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REVIEW

Immunological and biological dissection of normal and tumoral salivary glands

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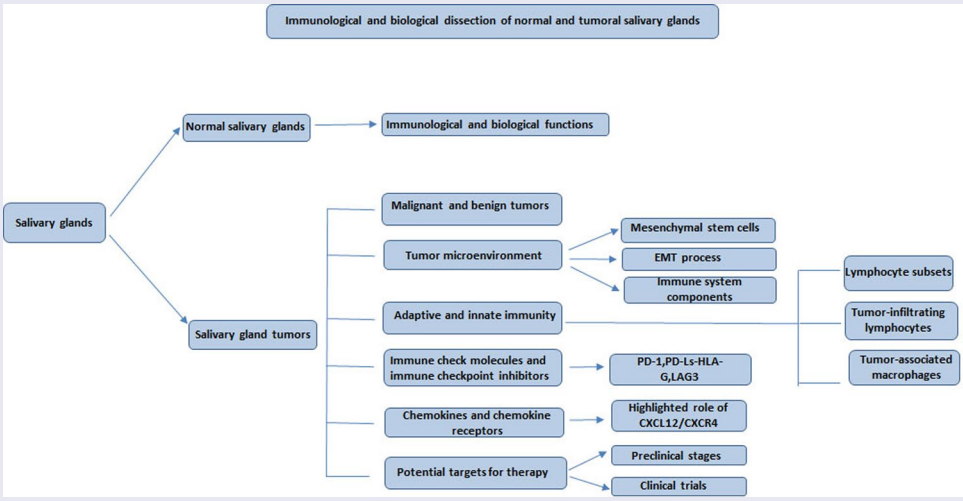
ABSTRACT

Salivary glands naturally play central roles in oral immunity. The salivary glands microenvironment inevitable may be exposed to exogenous factors consequently triggering the initiation and formation of various malignant and benign tumors. Mesenchymal stem cells are recruited into salivary gland microenvironment, interact with tumor cells, and induce inhibitory cytokines as well as cells with immunosuppressive phenotypes such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs). The immune components and tumor immune responses in malignant and benign SGTs are still under investigation. Immune responses may directly play a limiting role in tumor growth and expansion, or may participate in formation of a rich milieu for tumor growth in cooperation with other cellular and regulatory molecules. Immune checkpoint molecules (e.g. PDLs, HLA-G and LAG3) are frequently expressed on tumor cells and/or tumor-infiltrating lymphocytes (TILs) in salivary gland microenvironment, and an increase in their expression is associated with T cell exhaustion, immune tolerance and tumor immune escape. Chemokines and chemokine receptors have influential roles on aggressive behaviors of SGTs, and thereby they could be candidate targets for cancer immunotherapy. To present a broad knowledge on salivary glands, this review first provides a brief description on immunological functions of normal salivary glands, and then describe the SGT's tumor microenvironment, by focusing on mesenchymal stem cells, immune cell subsets, immune checkpoint molecules, chemokines and chemokine receptors, and finally introduces immune checkpoint inhibitors as well as potential targets for cancer therapy.



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Introduction

There are three paired major salivary glands (termed as parotid, sublingual and submandibular) as well as numerous minor salivary glands throughout the mouth and the aerodigestive tract. The main function of salivary glands is the saliva production, and proteins present in the saliva paly critical roles in human immunity. It has been accepted that a health and normal microbiota may largely contribute to salivary glands maintenance and function. It seems that saliva and its various composition naturally participate in shaping of a balanced microbiota.[1,2] Oral microbiota alterations and oral mucosa dysbiosis may contribute to chronic pre-cancerous lesions, neoplastic transformation and oral cancer development through inducing different mechanisms, such as pro-inflammatory responses, epithelial barrier alterations, epigenetic modulations, and cancerogenic metabolites.[3] A recent study showed that dysbiosis of salivary microbiome influenced on tumor cell proliferation specially in oral squamous cell carcinoma (OSCC) through the production of a panel of cytokines and chemokines (e.g. IL-6, IL-8, GM-CSF, TNF-α, and IFN-γ).[4] On the basis of these evidences, oral microbiota dysbiosis may dictate cancer development, and thereby microbiome profile can be clinically utilized as beneficial indicators for cancer screening and prognosis monitoring of head and neck cancers (HNCs) in general. Additionally, the use of probiotics and antimicrobial therapies might open new perspectives for cancer treatment.[5] Normal salivary gland microenvironments are composed of various types of cells, and tumors can result from abnormal proliferation in any type of these cells.[6] Tumors in these glands are rare. Salivary gland tumors (SGTs) are uncommon tumors that represent about 0.5% of all malignancies and 5% HNCs in humans. SGTs have particular importance among human neoplasia because they have the most complex histopathology of any tissue in the human body and represent an exceptional range of different tumor types.[7] Due to their rareness, very little information regarding the etiology of SGTs is available. Previous studies have shown that mesenchymal stem cells, immune cell subsets, immune checkpoint molecules and chemokine/chemokine receptor profiles could play important roles in both unfavorable biological behaviors and tumor progression in the patients with SGTs. To present a broad knowledge on normal and tumoral salivary glands, this review first provides a brief description on immunological and biological functions of salivary glands, and then describe the SGT's tumor microenvironment, by focusing on mesenchymal stem cells, immune cell subsets, immune checkpoint molecules, and chemokines and chemokine receptors, and finally introduces immune checkpoint inhibitors as well as potential targets for cancer therapy.

Immunological and biological function of salivary glands

The main function of salivary glands (SGs) is the production and the secretion of saliva. Among saliva proteins, immunoglobulins (IgA up to 90–98% and IgG up to 1–10%) and HSP70/HSPAs (70-kDa stress protein family) are implicated in both innate and adaptive oral immunity. Plasma cells residing in SGs produce IgA which then are secreted in saliva. In the oral cavity, saliva through IgA-mediated humoral immunity preserves oral surfaces.[8,9] As part of the oral humoral and mucosal immunity, SGs also contribute to oral tolerance to foods by the production of TGF-β.[10,11] In addition to B lymphocytes, macrophages, NK cells as well as αβ and γδ T cells may act as a mucosal immune network in SGs[12] . In a mouse model, tissue-resident memory T cells (T_{RM} cells) are permanently residents in SGs and provide effective local immunity during infection without requirement to local antigen presentation.[13] In fact, SGs act as a sink for both CD4 and CD8 T_{RM} lymphocytes to facilitate defense from local infection.[14] However, the mechanism of T cells migration to SGs tissue and their conversion into T_{RM} cells has not been well identified. Recently, It has been reported that α4β1 integrin promotes entry of activated T cells to SGs resulting in CD8 T_{RM} accumulation in SGs tissue.[15] Dendritic cells (DCs) in SGs could subdivide into $CD103^{+}$-$CD11b^{-}$ classical DC1 (cDC1s) and $CD103^{-}$-$CD11b^{+}$ classical DC2s (cDC2). Mouse cDC2s induce mucosal Th17 and/or Th2 responses in a manner dependent to IRF4 transcription factor.[16,17] $CD1c^{+}$ cDCs have been frequently reported in biopsies obtained from the human sublingual mucosa of SGs which are similar with those of mouse cDC2.[18] Additionally, human $CD1c^{+}$ cDCs present IRF4, produce IL-23 and promote Th17 cell responses.[19] In mouse model, cDC2 which is present in sublingual mucosa also induces $Foxp3^{+}$ Tregs in draining lymph nodes.[20] In addition, $CD64^{-}$, $CD11c^{+}$ cDCs (up to 10%) as well as $CD64^{+}$ macrophages (up to 90%) were recently found as professional antigen presenting cells (APCs) in murine SGs.[21] In sites of immune-mediated inflammation, CXCL10/CXCR3 axis is considered as a chemotactic factor in the recruitment of activated T cells especially Th1 lymphocytes. Epithelial cells in salivary glands constitutively present CXCR3-B to scavenger CXCL10. In this regard, CXCR3-B is able to recognize its ligands, but it could not stimulate

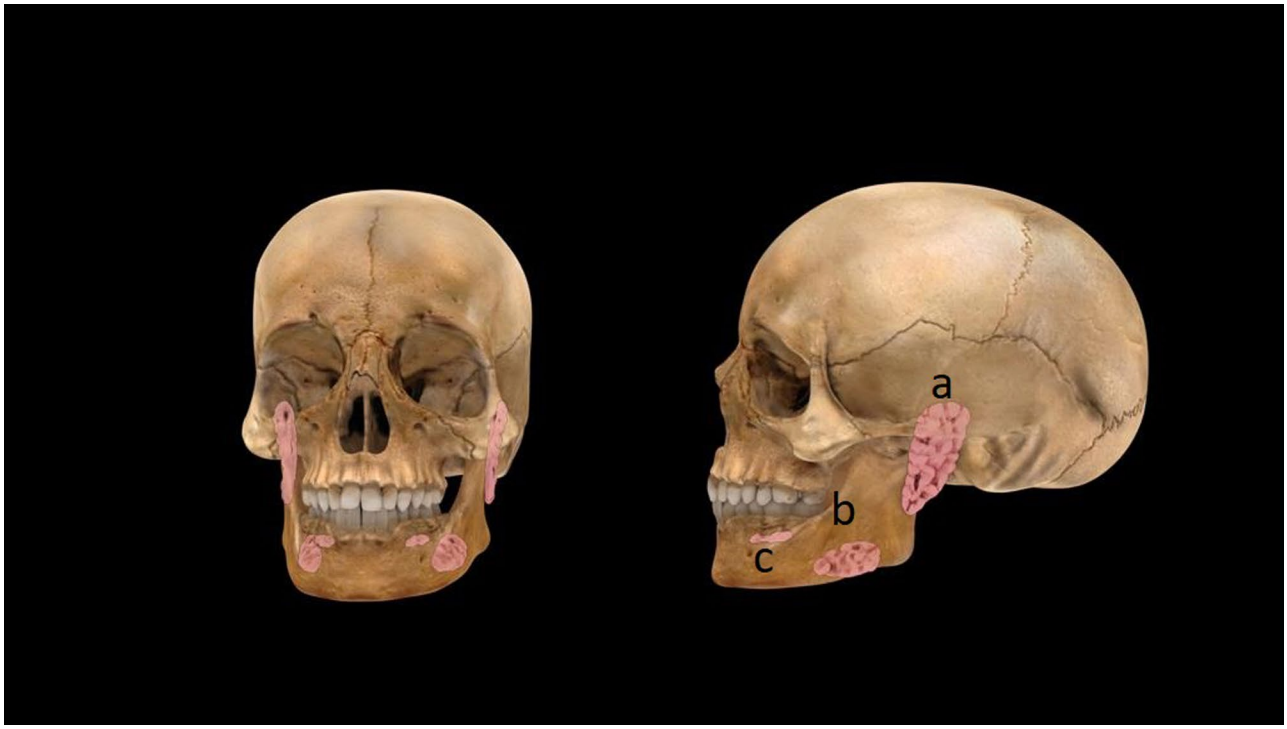

Figure 1. Anatomical location of major salivary glands tumors. Three major groups of salivary glands tumors are named based on their anatomical location: parotid tumors (a), submandibular (b) and sublingual (c) tumors.

chemotaxis and/or calcium mobilization on normal epithelial cells. However, it acts as a chemokine-scavenging receptor and retains the capacity to undergo post-ligand internalization of CXCL10.This process is naturally implicated in the epithelial tissue homeostasis. In Sjogren's syndrome (SS- an autoimmune disease in SGs), this mechanism is functionally impaired and causes extra recruitment of activated T cells to this region, situation that is associated with salivary gland destruction.[22]

Salivary gland tumors

Abnormal proliferation in salivary gland cells, chromosomal rearrangements, head and neck irradiation, oral infections, dedifferentiation and malignant transformation of benign tumors are associated with increased risk of salivary malignancies. Different factors, such as oncogenes, cell cycle regulators and angiogenesis factors may play a critical role in malignant transformation of salivary gland epithelium and may promote SGTs. Consequently, the tumors and epithelial cell transformation damage the salivary glands, lead to a limited or complete defect in saliva secretion, which importantly influence speech and taste sensitivity and feeding behavior.[23,24] As shown in figure 1, three major groups of SGTs are named by their anatomical location: parotid, submandibular, and sublingual. Parotid tumors are 63% of all SGTs.[25] SGTs could be benign or malignant. The Benign SGTs have been classified into 10 distinct histologic subtypes, with pleomorphic adenoma (PA) and warthin tumors (WT) as most common types. Malignant tumors have been categorized into 24 histologic subtypes; the most frequent malignant tumors (both major and minor SGTs) are mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC).[7, 26] PA, also referred to as benign mixed tumors, is a wolf in sheep's clothing. Although classified as a benign tumor, it may display some peculiar behaviors as well as problems in clinical courses because of its tendency to recurrence, malignant transformation and distant metastases.[27] WT, also named adenolymphoma is the second most benign SGTs. WT exhibits a unique histology, and is made up of epithelial cells, abundant lymphocytes, and lymphoid stroma with follicular configuration.[28] MEC is the most frequently diagnosed malignancy in both adults and children, comprising 34% of malignant SGTs.[29,30] Prognosis and biological behaviors of MECs depends on histological grade and tumor stage. Those defined as high grade are at significant risk for lymph nodes involvement and developing disease-progression and display a 0% to 43% five-year survival and possibly disease-related mortality.[31] Adenoid cystic carcinoma (ACC) is the second most malignant SGTs, represents ~10-15% of SGTs and nearly displays a lethal clinical course. ACC is

characterized by slow and relentless growth, great tendency of local recurrence, local infiltration, frequent perineural invasion as well as regional and distant metastasis.[32] Salivary duct carcinoma (SDC) is an aggressive type of SGTs that histologically is similar with ductal carcinoma of breast.[33] Myoepithelial carcinoma (MECA) and carcinoma ex pleomorphic adenoma (CEPA) are two other malignant subtypes of SGTs.[7]

Salivary gland tumor microenvironment: highlighted roles of mesenchymal stem cells and immune system components

The tumor progression, cancer behaviors and resistance in therapy in large number of cancers such as salivary gland tumors are directly affected by the tumor microenvironment components (TME).[34] In addition to cancer cells, the predominant cells that are found in TME are mesenchymal stem cells (MSCs), cancer/tumor-associated fibroblasts (CAFs or TAFs), tumor-associated-macrophages (TAMs), dendritic cells (DCs), endothelial cells, eosinophils, granulocytes, myeloid-derived suppressor cells (MDSCs), natural-killer (NK) cells, and B and T lymphocytes. A schematic view of TME components are shown in figure 2. TME components potentially play important roles in the initiation, promotion and invasion of cancer. MSCs, as the progenitors of stromal cells were reported to be recruited into salivary gland microenvironment in response to TGF-β generated by tumor cells.[35] Furthermore, chemokines and chemokine receptors may play influential roles in MSCs recruitment into salivary glands. In this context, MSCs could potentially home to salivary glands through CCR10 expression.[36] The recruited MSCs are initially differentiated into CAF/TAF-like phenotype and then they disband cell-cell connection and reduce E-cadherin in salivary gland tumor cells. The consequences of such conversions and interactions are in favor of cancer dissemination.[35, 37] In fact, MSCs in interaction with other TME components could promote epithelial–mesenchymal transition (EMT) in salivary gland tumors.[35] MSCs produce CXCL12, CCL2 and CCL5 to support tumor cell migration. In this regard, MSCs may participate in ACC invasion in a manner dependent on CXCR4/CXCL12 signaling pathway.[35] MSCs can also inhibit the adaptive and innate immune response against tumors through the recruitment of Treg cells and MDSCs and the production of factors related to tumor growth (CXCL8, PDGF, EGF, TGF-β, IL-10, IDO, VEGF, PGE2 and CD73).[37,38] The conflictive interactions between immune system components and TME components are documented as crucial drivers of tumor growth and progression.[39] It has been well known that inflammatory cytokines such as IL-6, IL-8 and TNFα, produced by tumor cells and immune cells, may induce EMT process.[40] It has been reported that a shift in immune components from neutrophil cells to macrophages as well as a change in immunosuppressive/exhausted markers (e.g. PD-L1, CTLA-4 and TIM3) occur during EMT process in different tumor microenvironments.[39–41] Interestingly, CD44 tumor cells in human head and neck squamous cell carcinoma (HNSCC) presented

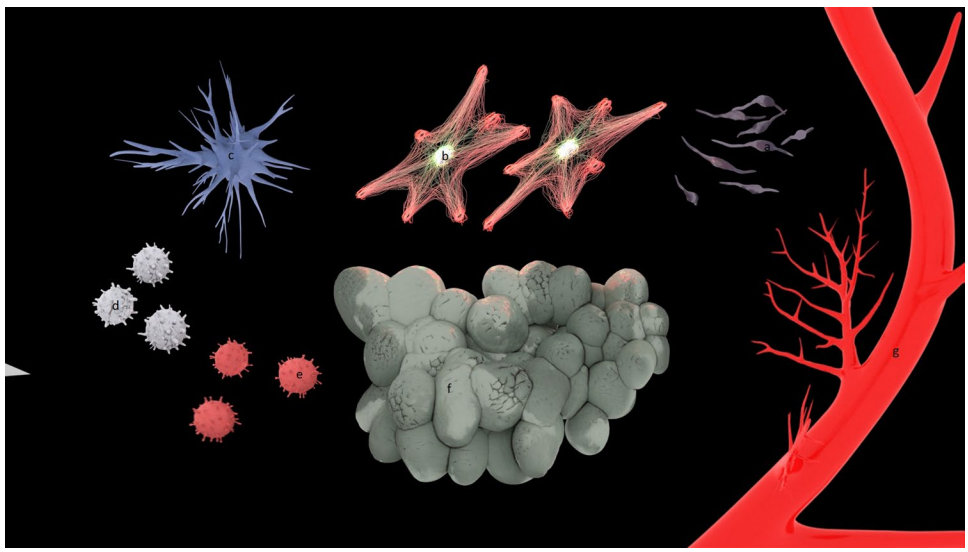

Figure 2. Tumor microenvironment (TME) in salivary gland tumors. TME is the environment around a tumor. They are often included mesenchymal stem cells (MSCs) (a), cancer-associated fibroblasts (CAFs) (b), immune cells such as dendritic cells (DCs) (c), cancer cells (f) and surrounding blood vessels (g). In some cases, B (d) and T (e) lymphocytes are also observed. Interactions between cancer cells and tumor surrounding cells promote the progression from primary sites to metastatic sites.

higher level of PD-L1 and exhibited EMT phenotype.[39] Recent evidence showed a reciprocal interaction between PD-L1 expression and EMT process in cancer. EMT could up regulate PD-L1 expression, and at the same time PD-L1 signaling pathway maintains EMT status.[42] CD44 is a key marker for cancer stem cells, and its overexpression was recently reported in ACC tumor cells.[43] Furthermore, TGF-β, a well-known driver of EMT in salivary gland tumors, was also able to induce surface expression of PD-L1 in difference cancer cells such as TE5, TE6 and TE11 cell lines.[44–46] As shown in esophageal squamous cell carcinoma and sorafenib-resistant hepatocellular carcinoma (HCC), PD-L1 up regulation reduced E-cadherin in cancer cells and promoted EMT.[46,47] As mention later, ACC tumor cells frequently express immune checkpoint molecules particularly PD-L1 and PD-L2.[70,74, 93–95] Similarly, it is proposed that immune check molecules in communication with other TME components could induce EMT in SGTs. EMT process commonly happens in both normal and tumoral salivary glands. Salivary glands are the best model to explore mechanisms involved in EMT.[48] EMT rate and tumor progression in SGTs depends upon expression level of molecules such as epithelial cadherin (E-cadherin), cytokeratin 8/18 (CK8/18), β-catenin and vimentin.[48–50] A recent study showed that the expression levels of EMT markers are variable in different tumor types of SGTs, and this could influence EMT activity in these types of tumors.[51] MEC and acinic cell carcinoma probably present a non-relevant activated EMT signaling because of higher expression of β-catenin and E-cadherin and lower expression of vimentin.[51] It has been shown that PD-L1 up regulation decreased the expression of E-cadherin, whereas PD-L1 knock down strongly increased the expression of this EMT marker in sorafenib-resistant HCC cells.[47] The EMT is necessary for ACC cells to acquire migratory or invasive capabilities. In addition to epithelial cadherin remodeling, B7-H3 through JAK2/STAT3 signaling pathway and C-kit (CD117 or stem cell factor receptor) by activating TGF-β potentially induce EMT in ACC.[45, 52] PA is the most common subtype of benign SGTs that frequently is characterized by peculiar properties. Approximately, 3-10% of all PA cases have an increased risk to develop a malignant parotid carcinoma in the future. Transformation to neoplastic myo-epithelial cells is probably accompanied by the genetic changes in expression of wilms' tumor-suppressor gene (WT1- a protein involved in mesenchymal–epithelial transition), results in morphological diversity as well as pathogenesis of PA.[53] Additionally, during EMT, neoplastic epithelial cells trans-differentiate into mesenchymal cells and lead to heterogeneity in PA tumors.[54,55] Furthermore, it has been reported that TWIST (a regulator of matrix metalloproteinases (MMPs)) may induce EMT process in PA, ACC or even in MEC.[56] The data support that TME components of salivary glands could be considered as good targets for SGT treatment. Regarding available data, combinational therapies with anti-PD-L1 and anti-EMT markers could be a suitable option for better clinical management of SGTs particularly those with ACC subtype that has few effective therapeutic choices.

Adaptive and innate immunity in malignant salivary gland tumors

It is now well accepted that during tumor development, tumor cells interact with surrounding microenvironment and suppress the immune cells aiming its growth and spread. Impaired immunity is in favor of tumor cells evasion, a situation which is reported to be associated with disease progression in various tumors.[57] Recently, the imbalance in immune cell subsets and/or cytokines has been reported in SGTs. Damar et al. investigated the percentages of total leukocytes and NLR (neutrophil to total lymphocyte ratio) in blood samples of patients with malignant and benign SGTs. It was revealed that the neutrophil mean percentage and NLR were significantly higher in malignant cases but the mean of lymphocyte percentage was significantly lower in comparison with benign SGTs. In malignant parotid gland, lymphocyte mean percentage and NLR were found to be significantly varied between high and low grade tumors suggesting that NLR could be employed as an inflammatory marker to differentiate high grade tumors from low grade ones.[58] Our previous study indicated that imbalance of Th17/Treg ratio and higher frequency of CTLA4 + CD4+ cells in peripheral blood of SGTs patients may contribute to disease progression.[59] It has been reported that Tregs inhibited effective antitumor responses in head and neck squamous carcinoma (HNSCC) and its prevalence was associated with higher tumor stage.[60] Our previous study also indicated a reduced levels of Th17 lymphocytes as well as an increased levels of Tregs in patients with malignant SGTs. Additionally, a positive correlation was observed between Th17 cell frequency and tumor size in these patients.[59] Th17 cells have been emerged as lymphocytes with dual function in cancer immunity. Anti-cancer and cancer-promoting effects of Th17 cells depends on tumor types, tumor environmental conditions and its high degree of plasticity.[61] Under inflammatory conditions, Th17 cells can be converted into IFN-γ-producing Th1-like cells that express T-bet.[62] These cells exert an

antitumor responses. Additionally, Th17 lymphocytes may recruit tumor-infiltrating CD8 + T lymphocytes and NK cells to the tumor milieu through producing CXCL9 and CXCL10.[63] By contrast, TGF-β produced by MDSCs or tumor cells can differentiate Th17 cells into Treg-like cells that express FOXP3. These cells suppress the immune response and stimulate tumor growth.[64] IL-17, an inflammatory cytokine produced by Th17 cells, may suppress antitumor immunity by regulating MDSCs function and generating Tregs.[64,65] IL-17 may also promote tumor growth through stimulating angiogenesis and anti-apoptotic factors.[66] Additionally, it has been reported that IL-17, an IL-4-rich microenvironment may trans-differentiate memory Th17 cells into Th17/Th2 cells.[67] Unlike Th1 cells and Tregs, the role of Th17/Th2 cells is not well understood in tumor immunity. The immune system in patients with cancer can be predicted by the balance of cytokine profile and/or the ratios of Th1/Th2 and Tc1/Tc2 in patient's peripheral blood. The role of Th and Tc lymphocytes subsets have not been fully investigated in SGTs. However, in our previous study, lower levels of Th1 and Tc1 cells as well as diminished ratios of Th1/Th2 and Tc1/Tc2 were found in patients with malignant SGTs. Moreover, mean florescent intensity (MFI) of IL-4 in type 2 T cells (both Th2 and Tc2) was significantly greater in these patients.[68] The data may propose that the lower level of type 1 T cells (Th1 and Tc1) might be due to from the more production of IL-4 by type 2 T cells in malignant patients. Therefore, it is proposed that the imbalance in Th and Tc subsets, at least in part, may contribute to SGTs progression. In this context, a lower plasma levels of IL-12 as well as a higher plasma levels of IL-6 and IL-10 have been reported in patients with advanced HNSCC than those with less advanced disease.[69] In addition, a positive correlation between tumor size and Tc2 lymphocytes was observed in malignant SGTs.[68] Similarly, it has been reported that higher expression of CD8 is related to smaller tumor size and lower recurrence rate in malignant SGTs.[70,71] Tumor size is a reliable prognostic factor in patients with SGTs. In SGT patients with a tumor size more than 4 cm the risk of locoregional or distant metastasis is reported to be higher, but the survival rate is shown to be lower.[72] Therefore, a positive interaction exist between T cell expansion and tumor growth in malignant SGTs. A more recent study investigated tumor-infiltrating immune cell populations as well as neoantigen landscape in three different tumor types of SGTs including MECA, ACC and SDC. SDC was found to be associated with highly immune cell infiltration, and exhibited higher mutational load. However, T cells were characterized by high levels of dysfunction. ACC was differentiated by a T cell exclusion phenotype, higher levels of immunosuppressive MDSCs and M2 macrophages and very low neo-antigen load. In terms of immune infiltration, MECA was more heterogeneous, demonstrating the entire range of immunity with some clustering closer to SDC and some clustering closer to ACC.[73] These date reveal that the mechanisms of immune suppression and immune escape probably vary by histological features, and thus definite immunotherapeutic lines are required for each SGT subtype. Sridharan et al. examined tissue of primary and metastatic ACC to evaluate patients for infiltrating immune cells and immune check molecules. Their analysis showed that most tumor cells expressed PD-L2, while infiltrating immune cells were infrequent. Furthermore, genes related to WNT/β-catenin signaling pathway was detectable in these tumors.[74] ACC tumor microenvironment, in another study, represented an increased level of PD-L2 and HLA-G as well as a reduced density of CD8+, GrB + TIL, CD1a and CD83 populations.[70] Such alterations in immune components lead to lower immunogenicity of ACC tumor microenvironment, and they might be linked to tumor escape and poor prognosis. Lower density of CD8 tumor-infiltrating lymphocytes (TILs) and DCs may reflect lower neo-antigen load and lower tumor mutation in ACC tumor microenvironment.[73] Furthermore, activating WNT/β-catenin signaling pathway in these tumor may explain lower density of infiltrating immune cells in ACC tumor microenvironment. Consistently, a positive correlation between this signaling pathway and absence of T cell gene expression was reported in melanoma.[75] The cross talk between tumor cells and TAMs through WNT/β-catenin signaling pathway may regulate pathways involved in polarization of M2 TAMs, and thereby may reinforce aggressive behaviors of tumors.[76–78] Interestingly, a high ratio of CD163-positive M2 TAMs to CD68-positive TAMs (M2 TAMs/TAMs) was reported in 6/8 (75%) of the SDCs with an immune-poor phenotype, and 13/13 (100%) with the immune-infiltrated phenotype. In other words, CD163-poisitive M2 TAMs represented a significant proportion of TAMs in the TME of SDCs.[34] Furthermore, the high infiltration of CD68-posiotive TAMs and CD163 positive-M2 TAMs were observed in ACCs. The increased proportion of these cells was found to be closely related to higher expression of CCL2 and CCR2. Notably, CCL2/CCR2 expression was associated with TAMs recruitment, M2 polarization and higher expression of Glial cell line-derived neurotrophic factor (GDNF) on TAMs. All of these features may seriously promote the proliferation, migration, and invasion of ACC cells which then contribute to generation of a suppressive tumor environment.[79] Conversely, CCR2

antagonist (RS504393, concentration of 50 ng/mL) greatly diminished the M2 polarization of TAMs in a xenograft mice model with ACC cells.[79,80] Similarly, antagonists of Wnt/β-catenin signaling (e.g. ICG-001) strongly reduced M2 polarization in HCCs, and strongly blocked CD24 + CD29+ tumor propagating cells in SGTs.[76, 78] The data suggest that SGTs could probably benefit from therapeutic targeting of TAMs and/or WNT/β-catenin signaling pathway in future. However, based on reviewing the literature, the ratio of MDSC/TAM population which is responsible for pathogenesis of variety of tumors have not been reported in salivary gland tumors. Reports on role of innate immunity in SGTs is very limited. A study exhibited that expression of CD56 was significantly augmented in high-grade malignant SGTs.[81] NK cells are frequently characterized by CD56 and CD16 markers. CD56 (NCAM) plays a role in interactions between NK cells and target cells and CD16 which is a low-affinity receptor for FcγRIII, mediates Ab-dependent cellular cytotoxicity (ADCC).[82] NK cells are the main components of the anti-tumor immune response; but their function may strongly be inhibited or even inverted by the immune suppressive milieu along with tumor development.[83] This may partially explain higher expression of CD56 in SGT patients with high grade. The role of NKT ($CD3^+$ $CD16^+$ $CD56^+$) cells has not been well investigated in head and neck cancers including SGTs. NKT cells have both positive and negative effects on immune system and tumor growth.[84] In generally, in contrast to HNSCC, the phenotype and function of innate and adaptive immune cells in SGTs have not been well investigated. Understanding of the immune response modulation in SGTs patients is important for overcoming immune suppression induced by tumors and generation of new immunotherapeutic strategies.

Adaptive and innate immunity in benign salivary gland tumors

PA and WT are the most common benign tumors. The cellular and molecular components of immune system has received less attention in benign SGTs than in malignant ones. Among molecules and cells involved in innate immunity only NK cells have been investigated through CD56 examination. In this context, CD56 expression, as detected by IHC, was found to higher in PA subtype.[81] NK cells are the first line of defense against cancer cells and rapidly exert anti-tumor response in early phases of innate immunity.[82] This data may reflect that anti-tumor response in benign SGTs is markedly mediated by NK cells. Our previous study revealed that immunosuppressive cells and co-inhibitory molecules (e.g. Tregs and CTLA-4) were higher in peripheral blood of patients with PA in comparison with healthy controls.[59] Additionally, our other study indicated a partial decrease in the ratios of Th1/Th2 and Tc1/Tc2 in patients with PA compared with healthy subjects. However, the difference was not significant.[68] Available data suggest that the anti-tumor response may be less inhibited in benign SGTs than malignant SGTs. However, further studies are required to clarify the role of immune system in PA subtype. WT is a well-defined salivary gland tumor which consists of epithelial cells, abundant lymphocytes, and lymphoid stroma with follicular configuration.[28] WT epithelium presented both MHC class I1 antigens and IL-1. Luminal tumor cells of WT might be modulated to function as antigen presenting cells (APCs) and they are able to present the luminal antigens to the underlying lymphoid organs.[85] Expression of MHC class I1 antigens by WT epithelium is similar to those observed in salivary glandular epithelium in Sjogren's syndrome.[86] Therefore, auto-antigens presentation, autoimmune T-cell induction and subsequent autoimmune pathogenesis might be proposed for WT. WT is typically known to have a mixture of oncocytic epithelial fragments and lymph node-like stroma as well as a clonal expansion of NK cells as well as B and T lymphocytes (both CD4 and CD8 cells).[28, 87] B lymphocytes in WT are mainly B-plasmacytoid type (CD79A), and mostly produce a high proportion of IgG and IgM(28). The presence of CD9 also named traspanin, a protein involved in differentiation, adhesion, and signal transduction has been reported in every WT.[88] WT exhibit unique properties regarding immune system components, and thereby immunotherapeutic lines might be more effective in clinical management of this tumor subtype.

Immune check molecules and immune checkpoint inhibitors in SGTs

The immune checkpoint molecules are controllers of the immune system. These molecules are expressed on the surface of immune cells and target cells. CTLA-4 and PD-1 are well known immune checkpoint molecules which interact with their ligands CD80/CD86, and PD-L1/2 respectively. Up-regulation of PD-1 in activated T cells is associated with immune-suppression and tumor evasion.[89] Compatibly, the up regulation of the other immune checkpoint molecules (e.g. PD-Ls, HLA-G and LAG3) in tumor cells and/or TILs participates in inducing of T cell exhaustion and immune tolerance, and finally provide tumor escape from the immune system.[90–92] Recently, HLA-G is considered as

a new member of immune check molecules, and its expression in cancers leads to immune tolerance.[91] Over-expression of PD-L1 has been reported in surgically removed tissues from SGTs patients, and PD-L1 expression rates were a prognostic factor in these patients.[93–95] In this context, PD-L1 was shown to be at relatively high levels at SGT subtypes that histologically categorized as high grade carcinoma including SDC, CEPA, SCC and large cell carcinoma (LCC). PD-L1up regulation was associated with HER2 over expression and aggressive behaviors in these tumors.[90] In contrast, those SGTs with lower expression of PD-L1 was accompanied by smaller tumor size.[71] Compared to PD-L1, PD-L2 has been less studied in SGTs. PD-L2 expression was highly detectable in ACC, SDC and MEC cases. PD-L2 expression in these tumors was importantly related to relapse.[71] Similarly, another study indicated that ACC tumor type strongly expressed PD-L2 and HLA-G while, they were negative or weakly expressed PD1, CTLA-4 and PD-L1.[70] In contrast, CTLA-4 positivity has been reported in SDC cases or even in ACC in another study.[71] Conflict data may be explained by difference in TNM stage and histological grade. In SGT microenvironment, it has been shown that PD-1 expression was negative in tumor cells while its expression was positive in TILs.[71, 74] Immune checkpoint inhibitors such as anti-CTLA-4 (ipilicmumab), anti-PD-1 (nivolumab and pembrolizumab) and anti-PDL-1 (atezolizumab) humanized monoclonal antibodies have widely been used in several solid malignancies including melanoma, breast, and lung and recently in recurrent metastatic salivary gland tumors.[96,97] Application of anti-PD-1 antibody pembrolizumab in SGTs patients with advanced disease and PD-L1-positive has shown promising results based on its anti-tumor activity and acceptable side effects.[97] However, the expression level of immune check molecules are frequently variable in SGTs. For example, in some studies PD-L1 was found to be highly dateable in tumor cells, while in the other studies it was detectable in only a minority of cells.[70, 93,94] Therefore, immune check inhibitors are not suitable for all SGTs and more analyzing is required to design therapeutic lines. A schematic view of the immune checkpoint molecules and immune checkpoint inhibitors are shown in figure 3. In addition to PD-Ls and HLA-G, a more recent study demonstrated the over expression of lymphocyte activation gene 3 (LAG3) in most SGTs particularly those with aggressive phenotype.[92] LAG3 is expressed on TILs in microenvironments of salivary gland tumors (particularly SDC tumor type), and its expression is associated with T cell exhaustion.[92] Recently, it has been shown that combinatory blockade of LAG3 and PD-1 may restore antitumor activity and improve clinical outcome in patients with cancer.[98] In respect to available data, therapeutically application of antibodies against the immune checkpoint molecules could be a promising strategy in management of some cases with malignant SGTs.[91, 96, 98]

Chemokine and chemokine receptor expression profile in salivary gland tumors

Chemokines and chemokine receptors are a complex network of regulatory ligands and receptors which interplay with immune and tumor cells to recruit inflammatory or non- inflammatory cells to the tumor niche. They also contribute to the tumor invasion and metastasis. Given similarities between cancer cell

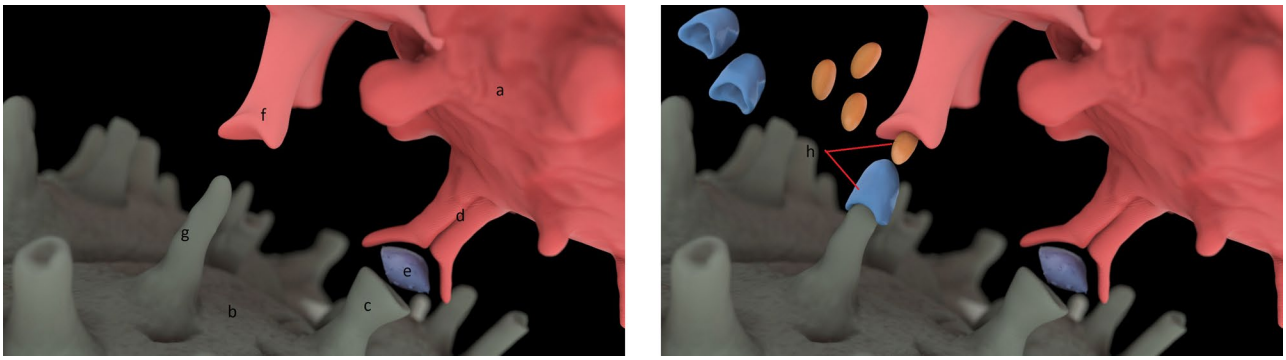

Figure 3. Immune checkpoints and immune checkpoint inhibitors in salivary gland tumors (SGTs). In suppressive tumor microenvironment, interactions between T cells (a) and cancer cells (b) promote tumor growth. After tumor antigen presentation (e), interactions between major histocompatibility complex (MHC) (c) and T cell receptor (TCR) (d) as well as interactions between programmed cell death protein 1 (PD1) (f) and programmed death-ligand 1 (PD-L1) (g) induce immune suppression and result in tumor escape and cancer progression. (Left figure). The targeting PD-1/PD-L1 protein-protein interaction by inhibitors such as Pembrolizumab (h, Right side) might be a promising strategy for cancer immunotherapy and restoring anti-tumor responses in SGT patients with advanced disease and PD-L1-positive.

spreading and leukocyte trafficking, chemokines and chemokine receptors are attractive targets in the field of cancer immunotherapy.[99] Among known chemokines and chemokine receptors, the CXC12/CXCR4 axis in SGTs pathogenesis has been more investigated. CXCL12, also known as stromal-derived factor-1 (SDF-1), is a vital α-chemokine that interacts to both CXCR4 and CXCR7 and controls the trafficking of normal and malignant cells.[100] IHC analysis by Uchida et al. revealed that CXCR4 protein was presented in either nucleus or cytoplasm of tumor cells in ACC (16 out of 20) and in MEC (4 out of 6) tissues. Additionally, this group by using quantitative RT-PCR and western blotting indicated that both CXCR4 protein and mRNA were up-regulated in ACC cell lines.[101] Another study by IHC displayed that the majority of MECs, ACCs and polymorphous low-grade adenocarcinoma (PLGA) showed higher expression of CXCR4 and CXCR7, whereas most PAs showed higher CXCR4 but lower CXCR7 expression. In MEC tumor type, the elevated level of CXCR4 was significantly related to advanced pathologic grade.[102] Klein Nulent eta.al showed a significant overexpression of CXCR4 in ACC of head and neck and its contribution to reduced recurrence-free survival (RFS). Additionally, their result indicated that expression of CXCR4 in the primary tumor was significantly elevated in tumors that recurred than those that did not recur.[103] Additionally, it has been reported that ACC cells presented CXCR4 and, in chemotaxis assays, they were responsive to CXCL12 and resulted in activating of Akt and ERK1/2 signaling pathways. It is suggested that CXCR4 signaling pathway may have a significant role in tumor cell survival program.[104] In addition to CXCR4/CXCL12, CCL5/CCR5 also investigated in SGT pathogenesis. In this regard, IHC and flow cytometry analysis showed that both CCL5 and CCR5 were over-expressed in ACC cases. Increase in their expression may contribute to peri-neural invasion (PNI) and advanced disease. Blockade of this chemotactic pathway was accompanied by suppression of invasion and PNI in ACC cells. Therefore, CCL5/CCR5 targeting may be promising strategy for the treatment of ACC cases with PNI presentation.[105,106]CCR7 is a key homing receptor that regulates the migration and the entry of leukocytes to secondary lymphoid tissue in response to CCL19 and CCL21. Accordingly, the expression of CCR7 by tumor cells leads to cancer cell dissemination as well as lymph node metastasis.[107] Our own previous study in tumor tissues of patients with benign and malignant SGTs indicated the overexpression of CCR7 and CCR4 transcripts in malignant SGT tissues compares to the benign ones. Immunohistochemistry analysis further confirms the result of gene transcript expression as CCR7 protein expression was observed to be significantly up-regulated in malignant tumors.[108] Higher expression of CCR7 in malignant SGTs may promote lymph node metastasis as reported in HNSCC.[109] Our previous study also indicated a significant inverse correlation between CXCL10 and tumor size in benign cases.[108] Regarding anti-antigenic activity of CXCL10, this chemokine could induce tumor regression in benign SGTs as reported in renal cell carcinoma.[110] Additionally, our study showed that CCL2 gene transcripts was lower in patients with positive lymph node involvement (LN^+) than those with LN^-.[108] Interestingly, another study in our center investigated CCL2 protein in serum of SGT patients and the results indicated a significant decrease in CCL2 in the patients with lymphoid involvement and advanced stages.[111] Lower CCL2 mRNA/protein level in SGTs may partially explain the sparse density of mononuclear cells in the salivary gland tumor niche. Several studies indicated a major role for CCL2 in the recruitment of leukocytes to the tumor microenvironment.[108, 111] Based on the above-mentioned points, chemokine and chemokine receptor expression profile have influential roles on clinical and biological behaviors of SGTs, and thereby they could be candidate targets in immunotherapeutic strategies.

Potential targets for SGT therapy

The therapeutic strategies in patients with SGTs are largely confined to surgery and/or chemo-radiation therapy. Distant metastases (mainly in lungs) are the major cause for treatment failure. Targeted therapies in SGTs have not yet been established, and are mostly restricted to clinical trials (See Table 1). It is necessary to develop new molecular biomarkers for clinical improving of the diagnosis, prognosis and therapeutic strategies for these patients. Targeted therapy clinical trials by using anti-PD-1 antibody pembrolizumab has currently begun in SGTs patients with advanced disease and PD-L1 positive.[97] It has been reported that mammalian target of rapamycin (mTOR) inhibitors (rapamycin and temsirolimus) led to complete regression in tumor-bearing mice. Human salivary gland acinic cell tumors present markers of activated mTOR signaling and thereby it is supposed that rapamycin therapy may be an effective therapeutic strategy.[112,113] Expression of genes related to β-catenin/Wnt and PI3K pathways was found to be up regulated in ACC cells.[74, 77] Up regulation of β-catenin/Wnt in tumors leads to polarization of M2 TAMs, reduction in CD8 effector T cell infiltration and an increase in Treg survival.[114] Therefore, targeting TAMs by CCR2

Table1. Potential therapeutic targets in salivary gland tumors.

Treatment strategy	Targets	Salivary gland tumors types	Development stage	Reference
Pembrolizumab	PD-1	Advanced, PD-L1-positive SGTs	Phase Ib	97
Dasatinib	Oncogenic protein tyrosine kinases e.g. cKIT	Recurrent/metastatic ACC and cKIT positive	Phase II	122
Molecular profiling, Pertuzumab + Trastuzumab	HER2	Advanced SGTs	Phase IIa	118
Pembrolizumab + Vorinostat	PD-1, HDAC	Recurrent/metastatic HNSCC and SGTs	Phase II	145
Trastuzumab + Docetaxel	HER2/neu	EGFR-positive SDC	Phase II	121
Lenvatinib	Multi-targeted tyrosine kinase e.g. VEGFR1-3	Recurrent/metastatic ACC	Phase II	120
Sorafenib	Raf serine/threonine kinases and multi-targeted tyrosine kinase e.g.VEGFR1-3	Recurrent/metastatic ACC	Phase II	119
Cetuximab	EGFR	Recurrent/metastatic SGTs	Phase II	138
Gefitinib	EGFR	Advanced SGTs	Phase II	139
U0126 and acyclovir	EGFR, MAPK/ERK and cytomegalovirus replication	MEC	Preclinical	141
Rapamycin	mTOR signaling pathway	Acinic cell carcinoma	Preclinical	112
CCR2 antagonist RS504393	CCR2-CCL2 axis and TAMs	ACC	Preclinical	79
ICG-001	Wnt/β-catenin signaling pathway	SGTs and HNCs	Preclinical	78
CUDC-101	HDAC, EGFR, HER2	MEC	Preclinical	140

SGTs, salivary gland tumors; ACC, Adenoid cystic carcinoma; MEC, Mucoepidermoid carcinoma; SDC, Salivary duct carcinoma; HNCs, Head and neck cancers; HNSCC, Head and neck squamous cell carcinomas; PD-1, Programmed cell death protein1; PD-L1, Programmed death-ligand 1; HDAC, Histone deacetylase; VEGF, Vascular endothelial growth factor receptor; EGFR, Epidermal growth factor receptor; MAPK/ERK, Mitogen-activated protein kinase/extracellular signal-regulated kinase; m TOR, Mechanistic target of rapamycin.

antagonist and inhibiting β-catenin/Wnt pathway by ICG-001 may improve immune responses and clinical outcome in SGTs (See Table 1).[78,79] TGF-β expression in SGT microenvironment might be associated with MSCs recruitment and PD-L1 expression.[35, 46] Regarding the high expression of TGF-β in ACC and MEC, it may be an ideal target in cancer therapy.[44, 115] C-kit is frequently up regulated in ACC, and its overexpression contribute to self-renewal of cancer stem cells and PNI invasion.[116] ACC is frequently characterized by *MYB* [v-myb myeloblastosis viral oncogene homolog (avian)] rearrangement and these may provide a platform for molecular targeted therapies in the future.[117] As summarized in Table 1, systematic therapy by molecular targets such C-kit, VEGF, EGFR, and HER2 could be considered as new promising therapeutic strategies in clinical trials.[118–122] HER2 expression in ACC is low while in CEPA is high.[123,124] Recently, our center have focused on Killer-cell immunoglobulin-like receptors (KIRs) genes as key receptors involved in development and function of human NK cells to determine their impact on genetic susceptibility to cancer. Two recent studies from our center revealed that KIR genes particularly KIR2DS4, KIR2DL2, and KIR2DS4del were linked with tumor progression and metastatic risk in tumors with head and neck origin.[125,126] KIR-based approach is now being initiated as therapeutic component in HNCs (NCT03341936). It has been reported that the genetic variations in KIR genes and/or human leukocyte antigen (HLA) ligands may predict the response to epidermal growth factor receptor (EGFR) therapy in HNCs.[127,128] Despite over expression of EGFR on head and neck tumor cells, the positive effect of EGFR inhibitors (e.g. cetuximab) was found only in a minority of the patients.[129] The evidences of the literature support this idea that the anti-tumor activity of cetuximab is mediated by inhibiting EGFR downstream signaling pathway and activating NK cells and DCs.[130–132] However, Treg-mediated suppression of NK cells might be a possible reason for cetuximab resistance in HNSCC.[129] Additionally, it has been reported that up-regulation of altered HLA-C and mutated HLA-A, KIR3DL2, and MHC class I polypeptide-related sequence A (MICA) on head and neck tumor cells may disrupt NK cell activation. Notably, anti-KIR2D monoclonal antibodies (e.g. lirilumab) increased NK-mediated killing of HNSCC cells.[128] The data suggest that immunogenic profiling and combination treatment strategies may overcome cetuximab resistance in HNSCC. SGTs have an increased EGFR gene copy number and high expression of extracellular signal-regulated kinase (ERK).[133] The EGFR signaling and mitogen-activated protein kinase (MAPK) components (Ras/Raf/MEK/ERK1/2 cascade) are implicated in aggressive behavior and poor prognosis of MECs, and MAP3K8 is associated with squamous cell carcinoma (SCC) development in murine salivary gland epithelial cells.[133,134] Accordingly, EGFR antagonists or MAPK/ERK inhibitors could be promising strategies for treatment of SGTs as reported in ACC cells.[135] It has been reported that a small number of patients with SDC particularly those with EGFR gene amplification exhibited a good response to EGFR

inhibitors.[136,137] However, the response rate for EGFR inhibitor monotherapy (e.g. cetuximab or gefitinib) was found to be relatively low in SGTs clinical trials.[138,139] Recently, the synergistic and potent cytotoxic effects of dual inhibition of EGFR and histone deacetylases (HDAC) have been shown in MEC cells.[140] Similar to those reported in HNCs, the combination therapy and immunogenic profiling may still have great promise to target SGT patients resistant to EGFR monotherapy. Human cytomegalovirus (hCMV) is a resident of the salivary gland duct epithelium, and plays an important role in tumorigenesis of MECs through up-regulation and activation of COX/AREG/EGFR/ERK signaling pathways.[141,142] Given the considerable pathway crosstalk, this type of malignant SGTs could benefit from the concurrent inhibition of MAPK/ERK kinase (U0126) and CMV replication (acyclovir).[141] MAPK and PI3K/AKT/mTOR signaling pathways are frequently involved in oncogenesis, cancer development, and drug resistance.[143] It has been shown that HDAC inhibitors (e.g. apicidin) induce apoptosis and autophagy in salivary MEC cells through inhibiting Insulin-like growth factor 1 receptor (IGF-1R) and regulating MAPK and AKT/mTOR pathways.[144] The combination therapy through HDAC inhibitors (e.g. vorinostat) and pembrolizumab against recurrent/metastatic HNSCCs and SGTs is currently under phase II clinical trials.[145] Potential therapeutic targets in SGTs are summarized in Table 1.

Conclusion

Salivary glands are naturally implicated in both innate and adaptive oral immunity. However, healthy microenvironment might be affected by formation of various malignant and benign tumors, and thereby functional failure might be due to epithelial cell transformation in these glands. Malignant and benign salivary gland tumors (SGTs) exhibit some aggressive and peculiar biological behaviors in clinical settings making them unpredictable and resistant against treatment processes. Despite many recent advances, the complete characteristics of malignant and benign SGTs in molecular and cellular levels are not fully understood due to their rarity, diversity, unavailability of cell lines, and lack of suitable animal models. A detailed analysis of molecular and cellular process underlying the development of these tumors may be essential in terms of diagnosis, prognosis, and immunotherapy. The investigation of immune cell subsets in peripheral blood is an easily available tool for monitoring clinical courses of patients with benign and malignant SGTs and for proposing probable therapeutic regimens against tumor growth. However, little data is available in the literature regarding the immune components and tumor immune responses in SGTs. Elevated levels of CTLA4 + CD4 + lymphocytes, Th2 lymphocytes and Tregs might be associated with suppressed or impaired anti-tumor responses in SGTs. Chemokines and chemokine receptors not only trigger recruitment of MSCs, MDSCs and Tregs into SGT microenvironment, but also they (particularly CXCR4, CCR5 and CCR7) could directly influence the aggressive behaviors such as PNI and metastasis. Additionally, over expression of PD-Ls, HLA-G and LAG3 on tumor cells and/or TILS supports the presence of an immunosuppressive microenvironment in SGTs, a situation which is associated with undesirable prognostic factor. Some targeted therapy clinical trials have currently begun in the patients with advanced SGTs. For example, pembrolizumab, an immune checkpoint inhibitor, showed some promising antitumor activity in SGT patients. However, SGTs still remain challenging cancers and merit more in-depth investigation in cellular and molecular levels to closely clarify interactions between immune system and TME components and to design immunotherapeutic lines with curative effect against SGTs.

Conflict of interest statement

Authors state that there was no conflict of interest.

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