Role of MIF/CXCL8/CXCR2 signaling in the growth of nasopharyngeal carcinoma tumor spheres

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Role of MIF/CXCL8/CXCR2 signaling in the growth of nasopharyngeal carcinoma tumor spheres

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Abstract

Macrophage migration inhibitory factor (MIF) and CXCL8 (also named IL-8) are strongly expressed in the tissues of nasopharyngeal carcinoma (NPC). However, their role in the growth of NPC has not been fully examined. This study aims to evaluate the functions of MIF and CXCL8 on the growth of NPC tumor spheres. The elevated expression of CXCL8 in tumor over normal tissues was confirmed in 37 pairs of biopsies from NPC patients. In the in vitro study, all the poorly differentiated NPC cell lines, including the EBV-positive C666-1, and the EBV-negative CNE-1, CNE-2, SUNE-1, HNE-1 and HONE-1 cells, were found to express CXCL8 and MIF. Therefore, the EBV-positive C666-1 cell was selected to examine for the role of MIF and CXCL8 in the growth of the NPC tumor spheres. Functional study showed that the growth of C666-1 tumor spheres, under the nutrient poor or growth factor supplemented culture conditions, could be inhibited by the CXCL8 specific peptide inhibitor. The growth of the tumor spheres could also be reduced by the CXCR2 specific inhibitor SB225002 or the PI3K/AKT inhibitor LY294002, indicating that the endogenously produced CXCL8 plays an autocrine role in the growth of the tumor spheres. Further mechanistic studies revealed that the gene expression of CXCL8 could be reduced by the MIF specific small interfering RNA (siRNA) or NF-κB
inhibitor parthenolide, and the growth of tumor spheres was also reduced after MIF siRNA transfection. Taken together, the present study highlights the role of MIF/CXCL8/CXCR2 axis in the growth of NPC tumor spheres. Chemotherapeutic interference of this signaling pathway may help to control the growth of the NPC tumor.
1. Introduction

Compared with the western countries, southern China has a high prevalence of nasopharyngeal carcinoma. The risk factors for the development of NPC are genetic factors, EBV infection and other environmental factors [1]. Recent genome-wide association study showed that the development of NPC is not only correlated with the HLA genotypes, but also associated with the genetic defects of certain genes such as TNFRSF19, MDS1-EV1 and CDKN2A-CDKN2B [2]. The oncogenic property of EBV in B-cell has been well documented. In NPC, the viral proteins LMP-1, EBNA-1 and virus-derived miRNA have been shown to regulate the growth of NPC. In addition to the genetic and viral factors, chemokines produced by the tumor cells and cells in the tumor microenvironment may also play a role in the development of NPC.

In the clinical NPC samples, the expression of chemokines, such as MIF, CXCL8 (IL-8), SDF-1α, MIP-3α, CCL-2, RANTES, and CXCL10 (IP-10), has been reported. The overexpression of chemokines was frequently used as biomarkers to correlate with the tumor metastasis and patient survival [3-6]. In addition to the chemotactic activity of these chemokines on the immune cells, CXCL8 has also been shown to induce tumor angiogenesis. In the breast tumor cell model, CXCL8 was found to play a novel role to regulate the self-renewal of breast cancer stem cells [7]. In spite of the
strong expression of MIF and CXCL8 in NPC tissues, the role of these two chemokines in the growth of NPC cells has not been examined in detail. In the present study, we show for the first time that the in vitro growth of NPC tumor spheres is regulated via the MIF/CXCL8/CXCR2 signaling pathways. Pharmacological intervention of the MIF/CXCL8/CXCR2 signaling may be a strategy to control the growth and metastasis of NPC.
2. Materials and Methods

2.1 Biopsies, cell lines and cell culture

Thirty seven pairs of tumor and normal biopsy tissues adjacent to the tumor biopsy tissues were collected from 37 NPC patients at the time of diagnosis of NPC in the Clinical Oncology Department, University of Hong Kong in Queen Mary Hospital with patients’ consent and approval by hospital’s ethics committee for the molecular study. The poorly differentiated EBV-positive C666-1 NPC cell (provided by Prof. Kwok-Wai Lo, CUHK) was cultivated in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (penicillin 50 Units/ml and streptomycin 50 µg/ml; GIBCO: 15070-063). The poorly differentiated EBV-negative CNE-1, CNE-2, HNE-1, HONE-1, SUNE-1 and well-differentiated HK-1 NPC cells were cultivated in DMEM medium (GIBCO) supplemented with heat-inactivated FBS (5%; GIBCO: 10270-106), heat-inactivated newborn calf serum (5%, GIBCO: 16010159) and antibiotics (GIBCO: 15070-063). All these cell lines had been genotyped by STR profiling and were obtained from the NPC AoE Cell Line Respository (HKU, HK). The non-tumorigenic immortalized normal nasopharyngeal epithelial cell line NP460 (obtained from Prof. Sai-Wah Tsao, HKU) was cultivated in 1:1 Defined KSFM/EpiLife (GIBCO). Cells were maintained at 37°C in a humidified 5% CO₂ incubator. All the cell lines had been authenticated
and were free of mycoplasma.

2.2 Antibodies and chemicals

CXCL8 was obtained from R&D System (Cat. No.: 208-IL-010). CXCL8 peptide inhibitor (Cat. No.: 62401) was purchased from ANASPEC, Inc, California [8]. Inhibitor LY294002, SB225002 [9], and parthenolide was purchased from Sigma, Merck, and Calbiochem, respectively. 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma. Antibodies against phospho-AKT (p-AKT Ser473), AKT, p65 and Snail were purchased from Cell Signaling. Antibodies against MIF and β-actin were purchased from Santa Cruz and Sigma, respectively.

2.3 TaqMan quantitative real time RT-PCR assay (Q-PCR)

Briefly, 37 pairs of tumor and surrounding normal biopsy tissues were collected from NPC patients at the time of primary diagnosis of NPC. RNA was extracted from paired tumor and normal biopsy tissues by RNeasy® mini kit (Qiagen). cDNA was synthesized from 500 ng of the extracted RNA by SuperScript III cDNA preparation kit (Life technologies). The cDNA samples were then made up to 100µL with RNase free water. TaqMan real time quantitative Q-PCR for RNA expression levels of CXCL8 was carried out in 1µL of diluted cDNA using ABI expression assay
under assay primer and probe ID Hs00174103_m1 in an ABI prism® 7000 Sequence
Detection System with an initial denaturation step at 95°C for 10 minutes followed by
40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All cDNA samples were also
subjected to Q-PCR analysis for ribosomal RNA, R18S (ABI expression assay primer
& probe ID number 4319143E) to give normalized relative expression for CXCL8.

2.4 Enzyme-linked immunosorbent assay (ELISA)

The secretion of CXCL8 was determined by Human IL-8 ELISA Kit
(RayBiotech). C666-1 cells (3x10^4 cells/well) were seeded onto wells of 96-well plates.
Cell free supernatant was collected at various times after incubation. The
concentration of CXCL8 in the culture supernatant was then performed according to
the manufacturer’s instructions. Each experiment was performed with three replicates.

2.5 Cell migration assay

C666-1 cells were initially serum-starved for overnight. 2x10^5 cells in 100 μl of
serum-free RPMI medium were then seeded onto the upper well of the transwell
migration chamber. Except otherwise stated, plain RPMI (300 μl) was added to the
lower chamber. After 24 hours of incubation at 37°C, cells remained in chamber were
removed by cotton swab and the cells migrated through the membrane were fixed in
4% paraformaldehyde for 15 minutes. The cells were then permeabilized with 0.2% Triton X-100 for 10 minutes and stained with 0.5 \( \mu \)g/ml DAPI for 10 minutes. The percentage of cells migrated across the membrane was determined under a fluorescent microscope.

2.6 Hanging drop assay

Hanging drop method was used to evaluate the growth of tumor spheres under the nutrient poor culture condition (Bohrnsen et al., 2009). C666-1 cells (5x10^{3}) in 20 \( \mu \)l of RPMI containing 1% FBS were inoculated onto each well of 96-well plates. The plates were then inverted and the tumor spheres were allowed to grow at 37°C for 7 days in a humidified CO\(_{2}\) chamber. All the tumor sphere images from each culture were captured and the size of tumor spheres was determined by ImageJ software.

2.7 Sphere formation assay

Sphere formation assay was used to evaluate the self-renewal capability of tumor cells under the growth factors supplemented and serum-free condition (Golestaneh et al., 2012). C666-1 cells (1x10^{4} per well) were seeded in DMEM/F12 (Invitrogen) supplemented with EGF (20 ng/ml), FGF (20 ng/ml) and IGF (20 ng/ml) in wells of 6-well ultra-low attachment plate (Corning). The cultures were then incubated in a
humidified chamber at 37°C for 7 days. The cultures were fed with EGF (20 ng/ml), FGF (20 ng/ml) and IGF (20 ng/ml) every 2 to 3 days during the 7 days of incubation period. All the tumor sphere images from each culture were captured and the size of the tumor spheres was determined by ImageJ software.

2.8 Detection of mRNA expression by semi-quantitative RT-PCR and SYBR Green Q-PCR

The mRNA expression was detected using the semi-quantitative RT-PCR and SYBR Green Q-PCR. Briefly, cellular RNAs were extracted by Tri Reagent (Molecular Research Center) according to the manufacturer’s protocol. Total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). For semi-quantitative RT-PCR, cDNA was subjected to PCR using Taq DNA polymerase (Life technologies). PCR products were analyzed using 2 % agarose gel. The band images were captured by the Gel Documentation system (BIO-RAD). Quantitative expression of mRNA was analyzed using the SYBR Green Q-PCR. cDNA was prepared as mentioned above. The real-time PCR reaction mixtures were amplified using Power SYBR Green Master Mix (Life technologies) in a real-time PCR system (Agilent Technologies: Mx3000P). The real-time PCR products were then analyzed
by the MxPro QPCR Software (Software version 4.10). Primers used were listed in Table 1.

Table 1 Primers used for PCR

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<th>Anti-sense</th>
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<td>CXCL8</td>
<td>ATGACTTCCAAGCTGGCCGT</td>
<td>TCTCAGCCCTCTTCAAAAAACT TCTC</td>
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<td>MIF</td>
<td>GTTCCTCTCCGAGCTACCC</td>
<td>GCAGCTTGTGTAGGAGCGGT TCTG</td>
</tr>
<tr>
<td>CXCR1</td>
<td>CCAGTCCAGTTTGCTATGAG</td>
<td>TGTAGGAGTACGATGAG CTG</td>
</tr>
<tr>
<td>CXCR2</td>
<td>AGGTCAGAAGTTTCATCGTCAAG</td>
<td>AAAGCTGTCACTCTCCATGTT AA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTTC</td>
</tr>
</tbody>
</table>

2.9 Western-blotting Analysis

Both the floating and adherent cells were harvested for protein analysis. After harvesting, cells were lysed by lysis buffer (250 mM Tris (pH 8), 1% NP-40 and 150 mM NaCl containing 1% phosphatase inhibitors cocktail and 0.25% protease inhibitors cocktail). Protein concentration was then determined by DC protein assay kit (Bio-Rad). The same amounts of protein were resolved on SDS-polyacrylamide gel using the Mini-PROTEAN 3 electrophoresis system (Bio-Rad) and the detached proteins were transferred to PVDF membranes (Millipore). After blocking with 5% non-fat dry milk, the membrane was then incubated with the corresponding primary and secondary antibodies. Signal from the protein bands was developed by incubating...
the membrane with Western Blot substrate (Labfrontier Co.Ltd.). The membrane was then exposed to the X-Ray film. The image was visualized using GBX Developer and followed by Fixer (Kodak).

2.10 Knockdown with small interfering RNA and Transfection

C666-1 cells (5x10^5 cells/well) were seeded onto fibronectin-coated 35-mm dishes for 24 hours. Cells were washed 2 times with RPMI and then transfected with 5 nM of si-Snail RNA (a pool of 4 siRNA: GCGAGCUGCAGGACUCUAA; AAUCGGAAGCCUAACUACA; GUGACUAACUAUGCAAUUAA; GAGUAUAUGGCUGUCACUCUG), si-MIF RNA (a pool of 4 siRNA: GGGUCUACAUCAACUUAUA; GCGCAGAAACGCUCCUACA; CAACUCCACCUCUGCUCUUA; and CAUGCUAACACCAACAGUG), si-p65 RNA (a pool of 4 siRNA: GGAUUGAGGAGAAACGUAA, CCCACGAGCUUGUAGGAAA; GGCUAUAACUCGCCUAGUG; and CCACACAACUGAGCAGUG) or si-Control RNA (UGGUUUACAGUGACUUA) (Dharmacon) by using 5 µl of Lipofectamine Reagent 2000 (Invitrogen) in RPMI. After 6 hours of transfection, the medium was replaced by fresh cRPMI medium and the cells were further incubated for 3 days. Cells were then harvested for further analysis.
2.11 Statistical analysis

Student t-test in SPSS 11 software was used to determine the level of significance. A p-value smaller than 0.05 was considered as statistically significant.
3. Results

3.1 Expression of CXCL8 in NPC tissues

Thirty seven pairs of tumor and normal biopsies were obtained from 37 NPC patients at the time of diagnosis of NPC. TaqMan real time quantitative RT-PCR for CXCL8 was performed in RNA samples extracted from these biopsy tissues. As shown in Figure 1, the expression level of CXCL8 in tumor tissues was approximately 20 folds higher than that of the surrounding normal tissues in NPC patients (p<0.0001 by Wilcoxon Signed Rank Test). This shows the preponderance of this inflammatory molecule in NPC tumor.

3.2 Expression of CXCL8 and CXCL8 receptors (CXCR1 and CXCR2) by NPC cells

In view of the significant expression of CXCL8 in the NPC tissues, we sought to determine the biological functions of CXCL8 in NPC cells. First of all, the expression of CXCL8, and its receptors CXCR1 and CXCR2 in various NPC cell lines was determined by RT-PCR. Results in Figure 2A showed that all the NPC cell lines expressed CXCL8 mRNA. Constitutive expression of CXCR1 (IL8RA) was also detected in all the NPC cell lines, but the expression of CXCR2 was only detected in the C666-1 and CNE-1 cells. Expression of CXCR1 and CXCR2 was not found in the
immortalized normal nasopharyngeal epithelial cell line NP460. Further ELISA analysis showed that the EBV-positive C666-1 cells also time-dependently produced and secreted CXCL8 into the culture medium (Figure 2B). The C666-1 cell line was then selected for the subsequent studies.

3.3 CXCL8 induces migration of C666-1 cells

A transwell migration assay was used to evaluate the functional activity of CXCL8 on C666-1. First, the effect of exogenously added CXCL8 on the migration of C666-1 was examined. Results in Figure 3A showed that CXCL8 dose-dependently enhanced the migration of C666-1 cells. Then the effect of endogenously produced CXCL8 was examined by incubating the C666-1 cells with CXCL8 blocking peptide in the upper chamber and the serum containing medium in the lower chamber. Results in Figure 3B showed that the migration of C666-1 cells across the membrane was significantly reduced by the blocking peptide (p<0.01). Furthermore, the migration of C666-1 could be inhibited by the PI3K/AKT inhibitor LY294002. Results from this study clearly indicated that the CXCL8-induced migration of C666-1 is through the PI3K/AKT signaling.
3.4 CXCL8 blocking peptide inhibits the growth of NPC tumor spheres via PI3K/AKT

Multi-layer tumor sphere culturing system is generally considered as an ideal system for the \textit{in vitro} study of tumor growth \cite{10}. The role of endogenously-produced CXCL8 on the growth of NPC tumor spheres was then determined by incubating the C666-1 cells with CXCL-8 peptide inhibitor. Figure 4A showed the representative size measurement of the tumor spheres. Results clearly showed that the numbers of tumor spheres were reduced by the CXCL8 peptide inhibitor. Western blotting analysis also revealed that the phosphorylation level of p-AKT was dose-dependently reduced by the CXCL8 peptide inhibitor (Figure 4B). Similarly, the growth of tumor spheres was reduced by the LY294002, an inhibitor of the AKT upstream PI3K kinase (Figure 4C). Results from these observations suggested that the endogenously produced CXCL8 appears to regulate the growth of tumor spheres via PI3K/AKT.

3.5 Inhibition of NF-\(\kappa\)B but not Snail resulted in the reduced expression of CXCL8

It has recently been shown that Snail, an activator of epithelial-mesenchymal transition, may be induced by LMP-1 of the EBV virus \cite{11}. And the expression of CXCL8 could be activated through the binding of Snail to E3/E4 E-boxes \cite{12}. The role of Snail in the expression of CXCL8 in the EBV-positive C666-1 cells was then
evaluated. In the control experiment, the expression of Snail in the C666-1 cells was reduced after the transfection with Snail siRNA (Figure 5A). However, the expression of CXCL8 mRNA was not changed (Figure 5B), indicating that the expression of CXCL8 is not regulated by Snail in C666-1. In addition to Snail, the expression of CXCL8 could also be regulated by NF-κB. It has also been shown that NF-κB is activated in EBV-infected NPC cells [13]. The C666-1 cells were then treated with NF-κB inhibitor parthenolide [14-16]. Results in Figure 5C showed that the expression of CXCL8 mRNA was dose-dependently reduced by parthenolide. Similarly, the expression of CXCL8 mRNA could also be reduced after the knockdown of p65 (a subunit of the NF-κB transcription complex) expression by the p65 siRNA (Figure 5D). These observations suggested that NF-κB is involved in the expression of CXCL8 in C666-1 cells.

3.6 Inhibition of CXCL8 results in a reduction of growth factor-induced growth of tumor spheres

To further establish the role of CXCL8 in the growth of C666-1, another tumor sphere culture condition, namely, a tumor sphere formation assay using growth factors (EGF, IGF and FGF) supplemented serum-free medium and ultra-low attachment condition, was used. Result from the representative experiment was shown in Figure 6.
CXCL8 peptide inhibitor was also found to effectively reduce the formation of tumor sphere in the culture. The total number of tumor spheres in the control group (N=257) was reduced after the CXCL8 inhibitor treatment (N=101). These findings together with the results from Figure 4A suggested that CXCL8 could act as autocrine to support the growth of tumor spheres formation. Since the average size of tumor spheres in the control group (mean = 52 µm) of the sphere formation assay was greater than the average size of tumor spheres grown under the hanging drop nutrient poor culture condition (mean = 22 µm, Figure 4A), the sphere formation assay was then selected for the subsequent studies.

3.7 Effect of CXCR2 inhibition on the growth of tumor spheres

Since C666-1 cells express both CXCR1 and CXCR2 receptor, we first determined their mRNA expression level by real-time PCR. Results in Figure 7A showed that the expression level of CXCR2 mRNA in C666-1 was significantly higher than the CXCR1 mRNA. In the functional study, we examined the effect of CXCR2 inhibition on the expression of p-AKT and the growth of tumor spheres. Results in Figure 7B showed that the expression of p-AKT was also reduced by the CXCR2 specific inhibitor SB225002. The effect was accompanied with the reduced in the number of tumor spheres (Figure 7C), indicating that CXCL8 plays an autocrine
function to regulate the growth of tumor spheres via the CXCR2 signaling.

3.8 MIF is involved in CXCL8-mediated growth of NPC tumor spheres

In addition to CXCL8, previous study showed that a high level of MIF expression was correlated with the increased expression of CXCL8 and the tumor metastasis in NPC patients [3]. However, the functional role of MIF in the growth of the NPC cells was not clear. In the present study, we sought to determine whether MIF plays a role in the regulation of expression of CXCL8 and also the growth of NPC cells. C666-1 cells were transfected with MIF siRNA. The expression of CXCL8 mRNA and the NPC tumor spheres growth were then measured after transfection. In the control experiment, the expression of MIF protein was time-dependently reduced after the MIF si-RNA transfection (Figure 8A). Results from the gene expression experiment clearly showed that the mRNA expression of CXCL8 was also significantly reduced in the MIF si-RNA transfected cells (Figure 8B). In the tumor sphere formation assay, both the mean diameter and the number of tumor spheres in the MIF siRNA transfected culture were reduced (Figure 8C). Results from these observations suggested that MIF is an important cytokine upstream of the CXCL8 in the regulation of the growth of tumor spheres.
3.9 Effects of CXCL8- and CXCR2-inhibitor on the growth of C666-1 cells incubated with exogenously added CXCL8

In addition to the autocrine production of CXCL8 by the tumor cells, CXCL8 may also be produced by other inflammatory cells in the tumor tissues. Next, we further examined whether CXCL8- or CXCR2-inhibitor would reduce the growth of C666-1 tumor sphere in the presence of exogenously added CXCL8. C666-1 cells were incubated with CXCL8 (25 ng/ml) and CXCL8 peptide inhibitor (50 μM) or CXCR2 inhibitor SB225002 (0.4 μM). This concentration of CXCL8 has been shown in Figure 3 to enhance the migration of C666-1. The concentration of the two inhibitors has also been shown in Figure 6 and 7C to effectively reduce the growth of C666-1 tumor spheres. Results in Figure 9 showed that exogenously added CXCL8 slightly further increased the size and number of the tumor spheres. The growth of C666-1 tumor spheres was significantly reduced by the CXCL8 peptide inhibitor or SB225002. This observation suggested that both CXCL8- and CXCR2-inhibitor could also inhibit the growth promoting action of exogenously-added CXCL8 in C666-1 cells.

4. Discussion
CXCL8 (IL-8) has long been considered as a proinflammatory cytokine responsible for the migration of neutrophils and macrophages. Recent studies showed that CXCL8 can promote the growth of both normal and malignant cells. These include the normal fetal mucosal cells [17], urothelial cells [18], and the malignant cells such as the prostate tumor cells [19;20] and melanoma cell lines [21]. In addition to the promotion of tumor cell growth, CXCL8 has also been shown to regulate the growth of breast [22] and colorectal [12] tumor cells with the stem cell properties. In case of NPC, patients with a high level of cytokines such as CXCL8 in peripheral blood were found to have worsen prognoses for overall survival [23]. Genetic polymorphism of the CXCL8 gene has also been implicated in the pathogenesis of NPC [24-26]. Mechanistic studies suggested that CXCL8 could promote metastasis [27] and tumor angiogenesis [3] in NPC.

In the present study, we employed two different tumor spheres formation assays, namely the hanging drop assay and the sphere formation assay, to demonstrate the growth promoting activity of CXCL8 in the EBV-positive C666-1 cells. In the hanging drop assay, the C666-1 tumor spheres were formed in the presence of 1% normal serum. In the sphere formation assay, C666-1 tumor spheres were grown in growth factors (EGF, IGF and FGF) supplemented serum-free medium. Both of these
two assay methods clearly showed that inhibition of the CXCL8 signaling has a significant impact on the growth of tumor spheres even under the condition of stimulation by the growth factor EGF, IGF and FGF. Although CXCL8 was found to be important for the growth of the C666-1 cells, the growth promoting function of CXCL8 on the other NPC cell lines (CNE-1 and HONE-1) was not observed in the previous study [27]. The discrepancy on the growth promoting activity of CXCL8 between these two studies may be related to the status of EBV viral infection in these cell lines. The association between EBV infection and the oncogenic development of NPC is well recognized. For the C666-1, the cells still maintain the status of EBV latent viral infection. However, the CNE-1 and HONE-1 cells lost the EBV virus. The presence of EBV has been shown to be important for the malignant transformation and survival of the infected NPC cells [28-30]. The EBV viral protein has been shown to regulate the expression of CXCL8 in the infected NPC cells [13;31]. In the present study, we found that the endogenously produced CXCL8 could support the growth of tumor spheres as inhibition of the CXCL8/CXCR2 signaling could reduce the formation of tumor spheres. In the EBV-negative HONE-1 cells, the tumor cells may adopt and addict on some other signaling pathways for growth and survival when the host cell lost the virus. And hence the growth of the tumor cells is no longer regulated by CXCL8 even under the condition of CXCL8 overexpression [27].
The role of AKT signaling in NPC needs to further be discussed. Previous studies on the clinical NPC samples showed that the EGFR/PI3K/AKT pathways is important in the pathogenesis of NPC [32]. EGF was found to induce EMT partly through the PI3K/AKT pathway [33]. Other growth factors such as Met have also been shown to be the upstream activator of AKT [34]. Using the AKT inhibitor, Kong and co-workers showed that AKT inhibition could reduce the number of the side population (putative stem cell population) in the EBV LMP-2A expressing cells [30]. In the present study, we found that the formation of NPC tumor sphere could be reduced through the inhibition of the CXCL8/CXCR2/AKT signaling. The result is reminiscent of the findings by Hinohara and co-workers that the expression of CXCL8 and the activation of AKT are involved in the formation of mammospheres in breast cancer [7], and the CXCL8/CXCR1/AKT signaling is important for the growth of breast cancer stem cells [22]. In the present study, we found that CXCR2 signaling is important for the growth NPC tumor spheres. It is worthy to note that the concentration of CXCR2 inhibitor SB225002 to reduced 50% of tumor sphere formation was lower than the concentration to reduce 50% expression of p-AKT. This observation suggested that CXCR2 might also activate other signaling pathways for the growth of the tumor spheres. In addition to AKT, CXCR2 has also been shown to
activate other signaling molecules such as ERK [35;36], PKC [37], and ras [38;39].

The role of these signaling in the growth of NPC tumor spheres is still under investigation. Taken together, since CXCR2 (IL8-RB) is also abundantly expressed by the NPC tumor [40], all these evidences suggested that CXCR2 may be an important signaling hub molecule to regulate the growth of NPC cells.

In addition to the modulation of cell migration, MIF and CXCL8 are also well known stimulators of angiogenesis [41;42]. In NPC, both MIF (69.5%) and CXCL8 (56.0%) were highly expressed in the clinical samples, and the expression was significantly associated with the increase angiogenesis and lymph node metastasis [3]. MIF has also been reported as one of the nine important biomarkers in NPC [43]. MIF is a pleiotropic cytokine and the growth promoting function of MIF has been reported in many cell types [44-46]. However, the role of MIF in the growth of NPC cells has not been studied in NPC. In the present study, we found that MIF siRNA knockdown resulted in the reduced expression of CXCL8 and the tumor spheres formation even in the present of the growth factors EGF, FGF and IGF. This observation indicates that MIF/CXCL8/CXCR2 is not only involved in the tumor cell migration and tumor angiogenesis, but also supports the growth of the NPC cells. Hence, pharmacological targeting of these chemokines may reduce the tumor cell migration, tumor
angiogenesis and the growth of the bulk tumor. Combined targeting of the MIF/CXCL8/CXCR2 signaling with other current therapies such as radiotherapy and chemotherapy may be a strategy to completely control the growth and metastasis of NPC.

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Differential activation and regulation of CXCR1 and CXCR2


Figure 1

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Figure 3

A

Control

25 ng/ml

50 ng/ml

100 ng/ml

CXCL8 (ng/ml)

Control 25 50 100

Migrated cells (%)

0 50 100 150 200 250

# # #
Figure 3

B

Control

CXCL8 peptide inhibitor

C

Control

LY294002

0 10 20

LY294002 (µM)

Migrated cells (%)

0 50 100 150

Control 10µM 20µM

Migrated cells (%)

0 100 150

# #

Control

LY294002 (µM)

# #

Control 10 20

Migrated cells (%)
Figure 4

A

![Image of control sample](image1.png)

23.1 µm

![Image of CXCL8 peptide inhibitor sample](image2.png)

19.2 µm

- **Control**
  - Mean: 22.7 µm
  - N=134

- **CXCL8 peptide inhibitor (50 µM)**
  - Mean: 20.5 µm
  - N=83
Figure 4 B

CXCL8 peptide inhibitor (µM)

<table>
<thead>
<tr>
<th>CXCL8 peptide inhibitor (µM)</th>
<th>Mol. Wt. (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 5 10 20 50</td>
<td>60</td>
</tr>
</tbody>
</table>

p-AKT

AKT

Band intensity (arb. unit)

CXCL8 peptide inhibitor (µM)

Band intensity (arb. unit)

PROOF
Figure 4

C

![Image of tumor spheres]

<table>
<thead>
<tr>
<th>Diameter (µm)</th>
<th>No. of tumor spheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.6</td>
<td>1</td>
</tr>
<tr>
<td>18.4</td>
<td>2</td>
</tr>
<tr>
<td>15.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Control
Mean: 23.7µm
N=141

LY294002 (10 µM)
Mean: 19.4µm
N=76

LY294002 (20 µM)
Mean: 19µm
N=87

Diameter (µm)
Figure 5

D

<table>
<thead>
<tr>
<th></th>
<th>si-Control</th>
<th>si-p65</th>
</tr>
</thead>
<tbody>
<tr>
<td>p65</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mol. Wt. (kDa)

65

45

Relative expression level of CXCL8 mRNA

![Graph showing relative expression level of CXCL8 mRNA]
Figure 6

No. of tumor spheres

Control
Mean: 52.8 µm
N=257

CXCL8 peptide inhibitor
Mean: 47.5 µm
N=101

54.9 µm

42.4 µm
Figure 7

A

![Graph showing fold-change for CXCR1 and CXCR2.]

B

![Western blot images for p-AKT and AKT with bar graph showing band intensity.]

- Mol. Wt. (kDa): 60
- Band intensity (arb. unit)
- SB225002 (µM): 0, 0.1, 0.5, 1
Figure 7

C

No. of tumor spheres

Control
Mean: 48.8
N=254

Mean: 42.2
N=188

Mean: 39.2
N=138

Mean: 30.9
N=42

Diameter (µm)
Figure 7

No. of tumor spheres

SB225002 (µM)

No. of tumor spheres

0 0.1 0.2 0.3 0.4

#  

##

PROOF
Figure 8

C

No. of tumour spheres

Diameter (µm)

Control
Mean 51.7 µm
N=251

si-MIF
Mean 37.5 µm
N=218

55.4 µm

38.5 µm
Figure 9

A

<table>
<thead>
<tr>
<th>No. of tumour spheres</th>
<th>Control</th>
<th>CXCL8</th>
<th>CXCL8 peptide inhibitor</th>
<th>CXCL8 + CXCL8 peptide inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean: 48.8 µm</td>
<td>Mean: 55.9 µm</td>
<td>Mean: 34.4 µm</td>
<td>Mean: 40.9 µm</td>
</tr>
<tr>
<td></td>
<td>N=285</td>
<td>N=317</td>
<td>N=125</td>
<td>N=113</td>
</tr>
</tbody>
</table>

Diameter (µm)

- Control: Mean = 48.8 µm, N=285
- CXCL8: Mean = 55.9 µm, N=317
- CXCL8 peptide inhibitor: Mean = 34.4 µm, N=125
- CXCL8 + CXCL8 peptide inhibitor: Mean = 40.9 µm, N=113
Figure 9

B

No. of tumour spheres

Control  CXCL8  CXCL8 + Peptide inhibitor  CXCL8 + Peptide inhibitor

No. of tumour spheres
0  100  200  300  400

#  ##  ##
Figure 9

C

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diameter (µm)</th>
<th>Mean</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.0 µm</td>
<td>53.6 µm</td>
<td>276</td>
</tr>
<tr>
<td>CXCL8 + SB225002</td>
<td>64.7 µm</td>
<td>64.1 µm</td>
<td>309</td>
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<tr>
<td>SB225002</td>
<td>10.8 µm</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>CXCL8 + SB225002</td>
<td>9.3 µm</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 9

No. of tumour spheres

Control  CXCL8  CXCL8 + SB225002  SB225002

# *
Figure legends

Figure 1. CXCL8 mRNA expression in paired NPC tumor and normal biopsy tissues. Fold change between tumor and the surrounding normal biopsy tissues was calculated by dividing the mean expression value of tumor versus normal tissues. Statistical analysis in the paired samples was performed by Wilcoxon Signed Rank test.

Figure 2. (A) Detection of CXCL8, CXCR1, and CXCR2 mRNA from various NPC cell lines. Exponentially growing cells were collected and total cellular RNA was prepared. Expression of CXCL8, CXCR1, CXCR2 and GAPDH mRNA were determined by PCR. (B) ELISA analysis of the kinetics of CXCL-8 secretion by C666-1. C666-1 cells (3x10^4 cells/well) were seeded onto wells of 96-well plate. Culture supernatant was collected at day-3, 4 and 5 after incubation. The level of CXCL8 was then determined and the results were expressed as mean ± S.D. * p<0.05

Figure 3. Effects of CXCL8 on the migration of C666-1 cells. (A) Exogenously added CXCL8 enhanced the migration of C666-1 cells. C666-1 cells were seeded onto the upper chamber and CXCL8 was added onto the lower chamber. The cultures were then incubated at 37°C for 24 hours for the cells to migrate across the membrane.
Upper panel: Images were captured by a fluorescence microscope. Scale bar = 100 µm. Lower panel: Quantitative analysis of transwell migration. (B) Inhibition of cell migration induced by the endogenously produced CXCL8. C666-1 cells were seeded together with CXCL8 peptide inhibitor (50 µM) onto the upper chamber and the serum containing medium was added onto the lower chamber. Left panel: Scale bar = 100 µm. Right panel: Quantitative analysis of transwell migration. (C) Effects of the PI3K inhibitor LY294002 on the migration of C666-1 cells. C666-1 cells were seeded together with LY294002 (10 - 20 µM) onto the upper chamber of a transwell. Upper panel: Images were captured by a fluorescence microscope. Scale bar = 100 µm. Lower panel: Quantitative analysis of transwell migration. Migrated cells were counted and results were expressed as mean ± SD from 3 replicates. # p < 0.01, ## p < 0.001.

**Figure 4.** Quantitative measurement of growth inhibition induced by CXCL8 inhibitor. (A) Hanging drop culture was set up as described in Materials and Methods. The cultures were incubated with or without CXCL8 peptide inhibitor for 7 days. Images of tumor spheres were captured and the size of spheroids was determined by ImageJ software. Results were presented as the total number of tumor sphere per culture. (B) Effect of CXCL8 peptide inhibitor on the phosphorylation level of AKT.
Total cell lysate was prepared at 24 hours after the CXCL8 inhibitor (1 - 50 μM) treatment. Expression of p-AKT was determined by Western Blots. ##, p<0.001. (C) Quantitative measurement of growth inhibition induced by the PI3K inhibitor LY294002. Hanging drop culture, with or without LY294002 (10 and 20 μM), was set up as described in Figure 3A. The size and the total number of tumor spheres were determined at 7 days after incubation. Scale bar = 50 μm.

**Figure 5.** Effects of Snail-knockdown or NF-κB inhibition on the expression of CXCL8 mRNA. (A) Western blotting analysis of snail protein expression by snail siRNA-treated C666-1 cells. (B) Expression of CXCL8 mRNA by snail siRNA-treated C666-1 cells. (C) Effects of NF-κB inhibitor parthenolide on the expression of CXCL8 mRNA. C666-1 cells were serum-starved in RPMI for overnight before the treatment with parthenolide (1 – 10 μM). Total RNA was collected at 24 hours after the treatment. The expression level of CXCL8 mRNA was determined by real-time PCR. * p<0.05. (D) Effects of p65-knockdown on the expression of CXCL8 mRNA. Upper panel: Western blotting analysis of p65 expression by p56 siRNA-treated C666-1 cells. Lower panel: Relative expression level of CXCL8 mRNA. #p < 0.01
Figure 6. CXCL8 inhibitor inhibits the growth of growth factor-induced tumor sphere formation. C666-1 cells (1x10^4) were grown in EGF, FGF and IGF supplemented DMEM/F12 (1:1) medium. The cultures were incubated with CXCL8 inhibitor (50 µM) in a humidified chamber at 37°C for 7 days. The cultures were fed with the supplement every 2 days. The number and the size profile of the tumor spheres were determined at day-7 after incubation. Scale bar = 50 µm.

Figure 7. (A) Quantitative measurements of expression of CXCR1 and CXCR2 mRNA. Exponentially growing C666-1 cells were collected and total cellular RNA was prepared. mRNA expression was determined by real-time PCR. Results were expressed as mean ± S.D from 3 replicates. ## p < 0.001. (B) Effect of CXCR2 inhibitor SB225002 on the phosphorylation level of AKT. C666-1 cells (5x10^5 cells/well) were serum-starved in RPMI medium for overnight before the treatment with SB225002 (0.1 - 1 µM). Total cell lysate was prepared at 24 hours after the treatment. The level of p-AKT was determined by Western blot. Value represented the arbitrary unit of band intensity. # p < 0.01. (C) Quantitative measurement of growth inhibition induced by CXCR2 inhibitor SB225002. C666-1 cells (1x10^4) were grown in EGF, FGF and IGF supplemented DMEM/F12 (1:1) medium in a 6-well ultra-low plate. The cells were then treated with SB225002 in a
humidified chamber at 37°C for 7 days. The size profile (upper panel) and number of tumor spheres per culture (lower panel) was determined. Scale bar = 50 µm. # p < 0.01, ## p < 0.001.

**Figure 8** Effect of MIF knockdown on C666-1. (A) Western blotting analysis of MIF protein expression in MIF siRNA-treated C666-1 cells. (B) Effects of MIF-knockdown on the expression of CXCL8 mRNA. Total cellular RNA was collected at 96 hours after the transfection. The expression level of CXCL8 mRNA was determined by real-time PCR. Results were expressed as mean ± S.D from 3 replicates. # p < 0.01. (C) Quantitative measurement of tumor spheres growth by MIF siRNA-treated C666-1 cells. The growth of tumor spheres was monitored using the spheroid formation assay. Scale bar = 50 µm.

**Figure 9** Effects of CXCL8 peptide inhibitor (A, B) and CXCR2 inhibitor SB225002 (C, D) on C666-1 cells incubated with exogenously added CXCL8. Tumor sphere cultures were set up as described in Figure 6 and 7. CXCL8 was added at a concentration of 25 ng/ml. The cells were treated with 50 µM CXCL8 peptide inhibitor (A, B) or 0.4 µM SB225002 (C, D). The cultures were then incubated in a humidified chamber at 37°C for 7 days. Scale bar = 50 µm. #: p < 0.05. ##: p < 0.001. *: The size of tumor spheres was smaller than 20 µm.