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Hot Topic

Biomarkers for immunotherapy response in head and neck cancer

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ABSTRACT

Preclinical data suggest that head and neck squamous cell carcinoma (HNSCC) is a profoundly immunosuppressive disease, characterized by abnormal secretion of proinflammatory cytokines and dysfunction of immune effector cells. Based on landmark phase III trials, two anti-Programmed Cell Death-1 (PD-1) antibodies, pembrolizumab and nivolumab have been approved for HNSCC by FDA and EMEA in the recurrent/metastatic setting; in addition, pembrolizumab has recently received FDA and EMEA approval as first line treatment. In clinical practice, only a minority of patients with HNSCC derive benefit from immunotherapy and the need for the discovery of novel biomarkers to optimize treatment strategies is becoming increasingly more relevant. Although currently only PD-L1 is widely used as a predictive biomarker for response to immune checkpoint inhibitors in HNSCC, there are many ongoing trials focusing on the identification of new biomarkers. This review will summarize current data on emerging biomarkers for response to immunotherapy in HNSCC.

Introduction

Harnessing immune system responses against cancer cells ushered a new era for oncology. Deciphering the mechanisms of cancer cell deregulation and resistance to conventional treatments resulted in preclinical evidence-based findings and translated into groundbreaking clinical results with substantial impact on survival and quality of life of some cancer patients even of terminal stage.

Immunotherapy outcomes are closely linked to the seven steps of Cancer-Immunity Cycle as proposed by Chen, Coukos and Mellman. According to this cycle, cancer cell eradication by the immune cells is a stepwise process beginning with cancer immune recognition and mounting of an adaptive immune response, to cancer cell elimination; each of them represents an eligible target for treatment, as well as a potential strategy for cancer immune escape [1]. In this context inhibition of PD-1/L1 checkpoint axis using monoclonal antibodies (mAbs) has shown a well-established efficacy in the treatment of numerous cancer types including head and neck squamous cell carcinoma (HNSCC) and has found wide clinical application in recent years, while novel approaches using combinations of checkpoint inhibitors with radiotherapy and/or chemotherapy, cytokine-based and/or adoptive T cell therapies are investigated in ongoing clinical trials [2].

Moreover, in 2016, the US Food and Drug Administration (FDA) approved two immune checkpoint inhibitors (ICI), the anti-PD-1 mabs, nivolumab (CheckMate141) (Opdivo, Bristol-Myers Squibb) and pembrolizumab (KEYNOTE-012) (Keytruda, Merck), as second line treatment of patients with recurrent/metastatic (R/M) HNSCC refractory to platinum-based therapy. A year later, Nivolumab was approved by the European Medicines Agency (EMEA) in the platinum refractory R/M setting while Pembrolizumab approval by EMEA in the same setting was restricted to those patients with PD-L1 Tumor Positive Score (TPS) ≥ 50% . In 2019, the FDA and EMEA approved pembrolizumab (KEYNOTE-048) for the first-line treatment of patients with R/M HNSCC who express PD-L1 Combined Positive Score (CPS) ≥ 1% . FDA approved Pembrolizumab with chemotherapy in patients with R/M HNSCC as a first line treatment regardless of PD-L1 score while EMEA restricted approval in patients with CPS PD-L1 expression ≥ 1% [3].

Despite the initial enthusiasm, the clinical benefit to ICI varies among R/M HNSCC patients with only 18% of them experiencing positive outcomes as shown in phase 1/2 KEYNOTE-012 [4]. ICI mechanism of action is not by direct cytotoxic effect as in traditional chemotherapy rendering more challenging their therapeutic response

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evaluation with the current radiographic response criteria like RECISTv1.1. Although ICI have better toxicity profile compared to EXTREME regimen, their immune-related adverse effects are entirely different to the traditional cytotoxic agents. Altogether, cost-benefit analysis of these new therapies emphasizes the necessity of effective patient selection. The need for predictive biomarkers in this setting has become imperative not only for providing the drug of choice with maximal patient benefit using a more individualized approach, but also for shedding light to newer combination therapeutic regimens and possible targets against this anatomically and biologically heterogeneous malignancy.

In July 2019, the Society for Immunotherapy of Cancer issued the first guidelines on immunotherapy for the treatment of HNSCC. The subcommittee among others has attested to the need for identification and understanding of factors with predictive value so we can maximise immunotherapy efficacy and spare toxicities in selected patients [5]. Aim of this review is to provide current knowledge for biomarkers in HNSCC.

Tumour related factors

PDL-1 expression

PD-1 checkpoint receptor expressed on activated T cells has been shown to promote immunosuppression upon interaction with its ligands, PD-L1 and PD-L2, located on cancer cells and immune infiltrating cells [6–8]. PD-L1 expression on immune cells in pre-treatment tumour biopsies is indicative of formerly elicited anti-tumour adaptive immune response [9] and has been associated with improved treatment outcomes [10]. Consequently, blocking PD-1/PD-L1 interaction with anti PD-1 or anti PD-L1 mAbs promotes the reactivation of immunity, resulting in durable anti-tumour effects in a fraction of patients with various solid tumours [11,12] Current evidence in HNSCC suggests that combined positive score (CPS), the number of PD-L1 positive cells including tumour, lymphocytes and macrophages, in relation to total tumour cells, offers a more effective evaluation than tumour proportion score (TPS), which measures PD-L1 expression on tumour cells alone [5].

The superiority of CPS versus TPS in correlation to clinical response to immunotherapy in HNSCC was first described in phase 3 KEYNOTE-048 trial supporting the use of CPS as the optimal biomarker for patient selection in this type of cancer [3,5]. This was a 1:1:1 prospective randomised trial in 882 patients (updated format presented in ASCO 2019) with incurable R/M HSNCC with pembrolizumab (P) (IgG4 humanised anti- PD-1 mab) vs. (P) + chemotherapy (C) vs. the EXTREME. At the second interim analysis (IA2), pembrolizumab significantly improved OS vs. cetuximab-chemotherapy in the CPS ≥ 20 (median 14.9 vs. 10.7 months, HR 0.61 [95% CI, 0.45–0.83]; p = 0·0007) and CPS ≥ 1 (12.3 vs. 10.3 months, 0.78 [0·64–0·96], p = 0·0086) populations and was non inferior in the total population (11.6 vs. 10.7 months, 0.85 [0.71–1.03]). P + C significantly improved overall survival (OS) vs. EXTREME in the total population (13.0 vs. 10.7 months, HR 0.77 [95% CI, 0.63–0.93], p = 0.0034) at IA2 and in the CPS ≥ 20 (14.7 vs. 11.0 months, 0.60 [0.45–0.82], p = 0.0004) and CPS ≥ 1 (13.6 vs. 10.4 months, 0.65 [0.53–0·0.80], p less than 0.0001) populations at final analysis. Neither pembrolizumab nor pembrolizumab-chemotherapy improved PFS at IA2. Overall, KEYNOTE-048 showed that P in CPS ≥ 20 and CPS ≥ 1 and P + C in the total population had superior efficacy to the standard of care, hence backed the FDA approval for pembrolizumab as first line treatment in the incurable R/M HNSCC in June 2019.

Similarly, phase 3 KEYNOTE-040, a multicentre, open-label prospective randomised trial that evaluated the clinical efficacy (OS) and adverse effects of 2nd line (P) vs investigator's choice of standard of care (SOC) treatment (weekly methotrexate, weekly cetuximab or Q3W docetaxel). In the intention-to-treat population, irrespective of PD-L1 status, median OS was 8.4 months (95% CI 6.4–9.4) with (P) vs. 6.9 months (5.9–8.0) with SOC (HR 0.80, 0.65–0.98; p = 0.0161). Among patients with a CPS ≥ 1, median OS was 8.7 months (95% CI 6.9–11.4) with (P) compared to 7.1 months (5·7–8·3) with SOC (HR 0.74; 95% CI: 0.58–0.93, p = 0.0070). Among patients with a CPS score of less than 1, median OS was 6.3 months (3.9–8.9) with (P) vs. 7.0 months (5.1–9.0) with SOC (HR 1.28; 95% CI: 0.8–2.07, p = 0.8476) [13].

PD-L1 expression, along with p16 status for HPV testing in oropharyngeal cancer, has been the most widely used biomarker for patient selection in head and neck cancer clinical trials to date. However, diverse results coming from multiple clinical studies have failed to establish PD-L1 infallibility in patient selection [4,14,15]. Longer overall survival (OS) was observed in phase 1b KEYNOTE 012 trial in 192 patients (60 patients in initial cohort and 132 in the expansion cohort) with PD-L1 ≥ 1. The median (95% CI) OS rates were also significantly different when CPS was used (PD-L1 +, 10 m [9–13 months] vs. PD-L1-, 5 [3–8 months]; one-sided P = 0.008), a finding confirmed also by long-term follow-up analysis, and additionally PD-L2 was associated with better overall response to treatment both on its own and co-expressed with PD-L1 (both ORR 23% vs. 10%). However, the authors noticed responses in PD-L1 – tumours in a significant portion of patients (9%) questioning the validity of PD-L1 as biomarkers for the use of (P) [4,16].

Nonetheless, in KEYNOTE 055, a phase II study on the use of (P) as 2nd line treatment for R/M HNSCC, although PD-L1 positive patients demonstrated, as expected, higher response rates, PD-L1 negative patients also had significant therapeutic benefit, suggesting that PD-L1 alone should not be used as a determinant for patient exclusion from immunotherapy and alternative biomarkers need to be explored [15]. Similar findings were seen in phase III CHECKMATE 141 trial investigating nivolumab treatment for R/M HNSCC, where although patients with > 1% TPS showed better PFS, there was no significant difference in overall survival (OS) between patients expressing and those not expressing PD-L1 [14,17].

Two more interesting issues pertained to PD-L1 evaluation are the intra-, inter-tumour heterogeneity and the differences in "cut-offs" defining positive or negative, as well as the reagents used for staining [5]. Recently, Rasmussen et al raised the intratumoural heterogeneity in PD-L1 after prospectively studying 33 whole surgical specimens of 16 patients with HNSCC [18]. They reported that with 1% cut off, 36% of the specimens were concordant with TPS and 52% with CPS, whereas a 50% cut-off value would yield a concordance of 70% with TPS and 55% with CPS. Defining a tumour as positive if just a single-one of the biopsies was positive, the negative predictive value (NPV) of a single negative core biopsy was 38.9 and 0% (1% cut off), and 79.9% and 62.8% (50% cut off) for TPS and CPS, respectively. This fact could account for the PD-L1- responders in ICI treatment. Also, Ratcliff et al. presented in ESMO 2016 their comparative study between 3 different PD-L1 staining assays used in HNSCC clinical trials to date (the Ventana SP263 assay in durvalumab (anti-PD-L1) clinical trials, the Dako 28–8 and Dako 22C3 assays, commonly used in nivolumab (Opdivo®) and pembrolizumab (Keytruda®) trials, respectively). After staining 108 tumour biopsies they found that overall percent agreement was > 90% [19].

Taken together, the SITC subcommittee comments that Tumour PD-L1 expression generally correlates with improved efficacy with anti-PD-1/PD-L1 ICIs in R/M HNSCC, with increased predictive value when including PD-L1 expression on tumour infiltrating immune cells *i.e.* CPS. Nevertheless, some patients who are PD-L1 negative still benefit from treatment with these agents [5].

Tumour mutational burden/ neo-antigens

Recently a significant number of studies have established the major role of neo-epitopes, resulting from non-synonymous mutations on tumour cells, in cancer immune recognition and specific T-cell activation [20,21]. Although tumours with high frequency of missense mutations

demonstrate increased density of infiltrating CD8 + T cells and are related to better outcomes [22], only a small proportion of these mutations results in the production of neo-antigens and only a part of these neo-antigens leads to T-cell recognition and reactivity [23]. Analysis of various types of tumours using RNA sequencing revealed that it is specifically immunogenic mutations, rather than whole mutational load, that are associated with better survival prognosis and lead to increased expression of *CD8A* and immune exhaustion markers (*PDCD1*, *CTLA4*) creating thus a favourable setting for immunotherapy response [22]. Consequently, as is challenging to predict the ultimate effect of mutations in anti-tumour immune activation, the prognostic value of TMB in respect to immune modulating treatments remains limited.

Nevertheless, TMB has been shown to be a promising biomarker for immunotherapy response by multiple studies. Increased mutational burden has been related to improved response to PD-1 inhibition and prolonged PFS in Non Small Cell Lung Cancer (NSCLC) [24], while research on anti-CTLA4 treatment in melanoma has depicted the association of TMB with clinical response and OS [25,26]. Another study focused on the effect of neo-antigen heterogeneity within both NSCLC and melanoma tumours revealed that high clonality of neo-antigen load predisposes to an inflamed TME and enhances the benefit of immunotherapy suggesting that multiple tumour subclones increase the risk of one of them escaping host immunity [27].

Regarding HNSCC, total mutational load, using a cutoff of ≥102 mutations per exome, was evaluated in KEYNOTE 012 trial and demonstrated a positive correlation with immunotherapy response [4,28]. Additional data from a cohort of 126 patients receiving anti-PD-1/L1 therapy have revealed that higher TMB was observed among responders and it was found to be a positive predictor among HPV-/EBV- patients of the same group. *NOTCH1* and *SMARCA4* mutations showed noticeably greater occurrence in responders in comparison to non-responders and when HPV/EBV viral status was taken into account the above results were only noticed among HPV-/EBV- responders. Moreover, microsatellite instability was higher among responders. In contrast, copy-number alterations indicated no association with response regardless of viral status [29].

Recently, two more studies shed light to the TMB prognostic role in HNSCC [5,8,30] analysing the RNA sequencing data from HNSCC by The Cancer Tumour Atlas (TCGA) and the Chicago Head Neck Genomics. They both concluded that TMB has no correlation with immune cell infiltrates. FDA has yet to recommend about TMB testing [5]. Also, given that microsatellite instability (MSI) is only reported in 1–3%, SITC subcommittee recommends against MSI testing [5].

Interferon-γ gene signature

Type I interferons (IFNs) are implicated in the mechanism by which tumour innate immune sensing results in spontaneous cytotoxic T cell recruitment, a key step for establishing an inflamed type of TME [31–33]. Specifically for HNSCC, the relation of interferons to ICI therapy was investigated in KEYNOTE-O12 trial where a six-gene IFN-γ signature (including *IDO1*, *CXCL10*, *CXCL9*, *HLA-DRA*, *STAT1*, *IFN*-γ gene expression) was examined in pretreatment biopsies. Results showed that IFN-γ gene signature exhibits statistically significant association with Best Overall Response (BOR) and PFS and could become a potential biomarker for patient exclusion from immunotherapy due to its high negative predictive value [4]. Research performed on mice using melanoma cell lines has shown that tumour- derived DNA located within the cytosol of host APCs induces IFN-β secretion through STING and IRF3 activation via the cytosolic DNA sensor cGAS suggesting that a functional STING pathway is essential for an endogenous T cell anti-tumour response and consequently for the effectiveness of immunotherapy [34]. The spontaneous formation of micronuclei within tumour cells is also involved in this process as micronuclear membrane has been shown to be prone to collapse resulting in cGAS relocalisation to the micronuclei and anti-tumour immune activation [35]. In an analysis performed by Ayers et al [36], *IFN*-γ gene expression profile of various types of cancers was examined and positively correlated to clinical response in patients treated with the anti-PD-L1 inhibitor pembrolizumab. STING 'agonists' have been developed in order to overcome immunosuppression caused by defective STING pathway and are currently investigated in early phase clinical trials. (NCT03010176, NCT02675439, NCT03172936).

Tumour microenvironment

Inflamed - Non-Inflamed

Tumour microenvironment (TME) and its implication in cancer development and progression is a promising source for developing predictive immunotherapy biomarkers. TME is classified by its immune cell components into three distinct phenotypes: inflamed tumours, immune-excluded and immune-desert. Inflamed tumours are characterized by abundant intratumoural and stromal infiltration with immune cells, whereas in immune-excluded phenotype immune cell presence is restricted to stroma and immune-desert phenotype is void of T-cells both in tumour bed and stroma [37]. Tolerogenic pathways such as PD-L1 and IDO overexpression stimulated by interferon-γ, in addition to FoxP3 + regulatory cell infiltration, driven by increased CD8 + presence within the TME, are identified as promoters of tumour immune escape for the T-cell inflamed subtype [38]. *CTLA4* methylation was proposed as an eligible IO biomarker in a melanoma study. While *CTLA4* expression was positively correlated with *IFN*-γ*/JAK/STAT1* pathway genes, *CTLA4* methylation was inversely correlated with *IFN*-γ expression and higher levels of methylated CTLA4 were associated with poor response to ICB therapy [39]. Elimination of immune inhibition with immunotherapeutic agents results in the reactivation of pre-existing effector T-cells within the TME rather than in the recruitment of new ones [9,40] which indicates why immunotherapy has been shown to exert positive clinical effects exclusively on T-cell inflamed tumours [41]. Conversely, in non-inflamed tumours immune evasion is achieved through complete T-cell exclusion from the tumour site due to the involvement of various oncogenic pathways. WNT-β-catenin pathway upregulation, impaired Basic leucine zipper transcriptional factor ATF-like 3 (BATF3) DCs, Myc overexpression, *LKB1* and *PTEN* gene mutations or deletion, *p53* inactivation, activating mutations of *IDH1/IDH2* and *FGFR3* genes and STAT3 and Peroxisome proliferator-activated receptor gamma (PPARγ) signalling amplification, all favour an immunosuppressive TME as shown in several preclinical studies on a variety of tumours [42].

Tertiary lymphoid structures (TLS) constitute yet another important promoter of anti-tumour immune response. By mimicking the normal function and architecture of secondary lymphoid organs, TLS have been shown to positively affect prognosis in the majority of solid cancers including HNSCC. Chronic inflammatory state, an invariable component of cancer pathophysiology, stimulates chemokine and cytokine expression within the TME, resulting in the recruitment of lymphocytes from a rich network of high endothelial venules [43] and their arrangement into TLS, with a follicular zone of B-cells organized in germinal centres, surrounded by a T-cell zone. TLS presence in stroma or tumour bed is associated with a direct and prolonged immune attack on the malignant cells. Consequently, in immune exhausted tumours, checkpoint inhibitors could reactivate TLS function and reattain their benefits while in non inflamed tumours, induced TLS generation might enhance anti-neoplastic effects [44].

The effect of tumour microenvironment in HNSCC

As TME structure, components and their effects vary among different malignancies, it is important to outline its specific characteristics present in HNC. Immune infiltration is notable in the majority of HNSCCs indicating an ongoing natural (spontaneous) immune response. However, the fact that even immune-inflamed tumours

ultimately manage to evade host immunity and continue to progress, insinuates that interactions between immune cells, tumour cells and their products within TME restrict adaptive immunity and eventually promote immunosuppression [30,45,46]. Chen et al. proposed a novel stratification of HNSC tumours regarding their immune cell composition and inflammatory marker expression. Using non-negative matrix factorization they were able to identify two distinct subtypes, named Active and Exhausted Immune Class, with the former incorporating characteristics related to favourable immunotherapy outcomes such as high levels of B-cell immune signatures, increased cytolytic activity and M1 macrophage infiltration in contrast with the latter which demonstrates activated stroma, M2 macrophage infiltration and activation of WNT/TGF-β, factors known to favour tumourigenesis [47].

Specific immune cell types such as CD3+,CD8 + and Foxp3 + tumor-infiltrating lymphocytes (TILs) are associated with better prognosis in HNSCC [48]. The positive effect of CD4 + Foxp3 + infiltrating T cells in survival and locoregional disease control might appear inconsistent to their immunosuppressive nature; however, it has been hypothesized that it derives from inhibition of tumour-promoting inflammatory factors, which are abundant in HNC and possibly a direct cytotoxic effect [49,50]. In addition, higher CD8 + T-cell infiltration has been observed among anti PD1/L1 therapy responders and has been proven to be an independent predictive factor for improved prognosis [29]. Supporting these findings, results from a study of PD-1 inhibition in melanoma suggest that tumour infiltration by CD8 + T cells under PD-1/PD-L1 inhibition as well as T-cell clonality are predictive of better therapeutic outcome [9]. TME infiltration by NK cells is also related to better prognosis and their anti-tumour effect may be even more crucial for non-inflamed tumours as they act independently to tumour antigen recognition and presentation [51]. Alternatively, the presence of myeloid-derived suppressor cells (MDSCs), which are attracted to tumour site by GM-CSF, IL-1 and IL-6 expression from cancer cells, M2 polarized tumour-associated macrophages (TAMs) and N2 tumour associated neutrophils (TANs) characterizes a tolerogenic tumour immune landscape [30] but also indicates potential targets for immunosuppression reversal. Data from preclinical research on mice revealed that inhibition of MDSC function against T cell proliferation with the addition of a PI3Kγ/δ inhibitor resulted in improved response to PD-L1 inhibition in T cell-inflamed tumours in contrast to non-inflamed [52]. Indoleamine 2,3-dioxygenase (IDO), a tryptophan metabolizing enzyme induced by inflammation, normally promotes immunosuppression in order to control harmful inflammatory responses. This otherwise beneficial effect, however, is also responsible for cancer immune evasion and IO resistance as IDO shows increased expression in tumour cells, tumour-related immune cells, dendritic cells and macrophages [53]. Tryptophan depletion and immune inhibitory tryptophan metabolites have been described as potential mechanisms by which IDO achieves tumour-specific T cell suppression and anergy as well as Tregs activation [54] and its expression has been associated with increased MDSC tumour infiltration, induced by Tregs [55]. Although results from clinical trial investigating IDO inhibitors in various cancers are far from promising [56], preclinical research on melanoma has shown that IDO promotes resistance to anti-CTLA4 therapy, while IDO inhibition reverses it [57] and IDO activity has also been associated with resistance to anti-PD-1 therapy in NSCLC [58]. Consequently, the value of IDO as an immunotherapy response biomarker remains to be determined.

Additionally, a specific subpopulation of tumour cells characterized by CD44 expression on their surface, known to display cancer stem cell properties, is implicated in therapy resistance and disease aggressiveness [59]. Research on their function and possible interactions within the TME revealed that CD44 + HNSCC cells have a negative interference with anti-tumour immunity by downregulating effector T-cell and Th1-cell activity and inducing regulatory T-cell and MDSC function [60]. Another proposed mechanism of CD44 + cell mediated immunosuppression is attributed to their increased PD-L1 expression which shields them from host immune responses and links them to disease recurrence, making CD44- rich tumours ideal targets for anti-PD-1 therapy, even after surgical intervention [61].

Exploring the possibility that immune cell distribution within TME might affect response to immunotherapy, Wood et al. assessed biopsies of 16 HNSCC patients at diagnosis and resection using RNA sequencing and immunohistochemistry assays. The results depicted a stable immune cell signature within various regions of the same tumours at different timepoints prior to treatment, contradicting the hypothesis that HNSCC immunotherapy failure could be attributed to immunological diversity within primary tumours [62]. Studies examining the impact of stromal TILs on patient survival have shown conflicting results. Vassilakopoulou et al demonstrated that stromal TIL (strTIL) abundance exerts positive impact on both disease free survival (DFS) and OS in HNSCC and associates with higher PD-L1 protein expression levels, suggesting a possible concordance with IO response [63]. Conversely, Badr et al., combining H/E stained slide evaluation with molecular analysis of immune cell signatures in treatment-naïve tumors, they showeda positive effect of intraepithelial TILs (ieTILs) on DFS, which was retained in HPV negative tumors after HPV status stratification, while strTILs failed to show association with survival [64]. An analysis by multiparametric flow cytometry in 34 patients with R/M HNSCC found that high co-expression of CD8 and PD-1/TIM3 checkpoints on tumour infiltrating T cells (inflamed tumours) was associated with longer survival, in comparison with non-inflamed tumours, from the time of starting anti PD-1 therapy. However, this result comes from a small subgroup of only 9 patients so further research towards this direction is mandatory before definite conclusions can be drawn [65]. Incorporation of the evaluation of all these heterogeneous factors into clinical practice would be yet another challenge. Analysing results from previous studies Bates et al. proposed a predictive computational model which utilizes expression of various factors, including PD-L1 and chemokines responsible for dendritic-cell migration as well as immunosuppressive biomarkers, in order to stratify HNSCC patients regarding their potential to respond to immunotherapy [66].

Molecular analysis of HNSCC tumours has identified significant correlations of specific immune cell subpopulations' infiltration with distinct gene expression patterns such as HPV, genetic alterations, neoantigens and smoking mutational signature as well as with survival. Notably, the HPV-related (atypical) molecular subgroup demonstrates the highest immune infiltration and cytolytic activity in addition to increased Treg/CD8 + Tcells ratio while the smoking related (classical) subgroup has the lowest levels of immune infiltration and IFN-γ signalling among HNSCCs. Regarding survival, adaptive immune response cell infiltrates and mutation dominated tumours exhibit better outcomes in comparison with tumours rich in components of innate immune response and copy number alterations [51]. Additionally, in a study focused on SCCs by Li et al., gene expression analysis using the Cancer Genome Atlas (TCGA) identified distinct immune entities with common characteristics and effect on prognosis. Tumours categorized as immune-cold had the lowest lymphocyte infiltration, TCR diversity and leukocyte/stroma ratio and highest aneyploidy and were associated with the worst prognosis while immune-hot tumours were characterized by the highest levels of lymphocyte infiltration, IFN-γ response, TCR diversity, M1/M2 ratio and cytolytic activity and the lowest levels of genomic aberrations and TGF-β expression and were related to the best prognosis. A third subtype demonstrated a molecular pattern consistent with immune-hot but tolerogenic tumours with high infiltration with M2 polarized macrophages, high TGF-β expression, TCR diversity and reactive stroma and relatively worse prognosis, while other subtypes with mixed characteristics showed association with intermediate outcomes [67]. These findings are promising and could point towards patients with the highest probability to benefit from IO and combination treatments.

Recently, a 12-gene chemokine gene expression (the Messina signature) was defined as T-cell inflamed phenotype (TCIP) in HNSCC in

analogy to melanoma studies [5,8]. TCIP-H tumours abounded PD-L1, PD-1, CTLA4, TIM3, CEACAM1, LAG3, CD206, FoxP3 along with M2 macrophages and FoxP3 + Treg conferring strong immunosuppressive milieu to CD8 + T-cells, albeit granzyme B and IFNγ levels were increased too. Interestingly, PD-L1 was not necessarily associated with CD8 + T cell infiltration, thus providing a plausible explanation why ICI in PD-L1 + tumours does not always elicit T-cell immune responses [5,8]. However no correlation with OS was found in multivariate analysis.

Host factors

HPV profile

HNSCCs are divided in two major subgroups in respect to their causative agents: HPV infection and tobacco use/alcohol consumption. HPV positivity has been identified as a favourable prognostic factor for survival in HNSCC patients treated with standard chemotherapy and radiotherapy by multiple clinical trials [68–72]. However, the value of HPV as a predictor of immunotherapy response remains to be determined. Although clinical trials so far have failed to show a clear association of HPV status with response to PD-1 inhibition therapy [4,14,15], viral protein expression within HPV + tumours is known to serve as a trigger for immune activation and implications of viral status in the effect of immunotherapy have been presented in a large number of studies. Well- defined differences have been observed regarding the immune landscape of HPV related and unrelated head and neck tumours making the former better candidates for IO. Specifically, HPV + cancers are characterized by marked increase of intratumoral CD8 + INFγ producing T cells, CD4 + Th1 cells, DCs and macrophages as well as higher chemokine (CXCL9, CXCL10, CXCL12, CCL17,CCL21) and proinflammatory cytokine (IL-2, IL-17, IL-23, IFN-γ) production and PD-L1 expression [73]. Additional data from transcriptomic analysis of 280 HNSCCs using TCGA showed that HPV-positive tumours demonstrate higher immunogenicity with larger infiltration of activated CD8 T cells in comparison with HPV-negative tumours. Moreover, although PD-1 and PDL-1 expression was not altered by HPV status, HPV-positive tumours had higher expression of CTLA-4 as well as Tregs infiltration and Tregs/CD8 ratio, leading to the conclusion that HPV positivity might enhance responsiveness to ICIs [51]. In accordance to this, further research has revealed that HPV + status in head and neck cancer prior to treatment translates into an inflamed tumour phenotype [74], while in another study, gene analysis performed on 544 head and neck tumours, revealed that higher expression of *CTLA-4*, *PD-1* and *TIM3* encoding genes was observed among HPV + compared to HPV- tumours, indicating that HPV positivity is linked to greater T-cell activation and immune exhaustion. In addition, TILs specifically in HPV + tumours were characterized by an increased B-cell signature [75]. PD-1 expression in correlation with HPV status was also examined in a study of 64 previously untreated HNSCCs. The results demonstrated PD-1 positivity in a considerably larger number of CD4 + and CD8 + infiltrating lymphocytes in HPV + compared to HPV- tumours. Remarkably, HPV + /high PD-1 lymphocytic infiltration subgroup presented with better overall survival suggesting that PD-1 constitutes a marker of T-cell activation and priory elicited anti-tumour immune response rather than immune exhaustion. Possible implications with immunotherapy were explored as anti- PD-1 mAb *in vitro* treatment of HPV + /high PD-1 TILs tumours resulted in increased IFN-γ secretion [76]. Another study showed overexpression of T-cell exhaustion genes in HPV + compared to HPV- tumours. Particularly, IDO-1 expression was associated with specific HPV antigenicity and combined PD-1/IDO-1 inhibition elicited HPV specific cytotoxicity, defining IDO-1 as an ideal target for reversal of immune exhaustion in HPV + HNSCC [77].

Both HPV positive and HPV negative HNSCC carries a high level of nonsynonymous mutations which have been implicated in the activation of immune response through MHC class I molecules [51]. However, each of these two tumour subgroups defined by HPV status has been associated with a distinct genetic profile and is characterized by different subsets of mutations. HPV + tumours are characterized by *PIK3CA* activating mutations, inactivating mutations of *CYLD* and amplification of *FGFR2* and *FGFR3* cell cycle genes while HPV- tumours show a prevalence of inactivating mutations in tumour suppressor genes *TP53*, *CDKN2A* [78]. Apolipoprotein-B mRNA editing catalytic polypeptide like (APOBEC), a family cytidine deaminase enzyme, has been associated with mutagenesis in HPV + HNSCC. It has been shown that specifically APOBEC3 enzymes demonstrate increased activity in HPV16 infected tumours [79] and in addition to participating in antiviral immunity, they promote tumorigenesis by driving a distinct set of mutations [80] including *PIK3CA* activating mutations, almost exclusively found in HPV + tumours [81,82]. Consequently, positive correlation of APOBEC overexpression to increased immune signalling might influence IO effect on HPV + tumours [81]. Therefore, tumour genetic profiling can help identify these mutations and further illuminate their potential of response. Moreover, p16 encoding gene (*CDKN2A*) is known to downregulate the activity of cyclin D1 and cyclin-dependent kinases resulting in cell cycle control, whereas *CDKN2A* mutations lead to overexpression of cyclin D1 (*CCDN1* gene) which is associated with poor prognosis [83]. Smoking associated HNSCCs demonstrate poor immune infiltration while they are characterized by a high mutational load. Thus the overall effect on IO response cannot be predicted [51].

Regarding the use of HPV status (p16) as biomarker for ICI, SITC subcommittee recommends that HPV status per se should not dictate the use of immunotherapy as there is no strong data that p16 + patients benefit excessively [5].

The microbiome

Microbiome exerts critical effects on anti-cancer therapy response and toxicity and it has emerged as a promising therapeutic target [84]. The role of host gut microbiota regarding its influence on IO outcomes has been a field of investigation for recent studies combining both clinical and preclinical research data. Microbiota and the immune system are on a constantly fluctuating dynamic relationship with significant impact on local and systemic immune responses. T-cell activation, involvement of pattern recognition receptors and small metabolite recruitment constitute possible mechanisms by which gut bacteria participate in anti-cancer immunity [85]. Specific genera among gut microbiome have been identified as IO response and toxicity predictors and fecal microbiota transplant (FMT) in mice has further enlightened these findings [86]. Research on lung, renal and urothelial cancer patients under treatment with ICI revealed that use of antibiotics right before or during therapy negatively affected median overall survival. *Akkermansia muciniphila* had the greatest association with response to ICI therapy and together with *Enterococcus hirae* increased responsiveness to ICI in previously non-responder mice following FMT. The immunological effect on these mice was depicted as increased CCR9 + CXCR3 + CD4 + T-cell tumour infiltration suggesting a stronger immune response [87]. Two similar studies on melanoma associated the presence of certain microbiome members (*Ruminococcaceae* spp., *Faecalibacterium* spp., *Bifidobacterium longum, Collinsella aerofaciens, Enterococcus faecius*) with response to ICI. Microbiome diversity was also greater among responders while higher levels of *Bacteriodales* were noticed in non-responders [88]. FMT of these microbes to previously germ-free mice lead to augmentation of IO efficacy, possibly achieved by increased CD8 + T cell and decreased Foxp3 + CD4 + T cell TME infiltration [88,89].

Oral cavity microbiome has been shown to have clear implication in HNSCC development and progression. *Fusobacteria* species have been found in abundancy in both primary and metastatic cancerous tissues whereas *Streptococcus* presence was limited [90]. Alcohol consumption and tobacco, the two major risk factors for HNC, as well as periodontitis

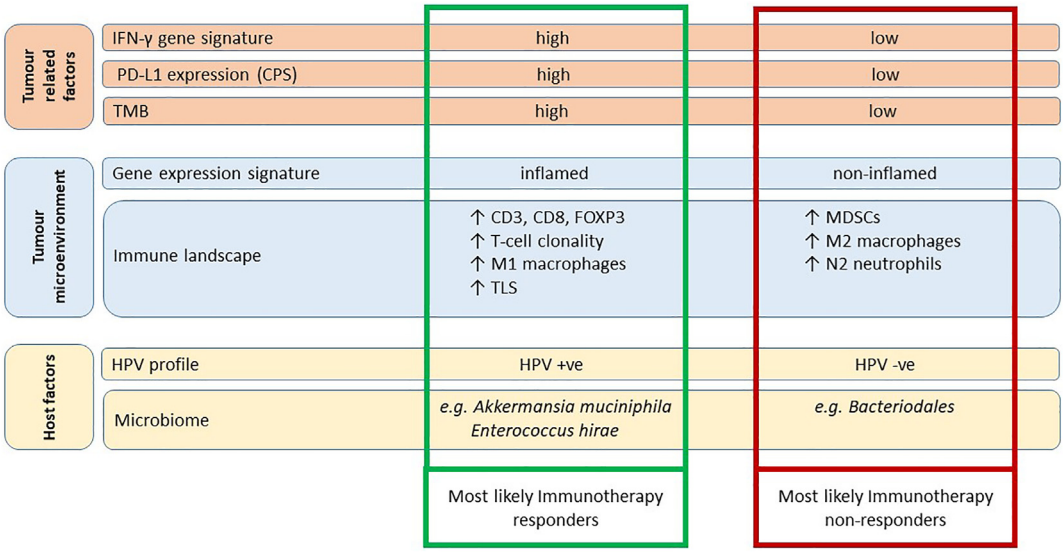

Fig. 1. Strategies to optimize response to immunotherapy depicted in the cancer immunity cycle.

and poor oral hygiene have been shown to modify oral microbiome composition [91,92]. In addition, certain bacterial species have been associated with late stage oral squamous cell carcinoma, specifically *F.periodonticum* abundance and *S.mitis* and *P.pasteri* scarcity commonly found in stage 4 OSCC [93]. Accordingly, the potential use of microbiome as an IO predictive biomarker for HNSCC remains to be elucidated with further research pointed to this direction.

Strategies to optimize response to immunotherapy are illustrated in Fig. 1.

Conclusions

Immunotherapy is a complex and rapidly evolving field that has the potential to provide substantial clinical benefit to patients with a variety of cancers. However, it becomes increasingly clear that a significant proportion of patients do not respond to widely used immunotherapies, such as immune checkpoint inhibitors. Currently, only PD-L1 is a validated biomarker used in clinical practice to guide treatment selection. Research should focus on improvement of patient selection, by implementing PD-L1 and by identifying new predictive biomarkers. In this context, tumor mutational burden, INF-γ signature, HPV status and the host's microbiome have emerged as potential predictors of immune response and are currently being evaluated in clinical trials. In addition, the tumor microenvironment represents an interesting source for the development of novel biomarkers. On the other hand, combination therapies will be required to increase treatment efficacy. Investigating cancer immunology by reverse translating to the laboratory from clinical studies is needed to bring benefit to a greater number of patients. Development of strategies for patients who lack preexisting immunity is also necessary. Such strategies will be able to fulfill the promise that immunotherapy brings to the advancement of oncology. Indeed, we are at the beginning of an exciting journey for patients and for scientific investigation.

Declaration of Competing Interest

AP has received honoraria and research funding from BMS, MSD, Merck Serono, Pfizer, KURA, Astrazeneca

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