpin RNA duplexes, or chemical modifications (e.g., 3′ triphosphates on the duplex) at the 3′ end of siRNA (167). The liposomal transfecting agent may also contribute to nonspecific toxicity (168). Hence, each siRNA construct and its delivery system should be carefully scrutinized with respect to its likelihood of soliciting a nonspecific IFN response that would negatively impact therapeutic outcome.

In a recent gene expression profile analysis, Jackson and coworkers suggested that siRNAs may exhibit silencing activities on unintended target sequences having less than 18-nucleotide homology with the intended target sequence (169). This apparent lack of fidelity may be explained by an inadequacy of transcriptome search (170). A more extensive evaluation of siRNA’s that had been designed for specific targets revealed multiple examples of other nucleotide sequence homology in addition to the intended target sequence (170). In fact, Snoke and Holen identified unintended target sequences with three or fewer mismatches in 75% of 359 published siRNA sequences (170), highlighting the potential risk of siRNA design based on limited sequence analysis.

The recent discovery of endogenous microRNAs (miRNAs) furthered misgivings regarding the off-target activity of siRNA. miRNAs are single-stranded RNAs of 21–25 nucleotides found in all multicellular organisms (171–173). In humans, 200–255 genes in the human genome encode miRNAs (172). miRNAs are generated from genome hairpin RNAs through processing by Drosher, and are believed to serve a regulatory function. miRNA inhibits the translation of mRNAs into proteins through imperfect base pairing with the target mRNA, but does not impede transcription or destroy mRNAs. It appears, however, that siRNAs “acting as miRNA” contribute minimally to off-target activity, as synergism between multiple, partially complementarity-bound miRNAs are needed for effective translational silencing (171). Furthermore, unique, target sequence-independent signatures of individual siRNAs remain a laboratory manifestation defined by gene array analyses. The impact of such off-target activity has not been evident in animal studies (152). Preliminary evidence suggests that in vitro off-target activity may be reduced further through chemical modifications, such as nucleotide selection in key positions, and the intentional introduction of mismatches at defined positions between the siRNA sense and antisense strands (174).

Development of siRNA technology is moving forward. Initial delivery vehicles will include nonviral strategies (i.e., cationic liposomes). Some of the initial targets will likely include similar genes identified for ASO development.

Concluding Remarks

In conclusion, a broad array of targeted gene-based therapies are under active clinical development in NSCLC. Common attributes of these therapies include remarkable safety with virtually no evidence of clinically significant off target effect.
outside of the target specificity. Tolerable toxicity, however, is observed in relation to delivery components. Further development is ongoing to reduce toxicity attributed to delivery of gene-based targeted therapeutics. Evidence of clinical activity has been demonstrated and further phase II and a phase III investigation is moving forward. At the same time quantitative proteomic and genomic technology is becoming more accessible, thereby enabling personalized attempts to match a particular targeted therapy with a unique cancer specific molecular signal.

References


Lung Cancer Resistance to Chemotherapy

David J. Stewart

Abstract Metastatic lung cancer remains incurable by chemotherapy. Several factors contribute to resistance to chemotherapy, including many factors that are adaptations of systems that evolved to protect normal cells from a hostile environment. Tumor cell characteristics, tumor cell interactions with extracellular matrix and stromal cells, and tumor physical characteristics all contribute to resistance. Resistance may arise from gene upregulation or downregulation as a downstream consequence of the oncogene mutations or tumor suppressor gene deletions that underlie tumorigenesis or may also arise due to tumor hypoxia or due to exposure to therapy. Host gene polymorphisms may alter resistance by determining the half-life or enzymatic activity of upregulated resistance factors. Resistance may arise from decreased drug delivery to tumor, impact of extracellular pH on drug uptake, altered drug uptake transporters or cell membrane characteristics, increased drug efflux or detoxification, decreased drug binding, altered drug targets, increased DNA repair, decreased proapoptotic factors, increased antiapoptotic factors, altered cell cycling or mitotic checkpoints, or altered transcription factors. This diversity of resistance mechanisms magnifies the challenges facing us in predicting patient prognosis and in overcoming resistance.

Keywords Lung cancer • Chemotherapy • Resistance

Lung Cancer and Resistance

As outlined elsewhere in this text, despite 20–50% of patients with advanced non-small cell lung cancer (NSCLC) and 60–80% of patients with extensive small cell lung cancer (SCLC) initially responding to chemotherapy, widely metastatic...