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REVIEW

Immunotherapy for head and neck cancer

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Abstract Head and neck cancer represents a challenging disease. Despite recent treatment advances, which have improved functional outcomes, the long-term survival of head and neck cancer patients has remained unchanged for the past 25 years. One of the goals of adjuvant cancer therapy is to eradicate local regional microscopic and micrometastatic disease with minimal toxicity to surrounding normal cells. In this respect, antigen-specific immunotherapy is an attractive therapeutic approach. With the advances in molecular genetics and fundamental immunology, antigen-specific immunotherapy is being actively explored using DNA, bacterial vector, viral vector, peptide, protein, dendritic cell, and tumor-cell based vaccines. Early phase clinical trials have demonstrated the safety and feasibility of these novel therapies and the emphasis is now shifting towards the development of strategies, which can increase the potency of these vaccines. As the field of immunotherapy matures and as our understanding of the complex interaction between tumor and host develops, we get closer to realizing the potential of immunotherapy as an adjunctive method to control head and neck cancer and improve long-term survival in this patient population.

Keywords Head and neck cancer · Head and neck squamous cell carcinoma · Tumor-specific antigens · Human papillomavirus · Antigen-specific immunotherapy · Tumor immunology

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Introduction

Significance of head and neck cancer and requirements for alternative treatments

Head and neck squamous cell carcinoma (HNSCC), with an estimated 600,000 cases reported annually, is the sixth most common cancer worldwide [1]. Despite recent treatment advances that have improved the quality of life of patients with head and neck cancer, the overall 5-year survival rate has not changed significantly in the last 25 years and remains approximately 50–59% [1]. These statistics demonstrate the need for innovative therapies, which not only improve functional outcomes but also impact long-term survival in these patients.

Immunotherapy represents a plausible approach for the control of head and neck cancer

One of the goals of adjuvant cancer therapy is to eradicate local regional microscopic and micrometastatic disease with associated minimal toxicity to surrounding normal cells. In this respect, immunotherapy is an attractive therapeutic approach. There are several advantages to exploiting the immune system to fight cancer. First, the immune system has the inherent capacity for specificity in identifying and killing neoplastic cells while sparing normal tissue. Second, the immune system demonstrates plasticity to evolve with the cancer cells. Both arms of the adaptive immune system, humoral and cellular, possess cells with a vast array of clonally distributed antigen receptors. The diversity of these receptors enables the immune system to recognize foreign and/or altered antigens and to discriminate self, or normal cells, from non-self, or cancerous cells. The humoral immunity generates

antibodies that can recognize and bind unique antigens and/or antigens overexpressed on the cell surface of head and neck cancers (for review see [2]). Furthermore, cell-mediated immunity, in particular T cell-mediated immunity, has specific T cell receptors that are capable of recognizing intracellular antigenic peptides uniquely expressed by head and neck cancers (for review see [3]).

Identifying head and neck cancer-specific antigens to develop antigen-specific immunotherapy

The single major obstacle in the application of the advances in fundamental immunology to cancer treatment has historically been the absence of suitable molecularly characterized tumor antigens. Prior to the molecular identification of the first human tumor-associated antigen (TAA) in 1991 [4], immunotherapists were forced to use undefined tumor antigens derived from tumor cell lines, tissues or their corresponding lysates. Subsequently, with the advancement of molecular genetics and the identification of a larges series of TAAs, antigen-specific immunotherapy became a reality. One of the main advantages of antigen-specific immunotherapy compared to other immunotherapeutic strategies is the ability to evaluate and monitor immune responses to targeted antigens and correlate these findings with clinical responses [5].

TAAs can be classified into several categories. There are those tumor antigens that are silenced in normal tissues but are reactivated in a certain group of tumors. These are referred to as tumor-specific shared antigens or germ cell antigens and include the MAGE genes. Differentiation antigens are expressed by the tumor cells as well as by the cells of origin of the tumor. These include gp100 and tyrosinase, which are expressed by melanoma cells and melanocytes. There are tumor-specific antigens which are genetically altered proteins unique to the tumor and which may be contributing to the malignant phenotype, such as p53 and CDK4. In addition, there are antigens expressed at some low level in normal tissues but overexpressed in tumors, such as HER-2/neu and epidermal growth factor receptor (EGFR). Lastly, there are viral antigens derived from oncogenic viruses, such as the human papillomavirus (HPV) E6 and E7 proteins, which may serve as targets for antigen specific immunotherapy (for review see [6]).

This article provides a review of identified head and neck tumor-associated antigens which can serve as potential targets for antigen-specific immunotherapy as well as discusses the immunotherapeutic strategies employed to target the humoral and cell-mediated immune responses. We will also discuss the current trends in immunotherapy which is shifting towards the development of strategies to enhance the potency of cancer vaccines targeted against head and neck cancer.

Identification of head and neck tumor-specific antigens (TSA) or tumor-associated antigens (TAA)

The identification and selection of an appropriate tumor antigen for the development of antigen-specific immunotherapy is critical. Several desired characteristics of a targeted tumor antigen include unique expression within the tumor or differential expression as compared to normal tissue or vital organs. A second desired characteristic is antigen expression by a majority of head and neck cancers, which broadens the applicability of the targeted therapy. Third, the tumor antigen should be constitutively expressed and be a requisite protein for tumor carcinogenesis, so that the tumor cannot evade the immune response by losing expression of the targeted antigen. Fourth, the tumor-specific or tumor-associated antigen should be highly immunogenic. Significant advances in molecular genetic technology are facilitating the identification of numerous TSAs in head and neck cancer, which try to meet all of the above criteria. Table 1 summarizes such tumor-associated antigens identified in head and neck cancers thus far.

HPV E6 and E7 proteins serve as model antigens for the development of immunotherapy for a subset of head and neck cancer

Of the various head and neck tumor-associated antigens identified, the human papillomavirus (HPV) E6 and E7 proteins are model antigens for the development of targeted immunotherapy for the reasons discussed above. First, recent studies have shown that HPV is associated with approximately 20–25% of all HNSCC and up to 60–70% of those tumors localized to the oropharynx in the United States (for review see [24]). Second, HPV type 16 has been found in more than 90% of HPV-positive HNSCC (for review see [25]). Third, the E6 and E7 proteins are constitutively expressed in HPV-associated malignancies and they play critical roles in tumor carcinogenesis. Therefore, the tumors are unlikely to lose expression of these critical genes in order to evade the immune system. Fourth, the E6 and E7 viral proteins are foreign antigens and, therefore, are highly immunogenic. Furthermore, since HPV type 16 is also associated with cervical and anogenital cancers, the same preventative and therapeutic vaccine strategies developed to prevent and/or treat HPV-associated cervical and anogenital cancers can also be used to prevent and/or treat HPV-associated head and neck cancers (for review see [26]).

Head and neck tumor-associated antigens identified by microarray analysis

While HPV targeted antigens account for 20–25% of all HNSCC, efforts in microarray analyses are facilitating the

Table 1 Potential tumor-associated antigens in head and neck cancer for targeted antigen-specific immunotherapy

Classification	Name of TSA	Function	Frequent head neck locations/cancer types	Expression levels/effects	References
Germ cell antigens	Melanoma antigen (MAGE-1)	Normal testicular protein	HNSCC	About 50% of HNSCC were shown to express the MAGE gene	[7, 8]
	Renal tumor antigen (RAGE-1)	Normal testicular protein	Larynx	8/28 tumor samples expressed RAGE-1	[9]
	RAGE-2	Normal testicular protein	Oropharynx	7/28 tumor samples expressed RAGE-2	[9]
	RAGE-4	Normal testicular protein	Hypopharynx	6/28 tumor samples expressed RAGE-4	[9]
	G antigen (RAGE-3, 4, 5, 6, 8)	Normal testicular protein	Hypopharynx	16/27 tumor samples expressed GAGE 3,4,5,6,8	[9]
Differentiation antigens	C-erbB-2	Receptor and intracellular signaling molecule	HNSCC	43/93 HNSCC patients expressed C-erbB-2	[10]
	Epithelial growth factor receptor (EGFR)	Receptor plays a critical role in cell survival and proliferation	HNSCC	EGFR is overexpressed in more than 90% of HNSCC	[11]
	Telomerase	Enzyme that adds DNA sequence repeats to the 3' end of DNA strands	HNSCC	Telomerase activity was elevated in 10/11 HNSCC cell lines	[12]
Mutated antigens	P53	Tumor suppressor gene	HNSCC	Aberrant P53 transcripts have been found in up to 80% of HNSCC	[13]
	Caspase 8 (CASP8)	Regulator of apoptosis	HNSCC	Mutated Caspase-8 protein is recognized by autologous cytolytic T cells	[14]
Overexpressed antigens	CyclinB1 (CCnB1)	Regulation of cell cycle	HNSCC	Peptides derived from Cyclin B1 were shown to be immunogenic in an HNSCC model	[15]
	Carcino-embronic antigen (CEA)	Oncofetal glycoprotein	HNSCC	Expression of CEA was found in a majority of tested HNSCC	[16]
	Psoriasis-associated fatty acid binding protein (E-FABP)	Proteins primarily involved in metabolism, promote metastasis	Pharynx	E-FABP is overexpressed in HNSCC	[17]
	Heterogeneous ribonucleoprotein H (hnRNP H)	Nuclear proteins, pre-mRNA binding-proteins, control gene expression upon alternative splicing	HNSCC	HnRNP H is overexpressed in HNSCC and lymph node metastases	[17]
	Growth factor receptor-bound Protein 2 (Grb2)	Proteins involved in signaling	HNSCC	Grb 2 is overexpressed in primary HNSCC tumors and in metastatic cells	[17]
	Squamous cell carcinoma antigen recognized by T cells (SART-1)	Regulate cell proliferation	HNSCC	HLA-A2601-restricted SART-1-associated peptides were described to be immunogenic in HNSCC cell lines	[18]
	Vascular endothelial growth factor (VEGF)	Signaling protein involved in vasculogenesis and angiogenesis	HNSCC	VEGF is overexpressed in HNSCC and associated with a high tumor proliferation rate and poor survival	[19]
Viral-associated antigens	Human papilloma virus (HPV) early gene 6/early gene 7 (E6/E7)	Viral oncoproteins	Oropharynx	HPV DNA has been detected in 20–30% of all HNSCC and approximately 60–70% of oropharyngeal cancers	[20, 21]
	Epstein-Barr Virus (EBV) latent membrane protein 1 (LMP1), EBNA1	Viral oncoproteins	Nasopharyngeal carcinoma	EBNA1 and LMP1 are expressed in EBV-associated tumors	[22, 23]

identification of other potential tumor antigens for targeted immunotherapy for the remaining HNSCC. Using gene microarray analysis, several genes highly expressed in 15 HNSCC primary tumor samples were identified [27]. These genes included Amphiregulin (AREG), Cadherin 3/P-Cadherin (CDH3), Kallikrein 10 (KLK10), Neuromedin U (NmU), and Secretory Leukocyte Protease Inhibitor (SLPI). AREG is a ligand for the type-1 EGFR [28] and is considered to play a critical role in cellular proliferation. Overexpression of AREG has been found in biliary tract, colorectal, breast [29], ovarian [30], pancreatic [31], and prostate [32] cancers. CDH3 is a cell adhesion molecule and has been shown, by microarray analysis, to be over-expressed in HNSCC [33], pancreatic carcinoma [34], and papillary thyroid cancer [35]. KLK 10 regulates cellular growth and has been shown to be overexpressed in ovarian cancer [36]. NmU is a G-protein receptor ligand and has been described as an ovarian cancer-associated antigen [37]. SLPI promotes cellular growth and has been reported to be overexpressed in lung, breast, oropharyngeal, bladder, endometrial, ovarian, and colorectal carcinoma [38–42]. While all of these genes are expressed in normal tissues at some low level, their expression levels are at least 10-fold higher in tumors [27]. In order to determine the general applicability and/or significance of these genes in head and neck tumors, their expression levels need to be confirmed in a larger sample of head and neck cancers. However, this study demonstrates how molecular genetic identification of altered and/or overexpressed genes within cancer cells can facilitate the identification of potential targets for the development of antigen-specific immunotherapy.

Head and neck tumor-associated antigens identified by SEREX analysis

Head and neck tumor-associated antigens can also be identified using serological analysis of recombinant cDNA expression libraries (SEREX). SEREX was developed to combine serological analysis with antigen cloning techniques to identify human tumor antigens eliciting autologous high-titer immunoglobulin G (IgG) antibody responses. SEREX involves the generation of cDNA libraries from tumors derived from cancer patients. Each cDNA strand is inserted into a plasmid and cloned into bacteria, which allows the expression of a single tumor antigen encoded by the cDNA. Autologous serum is then used to screen for seroreactivity against potential antigens, which can then be tested in larger-scale serological surveys of cancer patients and normal individuals. SEREX has identified a number of gene products that have known or suspected relevance to cancer development and that can serve as potential targets for cancer vaccines. Tumor antigens that have been identified using the SEREX technique include MAGE-A4, Integrin α6, and UBE3A [43]. The identification of SEREX-defined gene products that are recognized by the humoral immune system of subsets of cancer patients but not normal individuals emphasizes the potential of SEREX. Furthermore, the fact that a number of these genes are widely expressed in normal tissues indicates that cancer-specific recognition can occur in the absence of cancer-specific gene expression. The basis for this cancer-specific immunogenicity is still unclear and is one of the challenges that need further elucidation.

Humoral mediated antigen-specific immunotherapy

Although the humoral immune system can be used to identify potential tumor-associated antigens, there has been a renewed interest in using monoclonal antibodies (mAbs) for targeted immunotherapy. Several reasons for this resurgence include advancements in technology which has facilitated large-scale productions of clinical grade monoclonal antibodies which are highly specific to their antigenic targets. Second, mAbs are relatively safe and, in general, well tolerated compared to cytotoxic drugs (for review see [44]). Third, mAb based therapy has multiple mechanisms of action including inhibition of ligand-induced activation, induction of receptor degradation, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and/or complement-dependent cell-mediated cytotoxicity [45, 46]. Thus, the mAb not only blocks downstream activation of the targeted receptor on the cancer cells but can also induce cancer cell death.

To date, most of the mAb therapies developed target the EGFR which is overexpressed in more than 90% of HNSCC (for review see [11]). The epidermal growth factor receptor (EGFR; also known as HER1 and ErbB1) is a transmembrane tyrosine kinase receptor that plays a critical role in cell survival and proliferation. Activation of EGFR through ligand binding with the epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) leads to receptor dimerization, kinase activation, and autophosphorylation, which activates various cellular pathways involved in cellular proliferation, angiogenesis, metastases, and inhibition of apoptosis [47–49]. EGFR overexpression has been associated with an unfavorable prognosis [50, 51] and has been linked to early disease progression, poor survival and resistance to chemotherapy. Anti-EGFR antibodies such as cetuximab, which is a chimeric monoclonal antibody, act as a competitive antagonist to the receptor ligands.

In early clinical studies, single-agent activity of cetuximab was shown to be effective and safe in HNSCC [52–54]. The most common side effect was a skin rash and

less common side effects included fatigue, nausea, vomiting, diarrhea, mucositis, and hypersensitivity reactions. In a phase III randomized clinical trial that compared radiation therapy (RT) and cetuximab with radiation alone, patients with locally advanced HNSCC demonstrated better survival and locoregional control by 10–15%. Consequently, in February 2006, the Food and Drug Administration (FDA) approved cetuximab in combination with RT as a frontline treatment for patients with locally advanced HNSCC [55–57]. Additional trials have been undertaken to assess the feasibility of combining cetuximab with chemoradiation therapy. A phase II trial demonstrated that the combination of cetuximab with RT and cisplatin yielded a 3 year-overall survival, progression-free survival, and locoregional control rates of 76, 56, and 71%, respectively [58]. In addition, the combination of cetuximab with RT and gemcitabine in HNSCC yielded a complete response rate of 77% with 89% patient compliance to chemotherapy [59]. Due to the promising results observed in these early clinical trials, investigators are currently exploring the use of monoclonal antibodies directed against mutant EGFR as well as bispecific antibodies which target EGFR and immune effector cells in order to enhance antigen-specific immune responses. Table 2 summarizes the clinical trials using anti-EGFR specific monoclonal antibodies in head and neck cancer patients.

Another overexpressed tumor antigen which has been targeted by humoral mediated antigen-specific immunotherapy is the vascular endothelial growth factor (VEGF) which is a tumor secreted molecule that stimulates angiogenesis and lymphangiogenesis. High VEGF expression has been correlated with high expression of VEGF receptor in patients with head and neck cancers and coexpression of the protein and the receptor has been associated with a high tumor proliferation rate and poor survival [19]. These results suggest that an autocrine VEGF loop exists in head and neck cancer and supports the usefulness of VEGF-targeted therapy. Bevacizumab is a recombinant humanized anti-VEGF mAb which is currently being evaluated in patients with colorectal, renal, ovarian, and pancreatic cancers with promising observations including trends toward improved response rate, duration of response, and survival (for review see [19]). VEGF targeted monoclonal antibody therapy has yet to be explored in head and neck cancers; however, their applicability is intriguing either alone or in combination with anti-EGFR mAb therapy.

Table 2 Clinical trials using various monoclonal antibody derivatives targeting EGFR in head and neck cancer patients

Antibody derivative	mAb	Target molecule	Trial status	Effects/clinical outcome	References
Murine	EMD55900	EGFR	Phase I clinical trials	(1) 12 patients with advanced laryngeal and hypopharyngeal carcinoma tolerated antibody administration. (2) Good to excellent homogenous binding of EMD55900 to primary lesions and lymph node metastasis after 3 days.	[60]
Chimeric: (30%) murine and (70%) human sequences	Cetuximab; IMC-C225 IgG1	EGFR	Phase III clinical trials	(1) Significant increase in overall response rate for HNSCC patients treated with cisplatin and cetuximab (26%) versus HNSCC patients treated with cisplatin and placebo (10%). (2) Radiation with cetuximab resulted in better HNSCC patient survival and control of advanced cancer than radiation alone.	[54, 61]
Humanized: 90% human sequences	EMD 72000 IgG1	EGFR	Phase I/II clinical trials	(1) Maximum tolerated dose was 1,600 mg/week with fever and headache as dose-limiting toxicities. (2) 2/4 HNSCC patients showed a partial response.	[62]
	h-R3 IgG1	EGFR	Phase II clinical trials	In a study using h-R3 in combination with radiotherapy to treat locally advanced head and neck cancer patients, no severe toxicity or skin reactions were detected.	[63, 64]
	MDX-447 EGFR/FcRγ1	EGFR and anti-CD64	Phase I/II clinical trials	Most common side effects were headache, fever, chills, hypertension, myalgias, nausea, edema, fatigue, arrhythmia. Currently, a phase II trial in head and neck cancer is ongoing.	[65]
Human	ABX-EGF IgG2	EGFR	Phase II in a wide range of epithelial tumors	(1) No infusion-related or serious side effect reported with ABX-EGF antibody. (2) In a phase II trial for renal cell cancer, ABX-EGF showed modest activity.	[66]

Cell-mediated antigen-specific immunotherapy

Cell-mediated immunity is armed with multiple effector mechanisms capable of eradicating tumor cells. T cells are able to recognize TSAs in association with major histocompatibility complex (MHC) molecules and, upon recognition of the TSAs, they can become activated to directly lyse the tumor cells. Alternatively, anti-tumor immune responses can be achieved through the secretion of cytokines released by helper T cells (Th) which can navigate the ensuing immune response to activate macrophages, natural killer cells and cell mediated immunity or favor isotype switching in the humoral arm. Therefore, T cells play a critical role in mounting a successful anti-tumor immune response.

As stated previously, the HPV E6 and E7 viral antigens represent of the most promising tumor antigens identified to date for head and neck cancer cell-mediated immunotherapy. Thus, significant efforts in the development of antigen-specific immunotherapies for head and neck cancer have focused on the HPV E6 and E7 viral antigens. These strategies have explored the use of DNA, bacterial vector, viral vector, peptide, protein, dendritic cell and tumor-cell based vaccines.

Vaccines for HPV-associated head and neck cancers

DNA vaccines

DNA vaccines have been used in the clinical arena to elicit antigen-specific immune responses. Naked DNA is relatively safe, stable, cost efficient, and able to sustain reasonable levels of antigen expression within cells (for review see [67, 68]). In addition, since DNA vaccines do not elicit neutralizing antibodies in the vaccinated patient, they can be repeatedly administered with similar efficacy. However, several disadvantages to DNA vaccines are their relatively low transfection efficiency and poor immunogenicity. Unlike some bacterial or viral vectors, DNA vaccines also lack the intrinsic ability to replicate or spread to surrounding cells in vivo. Therefore, investigators have placed considerable efforts in devising strategies to enhance the potency of DNA vaccines. These strategies include exploring various vaccine administration techniques, which facilitate efficient targeting of the DNA to professional antigen presenting cells (APCs) such as dendritic cells (DCs), enhancing antigen processing and presentation by APCs, and modifying the DC to augment DC and T cell interactions. Table 3 summarizes some of the various strategies used to enhance the potency of DNA vaccines and Fig. 1 provides a schematic summarizing various mechanisms of DNA vaccine enhancement through modification of the DC.

Antigen processing and presentation in APCs can be enhanced through the linkage of the antigen-of-interest to intracellular targeting proteins of the MHC class I and II pathways. In preclinical studies, an HPV DNA vaccine encoding the E7 gene linked to the heat shock protein 70 (HSP70) demonstrated enhanced MHC class I processing and presentation of E7. Furthermore, mice vaccinated with the E7/HSP70 DNA vaccine generated significant levels of E7-specific CD8+ T cells which resulted in anti-tumor effects against an HPV-16 E7 expressing tumor model [88]. The promising results observed in the preclinical data led to a Phase I clinical trial using a naked DNA vaccine encoding the HPV-16 E7 gene linked to *M. tuberculosis* HSP70 (pNGVL4a-Sig/E7(detox)/HSP70). The naked DNA vaccine was administered to patients with advanced HPV-16 associated HNSCC at the Johns Hopkins Hospital.

Table 3 Various approaches to alter the properties of DCs to enhance the potency of DNA vaccines

Approaches	Methods	References
Efficient delivery of targeted antigen to antigen presenting cells such as DCs	Gene gun administration of DNA vaccine to epidermis where DCs are concentrated	[69–72]
	Linkage to antigen which facilitates intercellular antigen spreading	[73]
	Targeting to DCs by linkage to molecules capable of binding DCs	[74]
Enhancement of antigen processing and presentation in DCs	Use of intracellular targeting strategies to the MHC class I and II processing pathways for enhanced antigen presentation by DCs	[75–79]
	Codon optimization	[80, 81]
	Enhanced presentation of antigen through a MHC class I single chain trimer (SCT) composed of peptide, b2-microglobulin, and MHC class I heavy chain	[82]
Augmentation of DC and T cell interaction	Inhibition of DC apoptosis	[72, 83]
	Promotion of in vivo DC expansion	[84, 85]
	Co-expression of cytokines and stimulatory molecules	[76, 86, 87]
	Induction of helper T cell function	[79]

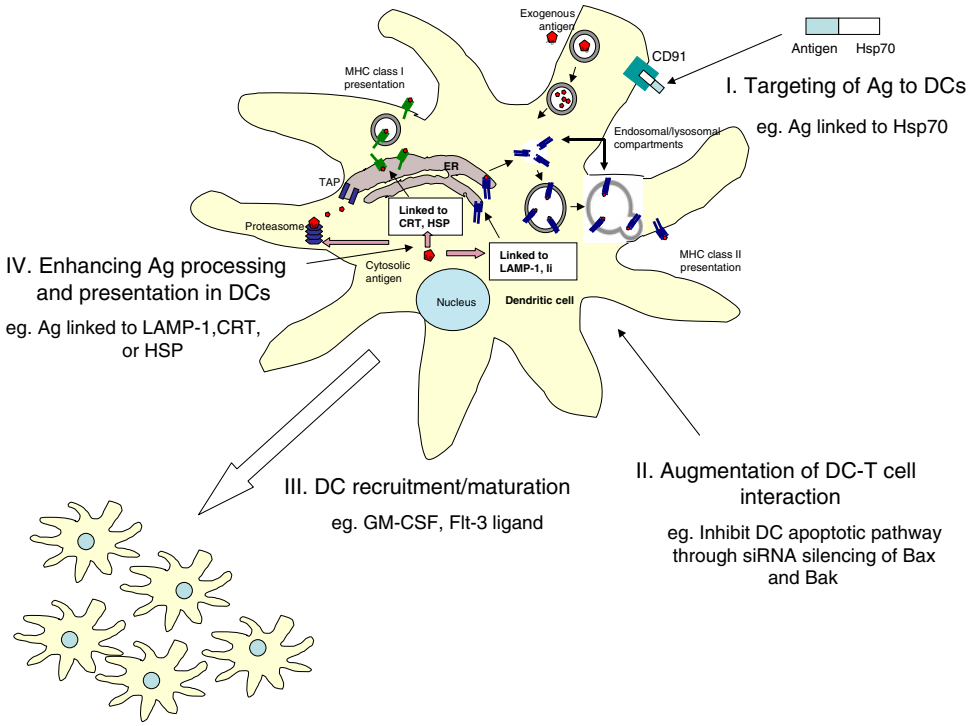

Fig. 1 Schematic diagram depicting the various strategies used to enhance the potency of DNA vaccines by modifying the properties of DCs. Various strategies aimed to enhance the potency of DNA vaccines through modification of the properties of DCs have been reported including: I. Efficient delivery of target antigens to DCs. For example, linking an antigen to heat shock protein 70 (Hsp70), which can then bind to scavenger receptors, such as CD91 on DCs. II. Augmentation of DC–T cell interaction. For example, the DC apoptotic pathway can be inhibited through the use of siRNA technology, which targets the pro-apoptotic proteins, Bax and Bak. III. Facilitate DC recruitment and/or maturation. For example, transfection of tumor cells with GM-CSF or FMS-like tyrosine kinase receptor (Flt-3) ligand can recruit and promote DC maturation. IV. Enhance antigen processing and presentation in DCs. For example, the linkage of an antigen to the sorting signal of lysosomal associated membrane protein type 1 (LAMP-1) or to calreticulin (CRT) can direct the antigen to the MHC Class II or Class I processing pathway, respectively

The DNA vaccine was well tolerated and a subset of the vaccinated patients who received the maximum dose of 4 mg of DNA/vaccination and a total of 4 vaccinations demonstrated detectable, systemic levels of E7-specific CD8+ T cell immune responses (Maura Gillison, personal communication).

Bacterial vectors

Bacteria, such as *Listeria monocytogenes*, *Salmonella*, *Lactococcus lactis*, *Lactobacillus plantarum*, and *Bacillus Calmette-Guérin*, have been used to deliver genes or proteins of interest to elicit antigen-specific immunotherapy (for review see [89]). Among these bacterial vectors, *L. monocytogenes* has emerged as a promising vector, which is able to elicit both CD8+ and CD4+ immune responses and induce regression of established tumors expressing a model antigen. *L. monocytogenes* is a gram-positive intracellular bacterium that usually infects macrophages. Unlike other intracellular pathogens, however, it can evade phagocytosis and endosomal compartmentalization within macrophages by secreting a factor, listeriolysin O, which allows it to escape into the cytoplasm of the macrophage. Thus, its presence in both the endosomal compartment and the cytoplasm allows it to deliver antigens of interest to both the MHC class I and II processing pathways, eliciting potent cellular immune responses from both the CD8+ and CD4+ T cell arms. Recently, it has been shown that a *Listeria*-based vaccine targeting E7 was capable of inducing the regression of solid implanted E7 expressing tumors in E7 transgenic mice and the vaccine was able to overcome central tolerance by expanding low avidity CD8+ T cells specific for E7 [90]. A phase I/II clinical trial is currently ongoing using the Listeria-based therapeutic HPV vaccine targeting the E7 antigen in patients with cervical cancer (Dr. Yvonne Paterson, personal communication). It is conceivable that similar bacterial based vaccines can be used in patients with HPV-associated head and neck cancers.

Viral vectors

Several viral vectors have also been used for vaccine development, including vaccinia virus (VV), adenovirus

(AdV), adeno-associated virus (AVV), alphavirus, and its derivative vectors, such as sindbis virus, semliki forest virus, and venezuelan equine encephalitis (VEE) virus (for review see [89]). Among these viral vectors, the VV, a member of the poxvirus family, has emerged as a promising viral vector to deliver genes and antigens of interest efficiently. Several VV vaccines have been tested in clinical studies. A phase I/II clinical trial using a recombinant VV encoding an HPV-16/18 E6/E7 fusion protein, termed TA-HPV, demonstrated that the vaccine was well tolerated and induced T cell mediated immune responses in patients with HPV-associated anogenital tumors [91–96]. Another recombinant VV encoding E2, called MVA-E2, has been tested in phase I/II clinical trials in patients with cervical cancer precursor lesions and genital warts. All vaccinated patients developed antibodies against the MVA-E2 vaccine and generated a HPV specific cytotoxic response against the papilloma-transformed cells which resulted in regression of high-grade lesions [97–99]. As the clinical trials using these vaccinia viral vectors encoding HPV antigens progress, their applicability to a subset of head and neck cancers will become more elucidated.

Peptide-based vaccines

Instead of gene delivery of tumor-associated antigens using DNA, bacteria, and/or viral vectors, antigenic peptides can be administered. Antigenic peptides can associate with the MHC class I or II molecules and this complex is presented on the cell surface of antigen presenting cells (APCs) to trigger cell-mediated immune responses against the antigen expressing tumor. In general, peptide-based vaccines are safe, stable, and easy to produce in large scale. In addition, since the peptide epitopes are precisely defined, specific immune responses can be monitored easily and correlated with clinical responses. However, a major limitation to peptide-based vaccines is the need to identify the immunogenic epitope of the tumor-associated antigen. This task is made even more difficult by the observation that the antigenic epitope with the highest binding affinity to the human leukocyte antigen (HLA) molecule does not necessarily correlate with its potential immunogenicity in vivo. Most peptide based vaccines have focused on antigenic peptides which bind the HLA-A2 molecule due to its high frequency of expression in up to 50% of the Caucasian population. However, once an immunogenic epitope is identified, the applicability of the peptide vaccine is limited to a group of select patients expressing the HLA molecule, making it difficult to carry out large scale vaccination treatment schemes. Another disadvantage to peptide vaccines are their relative poor immunogenicity as compared to bacterial or viral vaccine vectors. Consequently, most of the research in this area has focused on the co-administration of adjuvant immune-enhancing agents such as chemokines, cytokines, and costimulatory molecules to enhance the potency of the peptide vaccine (for a review, see [100]). Several phase I clinical trials using antigenic peptides derived from HPV E6/E7 have been conducted with various adjuvants, including incomplete Freund's adjuvant and Montanide ISA 51 adjuvant (for review see [89, 100]). From these clinical trials, it is clear that identification of the appropriate adjuvants and route of administration is important in order to maximize the immunological responses elicited from peptide-based vaccines.

Protein-based vaccines

The HLA restriction associated with peptide-based vaccines can be overcome with the use of whole protein-based vaccines, which harbor multiple immunogenic epitopes which can bind the various allelic HLA molecules. However, due to the poor immunogenicity of proteins, strategies, similar to those of peptide-based vaccines, have been investigated to enhance the potency of these vaccines. Studies have demonstrated that co-administration of chimeric GM-CSF molecules can lead to enhanced antigenic immune responses through the recruitment of antigen present cells [101, 102]. In addition, co-administration of immunostimulatory CpG oligodeoxynucleotides (ODNs) is able to enhance the potency of protein vaccines by stimulating macrophages to secrete IL-12 thus shifting the cytokine profiles to a Th1-type cell-mediated immune response [103, 104]. CpG ODNs are a promising alternative to complete Freund's adjuvant because they lack significant toxicity [105].

Dendritic cell based vaccines

Professional APCs, in particular DCs, play an important role in the generation of antigen-specific immunity. DCs are specialized APCs that express high levels of MHC and costimulatory molecules making them the most potent APC identified to date. Consequently, there has been intense interest in developing DC based cancer vaccines. A variety of methods for generating DCs, loading them with tumor antigens, and administering them to patients have been described. Strategies for loading DCs ex vivo include the application of proteins or peptides, apoptotic or necrotic tumor cells, tumor cell lysates, genetically engineered vectors, or cell fusion techniques. The advantage to DC based vaccines is the uniformity and control provided by ex vivo manipulation of the DCs that generate a pool of optimally activated APCs for stimulating immunity in vivo. DCs pulsed with recombinant HPV-16 and HPV-18 E7 proteins have been evaluated in patients with advanced

HPV-associated anogenital cancers [106]. In general, the vaccine was well tolerated with no significant local or systemic side effects and HPV antigen-specific T cell responses were observed in some of the patients [106]. At this early stage of clinical development, it is difficult to determine if DC vaccines represent a method of stimulating protective immunity in cancer patients that is superior to other vaccination strategies. In most studies, a fraction of patients, often less than half, exhibit immune responses against the vaccinating antigen. As investigators continue to explore the most effective route of administration, vaccination schedule, prime-boost regimens, and various maturation protocols, the potency of DC based vaccines will become better appreciated.

Tumor-cell based vaccines

Autologous tumor-cell based vaccines deliver a range of tumor antigens to the immune system that may not be present in single-target vaccines. However, since tumor cells are, in general, poorly immunogenic, studies have focused on strategies to enhance the potency of cell based vaccines including co-administration with adjuvants such as Bacille Calmette-Guérin (BCG), transduction of tumor cells with MHC or costimulatory molecules, and modification of tumor cell vaccines to secrete immunostimulatory cytokines. Transduction of immunostimulatory cytokines such as IL-2, IL-4, IL-12, IFN-γ, and GM-CSF have been evaluated in the clinical arena and, currently, GM-CSF transduced tumor cells represent one of the most promising cell based vaccine approaches. GM-CSF attracts DCs, which infiltrate the vaccination site, phagocytose released antigens from apoptotic tumor cells, and migrate to draining lymph nodes to prime antigen-specific immune responses. A limitation to autologous tumor vaccines is the labor-intensive preparation of an autologous vaccine for each individual patient which is time consuming and technically challenging. Thus, researchers have investigated the potential of allogeneic GM-CSF transduced tumor cell lines established in long-term culture. This overcomes the requirement to obtain tumor tissue from each patient. However, the use of allogeneic vaccines relies on an overlapping antigenic profile between the vaccine and the patient's own tumor (for review see [107]).

Another approach that has been investigated is the use of bystander GM-CSF releasing cells mixed with irradiated tumor cells [108] or GM-CSF-releasing microspheres that degrade over time, releasing a continuous controlled supply of GM-CSF in the vicinity of the tumor [109]. Fms-like tyrosine kinase 3 (Flt3-L)-transduced tumor vaccines can also recruit and activate DCs to the tumor bed and inhibit tumor growth in murine melanoma and lymphoma models [110]. Transduction of tumor cells with genes encoding MHC and/or co-stimulatory molecules, such as B7-1 [111, 112] have also been explored and found to enhance immunogenicity, leading to T cell activation and anti-tumor effects. While tumor-cell based vaccines have not been explored in head and neck cancers, this is an attractive approach which, merits further investigation. Table 4 summarizes the advantages and disadvantages of the various types of cancer vaccines. Figure 2 depicts the various mechanisms of action of antigen-specific immunotherapy; specifically illustrating how cancer vaccines and/or immunotherapeutic strategies employing the humoral and/or cell-mediated arms of the immune system can be used to control head and neck cancer.

Table 4 The advantages and disadvantages of different types of vaccines

Approaches	Advantages	Disadvantages
DNA-based	Safe, stable, cost efficient, allows multiple immunizations	Low immunogenicity
Bacterial vector-based Viral vector-based	High immunogenicity	Potential pre-existing immunity or neutralizing antibodies
	Variety of available vectors	Limited repeat immunizations, Toxicity hazard
Peptide-based	Safe	Low immunogenicity
	Relatively cost efficient	HLA restriction
Protein-based	No HLA restriction	Low immunogenicity
	Relatively cost efficient	Better induction of humoral responses than cell-mediated responses
Dendritic cell-based	High immunogenicity	High cost
	Controlled environment in generating a more uniform pool of activated DCs ex vivo	Labor-intensive
Tumor cell-based	Targeting of multiple tumor-associated antigens	High cost
	Useful when tumor antigen is unknown	Labor-intensive
		Possible weak antigen presentation by tumor cells due to down-regulation of MHC class I molecules

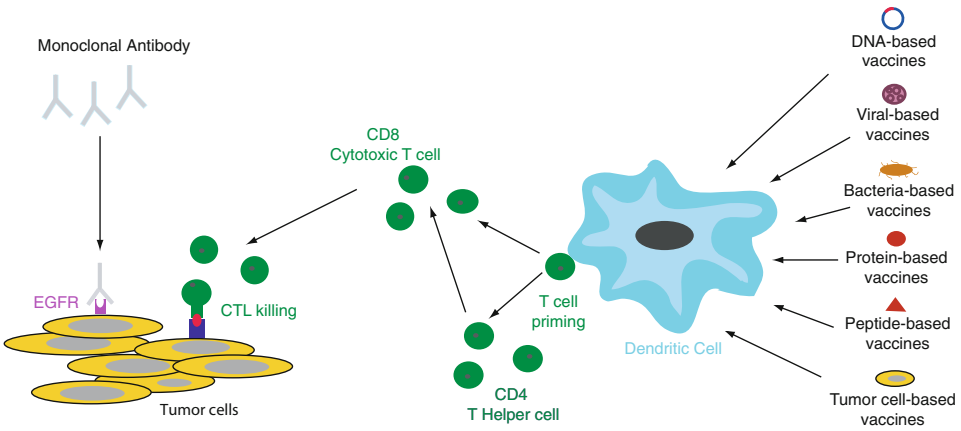

Fig. 2 Schematic diagram depicting the various mechanisms of action of antigen-specific immunotherapy to control head and neck cancer. Monoclonal antibodies have been used to target overexpressed cell surface receptors such as EGFRs. The binding of monoclonal antibodies to EGFR inhibits the function of EGFR, thus suppressing tumor cell proliferation, angiogenesis, and metastases while rendering the tumor susceptible to antibody and complement dependent cytotoxicity. DNA, viral, bacterial, protein, peptide, or tumor cell-based vaccines target DCs for antigen processing and presentation to effectively prime antigen-specific CD4+ and CD8+ T cells. The CD4+ T helper cells secrete cytokines to help activate cytotoxic T cells and the cytotoxic T cells, upon recognition of TSAs in association with MHCs can become activated to kill the tumor cells

Future directions

Early phase clinical trials have demonstrated the safety and feasibility of DNA, peptide, protein, bacterial, viral, DC, and tumor cell-based immunotherapies and the emphasis is now shifting toward the development of strategies which increase the potency of these vaccines by exploring various routes of administration, frequency of immunizations, co-administration of adjuvant immune-enhancing agents, and prime-boost vaccination strategies. In a preclinical model, a prime-boost regimen, consisting of an HPV E7 DNA vaccine followed by a live HPV E7 viral vector vaccine [113], elicited enhanced antigen-specific immune responses as compared to those obtained with a single vaccine alone. Another study found that mice primed with a Sindbis virus RNA replicon containing E7 linked to *M. tuberculosis* HSP70 (E7/HSP70) and then boosted with a vaccinia vector encoding E7/HSP70 generated strong E7-specific CTL responses as well as potent anti-tumor effects [114]. Investigators also found that the HPV-antigen-specific CD8+ T cell immune responses obtained from a protein-based vaccine could be enhanced by a heterologous booster immunization with a highly attenuated modified vaccinia virus Ankara (MVA) expressing the E7 protein [115]. Due to the enhanced efficacy of the vaccines using a prime-boost regimen in preclinical models, these combinatorial HPV vaccines have entered clinical trials. A clinical trial in patients with anogenital intraepithelial neoplasia demonstrated that a prime-boost regimen consisting of HPV-16 L2/E6/E7 fusion protein (TA-CIN) followed by a recombinant vaccinia virus encoding the E6/E7 fusion proteins of HPV types-16 and -18 (TA-HPV) could enhance HPV-16 antigen-specific T cell responses which correlated with clinical regression [116, 117].

Other strategies that are being explored include multimodality treatment options which combine immunotherapy with surgery, chemotherapy, and/or other biotherapeutic agents. Chemotherapy and immunotherapy have often been regarded as mutually exclusive; however, there is now increasing evidence that in appropriate immunologic settings, cancer drug-induced apoptotic death of tumor cells may trigger the generation of effective anti-tumor immune responses when combined with immunotherapy. A recent study demonstrated that a mild chemotherapeutic agent epigallocatechin-3-gallate (EGCG), the major polyphenol derived from green tea, can induce tumor cellular apoptosis and enhance antigen-specific T cell immune responses when combined with an E7 targeted DNA vaccine [74). These successful results have led to a phase I clinical trial at Johns Hopkins Hospital which combines oral EGCG administration with intradermal administration of a DNA vaccine, consisting of an immunostimulatory agent, calreticulin, linked to the HPV-16 E7 gene (CRT/E7), in patients with advanced HPV-associated head and neck squamous cell carcinomas (HPV-HNSCC) (S. Pai, personal communication). Similar, synergistic anti-tumor effects have been observed with cisplatin in combination with the CRT/E7 DNA vaccine in a preclinical HPV model (C.F. Hung, personal communication). Other studies have investigated the combination of recombinant E7 protein-based vaccines with CpG ODN adjuvant and chemotherapy, such as cisplatin. These combined strategies resulted in improved therapeutic anti-tumor effects against established E7-expressing tumors as compared to single modality treatments [118, 119].

These observed synergistic effects are attributed to the ability of certain chemotherapeutic agents to induce immunogenic cellular apoptosis with subsequent release of TAAs which can be processed and presented by the immune system to further expand the antigenic immune response beyond those targeted by the vaccine alone [74]. One can extrapolate these findings to other standard treatment modalities, such as RT, and one can predict that local treatment of tumors using RT in combination with immunotherapy may provide a feasible treatment option for cancer patients due to the effects of radiation-induced apoptosis and subsequent release of TAAs. Furthermore, this multi-modality treatment option is attractive since radiation treatment is usually limited to a defined field resulting in targeted tumor cell apoptosis with minimal damage to the host immune system.

Use of passive cellular immunotherapy, such as lymphokine-activated killer (LAK) cell transfer, in which patient's endogenous T cells are extracted and activated by IL-2 ex vivo and returned to the patient's bloodstream, or transfer of tumor-infiltrating lymphocyte (TIL) clones into patients, has yet to be performed in head and neck cancer patients. It has been demonstrated in preclinical models that the transfer of tumor-infiltrating lymphocyte (TIL) clones or TAA-reactive CTL clones, generated in vitro by autologous tumor stimulation or TAA-peptide stimulation, can result in tumor regression [120–123]. In human clinical trials, TIL expanded ex vivo and then adoptively transferred to melanoma patients with IL-2 resulted in objective responses in 34% of melanoma patients [124]. However, the application of this technology is currently limited by the ability to identify and isolate relevant antigen-specific CTL clones. As tumor-reactive CTLs from the peripheral blood of head and neck cancer patients are better defined, we foresee the ability to evaluate cellular immunotherapy in this patient population. Furthermore, once the T cell receptors of the tumor-reactive T cell clones are characterized, other potential immunotherapeutic strategies can be explored including genetic modification of patients' peripheral blood lymphocytes through the transfer of T cell receptor genes from TAA-specific T cell clones which can theoretically confer TAA-specific anti-tumor reactivity.

However, even with the successful application of passive cellular immunity, the full potential of immunotherapy will most likely be realized in multi-modality treatment regimens which combine immunotherapy with surgery and/or chemoradiation therapy. Each of these modalities provides unique strengths to the treatment regimen. Surgery is able to debulk large tumors. Chemotherapy and/or RT can induce tumor cell apoptosis of bulky tumors which may not be amenable to surgical resection due to associated functional deficits or attendant cosmetic deformity. Immunotherapy can provide long-term immune protection against tumor growth by inducing memory T cells that can be activated against microscopic persistent or recurrent disease. In addition, unlike any other current treatment option to date, the immune system can evolve with and adapt to evasive strategies developed by tumors. Therefore, it is the combination of these various treatment strategies, which will most likely impact the long-term outcomes in patients with head and neck cancer.

Conclusions

The identification and characterization of TSAs facilitate the development of novel therapeutic vaccine strategies for head and neck cancer. In this review, we have reviewed the tumor-associated antigens which represent potential targets for head and neck cancer vaccines and their application in various vaccine vectors. It is likely that effective immunotherapy against head and neck cancer will require a combination of therapeutic vaccines with innovative agents that are capable of overcoming the suppressive immune factors present in the tumor microenvironment. We foresee the benefits of immunotherapy will be appreciated in multi-modality treatment options which combine immunotherapy with surgery and/or chemoradiation therapy. As the field of immunotherapy matures and as our understanding of the complex interaction between tumor and host develops, we get closer to realizing the potential use of immunotherapy as an adjunctive method to control head and neck cancer and improve long-term survival in this patient population.

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