



Metastasis of nasopharyngeal carcinoma: What we know and do not know

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Abstract – Nasopharyngeal carcinoma (NPC) has the highest metastatic rate among head and neck cancers, with its underlying mechanism not yet fully unveiled. High- versus low-metastasis, NPC cell lines have been established. The footpad-popliteal lymph node metastasis model and other *in vivo* models have been stably used to study NPC metastasis. The histological appearance and the expression of epithelial-to-mesenchymal transition (EMT) markers might be helpful in selecting high-risk NPC patients for developing post-treatment metastasis. Tested EMT markers and their protein expression levels that correlate with patient disease-free survival in large patient cohorts include E-cadherin, N-cadherin, CD44, Twist, Snail, and Cyclin D1. Epstein-Barr virus (EBV) infection can trigger NPC metastasis from multiple angles via multiple signaling pathways. High endothelial venules are commonly seen in NPC tissues, with their role in NPC metastasis requiring clarification. The molecules that promote and inhibit NPC metastasis are introduced, with a focus on cytokines SPINK6, serglycin, interleukin 8 (IL8), Wnt family member 5A (WNT5A), and chemokine C-C motif ligand 2 (CCL2). Two videos showing NPC cells with and without SPINK6 knocked down are presented. Future directions for studying NPC metastasis are also discussed.

Key words: Nasopharyngeal carcinoma, Metastasis promoter, Metastasis inhibitor, High endothelial venule, Pre-metastatic niche, Epithelial-to-mesenchymal transition, EGFR, SPINK6, Video, Epstein-Barr virus, Time-lapse photography.

Background

The nasopharynx is covered with squamous epithelium, ciliated columnar epithelium, and some transitional regions of these two epithelial types [1]. Submucosal lymphoid tissue in the nasopharynx can be abundant, especially in the posterior superior wall and the roof, known as the adenoid [2]. Adenoid enlargement has been observed in children, and its atrophy is commonly seen in adults.

Nasopharyngeal carcinoma (NPC) is the most common malignancy of the nasopharynx with endemic regions in southern China and Southeast Asia [3, 4]. Although the Epstein-Barr virus (EBV) infection has not yet been conclusively confirmed

as the etiological factor of NPC tumorigenesis [5], its close relationship with NPC progression has been widely explored and EBV related biomarkers applied in clinical practice to predict NPC occurrence and recurrence [6–8]. Due to the hidden anatomical location of the nasopharynx, early NPC detection has always been a challenge, even in high-incidence areas, with screening for high-risk populations reported to help improve the early detection rate [9]. Consequently, most NPCs in endemic areas are diagnosed at a loco-regionally late stage, with about 10% of NPC patients suffer from distant metastasis at the time of diagnosis [10]. Although primary NPC is sensitive to radiotherapy and chemotherapy, 15–18.5% of new NPC patients without distant metastasis will eventually fail from loco-regional treatment caused by post-treatment metastasis of the tumor cells [11–13]. Metastasis is the main reason for treatment failure in NPC patients. This is a common challenge in our

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efforts to combat many cancer types as metastasis is the primary cause of cancer-related deaths [14].

Clinically, NPC's most common metastasis pattern is found at two organs/sites [15]. Among the five organs that are frequently colonized by cancer cells, the bone is the most common metastatic NPC site, followed by the lung, the liver, distant lymph nodes, and the brain [15]. Brain metastasis is extremely rare in NPC patients in endemic regions, with the mechanism still unclear [16]. According to the "seed and soil" theory [17], the interaction between NPC cells and the brain microenvironment, some critical features hamper the colonization and/or growth of NPC cells in brain tissues.

While anti-metastasis drugs are not available for many malignancies in current clinical practice, tremendous efforts have been spent over the last two decades trying to unveil the regulatory mechanisms of cancer cells' metastatic ability. Accumulating evidence, including that from NPC studies, shows that this malignant behavior of cancer cells is regulated by hundreds of gene products and multiple signaling cascades [18–22] and the interaction between the tumor microenvironment and tumor cells [23, 24]. This review article aims to introduce what we know about NPC metastasis from different angles and discuss the future of NPC research.

Cellular and animal models for studying NPC metastasis

It is critical to establish cellular and animal models for NPC metastasis. Our efforts [25] and others' [26] have provided two stable high-metastasis cellular clones, S18 and 5-8F, from two different NPC parental cell lines CNE2 and SUNE1, respectively. Two low-metastasis clones, S22 and S26, have also been established by our team [25]. These cellular clones have been widely used to explore the molecular mechanisms regulating NPC metastasis [27–37].

Metastasis is defined by the spread of cancer cells from one organ to other organs; therefore only *in vivo* models can be used to demonstrate this phenomenon convincingly. There are two major categories of murine models for studying metastasis [38]. One is the spontaneous model, where a primary tumor is generated, and metastatic lesions occur later in other organs. Two is the experimental metastasis model, where cancer cells are injected directly into the bloodstream to spread to distant organs without the formation of a primary tumor. In NPC studies, the widely used experimental metastasis model is the tail-vein injection of cancer cells into nude mice to generate pulmonary lesions. It is believed that the spontaneous metastasis model mimics the whole process of metastasis including the detachment of cells from the primary tumor, the invasion of cells into the surrounding normal tissues, intravasation, the survival of cancer cells in the bloodstream, extravasation, the colonization of cells in a recipient organ, the formation of micrometastasis, angiogenesis in the metastatic lesion, and the formation of macrometastasis. In contrast, the experimental metastasis model skips several initial steps of metastasis by releasing the cancer cells directly into the bloodstream.

The ideal spontaneous metastasis model generates a primary tumor in a respective organ, for example, via orthotopic

injection of breast cancer cells into a breast fat pad of a mouse to grow a primary tumor before pulmonary metastasis. However, there is no reliable orthotopic NPC mouse model for studying NPC metastasis. A convenient alternative is the spontaneous metastasis model for NPC study using a footpad injection before popliteal lymph node metastasis in a nude mouse [25, 39]. In such a model, NPC cells are subcutaneously injected into a hind footpad of a nude mouse to generate a primary tumor; metastasis will then occur in the sentinel lymph node (which is a popliteal lymph node in this model) two to four weeks after the initial inoculation of the cancer cells. Another widely used spontaneous metastasis model is via intrasplenic injection of NPC cells to generate liver metastasis [37].

Pathological morphology and metastatic propensity

According to the World Health Organization's (WHO) pathological classification, NPCs are divided into three types: keratinizing squamous cell carcinoma, non-keratinizing differentiated carcinoma, and non-keratinizing undifferentiated carcinoma [40]. However, this classification does not provide sufficient information to predict patient outcomes, especially the occurrence of post-treatment metastasis [41, 42]. A multi-center study proposed a new NPC pathological classification using a training cohort of 2716 patients, a retrospective validation cohort of 1702 patients, and a prospective validation cohort of 1613 patients [42]. With a total of 6031 patients, it is the most comprehensive study to date on NPC pathological classification. There are four types of NPC in this new classification based on morphologic features: epithelial carcinoma (EC), sarcomatoid carcinoma (SC), mixed sarcomatoid-epithelial carcinoma (MSEC), and squamous cell carcinoma (SCC) (Figure 1). Importantly, patients with sarcomatoid carcinoma have a relatively poorer overall survival rate [42]. Given that metastasis is the main reason for treatment failure in NPC, the metastatic ability of sarcomatoid NPC cells cannot be neglected. Interestingly, in our previously published NPC cohort [43], sarcomatoid NPC had a significant EMT status compared with other histological NPC types (Figure 2). Further studies to clarify the metastatic potential of sarcomatoid NPC are warranted for future selection of high-risk patients for distant metastasis, with this group of patients believed to benefit from anti-metastasis treatments.

Tumor microenvironment and pre-metastatic niche for NPC metastasis

High endothelial venules (HEVs) in NPC tissues and pre-metastatic regional lymph nodes

HEVs are unique blood vessels that facilitate the extravasation of lymphocytes from the bloodstream to lymphoid tissues (not including the spleen) [44]. HEVs are mainly localized in lymphoid organs, except for the spleen. HEVs express a specific mixture of glycoproteins, named peripheral lymph node addressin (PNAd), which are recognized by the membrane homing receptor L-selectin on the lymphocytes to fulfill their

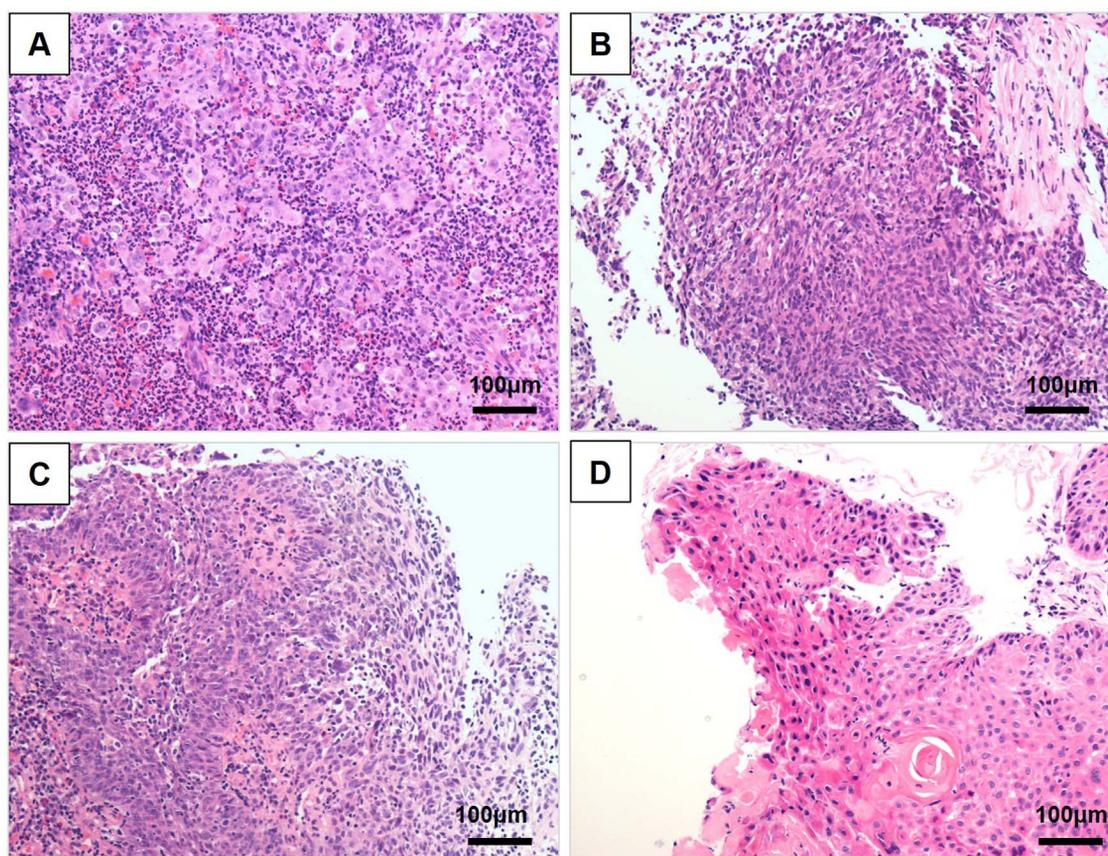


Figure 1. Representative morphologic character of tumor cells according to the new NPC classification. Typical NPC histological appearances. The epithelial carcinoma (EC) subtype shows round cells with prominent nucleoli and a pavement-like appearance, with a low nucleus: cytoplasm ratio (A). The sarcomatoid carcinoma (SC) subtype features irregular small cells and uniform medium-sized spindle cells (B). The mixed sarcomatoid-epithelial carcinoma (MSEC) subtype is characterized by a scattered infiltration of large, round cells in the spindle cell carcinomatous tissue without obvious boundaries (C). The squamous cell carcinoma (SCC) subtype shows well-differentiated keratinizing SCC with a large number of whorls and keratin (D).

immune function. However, our previous study has found that the primary tumor can remodel HEVs in the sentinel lymph node that drain the primary tumor before the arrival of NPC cells into vessels with an enlarged lumen, thinner wall, and the ability to transport large amounts of red blood cells, indicating a functional shift of HEVs from immune function to oxygen supply provider [25, 45]. After the arrival of the metastatic cancer cells, the metastatic colony further hijacks the functionally shifted HEVs to become part of the tumor vasculature with subsequent remodeling evident by partial to complete loss of PNA_d expression; this procedure is termed vessel co-option [46]. Following the engagement of tumor cells with HEVs, tumor cells can enter the bloodstream more efficiently, thereby generating distant metastasis [47].

Notably, the nasopharynx is a lymphoid organ with HEVs present in the nasopharyngeal mucosa (Figure 3). NPC cells are commonly close to the HEVs or even surrounding the HEVs. Cellular clusters inside the HEVs of NPC tissues are regularly observed, and these clusters can be made up of cancerous cells. The role of HEVs in NPC metastasis warrants further clarification.

Myeloid-derived suppressor cells

Many non-cancerous cell types have been reported to form a pre-metastatic niche in lung cancer, including bone marrow-derived cells, myeloid-derived suppressor cells (MDSCs), myeloid cells, monocytes, granulocytes, neutrophils, macrophages, regulatory T cells, endothelial progenitor cells, and hematopoietic progenitor cells [16]. Targeting the pre-metastatic niche formed by MDSCs has been suggested as a potential anti-cancer treatment strategy [48].

The important roles of MDSCs in NPC have been revealed. MDSC are a heterogeneous population of immune cells from the myeloid lineage that expand during infection, inflammation, and cancer progression [49]. A preliminary study found that MDSCs can promote NPC metastasis through over-expression of cyclooxygenase-2 (COX-2) in the cancer cells and subsequently activate beta-catenin/Transcription factor 4 (TCF4) signaling [50].

More efforts are needed to elucidate the cross-talk among multiple cell types underlying the formation of NPC pre-metastatic niches.

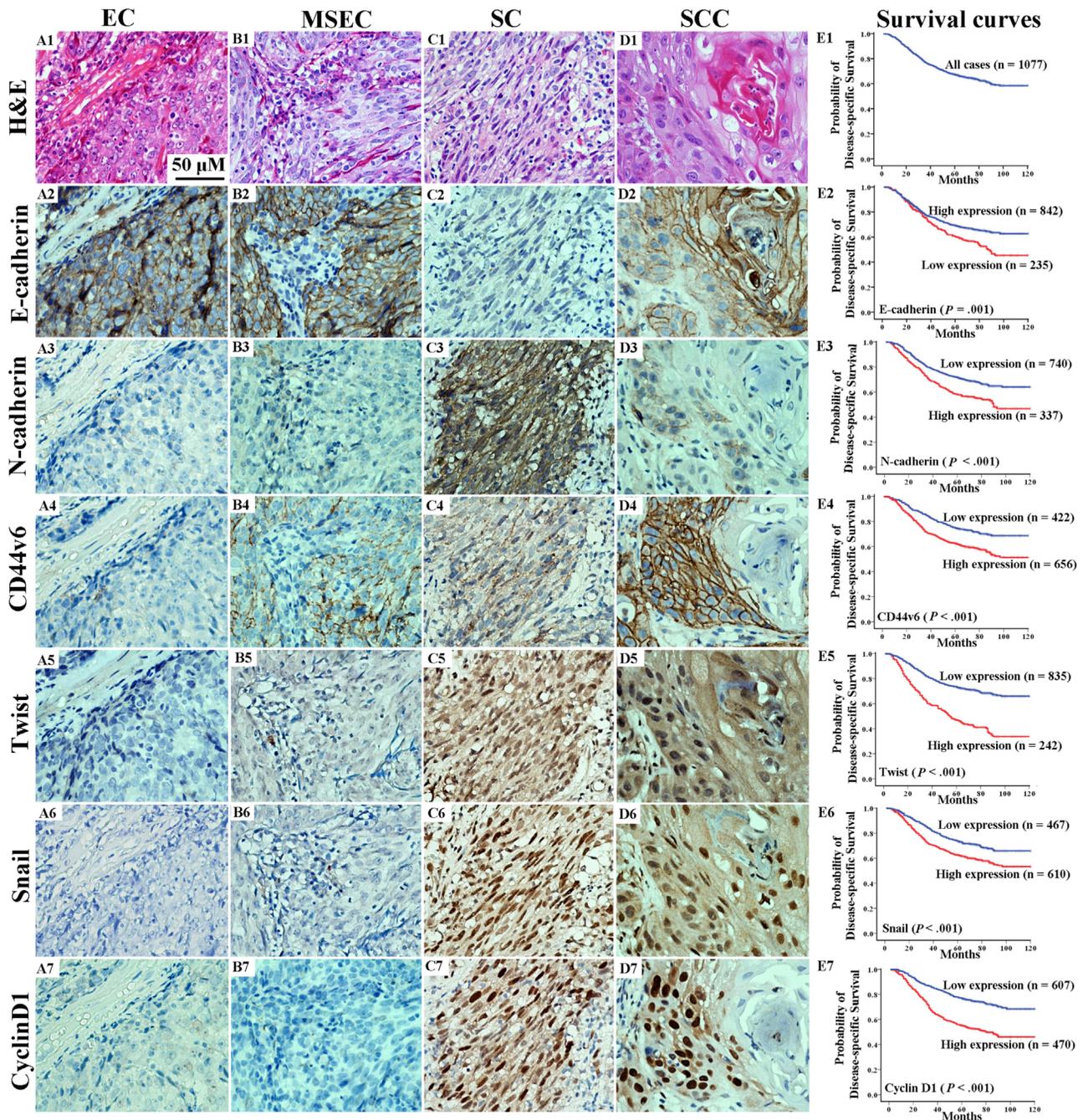


Figure 2. The expressions of epithelial-mesenchymal transition (EMT) markers in NPC and their correlations with patient disease-free survival. The hematoxylin and eosin (H&E) staining features of EC, MSEC, SC, and SCC subtypes (line 1, A1, B1, C1, and D1). Preserved E-cadherin expression in EC (A2), MSEC, (B2), and SCC (D2) in contrast with loss of expression in SC (C2) are shown in line 2. N-cadherin IHC staining with overexpression in SC (C3) and absent expression in EC (A3), MSEC (B3), and SCC (D3) are shown in line 3. CD44v6 IHC staining with a lack of expression in EC (A4) and expression in MSEC (B4), SC (C4), and SCC (D4) is shown in line 4. Twist IHC staining showing no expression in EC (A5) and MSEC (B5) and overexpression in SC (C5) and SCC (D5) (line 5). Snail IHC staining showing no expression in EC (A6) and MSEC (B6) and overexpression in SC (C6) and SCC (D6) (line 6). Cyclin D1 IHC staining with no expression in EC (A7) and MSEC (B7) and overexpression in SC (C7) and SCC (D7) (line 7). All histological images were taken using the same magnification shown in A1. Panel E1 shows the disease-free survival curve of all 1077 NPC patients. Panel E2-7 shows the survival curve of 1077 patients with IHC staining of EMT markers. The 5-year disease-free survival rates of NPC patients with low- vs. high-level expression. The indicated markers are: 60.8% vs. 69.4% for E-cadherin (E2), 71.9% vs. 58.3% for N-cadherin (E3), 75.1% vs. 62.7% for CD44v6 (E4), 73.5% vs. 47.1% for Twist (E5), 74.0% vs. 62.7% for Snail (E6), and 77.2% vs. 55.3% for cyclin D1 (E7). The P -values for all comparisons are less than 0.01. The largest difference in 5-year disease-free survival was in the Twist expression comparison, with a difference of 26.5%.

Epithelial to mesenchymal transition in NPC metastasis

The transition of epithelial cells to mesenchymal like-cells is called epithelial-to-mesenchymal transition (EMT). It is widely believed that EMT is the initial step in the cascade of cancer metastasis [51, 52]. Although, some controversial findings suggest that EMT is dispensable for metastasis [53]. In addition to the morphological alteration of the cells, EMT is more commonly determined by overexpression of mesenchymal markers and a loss of epithelial markers. Over a dozen EMT markers have been widely used in cancer research [54]. Among them, according to our large-cohort study findings, the elevated expressions of E-cadherin are correlated with better NPC patient prognosis. In contrast, the elevated expressions of N-cadherin, CD44v6, Twist, Snail, and Cyclin D1 are correlated with poorer NPC patient prognosis (Figure 2).

Based on our findings and other reports, the metastatic ability of NPC cells correlates with EMT [31, 37, 55, 56]. Suppressing EMT by CLCA2 and NOTCH2 can inhibit NPC metastasis in animal models [35, 57]. However, direct evidence of NPC metastasis inhibition by reversing EMT or inducing mesenchymal-to-epithelial transition is unconvincing, which is part of the much larger dilemma in cancer biology studies [58]. Moreover, some pro-metastasis cytokines do not induce typical EMT when they promote NPC metastasis. Serglycin, for example, can strongly promote NPC metastasis but only increases vimentin expression among various detected EMT markers [39]. These findings suggest that the metastatic ability of NPC cells could be both EMT-dependent and EMT-independent.

Epstein-Barr virus (EBV) infection and NPC metastasis

EBV is a latent infection in more than 90% of adults worldwide [59]. It is associated with NPC and lymphoid malignancies, such as Burkitt's lymphoma [60]. Almost all NPCs in endemic regions are associated with EBV infection [61]. The expression of the EBV genome in NPC cells comes from protein-coding and non-protein-coding genes. The former includes EBV latent membrane proteins (LMPs) and EBV nuclear antigens (EBNAs). The latter includes EBV-encoded small non-polyadenylated RNAs (EBERs) and microRNAs4 [62]. Two small EBV encoded RNAs, EBER1 and EBER2, are the most abundant viral transcripts in latent EBV-infected NPC cells [63]. The EBV miRNAs are mainly BamHI-A region rightward transcripts (BARTs) and BamHI fragment H rightward open reading frame 1 (BHRF1) miRNA clusters [64, 65]. In the latent 1–2 types, miRNAs encoded by BART are detectable, whereas miRNAs from BHRF1 are mainly detectable in latent type 3 and lytic infected cells [66]. EBV genes have been reported to be involved in NPC malignant progression, including EMT, cellular motility, angiogenesis [67], metastasis [68], and treatment resistance [66].

Target cells of EBV mainly include B lymphocytes and epithelial cells. EBV glycoproteins gHgL, and gB, are regarded as the core fusion machinery for membrane fusion of EBV with

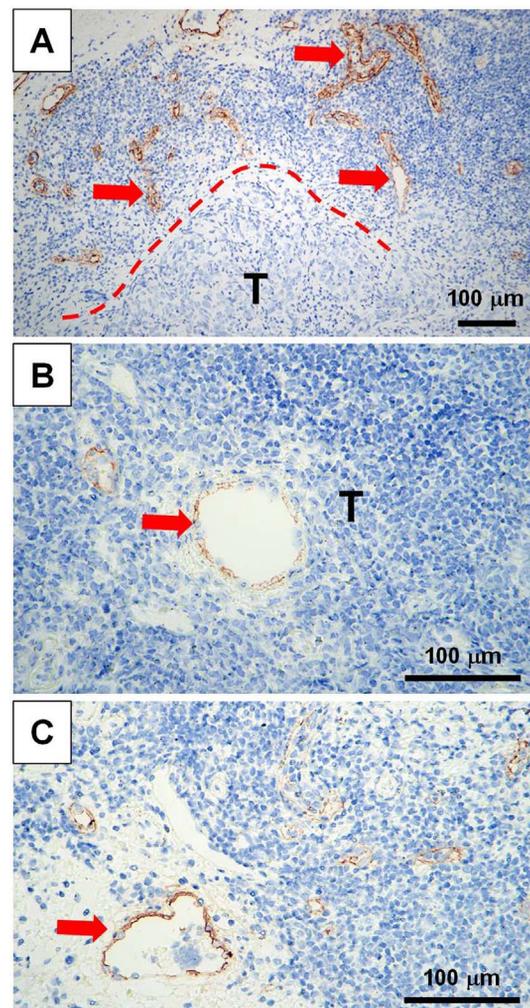


Figure 3. High endothelial venules in NPC tissues. High endothelial venules (HEVs) stained by MECA-79 antibody to visualize peripheral node addressin (PNAd). (A) The HEVs are indicated by arrows located in the periphery of the tumor nest (T). (B) One HEV is surrounded by tumor cells. (C) A NPC tissue section with an embolus cell cluster within a HEV (arrow).

all cell types [69]. EBV gB interacts with neuropilin 1, which may activate neuropilin 1-dependent EGFR (RTKS) signaling and promote EBV to enter nasopharyngeal epithelial cells through macropinocytosis and lipid raft-dependent endocytosis [70]. F-box only protein 2 (FBXO2) directly binds to gB N-glycosylation sites promoting gB degradation via the ubiquitin-proteasome pathway. A recent study found that depletion of FBXO2 promotes gB localization on the surface of nasopharyngeal epithelial cells, resulting in enhanced membrane fusion and viral entry [71].

Two small EBV encoded RNAs, EBER1 and EBER2, are the most abundant viral transcripts in latent EBV-infected NPC cells [63]. The overexpression of EBERs can trigger the secretion of pro-inflammatory cytokines, macrophage colony-stimulating factor (M-CSF), and monocyte chemo-attractant protein (MCP-1) via nuclear factor- κ B (NF- κ B) and IRF3 signaling pathways to form a pro-tumorigenic inflammatory

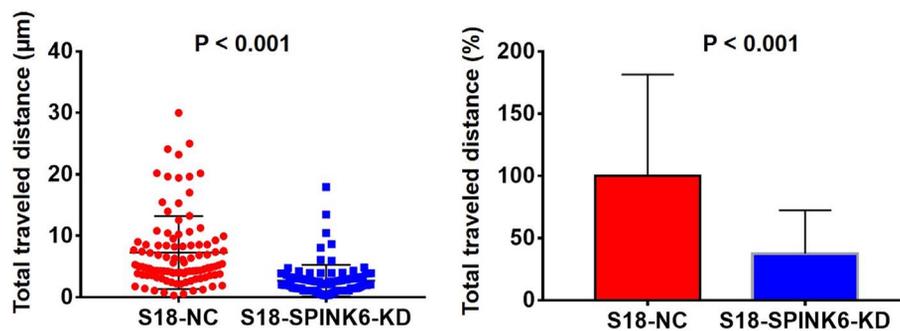


Figure 4. Migratory ability of NPC S18 cells is suppressed by knocking down SPINK6 expression. S18-NC and S18-SPINK6-KD cells (GFP-tagged) were seeded in a 96-well plate (2000 cells per well) and cultured in DMEM with 10% FBS in 5% CO₂ at 37 °C for 24 h. Next, the plate was placed under a Nikon ECLIPSE Ti2 Inverted Microscope System supplied with 5% CO₂ at 37 °C. Images of random fields at 100× magnification were taken automatically every 30 min for 72 h. A total of 145 sequential images of each field were taken, generating a video to display the movement of the cells at a speed of 15 FPS (frame/sec) (see Videos 1 and 2). The locations and moving tracks of the cells were analyzed using Image J software and the distance of movement between sequential images every 30 min were calculated. The videos displaying the movement of S18-NC and S18-SPINK6-KD cells are shown in Videos 1 and 2. The accumulated distance traveled in 72 h of 100 randomly selected cells from each cell line were calculated and are shown in A and B.

tumor microenvironment, which consequently promotes NPC development [72].

In an analysis of genome-wide profiling of EBV integration, the EBV genome can integrate into the human genome resulting in down-regulation of the genes coding tumor necrosis factor- α -induced protein 3 (TNFAIP3), Parkinson disease-2 (PARK2), and cyclin-dependent kinase 15 (CDK15) [73]. Recent findings revealed that all of these three gene products are either motility or metastatic inhibitors. TNFAIP3 inhibits the migration and invasion of NPC cells [74], PARK2 suppresses the metastasis of glioblastoma cells [75], and CDK15 is a metastatic breast cancer inhibitor [76].

The latent membrane protein 1 (LMP1) is an EBV-encoded protein and regulates biological abnormalities of NPC through various signaling pathways [69], including cell migration (SOCE, integrin- α 5 and N-cadherin) [77, 78] angiogenesis and permeabilization (SOCE) [79], necroptosis (RIPK1/3) [80], stemness (PI3K/AKT) [81], and metastasis [82]. A recent high-throughput sequencing study proved that LMP1 constitutively activated NF- κ B signaling [83], with LMP1-activated NF- κ B binding to the promoter region of the microRNA-203 gene downregulating miR-203, leading to EMT and potentially to metastasis propensity [84].

The transcriptional factor Twist, a highly conserved basic helix-loop-helix protein, is essential for early embryogenesis, which promotes EMT and plays an essential role in metastasis. LMP1 can induce Twist via the NF- κ B pathway activating the EMT program and promoting metastasis [85].

LMP2A is another membrane protein encoded by the EBV-LMP2 gene. It is consistently expressed in over 98% of NPCs [86] and activates PI3-K/Akt, JNK/SAPK, ERK-MAPK, and Wnt/beta-catenin signaling to regulate cell growth, apoptosis, and differentiation [67, 86], as well as competitively binding to Syk to suppress the interaction between SYK and ITGbeta4 to promote NPC cellular migration and invasion [87].

The EBV-encoded microRNAs that can activate invasion and metastasis of NPC cells include miR-BART6-3p,

miR-BART7-3p, miR-BART8-3p, miR-BART9, miR-BART13, and miR-18-5p [88].

Taken together, the EBV infection is an important factor in promoting NPC cellular motility and metastasis from multiple angles and involves multiple signaling pathways.

Metastatic promoters in NPC

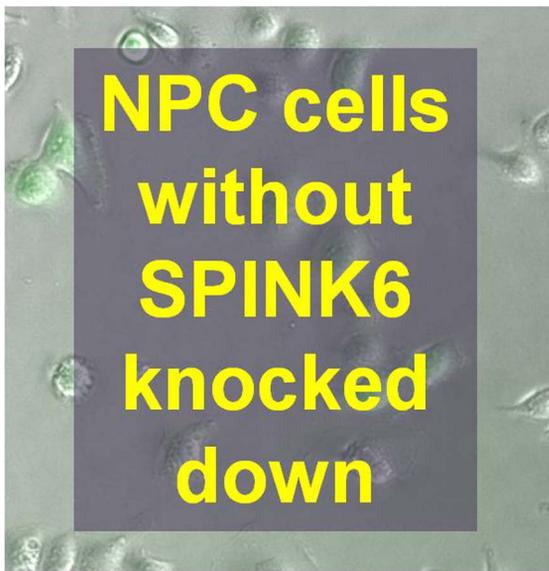
Cytokines and their receptors

Multiple cytokines are NPC metastasis promoters. These secreted molecules commonly act through both autocrine and paracrine manners in promoting NPC metastasis.

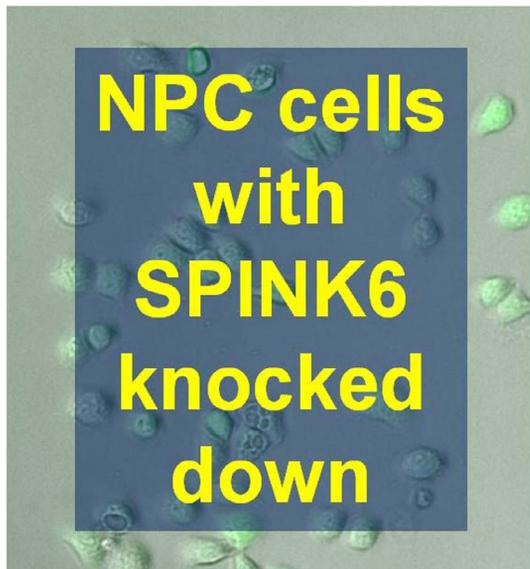
Kazal-type serine proteinase inhibitor 6 (SPINK6) possesses the normal physiological function of inhibiting several kallikrein-related peptidases (KLKs), specifically, KLK5, KLK7, and KLK14 [89]. Our group revealed that SPINK6 is also the ligand of epidermal growth factor receptor (EGFR) in NPC cells which induces the dimerization of EGFR and results in activating downstream AKT signaling for metastasis, and this function of SPINK6 is independent of its serine protease-inhibitory activity [37]. Using time-lapse photography, the total traveled distance of NPC S18 cells with or without SPINK6 knockdown is quantified in Figure 4 and shown in Videos 1 and 2.

Serglycin is a proteoglycan with its core peptide coded by the SRGN gene in humans. It is a family member of small proteoglycans with serine-glycine dipeptide repeats and modified with different types of glycosaminoglycan side chains [90]. In high-metastatic NPC cells, we found that serglycin promotes NPC metastasis through binding to its receptor CD44 and consequently activates MAPK/ β -catenin signaling [39, 91]. More interestingly, serglycin can also upregulate CD44 expression in an autocrine manner to maintain self-renewal of NPC cells [91].

As a member of the CXC chemokine family, interleukin 8 (IL8), alternatively known as CXCL8, is secreted by endothelial cells, neutrophils, tumor-associated macrophages,



Video 1. Migratory ability of NPC S18 cells transfected with the scrambled control shRNA (S18-NC). The time-lapse photography images described in Figure 4 of the NPC S18 cells without SPINK6 knocked-down (S18-NC). Notably, the cells are more active in comparison with Video 2. <https://vcm.edpsciences.org/10.1051/vcm/2021003#V1>



Video 2. Migratory ability of NPC S18 cells transfected with shRNA against SPINK6 (S18-SPINK6-KD). The time-lapse photography images described in Figure 4 of the NPC S18 cells with SPINK6 knocked-down (S18-SPINK6-KD). Notably, the cells are less active in comparison with Video 1. <https://vcm.edpsciences.org/10.1051/vcm/2021003#V2>

and cancer cells [92]. IL8 binds to two cellular membrane receptors CXCR1 and CXCR2, for its pro-inflammatory functions. We previously reported that high-metastatic NPC cells could secrete IL8 promoting NPC metastasis, with AKT signaling being the downstream activated pathway [31]. E-cadherin

has been regarded as an inhibitor of NPC metastasis [93, 94]. Another study of ours revealed that IL8 suppresses E-cadherin expression in NPC cells by enhancing E-cadherin promoter DNA methylation [95].

Wnt family member 5A (WNT5A) belongs to the large WNT family of cysteine-rich secreted glycoproteins and binds to Frizzled family receptors (Fzd) and low-density lipoprotein receptor-related protein 5/6 co-receptors (LRP5/6) [96]. We reported WNT5A promotes EMT in NPC cells, induces the accumulation of CD24⁻/CD44⁺ stem-like cells, and promotes NPC metastasis via protein kinase C (PKC) signaling [97].

Chemokine C–C motif ligand 2 (CCL2) is a cytokine that can bind to its receptor chemokine C–C motif receptor type 2 (CCR2) and trigger ERK1/2-MMP2/9 signaling for promoting NPC metastasis [98].

Other cytokines reported to promote NPC cellular motility in vitro, and hence are potential NPC metastasis-promoters, include TNFAIP2 [99], IL-6 [100], and IL-17A [101].

Cell-surface proteins as metastasis drivers

Epidermal growth factor receptor (EGFR) belongs to the ErbB receptor tyrosine kinase (TK) family, with its dysregulation found in multiple human cancers. The activation of EGFR by its native ligand EGF for deteriorating NPC metastasis has recently been confirmed [102], with PKM2 one of its key downstream molecules. SPINK6 is another ligand that can bind to EGFR and promote NPC metastasis [37].

Urokinase-type plasminogen activator receptor (uPAR) is a glycosyl phosphatidylinositol-anchored membrane protein possessing multiple functions, which its ligand uPA can activate through the binding of vitronectin [103]. We reported that uPAR promotes NPC cell growth and metastasis via activating the JAK-STAT pathway [27].

Ezrin is a member of the Ezrin-Radixin-Moesin protein family functioning as a linker between the plasma membrane and actin cytoskeleton, which is also known as a metastatic promoter in breast cancer [104]. Suppression of Ezrin expression results in an inhibition of NPC cellular migration and invasion [105], and its mRNA stability can be enhanced by circular RNA circARHGAP12 [106].

Glypican 6 is a putative cell surface co-receptor for growth factors. Its over-expression promotes NPC cellular motility, which might promote metastasis but is yet to be confirmed [107]. Another membrane protein, TSPAN8, has also been reported to promote NPC cellular motility via activating Akt/MAPK signaling [32].

Intracellular proteins as metastasis drivers

Two important intracellular proteins that activate the PI3K/AKT pathway and subsequently stimulate NPC metastasis are the non-receptor tyrosine kinase c-Src [108] and Ca²⁺-dependent phospholipid-binding protein annexin A1 [109]. Another intracellular protein that can also trigger AKT signaling for enhancing NPC cellular motility is FLJ10540 [110] and therefore possesses the potential to promote NPC metastasis. FLJ10540

Cdc42-interacting protein 4 (CIP4) is encoded by the *TRIP10* gene. Our group found that CIP4 is required for NPC cellular motility and metastasis. CIP4 promotes the activation of N-WASP that controls invadopodia formation. Meanwhile, CIP4 activates EGFR signaling resulting in an up-regulation of matrix metalloproteinase 2 (MMP2) [34].

Serine beta-lactamase-like protein (LACTB) is an enzyme encoded by the *LACTB* gene and located in the mitochondrial intermembrane space. LACTB's normal function is to regulate lipid metabolism. Our recent findings show that LACTB promotes NPC metastasis by activating ERBB3/EGFR-ERK signaling [111].

Zinc finger protein SNAI1 (SNAIL) is a transcriptional repressor that downregulates the expression of E-cadherin. The over-expression of SNAIL can promote NPC metastasis partially by down-regulating telomere length regulation protein TEL2 [112].

Non-coding RNAs

Accumulating evidence shows the important roles of non-coding RNAs in promoting NPC metastasis. Long non-coding RNA AFAP1-AS1 competes with endogenous RNA miR-423-5p to promote NPC metastasis through activating Rho/Rac signaling [113]. Long non-coding RNA FAM225A acts as ceRNA to sponge miR-590-3p/miR-1275 and upregulates ITGB3 to promote NPC metastasis [114]. Long non-coding RNA LINC01503 stimulates NPC metastasis by recruiting splicing factor proline-and glutamine-rich (SFPQ) to activate Fos-like 1 (FOSL1) transcription [115]. MicroRNA-494-3p promotes NPC cellular motility and growth by targeting Sox7 [116]. It is expected that more and more non-coding RNAs regulating NPC metastasis will be identified.

Metastatic inhibitors in NPC

In recent years, several NPC metastatic inhibitors have been revealed acting through a variety of mechanisms illustrating the complexity of NPC metastasis.

Transcriptional regulation

TEL2 belongs to E26-transformation specific transcription factors, which inhibit NPC metastasis by suppressing the expression of serpin family E member 1 (SERPINE1) [36].

Transcription factor zinc finger protein 582 (ZNF582) inhibits NPC metastasis via regulating the transcription and expression of adhesion molecules nectin-3 and neurexin 3 (NRXN3) [117].

Membrane receptor regulation

Toll-like receptor 3 (TLR3) activation inhibits NPC metastasis by down-regulating the expression of chemokine receptor CXCR4 [118].

Central pathway regulation

Shroom family member 2 (SHROOM2) inhibits NPC metastasis via RhoA-ROCK signaling-dependent and -independent mechanisms [119].

Depending on integrin β 1 and merlin interaction, CHL1 suppresses NPC metastasis by inhibiting the PI3K/AKT signaling pathway [120].

Nidogen-2 (NID2) suppresses NPC metastasis by inhibiting EGFR/Akt and integrin/FAK/PLC γ metastasis-related pathways [121].

The critical inhibitory effects of non-coding RNAs against NPC metastasis have also been discovered with the findings that NKILA, a long non-coding RNA, inhibits NPC metastasis via suppressing NF- κ B signaling [122], and MiR-99a inhibits NPC metastasis through targeting HOXA1 [123].

Other mechanisms

Other proteins and non-coding RNAs that can inhibit the migration and invasion of NPC cells and therefore possess the potential to inhibit NPC metastasis *in vivo* include TNFAIP3 [74], bactericidal/permeability-increasing-fold-containing family B member 1 (BPIFB1) [124], MYH10 [125], miR-30e-5p [126], miRNA-101 [127], and miR-451 [128].

Other oncogenes and tumor suppressor genes in NPC cells

Studies on chromosomal alterations in NPC cells have revealed the association between allelic losses on the short arms of chromosomes 3 and 9 and inactivation of several tumor suppressor genes, particularly p14, p15, and p16 [129–131]. Other genomic studies have found that HLA genes residing at the MHC region on chromosome 6p21 and other loci outside the MHC (e.g., TNFRSF19 on 13q12, MECOM on 3q26) are risk loci associated with NPC occurrence and development [10]. Recently, some molecular signatures that can predict NPC's prognosis independent of TNM staging have been identified. For locoregionally advanced nasopharyngeal carcinoma, a gene-expression signature of 13 genes has been generated to predict the risk of survival, the risk of distant metastasis and the effect of concurrent chemotherapy in NPC patients [132]. Consisting of five features (B7-H3TAIC, IDO-1TAIC, VISTA-TAIC, ICOSTAIC, and LAG3TAIC), the immune checkpoint-based signature (ICS) has also been proposed to predict the risk of overall, distant metastasis-free and disease-free in NPC, which was significantly linked to survival in patients with a high EBV-DNA load [133].

In addition to molecular signatures, an integrated molecular analysis identified two NPC-associated oncogenes, lymphotoxin beta receptor (LTBR) and Cyclin D1. LTBR encodes a member of the TNFR family, which activates the main signaling pathways, including NF- κ B and c-Jun N-terminal kinase. In a recent study, ectopic expression of LTBR correlated with upregulated NF- κ B activity in nasopharyngeal epithelia cells [134].

Cyclin D1 is a cell cycle regulating protein, which regulates the G1 to S-phase transition. It forms complexes with CDK4 or CDK6, subsequently phosphorylates the retinoblastoma tumor suppressor protein, and initiates DNA synthesis. It is overexpressed in more than 90% of primary NPCs. Array-based comparative genomic hybridization analysis identified Cyclin

D1 as the target oncogene in the 11q13.3 amplification of NPC [135]. Cyclin D1 transcription is regulated by the constitutively activated NOTCH3 signaling [136]. In vitro experiments have also demonstrated that cellular proliferation and metastasis are dependent on Cyclin D1 activation or other mechanisms for cell cycle inactivation (e.g., p16).

In the majority of NPCs, the PI3K-AKT signaling pathway is constitutively activated. The PIK3CA, located at 3q26.1, encodes the 110-kDa catalytic subunit of phosphatidylinositol 3-kinase (PI3K) and is a common oncogenic alteration in NPC. Phosphatidylinositol 3,4,5-triphosphate (PIP3) is generated as a second messenger when PI3K is coupled with the 85-kDa subunit. The PIP3, in turn, activates AKT signaling and a wide range of downstream targets that promote cell proliferation, inhibit apoptosis, and trigger cellular motility. Copy number gain and amplification of PIK3CA have been found in 75% and 20% of primary tumors, respectively, suggesting the critical roles of AKT signaling in NPC progression.

Future directions

It is evident that our understanding of NPC metastasis is limited, and a multitude of investigations are warranted. It is expected that the characterization of key molecules promoting or inhibiting NPC metastasis might be useful in identifying high-risk patients at the time of diagnosis, those prone to post-treatment distant metastasis, and developing targeted therapeutics for clinical intervention.

To use the well-established NPC cells with different metastatic abilities for anti-metastasis drug development is another plausible direction. Theoretically, these cells can be used to screen any compound library for identifying the lead anti-metastasis compounds. Of course, a live cell-based high throughput screening system needs to be established for this purpose, and more effective preclinical models need to be optimized to validate the identified lead compounds.

A better understanding of the pre-metastatic niche will help develop novel approaches to block the further spread of NPC cells from the sentinel or regional lymph nodes to distant organs. By doing this, the development of cancer progression can be significantly slowed down and patient survival meaningfully prolonged.

Taken together, metastasis in NPC is the bottleneck for further improvement of treatment outcomes. We can overcome this challenge by developing an early alert system for NPC metastasis based on our deeper understanding of its underlying mechanisms and developing anti-metastasis agents.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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