REVIEW

Nucleic acid targeting: towards personalized therapy for head and neck cancer

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In light of a detailed characterization of genetic aberrations in cancer, nucleic acid targeting represents an attractive therapeutic approach with significant translational potential. Head and neck squamous cell carcinoma (HNSCC) is a leading cause of cancer deaths worldwide with stagnant 5-year survival rates. Advances in conventional treatment have done little to improve survival and combined chemoradiation is associated with significant adverse effects. Recent reports have characterized the genetic alterations in HNSCC and demonstrated that mutations confer resistance to conventional and molecular targeted therapies. The ability to use specific nucleic acid sequences to inhibit cancer-associated genes including non-druggable targets facilitates personalized medicine approaches with less adverse effects. Additionally, advances in drug delivery mechanisms have increased the transfection efficiency aiding in greater therapeutic responses. Given these advances, the stage has been set to translate the information garnered from genomic studies into personalized treatment strategies. Genes involved in the tumor protein 53 and epidermal growth factor receptor pathways have been extensively investigated and many promising preclinical studies have shown tumor inhibition through genetic modulation. We, and others, have demonstrated that targeting oncogene expression with gene therapy approaches is feasible in patients. Other methods such as RNA interference have proven to be effective and are potential candidates for clinical studies. This review summarizes the major advances in sequence-specific gene modulation in the preclinical setting and in clinical trials in head and neck cancer patients.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a leading cause of morbidity and mortality worldwide and is primarily associated with tobacco and alcohol use.¹ Owing to late clinical presentation and advanced stage, HNSCC leads to over 300 000 deaths annually. Most patients require extensive, multidisciplinary treatment. However, even with aggressive treatment, the 5-year survival rate in the United States is only around 40% primarily due to the lack of effective therapeutic options for advanced-staged cancers.²

In patients with locally advanced disease, relapse is common despite early therapeutic intervention. In these patients, prognosis is poor and conventional treatment yields only 30–40% response rates with a median survival between 6 and 9 months.³ Traditional chemotherapy has low tumor specificity leading to significant adverse effects in patients. Further, a wide array of gene aberrations in HNSCC are implicated in therapeutic failure to conventional therapy.⁴ While some mutations are etiologic, others confer sensitivity or resistance to chemotherapy.^{5–7}

Expression of the variant III form of epidermal growth factor receptor (EGFR VIII) confers resistance to EGFR inhibitors and may explain the modest clinical response with these agents.^{8–10} Conversely, nearly one-third of HNSCC tumors harbor mutations in *PI3KCA* gene.¹¹ The oncogenic *PIK3CA* (E542K) mutation confers exquisite sensitivity to PI3K pathway inhibitor BEZ-325.¹² Given the heterogeneity in mutations, it would be difficult to develop small molecules or antibodies that specifically target each

oncogenic mutation.^{11,13,14} In this scenario, the use of targeted nucleic acid therapy to mitigate expression of mutated genes with high sequence specificity would facilitate personalized cancer therapy and combat treatment failures. In this manuscript, we discuss advances in gene delivery systems, and preclinical and clinical studies using nucleic acid targeted therapy for HNSCC.

GENE DELIVERY SYSTEMS

Direct intratumoral administration of therapeutic nucleic acids for proof-of-concept studies is feasible in easily accessible sites such as the oral cavity. However, difficulty in accessing deep-seated tumors, low transfection efficiency, and susceptibility to nuclease degradation are major limitations with injection of naked DNA or RNA.¹⁵ In order to facilitate systemic delivery and improve transfection efficiency, significant advances have occurred in vector development including liposomes, nanoparticles, and viral vectors (Figure 1). Liposome complexes protect nucleic acids from degradation and improve uptake in cells. A spherical lipid bilayer encapsulating the nucleic acid cargo fuses with the plasma membrane of cells, releasing the contents into the cytoplasm. *In vitro* studies show a transfection efficacy of up to 70%; however, the presence of serum components can decrease this by 35–45%.¹⁶ Despite the increase in efficiency, hematologic toxicity, hepatotoxicity, and innate immune responses have been associated with systemic therapy.^{17–19} In order to reduce toxicity and facilitate tumor specificity, next-generation liposomes carry

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Figure 1. Summary of the delivery vectors primarily tested for nucleic acid therapy in HNSCC. Naked DNA in the form of plasmids or oligonucleotides can freely diffuse across the plasma membrane and may enter the nucleus for expression. Microbubbles carrying nucleic acids ruptured at the tumor site by ultrasound release nucleic acids cargo that diffuses into tumor cells. Liposomes with or without targeted peptides fuse with the plasma membrane and deliver cargo into the cell. Polymer vehicles diffuse across the plasma membrane and release the payload into the nucleus or cytoplasm. Viral vectors can efficiently deliver exogenous nucleic acids into tumor cells. Oncolytic viruses specifically replicate and lyse target tumor cells.

tumor-targeted peptides in the lipid bilayer. For instance, liposomes conjugated to the transferrin ligand have a higher affinity for HNSCC cells with upregulated transferrin receptors than non-malignant cells. *In vitro* results suggest an increase in transfection efficiency from 10 to 50% when using transferrin ligand attachments.²⁰ Alternatively, the use of antitransferrin receptor single-chain antibody on the surface of cationic liposomes can increase specific uptake in tumor cells on systemic administration.²¹ The major challenge that persists with liposomes is hepatotoxicity upon clearance from circulation.

In an effort to decrease toxicity and increase transfection efficiency, other methods of gene delivery have been developed. Synthetic polymer complexes can diffuse across the plasma membrane or enter cells through endocytosis. Nucleotides dissociate from the polymer and exert their action within the cytoplasm or the nucleus. Common polymers used in HNSCC include polyethylenimine and glucosylated polyethylenimines.^{22–24} A challenge to the widespread use of these agents is the formation of complexes with blood components due to the high cationic charge.²⁵ Conjugation with hydrophilic polymers reduces this positive charge, increasing bioavailability. *In vitro* gene transfection efficiency can reach up to 47% while retaining *in vivo* antitumor efficacy.²⁶ Another novel mechanism for delivery involves systemically administered, ultrasound-guided microbubbles targeting HNSCC tumors. Vigorous mixing of aqueous solutions of lipid and nucleic acid generates microbubbles with a lipid shell and perfluorobutane gas interior. The average diameter of the microbubbles is 1–8 μm . Intravenously administered microbubbles are visualized using ultrasound imaging. As they perfuse the tumor, destruction of microbubbles with high-frequency ultrasound waves releases the nucleic acid cargo near tumor cells. Several preclinical studies demonstrated successful

microbubble-mediated transfer of nucleic acid to the tumor site.^{27–29} Cellular uptake of nucleic acids is facilitated by lipids in the shell, and by the acoustic frequency and force of cavitation of microbubbles.³⁰ In comparison to direct inoculation of tumors with antineoplastic agents or nucleic acids, this technique shows greater delivery efficiency and cellular uptake in *in vivo* models with HNSCC tumors.^{31,32} However, the use of microbubbles is limited to tumors that can be easily visualized using ultrasound imaging.

Significant advances in molecular engineering of viruses enable highly efficient transfer of transgenes into tumors. Adenoviruses and retroviruses are the two main classes of viral vectors tested in HNSCC. Adenoviruses are DNA viruses that infect both dividing and non-dividing cells. Exogenous DNA remains episomal without integrating into the genome. In contrast, retroviruses primarily infect actively dividing cells and stably integrate transgenes into the host genomic DNA with high efficiency. In addition to high efficiency of gene transfer, cell lysis during viral replication potentiates the therapeutic benefit of viruses. Wild-type adenoviruses have a low transfection efficiency;³³ however, modifications to the viral genome or surface structure have been employed to increase effectiveness.³⁴ In order to increase tumor specificity, oncolytic viruses are biochemically modified to target and replicate only within tumor cells or in cells expressing specific genes. In the following sections, we describe the use of gene delivery vectors in preclinical and clinical studies using nucleic acid-based therapy approaches in HNSCC.

DNA-BASED APPROACHES

There are over 1000 cancer clinical trials testing gene therapy approaches in various cancers (Figure 2). In the following sections,

Figure 2. Summary of the methods used for targeting gene expression in the treatment of HNSCC. Exogenous plasmid or viral DNA can stably integrate into the genomic DNA or exist as an episome within the nucleus. RNA polymerases (RNA pol) transcribe the foreign DNA into RNA fragments. Antisense RNA binds to target mRNA in the cytoplasm inhibiting transcription. RNAi-mediated translation inhibition is achieved after processing of double-stranded shRNA, siRNA, or miRNA precursor molecules by molecular machinery. Precursor miRNA are processed in the nucleus and subsequently exported into the cytoplasm. Dicer cleaves shRNA, siRNA, and miRNA precursors into 21–25 base oligomers that are loaded onto the RNA-induced silencing complex loading complex, which consist of Dicer, argonaute (Ago), and a dsRNA-binding protein. The guide strand (blue) binds complementary mRNA while the passenger strand is discarded. Perfect homology with the guide strand triggers target mRNA cleavage. Mismatch in a few bases results in translational repression through inhibition of ribosomal function. Abbreviations: RNAi, RNA interference; miRNA, microRNA.

we describe preclinical and clinical studies utilizing gene therapy for treatment of HNSCC.

Plasmid DNA

Preclinical studies. Plasmid expression vectors that knockdown oncogenes or restore tumor suppressor genes demonstrate antitumor efficacy in HNSCC preclinical models. Liposome-encapsulated antisense plasmid DNA reduced EGFR mRNA levels and inhibited HNSCC tumor growth³⁵ and angiogenesis when used in combination with antiangiogenic agent, endostatin.³⁶ The formulation was safe in animal models despite long-term persistence of exogenous plasmid DNA in plasma and organs distant from the injection site.^{37,38} Plasmid expression vector-mediated restoration of tumor suppressor p53 levels in HNSCC cell lines activated proapoptotic proteins, promoting tumor death and increased radiosensitivity.^{20,21} Immune-mediated antitumor effects are observed when human interleukin (IL-2)- and IL-27-encoding plasmid were transfected into HNSCC tumors.^{39,40} In another approach, toxin-conjugated IL-13 demonstrated specific tumor cell kill in cells transfected with exogenous interleukin-13 receptor $\alpha 2$.⁴¹ Although, plasmid expression vectors demonstrate feasibility, systemic delivery continues to be a major challenge in the widespread use of this approach.

Human trials. The majority of HNSCC clinical trials report use of plasmid expression vectors injected intratumorally using cationic liposomes (Table 1). A phase I clinical trial assessing the intratumoral injection of naked plasmid DNA with an antisense sequence targeting EGFR demonstrated a clinical response in 29% of patients, well above the response rate seen with other EGFR-inhibiting agents.⁴² The highest dose administered (1920 μ g DNA)

was well tolerated with no dose-limiting toxicities. Similar efficacy and safety profiles have been demonstrated with intratumoral injection of naked heat-shock protein 65 (Hsp65) DNA.⁴³ Hsp65 DNA produced a partial response in 4 of 14 HNSCC patients with minimal adverse effects. Although there is evidence of efficacy in both these clinical trials, tumors inaccessible for direct injection continued to grow.⁴² In order to protect DNA from serum nucleases and improve cellular uptake, several trials have tested cationic liposomes as carriers for gene delivery.

Intratumoral injection of cationic liposomes carrying plasmid DNA encoding *E1A*, an adenoviral gene with antineoplastic activity, in HNSCC tumors was well tolerated, demonstrated transgene expression and moderate tumor response with one of 24 patients experiencing complete remission.⁴⁴ Allovectin-7, a DNA–lipid complex containing human leukocyte antigen B7-encoding plasmids, has shown modest clinical response and minimal toxicity with intratumoral injection.^{45,46} Human leukocyte antigen B7 is a major histocompatibility complex class I antigen that elicits a broad antitumor immune response. Although results from phase II trials were encouraging, failure to meet key end points in a subsequent trial resulted in the discontinuation of Allovectin-7. Thus, although the proof-of-concept demonstrating antitumor efficacy by plasmid DNA expression vectors is established, the major limitation of susceptibility to serum nucleases remains.

Oligonucleotides

Oligonucleotides are short, single-stranded, 15–20-base sequences that bind complementary mRNA inhibiting translation primarily through RNase H-mediated degradation. Ideally, the complementary sequence needs to be unique to facilitate specific target

modulation. Frequently oncogenic mutations differ from wild-type sequences by a few bases. Development of oligonucleotide chemistries such that mismatch of even a single base pair would abrogate binding of antisense oligonucleotide to its target would be ideal for cancer therapeutics and personalized medicine.

Preclinical studies. Antisense DNA oligonucleotides (ASO) targeting EGFR demonstrate antitumor efficacy in HNSCC on intratumoral administration.⁴⁷ Further, the observed effects were sequence specific with lack of antitumor effects in control (sense) oligonucleotide-treated tumors. Substitution of nuclease-resistant phosphorothioate groups to alternate bases on the DNA backbone increases the serum half-life from approximately 5 min to up to 60 min.⁴⁸ Phosphorothioate-modified EGFR ASO potentiated the effects of docetaxel in HNSCC xenografts on systemic administration.^{49,50} However, the antitumor effects were modest given the relatively short serum half-life.

In order to increase the serum stability of ASOs, peptide nucleic acids (PNAs) were developed.⁵¹ These are nucleotide analogs containing *N*-(2-aminoethyl) glycine units in place of the sugar phosphodiester backbone. Despite longer serum half-lives, there is low cellular uptake due to the neutral charge of the backbone. In order to improve intracellular uptake, positively charged guanidinium residues were covalently linked to the peptide nucleic acid backbone (Figure 3). Uptake of guanidinium peptide nucleic acid oligomers was detected in tumors up to 4 h after systemic administration.⁴⁹ Guanidinium peptide nucleic acid designed to target EGFR has demonstrated sequence-specific target reduction and cytotoxicity in HNSCC cells. Further, the antitumor effects

observed are comparable to that of clinically relevant EGFR inhibitors. Changes in the position of the guanidinium group on the peptide nucleic acid backbone facilitated the development of guanidinium peptide nucleic acid that is conformationally preorganized into right-handed helices with higher target binding affinity and sequence specificity than earlier versions.⁵² This increased oligonucleotide serum stability makes systemic delivery of specific nucleic acid sequences for cancer therapy a possibility.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is upregulated in HNSCC and facilitates tumor progression by binding to specific promoter sequences of several tumorigenic genes. Leong and colleagues developed a double-stranded DNA oligonucleotide based on the STAT3-high serum inducible element promoter sequence.⁵³ The STAT3 decoy sequestered activated STAT3, preventing binding to the promoter of target genes.⁵³ When tested as monotherapy or in combination with erlotinib, STAT3 decoy has antiangiogenic and antitumor effects in xenograft tumors.^{54,55} In another study, guanine-rich oligonucleotides with the high serum inducible element formed G-quartet DNA structures with potent affinity for activated STAT3.⁵⁶ Further, intraperitoneal injections of polyethylenimine copolymers complexed to G-quartet oligomers induced antitumor effects in HNSCC xenografts. More recently, a third-generation ASO targeting Hsp27 was designed using locked nucleic acid technology modified with a ribose oxymethylene bridge to prevent serum degradation.⁵⁷ Intraperitoneal injection of Hsp27 locked nucleic acid led to increased radiosensitivity in HNSCC xenografts through dysregulation of DNA double-stranded break repair mechanisms.

Table 1. Published clinical data on nucleic acid targeting in the treatment of HNSCC

Phase	Technique	Target gene	Action	Vector	Biological response	Clinical response	Reference
0	Oligonucleotide	STAT3	Inhibition	—	Reduced levels of STAT3 target genes	—	60
I	Plasmid	Hsp65	Expression	—	Increased monocyte proliferation	PR in 4 of 14; SD in 1 of 14	43
I	Plasmid	HLA-B7	Expression	Liposome	Expression of HLA-B7 with increased apoptosis	PR in 4 of 9	45
I	Plasmid	EGFR	Inhibition	Plasmid	Decreased EGFR and STAT3 <i>in vitro</i>	29% with 2 CR, 3 PR, and 2 SD	42
I	ASO	eIF-4E	Inhibition	—	Reduction in cytoplasmic eIF-4E in 9 of 12 and 3 of 6 had reduced nuclear eIF-4E protein	22 patients assessed; 15 had PD, 7 had SD	59
II	Plasmid	E1A	Expression	Liposome	E1A expression in 14 of 15 specimens via RT-PCR	CR in 4.2%, PR in 8.3%, SD in 29.2%	44
II	Plasmid	HLA-B7	Expression	Liposome	—	SD or PR in 33%	46

Abbreviations: ASO, antisense oligonucleotide; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Figure 3. Chemical modifications of oligonucleotide backbones. Phosphorothioate-modified oligonucleotides (PTO) are formed by creating phosphorothioate bonds through the substitution of a sulfur atom for a non-bridging oxygen in the phosphate backbone. Peptide nucleic acids (PNAs) are DNA analogs with backbones consisting of *N*-(2-aminoethyl)-glycine units linked by peptide bonds. The addition of a positively charged guanidinium group to the PNA backbone (GPNA) facilitates oligomer uptake across cell membranes. Abbreviation: GPNA, guanidinium peptide nucleic acid.

Human trials. Clinical trials testing oligonucleotides in HNSCC treatment are in their infancy. The eukaryotic translation initiation factor 4e (eIF-4E) binds the 5' cap structure of cellular mRNA and is associated with tumor progression.⁵⁸ Intravenous administration of phosphorothioate ASOs with flanking methoxyethyl groups targeting eIF-4E- (LY2275796) was tested for toxicity in a phase I trial for multiple cancers including HNSCC.⁵⁹ One patient experienced grade 2 fatigue at the maximum dose tier of 1200 mg. The study defined the maximum tolerated dose as 1000 mg administered intravenously as a loading dose over 3 days followed by a weekly maintenance dose. Although a lower level of eIF-4E in the tumor indicated a biological response, there were no clinical responses achieved. Pharmacokinetic evaluation revealed the majority of ASOs distributed in tissues within 24 h of administration. The modifications to the nucleic acid backbone increased the terminal elimination phase half-life to 15 days (4% overall plasma exposure). To improve clinical responses, selection of patients with high tumor levels of eIF-4E or combined treatment with chemotherapy may be necessary.

The biological response of STAT3 decoy was tested in a phase 0, intraoperative trial.⁶⁰ Pharmacodynamic studies on intratumoral injection of STAT3 decoy oligomers at three doses revealed modulation of STAT3 target genes within 4 h of administration. There were no adverse effects reported. Although the results in human studies are limited, these early trials support proof-of-concept that oligonucleotides can be safe and are effective in modulating target gene levels in HNSCC.

RNA-BASED APPROACHES

The regulation of translation by small RNA is an evolutionary conserved process. Molecular machinery process double-stranded non-coding RNA into single-stranded fragments that bind to specific target sequences inhibiting mRNA translation. Initial studies primarily focused on use of RNA interference for knock-down of gene expression in order to determine the role of specific genes or pathways in tumorigenesis. However, the specificity and efficiency of target inhibition has made this an attractive therapeutic modality for cancer.

The most widely used classes of RNA interference for translational repression of single genes in HNSCC are small interfering RNA (siRNA) and short hairpin RNA (shRNA). The main difference between siRNA and shRNA is that siRNA regulate gene expression transiently (knockdown duration less than 2 weeks) while shRNA vectors are expressed after genomic integration.⁶¹ Within the cell,

molecular machinery process shRNA and siRNA to generate single-stranded RNA fragments that bind complementary sequences on mRNA, leading to their degradation (Figure 2). Multiple cancer-associated genes can be simultaneously downregulated by microRNA. The extent of homology of microRNA to its target dictates the mechanism of translation repression with highly homologous binding triggering target mRNA cleavage. Partial complementarity would inhibit translation of non-target mRNA through several mechanisms including inhibition of translation initiation (interfering with ribosome recruitment) and deacetylation of mRNA.⁶² Similar to microRNA, several reports demonstrate non-specific modulation of cellular mRNA by siRNA with partial sequence homology to the target indicating that siRNA may have off-target effects.^{63–65} Nevertheless, a deeper understanding of the mechanisms of translation inhibition could result in improved specificity of RNA interference approaches. Here we discuss use of these approaches in HNSCC preclinical models.

Small interfering RNA

Several reports describe siRNA modulation of target genes reduced HNSCC invasion, proliferation, metastatic dissemination, and enhanced response to therapy (Table 2). Invasion and motility of HNSCC cell lines was significantly decreased using protein kinase C ϵ siRNA.⁶⁶ Further, the use of hypoxia inducible factor-1 α and aurora A kinase siRNA induced apoptosis and reduced cell proliferation.^{67,68} Other factors have also been the target of treatment, notably the nuclear factor- κ B family of proteins, which control gene expression in response to several stimuli including cytokines, growth factors, and infection. Nuclear factor- κ B is an important target in HNSCC due to its implication in tumor growth and survival.⁶⁹ It is constitutively expressed in HNSCC and contributes to cisplatin resistance by preventing chromatin condensation through histone acetylation.⁷⁰ When used in combination with histone deacetylase inhibitors, systemic administration of siRNA targeting nuclear factor- κ B p65 (RelA), increased antitumor efficacy of cisplatin.⁷¹ Further, in combination with the proteasome inhibitor bortezomib, nuclear factor- κ B siRNA reduced tumor growth.⁷²

EGFR-targeted siRNA showed a 90% reduction in EGFR expression *in vitro* and enhanced sensitivity to cisplatin, 5-fluorouracil, and docetaxel.⁷³ To facilitate systemic administration, siRNAs conjugated to nanoparticles were tested in preclinical models of metastatic HNSCC. Ribonucleotide reductase subunit M2 (RRM2)-targeted siRNA conjugated to nanoparticles produced

Table 2. Putative RNAi targets associated with sequence-specific nucleic acid targeting in HNSCC

Putative target	Effects in HNSCC	Agent	Reference
AURKA	Increased apoptosis, paclitaxel sensitivity	siRNA	68
CD74	Decreased VEGF and MMP-9 expression, decreased cell proliferation	shRNA	78
Cyclin-D1	Decreased cell cycle progression, increased cisplatin sensitivity	shRNA	85
EGFR	Decreased cell proliferation, increased 5-FU and cisplatin sensitivity	siRNA	73
GLUT1	Decreased cell proliferation, increased apoptosis, increased cisplatin sensitivity	shRNA	84
HIF1 α	Increased apoptosis, decreased cell proliferation	siRNA	67
NF- κ B	Decreased cell proliferation	siRNA	69,72
NTSR1	Decreased invasion and migration	siRNA	117
PKC ϵ	Decreased invasion and motility	siRNA	66
RelA	Cell cycle arrest, increased sensitivity to HDI	siRNA	71
RRM2	Decreased tumor proliferation, increased apoptosis	siRNA	74
SET	Decreased E-cadherin and pan-cytokeratin, increased MMP-9 and -2 expression, decreased proliferation and increased cisplatin sensitivity	shRNA	79
SPHK1	Increased doxorubicin sensitivity, decreased AKT activation	shRNA	83
TERT/VEGF/Bcl-X _L	Increased apoptosis	shRNA	80,82,118

Abbreviations: AURKA, aurora A kinase; HIF1 α , hypoxia inducible factor-1 α ; PKC ϵ , protein kinase C ϵ ; TERT, telomerase reverse transcriptase; VEGF, vascular endothelial growth factor.

apoptosis in metastatic models on systemic delivery.⁷⁴ In a recent report, calcium phosphate-based nanoparticles with a hollow lipid-coated core and outer shell conjugated to anisamide ligands successfully delivered hypoxia inducible factor-1 α siRNA to sigma receptor expressing HNSCC cells on intravenous administration.⁷⁵ In combination with a photosensitizer photosan, hypoxia inducible factor-1 α siRNA potentiated the effects of photodynamic therapy in xenograft tumors. This study represents a significant advance in siRNA-mediated gene regulation as a therapeutic approach. However, addressing challenges associated with siRNA therapy including off-target effects, inflammation, and non-specific cytotoxicity would make this approach more feasible for therapeutic use.⁷⁶ While siRNA has not been used in human trials, repression of target protein expression using siRNA has been achieved *in vitro*, and *in vivo* studies have demonstrated biologic activity. These results suggest feasibility for their use with reasonable transfection efficiency and significant biological response.

Short hairpin RNA

Transfection or viral transduction of shRNA expression vectors results in integration into genomic DNA and RNA polymerase III-mediated transcription.⁷⁷ The double-stranded RNA fragments are transcribed in the nucleus with a short hairpin sequence at one end and transported into the cytoplasm where they follow the same molecular processing as siRNA.⁶¹ Several reports on HNSCC cells expressing target-specific shRNA describe the molecular mechanisms regulating tumor progression and response to therapy.

Reports on shRNA-mediated inhibition of several proteins including CD74 and the SET (suppressor of variegation, enhancer of zeste, and trithorax) oncogene demonstrate anti-proliferative effects in HNSCC.^{78–80} Further, local invasion can be controlled by targeting extracellular matrix modulators including metalloproteinase-9 and -11.^{78,81} While monotherapy has been efficacious in preclinical models, combination therapy may have synergistic effects. The use of three shRNAs targeting telomerase reverse transcriptase, proangiogenic vascular endothelial growth factor, and antiapoptotic Bcl-X_L genes decreased HNSCC xenograft growth, demonstrating the feasibility of a multitarget-approach.⁸²

Several reports demonstrate the role of oncogenes and oncogenic mutations in resistance to therapy. Cisplatin therapy is a vital component of current chemotherapy for HNSCC. However, patients frequently develop resistance to cisplatin. Recent studies have shown that shRNA-mediated downregulation of glucose transporter GLUT1 and cell cycle regulator cyclin-D1 increase sensitivity to cisplatin.^{83–85} Additionally, targeting sphingosine kinase 1 (SPHK1) increased tumor sensitivity to doxorubicin with a significant reduction in SPHK1 expression after transfection.⁸³ Similarly, the use of shRNA targeting mutant-tumor suppressor p53 conferred sensitivity to radiation in radio-resistant HNSCC.⁵ Although these studies are in preclinical development, developments in vectors including viral vectors would facilitate translation to the clinic. Together these studies provide insight into future applications for therapeutic gene targeting in HNSCC.

VIRAL METHODS

Viruses are effective transgene delivery agents in treatment of many disease processes ranging from metabolic disturbances to cancer. Of the several classes of viruses used to deliver transgenes, several reports use adenoviruses and herpesviruses in HNSCC preclinical models. While viruses are effective vectors for delivery of genetic material, their lytic life cycles confer the added advantage of tumor cell destruction. In this section, we discuss

the use of viruses for gene delivery in HNSCC and advances in oncolytic viruses for targeted destruction of cancer cells.

Viral vectors

Viruses are frequently used to transduce human cells due to their high efficiency of gene transfer. Depending on the type of virus, the exogenous nucleic acid either integrates into the host genome or remains episomal. Viruses used for gene transfer are generally replication-incompetent though there are exceptions.

Preclinical studies. Adenoviral vectors are the most widely used viral vectors for gene transfer in preclinical studies and clinical trials. Wild-type p53 delivered using recombinant adenoviruses lead to apoptosis in HNSCC tumor cells with up to 60% response in 48 h.^{86,87} This allows for reestablishment of the G1 block, increasing sensitivity to radiation and chemotherapy.^{88–90} Further, adenoviruses and adeno-associated viruses were used to deliver herpes thymidine kinase to tumor explants.^{91–93} Treatment with prodrug ganciclovir not only killed the transduced cells but also the adjacent bystander cells. In a recent study, recombinant vaccinia virus encoding the oncogene erbB2 was tested in a vaccination approach in a mouse model of salivary gland carcinoma.⁹⁴ Activation of antibody-dependent cellular cytotoxicity on intratumoral injection of the recombinant virus resulted in antitumor efficacy.

Human trials. Clinical trials have primarily focused on the use of adenoviruses for treatment of HNSCC (Table 3). Gene therapy using adenoviruses encoding wild-type p53 (Ad-p53) was tested in phase I human trials. In patients with incurable recurrent HNSCC, no dose-limiting toxicities or significant adverse events were observed using a single preoperative intratumoral injection of 10¹¹ plaque-forming units (pfu). Additionally there was a modest response in a number of patients with non-resectable tumors and one patient with resectable cancer showed complete response. Furthermore, there was an increase in survival rate by approximately 60% of individuals treated with combination Ad-p53 and chemotherapy.⁹⁰ A small-scale phase II trial assessing the efficacy of Ad-p53 in patients with resectable cancer demonstrated increased disease-free survival rates 1 year after resection.⁹⁵ The study enrolled 13 patients who received direct injection of 10¹² viral particles (vp) into the surgical margins intraoperatively and postoperatively via catheter instillation. There were minimal adverse events and 92% of patients were disease free after 1 year. Increased survival and improved response rates were reported in phase III clinical trials as well. The intra-arterial administration of combination Ad-p53 and chemotherapy improved survivability in patients with stage 3 HNSCC.⁹⁶ Response rates significantly increased in comparison to treatment with methotrexate for patients with either wild-type p53 or low-level expression of mutated p53.⁹⁷ Ad-p53 vectors are currently being tested in phase IV clinical trials.

Oncolytic viruses

Oncolytic viruses preferentially replicate in and lyse tumor cells that express or lack specific targetable genes. In theory, these agents lead to tumor destruction with minimal bystander damage. Newer generation oncolytic viruses carry exogenous genes that elicit antitumor effects or sensitize tumors to therapy.

Preclinical studies. Modified herpes simplex viral (HSV) and adenoviral vectors can carry up to 50 Kb of DNA transgene, and can preferentially replicate in and lyse tumor cells. Several HSV-based viral vectors were tested in preclinical models of HNSCC. HF10, HSV1716, and G207 vectors shows antitumor efficacy alone or with concomitant cisplatin in HNSCC preclinical models.^{98–100} Both *in vitro* and *in vivo* studies showed biologic activity and

Table 3. Published clinical data on viral vectors in the treatment of HNSCC

Phase	Technique	Target gene	Virus	Dose	Biological response	Clinical response	Reference
I	Adenovirus	p53	Ad-p53	1x10 ¹¹ pfu	P53 expression detected	PR in 2, SD in 6, PD in 9	119
II	Adenovirus+XRT	p53	Ad-p53	1x10 ¹² vp	—	Tumor reduction	120
II	Adenovirus+CRTX	p53	INGN 201 (Ad-p53)	1x10 ¹² vp	—	92% 1-year progression-free survival	95
III	Adenovirus+CTX vs CTX	p53	Ad-p53	1x10 ¹² vp	Increased p53, Bax and decreased Bcl-2	82% RR with 48% CR	96
I	OV	TNF-α	TNFERade Biologic	4x10 ¹¹ pfu	TNF-α expression in all biopsy specimens	83.3% RR: CR in 5, PR in 5, SD in 2, PD in 3	116
I	OV	—	ONYX-105	1x10 ¹¹ pfu	—	No objective clinical response	107
I	OV	—	HSV1716	1x10 ⁵ or 5x10 ⁵ pfu	HSV DNA in tissue biopsy of 2 patients	—	106
II	OV	—	ONYX-015	2x10 ¹¹ vp	PCR detected virus in 41% of patients 24 h after injection and 9% after 10 d in first cycle	Regimen 1—SD in 41%, PD in 45% PR/CR in 14% PR/CR Regimen 2—CR in 10%, SD in 62%, PD in 29%	108
II	OV+CTX	—	ONYX-015	1x10 ¹⁰ pfu	—	CR in 33%, PR in 33%, mR in 11%, SD in 22%	109
I	OV	GM-CSF	OncoVEX ^{GM-CSF}	1x10 ⁶ pfu	GM-CSF detected 48 h after injection;	SD in 3 of 17	114
II	OV	GM-CSF	OncoVEX ^{GM-CSF}	1x10 ⁶ pfu/mL (up to 6 mL)	Seroconversion of all patients after injection	26% RR; CR in 8 of 50, PR in 5 of 50	112

Abbreviations: OV, oncolytic virus; XRT, radiotherapy; CTX, chemotherapy; CRTX, chemoradiotherapy; pfu, plaque-forming units; vp, viral particles; RR, response rate; CR, complete response; PR, partial response; mR, minor response; SD, stable disease; PD, progressive disease.

destruction of all tumor cell lines. Further, mice with HNSCC xenografts had a statistically significant increase in survivability at 100 days.⁹⁹ The ONYX class of viruses are E1B-gene-deleted adenoviruses that selectively replicate within cells lacking wild-type tumor suppressor p53 and have demonstrated antitumor efficacy *in vitro* and *in vivo*.¹⁰¹ Intravenous injection of ONYX-015 leads to tumor localization and produces antitumor effects in nude mice bearing xenograft tumors with a viral replication reported in 100% of tumors. Furthermore, there was a reduction in tumor growth up to 50% though no appreciable effect on the growth of distant established tumors was observed.¹⁰² The main obstacle to HSV and adenoviral vectors lie in the endemic nature of the viruses with more than 70% of the population having a pre-existing immune response that specifically inactivates viral particles and cells that express viral proteins.¹⁰³ Preclinical studies with other classes of oncolytic viruses are underway.

In order to facilitate antitumor immune-mediated tumor kill, a fusogenic vesicular stomatitis virus encoding murine IL-12 was developed, which lysed HNSCC more effectively than virus lacking the transgene leading to significantly increased survival of mice.¹⁰⁴ Another study with measles virus engineered to replace the viral attachment protein with a single-chain antibody targeting EGFR demonstrated increased tumor specificity.¹⁰⁵ In addition, the virus carried the cytosine deaminase/uracil phosphoribosyltransferase (CD/UPRT) gene. CD/UPRT converts prodrug 5-fluorocytosine to 5-fluorouracil, which can diffuse from dying cells, significantly increasing antitumor effects in non-infected bystander replicating tumor cells. HNSCC xenograft tumors responded well to intratumoral injections of the recombinant viral particles followed by prodrug treatment. These studies demonstrate efficacy of transgene expression by oncolytic viruses for added therapeutic benefit.

Human trials. Multiple human trials using oncolytic viruses have shown biologic or clinical response (Table 3). Intratumoral injection into subcutaneous nodules of a conditionally replication-competent HSV vector called HF10 in two patients with metastatic HNSCC demonstrated antitumor response with

minimal adverse effects.¹⁰⁰ Although preoperative intratumoral injection of 10⁵ pfu of another HSV vector, HSV1761, was well tolerated with no serious adverse effects, there was no biological effect of therapy.¹⁰⁶ ONYX-015 has demonstrated safety with injection of 10¹¹ pfu.¹⁰⁷ Stable disease and modest antitumor activity was achieved with intratumoral administration of ONYX-015. In a small-scale trial of nine patients, 10¹⁰ pfu of ONYX-015 was administered intratumorally with systemic cisplatin and 5-FU leading to complete or partial regression in all subjects.¹⁰⁸ The study was expanded to a larger phase II trial that reported 63% of individuals with regression.¹⁰⁹ The commercial version of ONYX-015 (ONYX-H101) has been tested in humans and is approved for human use in China.¹¹⁰

Significant advances in the development of oncolytic viruses have enabled the expression of transgenes that trigger antitumor responses in addition to cell lysis. The granulocyte macrophage colony-stimulating factor (GM-CSF) recruits and activates leukocytes including neutrophils and monocytes to elicit potent antitumor effects. GM-CSF was the first cytokine incorporated into an oncolytic virus in order to harness the both immune and virus-mediated tumor cell kill. OncoVEX^{GM-CSF} is a modified HSV-1 encoding GM-CSF tested in several clinical trials.^{111–113} In a phase I/II trial, intratumoral injections of OncoVEX^{GM-CSF} in combination with chemoradiotherapy demonstrated a pathologic complete response rate of 93% (*n* = 17) in late stage HNSCC.¹¹⁴ Pharmacodynamic analyses demonstrated virus replication in injected and uninjected tumors. This study is the first to demonstrate the efficacy of using transgene-expressing oncolytic viruses in combination and standard curative therapy.

In another study, tumor necrosis factor alpha (TNF-α) an immune system stimulator, chemotherapy and radiation sensitizer expressed by an oncolytic virus was tested for antitumor effects in recurrent previously treated HNSCC patients. AdGV.EGR.TNF.11D (TNFERade Biologic) is a replication-deficient adenovirus encoding TNF-α under the control of radiation-inducible Egr-1 promoter.¹¹⁵ Dose-limiting toxicity in 3 of 14 patients experiencing thrombotic events on intratumoral injections of TNFERade Biologic at a maximum dose of 4x10¹¹ pfu combined with chemoradiotherapy

helped establish the maximum tolerated dose.¹¹⁶ Although phase I trials are not designed to assess efficacy, the response rate of 83%, with one patient surviving for 3 years underscores the therapeutic potential of this approach. Further developments facilitating systemic administration and tumor-specific targeting would facilitate the use of oncolytic viruses in the treatment of inaccessible metastatic disease.

CONCLUSIONS

HNSCC remains a major cause of mortality despite advances in treatment. As more therapeutic targets are identified through cancer genome sequencing studies, nucleic acid targeted therapy has the potential to decrease mortality, increase survival rates, and improve quality of life in patients with HNSCC. The field of nucleic acid targeting is rapidly advancing with new avenues of treatment unfolding. A variety of delivery techniques have been employed for multiple therapy modalities with positive results in both preclinical studies and human trials. Most methods of nucleic acid delivery have been safe in human trials with reduction in target gene expression and evidence of antitumor efficacy.

While there have been clinical trials assessing safety and efficacy of viral vectors, less clinical data are available for the use of antisense oligonucleotides and RNA interference methods. The development of oligonucleotides that target-specific gene mutations within a given tumor could provide a patient-centered approach to therapy. At the very least, nucleic acid targeting has a crucial role in adjunct treatment for HNSCC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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