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Gene Therapy for Oral Cancer

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Abstract – New treatment approaches are needed to improve the effectiveness of oral cancer treatment, since surgical resection of the tumor in oral region causes various oral dysfunctions. The molecular biology of oral cancer has been progressively delineated. Concurrently, gene therapy techniques have been developed that allow targeting or replacement of dysfunctional genes in cancer cells, offering the potential to treat a wide range of cancer. Oral carcinoma is attractive target for gene therapy because of its accessibility. In this article, we review the current status of gene therapy as applied to oral carcinoma.

Keywords □ cancer gene therapy, oral cancer, oral squamous cell carcinoma, gene transfer

INTRODUCTION

Carcinomas of the oral cavity are among the 10 most common cancers in the world (Parkin *et al*., 1993). The World Health Organization (WHO) predicts a continuing worldwide increase in the incidence of oral cancer, extending this trend into the next several decades. Most malignant tumors of the oral cavity are squamous cell carcinomas, followed by adenocarcinomas and rarely other types of malignant tumors.

The early stage oral cancers are highly curable by surgery or radiation therapy. Advanced cancers are generally treated by surgery followed by chemoradiation therapy. However, the results of standard therapy, even by employing increasingly radical surgery and various forms of radiotherapy and chemotherapy, have remained at a stable level (Bettendorf *et al*., 2004).

Since the primary modality for oral cancer therapy is surgery, wide surgical resection of the tumor region causes various oral dysfunctions. Therefore, new treatment strategies are urgently required. Gene therapy has proved promising in many cancers. Moreover, tumor in oral cavity is attractive target for gene therapy because of its accessibility. Gene therapy offers the potential to treat a wide range of inherited and acquired human diseases. In general, this approach involves the treatment of disease by introducing new genetic instructions to compensate for abnormal genes or to convey a new function. The number of clinical gene therapy trials continues to grow, with almost 650 worldwide (Emery, 2004). These trials are predominantly focused on the treatment of cancer. In this article, we review the current status of gene therapy as applied to oral carcinoma.

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DELIVERY OF THERAPEUTIC GENES

Table I lists both viral and nonviral vectors commonly used in gene transfer studies for the treatment of oral cancer.

Adenoviral vector

Recombinant adenoviral vectors (AV)[1] are the most extensively tested vectors for the oral cancer gene therapy. Adenovirus serotype 5 is the most commonly used vector. The virus enters into the cells primarily by binding to the cell surface coxsackie-adenovirus receptor (CAR) through a knob domain present on its fiber proteins. Following CAR binding, an additional interaction between the penton base of adenovirus capsid and áíâ3 integrins of cell is required for internalization of virus. This results in receptor-mediated uptake of the virus into endosomes from which it escapes into the cytoplasm. Although AV does not integrate into the host genome, the viral episome is stable in cells and will last for essentially the life of the cell. Initial AVs were engineered for gene therapy by deleting the E1A region and rendering them replication incompetent. The desired gene of interest can then be fitted in its place, allowing for about 7.5 kb of therapeutic gene to be inserted (Majhen and

Table I. Representative Delivery Systems Used for Therapeutic Gene Transfer of Oral Cancer

Source	Advantages	Disadvantages
Adenovirus (14 cases)	wide host range highly efficient gene transfer capsid stable high titre cell cycle independent	immunogenicity hard to avoid the formation of replication competent virus in case of incompetent viral vector
Liposome (6 cases)	few limits on transgene size potential for targeting safe	low gene transfer transient expression
Plasmid DNA (2 cases)	safe	low gene transfer transient expression
AAV (2 cases)	potential genome integration capsid stable wide host range safe cell cycle independent	inefficient production
Lentivirus (2 cases)	genome integration cell cycle independent	no immune response safety concerns inefficient production
Vacccinia virus (1 case)	wide host range big insert size highly efficient gene transfer	immunogenicity

Ambrioviæ-Ristov 2006). There are several advantages of recombinant AVs. AV can infect dividing and nondividing cells in wide range of tissues with high transduction efficiency. They can consistently be propagated in a stable fashion to high titers that permit its use *in vivo*. However, one disadvantage is to elicit a dose-dependent host immune response that prevents a second dosing (Chung, 2003).

Cationic liposomes

As a nonviral vector, liposomes are comprised of synthetic phospholipid bilayers containing an aqueous solution in the middle that contains the DNA. Positively charged cationic lipid binds to the negatively charged DNA and forms a lipid-DNA complex. This allows for fusion of the liposome with the target cell membrane, endocytosis, and delivery of the DNA into the cytoplasm. This method has several advantages which include transferring very large DNA and targeting the specific tissues by modifying the liposome moieties. Liposomes are generally safe and nonimmunogenic, with a very low incidence of chromosomal integration. However, the use of this system is limited by a very low level of gene transfer and the transient therapeutic gene expression in highly dividing cells (Nishikawa and Hashida, 2002).

Plasmid DNA (Naked DNA)

The therapeutic gene is inserted in an expression plasmid, prepared in DNA form and administered to patients by subcutaneous or intramuscular infection. The advantages of naked DNA include the ease of large-scale production and lack of specific immune response to the vector itself. However, this method is plagued by very low transduction efficiency (Cheng *et al*., 1993).

Adeno-Associated viral vector

Adeno-Associated viral vector (AAV) are encapsidated parvoviruses with small (5 kb) linear single stranded DNA genomes that encode only two gene products, *i e.*, *cap* and *rep* genes. As the name implies, AAV is dependent on the presence of adenovirus for replication. Since the space is limited in the AAV vector, only about 4.5 kb of therapeutic gene may be inserted. When producing the vector, it is propagated along with a helper virus and a packaging cell line capable of supplying the cap and rep proteins. Of note, high concentrations of rep proteins are toxic to both helper viruses and the packaging cell line, which makes it difficult to achieve high titers. Despite its limitations, AAVs possess several properties that make it a popular vector for gene therapy. It is non-pathogenic to humans, therefore, extremely safe for clinical use. It is capable of infecting both dividing and non-dividing cells. They have a reasonable range of tropism for various cell types and are able to target various different kinds of tissues. As similar to retroviral vectors, they can lead to stable expression because of its poten-

tial to integrate into the target cell genome (Monahan and Samulski, 2000).

Lentiviral vector

Lentiviruses are enveloped RNA viruses and are members of the retrovirus family. Most lentivirus vectors that have been tested for gene therapy were developed from human immunodeficiency virus (HIV). Lentiviral vectors have several advantages that make them attractive candidates for gene therapy. They are capable of supporting long-term expression due to the chromosomal integration and infecting non-dividing cells (Naldini *et al*., 1996).

Vaccinia viral vector

Once used as a vaccine against smallpox, vaccinia virus is currently used in gene therapy as a vector. One advantage of vaccinia virus is its large capacity which can carry at least 25 kb of heterologous DNA, allowing the simultaneous expression of different foreign genes. High transduction efficiency at low multiplicity of infection (MOIs) is characteristic of vaccinia viral vector. The large genome size allows it to encode many vector gene proteins that may interact with the host protein and elicit an immune response (Carroll *et al*., 1997).

ANTICANCER GENE THERAPY STRATEGIES

Strategies for cancer gene therapy include prodrug activation by suicide genes; inhibition of activated oncogenes; inhibition of angiogenesis; transfer of tumor suppressor genes; oncolytic vector; transfer of cytokine gene transfer. Table II summarizes

Table II. Gene Therapy Protocols Developed for Oral Cancer

Reference	Therapeutic gene	Delivery	Research stage	Comments
Yamamoto et al., (2007)	EpCAM siRNA	cationic liposome	in vitro	
Drugs in R&D, (2007)	p53	AV	Phase II	
Neves *et al*., (2006)	HSV-tk	cationic liposome	in vitro in vivo	transferrin-associated lipoplexes
Jiang et al., (2006)	survivin siRNA	lentiviral vector	in vitro in vivo	
Young *et a*l., (2005)	HSV-tk	cationic liposome	in vitro	
Yanamoto *et al*., (2005)	p53R2 siRNA	cationic liposome	in vitro	5-FU sensitivity incresed
Xu *et al*., (2005)	MDR1 siRNA	scAAV	in vitro	
Kudo *et al*., (2005)	Skp2 siRNA	plasmid vector	in vitro	
Harada *et al*., (2005)	mutant $p27^{Kip}$	plasmid vector	in vivo	
Konopka *et a*l., (2005)	HSV-TK	cationic liposome	in vitro	
Morley *et al*., (2004)	ONYX-015	AV	phase II	oncolytic vector
Darby Weydert *et al*., (2003)	MnSOD	AV	in vitro in vivo	+ BCNU
Dehari *et a*l., (2003)	IL-2,	AV	in vitro in vivo	integrin binding motif (RGD)
Dasgupta *et al*., (2003)	IL-2	vaccinia virus	in vivo	replication competent
Ding *et al*., (2003)	endostatin	AV/	in vitro in vivo	
Matsumoto *et al*., (2002)	angiostatin	cationic liposome	in vivo	
Chen *et al*., (2002)	NF-kappaB inhibitor	AV	In vitro	
Pang *et al*., (2001)	HIV-1 Vpr	Lentiviral vector	in vivo	
Fukui *et al*., (2001)	HSV-TK	AAV	in vitro	
Gibson *et al.*, (2000)	anti-bcl-2 ribozyme	AV	in vitro	
Shillitoe *et al*., (2000)	HSV-TK	AV	in vitro	HPV-virus promoter
Ganly *et al*., (2000)	ONYX-015	AV	Phase I	oncolytic vector
Khuri *et al*., (2000)	ONYX-015	AV	Phase II	-oncolytic vector -combination with cisplastin and 5-FU
O'Malley *et al.*, (1999)	IL-2	AV	in vivo	
Sewell *et a*l., (1997)	HSV-TK	AV	in vivo	
O'Malley *et al.*, (1996)	HSV-TK, IL-2	AV	in vivo	
Clayman *et al.*, (1995)	LacZ	AV	in vivo	transduction test

gene therapy protocols that have been conducted for the treatment of oral cancer during last decade.

Suicide gene therapy

Suicide gene therapy or gene-directed enzyme prodrug therapy (GDEPT) attempts to transduce tumor cells with a gene coding for an enzyme capable of activating an inactive prodrug into an active cytotoxic drug. The herpes simplex virus thymidine kinase (HSV-tk) gene has been the most commonly studied suicide gene. This enzyme can specifically bind and phosphorylate nucleoside analogues such as acyclovir and ganciclovir (GCV), whereas the endogenous mammalian thymidine kinase cannot. Cells, which express HSV-tk and subsequently try to replicate, incorporate phosphorylated GCV, which blocks DNA synthesis and causes cell death. Since cancer cells divide more rapidly than normal cells, this treatment preferentially kills tumor cells transduced with the gene rather than normal cells. The antitumor effect is dependent on a biochemical bystander effect, which is mediated by gap junctions between cells. The toxic metabolite is able to pass from a tranduced cell into a non-transduced cell *via* gap junctions. Therefore, only 10-20% of tumor cell transduction is required for efficacy (Portsmouth *et al*., 2007).

Numerous studies have been reported regarding the antitumor effect of the HSV-tk/GCV approach on human oral carcinoma. In these studies, researchers utilized various delivery methods including cationic liposome (Neves *et al*., 2006; Young *et al*., 2005; Konoptka *et al*., 2005), AAV (Fukui *et al*., 2001) and AV (Yanamoto *et al.,* 2005; Shillitoe *et al*., 2000; Sewell *et al*., 1997; O' Malley *et al*., 1999). Recent *in vivo* study of a animal model for oral squamous cell carcinoma have shown a significant inhibition of tumor growth upon injection of cationic liposome, *i. e*., transferrin-associated lipoplexes, carrying HSV-tk followed by intraperitonal injection of GCV, as compared to controls (Neves *et al*., 2006).

Immunotherapy-cytokine genes

Many tumors escape from the immune system by being not immunogenic or actively suppressing the immune response against themselves (Pettit *et al*., 2000). Cancer immune gene therapy attempts to overcome the repressed immunity by *in vivo* transfection of tumor cells with immune response stimulating molecules, transfecting of effector cells or application of *ex vivo* transfected tumor cells for vaccination. Utilized genes for transfection are usually potent immune initiating or supporting factors, like IL-2 that is strongly involved in the induction phase of the immune response.

While O'Malley *et al*., (1999) described on limitations of adenoviral interleukin-2 gene therapy, same group considered it as a potential component of combination therapy for oral cancer. Dehari *et al.*, (2003) observed the enhanced antitumor effect of RGD fiber-modified AV containing IL-2 gene in an *in vivo* xenograft model of oral squamous cell carcinoma in nude mice, compared to those of unmodified AV with IL-2 gene. According to the report of Dasgupta *et al*., (2003), a recombinant vaccinia virus expressing IL-2 generated effective antitumor responses in an orthotopic murine model. Lymphocytes as well as macrophages appeared to play an important role in this antitumor immunity.

Oncogene inhibition therapy

Targeting the mRNA displays greater possibility. Approaches like hammerhead ribozymes and small interfering RNA (siRNA) or exon skipping raise hope to selectively silence or correct the mutant allele. Gibson *et al.,* (2000) tested a hammer-head ribozyme which has been designed to cleave the bcl-2 transcript. A gene encoding the ribozyme was cloned into an AV and transferred to the human oral cancer cell lines. The growth of cancer cell line was reduced.

RNA interference (RNAi) has emerged as one of the most important discoveries of the last years in the field of molecular biology. RNAi has largely been exploited as a powerful tool to uncover the function of specific genes and to understand the effects of selective gene silencing in mammalian cells both *in vitro* and *in vivo* (Novina and Sharp, 2004). RNAi is a strategy in which double strand (dsRNA) molecules are transferred into a cell. The antisense and sense RNA strands as well as the dsRNA molecules themselves have inhibitory effects on the exon sequences in mature target homologous mRNA. The dsRNA is cleaved into 21- to 25-bp siRNAs by dicer, a RNA nuclease with a helicase domain and dual RNAse III motifs that specifically cleave dsRNAs, which results in production of multiple effector molecules from a single dsRNA molecule. The siRNA-dicer complex can further recruit additional components to form an RNA-induced silencing complex in which the unwound siRNA base pairs with complementary mRNA and guides the RNAi machinery to target mRNA, resulting in the effective cleavage of the target mRNA, at sites 21 to 25 nucleotides apart only within the region of identity with the dsRNA, and subsequent catalytic degradation of potentially multiple target mRNAs (Masiero *et al*., 2007). RNAi can be induced by direct introduction of chemically synthesized

siRNA into the cell or by the use of plasmid and viral vectors encoding for siRNA allowing a more stable RNA knockdown.

The p53R2 gene product causes an increase in the deoxynucleotide triphosphate (dNTP) pool in the nucleus, which facilitates DNA repair and synthesis. RNAi-mediated p53R2 reduction selectivity inhibited growth and enhanced chemosensitivity in oral cancer cell lines, suggesting that targeting of p53R2 could be useful for oral cancer gene therapy (Yanamoto *et al.,* 2005). Epithelial adhesion molecule (EpCAM) is a transmembrane glycoprotein involved in intercellular adhesion. EpCAM is found to be overexpressed in the majority of human epithelial carcinoma including tongue cancer. RNAi-mediated EpCAM reduction decreased the invasion potential and proliferation activity (Yanamoto *et al*., 2007). Xu *et al*., (2005) demonstrate that a self-complementary recombinant AAV vector efficiently delivers siRNA into multidrug-resistant (MDR1) oral cancer cells and suppresses MDR1 gene expression. This results in rapid, profound, and durable reduction in the expression of the P-glycoprotein multidrug transporter and a substantial reversion of the drug-resistant phenotype. Kudo *et al.*, (2005) showed that Skp2 siRNA transfection decreased Skp2 protein and induced the accumulation of p27 protein in oral squamous cell carcinoma cells. Moreover, p27 protein in Skp2 siRNA-transfected cells is more stabilized than that in control siRNA-transfected cells. Interestingly, Skp2 siRNA inhibited the cell proliferation of oral squamous cell carcinoma cells both *in vitro* and *in vivo*. Jiang *et al*., (2006) showed that survivin was persistently and markedly reduced by lentivirus-mediated RNAi. The growth of KB cells was decreased and the apoptosis rate induced by vincristine was increased. The survivin-knockout KB cells were also more susceptible to adriamycin than the control. In the xenograft model, the development of tumors as well as the growth of established tumors was inhibited.

Anti-angiogenesis gene therapy

Angiogenesis is an essential process for development and growth of tumor. The possibility of inhibiting tumor growth by blocking the formation of new tumor vessels has been tested. A few studies with anti-angiogenesis strategy have been performed. Matsumoto *et al*, (2002) used liposomes complexed to angiostatin cDNA and targeted to human squamous cell carcinoma cell lines *in vivo*. Tumor cells expressing angiostatin after local gene transfer showed markedly reduced vascularity and contained many apoptotic tumor cells. Ding *et al*., (2003) showed the inhibition of tongue cancer development in nude mice transfected with adenovirus carrying human endostatin gene.

Oncolytic vector

Oncolytic vectors replicate in the tumor cells and subsequently cause their lyses. The defective p53 pathway has been used for the advantages of oncolytic adenoviruses. ONYX-015, is a well-characterized modified adenovirus which differs from the wild-type adenovirus in that its E1B-55K gene is deleted. In wild-type viruses, the product of this gene inactivates normal p53, a molecule which inhibits viral replication and tumor development. Consequently, the ONYX-015 can only replicate in cells in which normal p53 function is otherwise suppressed (Bischoff *et al*., 1996).

Morley *et al*., (2004) conducted a Phase II trial in which ONYX-015 was administered to 15 patients *via* a direct intratumoral injection before surgery for untreated oral squamous cell carcinoma. The virus replicates selectively in tumor as opposed to normal tissue after this direct injection. No adverse effects of viral injection were noted. The data support the concept that ONYX-015 is replication deficient in normal, compared with cancerous, tissues and has potential as a selective anticancer agent against tumor tissues. Two other clinical trials of ONYX-015 were conducted in patients with recurrent head and neck cancer including cancers in oral region as follows. Ganly *et al*., (2000) undertook a Phase I study and demonstrated that intratumoral administration of ONYX-015 is feasible, well tolerated, and associated with biological activity. Khuri *et al*., (2000) observed a better therapeutic benefit of ONYX-015 and chemotherapy (cisplatin and 5-FU) combination therapy in Phase II study.

Tumor suppressor gene

Tumor suppressor genes are cell division inhibiting parts of the cell cycle. The p53 tumor suppressor gene is deleted or mutated in many tumor cells and is one of the most frequently mutated genes in human tumors. The p53 protein is one of the most intricate elements in the apoptotic signaling cascade, and a mutation in the gene encoding it is believed to result in a decreased ability of cell apoptosis. Phase II and III clinical trials for various cancers have been conducted to replace this gene *via* AV p53 gene therapy, hoping to result in the increased apoptosis where INGN 201 (Ad5CMV-p53, AV containing wild type p53 under the control of CMV promoter) was administered. In April 2004, the Southwest Oncology Group initiated a phase II clinical trial using INGN 201 for the treatment of stage III or IV squamous cell carcinoma of the oral cavity that is able

to be removed surgically. The study assessed the feasibility, efficacy and safety of administering INGN 201 at the time of surgery for suppression of remaining tumor cells, followed by a combination of chemotherapy and radiation therapy. In November 2003, a 6-month phase I/II study with p53 gene therapy administered in the form of an oral rinse or mouthwash for patients with oral premalignancies has been initiated. It was the first trial to investigate the effect of this treatment on oral lesions that are at high risk for developing into full cancers. All phase I and II clinical studies for oral carcinoma have established the safety and clinical efficacy of gene therapy, *i.e*., prolong survival and delaying time to disease progression, in the treatment of oral carcinomas in humans, especially in combination with chemotherapy and radiotherapy (Adis International Limited, 2007).

Others

Harada *et al*., (2005) carried out an experiment of transferring mutant type $p27^{Kip1}$ gene. These results suggest that it is possible to transfer the plasmid DNA containing mutant-type $p27^{Kip1}$ into oral cancer xenografts using electroporation and to suppress the growth of tumors. Darby *et al*., (2003) demonstrated the inhibition of oral cancer cell growth by manganese superoxide dismutase (MnSO) delivered by AV plus 1,3-*bis*-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (BCNU) treatment. The stress-responsive transcription factor nuclear factor kappa B (NF-kappaB), which has been found to be associated with oral cancer development, plays an essential role in the suppression of tumor necrosis factor (TNF)-mediated apoptosis. Therefore, Chen *et al*., (2002) carried out an experiment to test the efficacy of a NF-Kappa B inhibitor. They reported that an adenovirus-mediated gene transfer of NF-kappaB inhibitor, super-repressor I kappa B alpha (Adv-SR-IkappaBalpha), blocked TNF-induced NF-kappaB activation and sensitized oral squamous cell carcinoma cells to TNF killing. A single intratumoral injection of the Vpr lentiviral vector significantly reduced the primary tumor volume as well as completely regressed tumors in more than 40% of animals (Pang *et al*., 2001)

CONCLUSIONS

Around 30 gene therapy protocols for oral cancer have been conducted, since the first transduction test study of AV containing reporter gene reported on 1995. AV has been most extensively evaluated in oral cancer gene transfer. This vector can transfer genes into target cells with very high efficiency. However, the fatal complication of AV in clinical gene therapy trial of ornithinetranscarbamylase deficiency in USA has necessitated additional safety requirements for human gene therapy trials using AV. Immunogenicity of AV has to be solved before it can be administered repeatedly. In addition, intensive researches on nonviral vectors that have higher transduction efficiency are encouraged as an alternative. More studies have utilized cationic liposome to deliver therapeutic genes to oral cancer cells as seen in Table II. In general, prompt research in gene delivery systems, particularly in the development of transduction-targeted, transcription-targeted and replication-competent viral vector for the treatment of oral carcinoma are highly encouraging.

HSV-tk/GCV remains the most widely used systems in oral cancer gene theapy. While the ideal suicide gene/prodrug combination also remains to be discovered, already developed suicide gene approaches, *e.g.,* cytosine deaminase/5-fluorouracil, need to be applied for oral cancer. Although the number of studies is limited, combinational studies indicated that oral cancer gene therapy may have useful synergy with chemotherapy as seen in other types of cancer. It seems that addition of gene therapy to conventional therapies would improve their effectiveness.

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