

CXCL12 genetic variants as prognostic markers in nasopharyngeal carcinoma

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Abstract: The chemokine receptor 4/chemokine ligand 12 (CXCR4/CXCL12) axis plays an important role in tumorigenesis, metastasis, and recurrence of tumors. Its single nucleotide polymorphisms (SNPs) are associated with patient survival in several types of cancer. However, the prognostic value of SNPs in nasopharyngeal carcinoma (NPC) has not been fully investigated. This retrospective study assessed the relationships between CXCR4 rs2228014 and CXCL12 rs1801157 polymorphisms and patient outcome in 222 patients newly diagnosed with NPC. The analysis found no significant correlation between the presence of both SNPs and clinicopathological factors. However, univariate analysis showed that N classification, clinical stage, and the CXCL12 rs1801157 polymorphism were significantly associated with distant metastasis-free survival ($P=0.018$, 0.028 , and 0.013 , respectively) and progression-free survival ($P=0.007$, 0.046 , and 0.021 , respectively). After adjusting clinicopathological factors, multivariate analysis identified CXCL12 rs1801157 as an independent prognostic factor for distant metastasis-free survival and progression-free survival (hazard ratio: 3.332; 95% confidence interval: 1.597–6.949; $P=0.001$ and hazard ratio: 2.665 95% confidence interval: 1.387–5.119; $P=0.003$, respectively). Our results suggest that CXCL12 rs1801157 AA genotype might serve as a potential prognostic factor in patients with NPC.

Keywords: nasopharyngeal carcinoma, CXCR4, CXCL12, polymorphism, prognosis

Introduction

Nasopharyngeal carcinoma (NPC) is the most common nasopharyngeal malignancy, with an extremely unbalanced endemic distribution. According to the International Agency for Research on Cancer, 84,400 cases of NPC were newly diagnosed in 2008, with 51,600 mortalities worldwide. Approximately 40% of these occurred in the People's Republic of China.¹ Despite combined applications of magnetic resonance imaging (MRI), intensity-modulated radiation therapy, and chemotherapy, distant metastasis occurs in 20%–30% of patients and is the main cause of treatment failure in NPC.²

Tumor, node, metastasis (TNM) classification is widely used for prognosis evaluation and establishment of treatment strategy. However, due to the heterogeneity of the tumor in NPC, patients at the same TNM stage who are undergoing similar treatments frequently experience different clinical outcomes, suggesting that the TNM staging system is inadequate for prognostic stratification in NPC.³ Therefore, the development of new prognostic markers is required as an adjunct to traditional staging techniques in order to develop patient-specific treatment strategies for patients with NPC.

It is generally recognized that chemokines and their receptors play an important role in the metastatic process by guiding the migration of receptor-bearing tumor cells to secondary tissues where their ligands are expressed.⁴ Chemokine receptor 4 (CXCR4) is widely expressed in malignant cells, and its sole ligand is chemokine

ligand 12 (CXCL12).⁵ The CXCR4/CXCL12 axis facilitates tumor metastasis by directly guiding tumor cells to metastatic sites. As such, it has been implicated in promoting growth, proliferation, and angiogenesis of tumor cells.⁶ Increased expression of CXCR4 and CXCL12 has been associated with poor prognosis in several tumors.^{7,8}

The *CXCR4* gene is located on chromosome 2q2⁹ with a silent single nucleotide polymorphism (SNP) at codon 138, named rs2228014.¹⁰ The *CXCL12* gene is located on chromosome 10q11.1,¹¹ with a functional SNP rs1801157 in the 3'-untranslated gene region.¹² Both polymorphisms have been associated with an increased risk of cancer and poorer prognoses in colorectal,¹³ endometrial,¹⁴ and lung cancer.¹⁵ However, little is known about the prognostic value of these two polymorphisms in NPC.

Genetic background may contribute to the differences in the level and activities of CXCR4 and CXCL12 between patients. To explore the hypothesis that *CXCR4* rs2228014 and *CXCL12* rs1801157 polymorphisms may be associated with clinical outcome in patients with NPC, we retrospectively investigated the relationship between these two polymorphisms and the clinicopathological characteristics of 222 untreated patients with NPC from a single endemic area in the People's Republic of China and their association with prognosis.

Materials and methods

Patient selection, treatment, and follow-up

A total of 222 patients, diagnosed with NPC at the Sun Yat-sen University Cancer Center (Guangzhou, People's Republic of China) between December 2000 and March 2007, were enrolled in this study. The selection criteria were as follows: ethnic Han Chinese; newly diagnosed with histologically confirmed NPC and with no distant metastasis; no history of any other malignant disease; Karnofsky score ≥ 70 ; no prior treatment for NPC; treated with radiotherapy at our Cancer Center; underwent regular follow-ups; and availability of periphery blood samples. All medical records were reviewed retrospectively, and all patients were restaged according to the seventh edition of the UICC/AJCC TNM classification system. Written informed consents had been given by all patients, and the research was approved by the Center's ethics committee.

Before enrollment, each patient underwent a complete pretreatment evaluation, including patient history, physical examinations, hematological and biochemical profiling, MRI of the head and neck, chest radiography, abdominal sonography, and whole-body bone scan. All patients received definitive radiotherapy following established methods.¹⁶

The accumulated dose to the primary tumor was 64–80 Gy; the accumulated dose to the involved areas of the neck was 60–62 Gy, and it was 50 Gy to the uninvolved areas. Concurrent chemotherapy consisted of cisplatin administered weekly or on weeks 1, 4, and 7 of radiotherapy. Neoadjuvant and adjuvant chemotherapy consisted of cisplatin with 5-fluorouracil or taxanes administered every 3 weeks for three cycles.

Follow-up was carried out every 3 months during the first year and every 6 months thereafter, until the final follow-up or death. Follow-up was concluded on May 2015. The median follow-up period was 86.6 months (range: 3.1–152.9 months). Distant metastasis-free survival (DMFS) and progression-free survival (PFS) were selected as the endpoints of this study. DMFS was calculated from the date of enrollment to the date of distant metastasis or the last follow-up. PFS was calculated from the date of enrollment to the date of any form of tumor progression or to the last follow-up.

DNA extraction and genotyping

Peripheral blood (2 mL) was collected at the time of enrollment for genotyping. DNA was obtained from peripheral lymphocytes using the QIAamp DNA Blood Midi Kit (QIAGEN; Valencia, CA, USA) according to the manufacturer's instructions. The primers for the polymerase chain reaction (PCR) analysis of *CXCR4* and *CXCL12* polymorphisms were designed using Primer 5.0 software and synthesized commercially by BGI (Shenzhen, People's Republic of China). The primer sequences were as follows: *CXCR4* rs2228014; forward: 5'-TCCGTGAAGAAAATGCTAAT-3'; reverse: 5'-AACACAACCACCCACAAGTC-3' and *CXCL12* rs1801157; forward: 5'-TCTTTCCACGGAGCCACT-3'; reverse: 5'-TCATTTCTGCTTGGTGC-3'.

PCR amplification was performed in a 30 μ L reaction volume containing 2 μ L genomic DNA, 2 μ L 2.5 mM dNTPs, 3 μ L 10 \times rTaq buffer, 1 μ L of each primer (10 mM), and 0.2 μ L rTaq. PCRs were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 20 seconds at 95°C, annealing at 65°C for 20 seconds, polymerization at 72°C for 30 seconds, with a final polymerization step at 72°C for 5 minutes. The PCR products were purified and sequenced by BGI, and the sequence data were analyzed using DNASTAR analysis programs.

Statistical analysis

All the analyses, with the exception of the Schoenfeld residual plot test, were performed using v17.0 software (SPSS Inc., Chicago, IL, USA). Pearson's chi-squared test (χ^2) was

used to determine correlations between the two SNPs and the patients' clinicopathological features. The χ^2 test was also used to test the Hardy–Weinberg equilibrium. The Kaplan–Meier method was used to estimate the actuarial rates, and the survival curves were compared by the log-rank test. Schoenfeld residual plot test was applied to test the proportional hazard assumption, using the Stata statistical software (STATA 12; StataCorp LP, College Station, TX, USA).

Univariate and multivariate analyses were performed using a Cox proportional hazards model in order to calculate hazard ratios (HRs) and determine the independent significances of explanatory variables by backward elimination. The following parameters were selected as the covariates in the multivariate analysis: age, sex, smoking status, alcohol consumption, histological type, radiotherapy technique, chemotherapy, clinical stage, and *CXCL12* rs1801157. Two-tailed *P*-values <0.05 were considered statistically significant.

Results

Patient characteristics and clinical outcomes

The distribution of the clinicopathological features of the patients are summarized in Table 1. The median age was 48 years (range: 14–80 years). Two-dimensional radiotherapy was administered to 208/222 (93.7%) patients, and intensity-modulated radiation therapy was administered to 14/222 (6.3%) patients. A total of 150/222 (67.6%) patients received chemotherapy. The distribution of clinical stages was similar to those observed in other studies performed in our cancer center. At the final follow-up, 54 patients (24.3%) had died, and the 5-year DMFS and PFS rates were 82.8% and 74.4%, respectively.

Correlation of *CXCR4* and *CXCL12* polymorphisms with patients' clinicopathological features

Genotyping showed that the distribution of the *CXCR4* rs2228014 genotype within the 222 enrolled patients was 70.3% (149/222) CC (homozygous wild allele), 25.5% (54/222) CT, and 4.2% (9/222) TT, respectively. The distribution of *CXCL12* rs1801157 genotype was 51.9% (109/222) GG (homozygous wild allele), 39.1% (82/222) GA, and 9.0% (19/222) AA. The completion rates of these two polymorphisms were 94.6% and 95.5% for *CXCR4* rs2228014 and *CXCL12* rs1801157, respectively. Representative sequencing chromatograms from randomly chosen cases were used to illustrate these distributions (Figure 1).

Table 1 Clinicopathological characteristics of patients with NPC who were enrolled in this study (n=222)

Characteristic	Number of patients (%)
Age	
≥48 years	115 (51.8)
<48 years	107 (48.2)
Sex	
Male	175 (78.8)
Female	47 (21.2)
Smoking status	
Ever	103 (46.4)
Never	119 (53.6)
Alcohol consumption	
Ever	49 (22.1)
Never	173 (77.9)
Histology	
WHO I type	1 (0.5)
WHO II type	12 (5.4)
WHO III type	209 (94.1)
Radiotherapy	
IMRT	14 (6.3)
2D-RT	208 (93.7)
Chemotherapy	
Yes	150 (67.6)
No	72 (32.4)
T classification	
T ₁₋₂	93 (41.9)
T ₃₋₄	129 (68.1)
N classification	
N ₁₋₂	170 (76.6)
N ₃₋₄	52 (23.4)
Clinical stage	
I–II	79 (35.6)
III–IV	143 (64.4)

Abbreviations: NPC, nasopharyngeal carcinoma; WHO, World Health Organization; IMRT, intensity-modulated radiotherapy; 2D-RT, two-dimensional radiotherapy.

Although the genotype frequencies for these two polymorphisms were confirmed to be in Hardy–Weinberg equilibrium for patients with NPC, there were no statistically significant associations between genotypes and sex, age, smoking status, alcohol consumption, histology, radiotherapy technique, chemotherapy, T classification, N classification, or clinical stage (data not shown).

Univariate analysis

In relation to patients' clinicopathological features, DMFS (HR: 1.034; 95% confidence interval [CI]: 1.006–1.064; *P*=0.018 and HR: 1.037; 95% CI: 1.004–1.071; *P*=0.028, respectively) and PFS (HR: 1.032; 95% CI: 1.009–1.057; *P*=0.007 and HR: 1.026; 95% CI: 1.000–1.052; *P*=0.046, respectively) were both significantly associated with N classification and clinical stage, as expected. In contrast, none of the other tested characteristics (age, sex, smoking status, alcohol consumption, histological type, radiotherapy

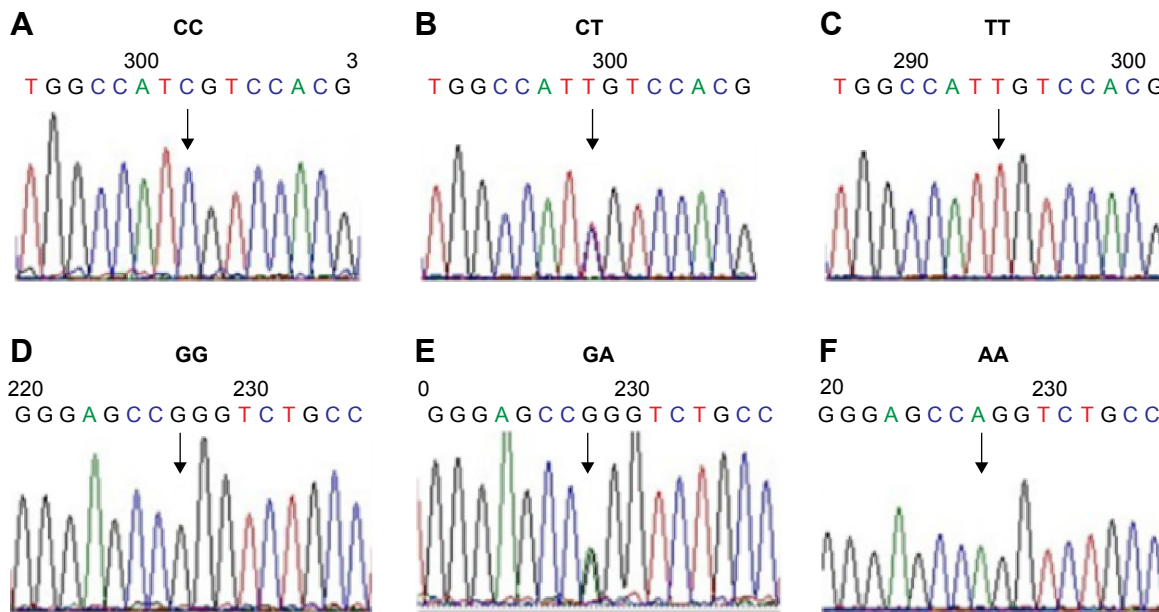


Figure 1 Sequencing chromatograms of *CXCR4* rs2228014 and *CXCL12* rs1801157.

Notes: (A–C) Shows the chromatograms for *CXCR4* rs2228014 CC, CT, and TT genotypes, respectively. (D–F) Shows the chromatograms for *CXCL12* rs1801157 GG, GA, and AA genotypes, respectively. The results represent randomly selected samples.

Abbreviations: *CXCR4*, chemokine receptor 4; *CXCL12*, chemokine ligand 12.

technique, chemotherapy, and T classification) has a significant effect on either DMFS or PFS.

In relation to genotype, univariate analysis found no significant association between *CXCR4* genotypes and DMFS or PFS ($P=0.998$, $P=0.833$, respectively; Table 2). In contrast, a close association was found between *CXCL12* GG and GA genotypes and improved prognosis, compared to patients carrying the *CXCL12* AA genotype ($P=0.044$ and $P=0.062$ for DMFS and PFS, respectively; Table 2, Figure 2A and C). The significance of this association was increased when patients carrying G alleles (GG + GA) were combined for further analysis, compared to patients carrying the *CXCL12* AA genotype ($P=0.013$ and $P=0.021$ for DMFS and PFS, respectively; Table 2, Figure 2B and D).

Multivariate analysis

After adjusting for clinicopathological features (age, sex, smoking status, alcohol consumption, histological type, radiotherapy technique, chemotherapy, and clinical stage), multivariate analysis using Cox proportional hazards model confirmed that the *CXCL12* rs1801157 polymorphism was a significant independent prognostic factors for DMFS (HR: 3.332; 95% CI: 1.597–6.949; $P=0.001$) and PFS (HR: 2.665; 95% CI: 1.387–5.119; $P=0.003$) in patients with NPC (Table 3). Clinical stage was also identified as an independent prognostic factor for DMFS (HR: 2.873;

95% CI: 1.378–5.988; $P=0.005$) and PFS (HR: 1.806; 95% CI: 1.033–3.158; $P=0.038$; Table 3).

Discussion

To date, there has been considerable research in identifying new biomarkers for more accurate prognostic assessment in NPC. Tumor-derived markers, including Epstein–Barr virus DNA,¹⁷ VEGF,¹⁸ and IL-8¹⁹ have been proposed as potential candidates. Germline polymorphism is a common approach to explore the value of host-derived factors. The advantages of SNP genotyping, compared to gene expression profiling, include less variability between laboratories and across platforms and the ability to analyze both tissue samples and peripheral blood.²⁰

CXCL12 is believed to attract tumor cells by binding to its sole receptor *CXCR4*, which is widely expressed in tumor cells.⁵ The *CXCR4*/*CXCL12* axis has multiple functions in tumorigenesis, including tumor growth, angiogenesis, distant metastasis, and survival.⁶ However, the prognostic value of *CXCR4* and *CXCL12* genetic variants in patients with NPC is unclear. Therefore, the aim of this study was to investigate the association between two specific *CXCR4* and *CXCL12* genetic polymorphisms, namely *CXCR4* rs2228014 and *CXCL12* rs1801157, and the clinical outcomes of 222 patients with NPC in a single endemic area.

Activation of *CXCR4*/*CXCL12* in the tumor microenvironment has been implicated in tumor growth and

Table 2 Univariate analysis of DMFS and PFS in patients with NPC (n=222)

Variable	DMFS			PFS		
	HR	95% CI	P-value	HR	95% CI	P-value
Age			0.290			0.342
<48 years	1.000			1.000		
≥48 years	1.384	0.758–2.526		1.269	0.776–2.075	
Sex			0.360			0.110
Male	1.000			1.000		
Female	1.459	0.650–3.274		1.776	0.879–3.592	
Smoking status			0.875			0.959
Never	1.000			1.000		
Ever	1.046	0.586–1.837		0.988	0.624–1.565	
Alcohol consumption			0.656			0.646
Never	1.000			1.000		
Ever	0.851	0.413–1.754		1.137	0.661–1.957	
Histological type			0.743			0.631
WHO I	1.000			1.000		0.631
WHO II	4,416.604	0.000–8.29E89		4,441.070	0.000–7.438E73	
WHO III	2,956.944	0.000–5.538E89		2,955.821	0.000–4.941E73	
Radiotherapy			0.523			0.417
2D-RT	1.000			1.000		
IMRT	0.630	0.152–2.604		0.619	0.194–1.972	
Chemotherapy			0.280			0.951
No	1.000			1.000		
Yes	1.444	0.741–2.813		0.984	0.587–1.648	
T classification			0.425			0.572
T ₁₋₂	1.000			1.000		
T ₃₋₄	1.011	0.984–1.040		1.007	0.984–1.030	
N classification			0.018			0.007
N ₀₋₁	1.000			1.000		
N ₂₋₃	1.034	1.006–1.064		1.032	1.009–1.057	
Clinical stage			0.028			0.046
I–II	1.000			1.000		
III–IV	1.037	1.004–1.071		1.026	1.000–1.052	
CXCR4 rs2228014			0.998			0.833
TT	1.000			1.000		
CT	0.955	0.229–3.980		1.507	0.367–6.194	
CC	0.958	0.214–4.285		1.403	0.324–6.075	
CXCL12 rs1801157			0.044			0.062
AA	1.000			1.000		
GA	0.384	0.171–0.864		0.425	0.208–0.871	
GG	0.370	0.158–0.866		0.486	0.234–1.008	
GA + GG	0.378	0.176–0.811	0.013	0.451	0.230–0.885	0.021

Note: $P < 0.05$ was considered statistically significant.

Abbreviations: DMFS, distant metastasis-free survival; PFS, progression-free survival; NPC, nasopharyngeal carcinoma; HR, hazard ratio; CI, confidence interval; WHO, World Health Organization; 2D-RT, two-dimensional radiotherapy; IMRT, intensity-modulated radiotherapy; T, tumor; N, node; CXCL12, chemokine ligand 12; CXCR4, chemokine receptor 4.

distant metastasis.^{21,22} In vitro and in vivo studies have been supported by retrospective studies showing a close relationship between expression of CXCR4 and CXCL12 and disease stage and prognosis in several malignancies, including melanoma,²³ prostate cancer,²⁴ and pancreatic cancer.²⁵ Although high expression of CXCR4 has been linked to metastatic progression and poor prognosis in patients with NPC,^{8,26} details of the role and molecular

mechanisms of the CXCR4/CXCL12 axis in NPC remains scarce.

Differences in genetic polymorphisms may contribute to the variability in CXCR4 and CXCL12 expression between individuals. A significant association between CXCL12 rs1801157 genotypes and susceptibility and prognosis in breast cancer and several other malignancies have been reported.^{14,27} However, the influence of the CXCL12

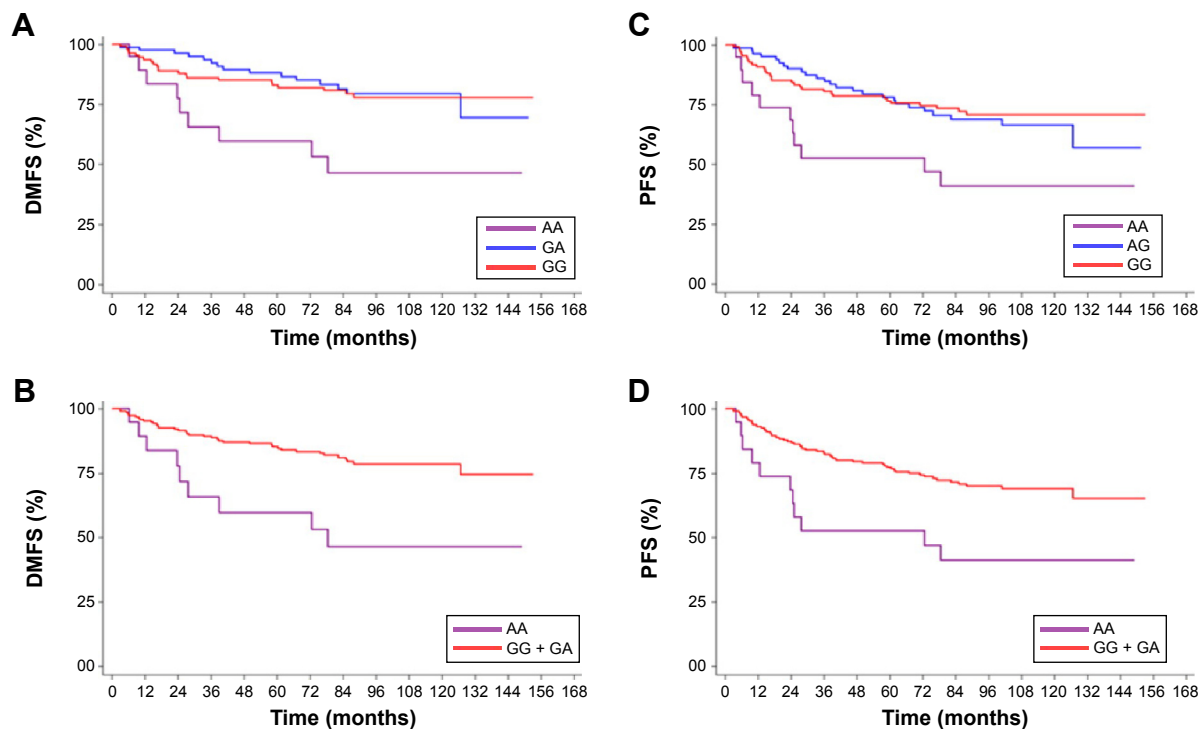


Figure 2 Kaplan–Meier curves of DMFS and PFS for the different *CXCL12* rs1801157 genotypes.

Notes: (A and C) Compare GG, GA, and AA genotypes for DMFS and PFS. (B and D) Compare GA + GG and AA genotypes for DMFS and PFS. *P*-value was calculated by log-rank test.

Abbreviations: *CXCL12*, chemokine ligand 12; DMFS, distant metastasis-free survival; PFS, progression-free survival.

rs1801157 polymorphism in the expression of the *CXCL12* protein remains controversial. Studies have shown that *CXCL12* rs1801157 homozygous AA genotype was correlated with the increased levels of protein expression,^{12,27} a higher risk of cancer, and poorer clinical outcome in several malignancies. However, a conflicting result was found in breast cancer, with significantly lower expression levels of *CXCL12* mRNA in peripheral blood samples in patients carrying *CXCL12* rs1801157 A alleles compared to those with the GG alleles.²⁸ Consequently, further research is required to determine the molecular mechanisms that underline *CXCL12* rs1801157 polymorphism and gene expression.

Table 3 Multivariate analysis for DMFS and PFS in patients with NPC (n=222)

Endpoint	Variable	HR	95% CI	<i>P</i> -value
DMFS	Clinical stage	2.873	1.378–5.988	0.005
	<i>CXCL12</i> rs1801157	3.332	1.597–6.949	0.001
PFS	Clinical stage	1.806	1.033–3.158	0.038
	<i>CXCL12</i> rs1801157	2.665	1.387–5.119	0.003

Note: *P*<0.05 was considered statistically significant.

Abbreviations: DMFS, distant metastasis-free survival; PFS, progression-free survival; NPC, nasopharyngeal carcinoma; HR, hazard ratio; CI, confidence interval; *CXCL12*, chemokine ligand 12.

Our study reveals that the *CXCL12* rs1801157 AA genotype was significantly associated with poor DMFS and PFS compared to GG and GA genotype, which are consistent with recent studies of other malignancies. *CXCL12* AA genotype and *CXCR4* TT genotype contributed to higher odds ratio for lung cancer, and patients carrying *CXCL12* AA or *CXCR4* TT genotype exhibited a tendency to advanced cancer stage and poorer prognosis compared with those with other genotypes.¹⁵ Similar findings have been reported in colorectal cancer and esophagogastric cancer.²⁹ We hypothesized that the G → A mutation in *CXCL12* rs1801157 could alter gene expression, thereby influencing the migration and metastasis in NPC. The earlier studies indicate that *CXCL12* rs1801157 has great potential as a novel prognostic biomarker in several malignancies, and the critical effect of *CXCL12* in NPC progression is of great value for further exploration.

CXCR4 rs2228014 is a silent SNP. Patients carrying T allele have been linked to higher cancer risk and poorer prognosis in endometrial carcinoma and non-small-cell lung cancer than those carrying C alleles.^{14,15} However, our results found no significant relationship between the *CXCR4* rs2228014 genotypes and prognosis in NPC. It has been suggested that this silent SNP may be linked to an as yet

unknown functional mutation that can modify gene expression, induce differences in transfer RNA (tRNA) selection, and alter RNA stability, folding, or splicing.³⁰

The CXCR4/CXCL12 axis is attracting increasing interest as a potential target for the development of anticancer therapeutics.³¹ To date, over 15 new drugs targeting the CXCR4/CXCL12 axis have been developed.³² AMD3100, a CXCR4 antagonist also known as plerixafor (Mozobil, Sanofi US, Bridgewater, NJ, USA), has been approved for use in patients with non-Hodgkin's lymphoma and multiple myeloma, by the Food and Drug Administration.^{33,34} Phase I and II clinical trials have been carried out on other drugs, including TG-0054, a CXCR4 inhibitor, and CTCE-9908,³⁵ a CXCL12 peptide analog. These developments promote the therapeutic potential of the CXCR4/CXCL12 axis against NPC.

Conclusion

This study has demonstrated that the *CXCL12* rs1801157 genotype was significantly associated with poorer DMFS and PFS in patients with NPC, compared to *CXCL12* rs1801157 GG and GA genotypes. This suggests that the inhibition of the CXCR4/CXCL12 signaling pathway may provide a novel therapeutic strategy for the treatment of patients with NPC. The study had the following limitations: the cohort was small, so the results will need to be validated using larger sample sizes, and the details of the underlying mechanisms were not investigated. Despite these limitations, the close association between the *CXCL12* rs1801157 polymorphism and prognosis in patients with NPC warrants further investigation and has demonstrated its potential as a promising therapeutic target in NPC.

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Disclosure

The authors report no conflicts of interest in this work.

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