

Association of *SDF-1* and *CXCR4* Polymorphisms With Susceptibility to Oral and Pharyngeal Squamous Cell Carcinoma

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Abstract. Background/Aim: Long-term exposure to betel quid (BQ)-, cigarette-, and alcohol-induced chronic inflammation is a crucial risk factor for oral and pharyngeal squamous cell carcinoma (OPSCC) progression. We analyzed the genotypes of stromal-cell-derived factor-1 (*SDF-1*) and CXC-chemokine receptor-4 (*CXCR4*) and determined the association between their polymorphisms and the risk of OPSCC. Materials and Methods: This study consisted of 452 patients with pathologically proved OPSCC and 424 sex- and age-matched cancer-free controls. The genotypes of *SDF-1* and *CXCR4* were detected through the TaqMan real-time polymerase chain reaction (PCR) method. Results: Our data indicated that the C

allele and C/C genotypes of *CXCR4* were significantly associated with OPSCC [adjusted odds ratio (AOR)=1.41, 95% confidence interval (CI):1.02-1.96, $p=0.037$ and AOR=1.51, 95% CI:1.05-2.17, $p=0.028$, respectively] and OSCC (AOR=1.41, 95%CI:1.00-2.00, $p=0.049$ and AOR=1.49, 95%CI:1.01-2.20, $p=0.044$, respectively) risk. Patients with genetic polymorphisms of the genotype combination *SDF-1/CXCR4* had a higher risk of OSCC (p trend=0.033). We analyzed the effects of *CXCR4* genetic variants on susceptibility to OPSCC in patients with different risk habits of BQ chewing, tobacco smoking and alcohol consumption, and revealed that C/T+T/T genotypes exerted an increased risk only in patients with one (AOR=2.68, $p=0.036$) or two risk habits (AOR=2.02, $p=0.027$) compared to patients with the C/C genotype. Conclusion: We concluded that *CXCR4* C>T can be used as a genetic marker of susceptibility to OPSCC, particularly in OPSCC patients with one or two types of risk habits with a synergistic effect.

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Oral and pharyngeal cancer is an critical public health issue worldwide and leads to cancer morbidity and mortality worldwide (1). Squamous cell carcinoma (SCC) accounts for >90% of all oral and pharyngeal cancers (2). Three types of substance use, namely betel quid (BQ) chewing, cigarette

smoking, and alcohol drinking, have been identified as major risk factors for oral and pharyngeal SCC (OPSCC) (3, 4). BQ ingredients induce oral keratinocyte to secrete tumour necrosis factor (TNF)-alpha and interleukin (IL)-6, which may provoke oral and pharyngeal mucosal inflammation (5). Cigarette smoking causes a drastic change in immunity that leads to increased constitutive inflammation and suppressed antitumoral immune cell responses (6). It also causes inappropriate priming and activation of monocytes and neutrophils (7). Acetaldehyde, rather than alcohol itself, is responsible for the carcinogenic effect of alcohol consumption through induction of inflammation and enhancement of cell injury (8, 9). Therefore, long-term exposure to BQ, cigarette, and alcohol may induce OPSCC through chronic inflammation of oral and pharyngeal mucosa. However, only a fraction of cigarette smokers, alcohol drinkers, or BQ chewers develop OPSCC, suggesting the existence of genetic susceptibility to this type of cancer (10). Our previous studies have shown that the interaction of genetic polymorphisms of inflammation-related genes with substance use were associated with the risk of OPSCC (11, 12).

Chemokines are mediators of acute and chronic inflammation (13). They have low molecular weights (approximately 8-17 kDa) and were originally defined as potent attractants for leukocytes (13). Stromal-cell-derived factor-1 (SDF-1) is produced by stromal cells of mesenchymal origin, including fibroblasts, osteoblasts, and endothelial cells, and by haematopoietic stem/progenitor cells and endothelial stem/progenitor cells of the bone marrow (14-16). SDF-1 is a powerful chemoattractant for monocytes and T cells and regulates the recruitment of lymphocytes from the blood to the endothelium (17, 18). Two studies have demonstrated that SDF-1 expression is correlated with the invasion and metastasis of head and neck SCC (19-21). The AA genotype of the single nucleotide polymorphism (SNP) at position 801, 3'-untranslated region (3'-UTR), leads to higher SDF-1 secretion (22). The chemokine SDF-1 is the sole ligand of CXCR4, which is widely expressed in monocytes, neutrophils, lymphocytes, epithelial cells, and a majority of tumours, including those of epithelial, mesenchymal, and hematopoietic origin (23, 24).

Previous studies have revealed that the SDF-1/CXCR4 axis contributes to cell motility in OSCC and is well correlated with OSCC progression (25-28). An immunohistochemical study has demonstrated that all SCC tissues of the tongue express CXCR4, whereas no CXCR4 expression could be detected in almost any normal epithelial tissues of the tongue (29). Another study has revealed that the expression levels of CXCR4 significantly decreases during cerebellar development and is specifically upregulated in medulloblastomas of the desmoplastic/ nodular subtype (30). These results imply that

CXCR4 expression is essential for the development of the cerebellar cortex and in the pathogenesis of medulloblastomas. The same study has also identified two variants that may affect the binding of SDF-1 by changing codons 53 and 97, which are located in the transmembrane region (30). In addition, a silent sequence variant with a C/T substitution (+414 C > T; rs2228014) has been found in medulloblastomas, with no association between the +414 C>T variant and the subtype of medulloblastomas (30). Until now, numerous studies have reported that *SDF-1* rs1801157 and *CXCR4* rs2228014 are significantly associated with susceptibility to carcinogenesis in humans (28, 31-38). However, the association of *SDF-1* and *CXCR4* polymorphisms with the risk and the clinicopathological development of OPSCC remain inconclusive and controversial (39, 40). Teng *et al.*, have reported that patients with the A/G genotype of SDF-1 had a 1.86-fold higher risk of oral cancer than those with the G/G genotype (39). Vairaktaris *et al.*, have reported that the G/A genotype of SDF-1 had no significant impact on susceptibility to oral cancer, but patients with this genotype had a 0.33-fold lower risk of oral cancer in the advanced stage (40).

In Taiwan, approximately 2 million people have the habit of chewing BQ. Most BQ chewers are also cigarette smokers (86%) or alcohol drinkers (74%) (41). The effects of the interactions of the *SDF-1* and *CXCR4* genetic variants with the habits of BQ chewing, tobacco smoking, and alcohol consumption on the risk of OPSCC remain uncertain. Therefore, we investigated the relationship between *SDF-1/CXCR4* genetic variants and susceptibility to OPSCC. We also evaluated the effect of interactions between *SDF-1/CXCR4* genetic variants and substance use on OPSCC risk.

Materials and Methods

Participants. In total, 452 patients with primary OPSCC were recruited from the Department of ENT and Dentistry at the Kaohsiung Veterans General Hospital (KSVGH). These OPSCCs included 344 (76.1%) oral cavity, 72 (15.9%) oropharynx, and 36 (8.0%) hypopharynx cancers. In addition, 424 non-cancer controls were also recruited in this study. The detail process and information is described in our previous study (12). The study protocol was approved by the Institutional Review Board of KSVGH (VGHKS10-CT11-17). The methods were carried out in accordance with the approved guidelines and all patients provided informed consent.

Polymorphism genotyping. Genomic DNA of whole-blood samples was extracted and purified using the QIAamp® DNA extraction kit (Qiagen Sciences®, Germantown, MD, USA). Genotypes of *SDF-1* G>A (rs1801157) and *CXCR4* C>T (rs2228014) were detected using the TaqMan real-time polymerase chain reaction (PCR) method and were subsequently analysed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The genotyping assay was performed as previously described (12).

Statistical analysis. The Hardy–Weinberg equilibrium of *SDF-1* and *CXCR4* was evaluated in the controls by using a chi-squared test with 1 df. The crude odds ratios (ORs) and 95% confidence intervals (CIs) of *SDF-1* and *CXCR4* polymorphisms between the patients and controls were calculated through logistic regression. Following further adjustment of various confounders, the association of allele types and genotypes of the SNPs in *SDF-1* and *CXCR4* with the risk of OPSCC was evaluated using multiple logistic regression models. Because only one female participant was present in the pharyngeal SCC (PSCC) group, sex was not included in the multiple logistic regression models for analyzing the PSCC risk. The association between clinicopathological outcome and polymorphisms was estimated using the Kaplan–Meier method. The statistical analysis has been described in detail in our previous study (12). The statistical software packages SPSS (version 12.0, SPSS Inc., Chicago, IL, USA) and SAS/Genetics (version 9.1.3, SAS Institute, Inc., Cary, NC, USA) were used for all statistical analyses. Differences with $p < 0.05$ were considered significant.

Results

Demographic and substance-use features of the participants. This case–control study consisted of 452 patients with pathologically proved OPSCC [comprising 344 with oral cavity SCC (OSCC) and 108 with PSCC] and 424 sex- and age-matched cancer-free controls. The controls and three patient groups were well matched in terms of age and sex, but the PSCC group had a slightly higher proportion of male participants compared to the control group ($p = 0.086$) (Table I). As expected, BQ chewing, cigarette smoking, and alcohol drinking were associated with an increased risk of OSCC, PSCC, and OPSCC. The PSCC group contained a considerably greater proportion of heavy smokers and heavy drinkers compared to the control (Table I) and OSCC groups (heavy smoker *vs.* never smoker, $COR = 5.07$, $95\%CI = 2.12-12.15$, $p < 0.001$; heavy drinker *vs.* never drinker, $COR = 6.27$, $95\%CI = 3.15-12.49$, $p < 0.001$, data not shown). In addition, most substance users (73.80%) use more than one type of substance. The combined effect of the three types of substance use (namely BQ chewing, cigarette smoking, and alcohol drinking) on OPSCC risk was, therefore, evaluated in this study. Compared to those without any substance use (habit free), participants with two or more types of substance use had an increased risk of OSCC ($COR = 3.64-9.39$, $95\%CI = 2.28-14.98$), PSCC ($COR = 11.17-50.15$, $95\%CI = 3.26-165.09$), and OPSCC ($COR = 4.22-12.53$, $95\%CI = 2.69-19.66$) (Table I). However, participants using only one type of substance did not have an increased risk of various types of OPSCC. The consumption volume was significantly lower in participants using only one type of substance than in those using more than one type of substance ($p < 0.001$ for smoking and $p = 0.019$ for drinking), except for BQ chewing ($p = 0.57$). Only 16 participants had only BQ chewing habit, thus, the statistical power might be insufficient for the difference.

Association of genotype and allele type of *SDF-1* G>A (*rs1801157*) and *CXCR4* C>T (*rs2228014*) with OPSCC risk. The genotypic frequencies of *SDF-1* G>A and *CXCR4* C>T among the controls did not differ from the expected distributions based on the Hardy–Weinberg equilibrium ($p = 0.857$ and $p = 0.799$, respectively). The genotypic and allelic frequencies of *SDF-1* G>A and *CXCR4* C>T among the different patient groups and controls are presented in Table II. We found that the genotypic and allelic types of *SDF-1* G>A were not correlated with the risk of OSCC, PSCC, and OPSCC. However, compared to the participants with the C/T+T/T genotype or the T allele of *CXCR4* C>T, those with the C/C genotypes or the C allele exhibited an approximately 1.41- to 1.49-fold increased risk of OSCC [adjusted OR (AOR) = 1.49, $95\%CI = 1.01-2.20$, $p = 0.044$; AOR = 1.41, $95\%CI = 1.00-2.00$, $p = 0.049$; respectively] and OPSCC (AOR = 1.51, $95\%CI = 1.05-2.17$, $p = 0.028$; AOR = 1.41, $95\%CI = 1.02-1.96$, $p = 0.037$; respectively). A significant linear trend was also observed for the increasing risk of OSCC and OPSCC as the number of C alleles at the locus of *CXCR4* C>T increased (p for linear trend = 0.047 and 0.036, respectively).

Combined genotypes of *SDF-1* G>A and *CXCR4* C>T between OPSCC patients and controls. When analysing the genotypic combination of two SNPs between patients and controls (Table III), we regarded the G allele of *SDF-1* G>A and the C allele of *CXCR4* C>T as the risk alleles on the basis of the aforementioned preliminary results. The various genotypic combinations were categorised into three groups according to the number of risk alleles. Group I had a genotypic combination of none, one, and two risk alleles, such as *SDF-1-AA/CXCR4-CC*, *SDF-1-AG/CXCR4-CT*, *SDF-1-GG/CXCR4-TT*, *SDF-1-AA/CXCR4-CT*, *SDF-1-AG/CXCR4-TT*, and *SDF-1-AA/CXCR4-TT*. Group II had a genotypic combination of three risk alleles, such as *SDF-1-AG/CXCR4-CC* and *SDF-1-GG/CXCR4-CT*. The genotypic combination of *SDF-1-GG/CXCR4-CC* with four risk alleles was classified as group III. Participants carrying four risk alleles had an increased susceptibility to OSCC as compared to participants carrying none, one, or two risk alleles (AOR = 2.02, $95\%CI = 1.02-4.00$, $p = 0.044$). We also found that participants carrying more risk alleles in the combined genotypes of *SDF-1* and *CXCR4* SNPs had a higher risk of OSCC (p trend = 0.033). However, the combined genotypes for *SDF-1* and *CXCR4* SNPs were not associated with the risk of PSCC and OPSCC.

Interactions between three types of substance use and *CXCR4* C>T. We evaluated whether there was any interaction between the *CXCR4* +414 C>T and the three types of substance use, namely BQ chewing, cigarette smoking, and alcohol drinking (Figure 1). Compared to the assumed lowest risk category, namely the carriers of

Table 1. Distribution and odds ratios for OPSCC cases and cancer-free controls by selected demographic and lifestyle risk factors.

Factor/category	Controls (n=424)			Cases (n=452)			Cases vs. controls			
	Number (%)	OSCC (n=344)	OPSCC (n=452)	Number (%)	OSCC (n=108)	OPSCC (n=452)	COR (95%CI)	p-Value	COR (95% CI)	p-Value
		Number (%)	Number (%)		Number (%)	Number (%)				
Age mean±SD (yrs)	51.2±11.0	52.1±11.4	51.9±11.0	51.3±9.6	51.9±11.0	51.9±11.0	0.259 ^c	1.00	0.924 ^c	0.331 ^c
<40	52 (12.3)	41 (11.9)	55 (12.2)	14 (13.0)	55 (12.2)	55 (12.2)	1.00	1.00	1.00	1.00
40-49	135 (31.8)	109 (31.7)	143 (31.6)	34 (31.5)	143 (31.6)	143 (31.6)	1.02 (0.63-1.66)	0.923	0.94 (0.47-1.88)	0.852
50-59	153 (36.1)	118 (34.3)	160 (35.4)	42 (38.9)	160 (35.4)	160 (35.4)	0.98 (0.61-1.57)	0.927	1.02 (0.52-2.02)	0.955
≥60	84 (19.8)	76 (22.1)	94 (20.8)	18 (16.7)	94 (20.8)	94 (20.8)	1.15 (0.69-1.92)	0.600	0.80 (0.37-1.74)	0.566
Gender										
Female	22 (5.2)	26 (7.6)	27 (6.0)	1 (0.9)	27 (6.0)	27 (6.0)	1.00	0.180	1.00	0.086
Male	402 (94.8)	318 (92.4)	425 (94.0)	107 (99.1)	425 (94.0)	425 (94.0)	0.67 (0.37-1.20)	0.180	5.86 (0.78-43.94)	0.086
BQ chewing (pack ^a -years)										
Never-chewer	322 (75.9)	91 (26.5)	115 (25.4)	24 (22.2)	115 (25.4)	115 (25.4)	1.00	<0.001	1.00	<0.001
Light (0.21-13.80)	50 (11.8)	74 (21.5)	91 (20.1)	17 (15.7)	91 (20.1)	91 (20.1)	5.24 (3.42-8.03)	<0.001	4.56 (2.29-9.09)	<0.001
Heavy (>13.80)	52 (12.3)	179 (52.0)	246 (54.4)	67 (62.0)	246 (54.4)	246 (54.4)	12.18 (8.28-17.93)	<0.001	17.29 (9.97-29.98)	<0.001
Smoking (pack ^b -years)										
Never-smoker	162 (38.2)	69 (20.1)	75 (16.6)	6 (5.6)	75 (16.6)	75 (16.6)	1.00	0.019	1.00	0.003
Light (0.150-23.40)	131 (30.9)	89 (25.9)	109 (24.1)	20 (18.5)	109 (24.1)	109 (24.1)	1.60 (1.08-2.36)	0.019	4.12 (1.61-10.56)	0.003
Heavy (>23.40)	131 (30.9)	186 (54.1)	268 (59.3)	82 (75.9)	268 (59.3)	268 (59.3)	3.33 (2.33-4.78)	<0.001	16.90 (7.15-39.95)	<0.001
Drinking (gram-years)										
Never-drinker	270 (63.7)	124 (36.0)	135 (29.9)	11 (10.2)	135 (29.9)	135 (29.9)	1.00	<0.001	1.00	<0.001
Light (1.429-1053.0)	77 (18.2)	105 (30.5)	138 (30.5)	33 (30.6)	138 (30.5)	138 (30.5)	2.97 (2.07-4.27)	<0.001	10.52 (5.08-21.78)	<0.001
Heavy (>1053.0)	77 (18.2)	115 (33.4)	179 (39.6)	64 (59.3)	179 (39.6)	179 (39.6)	3.25 (2.27-4.65)	<0.001	20.40 (10.25-40.59)	<0.001
Combined substance use (SU)										
Never	127 (30.0)	36 (10.5)	39 (8.6)	3 (2.8)	39 (8.6)	39 (8.6)	1.00	0.922	1.00	0.813
Use one type of substance	141 (33.3)	41 (11.9)	45 (10.0)	4 (3.7)	45 (10.0)	45 (10.0)	1.03 (0.62-1.71)	0.922	1.20 (0.26-5.47)	0.813
Use two types of substance	91 (21.5)	94 (27.3)	118 (26.1)	24 (22.2)	118 (26.1)	118 (26.1)	3.64 (2.28-5.83)	<0.001	11.17 (3.26-38.20)	<0.001
Use three types of substance	65 (15.3)	173 (50.3)	250 (55.3)	77 (71.3)	250 (55.3)	250 (55.3)	9.39 (5.89-14.98)	<0.001	50.15 (15.23-165.09)	<0.001

OSCC: Squamous cell carcinoma of the oral cavity; PSCC: squamous cell carcinoma of the oropharynx and hypopharynx; OPSCC: squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx; COR: crude odds ratios; p-Value is estimated by logistic regression. ^aTwenty betel quids per pack; ^bTwenty cigarettes per pack. ^cp-Value is estimated by the t-test.

Table II. Distribution and adjusted odds ratios for OPSCC cases and cancer-free controls by various genotypic and allelic frequencies of *SDF-1* and *CXCR4*.

Chemokines SNPs	Genotype	Cases (n=452)			Cases vs. controls						
		Controls (n=424)		OPSCC (n=452)	OSCC vs. controls		PSCC vs. controls		OPSCC vs. controls		
		Number (%)	OSCC (n=344)		PSCC (n=108)	AOR ^a (95%CI)	p-Value	AOR ^b (95%CI)		p-Value	
SDF-1 (-801G>A) rs1801157	G/G	218 (51.4)	185 (53.8)	52 (48.1)	237 (52.4)	1.00	1.00	1.00	1.00	1.00	1.00
	G/A	171 (40.3)	133 (38.7)	43 (39.8)	176 (38.9)	0.90 (0.63-1.28)	0.549	1.12 (0.64-1.99)	0.687	0.89 (0.64-1.25)	0.511
	A/A	35 (8.3)	26 (7.6)	13 (12.0)	39 (8.6)	0.92 (0.49-1.73) <i>p</i> for linear trend =	0.791	1.55 (0.64-3.77) <i>p</i> for linear trend =	0.336	1.04 (0.58-1.85) <i>p</i> for linear trend =	0.899
3'-UTR	G allele	607 (71.6)	503 (73.1)	147 (68.1)	650 (71.9)	1.00	1.00	1.00	1.00	1.00	1.00
	A allele	241 (28.4)	185 (26.9)	69 (31.9)	254 (28.1)	0.93 (0.71-1.21)	0.593	1.21 (0.81-1.82)	0.353	0.97 (0.75-1.24)	0.785
	C/C	299 (70.5)	264 (76.7)	82 (75.9)	346 (76.5)	1.00	1.00	1.00	1.00	1.00	1.00
CXCR4 (+414C>T) rs2228014	C/T	115 (27.1)	76 (22.1)	23 (21.3)	99 (21.9)	0.68 (0.45-1.01)	0.054	0.61 (0.32-1.15)	0.125	0.66 (0.45-0.97)	0.032
	T/T	10 (2.4)	4 (1.2)	3 (2.8)	7 (1.5)	0.60 (0.16-2.24) <i>p</i> for linear trend =	0.444	1.71 (0.30-9.70) <i>p</i> for linear trend =	0.546	0.68 (0.21-2.21) <i>p</i> for linear trend =	0.519
	C/T + T/T	125 (29.5)	80 (23.3)	26 (24.1)	106 (23.4)	0.67 (0.46-0.989)	0.044	0.67 (0.36-1.22)	0.191	0.66 (0.46-0.96)	0.028
Ile>Ile	C allele	713 (84.1)	604 (87.8)	187 (86.6)	791 (87.5)	1.00	1.00	1.00	1.00	1.00	1.00
	T allele	135 (15.9)	84 (12.2)	29 (13.4)	113 (12.5)	0.71 (0.50-0.999)	0.049	0.77 (0.46-1.32)	0.343	0.71 (0.51-0.979)	0.037

OSCC: Squamous cell carcinoma of the oral cavity; PSCC: squamous cell carcinoma of the oropharynx and hypopharynx; OPSCC: squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx; ^aAOR: adjusted odds ratio; adjusted for sex (male vs. female), age (40-49, 50-59, ≥60 vs. <40), BQ chewing (0.21-13.80, ≥13.80 vs. never-chewing), cigarette smoking (0.15-23.40, ≥23.40 vs. never-smoking) and alcohol drinking (1.429-1053.0, ≥1053.0 vs. never-drinker); *p*-value is estimated by multiple logistic regression. ^bAOR: adjusted odds ratio; adjusted for age (40-49, 50-59, ≥60 vs. <40), BQ chewing (0.21-13.80, ≥13.80 vs. never-chewing), cigarette smoking (0.15-23.40, ≥23.40 vs. never-smoking) and alcohol drinking (1.429-1053.0, ≥1053.0 vs. never-drinker); *p*-value is estimated by multiple logistic regression.

C/T+T/T genotypes and without any substance use, the risk gradually increased for those carrying the C/C genotype and having a habit of one (AOR=1.79, $p=0.174$), two (AOR=7.68, $p<0.001$), or three (AOR=20.49, $p<0.001$) types of substance use. However, for those carrying C/T+T/T genotypes, the risk was increased only in participants with two (AOR=3.81, $p=0.005$) or three (AOR=17.68, $p<0.001$) types of substance use. Furthermore, compared to those carrying C/T+T/T genotypes, patients carrying the C/C genotype were at an increased risk only if they engaged in one (AOR=2.68, $p=0.036$) or two (AOR=2.02, $p=0.027$) types of substance use. Therefore, CXCR4 +414C>T did not have any effect on OPSCC risk either in participants without any substance use or in those with the three types of substance use.

Association of genotypes of SDF-1 G>A (rs1801157) and CXCR4 C>T (rs2228014) with the clinicopathological outcomes of OPSCC. We further examined the association of SDF-1 G>A (A/A+A/G vs. G/G) and CXCR4 C>T (T/T+C/T vs. C/C) with the clinicopathological characteristics of the OPSCC patients. Our results revealed that the genotypes of SDF-1 and CXCR4 did not significantly affect the clinicopathological features of the patients (Tables IV and V). Furthermore, a multivariate analysis showed that the G/G type of SDF-1 was not significantly correlated with the disease-specific survival (DSF) time of OSCC [A/A+A/G vs. G/G, adjusted hazard ratio (AHR)=0.79, 95%CI=0.51-1.23, $p=0.298$], PSCC (AHR=1.33, 95%CI=0.60-2.96, $p=0.484$), and OPSCC (AHR=0.97, 95%CI=0.66-1.42, $p=0.858$). In addition, no significant difference was also found between DSF and the genotype of CXCR4 C>T in OSCC (T/T+C/T vs. C/C, AHR=0.89, 95%CI=0.53-1.47, $p=0.642$), PSCC (AHR=1.05, 95%CI=0.42-2.66, $p=0.914$), and OPSCC (AHR=0.91, 95%CI=0.58-1.41, $p=0.660$), even in the combined genotypes of SDF-1 and CXCR4 variants (Table VI). No significant correlation was observed between SDF-1 and CXCR4 variants and prognostic outcomes of OPSCC. Based on the present study, CXCR4 C>T can be used as a genetic marker of susceptibility to OPSCC, which exerts a synergistic effect on OPSCC patients with the one or two types of risk habits.

Discussion

In this study, we found that the C allele and C/C genotypes of CXCR4 at locus +414 were significantly associated with OPSCC risk, particularly OSCC. However, the combined genotypic types of SDF-1/CXCR4 were not correlated with OPSCC risk, except for OSCC. In addition, the risk of OPSCC increased gradually in participants with a habit of two or more types of substance use compared to those without a habit of any substance use. Regarding the

Table III. Effect of combined genetic polymorphisms of SDF-1 and CXCR4 on OPSCC cases and cancer-free controls. We regard the G allele of SDF-1 -801G>A and the C allele of CXCR4 +414C>T as dangerous alleles.

Group	Combined genotypes of Ligand/receptor SNP		Controls (n=424)		Cases (n=452)		Cases vs. controls								
			OSCC (n=344)		PSCC (n=108)		OSCC vs. controls		PSCC vs. controls		OPSCC vs. controls				
			Number (%)	Number (%)	Number (%)	Number (%)	AOR ^a (95%CI)	p-Value	AOR ^b (95%CI)	p-Value	AOR ^a (95%CI)	p-Value			
I	SDF-1	CXCR4	AA	23 (5.4)	18 (5.2)	8 (7.4)									
			AG	45 (10.6)	28 (8.1)	12 (11.1)	40 (8.8)								
			GG	4 (0.9)	4 (1.2)	2 (1.9)	6 (1.3)	1.00	0.718	1.00	0.812	1.00	0.133	1.00	0.090
			AA	10 (2.4)	8 (2.3)	5 (4.6)	13 (2.9)								
			AG	4 (0.9)	0 (0)	1 (0.9)	1 (0.2)								
			AA	2 (0.5)	0 (0)	0 (0)	0 (0)								
II	AG	CC	122 (28.8)	105 (30.5)	30 (27.8)	135 (29.9)	1.31 (0.68-2.52)	0.427	0.46 (0.19-1.07)	0.072	1.02 (0.55-1.88)	0.953			
			60 (14.2)	40 (11.6)	6 (5.6)	46 (10.2)	2.02 (1.02-4.00)	0.044	0.85 (0.35-2.07)	0.718	1.64 (0.86-3.13)	0.133			
			154 (36.3)	141 (41.0)	44 (40.7)	185 (40.9)	<i>p</i> for linear trend= 0.033	<i>p</i> for linear trend= 0.812	<i>p</i> for linear trend= 0.090						

OSCC: Squamous cell carcinoma of the oral cavity; PSCC: squamous cell carcinoma of the oropharynx and hypopharynx; OPSCC: squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx. ^aAOR, adjusted odds ratio; adjusted for sex (male vs. female), age (40-49, 50-59, ≥60 vs. <40), BQ chewing (0.21-13.80, ≥13.80 vs. never-chewing), cigarette smoking (0.15-23.40, ≥23.40 vs. never-smoking) and alcohol drinking (1.429-1053.0, ≥1053.0 vs. never-drinker); *p*-value is estimated by multiple logistic regression. ^bAOR, adjusted odds ratio; adjusted for age (40-49, 50-59, ≥60 vs. <40), BQ chewing (0.21-13.80, ≥13.80 vs. never-chewing), cigarette smoking (0.15-23.40, ≥23.40 vs. never-smoking) and alcohol drinking (1.429-1053.0, ≥1053.0 vs. never-drinker); *p*-value is estimated by multiple logistic regression.

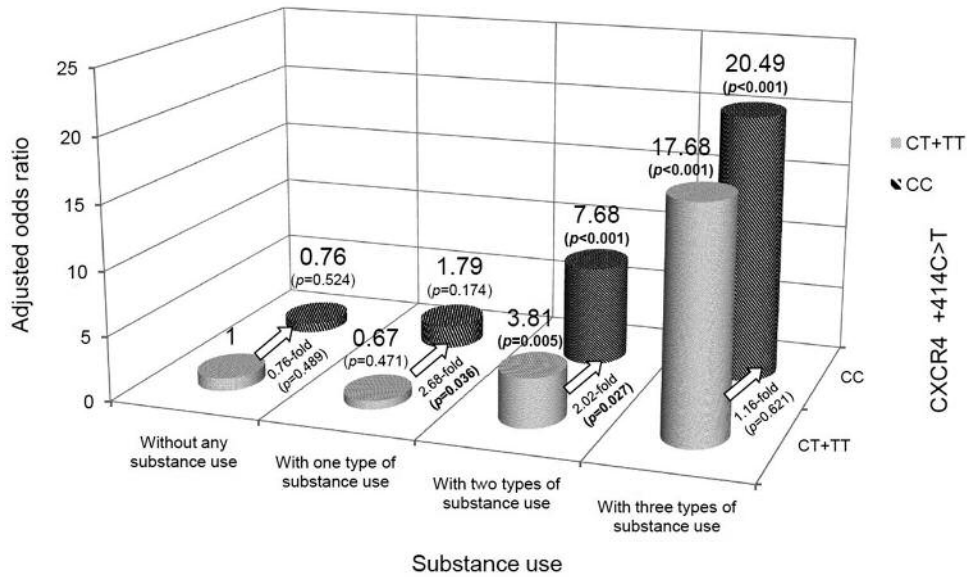


Figure 1. Association of SDF-1 and CXCR4 polymorphisms with susceptibility to OPSCC was evaluated in combination with different types of substance use. Patients with OPSCC were classified into four groups depending on their substance use (BQ chewing, smoking, and drinking). The effect of CXCR4 C>T (CT+TT and TT) on susceptibility to OPSCC among the groups was evaluated using a multiple logistic regression model.

Table IV. Relationship between SDF-1 G>A and clinicopathological parameters of OPSCC patients.

Variables	OPSCC (n=380)					
	Genotype (%)		COR (95%CI)		AOR (95%CI)	
	A/A + A/G	G/G	for A/A + A/G vs. G/G	p-Value ^a	for A/A + A/G vs. G/G	p-Value ^a
Cell differentiation						
Well	20 (39.2)	31 (60.8)	1.00		1.00	
Moderate+Poor	159 (48.3)	170 (51.7)	0.69 (0.38-1.26)	0.227	0.70 (0.38-1.28)	0.245 ^b
AJCC pathological stage						
I + II	99 (45.6)	118 (54.4)	1.00		1.00	
III+ IV	80 (49.1)	83 (50.9)	0.87 (0.58-1.31)	0.504	0.89 (0.59-1.33)	0.563 ^c
T classification						
T ₁ +T ₂	135 (48.2)	145 (51.8)	1.00		1.00	
T ₃ +T ₄	44 (44.0)	56 (56.0)	1.19 (0.75-1.88)	0.469	1.31 (0.81-2.13)	0.271 ^d
N classification						
N ₀	119 (44.4)	149 (55.6)	1.00		1.00	
N ₁ +N ₂ +N ₃	60 (53.6)	52 (46.4)	0.69 (0.45-1.08)	0.103	0.68 (0.42-1.10)	0.117 ^e
Recurrence (postoperative)						
No	122 (48.8)	128 (51.2)	1.00		1.00	
Yes	57 (43.8)	73 (56.2)	1.22 (0.80-1.87)	0.359	1.25 (0.81-1.92)	0.309 ^f
Metastasis (postoperative)						
No	167 (46.6)	191 (53.4)	1.00		1.00	
Yes	12 (54.5)	10 (45.5)	0.73 (0.31-1.73)	0.473	0.79 (0.33-1.88)	0.588 ^f

OPSCC: Squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx; AJCC: American Joint Committee on Cancer; COR: crude odds ratio; AOR: adjusted odds ratio. ^ap-Value was estimated by Logistic regression. ^bLogistic regression model adjusted for AJCC pathological stage (stage III, stage IV vs. stage I, stage II). ^cLogistic regression model adjusted for cell differentiation (well vs. moderate + poor). ^dLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), N classification (N₁+N₂+N₃ vs. N₀). ^eLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), T classification (T₃+T₄ vs. T₁+T₂). ^fLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), AJCC pathological stage (stage III+stage IV vs. stage I + stage II).

Table V. Relationship between CXCR4 C>T and clinicopathological parameters of OPSCC patients.

Variables	OPSCC (n=380)					
	Genotype (%)		COR (95%CI)		AOR (95%CI)	
	T/T+C/T	C/C	for T/T + C/T vs. C/C	p-Value ^a	for T/T + C/T vs. C/C	p-Value ^a
Cell differentiation						
Well	9 (17.6)	42 (82.4)	1.00		1.00	
Moderate+ Poor	81 (24.6)	248 (75.4)	0.66 (0.31-1.41)	0.279	0.65 (0.30-1.40)	0.273 ^b
AJCC pathological stage						
I+II	52 (24.0)	165 (76.0)	1.00		1.00	
III+IV	38 (23.3)	125 (76.7)	1.04 (0.64-1.67)	0.883	1.06 (0.66-1.71)	0.817 ^c
T classification						
T ₁ +T ₂	63 (22.5)	217 (77.5)	1.00		1.00	
T ₃ +T ₄	27 (27.0)	73 (73.0)	0.79 (0.47-1.32)	0.364	0.71 (0.41-1.23)	0.221 ^d
N classification						
N0	65 (24.3)	203 (75.7)	1.00		1.00	
N ₁ +N ₂ +N ₃	25 (22.3)	87 (77.7)	1.11 (0.66-1.88)	0.686	1.31 (0.74-2.29)	0.354 ^e
Recurrence (postoperative)						
No	62 (24.8)	188 (75.2)	1.00		1.00	
Yes	28 (21.5)	102 (78.5)	1.20 (0.72-2.00)	0.478	1.22 (0.73-2.02)	0.447 ^f
Metastasis (postoperative)						
No	83 (23.2)	275 (76.8)	1.00		1.00	
Yes	7 (31.8)	15 (68.2)	0.65 (0.26-1.64)	0.358	0.65 (0.25-1.66)	0.365 ^f

OPSCC: Squamous cell carcinoma of the oral cavity, oropharynx and hypopharynx; AJCC: American Joint Committee on Cancer; COR: crude odds ratio; AOR: adjusted odds ratio. ^ap-Value was estimated by Logistic regression. ^bLogistic regression model adjusted for AJCC pathological stage (stage III, stage IV vs. stage I, stage II). ^cLogistic regression model adjusted for cell differentiation (well vs. moderate + poor). ^dLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), N classification (N₁+N₂+N₃ vs. N0). ^eLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), T classification (T₃+T₄ vs. T₁+T₂). ^fLogistic regression model adjusted for cell differentiation (moderate + poor vs. well), AJCC pathological stage (stage III+stage IV vs. stage I + stage II).

combined effect of CXCR4 C>T and substance use, we found that the effect of CXCR4 C>T on OPSCC risk increased only when participants had the habit of one or two types of substance use. To the best of our knowledge, the combined effect of CXCR4 C>T and three types of substance use on the susceptibility to OPSCC has not been previously reported.

In the present study we did not observe an association between SDF-1 G>A and OPSCC risk, including OSCC and PSCC. A study conducted in central Taiwan by Teng *et al.*, has showed that the heterozygous genotype of G/A at SDF-1 G>A is associated with an increased risk of oral cancer, but the A/A genotype at SDF-1 G>A did not have any effect on oral cancer risk (39). Analysis based on chi-square test revealed that the genotypic frequencies of the controls are significantly different between the study by Teng *et al.*, and our study. In the study by Teng *et al.*, the frequencies of the G/G, A/G, and A/A genotypes at SDF-1 G>A were 58.5%, 28.5%, and 13.0%, respectively, whereas in our study, they were 51.4%, 40.3%, and 8.3%, respectively ($\chi^2=12.03$, $p=0.002$). However, our genotypic frequencies of the control are consistent with those of Lee *et al.*, in Taiwan (the

genotypic frequencies were 52.1%, 41.5%, and 6.4%; $X^2=0.93$, $p=0.629$) (42). SDF-1 G>A in the 3'-UTR, whose allele A is regarded a target of cis-acting factors, has been shown to have the ability to up-regulate SDF-1 expression in prostate cancer (22). By contrast, we observed no correlation between the serum expression levels of SDF-1 and its genotype, which was consistent with the finding of Viswanathan *et al.* (43). These contradictory results might be attributed to the different cancer types and sample sources. Viswanathan *et al.*, and us have both examined circulating SDF-1 in the serum through ELISA, but Hirata *et al.*, have analyzed the expression levels of SDF-1 in prostate cancer tissue immunohistochemically (22). These findings must be confirmed by additional experiments in the future.

A silent SNP of CXCR4 (CXCR4 +414C>T) has been revealed to be located at the locus of +41434. Teng *et al.*, have reported that no significant association existed between CXCR4 C>T and oral cancer risk (39). However, the results from our study (in which the number of participants is >2-fold greater compared to the study of Teng *et al.*) show that the C allele and C/C genotypes of CXCR4 C>T are associated with an increased risk of OPSCC and OSCC. A

Table VI. Association between the SDF-1/CXCR4 genetic variation and the survival of patients with OPSCC.

Variables	OSCC (n=305)				PSCC (n=75)				OPSCC (n=380)			
	N (%)	5 year disease-specific survival rate %	AHR (95%CI)	p-Value ^a	N (%)	5 year disease-specific survival rate %	AHR (95%CI)	p-Value ^b	N (%)	5 year disease-specific survival rate %	AHR (95%CI)	p-Value ^a
SDF-1 (G>A)												
A/A+A/G	142 (46.6)	65.5%	1.00		37 (49.3)	56.8%	1.00		179 (47.1)	63.6%	1.00	
G/G	163 (53.4)	68.6%	0.79	0.298	38 (50.7)	51.8%	1.33	0.484	201 (52.9)	65.8%	0.97	0.858
			(0.51-1.23)				(0.60-2.96)				(0.66-1.42)	
CXCR4 (C>T)												
T/T+C/T	71 (23.3)	62.0%	1.00		19 (25.3)	51.1%	1.00		90 (23.7)	60.2%	1.00	
C/C	234 (76.7)	68.4%	0.89	0.642	56 (74.7)	56.0%	1.05	0.914	290 (76.3)	66.0%	0.91	0.660
			(0.53-1.47)				(0.42-2.66)				(0.58-1.41)	

^ap-values were adjusted cell differentiation (moderate + poor vs. well), AJCC pathological stage (stage III, stage IV vs. stage I, stage II), smoking (smoking vs. never-smoking), BQ chewing (chewing vs. never-chewing), and drinking (drinking vs. never-drinking) by multiple Cox's regression.

^bp-values were adjusted for AJCC pathological stage (stage III, stage IV vs. stage I, stage II), smoking, BQ chewing, drinking by multiple Cox's regression.

comparison of genotypic frequencies at CXCR4 C>T among the controls revealed no differences between our results and those of Teng *et al.*, and Chang *et al.*, (39, 44) (the frequencies of the C/C, C/T, and T/T genotypes at CXCR4 +414C>T were 71.5%, 27.1%, and 1.4%, respectively, in Teng *et al.*; 70.5%, 27.1%, and 2.4%, respectively, in our study; and 72.0%, 26.4%, and 1.6% in Chang *et al.*). Regarding the genotypic frequencies at CXCR4 C>T among patients, no differences were observed between the results of Teng *et al.*, and our results. The binding of SDF-1 to CXCR4 induces intracellular signalling through several distinct pathways, such as chemotaxis, inflammation, and proliferation (23, 45). CXCR4 C>T has been found to affect codon 97 with a substitution of negatively charged aspartic acid (D) with neutral asparagine (N). This mutant codon is located in the second transmembrane region, a part of the receptor, which is relatively close to the cell surface and possibly important for SDF-1 binding (30). Brelot *et al.*, have demonstrated that substitutions of acidic residues in the membrane-spanning domain (D97N) affects SDF-1 binding and impairs or stops receptor activation (46). Therefore, we speculated that CXCR4 C>T might, at least in part, lead to a decrease in the trend of SDF-1/CXCR4 signalling. Many cancers arise from sites of chronic irritation and inflammation (47). As mentioned, long-term exposure to BQ, cigarette, or alcohol may cause injury to oral-cavity cells. The result attracts diverse leukocyte-expressing CXCR4 populations – for example, neutrophils, macrophages, and lymphocytes – all of which are capable of producing an assorted array of cytokines, cytotoxic mediators, including

reactive oxygen species, serine, and cysteine proteases (*i.e.*, MMPs), and soluble mediators of cell killing (*i.e.*, TNF-alpha, ILs, and interferons) (47, 48). It is now becoming clear that the tumour microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant of the neoplastic process, fostering proliferation and migration (47). Therefore, CXCR4 on leukocyte population cells plays a crucial role in a developing neoplasm.

Risk habits, such as tobacco use, alcohol consumption, and BQ chewing have been identified as major risk factors for head and neck cancer (4). As described in the previous paragraph, these risk factors exert profound effects on multiple components of the inflammatory system. Chemokines accumulate in the microenvironment of tissues affected by chronic inflammation. If persistent, these inflammatory factors have the capacity to induce cell proliferation and to promote prolonged cell survival through the activation of oncogenes and inactivation of tumour-suppressor genes (49). We speculated that the C/C genotype of CXCR4 C>T might increase tumour development through the modulation of inflammatory cell aggregation and the suspension of cell proliferation. Therefore, we further characterised the combined effect of BQ cigarette, alcohol, and CXCR4 C>T. Our data indicate that compared to the assumed lowest risk category, such as the carriers of C/T+T/T genotypes without the habit of any type of substance use, the risk was gradually increased for those carrying the C/C genotype and with the habit of two or three types of substance use, placing the last ones in the higher

risk category. The results were in accordance with our expectations. Compared to the C/T+T/T genotypes, the C/C genotype showed a 2.68-fold and 2.02-fold risk of OPSCC among participants with the habit of one or two types of substance use, respectively. Accordingly, the role of *CXCR4* C>T cannot be ignored, particularly in individuals with one or two types of substance use.

In conclusion, our study suggests that *SDF-1* G>A and *CXCR4* C>T polymorphism plays a role in the development of OSCC and OPSCC. However, the combination of *SDF-1* G>A and *CXCR4* C>T did not correlate with OPSCC risk, except for OSCC. Moreover, OPSCC risk was gradually increased in participants with a habit of using two or more of the aforementioned substances as compared to those without a habit of using any of these substances. With regards to the combined effect of *CXCR4* C>T and substance use, the effect of *CXCR4* C>T on OPSCC risk increased only when participants had the habit of using one or two of the aforementioned substances.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

SJH, YKT and YHL conducted this study and drafted the manuscript. SJH performed the genotype analysis. PCW, JHL, CCL, CMY, CCW, LMY, YCL and MHL assisted in clinical samples' collection and manuscript drafting. LPG and HHL performed the statistical analysis. LPG and KWT supervised the study and edited the manuscript.

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