Review Article

Classifying the tumor microenvironment to stratify nasopharyngeal carcinoma patients

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Abstract: Nasopharyngeal carcinoma (NPC) tumors are often presented with a large number of lymphocyte infiltration at primary sites. Conventional therapeutics, such as chemotherapy and radiotherapy, primarily target cancer cells without amelioration of the tumor-protective and immunosuppressive microenvironment, which leads to immune escape and therapeutic resistance. The progression of NPC is a mutual evolution process of cancer cells and tumor microenvironment (TME), in which contains extracellular matrix, stromal cells, cancer-associated fibroblast, NK cells, T cells, B cells, dendritic cells, endothelial cells etc. TME plays important roles in tumor development, invasion, and metastasis. Considering the multiplicity of stromal compositions and complexity of cell-cell communications, characterizing TME facilitates the identification of predictive or diagnostic biomarkers, and might be useful supplements to tumor staging and other prognostic criteria. With the advancement of single-cell sequencing technique, more and more integrative studies have been conducted recently to decipher the composition of subpopulations and functional dynamics in the TME, to dissect the pathogenic hierarchies and illustrate the infiltrating-immune surveillance system that determines tumor progression, and explore the correlation between TME and clinical outcomes in NPC. In this review, we aim to depict the compositions of TME and their clinical relevance, and summarize novel gene signatures and stratification strategies based on single-cell sequencing technology.

Keywords: Tumor microenvironment (TME); nasopharyngeal carcinoma (NPC); prognosis

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Implication of TME in nasopharyngeal carcinoma (NPC) prognosis

In current practice, TNM classification of malignant tumors seems to be the well-recognized standard of tumor staging. It is based on the tumor burden (T), regional lymph node involvement (N), and distant metastasis (M). However, the prognostic information offered by the TNM classification is limited and cannot predict the response to treatment. Recent studies indicate that the development of NPC is affected by the tumor microenvironment (TME) and the host immune status, which is the basis for the use of TME and immunological biomarkers to predict treatment response and patient outcomes. Increasing data indicates that TME classification, especially immunological classification, has a predictive value for prognosis and could be as a useful supplement to TNM classification.

The composition of TME

Tumors are not simple aggregations of tumor cells that

exist in isolation, and they also contain a complex system consisting of stromal cells, neural and neuroendocrine cells, endothelial cells (EC), immune cells, and extracellular matrix (ECMs). All these components constitute the TME, which is characterized by complex connections and interactions between cells and ECM components. These cells play important roles in tumor development, invasion, and metastasis. In this complex network, there is a large amount of information and material exchange. It is now known that chemokines, enzymes, growth factors (GF), cytokines, metabolic intermediates and metabolites of matrix proteins are present in the TME. The recruitment and activation of immune cells induced by the interaction between tumor cells and the microenvironment affect the development of tumors and the prognosis of patients. In recent years, increasing numbers of studies have focused on the TME, including large-scale analysis of stromal and immune cell types, which indicate that the TME has an important predictive value for evaluating the prognosis and response to treatment (*Table 1*). In addition, classification of the TME and immune cell infiltrates may be useful supplements to tumor staging and other prognostic criteria (56,57).

Many studies (1,15,38,58,59) have suggested the crucial role of TME in the pathogenesis of NPC (NPC). EBV-positive NPC tumors are often infiltrated by a large number of leukocytes, which are involved in the early stages of cancer development (60). EBV-infected epithelial cells secrete cytokines and chemokines, or release tumor exosomes, thereby participating in the formation of the TME and the exchange of information between cells, to suppress immune surveillance and promote tumor development and metastasis (58).

ECM

The components of the TME are similar to normal or inflamed tissue, including the ECM, non-immune/inflammatory stromal cells, and immune cells. The ECM is composed of hyaluronic acid, proteoglycans and other matrix proteins, along with cytokines, hormones and GF secreted by cells nearby (61). The ECM can influence tumor development by altering the microenvironment (57). For example, the ECM can provide a hypoxic or acidic microenvironment, which gives a survival advantage to tumor cells that obtain energy through anaerobic glycolysis compared to normal cells that require aerobic oxidation. Additionally, the abundant ECM in the TME can promote tumor growth by activating the pro-survival phosphoinositide 3-kinase (PI3K) signaling pathway (56). Moreover, interactions between the ECM and lymphocytes have a key impact on the movement and positioning of immune cells (62), which can help tumor cells evade immune surveillance. For example, ECM deposition and increased density can reduce lymphocyte replacement, limit T cell movement, and thereby suppress the anti-tumor effect of the body's immune response (56). A recent study shows that, in the NPC microenvironment, ECM is one of the key components mediating immunosuppressive functions. The main ECM components, such as collagen and fibronectin, are beneficial to angiogenesis and inhibit tumor cell apoptosis. In addition, the tumor parenchymal barrier constituted by the ECM is a physical protective barrier for tumor cells, which can attenuate T cell infiltration and drug penetration (63).

Stromal cells

Stromal cells mainly include vascular ECs, pericytes, mesenchymal stem cells (MSCs), fibroblasts, neural/neuroendocrine cells, and adipocytes. Perivascular cells, EC, and smooth muscle cells are the main components of blood vessels. A study of breast cancer tumors (64) found that neovascular tips are enriched with active TGF-β1 and periostin, which can promote growth. ECs are involved in tumor proliferation, invasion, and metastasis, while pericytes can prevent lymphocyte infiltration and effectively down-regulate the inflammatory response at the tumor site (65). MSCs can differentiate into a variety of other stromal cells, such as fibroblasts, pericytes, adipocytes, osteocytes, and chondrocytes. They also have immunoregulatory functions, such as secreting cytokines, GF and immune receptors, regulating the tissue microenvironment (66). For example, In the NPC microenvironment, fibroblasts can secrete a variety of GF that promote tumor progression or immunosuppression, including EGF, FGF, TGF-β, CSF, and IGF1 (67).

Cancer-associated fibroblasts (CAFs)

As the main non-inflammatory stromal cell type in the TME, CAFs are heterogeneous cells from multiple sources that also synthesize the ECM. Studies suggest that CAFs may also hinder the anti-tumor immune response (68,69). By producing TGF-β, malignant cells can activate adjacent CAFs, which in turn release large amounts of interleukins and chemokines, such as IL-1, IL-6, IL-8, IL-22, CXCL

Table 1 Specific subpopulations in tumor microenvironment and their correlation with clinical outcomes in NPC

Cell type	Cell marker & level	Overall outcome	Clinical outcome	References
TAMs	High CD163 expression	Undefined	No significant differences detected in pooled study	(1-6)
	High α-SMA$^+$ CAFs	Unfavorable	Reduced OS, PFS and DMFS	(1,7)
	CD68$^+$	Undefined	No significant differences detected in pooled study	(1,2,5,6,8,9)
	High density of CD206$^+$ M2-like TAMs	Unfavorable	Reduced OS and DFS	(6)
	High HO-1 expression	Unfavorable	Reduced OS and PFS; poor response to radiotherapy	(3)
	CD68$^+$ CCL18$^+$	Unfavorable	Reduced OS and DFS	(6,9)
TILs	High CD3 TIL infiltration	Favorable	Better OS but not DFS in pooled study, despite different results in different studies	(2,10-14)
	High CD8$^+$ TIL density	Favorable	Better OS but not PFS or DFS in pooled study, despite different results in different studies	(2,10,13-22)
	Higher CD4/CD8 ratios	Favorable	Better 5-year DMFS, lower distant metastasis rate	(23)
	High PD-L1 expression	Favorable	Better OS in pooled study, despite different results in different studies	(11,13,20,21,24-36)
	AFAP1-AS1 and PD-1 co-expression	Unfavorable	Reduced OS; more likely to have distant metastasis	(37)
	High PD-1 expression	Undefined	No statistically significant differences existed between the expression level of PD-1 in TILs and the OS in pooled study	(13,15,28,33,35,37-40)
	CD80 and CD86 expression	Undefined	Appears to be a marker of better survival, no significance after adjustment	(41)
	High density of NK cells	Favorable	Better OS and PFS	(15)
		Unfavorable	Reduced 2-year OS (a higher proportion of infiltrated NK cells in the NK-high cohort co-expressed PD-1)	(38)
	High FoxP3/CD8$^+$ ratios	Favorable	Better PFS in early-stage patients	(18)
	High expression of FoxP3 and GrB	Favorable	Better OS and PFS	(18)
	High ICOS expression	Favorable	Better OS	(42)
	High FoxP3$^+$ Tregs	Undefined	No significant differences detected in pooled study	(2,15,18,39,43)
B cells	High CD19$^+$ B cell	Favorable	Better OS and PFS	(44)
	High memory B cells	Favorable	Better OS	(45)
Neutrophils	High ANC of pre-RT (>7×10^9/L) and a high increase (>5×10^9/L) of ANC during RT number of neutrophils	Unfavorable	Reduced OS	(46)
		Unfavorable	Reduced OS and PFS	(15)
Mast cells	High mast cells infiltration	Unfavorable	Reduced OS and PFS	(15,47)

Table 1 (*continued*)

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Cell type	Cell marker & level	Overall outcome	Clinical outcome	References
Dendritic cells	High density	Favorable	Improved survival	(48-50)
Combined	$CD163^{-}$/$FoxP3^{-}$	Unfavorable	Reduced OS	(43)
	$CD206^{-}$/$FoxP3^{-}$	Unfavorable	Reduced OS	(43)
	$CD68^{-}$/$FoxP3^{-}$	Unfavorable	Reduced OS	(43)
	NK cells combined with mast cells infiltration	Unfavorable	more recurrence or metastasis	(15)
	Neutrophil-lymphocyte ratio	Unfavorable	Reduced OS, PFS and DMFS	(51-53)
Others	NO	Favorable	Better RFS	(54)
	IL-35	Unfavorable	Reduced OS	(55)

NPC, nasopharyngeal carcinoma; CAFs, cancer-associated fibroblasts; TAMs, tumor-associated macrophages; HO-1, high heme oxygenase-1; TILs, tumor infiltrating lymphocytes; NO, nitric oxide; NK, natural killer; RFS, recurrence-free survival; OS, overall survival; PFS, progression-free survival; DMFS, distant metastasis-free survival; DFS, disease-free survival.

and CCL (70). In addition, CAFs can enhance tumor progression by promoting angiogenesis or through the interaction of different cytokines and chemokines with inflammatory and neuroendocrine cells. The accumulation of CAFs is usually associated with a poor prognosis (56). A study of 154 NPC patients found a significant relation between the M2 tumor-associated macrophages (TAMs) and CAFs density, which can be used as an independent predictor of patient outcomes. This study stratified the patients into three groups based on the CD163 and α-SMA expression levels, which was further confirmed by a validation test (1).

Immune and inflammatory cells

A large number of immune and inflammatory cells are also present in the TME, such as natural killer (NK) cells, T cells, B cells, and dendritic cells (DCs). In addition, other myeloid precursor cells such as neutrophils, macrophages and mast cells infiltrate the TME attracted by the inflammatory chemokines produced by tumor cells. The invasive margin of cancer may include tertiary lymphatic structures (TLS), which have similar characteristics to peripheral lymph node tissue. TLS act as a functional area to induce immune cell activation and differentiation, where plasma cells can produce a large number of specific antibodies, which is related to the induction of the adaptive immune response (71). From the perspective of tissue distribution, immune cells have a specific enrichment patter in the tumor, which is also closely related to their functions. For example, immature DCs are mainly located in the central area of the tumor, while mature DCs penetrate into the edge that is close to T cells. B cells exist in the TLS and infiltration margins, while NK cells are scattered in the stroma and infiltration margins (characteristics of tertiary lymphoid structures in primary cancers). In addition, in the TME of NPC , B lymphocytes, NK cells, monocytes, and neutrophils can also be found (72).

NK and NKT cells

As part of the innate immune system, NK cells ($CD56^{+}$/$CD3^{-}$) play an important role in preventing infection and cancer. NKT ($CD56^{+}$/$CD3^{+}$) cells, like its name, share multiple markers of both NK cells and T lymphocytes, and express NK group 2D (NKG2D) receptors (73). In terms of cancer prognosis, NK cell infiltration showed inconsistent results. A study of 9 types of immune cells in 197 NPC patients showed that a high density of NK cells is correlated with favorable OS and PSF (15). However, another study suggested that patients with higher levels of tumor-infiltrating NK cells ($PD\text{-}1^{+}$/NK^{high}) have a poorer 2-year survival (38). This controversy may partly be due to the presence of different subtypes of NK cells in NPC. One case report described an advanced NPC patient with intracranial metastases that progressed during conventional

therapy. After NK cell transfer therapy combined with chemoradiotherapy, the patient maintained long-term tumor control (74).

Tumor infiltrating lymphocytes (TILs)

TILs are a group of T cells that surround malignant cells and consist mainly of $CD4^+$ and $CD8^+$ T cells (75). $CD4^+$ T cells include $CD4^+$ Th cells and $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells, which mediate anti-tumor immune response and suppress anti-tumor immunity, respectively (76,77). There are many infiltrating lymphocytes in NPC tumor stroma including anti-LMP1 and -LMP2 cytotoxic T cell precursors. However, these precursors do not kill tumor cells normally, which may due to the immunosuppressive microenvironment (72). Another study shows that among infiltrating lymphocytes, $CD3^+$ T lymphocytes usually account for more than 50% (59). The massive infiltration of leukocytes has been shown to be a shared characteristics of primary NPC tissues, but it is less frequently presented in metastatic lesions (78). This phenomenon suggests that the infiltration of leukocytes may have a more important role in the NPC development, whereas lymphocyte infiltration may have less effects on late and metastatic lesions. It is worth noting that in NPC, lymphocyte infiltration does not seem participate in eliminating tumor cells, but instead promotes tumor development (72). There is abnormal T cell activity in patients with undifferentiated NPC: peripheral $CD4^+$ T cells of these patients are deficient in producing IL-2, whereas higher level of IL-10 were detected, which suggests a Th-1/Th-2 imbalance (79). TILs expanded under $IL\text{-}2^{low}$ conditions lack cytotoxic activity, so they can neither lyse autologous EBV-infected NPC cells nor produce IFN-γ (80). Although EBV-specific cytotoxic T lymphocytes (CTLs) from the patient's blood can be reactivated, these activated CTLs will be selectively non-functional at the NPC tumor site (80). Thus, to reverse the immunosuppressive effect of the TME may provide an option for effective NPC treatment.

Studies have shown that in Hodgkin lymphoma, the expression of EBNA1 induces the production of CCL20 and the migration of Treg cells (81). High levels of CCL20 (MIP-3α) have also been detected in NPC patients. This may be the cause of Treg migration to the TME (82,83). The PBMC and TIL of NPC patients were found to contain a higher proportion of $CD25^+$ Treg cells than healthy controls (83). In addition, interferon (IFN)-γ, IL-10, IL-8 and IL-6 levels were elevated, whereas IL-2 levels were decreased in NPC biopsies, which may contribute to immune cell recruiting and regulation of cellular immunity (80,82,84,85).

In 2018, a cohort study of 1,490 NPC samples investigated whether TIL density can be used to assess the prognosis of patients with NPC. This study proved that high TIL infiltration is significantly associated with longer overall survival (OS), disease-free survival (DFS), distant metastasis-free survival (DMFS), and local recurrence-free survival, and it can be used as an independent prognostic indicator for patients with non-disseminated NPC (86). In addition, a study conducted in 2016 involving 719 patients suggested that the CD4/CD8 ratio can effectively predict the distant metastasis risk, whereby higher CD4/CD8 ratio correlated with better 5-year DMFS. High $CD3^+$ or $CD8^+$ T cell infiltration predicted better OS, but not DFS in this study (2).

B cells

B cells have also been found in the TME. B cells can aggregate at tumor margins or into complex immune aggregates, which are often related to the formation of ectopic lymphoid tissues, and have a positive effect on the differentiation of tumor-specific B- and T-cell responses (87,88). However, the prognostic value of B cell infiltration in NPC is not well-established. CXCL13, which interacts with the CXCR5 receptor in B cells, is considered to be one of the most effective chemoattractants for B cells. As a consequence, the expression of CXCL13 may affect tumor progression by recruiting more B cells to the tumor site (89,90). The increase of CXCL13 expression at the tumor site has been shown to be associated with better survival in other tumors, such as colorectal and breast cancer (91,92). In NPC, a recent study on $PD\text{-}1^+CXCR5^-CD4^+$Th-CXCL13 cell subsets suggested that an increase in Th-CXCL13 cell subsets is associated with better prognosis in NPC patients, while the infiltration of $CD38^+$ plasmablasts and $CD138^+$ plasma cells in the TME is associated with better survival outcomes, suggesting a cooperative role of B cells in the TME (93).

DC cells

DC cells are considered to act as a bridge between innate and adaptive immunity. They are also the pioneer cells that migrate the fastest to the tumor site due to attraction by chemokines (CXCL12 and CXCL8), vascular endothelial

growth factor (VEGF) and hepatocyte growth factor (HGF), and antimicrobial peptides (β-defensin) that are secreted by tumor and stromal cells. After immature DCs interact with an antigen, they will migrate to the secondary lymphoid organs where lymphocytes are enriched, and act as antigen presenting cells (APCs) to further induce specific immune responses (94,95). Although DCs may be present in NPC, there is not much research on their role in this malignancy. A study of tumor samples from untreated NPC patients demonstrated that CD207 expression was higher in $CD1c^+$ DCs from NPC lesions, comparing to $CD141^+$ and $CD141^-$ $CD1c^-$ DCs, and $CD123^+$ DCs, suggesting that $CD207^+$ DCs might serve as therapeutic targets for cross-presentation of tumor antigens (96). Studies have shown that high infiltration of DCs, believed to mediate T-cell interactions, is association with improved patient outcomes (48-50).

TAMs

The EBV-related genes and cytokines expressed by NPC cells also play an important role in promoting the secretion of pro-inflammatory cytokines. LMP1 encoded by EBV can induce CXCL-10 expression through NF-κB signaling (97). IL-18 can be released by NPC cells, forming a positive feedback loop to exacerbate leukocyte infiltration and inflammation (97,98). Under the circumstances, $CD68^+$ macrophages can be stimulated to produce IL-12 and IL-18, and further stimulate both T and NK cells. Once activated, macrophages can differentiate into M1 and M2 phenotypes (99). M1 macrophages have strong antigen presentation ability, and they participate in antitumor immune response along with Th1 cells. By contrast, the immune-suppressive M2 macrophages produce IL-10, which is essential for initiating Th2-type responses and suppressing CTL responses. In the process of tumor development, the pre-invasive TME has anti-tumor properties, mainly including M1 and Th1 cells, leading to the production of inducible NO synthase (iNOS), IFNγ, and IL-12. The transition toward an aggressive TME is marked by the emergence of tumor-promoting properties, characterized by the transition from Th1 to Th2 cells and M1 to M2 cells (100). In EBV-positive NPC biopsies, $CD68^+$ TAM and $FoxP3^+$ Treg cells was found to be increased in EBV-positive NPC specimens. Treg cells are likely to promote primary NPC cells migration by RANKL, which leads to metastasis and poor survival (43). However, in a systematic analysis conducted in 2021 on the prognostic value of macrophage and lymphocyte infiltration, there was no prognostic significance of $CD68^+$ or $CD163^+$ macrophages (2). However, a large cohort study is needed to confirm these findings. A recent study of primary antioxidant enzyme heme oxygenase-1 (HO-1), showed that it plays a crucial role in the polarization of M2 macrophages, suggesting that its expression is highly correlated with $CD163^+$ status. Moreover, HO-1 exhibited higher accuracy in the prognosis of NPC patients than CD163 (3).

Neutrophils

Neutrophils are one of the first immune cells to be recruited to inflamed tumor tissues (101). Neutrophils can form a network structure and release reactive oxygen species (ROS) that can damage DNA. Neutrophil extracellular traps (NETs) can be formed in the TME, where they play a tumor-promoting role (102,103). The EBV lytic trans-activator Zta was found to upregulate prostaglandin E2 (PGE2) and granulocyte-macrophage colony stimulating factor (GM-CSF) (104) to promote the production of IL-10 from neighboring monocytes in the TME (104), effectively inhibiting the cytotoxic function of activated $CD8^+$ T cells (105). In addition, Zta can induce NPC cells to express IL-8, which is a powerful chemical attractant for neutrophils (106).

A survey of 1,753 patients with NPC showed that a high absolute neutrophil count (ANC) before radiotherapy ($>7\times10^9$/L) and large ANC increase during radiotherapy ($>5\times10^9$/L) were significantly correlated with poor OS and can be regarded as an independent prognostic factor (46). Similarly, high infiltration of neutrophils was found to be associated with poor prognosis (15). Another study found the neutrophil-to-lymphocyte ratio (NLR) was correlated with T stage in stage II NPC patients, whereby high NLR was significantly associated with poor prognosis. The study suggests that NLR can be used as an independent prognostic indicator in stage II NPC patients (107).

Other factors

In addition to the above-mentioned cellular components, the presence of cytokines and other substances in tumor tissues also has a potential prognostic value. High expression of IL-35, which is correlated with EBV-induced gene 3 (EBI3) and IL-12p35, was found to be associated with the progression of NPC, and patients with high EBI3 or p35 staining were more likely to have a more advanced

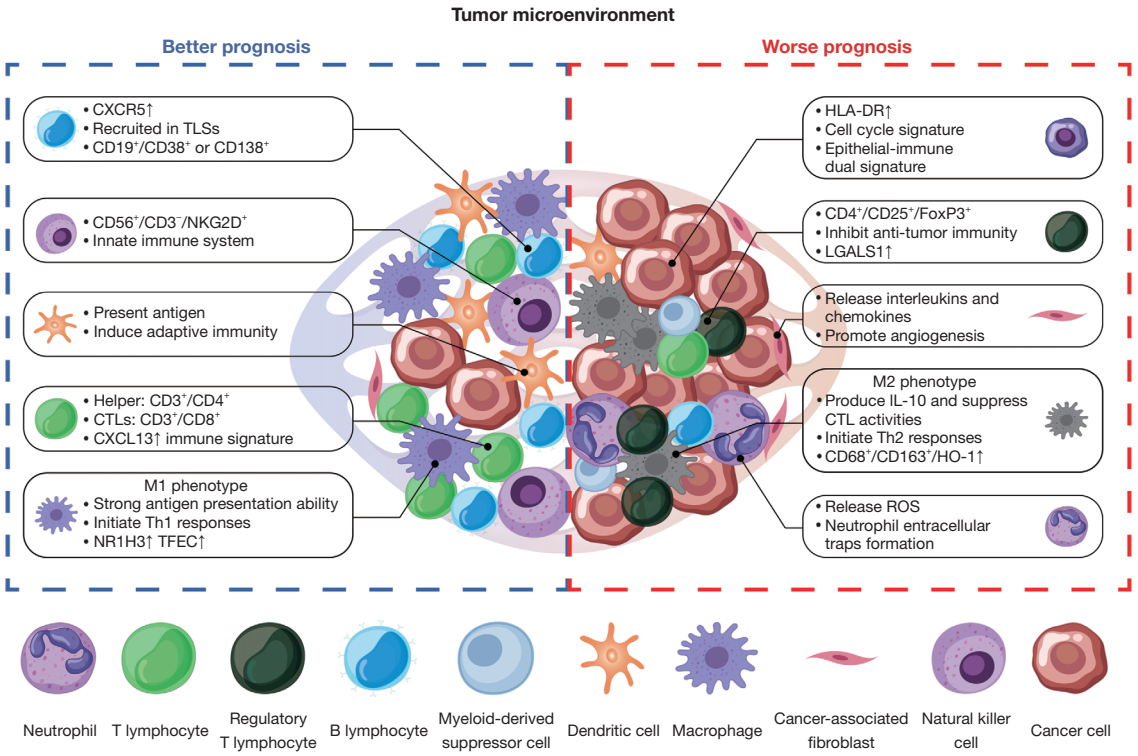

Figure 1 Cellular components of the TME of NPC that influence tumor progression and the patients' prognosis. The left panel shows the markers and key functions of major cell types, such as B cells, NK cells, CTLs and M1-like TAMs, which indicate a better prognosis of NPC. The right panel shows the markers and key functions of major cell components, such as dual epithelial-immune signature NPC cells, Tregs, fibroblasts, neutrophils, and M2-like TAMs, which indicate worse prognosis of NPC. TME, tumor microenvironment; NPC, nasopharyngeal carcinoma; NKs, natural killer cells; CTLs, cytotoxic T lymphocytes; TAMs, tumor-associated macrophages; HLA-DR, human leukocyte antigen DR.

tumor and shorter survival. By contrast, nitric oxide (NO) was found to act as a key mediator of tumor growth, and it is involved in the IL-6/NOS2 inflammatory signal pathway. It was also found that elevated levels of serum nitrite are linked to better recurrence-free survival (54).

Application of scRNA-seq in the evaluation of survival and prognosis in NPC

High PD-L1 expression of tumor cells and abundant infiltration of non-malignant lymphocytes (about 50% of samples with >70% stromal TILs or >10% intratumoral TILs) are major characteristics of NPC (11,13,21,29,86). Several important single-arm trials evaluating anti-PD-1 monoclonal antibodies in recurrent or metastatic NPC showed promising clinical outcomes (108-110). However, PD-1 inhibition benefits only a subset of patients, which proposes challenge of identifying biomarkers associated with immunotherapy response for different patient subgroups. Unfortunately, no strong evidence currently supports the correlation between response rate to anti-PD-1 monoclonal antibodies and known biomarkers such as expression levels of typical marker genes in tumor or immune cells. Nonetheless, more large-scale clinical studies and preclinical investigations are warranted to identify and validate potential therapeutic biomarkers and major subtypes in TME (e.g., TIL infiltration level, immune gene signatures, immune molecular subgroups) (111-114), which might provide an insight into NPC treatment response or resistance.

Understanding intratumoral heterogeneity is one of the greatest difficulties in cancer biology, diagnosis, and therapy (115). Developments in scRNA-seq technologies have now provided a powerful tool to explore different cell subpopulations in the TME, dissect the pathogenic hierarchies, identify novel driver genes, and illustrate the

infiltrating-immune surveillance system that determines tumor progression (116-119) (*Figure 1*). The comprehensive single-cell transcriptome atlas of the multicellular ecosystem of the NPC TME (TME) lays a new foundation for the development of diagnosis and precision therapies (120).

Subtype-specific gene signatures

By revealing the cellular heterogeneity of the TME by scRNA-seq, several intratumoral and immune subtype-specific gene signatures have been identified as potential biomarkers for the prognosis of NPC. Recently, the role of cell cycle regulation in tumorigeneses and progression has been well characterized in NPC that the cell cycle status of malignant cells could be an important factor contributing to the heterogeneity of tumors (121-125), and several reagents targeting various cell-cycle regulators have been tested in preclinical trials and clinical therapy in cancer patients (126-128). Zhao *et al.* observed that some cell cycle genes related to cell proliferation were significantly increased in EBV-positive NPC cells (129). Furthermore, survival analyses from another study revealed that high cell cycling scores significantly predicted poor survival in a clinical NPC patient cohort, which suggests that the cell cycling signatures may reflect a highly proliferative and aggressive status, reflecting the aggressiveness of NPC (130). Overall, cell cycle modulators are considered as attractive potential therapeutic targets in these NPC patients. Since radiotherapy, one of the most common treatments for NPC, induces cell cycle arrest and apoptosis (131), the combination of radiotherapy and chemotherapy with cell cycle inhibitors may be a more effective treatment modality for EBV-positive NPC. Intriguingly, the cell cycling signature did not predict survival in head and neck squamous cell carcinoma (HNSCC) (130), which means that cell cycling signatures may be a tumor-specific phenomenon of NPC.

Immune cells, particularly T and B lymphocytes, play important roles in the immunological surveillance that can potentially eliminate tumor cells (132-134). Previous studies have shown that higher levels of particular lymphocyte subpopulations in the peripheral blood were associated with tumor development and poor prognosis in NPC patients (133,135). It was also reported that an early clinical N stage and high CD4/CD8 ratio may serve as better predictors of outcomes in patient s with distant metastasis (23). Additionally, an immune-enriched subtype, identified through virtual microdissection of gene expression profiles, was found to predict the clinical outcomes and immunotherapy responses of suitable patients (136). However, there are also reports that CD8 and PD-L1 expression levels of tumor-infiltrating immune cells are not correlated with clinical outcomes (20,21). Consequently, more evidence is needed to identify biomarkers predicting the effectiveness of immune checkpoint inhibitors (ICIs) in NPC.

Heterogeneous immune cells have been characterized using transcriptional profiling at single-cell resolution in several cancers, revealing that certain subtypes of immune cells and gene signatures in the TME are important for tumor progression and sustained treatment responses (129). Profound infiltration of lymphocytes has been observed in histological biopsies of NPC. Moreover, a high density of TILs was associated with favorable survival outcomes in patients with NPC (129). Furthermore, Gong *et al.* found that CXCL13 and LGALS1 were specifically upregulated in NPC-derived T cells, illustrating the molecular variation between T cell infiltrates in the tumor and non-malignant microenvironments (137). Higher expression of CXCL13, together with a higher fraction of $CXCR5^+$ B cells, was found to be correlated with better progression-free survival (PFS) in NPC patients, suggesting the CXCL13-high T cells might be of great importance in tumor immune modulation. While LGALS1 was reported to have an immunoregulatory function in various immune cells, a higher level of immunosuppressive Tregs, instead of resting Tregs, was identified in LGALS1-high patients, and higher expression of LGALS1 was associated with poor PFS, indicating that LGALS1 might be a key regulator in Treg activation. Among other immune cell signatures, high expression of the specific genes of macrophages, DCs, DC1, NK cells, and plasma cells significantly predicted a favorable prognosis in NPC (130).

Interestingly, the epithelial-immune dual feature of NPC tumor cells, which is mainly characterized by the expression of IFN response genes, has been defined recently (138). Higher expression of HLA-DR on tumor cells was correlated with poorer OS, indicating that the epithelial–immune dual feature was closely associated with the prognosis in NPC. The tumor cells with high immune scores (ISs) increased the expression of PD-1, LAG-3, TIM-3 in both $CD4^+$ and $CD8^+$ cells, while reducing IFN-γ secretion in TILs, indicating that IS-high tumor cells may induce the exhaustion of TILs. These findings suggest that the ISs of tumor cells can serve as a potential biomarker for assessing the prognosis of NPC patients. As an alternative form of molecular subtyping, patient stratification based on

the TME hierarchy can serve as an indicator that is closely associated with treatment responses and prognosis in NPC.

In addition to traditional well-characterized immune cells and tumor cells, some cell types with low abundance in the TME of NPC may nevertheless be of great importance. Of note, Chen *et al.* found increased expression of NR1H3 and TFEC, which facilitate the differentiation and maturation of monocyte-derived macrophages (130). Further survival analyses revealed an association between high expression levels of NR1H3 and TFEC and improved outcomes in NPC patients. These results support the idea that NR1H3 and TFEC may promote antitumor immunity in NPC.

By dissecting tumor heterogeneity using scRNA-seq, functional cell types that are associated with tumor state and clinical outcomes of NPC patients can be identified. Subtype-specific gene signatures may be a strong indicator for survival and prognosis. Importantly, the relationship between NPC-specific characteristics and patient stratification and survival have been well validated, which makes great contribution to the development of personalized prognostic and therapeutic approaches. However, whether the gene signatures identified based on specific subtypes can be simply applied to bulk NPC samples still needs further validation in larger cohort studies.

Deconvolution based on the cell-type composition

Gene expression analyses of bulk tissues ignore the cell type composition as an important confounding factor, resulting in a loss of signals from rare cell types. However, scRNA-seq has limited value in clinical practice due to its high cost and long processing time. Thus, using computational deconvolution methods to infer the abundance of different cell types and/or cell type-specific expression profiles in heterogeneous samples without performing physical cell sorting is of great significance for the broader clinical application of these findings (139).

Based on the computational deconvolution methods, Gong *et al.* leveraged a RNA sequencing data of 88 NPC patients with clinical information for deconvolution using an annotated signature matrix containing 733 representative genes for selected major subpopulations of T cells, B cells and myeloid cells to estimate the abundance of immune-cell subtypes (137). The patients were further clustered into three groups based on immune-cell abundance according to the activation status of tumor immune microenvironment (TIME). The results indicated that a higher fraction of exhausted T cells, plasma B cells, DCs and macrophages was correlated with a better prognosis in NPC patients. On the other head, abundance of double-negative B cells and MDSCs in NPC patients was predictive of worse PFS. Moreover, other than cell type proportions, marker gene signatures such as low T-immune scores or inactivated TIME were found to be associated with worse prognosis (137).

Instead of adopting selected major subpopulations in a certain cell-type, Jin *et al.* partitioned 140 primary NPC tumors from treatment-naïve patients into 5 groups (G1-G5) according to their notable differences in NPC TME composition and analyzed their correlation with PFS. Tumors from G4 and G5 exhibiting relatively lower immune cell-associated signatures and were associated with a shorter PFS outcome, whereby the G5 subgroup with a higher expression of the fibroblast-associated signature had an even worse prognosis. Tumors from G1, G2 and G3 had relatively higher abundance of immune cells and presented a better prognosis. Interestingly, the G2 subgroup with a remarkably higher fraction of fibroblasts was correlated with a shorter PFS than the G1 and G3 subgroups. These data indicated that the affiliation among several major cell-types mutually influenced tumor progression (138).

Currently, there is still a lack of representative clinical models for prognostic risk stratification, survival prediction and therapeutics and drug treatment evaluation in NPC. The incorporation of functional modules into the single cell-based devolution method enhanced its reliability as an integrated and feasible model to classify cancer subtypes, as well as predict the survival and therapeutic outcomes, but it is still of great necessity to validate these findings in larger clinical cohorts.

Conclusions and outlook

Tumor formation involves the co-evolution of neoplastic cells together with ECM, stromal cells, tumor vasculature and immune cells. Tumor progression and eventual metastasis is not determined solely by tumor cells, but also by the alterations of different cell types in TME. Heterologous cell types within tumors can actively influence therapeutic response and shape resistance, therefore evaluating tumors as complete organs together with crucial cell components in TME is of vital importance.

More and more studies (130,137,138) have corroborated the idea that specific NPC-specific signatures were closely associated with patient survival, indicating that the relative abundance of various stromal subpopulations and immune activation status in NPC patients directly

influence tumor development and treatment outcomes. A pharmacogenomics-based precision medicine approach by integrating genomics with a drug sensitivity test has been conducted recently to investigate the correlation between biological features of different subtypes of NPC and their responses to therapeutic drugs (140), enlightening the application prospects of genomics-based precision medicine. The emerging scRNA-seq studies of NPC provide new opportunities for identifying novel signatures as prognostic biomarkers and pathogenic cell components as therapeutic targets. The minor subpopulations residing in the NPC microenvironment might also influence the clinical outcomes (63,114). Hence, single-cell sequencing technology is an important and powerful tool for the in-depth identification and characterization of minor subpopulations within the heterogeneous tumor mass.

Since scRNA-seq became more and more conventional, technological advancements with unprecedented cellular resolution such as single cell spatial transcriptomics, advanced tissue histology approaches and new molecular immune profiling methods provides useful tools to dissect the complex cell-to-cell interplays within TME, which may support better theoretical basis during immunotherapy and drug development. Spatial transcriptomic approaches are addressing the main drawback of scRNA-seq that the significant intra-tissue information (shape, cell-cell and cell-matrix interactions) after dissociation was abandoned (141). Recent study suggested that compartmentalization of potential immune suppression and pathogenic subpopulation-enriched gene networks revealed by single cell spatial transcriptomics may be associated with tumor subclones and tumor progression in squamous cell carcinoma patients (142). We are entering a new era of precision immunotherapy that utilizes the unearthing of predictive biomarkers, understanding of cell components in microenvironment, and design of combination therapies with better clinical application prospects. Hopefully, with the development of powerful techniques, comprehensive workflows will be established to investigate the biological mechanisms underlying its pathogenesis, refine screening and staging strategies, identify biomarkers related to prognostic risk stratification, optimize treatment strategies for the different patient subgroups, and develop novel therapeutics and drug treatment.

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