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Review

How patients with an intact immune system develop head and neck cancer

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ABSTRACT

Although the adaptive immune system can detect and eliminate malignant cells, patients with intact and fully functional immune systems develop head and neck cancer. How is this paradox explained? Manuscripts published in the English language from 1975 to 2018 were reviewed using search inputs related to tumor cell antigenicity and immunogenicity, immunodominance, cancer immunoediting and genomic alterations present within carcinomas. Early in tumor development, T cell responses to immunodominant antigens may lead to the elimination of cancer cells expressing these antigens and a tumor composed to tumor cells expressing only immunorecessive antigens. Conversely, other tumor cells may acquire genomic or epigenetic alterations that result in an antigen processing or presentation defect or other inability to be detected or killed by T cells. Such T cell insensitive tumor cells may also be selected for in a progressing tumor. Tumors harboring subpopulations of cells that cannot be eliminated by T cells may require non-T cell-based treatments, such as NK cell immunotherapies. Recognition of such tumor cell populations within a heterogeneous cancer may inform the selection of treatment for HNSCC in the future.

Introduction

Genomic rearrangement of T cell receptor (TCR) genes produces an estimated 10^{13} different T cell clonotypes giving the adaptive immune system the capacity to respond to a large number of unique antigens [1]. These antigens, presented to T cells via major histocompatibility complex (MHC) molecules, can be derived from foreign pathogens or, in the case of cancer, from altered endogenous proteins. When altered or mutated endogenous proteins are processed and presented on MHC with subsequent recognition by T cells, these antigens are termed neoantigens. Head and neck squamous cell carcinomas (HNSCCs) typically harbor many genomic alterations and thus, HNSCCs are predicted to harbor many neoantigens [2–4]. So how do cancers with many genomic alterations and neoantigens escape T cell recognition and elimination? One evasive mechanism employed by malignant cells is the expression of chemokines and cytokines that generate an immunosuppressive tumor microenvironment blocking the function of effector immune cells [5–7]. However, development of an immunosuppressive tumor microenvironment may primarily be a mechanism of immune suppression in later-stage tumors that have accumulated a mass of tumors cells that produce chemokines sufficient to mediate chemotaxis of immunosuppressive cells [5].

Early in tumor development, it is likely that variable *antigenicity* of tumor cells drives the formation of a tumor composed of tumor cells that are able to escape immunity [8]. The genomic instability of malignant cells results in a high degree of cancer cell heterogeneity [9]. Some tumor cells harbor strong (immunodominant) T cell antigens that are likely eliminated by the host's immune system early in tumor development [10]. This process, termed immunoediting, leads to the development of clinically relevant tumors that only harbor weaker (immunorecessive) antigens [11]. Complicating this further, antigen-specific T cells tend to develop against immunodominant and not immunorecessive antigens, even when the immunodominant antigen has been removed from the tumor via immunoediting [12–14]. Therefore, some tumors may be susceptible to immune detection and elimination if T cell responses against immunorecessive antigens develop, either naturally or after immunotherapy designed to activate T cell immunity.

Aside from differences in tumor cell antigenicity driving immune escape, differences in *immunogenicity* (or how well the tumor cell is recognized by T cells) may also be playing a role. Through genomic instability and selection pressure in the face of anti-tumor immunity, tumor cells that harbor genomic or epigenetic alterations that interfere with antigen processing and presentation, interferon responses or susceptibility to T cell killing may also be selected by the immune system [15–18]. Translationally, this is more ominous and means that some cancers may simply be resistant to immunotherapy designed to activate T cell immunity, even if they harbor T cell antigens. Here, we review the underlying genomic alterations present within tumor cells that lead

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to resistance to T cell recognition and killing. Recognizing and fully elucidating these intrinsic mechanisms of resistance is required to begin to devise means of reversing these processes to enhance the efficacy of therapies relying on cytotoxic T cell responses.

How should antigen-specific T cell immunity develop?

Analysis of how tumor cells evade T cell detection and elimination requires an understanding of the normal biology. The process of T cell detection of infected or cancerous cells begins when clonally expanded antigen-specific T cells recognize antigen presented on the surface of target cells via MHC class I (MHC I). To maximize chances that they see the antigen for which they are specific, activated T cells express interferon gamma (IFNγ) that in turn potently induces MHC class I expression on tumor cells through the IFN receptor/JAK/STAT signaling pathway [19]. Integrins and cell adhesion molecules strengthen the immunological synapse and "hold" T cells to target cells for a period of time to allow effector function and killing. Antigen:MHC complex binding to the antigen specific T cell receptor (TCR) triggers rapid release of preformed granules containing perforin and granzymes. Upon cell entry, granzymes mediate the early (first 8–16 h) cytotoxic effects of T cells by acting as potent proteases and activating caspase-independent apoptosis [20,21]. Secondary mechanisms of T cell killing that occur after granule exocytosis include expression of tumor necrosis factor (TNF)-superfamily ligands such as TNFα, Fas ligand and TRAIL. The ligands bind different TNF family receptors to activate extrinsic apoptosis pathways. One or more genomic alterations or epigenetic expression defects in any of these biologic processes could act as a mechanism of resistance to T cell recognition and killing.

This review will categorically explore intrinsic mechanisms of resistance to T cell recognition and killing that have been identified in head and neck cancers:

(1) altered antigen processing and presentation
(2) absent or defective IFNγ receptor expression or downstream signaling
(3) defective formation of the immunologic synapse
(4) neutralization of granzyme released from T cells
(5) resistance to death receptor-induced extrinsic apoptosis
(6) caspase mutations
(7) tumor cell intrinsic cell cycle alterations in response to cytotoxic insults

Discussion

Alterations in antigen processing and presentation

For CTLs to recognize an antigen on MHC I, the cell must first generate peptide to serve as antigen. Fig. 1 demonstrates critical steps in this process and common genomic alterations that disrupt antigen processing and presentation. Broadly, antigen processing and presentation requires successful cleavage of proteins to appropriate size, loading of the antigen onto the MHC I molecule and trafficking of the complex to the cell membrane. Defects in this process have been documented as a common cellular feature allowing for tumor evasion of CTL recognition and subsequent killing [22]. Antigens are most commonly peptides of 8–10 amino acids generated via ubiquitination and degradation by the multi-catalytic proteasome complex [23]. Critical subunits of the multi-catalytic proteasome complex are IFNγ inducible [24] and loss of response to IFNγ may downregulate this process [15].

Next, antigens are assembled with MHC I and β2-microglobulin (B2M) in the endoplasmic reticulum. The MHC I peptide loading complex (PLC) is comprised of MHC I, the peptide transporter (TAP), the bridging factor tapasin, endoplasmic reticulum aminopeptidase and chaperones calreticulin, calnexin and ERp57 [25,26]. Loss or down regulation of TAP1 results in a global decrease in MHC I cell surface expression [17,24,27]. Indeed, alterations in TAP1 expression occur in HNSCC at a rate of about 34% [28] with downregulation of TAP1 and tapasin occurring in both primary and metastatic lesions [29]. Beyond the processing and loading machinery, issues may arise within the MHC I:B2M complex [27]. Two copies of the B2M gene are present in each cell and only one copy is needed for MHC I antigen presentation [30]; thus, loss of both functional B2M genes is required for loss of MHC I antigen presentation. Loss of both genes for this support protein renders tumor cells completed unresponsive to IFNγ stimulation of MHC I expression and unable to present antigens [31,32].

Similarly, the absence of structurally sound MHC Class I molecules may arise due to total or partial loss of one copy of chromosome 6, which encodes for MHC I heavy chains or loss of MHC allospecificity [33]. Transcription of MHC I may be suppressed due to hypermethylation of DNA CpG islands, specifically of HLA-A, B and C promoter regions [34], and histone hypoacetylation [33]. Transcription can be restored by DNA demethylating agents (5-azacytidine and decitabine) and histone deacetylases [22]. Thus, mutations or downregulation of either B2M or MHC I allows tumor cells to evade CTL recognition and elimination [18]. Clinically, loss of heterozygosity on chromosome 6p21, has been documented in HNSCC at a frequency of 36% [24] and MHC class I downregulation is associated with clinical reduction in disease free interval and overall survival [28]. Finally, 80% of HNSCC are EGFR positive and EGFR-induced MHC I downregulation may contribute to resistance to T cell killing [35].

Absent or defective IFNγ receptor expression or downstream signaling

Cytotoxic T cells express interferon-γ which induces upregulation of MHC I expression in tumor cells [36]. Binding of IFNγ to IFNγ receptor 1 and receptor 2 induces heterodimerization of the two components. Next, JAK2 phosphorylates the receptor allowing for binding of STAT1 proteins. STAT1 is phosphorylated, forms a homodimer and traffics to the nucleus [25]. Once in the nucleus, STAT1 interacts with interferon regulatory factor 1 (IRF-1) to activate a wide variety of cellular responses including specific and nonspecific mechanisms of defense against viral infection and malignant transformation [37]. Specifically, IFNγ is a major cytokine responsible for upregulation of MHC I, including induction of proteins required for MHC I:Antigen loading and processing such as TAP1, tapasin, calreticulin, calnexin and ERp57 [25]. Disruption of any of these components can result in defective IFNγ signaling and a decrease in or absence of MHC I presentation on the tumor cell surface [15–17].

Both HPV-positive and -negative HNSCC are enriched for somatic mutations in the IFNγ receptor [38]. Mutations in the JAK proteins have been widely documented in various cancer types, including melanoma [39], and can produce a variety of phenotypes. Homozygous loss of function mutation in JAK1 leads to resistance to IFNγ signaling in addition to loss of IFNα and IFNβ response. In contrast, JAK2 mutations result in cells that are unresponsive to IFNγ only. Both mutations were documented in human melanoma lines and were correlated with initial tumor regression on immune checkpoint blockade with later disease progression [40]. In tumor lines with functional JAK proteins, IFNγ signaling can be constitutively inhibited by suppressor of cytokine signaling-3 (SOCS3). SOCS3 inhibits JAK-STAT signaling through prolonged and extensive phosphorylation of STAT1 at the S727 site thus inhibiting homodimerization translocation to the nucleus [41]. In contrast, Src homology 2 domain-containing phosphatase (SHP)-2 overexpression dephosphorylates pSTAT1, also resulting in inhibited homodimerization and decreased expression of MHC I APM components [42,43]. When taken together, phosphorylation of STAT1 must be tightly regulated for proper functioning of the IFNγ induced JAK-STAT pathway and dysregulation results in abrogation of pathway function. Finally, within the nucleus the STAT1 complex interacts with interferon regulatory factor 1 (IRF-1) to activate IFN inducible genes. Methylation of IRF-1 prevents binding by STAT1 and transactivation of IFN

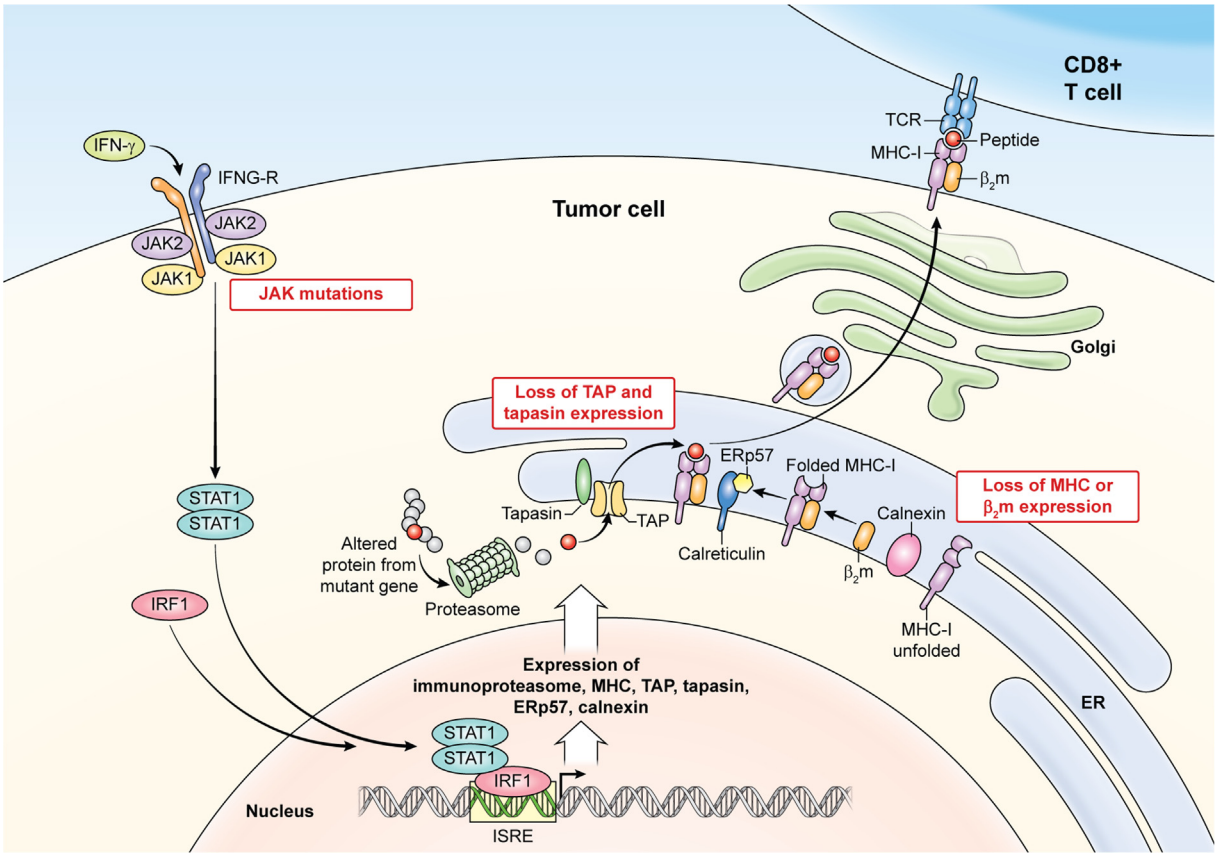

Fig. 1. Frequent genomic alterations present within head and neck squamous cell carcinomas that lead to escape from T cell immunity. Illustration of common genomic alterations identified within head and neck squamous cell carcinomas that result in defective antigen processing and presentation. IFN-γ, interferon-γ; IFNG-R, interferon-γ receptor; JAK, janus kinase; STAT, signal transducer and activator of transcription; IRF1, interferon regulatory factor-1; ISRE, interferon-sensitive response element; TAP, transporter 1, ATP binding cassette subfamily B member; MHC-1, major histocompatibility complex-1; β_2M, β-2-microglobulin; TCR, T-cell receptor.

inducible genes [30]. Encouragingly, demethylating agents have been shown to restore IFNγ induced MHC I expression [44].

Defective formation of immunologic synapse

Immune cells communicate directly with each other via cell-cell contacts also known as the immune synapse. Cytotoxic cells of the immune system also use this synapse during direct attacks on infected and tumor cells [45]. Formation of the functional synapse requires the assembly of various structural components clustered around the central T-cell receptor, including the critical leukocyte function associated antigen-1 (LFA-1) which is activated when bound to intercellular adhesion molecule 1 (ICAM-1) on the antigen presenting cell [46]. This interaction is especially critical in antitumor immune function (8666900) and can activate CD8+ T-cells in the absence of classical costimulatory molecules such as CD80 and CD86 [47,48]. It would then logically follow that down regulation of ICAM-1 would confer resistance to cytotoxic lymphocyte killing. Indeed, discovery of this key interaction was first described in melanoma cell lines and multiple groups have shown that ICAM-1 expression enhances *in vivo* lymphocyte adhesion in murine fibrosarcoma models [49,50] and in breast, gastrointestinal and hematologic malignancies [51–53]. However, establishing patterns of ICAM-1 expression in head and neck cancer has proved difficult with varying expression levels among patient samples and between cancer types [54,55]. Patients with nasopharyngeal carcinoma and adenoid cystic carcinoma of the head and neck were demonstrated to have elevated soluble ICAM-1 in their sera as compared with normal controls [54,56]. In the case of nasopharyngeal carcinoma, increased levels of soluble ICAM-1 were correlated with recurrence and poor prognosis [57] but in adenoid cystic carcinoma ICAM-1 down-regulation was found to coexist with increased distant metastasis and poor prognosis [56]. Thus, from a mechanistic stance down regulation of ICAM could confer resistance to CTL killing but *in vitro* evidence is lacking, and clinical correlates have yet to be fully elucidated.

Neutralization of granzyme release from T cells

After successful recognition and binding of the TCR to MHC I, T-cells can induce apoptosis of cancer cells via the granule exocytosis pathway, important in early onset target cell apoptosis [20,21,58]. Activated lymphocytes secrete perforin, a membrane permeability protein, and granzymes, proteolytic enzymes that activate caspases [59], from cytotoxic granules toward the target cells. It is not fully understood whether release of perforin into the immunosynapse forms pores directly on the tumor plasma membrane to allow the entry of granzymes into the tumor cell or if the granules enter target tumors cells through receptor-mediated endocytosis followed by the subsequent perforin-dependent release of granzyme content from the endosome into tumor cytosol [60]. It is known, however, that calcium-dependent polymerization of perforin leads to loss of plasma osmotic stability and leads to the channel-like pore formation [61]. After pore formation, granzyme B is the main effector enzyme. Granzyme B causes target cell apoptosis directly through cleavage of BID, a member of the pro-apoptotic BCL-2 family [62,63] resulting in cytochrome c release. Thus, granzyme B induced apoptosis acts in a caspase independent manner [64].

Tumor cells may be resistant to granzyme mediated apoptosis due to expression of the serine protease inhibitor (serpin) PI-9, or the murine

counterpart SPI-6, which inactivates the effects of Granzyme B [59]. Normally, PI-9 is expressed in immune privileged sites, such as in lymphoid tissue, on T lymphocytes, and on NK cells, possibly as a mechanism to inactivate immune cell self-destruction by Granzyme B [65,66]. PI-6 is expressed in human melanoma, breast, cervical, and colon carcinomas and, notably, is not detectable in normal melanocytes, breast or cervical epithelium, or colon tissue [66]. PI-9 expression on tumor cells is clinically associated with poorer outcomes in melanoma patients with metastatic disease [67].

Resistance to death receptor-induced extrinsic apoptosis

Late onset apoptosis mediated by T cells is largely induced through "death receptors" activated by tumor necrosis factor (TNF) receptor superfamily ligands such as Fas receptor (CD95/Apo-1) and death receptor (DR) 4 or 5, the receptors for tumor necrosis factor related apoptosis inducing ligand (TRAIL) [21,68,69]. Specifically, T cells express surface FAS ligand (FasL or CD95L) which binds to Fas on cancer cells. Ligand binding to Fas leads to the recruitment of Fas-associated death domain (FADD) and procaspase-8 to form the death inducing signaling complex (DISC), which in turn recruits and cleaves procaspases to form active caspases resulting in cell apoptosis [21,70].

Fas is expressed in normal squamous epithelia, including gingival and basal cell layers. Malignant cells of head and neck cancer have variable expression of both Fas and FasL [70]. In tobacco related intraoral carcinoma, for example, tumor cells downregulate Fas receptor and upregulate FasL, which confers not only resistance to CTL killing but provides a mechanism of tumor induced killing of anti-tumor immune cells that also express the Fas receptor. FasL expression is high in poorly differentiated oral squamous cell carcinoma and negatively correlated with the degree of apoptosis in these tumors [70].

The main mechanisms of tumor-intrinsic resistance to Fas -mediated apoptosis are overexpression of c-FLIP and expression of decoy receptors. c-FLIP, or cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein, is a relatively proximal anti-apoptotic protein that interacts with the Fas induced apoptosis pathway [71]. c-FLIP resembles procaspase-8 but lacks the catalytic site required for downstream death-inducing signaling complex formation and completion of apoptosis. An increased ratio of c-FLIP to caspase-8 expression is associated with increased resistance to Fas-mediated apoptosis [72]. Clinically, c-FLIP overexpression associated with higher clinical stage and lymph node metastases specifically in HNSCC [73]. Cells can be resensitized to Fas mediated apoptosis by decreasing or silencing c-FLIP providing and possible therapeutic target. Silencing of c-FLIP utilizing the small molecule inhibitor MLN4924 has been pre-clinically studied in HNSCC [74] and melanoma [72,75], and clinical trials across a variety of cancer types are in progress. In oral squamous cell carcinoma, the PI3/AKT signaling cascade can enhance Fas-mediated apoptosis through downregulation of cFLIP [76]; however therapeutic benefits of this interaction have yet to be explored.

Notably, c-FLIP overexpression does not inhibit granzyme B-mediated apoptosis [77], but *in vivo* c-FLIP overexpression does result in tumor immune escape, suggesting that evasion Fas-dependent apoptosis may be a more influential mechanism for *in vivo* tumor immune escape than Granzyme B-mediated apoptosis [78,79]. In a mouse model of viral FLIP (v-FLIP), tumor immune escape only occurred in immune competent mice and the effect of v-FLIP was not seen in mice lacking B and T cells further supporting the hypothesis that FLIPs effects on Fas-mediated apoptosis is directly related to cytotoxic lymphocyte killing [80]. Finally, the possibility exists that c-FLIP's overall impact on cancer severity is related to c-FLIP's activating effects on the NFκB, MAPK, and ERK pathways [71,81,82] in addition to increased resistance to CTL killing; however, these effects require further study.

Decoy receptors are members of the TNF receptor family that morphologically mimic true death receptors but lack a functional death domain [79,83]. As a result, they competitively bind to FasL, and prevent Fas death receptor-induced apoptosis. Soluble Fas (sCD95) and decoy receptor 3 (DcR3) are two decoy receptors that prevent FasL binding [83,84]. sCD95 is expressed in many cancer types, including melanoma [85] and is specifically associated with a worse prognosis in melanoma [86]. DcR3 expression and function has been described in several cancer types [87]. Tumor necrosis factor related apoptosis inducing ligand (TRAIL) is another member of the TNF superfamily expressed by cytotoxic lymphocytes that can initiate apoptosis by binding to its associated death receptors DR4 and DR5 [69]. These receptors induce apoptosis via the same mechanisms as the Fas mediated pathway; however, TRAIL mediated signaling can also induce cleavage of the proapoptotic BH3 protein Bid creating the active, truncated Bid (tBid), which in turn interacts with Bax and Bak at the mitochondrial membrane to release apoptotic factors, including cytochrome c [69,88].

HNSCC cells demonstrate resistance to TRAIL-death receptor mediated apoptosis via a variety of mechanisms. Beginning at the receptor site, resistance may be conferred by polymorphisms in DR4 resulting in loss of receptor function due to dominant negative expression of the mutated protein. Down regulation of DR5 receptor expression via histone deacetylation also confers resistance to CTL mediated killing [89]. Malignant cells may also express TRAIL receptors DcR-1 and DcR-2, decoy receptors that do not possess an intracellular death domain and cannot form signaling complexes to induce apoptosis. These receptors have been documented in several cancer types including HNSCC [90].

Caspase mutations

Caspase 8 is involved death receptor mediated mechanisms of cytotoxic T-lymphocyte killing and, to a lesser extent, granzyme B mediated apoptosis [91]. Caspase 8 mutations are associated with tumor resistance in many cancer types, including head and neck cancers [92,93]. Caspase 8's critical role in the extrinsic apoptosis pathway likely means that mutated Caspase 8 results in a global decrease in extrinsic apoptosis pathway activation. In HNSCC, mutated procaspase-8 can be constitutively bound to FADD and can impair recruitment of DISC components. Finally, tumors bearing caspase 8 mutations are further associated with enhanced tumor migration, invasion, and growth [93].

Intrinsic cell cycle alterations in response to cytotoxic insults

Many tumors including HNSCC harbor mutations in proteins such as p53, p21 and Rb that affect the G1/S cell cycle checkpoint [94,95]. As a result, cancer cells become more dependent upon the G2/M cell cycle checkpoint for cellular and DNA repair after exposure to cytotoxic or genotoxic insults. This gives cells time to repair injury before segregation of damaged DNA during mitosis. Cell cycle pause after insult has been a recognized phenomenon following chemotherapy and ionizing radiation for some time [96]. More recent work has demonstrated that granzyme B and TNF-α similarly activate the G2/M cell cycle DNA damage checkpoint during early and late phases of T cell killing [97–99]. This intrinsic mechanism of resisting damage and induction of apoptosis reduces tumor cell susceptibility to T cell killing. Reversal of G2/M cell cycle arrest via Wee1 kinase inhibition makes them more susceptible to *in vitro* and *in vivo* T-cell killing in multiple pre-clinical models including models of HNSCC [99,100].

Clinical repercussions of altered tumor cell immunogenicity

In tumors composed of cells that harbor intact IFN responses and T cell recognition pathways, lack of development of T cell responses to immunorecessive antigens may be overcome with immunotherapies such as immune checkpoint blockade [101]. However, the mechanisms of altered tumor cell immunogenicity described above suggest that a tumor cell could evade T cell recognition and destruction despite

harboring one or more T cell antigens. Accordingly, recent genomic studies exploring predictors of response to immune checkpoint inhibitors have demonstrated tumors harboring alterations in IFNγ response genes and genes encoding for JAK1/2 and β2M do not respond to treatment [39,40,102,103]. If defects in immunogenicity are detected within a tumor, what other immunotherapy approaches could be effective? It likely depends upon which pathways are altered. If a tumor harbors defects in T cell antigen processing and presentation, defective immune synapse formation or response to IFN, these primarily alter TCR recognition of intracellular antigen presented via MHC I. Thus, immunotherapy that does not rely on TCR signaling such as NK cellular therapies (which are MHC and antigen independent) or chimeric antigen receptor (CAR) engineered T cellular therapies (which recognize a cell surface epitope similar to an antibody) may be effective. Conversely, if tumors harbor defects in responses to granzymes or TNFR superfamily ligands (FasL, TRAIL) or caspase mutations, then any form of immunotherapy is likely to be ineffective since both T and NK cellular therapeutics ultimately use the same mechanisms to destroy a tumor cell. In these situations, standard anti-cancer approaches such as surgical resection or localized ionizing radiation may be the only rational approach. Ultimately, integration of genomic characterization of tumors with different T and NK cell-based immunotherapy approaches is needed to determine if identification of such alterations predicts responses. If they do, this would be a major advance toward the ability to personalize treatment approaches for patients with HNSCC.

Conclusions

Recent advances in cancer immunotherapies have indeed provided exciting therapeutic options for HNSCC patients; however, current FDA-approved therapies stimulate endogenous immunity being blocked by immune checkpoints only. This can certainly benefit some patients. Yet, many tumors harbor cells with defects in antigen processing and presentation, responses to IFN, and direct susceptibility to T cell killing through granzymes and TNFR superfamily ligands. As a result, some tumors may harbor a population of cells that simply cannot be detected and/or destroyed by T cells. Only through complete genomic characterization of tumors and a thorough understanding of how immune cells detect and eliminate tumor cells can we hope to prospectively assign specific immunotherapy or standard anti-cancer therapy approaches to individual patients in a personalized fashion. As more immunotherapy tools are developed that are independent of T cells, such as NK cellular therapies, integration of genomic characterization into prospective trials to determine which tumor genomic alterations predict responses to which immunotherapy becomes critical. Such ability is a major goal of the immunotherapy research community in order to minimize treatment of cancer patients with treatment approaches that have little to no chance of patient benefit.

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Conflict of interest statement

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