A clinical study on the factors associated with nasopharyngeal carcinoma among the Chinese population

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Abstract. Nasopharyngeal carcinoma (NC) arises from the nasopharynx epithelium and the majority of NC cases globally are within China and Southeast Asia. Both short palate lung and nasal epithelium clone 1 (SPLUNC1) and myelodysplasia syndrome 1-ectopic viral integration site 1 (MDS1-EVI1) play an important role in carcinogenesis and have been found to be associated with nasopharyngeal carcinoma. In spite of their role in NC, the association between these genes and their polymorphisms in the development of NC has thus far not been studied. In the present study, the relationship between SPLUNC1 (rs2752903, T>C) and MDS1-EVI1 (rs6774494, G>A) polymorphisms and their role in the development of NC among the Chinese population were investigated. From a Chinese population of 1,059 patients with NC and 891 controls, genotype frequencies and the distribution of SPLUNC1 and MDS1-EVI1 polymorphisms were analyzed for possible susceptibility to NC. It was observed that those with MDS1-EVI1 CC (OR, 2.76; 95% CI, 1.96-3.81) and MDS1-EVI1 CT (OR, 1.51; 95% CI, 1.22-2.14) polymorphisms had an increased risk of developing NC. Those with SPLUNC1 AA genotypes also observed a higher risk for NC compared with SPLUNC1 GG genotypes (OR, 2.15; 95% CI, 1.62-3.15). When observing the gene-gene interaction between SPLUNC1 and MDS1-EVI1 polymorphisms, it was found that the presence of both SPLUNC1 CC and MDS1-EVI1 AA alleles was associated with a higher risk for NC compared with those who did not carry both alleles (OR, 6.75; 95% CI, 3.41-12.11). The present study suggested that the association between SPLUNC1 (rs2752903, T>C) and MDS1-EVI1 (rs6774494, G>A) polymorphisms may be a potent risk factor in the occurrence of NC.

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Introduction

Nasopharyngeal carcinoma (NC) is a type of cancer that arises from the nasopharynx epithelium. NC is an uncommon type of cancer that has a variable geographical distribution (1). As of 2012, there were >86,500 cases of NC globally, accounting for 0.6% of all diagnosed cancers (2,3). Notably, >70% of new cases were diagnosed in East and Southeast Asia (4). Furthermore, incidences of NC cases are also common among the population of Southern China and other Chinese populations in Hong Kong, Singapore and Taiwan, which may be due to polygenic events and genetic factors (5). Therefore, identification of the genes causing susceptibility to NC may aid in the identification of individual communities and populations that have an increased risk of developing NC. This could lead to further understanding of the pathogenesis and malignancy of NC (6,7). Previous linkage analysis has revealed several chromosomal regions that contain various genes susceptible to NC (8). Studies from China have reported the susceptibility of chromosome regions 6p22, 4p15.1-q12 and 3p21.31-21.2, and their involvement in human leukocyte antigens and pathogenesis in NC (9,10). Despite advances in cancer research, the genomic alleles that are responsible for cancer susceptibility are not fully understood (11). In the present study, it was investigated whether functional polymorphisms in short palate lung and nasal epithelium clone 1 (SPLUNC1) and its interaction with myelodysplasia syndrome 1 (MDS1) may have an influence on the risk or severity of nasopharyngeal carcinoma among the Chinese population. The SPLUNC1 gene encodes a secreted protein that is present at the surface of the nasopharyngeal epithelium, which acts as an innate immunity defensive molecule and has previously been identified to be a potent risk factor for NC (12,13). SPLUNC1 also acts as a potent tumor suppressor gene, which increases the susceptibility to NC, however, its underlying mechanism is not fully understood (14). MDS1-ectopic viral integration site 1 (EVI1) is encoded by the MDS1 and EVI1 Complex Locus (MECOM) gene and is comprised of three different proteins: EVI1, MDS1 and MDS1-EVI1 (15). EVI1 is a transcription factor that has the ability to suppress TGF-β, thereby promoting tumor growth (16), and the imbalance of EVI1 and MDS1-EVI1 proteins is considered to be an important factor for the pathogenesis of NC (17). Indeed, to the best of our knowledge, the gene-to-gene interaction between SPLUNC1 and MDS1-EVI1 polymorphisms has not been previously studied. The significant

gene-to-gene interaction impact on other types of cancer may affect the function of SPLUNC1 and MDS1-EVI1, which may play a crucial role in the development of NC. Moreover, these polymorphisms may cause individual vulnerability towards carcinogenesis. The aim of the present study was to investigate the relationship between polymorphisms in SPLUNC1 (rs2752903, T>C) and MDS1-EVI1 (rs6774494, G>A), and the risk of developing NC among the Chinese population.

Materials and methods

Ethical approval. All procedures were carried out in accordance with the Declaration of Helsinki 1964 and its later amendments. Written informed consent was provided by all patients and participants. Consent was obtained from each subject regarding their personal information on demographic factors, any history of medical information, tobacco smoking and alcohol consumption using a standard questionnaire. All protocols and procedures were approved by the Hospital Ethical Research Committee of the Jining Medical University (Jining, Shandong, China; approval no. JMU/AH/OncDept/2017-34GA2).

Study participants. The study population consisted of 1,059 patients (mean age, 42.3±0.26 years; age range, 31-72 years) with NC enrolled at the Affiliated Hospital of Jining Medical University (Jining, Shandong, China). All patients were of Han Chinese ethnicity and residents in Jining and the surrounding regions. Patients were newly diagnosed with NC, which was confirmed by pathological examination between July 2014 and June 2019, with a response rate of 94%, where 6% of the patients were either shifted to other hospital or left the city and were lost during follow-up. Patients treated with chemotherapy and radiotherapy with other types of cancer prior to surgery were not included in the study. Cancer staging was carried out based on the Tumor, Node, Metastasis (TNM) staging system, according to the American Joint Committee on Cancer system (AJCC) Cancer Staging Manual, 2009. The staging was determined by one senior pathologist and one doctor of the hospital based on pathological examination of the tissues (18). A total of 891 control participants (mean age, 44.1±0.17 years; age range, 30-65 years) were randomly selected from the same region within the Jining area from a cancer screening community program carried out during the same period, with a response rate of 90%, where and 10% of the patients moved out of the city and were lost during the follow-up. Control participants were selected based on those who had no episodes of any type of cancer, regardless of the age or sex of the participants. Furthermore, the association between a functional polymorphism in SPLUNC1 and its interaction with MDS1, which may influence NC susceptibility, were examined based on age, sex, smoking and alcohol consumption status and TNM classification.

Polymorphism genotyping. In total, ~5 ml peripheral blood were drawn into a tube from the study participants. Genomic DNA was then extracted using the TIANamp Blood DNA kit (cat. no. DP318; Tiangen Biotech Co., Ltd.) by following the manufacturer's protocol, and the genotypes examined following a PCR-based protocol. Initially, the genotyping was carried out without knowing the status of the case subjects and the control subjects. The genotypes of SPLUNC1 (rs2752903, T>C) were analyzed using the PCR-restriction fragment length polymorphism method, according to the protocol by Ara *et al* (19). The forward primer, 5'-TTGCCG TCCCAAGCAATGGATGA-3' and reverse primer, 5'-TCT GGGAAGGGACAGAAGATGAC-3', were used to amplify and sequence the target region containing the T/C site (Qiagen, Inc.). PCR amplification was carried out by mixing the reaction mixture, which was comprised of the template DNA, 0.5 μM both the forward and reverse primers. PCR amplification was carried out with a 25 μl master mix that contained ~120 ng template DNA, both forward and reverse primers (~0.5 μM), dNTP (0.4 mM), $MgCl_2$ (1.7 mM) and 2U Taq polymerase (Promega Corporation). The PCR thermocycling conditions were as follows: Denaturation at 92˚C for 3 min; 32 cycles of annealing at 93˚C for 35 sec, 55˚C for 25 sec and 72˚C for 25 sec; and a final elongation step at 72˚C for 5 min. MDS1-EVI1 (rs6774494, G>A), genotypes were analyzed using amplification-refractory mutation system-PCR, which is an economical method for genotyping single-nucleotide polymorphism. The amplification was carried out using the following primers: MDS1-EVI1 forward, 5'-TTGAGGCCCGTTTAGATACCA-3' and reverse, 5'-CTTTCCTTGGAGCAATGTAGTT-3' for the MDS1-EVI1 G-allele, and forward, 5'-ACTATCAGGACA CGCC-3' and reverse, 5'-TAACCACCGAATGGTG-3' for the MDS1-EVI1 A-allele (Qiagen, Inc.). PCR amplification was carried out by mixing a 15 μl reaction mixture that contained ~20 ng template DNA, 0.5 μM of both the forward and reverse primers, 0.3 mM dNTP, 1.6 mM $MgCl_2$ and 3U Taq polymerase supplemented with Q-solution (Qiagen, Inc.). The PCR thermocycling conditions were as follows: Denaturation at 95˚C for 10 min; 32 cycles of annealing at 93˚C for 32 sec, 57˚C for 25 sec and 72˚C for 30 sec; and a final elongation step at 72˚C for 8 min.

Statistical analysis. The genotype frequencies and the distribution between the patient and control groups were compared by departures from Hardy-Weinberg equilibrium by gene counting and tested using a two-sided χ^2 test (20). The parameters which constitute the departure from Hardy-Weinberg include allele frequency and number of genotypes. Smokers were defined as participants who smoked up to 1 year before the date of a cancer diagnosis for patients, or the date of interview for controls. Alcohol drinkers were defined as participants who drank alcohol ≥ once per week for >6 months. Unconditional logistic regression analysis was used for calculating the odds ratio (OR) and 95% CI to estimate the association between the SPLUNC1 and MDS1-EVI1 polymorphisms and the risk of developing NC. Gene-gene interaction analysis was tested for its null hypotheses from the multiplicative joint effect models based on the logistic regression model. χ^2 test was used to estimate the correlation between the genotypes and their clinical parameters. P-values, ORs and 95% CIs were calculated and adjusted for sex, age, alcohol and tobacco use where appropriate. All statistical tests were two-sided and $P<0.05$ was used as the criterion of statistical significance. All analyses were performed using SPSS version 21 (IBM Corp.).

Table I. Characteristics of patients with NC (n=1,059) and controls (n=891) in the Chinese population.

Characteristics	Patients with NC, n (%)	Controls, n (%)	χ^{2a}	P-value
Age, years			6.01	0.014
>40	483 (45.61)	456 (51.18)		
≤40	576 (54.39)	435 (48.82)		
Sex			4.075	0.43
Male	662 (62.51)	517 (58.02)		
Female	397 (37.49)	374 (41.98)		
Smoker	359 (33.90)	288 (32.32)	0.54	0.46
Alcohol drinker	463 (43.72)	273 (30.64)	35.23	<0.00001
Clinical stage				
I	249 (23.51)			
II	419 (39.57)			
III	359 (33.90			
IV	32 (3.02)			
Local tumor invasion (T-classification)				
T1	186 (17.56)			
T2	539 (50.90)			
T3	229 (21.62)			
T4	105 (9.92)			
Lymph node involvement (N-classification)				
N0	239 (22.57)			
N1	512 (48.35)			
N2	221 (20.87)			
N3	87 (8.22)			
Presence of metastasis				
Yes	480 (54.67)			
No	579 (45.33)			

[a]χ^2-Chi squared test; P-value was obtained by χ^2 test. NC, nasopharyngeal carcinoma.

Table II. Characterization of the study population (n=1,950) based on history of tobacco and alcohol consumption in patients with NC (n=1,059) and controls (n=891).

Serial no.	Characteristics	Population, n
1	Patients with NC with no history of tobacco or alcohol use	237
2	Patients with NC with history of tobacco or alcohol use	822
3	Controls with no history of tobacco or alcohol use	330
4	Controls with history of tobacco or alcohol use	561

NC, nasopharyngeal carcinoma.

Results

In the present study, the effect of SPLUNC1 CC and MDS1-EVI1 AA genotypes on the susceptibility of NC in the investigated province, comprised of 1,059 patients and 891 controls, was investigated. As demonstrated in Table I, the NC and controls groups were comparable in terms of the sex composition (P=0.43), they differed significantly in comparing both age groups (P=0.014). The younger age group (>40) has a lower number of NC patients compared with the control (45.61 vs. 51.18%), where whereas the older age group (≤40) has a higher number of NC patients compared with the control (54.39 vs. 48.82%). The number of patients with NC was almost comparable to the controls with respect to those cases who have a history of smoking. However, for patients with NC, there was a higher number of alcohol drinkers compared with the controls (43.72 vs. 30.64%). It was also observed that 23.51, 39.57, 33.90 and 3.02% patients with NC were classified as TNM stage I, II, III and IV, respectively. Table II presents the details of the investigated population based on history of

Table III. Genotype frequencies of SPLUNC1 and MDS1-EVI1 among patients with NC (n=1,059) and controls (n=891) and their association with the risk of developing NC.

Genotypes	Patients with NC, n (%)	Controls, n (%)	OR (95% CI)	χ^2	P-value
SPLUNC1 (rs2752903, T>C)				39.884	<0.00001
TT (Reference)	234 (22.10)	298 (33.45)	1.0		
TT Smoker (Reference)	75 (7.08)	59 (6.62)			
TT Alcohol drinker (Reference)	103 (9.73)	74 (8.31)			
CT	552 (52.12)	428 (48.04)	1.51 (1.22-2.14)		0.014
CC	269 (25.78)	165 (18.52)	2.76 (1.96-3.81)		0.046
CT + CC	821 (77.9)	593 (66.56)	1.43 (1.04-1.91)		0.021
CT + CC Smoker	284 (26.82)	229 (25.70)			
CT + CC Alcohol drinker	360 (33.99)	199 (22.33)			
MDS1-EVI1 (rs6774494, G>A)				50.24	<0.00001
GG (Reference)	224 (21.15)	266 (29.85)	1.0		
GG Smoker (Reference)	69 (6.52)	56 (6.29)			
GG Alcohol drinker (Reference)	91 (8.59)	71 (7.97)			
AG	514 (48.54)	467 (52.41)	1.37 (1.19-2.06)		0.21
AA	321 (30.31)	158 (17.73)	2.15 (1.62-3.15)		0.018
AG + AA	835 (78.85)	625 (70.14)	1.53 (1.00-2.36)		0.032
AG + AA Smoker	290 (27.38)	232 (26.04)			
AG + AA Alcohol drinker	372 (35.13)	202 (22.67)			

NC, nasopharyngeal carcinoma; OR, odds ratio; SPLUNC1, short palate lung and nasal epithelium clone 1; MDS1-EVI1, myelodysplasia syndrome 1-ectopic viral integration site 1.

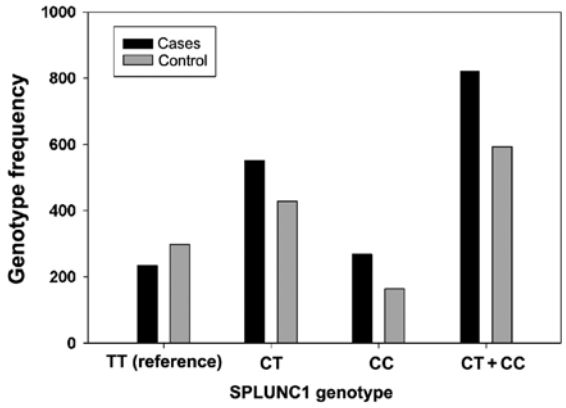

Figure 1. Bar graph representing the association between SPLUNC1 polymorphisms and nasopharyngeal carcinoma for TT (Reference), CT, CC and CT + CC genotypes. TT genotype denotes the reference group. SPLUNC1, short palate lung and nasal epithelium clone 1.

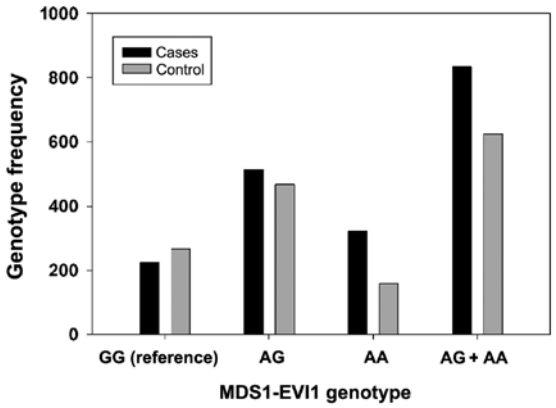

Figure 2. Bar graph representing the association between MDS1-EVI1 polymorphisms and nasopharyngeal carcinoma for GG (Reference), AG, AA and AG + AA genotypes. GG genotype denotes the reference group. MDS1-EVI1, myelodysplasia syndrome 1-ectopic viral integration site 1.

tobacco and alcohol consumption, including both controls and patients (n=1,950). Table III shows the genotyping results and frequencies of SPLUNC1 and MDS1-EVI1 polymorphisms in patients with NC and controls. In both cases, the genotypic frequency does not deviate from the expected Hardy-Weinberg equilibrium. Furthermore, the distributions of the genotypes that were compared with the NC cases and control are presented in Table III and graphically presented in Figs. 1 and 2. It was observed that the SPLUNC1 CT + CC genotypes may be a genetic risk factor for NC (Fig. 1). By contrast, it was observed that the MDS1-EVI1 AG + AA genotypes may be a potential risk factor for NC among the studied population (Fig. 2). The frequencies of the SPLUNC1 TT, CT and CC genotypes in patients with NC were 22.10, 52.12 and 25.78% respectively, which was significantly different compared with the controls (Table III). This difference was particularly observed in the CC genotype (25.78 patients vs. 18.52% controls). The CC homozygote was also the most dominant genotype. Based on logistic regression analysis with MDS1-EVI1, the AA allele was observed to have a more significant risk for NC compared with patients carrying the AG allele (AA allele; OR, 2.15; 95% CI, 1.62-3.15). Thus,

Table IV. Risk of NC associated with SPLUNC1 and MDS1-EVI1 genotypes in patients with NC (n=1,059) and controls (n=891).

Genotype					
SPLUNC1	MDS1-EVI1	Patients with NC, n (%)	Controls, n (%)	OR (95% CI)	P-value
TT	GG	51 (4.82)	79 (8.87)	1.00	
	AG	135 (12.75)	163 (18.29)	1.28 (0.87-2.51)	0.072
	AA	47 (4.44)	54 (6.06)	1.59 (0.93-3.16)	0.063
CT	GG (Reference)	109 (10.29)	148 (16.61)	1.24 (0.83-2.42)	0.002
	AG	254 (23.98)	208 (23.34)	2.24 (1.87-3.43)	0.059
	AA	117 (11.05)	81 (9.09)	2.81 (1.42-5.27)	0.017
CC	GG (Reference)	85 (8.03)	47 (5.27)	2.76 (1.28-4.81)	0.0078
	AG	152 (14.35)	85 (9.54)	2.97 (1.54-5.69)	0.087
	AA	109 (10.29)	26 (2.92)	6. 75 (3.41-12.11)	0.002

NC, nasopharyngeal carcinoma; OR, odds ratio; SPLUNC1, short palate lung and nasal epithelium clone 1; MDS1-EVI1, myelodysplasia syndrome 1-ectopic viral integration site 1.

indicating that the MDS1-EVI1 AA allele is the higher-risk allele. The present study also observed no direct relationship between the age group, tumor invasion stage and lymph node involvement at the time of diagnosis and polymorphisms of these genes. Moreover, the logistic regression analysis also showed that subjects with SPLUNC1 CC genotypes had a significantly higher susceptibility to NC compared with the TT genotype (OR, 2.76; 95% CI, 1.96-3.81) when adjusted for sex, age, smoking and drinking status. However, in the present analyses, there was no correlation between sex, tobacco smoking and alcohol consumption on the risk of lymph node related to the CT + CC genotype. Furthermore, the gene-gene interaction between SPLUNC1 and MDS1-EVI1 polymorphisms was analyzed (Table IV). This analysis found that patients with NC that have the SPLUNC1 CC genotype were more likely to have the MDS1-EVI1 AA genotype compared with the controls (10.29 vs. 2.92%; P<0.002). Moreover, the SPLUNC1 CC genotype alone was also associated with an increased risk in developing NC (OR, 2.76; 95% CI, 1.28-4.81; P<0.0078) when compared between patients with NC and control individuals. In the case of control subjects, the MDS1-EVI1 AA genotype did not show a higher risk compared with the SPLUNC1 CC genotype (OR, 6.75; 95% CI, 3.41-12.11; P<0.002; Table IV). However, the presence of both SPLUNC1 CC and MDS1-EVI1 AA genotypes was associated with a markedly higher risk for NC compared with others whom do not carry both the genotypes (OR, 6.75; 95% CI, 3.41-12.11). Overall, this analysis suggested that there was a multiplicative interaction between the genotypes of SPLUNC1-CC and MDS1-EVI1-AA, which was associated with an increased risk of developing nasopharyngeal carcinoma.

Discussion

In the present investigation, it was observed that SPLUNC1 and MDS1-EVI1 polymorphisms may trigger the development of nasopharyngeal carcinoma among the Chinese population. By examining 1,059 patients with NC and 891 controls, the study showed that SPLUNC1 CC genotype increases the risk for the development of NC. Whereas MDS1-EVI1 AA genotypes were observed to be associated with an increased risk of developing NC. Moreover, the gene-gene interaction between SPLUNC1 and MDS1-EVI1 polymorphisms increased the risk for NC in an additive manner. This suggests that the case subjects carrying both of these genotypes are associated with higher risks for the occurrence of NC. The results of the present study implied the risk between the association of SPLUNC1 and MDS1-EVI1 polymorphisms and NC. This increased risk may be due to the importance of these genes in providing genomic integrity and suppressing cancerous cells (21,22).

Several genes have been previously reported to be associated with nasopharyngeal carcinoma, such as cytochrome P450 2E1, arylamine N-acetyltransferase 2, SPLUNC1, DNA repair protein XRCC1, DNA repair protein RAD51 homolog 2, MDS1-EVI1 and interleukins (23). Liu *et al* (24) reported on PLUNC proteins and deduced that the expression level of nasopharyngeal carcinoma-related protein/SPLUNC1 was higher in nasopharynx cells under chronic inflammation. They also observed that SPLUNC1 played a major role in the differentiation of NC cells, and might be an important antimicrobial protein involved in innate immunity defense (24). SPLUNC1 was found to be responsible for inhibiting the Toll-like receptor 9/NF-κB signaling pathway and reduced the inflammatory microenvironment in Epstein-Barr virus-associated NC (25). Whereas, Zhang *et al* (26) observed that von Ebner minor salivary gland protein and SPLUNC1 genes were expressed in nasopharyngeal epithelial tissue and the trachea, based on cDNA microarray hybridization. In addition, it has been suggested that SPLUNC1 is associated with the prognosis of NC and that the positive expression of SPLUNC1 in NC cases infers an improved prognosis (27). Yew *et al* (28) reported that the single nucleotide polymorphism rs1407019, which lies within the intronic enhancer region of SPLUNC1, was associated

with susceptibility to NC, in which the A allele was responsible for an increased risk for NC.

The association between the mutation of MDS1-EVI1 and tumor susceptibility has also been reported by Heller *et al* (29). Moreover, Wilson *et al* (30) observed that mice lacking an inactivating mutation in the MDS1-EVI1 allele tended to develop a lower rate of tumor growth compared with mice harboring the MDS-EVI1 allele. The overexpression of SPLUNC1 also gives rise to the inactivity of MDS1-EVI1, which has been evidenced in various tumor types (31). Moreover, there are reports that have suggested that polymorphisms among the SPLUNC1 and MDS1-EVI1 genes lead to functional consequences (14). For example, previous reports have suggested MDS1-EVI1 as a proto-oncogene, and its overexpression was associated with leukemia and myelodysplastic syndrome (32). In addition, a case study on SPLUNC1 polymorphisms reported that two promoter single nucleotide polymorphisms, rs2752903 and rs750064, were significantly associated with NC in a Cantonese-speaking Chinese population from Guangdong province in China (14). Moreover, the aberrant expression of MDS1-EVI1 has been reported in various cancer studies (32,33). However, its role in NC has not been extensively studied, and the present study could not draw any significant relationship between SPLUNC1 and MDS1-EVI1 genotypes on the prognostic status of nasopharyngeal carcinoma (33). These findings suggest that the polymorphisms between SPLUNC1 and MDS1-EVI1 genes may not serve as an absolute marker for prognosis of NC. Therefore, it is recommended that investigations on a large number of patients with prospective follow-up clinical measures are performed. One limitation of the present study was that the participants were from one community admitted to one hospital. In future studies, these findings should be verified in other populations with high cases of patients with NC.

In conclusion, the association between SPLUNC1 (rs2752903, T>C) and MDS1-EVI1 (rs6774494, G>A) polymorphisms exhibited a higher risk of developing NC. The present study suggested a gene-to-gene interaction with MDS1-EVI1 (rs6774494, G>A) polymorphisms. The study also observed that SPLUNC1 CT + CC genotypes are a genetic risk factor for the development of NC among the studied Chinese population. Therefore, the present study provided useful information for the development of screening for nasopharyngeal carcinoma and its treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZG, DH, WC, HL and CZ performed the experiments, the data collection and the statistical analysis. DH, WC, HL and CZ interpreted the data and proofread the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were carried out in accordance with the Declaration of Helsinki 1964 and its later amendments. Informed consent was provided by all patients and participants. Consent was obtained from each subject regarding their personal information on demographic factors, any history of medical information, tobacco smoking and alcohol consumption using a standard questionnaire. All protocols and procedures were approved by the Hospital Ethical Research Committee of the Jining Medical University (Jining, Shandong, China; approval no. JMU/AH/OncDept/2017-34GA2).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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