

Immune Escape Mechanisms and their Clinical Relevance in Head and Neck Squamous Cell Carcinoma

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Abbreviations

β_2 -m, β_2 -microglobulin; CAF, cancer associated fibroblast; CSC, cancer stem cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECM, extracellular matrix; EGF-R, epidermal growth factor receptor; ER, endoplasmic reticulum; FDA, Food and Drug Administration; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; ICP immune checkpoint; ICPi, immune checkpoint inhibitor; IFN, interferon; LMP, low molecular weight protein; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cell; mTOR, mammalian target of rapamycin; MSI, multispectral imaging; NK, natural killer; OS, overall survival; PBL, peripheral blood lymphocytes; PBMNC, peripheral blood mononuclear cells; PD1, programmed death receptor 1; PD-L1, programmed death ligand 1; PFS, progression-free survival; PI3K, phosphatidyl-linositol-3-kinase; R/M, recurrence and or metastatic; STAT, signal transducer and activator of transcription; TAA, tumor-associated antigen; TAM, tumor associated macrophages; TAP, transporter associated with antigen processing; TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte; TLS, tertiary lymphoid structure; TME, tumor microenvironment; Treg, regulatory T cell; TSA, tumor-specific antigen; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor.

Abstract

Immunotherapy has been recently approved for the treatment of relapsed and metastatic head and neck squamous cell carcinoma (HNSCC). However, the response of patients is limited and the overall survival remains short with a low rate of long-term survivors. There exists growing evidence that immune escape mechanisms play an important role for the low efficacy of immunotherapies in this disease. These are caused by diverse complex processes characterized by (i) changes in the expression of immune modulatory factors in tumour cells, (ii) alterations in the frequency and composition of immune cell subpopulations in the tumour microenvironment and peripheral blood leading to reduced innate and adaptive immune responses, (iii) impaired homing of immune cells to the tumour site as well as (iv) the presence of immune suppressive soluble and physical factors in the tumour microenvironment. We here summarize the major immune escape strategies of HNSCC lesions, the role of the tumor microenvironment in this process, the clinical relevance of HNSCC-induced immune tolerance, currently employed immunotherapeutic approaches and possibilities to overcome resistance to immunotherapy thereby improving the HNSCC patients' survival.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer worldwide with 700.000 new cases diagnosed in 2019 with a mortality rate ranging between 40 – 50 % [1] [2]. The development of HNSCC has been correlated to life-style risk factors like smoking and alcohol consume as well as to infections with human papilloma viruses (HPV) [3] [4]. Conventional treatment for HNSCC comprises of surgery, radio- and/or chemotherapy, which is associated with a substantial high morbidity and toxicity rate. Therefore, more effective therapies for the treatment of this disease are urgently needed. Recently, a number of targeted therapies using either monoclonal antibodies or tyrosine kinase inhibitors directed against the epidermal growth factor receptor (EGF-R), the vascular endothelial growth factor (VEGF) and its receptor (VEGF-R), the phosphatidyl-inositol-3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) has been approved for the treatment of HNSCC, such as e.g. cetuximab, panitumumab, zалутумумаб, gefitinib, erlotinib, lapatinib, bevacizumab, sorafenib, rapamycin and everolimus, with significant, but still limited response rates ranging between 10 – 15 %. The low efficacy of these targeted therapies might depend on the molecular and immunological make up as well as the heterogeneity of HNSCC lesions with a number of genetic drivers of oncogenesis altering also the anti-tumoral immune responses. During the last years, immunotherapy approaches have been shown to improve the survival of HNSCC patients, which include immunomodulatory antibodies, vaccines, oncolytic viruses and adoptive cell therapy. Treatment with immune checkpoint inhibitors (iCPI) targeting the programmed death receptor 1 (PD-1), like nivolumab and pembrolizumab and its ligand PD-L1 (atezolizumab), had significant clinical effects in a subset of HNSCC patients. However, the majority of HNSCC patients are resistant to these immunotherapies. This might be due to HNSCC (stem) cell mediated progression, recurrence or metastases, but the underlying molecular mechanisms of resistances are so far mainly unknown [5-7]. It is currently being discussed whether cellular and soluble factors of the tumor microenvironment (TME), alterations of the immunogenicity of tumor cells including cancer stem cells (CSC) *per se*, the composition of peripheral blood mononuclear cells (PBMNC) and/or the presence of immune suppressive mediators might lead to immune evasion.

Approximately 50 years ago, research suggested that pre-malignant cells frequently occur, but are spontaneously eliminated by the immune system, before an invasive tumor can develop thereby preventing neoplastic transformation. This hypothesis was supported by epidemiological data demonstrating that patients infected with the human immunodeficiency virus and patients receiving immune suppressive therapy were more prone to cancer [8] [9]. Furthermore, tumor patients can develop spontaneous cancer regressions [10] as well as an increase of HNSCC

incidence was detected in transplanted as well as immune suppressed patients [11]. These data led to the assumption that tumors can evade immune responses by developing diverse immune escape strategies, which will allow the selection for and escape of non-immunogenic cells from immune surveillance by inhibiting their cytotoxic functions of immune cells via distinct mechanisms [12]. Thus, there exists an interplay between the immune system and tumors, which was defined as cancer immunoediting [13-16]. Next to the direct immune escape mechanisms of tumors, an immune suppressive network within the TME was described. This observation represents the basis for the development of novel individualized immunotherapies for cancer. In this review, the diverse processes leading to tumor immune escape in HNSCC and their impact on immunotherapies will be discussed.

Immune escape mechanisms of tumors

It is generally accepted that an effective T cell activation is required for a proper anti-tumor response [17], which consists of two distinct signals. The first signal is mediated by the interaction of the T cell receptor (TCR) with tumor antigens presented by human leukocyte antigens (HLA) class I molecules, the second by the interaction of co-stimulatory receptors with their ligands resulting in T cell proliferation, cytokine secretion and cytotoxicity. A pre-existing intratumoral anti-tumor immune response has been associated with a favourable outcome and responsiveness to immunotherapies [18].

Tumor antigens can be classified into tumor-associated antigens (TAAs) or tumor-specific antigens (TSA) [19] [20]. TAAs are expressed by both malignant and healthy cells, while TSA are expressed only by tumor cells. TSA and TAA are proteins, from which T cell epitopes are generated by the multicatalytic proteasome complex consisting of the constitutive subunits X, Y and Z as well as the interferon (IFN) inducible low molecular weight proteins (LMP) 2, 7 and 10. The yielded peptides are transported via the transporter associated with antigen processing (TAP) subunits TAP1 and TAP2 from the cytosol into the endoplasmic reticulum (ER) and then loaded onto HLA class I molecules. The trimeric complex consisting of the MHC class I heavy chain (HC), β_2 -microglobulin (β_2 -m) and the respective TAA/TSA derived epitope is then shuttled via the trans-Golgi to the cell surface and presented to CD8 $^+$ cytotoxic T lymphocytes (CTL) [21] [22]. Complete loss or downregulation of HLA class I surface expression leads to evasion of tumor cells from CTL recognition. The loss or downregulation of HLA class I expression is often mediated by an abnormal expression and function of major components of the HLA class I antigen processing machinery (APM). This can be either due to structural abnormalities or due to deregulation at the

transcriptional, epigenetic, post-transcriptional as well as post-translational level of APM components. In addition, the loss of adhesion molecules, e.g. ICAM-1, and the frequency of tumor infiltrating lymphocytes (TILs) interferes with anti-tumoral responses [23, 24]. Furthermore, non-classical HLA class I antigens, like HLA-G and HLA-E, may be expressed in tumors at a high frequency leading to tolerance not only against CTL, but also against natural killer (NK) cell-mediated cytotoxicity. In addition, immune suppressive ligands, like PD-L1 and nectin 4, and apoptosis-inducing ligands, such as the fas ligand and TRAIL, have been reported to be expressed by tumor cells and/or host's myeloid cells, which results in apoptosis or anergy of T and/or NK cells.

The composition and frequency of immune cells within the TME and peripheral blood plays an important role in tumorigenesis. While NK cells, CD4⁺ and CD8⁺ T cells, dendritic cells (DC) and proinflammatory M1 macrophages promote anti-tumor immune responses, heterogeneous populations of myeloid-derived suppressor cells (MDSC), FOXP3⁺ regulatory T cells and M2 macrophages counteract tumor immunity [17] [25]. The complexity of the immune system is reflected by the composition of the immune cell infiltrate, the amount of inflammation and the frequency of TILs, which affect the overall survival (OS) of patients. These observations resulted in the categorization of inflamed, immune desert and immune excluded tumor phenotypes [26]. Tumors may also secrete various immune suppressive and anti-apoptotic factors like TGF- β , IL-10, PGE2 and IL-6 as well as release immune suppressive extracellular vesicles (EV), such as micro-vesicles and exosomes and different metabolites including arginase, adenosine, indolamin-2,3-dioxygenase (IDO) and nitric oxygen species (NOS) [27][28]. Furthermore, the immune system is also affected by metabolic changes in the TME due to hypoxic and acidic conditions [29].

The role of classical and non-classical HLA class I and antigen presentation and processing in HNSCC immunotherapies in HNSCC

The expression of classical HLA class I antigens was shown to be diminished at a high frequency varying from 20 up to 80% of HNSCC lesions and cell lines whereas β_2 -m expression was absent in approximately 16 % of tumors [30] [31] [32] [33]. There was no difference in the frequency of HLA class I abnormalities between HPV⁻ and HPV⁺ tumors [34]. Interestingly, the loss or downregulation of HLA class I and β_2 -m expression was correlated with the loss of chromosomal regions at 6p21.3 and loss of heterozygosity (LOH) at 6p21.3 and 15q [32, 35].

Reduced expression of HLA class I antigens was associated with a downregulation in the expression of various APM components. While TAP1, LMP2, LMP7 and β_2 -m expression were downregulated in up to 70% of HNSCC lesions, calnexin and ERp57 were only downregulated in approximately 25% of lesions [33, 36, 37]. The extent of HLA class I APM defects was associated with a low CD8 $^+$ T cell infiltration. It has been also demonstrated that overexpression and activation of epidermal growth factor receptor (EGF-R), which is expressed at a high frequency in HNSCC specimens, can lead to a downregulation of HLA class I by affecting downstream pathways, such as STAT1, MAPK and PI3K/AKT. This impaired HLA class I expression could be reversed by treatment with respective inhibitors or by IFN- γ [38]. In contrast, HLA class II expression was only found in a minority of HNSCC lesions, but at a high frequency in stromal cells. Interestingly, HLA class II expression was higher in HPV $^+$ compared to HPV $^-$ tumors [39].

Next to classical HLA class I antigens, the non-classical HLA-G expression was detected in more than 50% of HNSCC lesions, while healthy controls lacked HLA-G expression [40] [41] [32] [33] [37]. A higher frequency of HLA-G-expression was found in HPV $^+$ HNSCC when compared to HPV $^-$ HNSCC [42]. Furthermore, significantly higher HLA-G expression levels were detected in metastases compared to primary tumors [43]. HLA-E was recently also found to be expressed on HNSCC [44]. Regarding PD-L1 expression, its frequency highly varies in the HNSCC tumors depending on the intratumoral localization and the tumor specification as PD-L1 expression was higher in the invasive tumor margin than in the tumor center and higher in HPV $^+$ than in HPV $^-$ HNSCC [34]. As for HLA-G, PD-L1 expression was more frequent in metastases than in primary tumors [45]. Interestingly, the metabolic tumor volume and the glycolytic activity correlated significantly positive with PD-L1 expression [46].

Tumor microenvironment and HNSCC

A number of soluble and cellular factors within the TME of HNSCC have been described to be involved in the immune escape of this disease [47]. Furthermore, the TME has the ability to adapt to environmental demand depending on the tumor metabolism and hypoxia. This reprogramming not only determined the fate and functions of tumor cells, but also of immune cells. Both factors are known to play key roles in the course of HNSCC and to support the progression of this disease [48]. Indeed, due to hypoxia and acidic conditions HNSCC switch to the glycolytic metabolism, which further reduces the pH, which is mediated by the production of lactic acid [49][50]. Importantly, these metabolic alterations in the TME not only affect the HNSCC lesions by altering

the expression of immune modulatory molecules, such as e.g. an increased expression of PD-L1, but also change the immune cell repertoire and activity. This leads to a reduced T cell activation, proliferation, cytotoxicity and diminished antigen processing of DC with simultaneous attraction and accumulation of immune suppressive cells (Treg, MDSC and TAMs) [51] [52]. This is accompanied by a high frequency of CD4⁺ Tregs, which was associated with an increased expression of TNF receptor family members, but a reduced expression of genes involved in the IFN pathway. Interestingly, OX40, PD-1 and CTLA were enriched in T cells isolated from HNSCC [53]. MDSC were found to be recruited in HNSCC, but their role in this disease has not yet been analysed in detail. Moreover, the hypoxic condition induces a stromal accumulation of TAMs, which are responsible for the epithelial mesenchymal transition (EMT) in this disease and might represent an immunotherapeutic target for HNSCC patients [54]. Furthermore, the hypoxia-induced release of VEGF and various chemokines could induce monocytes to differentiate into TAMs, while the secreted VEGF can also cause an abnormal angiogenesis [55] [56]. This angiogenic switch further contributes to the maintenance of the hypoxic TME, additionally lowering the tissue pH.

Last but not least, remodelling of the extracellular matrix (ECM) mediated by cancer-associated fibroblasts (CAFs) occurs and leads to the protection of tumor cells by a biophysical barrier to effector T cells and to resistance against monoclonal antibodies (mAbs) targeting EGF-R [57]. Interestingly, late stage HNSCC consists of more than 80 % CAFs, which secrete HGF thereby inducing a glycolytic switch in tumor lesions [58]. Furthermore, CAFs promote an immune suppressive TME through the induction of a pro-tumoral phenotype in macrophages [46]. This further reflects that different immune escape mechanisms are dependent of each other and crossover thereby creating a redundant and highly efficient immune suppressive system.

Recently, the immune landscape of HNSCC was analysed and genetic profiles were identified, which delineate tumors in immune active and immune exhausted phenotypes [59]. This gives information on the immune status of HNSCC patients, which could be used for the development of novel (immuno)therapies [60]. Combination of single cell RNA sequencing (scRNA-seq) with multispectral imaging (MSI) that allow to characterize the spatial localization of immune cells and their cellular neighbourhood within the TME will give deeper insights into the immune cell repertoire of HNSCC, their transcriptional states and differentiation trajectories as well as into the cellular crosstalk in the TME with potential relevance to tumor progression [61-63].

In depth analyses of the composition of innate and adaptive immune cells in the TME of patients in HPV⁻ as well as HPV⁺ HNSCC lesions highlighted differences in the immune cell repertoire in

dependence of the HPV status of the tumor specimen, which might have also implications for their treatment with immunotherapies. The distinct immune cell repertoire in the TME might be associated with viral antigens leading to the activation of primarily innate immune responses followed by adaptive immune responses mediated by T and B lymphocytes. Furthermore, single cell analyses demonstrated subpopulations of immune cells in particular of exhausted CD8⁺ T cells with different biological functions [64].

Correlation of the efficiency of (immuno)therapies with immune escape mechanisms

It has been demonstrated that established therapies for HNSCC patients, such as radiotherapy, chemotherapy as well as mAbs directed against the EGF-R affect the TME. Since CD8⁺ TILs exhibit a high frequency of PD1 and TIM-3 expression accompanied by high levels of granzymes and perforin and is associated with the clinical outcome, a regulatory role for TIM-3 and PD1 was suggested in cetuximab-promoting cytolytic activities of CD8⁺ TILs. Furthermore, the increased frequency of PD-1⁺ and TIM-3⁺ CD8⁺ TILs was inversely correlated with the clinical outcome of cetuximab therapy [65].

Treatment of HNSCC patients with immunotherapies increases the overall survival (OS) of HNSCC patients suggesting that the immune system might be targeted by these drugs to achieve clinical benefits for these patients. Despite 20-30 % of HNSCC patients treated with mAbs directed against PD1 or its ligand PD-L1 have a better OS the efficacy is still limited [66]. Thus, there is an urgent need to increase the knowledge of the complex biology of this disease, in particular of immune escape mechanisms, anti-tumoral immune responses and the composition of the TME in order to improve treatment efficacy [67]. These data might help to identify features associated with responsiveness to immunotherapy and might also lead to the design of novel treatment regimens using single or multi-agent immunotherapies alone or in combination with standard therapies. Some drugs targeting these distinct mechanisms are currently in the clinical development or already approved for the treatment of HNSCC [1]. Next to targeting the PD1/PDL1 pathway, a number of novel immunotherapeutic targets are currently in preclinical studies and clinical phase I and II trials as single agent or in combination with other checkpoint molecules. These include drugs targeting LAG-3, TIM-3 and ICOS [68].

Impact of immune escape mechanisms on HNSCC patients' outcome

Both, the direct immune escape mechanisms of tumor cells as well as the composition of the TME, have an impact of the outcome and prognosis of the HNSCC patients. Recently, tertiary lymphoid structures (TLS) with a high frequency of B cells were identified in HNSCC and found to be associated with an improved survival like in other tumor entities, whereas high frequencies of intratumoral B-lymphocytes rather indicated an adverse outcome [69] [70]. Germinal center derived B cells were present during disease progression of HPV⁺ HNSCCs and with a reduced frequency in HPV⁻ HNSCC [71] [72].

High levels of TILs have been associated with improved survival in HPV⁻ HNSCC [73]. The frequency of both CD8⁺ and CD3⁺ T cells, have been associated with an increased OS after chemoradiotherapy in HPV⁺ and HPV⁻ HNSCC [74] [75] [76]. In several studies, HNSCC have been shown to possess a high degree of Treg infiltration [70] [77] [78], which correlates with a favourable OS [70] [78]. This might reflect the downregulation of inflammation, which triggers the initiation of carcinogenesis [79].

In a more recent multicenter study of patients with HNSCC after post-operative chemoradiotherapy, a high CD8 TIL density in the tumor periphery, tumor stroma, and tumor cell area was predictive for improved OS [74]. In another study, only stromal TIL infiltration was associated with increased OS [80]. Concerning the clinical relevance of PD-L1 expression on the outcome of HNSCC patients, there exist controversial data. In a laryngeal HNSCC cohort, high PD-L1 expression assessed by Automated Quantitative protein Analysis (AQUA) positively correlated with disease outcome [81]. In a recent report by Yang and co-authors, PD-L1 was shown to correlate with improved progression-free survival (PFS), but not OS in patients with advanced HNSCC. As expected, patients with combined low frequency of TILs and high expression of PD-L1 were characterized by dismal survival [82]. Another retrospective analysis assessing the PD-L1 expression in a large cohort of patients demonstrated that high PD-L1 expression was the strongest predictor of worse patients' outcome independent of tumor stage and distant metastases [83]. In cancers of the oral cavity, increased PD-L1 expression has also been shown to correlate with a poor patients' survival [84]. Furthermore, TIM-3, LAG-3, IDO and CTLA4 expression was negatively correlated with OS [85].

Therapeutic strategies to overcome immune escape mechanisms in HNSCC patients

So far, the OS for patients with recurrent/metastatic (R/M) disease is only 10-13 months [86] [87] [88][88, 89] when applying the current standard of care for locally recurrent disease (without

surgical or radiation treatment options) and/or metastatic disease in the first-line setting with platinum-based double chemotherapy and cetuximab. Furthermore, second-line treatment options with cetuximab, methotrexate and taxane demonstrated a response rate between 10–13 % and median PFS of 2-3 months without any obvious improvement in OS [90] [91]. The data from Checkmate 141 and KEYNOTE-040 provided evidence for the use of single agent anti-PD-1 immunotherapy for the treatment of R/M HNSCC patients with disease progression after platinum-based chemotherapy. Furthermore, the KEYNOTE-048 study using anti-PD-1 therapy in patients with R/M HNSCC who had not received prior treatment with platinum-based chemotherapy also revealed a clinical benefit suggesting the use of pembrolizumab as an appropriate first-line treatment for PD-L1⁺ HNSCC [92]. In 2016, the US Food and Drug Administration (FDA) received the first immunotherapeutic approvals for the treatment of HNSCC patients with iCPI, namely the anti-PD-1 immune checkpoint inhibitors nivolumab and pembrolizumab, for the treatment of patients with HNSCC that are refractory to platinum-based regimens. The European Commission followed in 2017 with the approval of nivolumab and shortly thereafter with the approval of pembrolizumab monotherapy for the treatment of recurrent or metastatic HNSCC in adults, whose tumors express PD-L1 with a ≥ 50% tumor proportion score and that have progressed after platinum-containing chemotherapy. In 2019, the FDA granted approval of pembrolizumab as first-line treatment for patients with metastatic or unresectable, recurrent HNSCC, in combination with platinum and fluorouracil and of pembrolizumab as a single agent for patients with HNSCC, whose tumors express a PD-L1 combined positive score ≥ 1. These approvals marked the first new therapies for these patients since 2006 as well as the first immunotherapeutic approvals in this disease.

However, to improve immunotherapy a much better understanding of emerging immunotherapies, including appropriate patients' selection, therapy sequence, response monitoring, adverse event management and biomarker testing, is required to guide improvements in care. In order to address these issues, the Society for Immunotherapy of Cancer (SITC) established the Cancer Immunotherapy Guideline - Head and Neck Cancer subcommittee to provide evidence-based recommendations on how to incorporate immunotherapies into practice for the treatment of patients with HNSCC [1].

In this context, insights into the complex biology of this disease in particular of the anti-tumoral immune response and the TME is urgently required. This knowledge might help to identify features associated with non-responders and responders as well as the development of resistance to the immunotherapies. Based on this information, novel treatment regimens using

single or multi-agent immunotherapies alone or in combination with standard therapies could be developed. Next to the PD1/PD-L1 pathways, some drugs targeting these distinct mechanisms are currently in preclinical studies, in clinical phase I and II trials as single agent or in combination with other checkpoint molecules or already approved for the treatment of HNSCCs. These include antibodies directed against LAG-3, TIM-3, TIGIT and ICOS [68] [93] [94] [95]. In addition, a bifunctional fusion protein to block PD-L1 and β receptor has been applied in a phase I trial demonstrating a clinical activity [96].

Further approaches address the natural killer antibody-dependent cell cytotoxicity and the use of NK cell therapy. Since the expression of HLA-I is reduced up to 80% compared to the surrounding healthy tissues low/neg. [30-32], NK cells might have the power to eliminate the HLA-I HNSCC cells via the missing self-recognition. However, HNSCCs express high levels of HLA-G and HLA-E [40] [44], which inhibits both NK and CD8 $^{+}$ cells via ILT2/4 and/or NKG2A [97, 98]. Therefore, mAbs targeting NKG2A, such as monalizumab could be employed for the treatment of HNSCC by blocking the interaction with HLA-E and/or HLA-G. Indeed, a phase II study of monalizumab combined with cetuximab showed an excellent activity in previously treated HNSCC patients including some previously exposed to cetuximab [44].

Conclusions

It is obvious that HNSCC are able to develop or use a multitude of mechanisms to escape T and/or NK cell-mediated immune surveillance. Thus, one single strategy might be often not sufficient to mount proper immune responses, since HNSCC evade immune recognition by different approaches, which explain the limited efficacy of immune therapies in this disease. Increased insights to the immune escape mechanisms will help in the design of novel as well as optimized immune therapeutic strategies, which either have to be directed against tumor cells or revert the immune suppressive TME.

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Author's contributions

Barbara Seliger conceptualized and wrote the review, Claudia Wickenhauser was responsible for the clinical part, all authors worked on the manuscript, discussed the contents and corrected the manuscript.

Conflict of interest

The authors have no conflict of interest.

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