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Cancer stem-like cells of nasopharyngeal carcinoma express CXCR4 and display highly invasive activity

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Running Title: CXCR4+ NPC tumorsphere cells exhibit metastatic potential

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Abstract

Background: Nasopharyngeal carcinoma (NPC) patients with distant metastasis have a poor response to conventional first-line chemoradiotherapy and a poor prognosis. Growing evidence suggests that a subpopulation of cancer stem cells (CSCs) with certain markers is essential for cancer metastasis. However, the unique subset of CSCs that drives NPC metastasis is still unclear. **Methods:** Immunohistochemistry was used to analyze the expression characteristics, distribution and relationship of CXCR4 with clinical parameters in nasopharyngeal carcinoma. The expression characteristics of CD133 and CXCR4 in nasopharyngeal carcinoma tumorspheres were analyzed by flow cytometry. Through a magnetic activated cell sorting system, CXCR4-positive and CXCR4-negative tumorsphere cells were obtained, and then, the differences in invasion (in vitro and in vivo), metastatic tumor formation, stemness and EMT characteristics of the two cell types were compared. SDF-1 and its inhibitor were used to explore the roles of stromal-cell derived factor-1 (SDF-1) in the metastatic process. **Results:** In 71 cases of human nasopharyngeal carcinoma, CXCR4 expression was correlated with T stage and N stage. The CXCR4-positive rate of the tumor cells in the T3-4 group was higher than that in the T1-2 group (60.6% vs 28.9%, P =0.009), and that in the N2-3 group was higher than that in the N0-1 group (76.5% vs 13.5%, P=0.000). The CXCR4 expression level was higher in cells with high metastasis or low differentiation. The culture of tumor spheres enriched CD133-positive and CXCR4-positive cells, and most CD133-positive cells also expressed CXCR4 in NPC. The secondary tumorsphere-forming rate of the CXCR4-positive tumorsphere cells was higher than that of the CXCR4-negative tumorsphere cells, and most of the CXCR4-positive tumorsphere cells had a spindle morphology. The CXCR4-positive tumorsphere cells had stronger migration and invasion ability in vitro and metastatic tumor formation ability in mice, which depends on the SDF-1/CXCR4 axis, than the CXCR4-negative tumorsphere cells.Compared with those of the CXCR4-negative tumorsphere cells, the mRNA expression levels of Oct4, Nanog, Sox2, Snail, Twist, Vimentin and N-cadherin in the CXCR4-positive tumor tumorsphere cells were increased, while E-cadherin decreased, and the protein expression levels of Vimentin and N-cadherin increased, while that of E-cadherin decreased. **Conclusion:** Therefore, CXCR4 may be a marker of nasopharyngeal metastatic tumor stem cells, and CXCR4 may be involved in tumor stem cell migration and invasion by regulating the EMT process.

These results suggest that targeting the SDF1/CXCR4 axis may interfere with nasopharyngeal carcinoma tumor stem cell migration and invasion and provide new treatment strategies.

Keywords: NPC; Metastatic tumor stem cell; SDF1; CXCR4; EMT.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor occurring in the epithelium of the nasopharynx (top and sidewall of the nasopharynx). This disease shows a distinct geographical distribution and ethnic susceptibility. NPC is mainly found in South China and Southeast Asia, especially in the Guangdong and Guangxi provinces, and is the leading head and neck malignant tumor[1]. Radiotherapy and chemotherapy are the main treatments for advanced NPC. Due to the development of diagnostic and treatment technology, the survival time of patients with nonmetastatic NPC has been significantly improved. However, approximately 15% of patients already have distant metastases at their first visit [2]. Patients with distant metastasis of NPC have a poor response to conventional first-line chemoradiotherapy and a poor prognosis[3]. Therefore, it is necessary to further explore the mechanism of NPC metastasis. Understanding the pathophysiological mechanisms involved in distant metastasis of NPC is important for the discovery of new therapeutic and prognostic approaches.

Cancer stem cells (CSCs) have the ability to self-renew and differentiate[4,5].Tumorsphere culture technology is an important method for enriching tumor stem cells and is widely used in basic research. Cells isolated from the tumorspheres are believed to show self-

renewal and tumor formation and are widely believed to have the characteristics of stem cells[6,7].Thomas Brabletz et al[8]. proposed that there are two dynamic types of tumor stem cells: stable tumor stem cells and metastatic cancer stem cells (MCSCs). Metastatic tumor stem cells not only possess the characteristics of stem cells but also acquire the ability to invade through the EMT process. In this process, Snail, Twist, Zeb and other transcription factors located upstream of EMT alter the adhesion, migration and invasion of tumor cells by regulating the expression of E-cadherin, N-cadherin and Vimentin. Moreover, these transcription factors interact with stem cell-related genes such as SOX2, OCT4 and Nanog to influence the two dynamic patterns of tumor stem cells. Signaling pathways such as Wnt and TGF play an important role in the EMT process [9,10].

Studies further showed that a subpopulation of CSCs with the CD133 and CXCR4 markers is essential for colon and pancreatic cancer metastasis [11,12]. However, it is still not clear which unique subset of CSCs can drive NPC metastasis. We hypothesized that there may be a subset of NPC stem cells capable of forming metastases in addition to tumorigenesis. However, this hypothesis needs to be proven in MCSCs isolated from CSCs.

Initial studies have suggested that $CXCR4^+$ NPC cells have a stronger capability for invasion and metastasis [13-15]. CXCR4 may not be the only marker, but it remains the most widely reported marker of MCSCs in many types of cancer [11,12,16]. In this study, we further assessed the expression of CXCR4 in NPC tumorspheres and investigated the role of CSC subsets expressing this marker in metastasis.

The CXCR4 chemokine receptor and its ligand CXCL12 (SDF-1) are one of the best-studied chemokine systems in tumor biology. In gliomas, the CXCL12/CXCR4 axis regulates multiple mechanisms that sustain tumor proliferation, migration and angiogenesis [17-19]. CXCL12 is the only known chemokine that binds CXCR4. Recent studies suggest that CXCR4 plays an important role in tumor metastasis and progression[20-22].

In this study, we identified a subpopulation of CXCR4+ NPC-CSCs that is capable of forming metastases and CXCR4 signaling as a potential target for novel therapeutic strategies in NPC.

Methods

Cell culture

The immortalized nasopharyngeal epithelial cell NP69 and NPC cell lines (6-10B, 5-8F, CNE1, CNE2 and Sune1) were obtained from the Cancer Research Institute of Southern Medical University (Guangzhou, China). In this study, these cell lines were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with Penicillin-Streptomycin solution (100 units/ml penicillin and 100 mg/ml streptomycin) and bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel). The cell incubation condition requires a sterile environment in a humidified incubator under 95% air and 5% CO2 at 37°C.

Tumorsphere culture and count

The cells were collected, washed to remove serum three or four times with PBS, digested into single cells and suspended in a special serum-free medium. This medium consists of DMEM/F12 (Biological Industries) as the basic component, and contains 2% B27 (1:50 dilution), 20ng/ml epidermal growth factor and 10ng/ml basic fibroblast growth factor. These cytokines were procured from PeproTech company (Rocky Hill). The cells were administered in ultralow attachment 6-well plates (Corning, Inc., Corning, NY, USA) in serum-free medium with a density of 500 cells /ml and stored in a humidified incubator at 37°C under 5% CO_2 for 7 days. After the experiment, several lines were drawn on the bottom of the six-well plate, and the number of tumor spheres with more than 50 cells could be directly observed and counted under an inverted microscope. Each sphere was approximately 100 μm in diameter.

RT-qPCR

We cultured adherent cells for 24 h and tumorsphere cells for 7 days and then collected the cells. After that, according to the instruction manual of the manufacturer, We extracted RNA using RNAiso Plus Extraction Kit (TaKaRa Bio, Inc., Otsu, Japan). Data acquisition of the concentration of total RNA was performed using a spectrophotometer (Thermo NanoDrop 2000, Thermo Fisher Scientific, Inc.). Extracted RNAs were reverse transcribed using a reverse transcriptase reagent kit (Takara Biotechnology, Japan). Then, we conducted qPCR using RT-qPCR Master Mix reagent kit (Takara Biotechnology, Japan). The primer sequences used for octamer binding transcription factor

4 (Oct-4), sex determining region Y box 2 (Sox2), Nanog, β-catenin, Snail, Vimentin, E-cadherin, Twist, CXCR4 and GAPDH are reported in Table I. GAPDH expression was used as the reference standard for internal control. The PCRs included initial denaturation at 95°C for 2 min, followed by 42 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 sec, and a final incubation at 72°C for 30 sec. The data obtained from RT-qPCR experiments were analyzed by 2□ΔΔCq method in this study.

Western blot analysis

A protein extraction kit (Beyotime Biotechnology, China) was used to lyse the cells to obtain whole cell protein. The BCA reagent kit (Beyotime Biotechnology, China) was used to detect the proteins concentration.Total target proteins were separated using a 10% SDS PAGE gel and transferred to a PVDF membrane (Millipore, Boston, USA). The membranes were blocked in Tris-buffered saline contain 3% BSA (bovine serum albumin, Sigma-Aldrich) at room temperature for 1 h, and incubated at room temperature 2 h or 4°C overnight with the following primary antibodies (all antibodies purchased from Abcam, USA): GAPDH (ab9485; 1:2,500), Vimentin (ab45939; 1:1,000), N-cadherin (ab18203; 1:1,000), E-cadherin (ab15148; 1:1,000), and CXCR4 (ab74012; 1:1,000). Unbound antibody was washed and removed in TBST solution 4 times, 10 min each time. The PVDF membranes were incubated for 1 h with goat anti-rabbit secondary antibody combined with HRP (BS13278; 1:5,000; Bioworld Technology, USA), then washed with TBST solution for 3 times. The immunoblots were observed using an enhanced chemical illuminant reagent (Pierce; Thermo Fisher Scientific, USA) and GAPDH expression was used as an internal

control. All immunoblots images were then analyzed via software suite (Image Lab software 3.0, Bio-Rad Laboratories, Inc.).

Flow cytometry analysis and sorting

Anti-CD133-phycoerythrin antibody (Dilution ratio 1:10, Miltenyi Biotec GmbH, Germany) was used to identify the CD133 protein on the cell surface. Antibodies against the human antigen CXCR4-APC (Miltenyi Biotec, Germany) were purchased commercially. The adherent and tumor spheres cells were collected and gently decomposed into a single cell suspension by 0.25% trypsin, fixed with 4% paraformaldehyde (PFA) and stained with CD133-PE or CXCR4-APC in dark room at 4°C for 30 minutes. Then, these stained cells were detected by a flow cytometer (Becton Dickinson). FlowJo software suite (FlowJo LLC, Ashland, USA) was used for data analysis. CXCR4-positive cells were isolated from the adherent cells and tumorspheres using a CXCR4-biotin antibody (1:100, eBioscience, USA) and a magnetic activated cell sorting system (cat. MSPB-6003, MACS, MagniSort®, eBioscience, USA) according to the instruction manual of the manufacturer. Isolated cells were cultured in RPMI-1640 complete medium and serum‑free DMEM/F12, respectively. Then, they were used for further experiments.

Migration and invasion assays

We evaluated the cell migration and invasion by Transwell analysis in a 24-well plate. These Transwell filters (8-mm pore size) were coated without or with Matrigel (50 μL/well; BD Biosciences) in a concentration of 1 mg/mL. Cells resuspended in serum-free medium

were inoculated at $1x10^5$ cells/well into the top chamber of a 24-well plate, and 500 μL of culture medium containing DMEM/10% FBS was placed in the lower well. The chambers were incubated for 48 hours in a humid incubator. Those cells that remained on the Transwell's upper surface were scrubbed away. The cells that penetrate the filter on the other surface of Transwell were fixed with methanol solution, stained with 0.5% hematoxylin solution and washed. When they were completely dry, cells from five random microscopic fields were counted using a hematocytometer under an inverted microscope.

Immunohistochemistry

The 5-μm-thick pathological tissue sections were baked at 72℃ for 1h and were dewaxed in xylene 3 times for 10 min each times. We further treated these tissue sections with a gradient concentration of ethanol solution. To repair the antigen, we placed the slides in a citric acid buffer at pH 6.0 and heated them in a pressure cooker for 30 minutes. We incubated the samples using 3% hydrogen peroxide solution for 20 minutes in order to inhibit endogenous peroxidase. These tissue sections were incubated with primary antibodies of appropriate concentration at 4°C overnight. The primary antibodies included anti-CXCR4 (1:100, ab74012, Abcam) and anti-CK (1:200, #54135S, CST). The secondary antibodies were selected for further incubation according to the species of the primary antibodies. In this study, the secondary antibodies were biotinylated goat anti-rabbit IgG (LSAB, IHC detection system kit, IHC001, Bioss). These sections then were washed in PBS, and incubated with HRP conjugate for 20 min. Immunoreaction was visualized with

30-50 μl of DAB solution as a chromogen (Bioss). Then, the sections were further stained with hematoxylin. Finally, the results were analyzed under an inverted microscope.

Tail vein metastatic surrogate assay

All animal experiments in this study were performed in strict accordance with the Guide for the Use and Care of Laboratory Animals, which was approved by the Experimental Animal Ethics Committee of Southern Medical University. Single 5-8F CXCR4-positive and CXCR4-negative cells were injected into the caudal vein of BALB/c nude mice in the cell population (5x10^5). The mice were divided into four groups based on the cell type and the use of a CXCR4 inhibitor (AMD3100). The animals were kept in microisolator cages in SPF laboratory animal facility of Southern Medical University. This specified pathogen-free facility was under constant temperature (20°C) and humidity (50%) condition with 12 hour light/dark cycle. The mice were observed and recorded the fluorescence intensity of metastatic lesions using IVIS (In Vivo Imaging System) and then euthanized 4 weeks after the cells were injected.

Statistical analysis

The data are presented in the form of mean ±SD unless specified otherwise.

The tumor/metastasis formation rates were compared using the Fisher’s exact test in vivo mouse models. Statistical methods were used to analyze the relationship between CXCR4+ cell content and various clinical factors. "Age" is a continuous variable that is described as the mean (SD). The differences between groups were statistically analyzed

using Student's t-test. Fisher's exact test was used to analyze categorical variables, including M status, N status, sex and location. Permutation test was used to analyze ranked variables, including T status and grading in TNM (tumor-node-metastasis) staging. The randomly permuting sample such as high versus low CXCR4+ cell content was tested with Permutation test. All statistical analyses were performed using the SPSS 17.0 software suite (SPSS, Inc., Illinois). All tests were 2-tailed and a P value less than 0.05 was considered to indicate a statistically significant difference. Experiments were repeated at least 3 times in independent.

Results

The expression level and significance of CXCR4 in clinical NPC tissues

To further verify the role of CXCR4-positive cells in tumor cell invasion and metastasis in clinical patients, we performed immunohistochemical staining of 71 confirmed NPC tissues and 5 nontumor pathological tissues from individuals with rhinitis, and statistical analysis was performed on the results. We obtained 76 paraffin wax-embedded specimens from the Department of Pathology, Pearl River Hospital. None of the patients received chemoradiotherapy prior to biopsy. Among the 71 samples of NPC, 60 were primary tissue and 11 were lymph node tissue. Two of the cases had distant metastasis (1700065, left submaxillary; 1701840, right eyebrow arch), and 69 cases had no distant metastasis. Detailed clinical parameters are shown in Table 1.

We found that CXCR4 expression was negative in rhinitis tissues as a negative control

(Figure 1A). In 71 cases of NPC, CXCR4 was expressed at different levels, and the expression level, including negative expression (Figure 1B), weakly positive expression (Figure 1C) (positive cells make up less than or equal to 10% of all tumor cells) and strong positive expression (Figure 1D) (positive cells make up more than 10% of all tumor cells). To identify these cells as NPC cells, we performed PAN-CK staining on these tissues, and the results showed that CXCR4 expression partially overlapped with that of PAN-CK, and CK in the positive region of CXCR4 was also positive (Figure 1E-H). These results confirmed that the CXCR4-positive cells were NPC cells. Then we found that CXCR4 expression was related to NPC clinical parameters. CXCR4 expression was correlated with T stage (r=0.318) and N stage (r=0.634). The tumor cell positive rate of CXCR4 in the T3-4 group was higher than that in the T1-2 group (60.6% vs 28.9%, P =0.009), and the tumor cell positive rate of CXCR4 in the N2-3 group was higher than that in the N0-1 group (76.5% vs 13.5%, P=0.000) (Table 1).

CXCR4 was strongly positive in the pathological tissues of the primary lesions in two cases of distant metastasis (NO.1700065, left submandibular; NO.1701840, right eyebrow arch). Among the 11 lymph nodes with NPC metastasis, CXCR4 was positively expressed, and 8 cases were strongly positive (not shown). These results suggest that CXCR4 is closely related to the metastasis of NPC.

We found that CXCR4 was expressed in the cell membrane and cytoplasm of typical CXCR4-positive cells, and the morphology of CXCR4-positive cells was mostly spindle-

shaped (Figure1 I and Figure1 I-enlarged). In some specimens, CXCR4-positive cells were tightly clustered at the edge of tumor tissues (Figure1 J). In addition, CXCR4-positive cells were distributed in the invasion path of cancer cells (Figure 1K and 1L). In lymph node metastatic carcinoma, CXCR4 immunohistochemical staining revealed a single CXCR4-positive cell in the micrometastatic lesion (Figure 1M and 1N). In metastatic lymph node tissues, most tumor cells expressed CXCR4, and the CXCR4-positive cancer cells showed a trend of invasion (Figure 1O). In the vascular tissue, tumor cells could be observed and expressed CXCR4 (Figure 1P).These phenomena suggest that CXCR4-positive cells may play an important role in the early stage of NPC metastasis, vascular metastasis and distant lesion formation.

The CXCR4 expression level is higher in cells with high metastasis or low differentiation

To detect the effect of CXCR4 on the migration and invasion of NPC cells, we detected CXCR4 expression in several NPC cell lines. The results showed that CXCR4 expression increased in all 5 NPC cell lines compared with the immortalized NP69 cell line. Among them, 6-10B is a poorly metastatic cell line, 5-8F is a highly metastatic cell line, CNE1 is a highly differentiated cell line, CEN2 is a poorly differentiated cell line, and SUNE1 cells have no obvious metastatic and differentiation characteristics.

qRT-PCR data showed that the CXCR4 expression levels were higher in the 5-8F, CNE2 and SUNE1 cell lines than in the 6-10B and CNE1 cell lines (Figure 2A). That is, the CXCR4 expression level is higher in cells with high metastasis or poor differentiation than in those with low metastasis or high differentiation. These results suggest that CXCR4 may

be involved in the metastasis and differentiation of NPC cells. The above results were further verified by Western blots at the protein level(Figure 2B).

Tumorspheres show enrichment of CD133-positive and CXCR4-positive cells, and most CD133-positive cells also express CXCR4 in NPC

Flow cytometry was performed to detect the expression levels of CXCR4 and the NPC stem cell marker CD133 in the tumorspheres of NPC. The final result of the above data was obtained by subtracting the corresponding control data from that of the experimental group.

The data showed that in 5-8F cells (Figure 2C and 2D), the proportions of CD133 and CXCR4 positivity in adherent cells were 0.735% and 14.375%, respectively. The proportions of CD133 and CXCR4 positivity in tumorsphere cells were 4.793% and 85.345%, respectively; the proportions of CD133 and CXCR4 double-positive staining in adherent cells and tumorsphere cells were 0.889% and 4.760%, respectively. In SUNE1 cells (Figure 2E and 2F), the proportions of CD133 and CXCR4 positivity in adherent cells were 3.985% and 17.998%, respectively. The proportions of CD133 and CXCR4 positivity in tumorsphere cells were 10.927% and 85.345%, respectively. The proportions of CD133 and CXCR4 double-positive staining in adherent cells and tumorspheres in SUNE1 cells were 4.010% and 10.900%, respectively.

The data showed that the proportion of CD133-positive cells in the 5-8F and SUNE1

tumorspheres was significantly higher than that of adherent cells. Moreover, the data showed that the proportion of CXCR4-positive cells in the tumorsphere was also higher than that of adherent cells, and most CD133-positive cells also expressed CXCR4. The above data suggested that the tumorspheres have the ability to enrich stem cells and CXCR4-positive cells, suggesting that CXCR4 may also be a marker of nasopharyngeal cancer stem cells.

The tumorsphere-forming rate of the CXCR4-positive tumorsphere cells is higher than that of the CXCR4-negative tumorsphere cells, and the CXCR4-positive tumorsphere cells have a spindle morphology

To observe the morphology of the CXCR4-positive tumorsphere cells, we obtained 5-8F CXCR4-positive tumorsphere cells by magnetic bead sorting and cultured them in serum-free or complete medium, and the morphology of the cells was observed 72 hours later. The results showed that the CXCR4-positive tumorsphere cells in serum-free medium were mostly fusiform and some were polygonal (Figure 3A), while almost all were polygonal in complete medium (Figure 3B). These results indicated that the CXCR4-positive tumorsphere cells had spindle characteristics. The data also indicated that the CXCR4-positive tumorsphere cells mostly differentiated into ordinary tumor cells after adherent culture in complete culture medium.

The tumorsphere-forming rate of the 5-8F CXCR4-positive tumorsphere cells was higher than that of the CXCR4-negative tumorsphere cells and unscreened adherent cells.

However, there was no significant difference in the tumorsphere formation rate between the unscreened adherent cells and the CXCR4-negative tumorsphere cells (Figure 3C). Consistent results were obtained in SUNE1 cells. The above results showed that the CXCR4-positive tumorsphere cells had the characteristics of spindle cells and metastatic tumor stem cells.

The CXCR4-positive tumorsphere cells have enhanced in vitro migration and invasion

To further clarify the effect of CXCR4 on the migration and invasion of tumorsphere cells, we conducted in vitro migration and invasion experiments. The results (Figure 4A and 4B) showed that the CXCR4-positive tumorsphere cells in the 5-8F and SUNE1 cell lines had stronger migration and invasion than the CXCR4-negative tumorsphere cells. After treatment with the CXCR4 ligand SDF-1, the migration and invasion of the CXCR4-positive cells in the SDF-1 group was enhanced compared with that in the untreated group. In the SDF-1-treated group, the CXCR4-positive tumorsphere cells also had stronger migration and invasion than the CXCR4-negative tumorsphere cells. The migration and invasion of the CXCR4-negative cells after SDF-1 treatment did not change significantly. The above results indicated that the CXCR4-positive tumorsphere cells had stronger migration and invasion, which was mediated through the SDF-1/CXCR4 axis.

The CXCR4 subgroup of tumor stem cells has stronger metastatic tumor formation than the CXCR4-negative group

To clarify the role of CXCR4 in the process of tumorsphere cell metastasis, we carried out

a 5-8F tumorsphere cell metastasis experiment in mice through tail vein injection. The results (Figure 5) showed that the CXCR4-positive tumorsphere cells had stronger metastatic tumor formation ability than the CXCR4-negative tumorsphere cells in the cell population (5x10^5). Moreover, a CXCR4 inhibitor (AMD3100, 2.5 μg/g, intraperitoneal injection every two days for a total of 10 times) was used in the CXCR4-positive group and the CXCR4-negative group. The results showed that the formation of metastatic tumors in vivo was reduced when the inhibitor was added. These data suggested that the CXCR4 subgroup of tumor stem cells has stronger metastatic tumor formation than the CXCR4-negative group, which was mediated through CXCR4.

CXCR4 participates in the EMT process of NPC cells

To further explore the mechanism of CXCR4 in stem cell stemness maintenance and metastasis, we tested the CXCR4-positive and CXCR4-negative tumorsphere cells for stemness-related genes and EMT-related genes. The results(Figure 6) showed that compared with those in the CXCR4-negative tumorsphere cells, the expression levels of the Oct4, Nanog and Sox2 genes in the CXCR4-positive tumor sphere cells were increased, and the expression levels of the Snail and Twist genes, which are located upstream of the EMT process, were also increased. The expression of the mesenchymal-related genes Vimentin and N-cadherin increased, while that of E-cadherin decreased. These results suggested that the CXCR4-positive tumorsphere cells have characteristics of tumor stem cells and may be involved in the migration and invasion of NPC cells and the formation of metastatic tumors through EMT, showing the characteristics of metastatic

tumor stem cells.

Discussion

Distant metastasis is the leading cause of death from NPC. The tumor stem cell theory holds that tumor stem cells are the root cause of drug resistance, metastasis and recurrence[6,7]]. Brabletz et al.[8] proposed that tumor stem cells can be divided into stable tumor stem cells and transplanted tumor stem cells. Thomas Brabletz et al.[8] proposed that metastatic tumor stem cells are mainly responsible for tumor metastasis. Subsequently, Baccelli et al.[23] found that primary breast cancer CTCs contained metastatic tumor initiation cells (MICs), which led to multiple metastases of breast cancer in bone, lung and liver. These MICs are EPCAM (+) CD44 (+) CD47 (+) MET(+) rather than simply EPCAM (+). Hermann et al.[12] demonstrated that a subpopulation of CD133+ CXCR4+ CSCs is essential for pancreatic cancer metastasis. In theory, we can inhibit metastatic tumor stem cells to block metastasis and prolong the patient's survival time. However, it is not clear whether metastatic stem cells also exist in NPC.

In this study, we showed that CXCR4-positive tumorsphere cells had the characteristics of metastatic tumor stem cells, which may be involved in the migration, invasion and metastasis of NPC tumor stem cells. In vitro and in vivo experimental results and

immunohistochemical staining results of clinical specimens indicated that CXCR4 may be a marker of potential NPC metastatic tumor stem cells. This finding provides a new therapeutic target for early intervention of NPC from the perspective of metastatic tumor stem cells.

We performed flow cytometry on tumorsphere cells and adherent NPC cells and found that compared with that of adherent cells, the proportion of tumorsphere cells labeled with CD133, a classic marker of NPC[24], was increased, and the proportion of CXCR4-labeled cells was significantly increased. This result prompted us to examine the role of CXCR4 in tumor stem cells. Previous studies have found that CXCR4 is involved in biological processes such as granulocyte migration in the inflammatory response, embryonic development, hematopoietic stem cell migration and homing. The SDF1/CXCR4 axis is also closely related to cell migration, invasion, metastatic tumor formation and prognosis in a variety of tumors [25,26]including NPC [27-29]. And Tan HX et al. effectively blocked the liver metastasis of colon cancer by blocking the SDF-1/CXCR4 axis, which was confirmed in the studies [30].Therefore, we speculated that CXCR4 might be involved in the migration of NPC stem cells. CXCR4-positive tumorsphere cells may have the characteristics of metastatic tumor stem cells. Our results showed that CXCR4-positive cells play an important role in the migration, invasion and metastasis of NPC stem cells.

Tumor metastasis is a highly selective process. Only tumor cells with specific abilities can successfully colonize distant organs [31]. The complete process requires tumor cells to

first invade the adjacent organs, spread to the vasculature, circulate to specific organs and successfully colonize the organs. A widely accepted view is that metastatic tumors are formed gradually by the expansion of a single tumor cell [32,33]. We observed a series of NPC metastases in a series of clinical tissue specimens. For example, In the metastatic lymph node tissue, we observed only one CXCR4-positive cell in the middle of a small mass of tumor cells. We speculated that CXCR4-positive cells expanded to form this tumor cell mass after migration to lymph nodes through vascular vessels, which is characteristic of metastatic tumor stem cells. The above observed features of CXCR4-positive cells are consistent with the previously reported features of metastatic tumor stem cells; that is, tumor stem cells migrate to vessels through the EMT process, settle in distant lesions, and eventually proliferate to form metastatic lesions[34].

CXCR4 is a chemokine receptor; thus, through what mechanism does it participate in the migration of nasopharyngeal cancer stem cells? The EMT process is one of the main mechanisms by which tumor cells acquire stem capacity [35-37]. Metastatic tumor stem cell theory holds that EMT is an important way for CSCs to transform into metastatic tumor stem cells[38]. And our results showed that CXCR4 may regulate the EMT process by regulating the related signaling pathways dependent on Snail and Twist.

NPC is a kind of EBV-associated malignancy [2]. At first, we considered using an EBV positive cell line as the experimental object. C666-1 is stable EBV positive nasopharyngeal cell line established by the faculty of the University of Hong Kong [39]. We have obtained

the use of this cell line from Prof. GSW Tsao of HKU. However, subsequent studies showed that C666-1 cells had weak ability of tumor sphere formation. Therefore, C666-1 was not selected as the research object in this study.

Conclusions

Based on the theory of metastatic tumor stem cells, targeted metastatic stem cells can block the formation of metastatic tumors. Our animal experiments showed that the tumor formation rate of the mice treated with the CXCR4 inhibitor ADM3100 was reduced. Therefore, both in vitro and in vivo experiments confirmed that CXCR4-positive tumorsphere cells had the characteristics of metastatic tumor stem cells. Intervention in the SDF-1/CXCR4 axis may be an effective method to reduce the migration of nasopharyngeal CSCs and inhibit the formation of metastatic tumors. CXCR4 is expected to be an important target for the clinical treatment of NPC.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' contributions

Kaitai Yao and Lin Chen conceived the project and drafted the manuscript. Zhenwei Zhu designed the study and analyzed the data. Jingyu Li and Jingxian Liu performed the IHC experiments and analyzed the images. Yuan He and Yan Xue performed the animal experiments. Xiangchan Hong organized the patients' information. Zeying Jiang, Dangu Luo and Pei Guo revised the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

list of abbreviations

abbreviations	full name
CSCs	subpopulation of cancer stem cells
NPC	nasopharyngeal carcinoma
EMT	Epithelial-to-mesenchymal transition
MCSCs	metastatic cancer stem cells
FBS	Fetal bovine serum
cDNA	Complementary DNA
DMSO	Dimethyl Sulfoxide
h	Hour
IHC	Immunohistochemistry
Mean±SE	Mean±standard error
mg/ml	Milligram/milliliter
mRNA	Messenger RNA
MET	Mesenchymal-to-epithelial transition
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Tris	Tris-base
WB	Western blot

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Table 1 Clinical characteristics of 71 human NPC tissues for IHC analyses.

Clinical factor	Total cases	CXCR4 negative	CXCR4 positive	CXCR4 strong positive	Positive rate	P^a	Correlation coefficient	P^b
Gender								
Male	45	25	8	12	44.4%	0.530	0.021	0.864
Female	26	15	4	7	42.3%			
Age								
＜50	28	14	6	8	50%	0.199	-0.129	0.282
≥50	43	26	6	11	39.5%			
TNM stage[c]								
T status								
T1-T2	38	27	5	6	28.9%	0.009	0.318	0.007
T3-T4	33	13	7	13	60.6%			
N status								
N0-N1	37	32	5	0	13.5%	0.000	0.634	0.000
N2-N3	34	8	7	19	76.5%			
M status								
0	69	40	10	12		-	-	-
1	2	0	0	2	100%			
Lymph node metastasis NPC tissue	11	0	3	8	100%	-	-	-

[a]Fisher's exact test，[b]Spearman correlation analysis; [c]TNM is a cancer staging system and was assessed according to the 8th edition of the UICC (International Union against Cancer);TNM, tumor-node-metastasis

Table 2. A list of primers used in the qRT-PCR reactions

Gene primer name		Sequence (5’-3’)
Sox2	Sense	AGAACCCCAAGATGCACAAC
	Antisense	ATGTAGGTCTGCGAGCTGGT
Nanog	Sense	CAAAGGCAAACAACCCACTT
	Antisense	ATTGTTCCAGGTCTGGTTGC
Oct	Sense	AGTGAGAGGCAACCTGGAGA
	Antisense	CAAAAACCCTGGCACAAACT
Snail	Sense	CAGTGGGAGACCTCGAGAAG
	Antisense	TCCCTCGGAACATCAGAAAC
Beta-catenin	Sense	CATTACAACTCTCCACAACC
	Antisense	CAGATAGCACCTTCAGCAC
N-cadherin	Sense	ACAGTGGCCACCTACAAAGG
	Antisense	CCGAGATGGGGTTGATAATG
Vimentin	Sense	GAGAACTTTGCCGTTGAAGC
	Antisense	GCTTCCTGTAGGTGGCAATC
Twist	Sense	CAGCGCACCCAGTCGCTGAA
	Antisense	CCAGGCCCCCTCCATCCTCC
GAPDH	Sense	GAAGGTGAAGGTCGGAGTC
	Antisense	GAAGATGGTGATGGGATTTC

Figures and figure legends

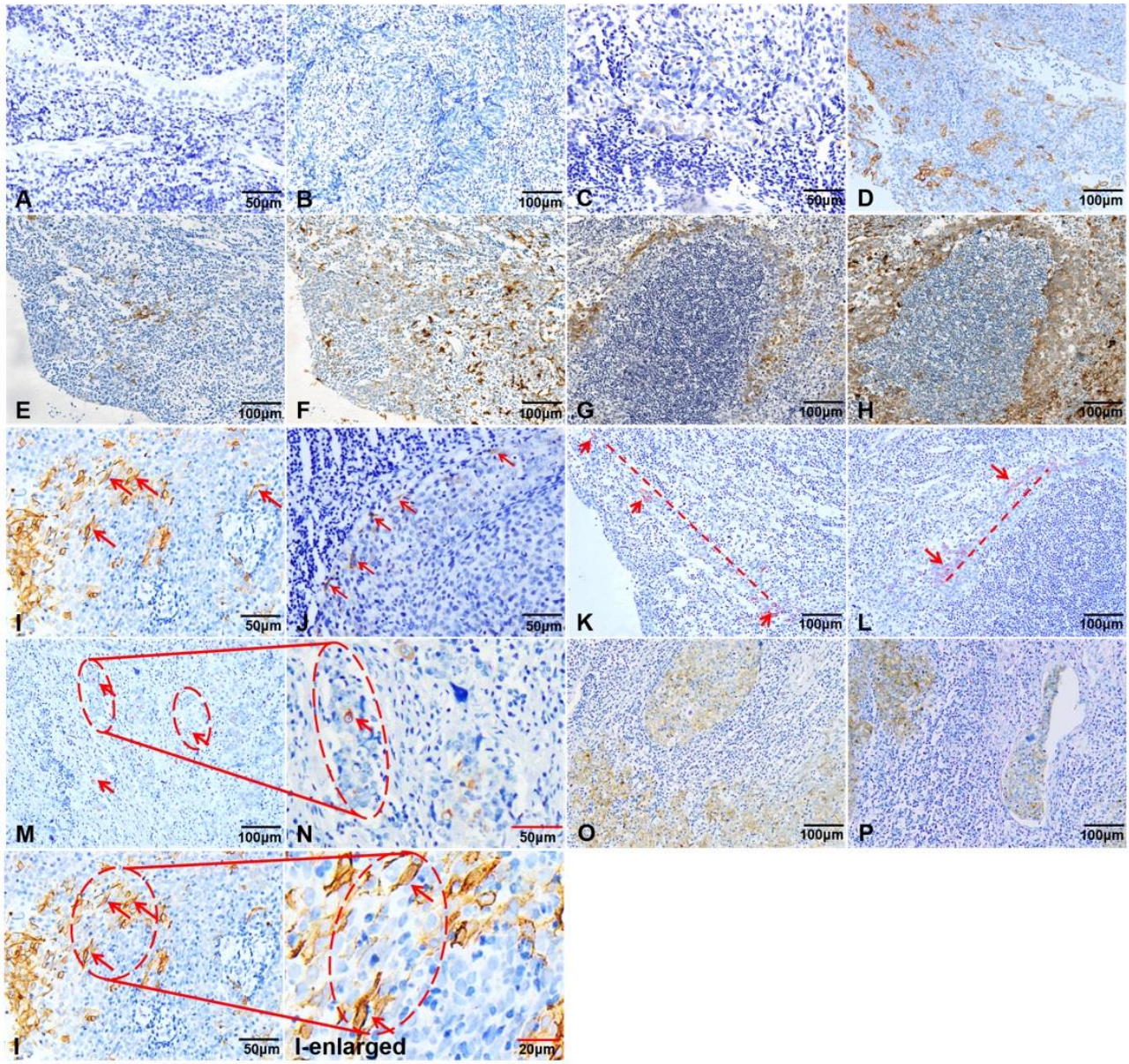

Figure 1 CXCR4-positive NPC cells play an important role in the early stage of nasopharyngeal carcinoma metastasis, vascular metastasis and distant lesion formation. Positive cells are usually spindle-shaped.

(A) Nasopharyngeal rhinitis tissue does not express CXCR4. (B-D) Different levels of CXCR4 expression in nasopharyngeal carcinoma tissues, (B) negative expression, (C) weakly positive expression (positive cells make up less than or equal to 10% of all cancer

cells) and (D) strong positive expression (positive cells make up more than 10% of all cancer cells). (E, G) CXCR4 immunohistochemical staining in nasopharyngeal carcinoma tissues and lymph node metastases. (F, H) Pan-CK immunohistochemical staining in nasopharyngeal carcinoma tissues and lymph node metastases of nasopharyngeal carcinoma in serial sections (E-F, G-H). The expression of CXCR4 and PAN-CK in nasopharyngeal carcinoma tissues and lymph node metastasis tissues partially overlapped, and CK of CXCR4-positive cells was also positive, indicating that CXCR4-positive cells were nasopharyngeal carcinoma cells. (I) CXCR4 is expressed in the cytoplasm or cell membrane of nasopharyngeal carcinoma cells. Positive cells are usually spindle-shaped. (J) CXCR4-positive cells were tightly clustered at the edge of tumor tissues. (K-L) CXCR4-positive NPC cells seem to form a pathway (red line). (J-L) These phenomenon suggests that CXCR4 molecules maybe involved in the migration of NPC cells. (M-N)In lymph node metastatic cancer tissues, CXCR4 immunohistochemical staining revealed a single or a small number of CXCRA-positive cells in the micrometastatic foci. (O) In the lymph node tissue, most of metastatic NPC cells expressed CXCR4. (P) CXCR4-positive NPC cells were observed in the vasculature.

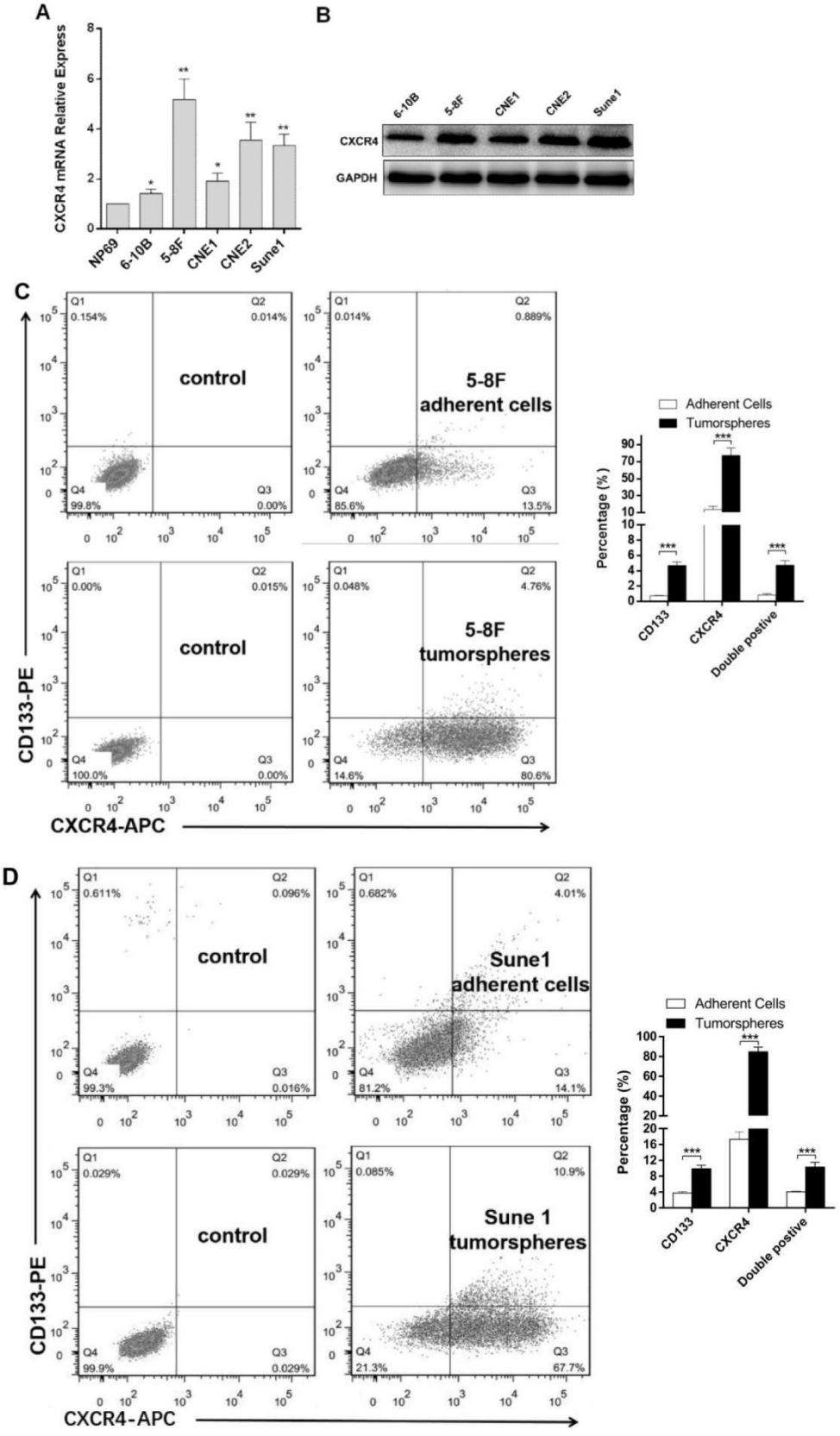
A
CXCR4 mRNA Relative Express
NP69
6-10B
5-8F
CNE1
CNE2
Sune1
B
6-10B
5-8F
CNE1
CNE2
Sune1
CXCR4
GAPDH
C
control
5-8F adherent cells
control
5-8F tumorspheres
CD133-PE
CXCR4-APC
Adherent Cells
Tumorspheres
Percentage (%)
CD133
CXCR4
Double postive
D
control
Sune1 adherent cells
control
Sune 1 tumorspheres
CD133-PE
CXCR4-APC
Adherent Cells
Tumorspheres
Percentage (%)
CD133
CXCR4
Double postive

Figure 2. The expression levels of CXCR4 and CD133 in nasopharyngeal carcinoma cells.

(A) The expression level of CXCR4 was detected by qPCR.

(B) The expression level of CXCR4 was detected by Western blots.

(C) The percentage of CD133 and CXCR4 in 5-8F adherent cells and tumorspheres. The proportion of positive cells increased significantly in the tumorspheres compared with adherent cells. Most CD133-positive cells were distributed on the positive side of CXCR4.

(D) We obtained similar results in the Sune1 cell line.

Each bar represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

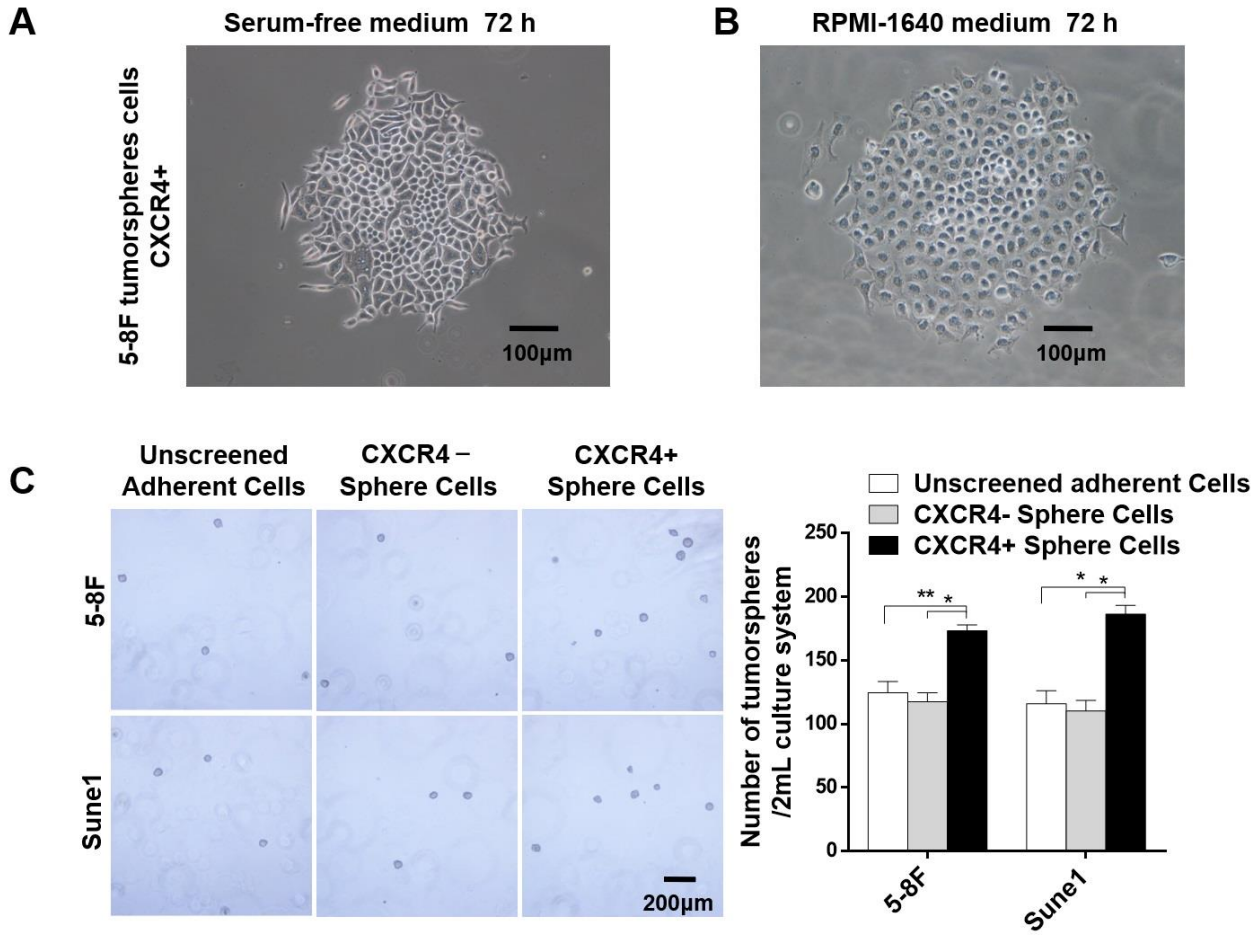

Figure 3 Morphological characteristics of CXCR4- and CXCR4+ tumorspheres.

(A) CXCR4-positive tumorsphere cells in serum-free medium were fusiform. (B) The cells were differentiated and lost their spindle characteristics after being cultured in complete medium. (C) The sphere-forming ability of CXCR4-positive tumorsphere cells was stronger than that of negative tumorsphere cells and adherent cells.

Each bar represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

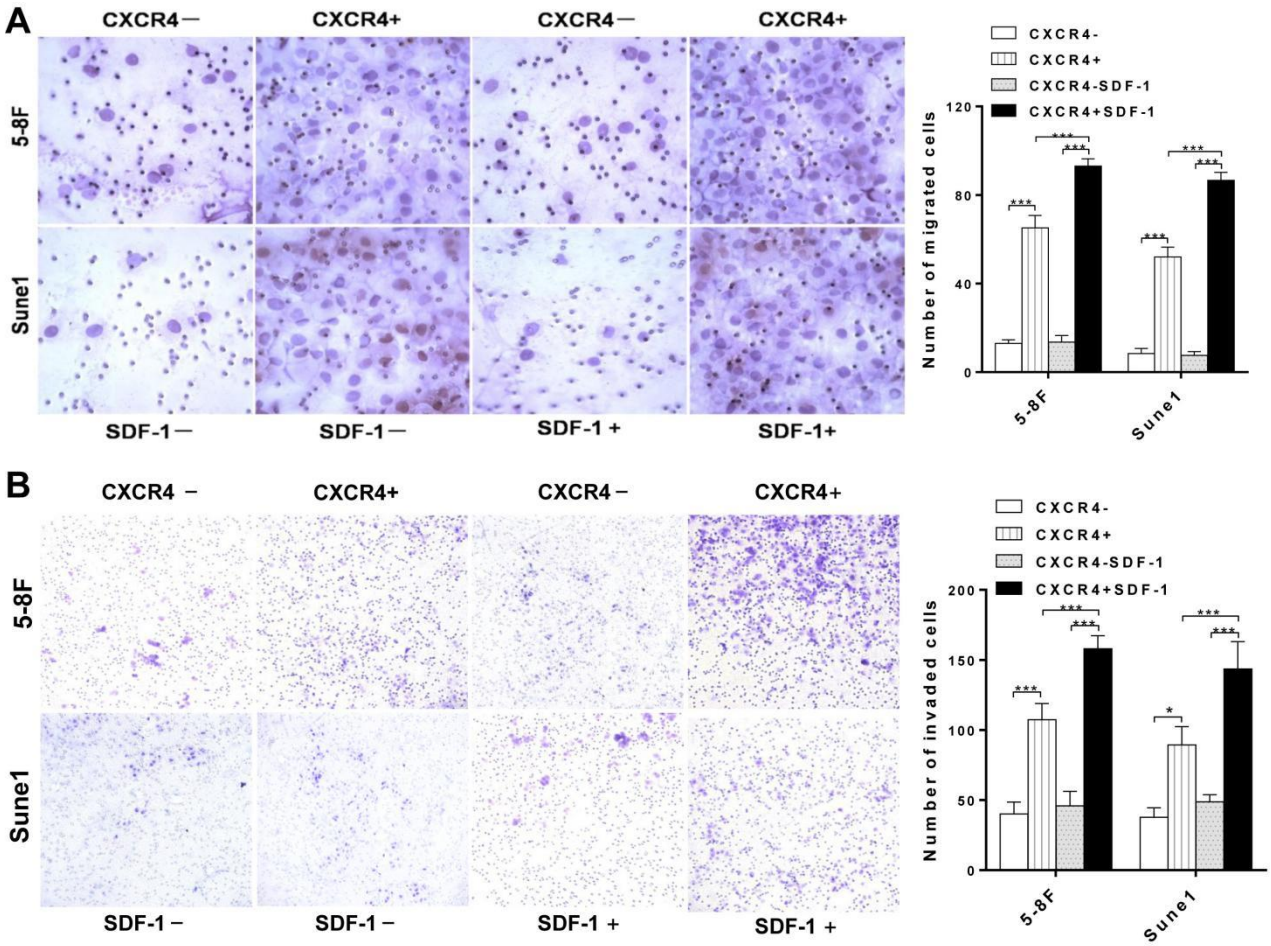

Figure 4. Enhanced migration of CXCR4-positive tumorsphere cells in vitro.

(A) In 5-8F and SUNE1 cells, CXCR4-positive tumorspheres showed stronger migration than CXCR4-negative tumorspheres with or without SDF-1 treatment. SDF-1-treated CXCR4-positive tumorspheres showed stronger migration than untreated CXCR4-positive tumorspheres. There was no significant difference in migration between the SDF-1-treated CXCR4-negative tumorspheres and the untreated CXCR4-negative tumorspheres. These results suggested that CXCR4-positive tumorspheres had stronger migration than CXCR4-negative tumorspheres, which depends on the SDF-1/CXCR4 axis.

(B) The statistical analysis of Figure A.

(C) In 5-8F and SUNE1 cells, invasion experiments obtained results consistent with

migration experiments.

(D) The statistical analysis of Figure C.

Each bar represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

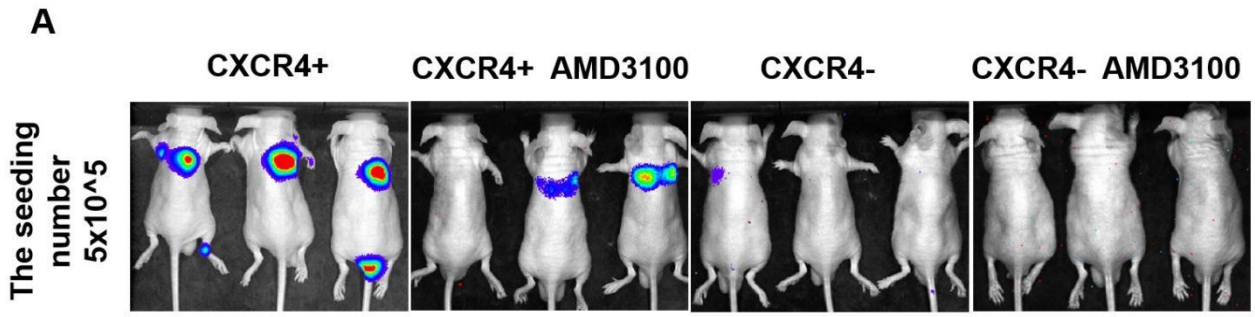

Figure 5. Metastatic tumor formation experiment in mice injected through the tail vein. CXCR4-positive tumorsphere cells had stronger metastatic tumor formation than CXCR4-negative tumorsphere cells in the cell population (5x10^5). Moreover, a CXCR4 inhibitor (AMD3100, 2.5 μg/g, intraperitoneal injection every two days for a total of 10 times) was used for the CXCR4-positive group and the CXCR4-negative group. The results showed that the formation of metastatic tumors in vivo was reduced when the inhibitor was added. These data suggest that the CXCR4 subgroup of tumor stem cells has stronger metastatic tumor formation than the CXCR4-negative group.

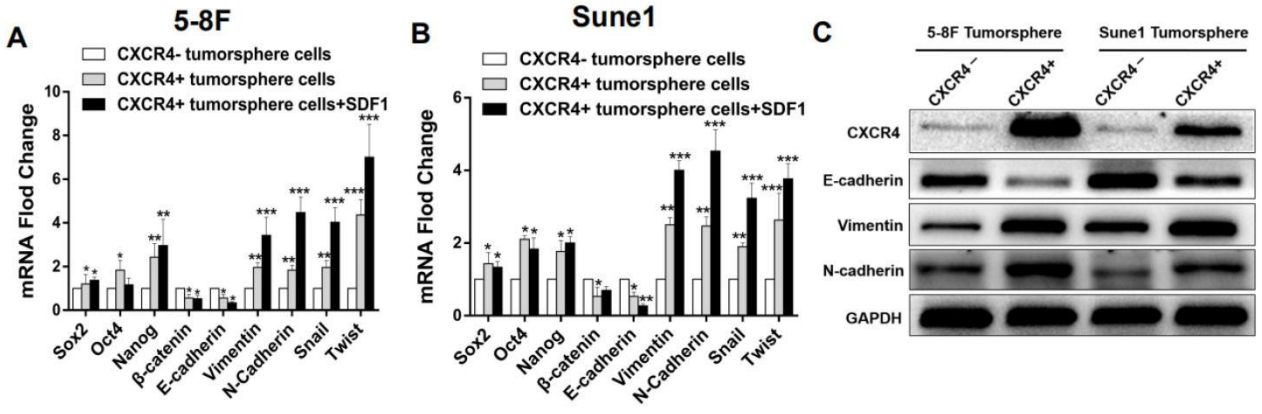

Figure 6. CXCR4 participates in the EMT process of nasopharyngeal carcinoma cells

(A-B) Compared with those of the CXCR4-negative tumorsphere cells, the mRNA expression levels of the Oct4, Nanog and Sox2 genes in the CXCR4-positive tumor tumorsphere cells were increased, and the mRNA expression levels of the Snail and Twist genes, which are located upstream of the EMT process, were also increased. The mRNA expression levels of the mesenchymal-related genes Vimentin and N-cadherin increased, while E-cadherin decreased. (C) The protein expression levels of the mesenchymal-related genes Vimentin and N-cadherin increased, while E-cadherin decreased.

These results suggest that CXCR4-positive tumorsphere cells have the characteristics of tumor stem cells and may be involved in the migration and invasion of nasopharyngeal carcinoma cells and the formation of metastatic tumors through the EMT process, showing the characteristics of metastatic tumor stem cells.

Each bar represents the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.