

Association of the rs2071559 (T/C) polymorphism with lymphatic metastasis in patients with nasopharyngeal carcinoma

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Abstract. Vascular endothelial growth factor (*VEGF*) and its receptor, *VEGFR2*, serve a critical role in angiogenesis and lymphangiogenesis, which are involved in the initiation and progression of malignancies. Specific single nucleotide polymorphisms of *VEGF* and *VEGFR2* have been shown to modulate gene expression and influence malignancy aggressiveness. The aim of the present study was to determine whether the *VEGFR2* rs2071559 (T/C) polymorphism is associated with the risk of developing nasopharyngeal carcinoma (NPC) and the aggressiveness of NPC in a southern Chinese population. A case-control study comprising 171 NPC patients and 184 healthy individuals was performed. Genotyping of the rs2071559 polymorphism was performed by quantitative polymerase chain reaction using TaqMan probes. Genotype and allele distribution of the rs2071559 polymorphism was not associated with the risk of NPC following adjustment for age, sex and ethnicity by multivariate logistic regression analyses. Regional lymph node metastasis was significantly correlated with the rs2071559 C allele and the related genotypes (OR 0.402, 95% CI 0.193-0.835, P=0.016; and OR 0.347, 95% CI 0.145-0.829, P=0.024, respectively). No correlations between genotype or allele distribution and the primary tumor size, distant metastasis, clinical stage, or histological type were observed. The rs2071559 polymorphism was shown to have an association with lymphatic metastasis in patients with NPC; however, the precise molecular mechanism should be elucidated in additional studies.

Introduction

Nasopharyngeal carcinoma (NPC) is commonly observed in southern China, particularly in the Pearl River delta area and the Xijiang River basin in the Guangdong and Guangxi provinces, with an incidence rate as high as 25-50 per 100,000 between 2000 and 2002 (1,2). The etiology of NPC is considered a result of unique interactions among environmental and genetic factors, but a detailed mechanism is yet to be elucidated. Studies have shown that gene polymorphisms not only influence gene expression or the function of the encoded protein, but may also affect individual susceptibility to NPC or the severity of the disease (3).

In a New England Journal of Medicine article published in 1971, Judah Folkman first proposed the hypothesis that angiogenesis was essential to cancer cell growth, and that angiogenesis inhibition could be a target for cancer therapy (4). A substantial body of preclinical and clinical evidence shows that vascular endothelial growth factor (*VEGF*) and its receptors serve a crucial role in tumor-related angiogenesis (5,6). Vascular endothelial growth factor receptor-2 (*VEGFR2*, also known as kinase insert domain-containing receptor) is considered to be the most important type of receptor for angiogenesis during tumor invasion (7). Numerous studies have shown that *VEGFR2*, not *VEGF* or *VEGFR1*, is associated with tumor prognosis and response to therapy (8,9). Specific single nucleotide polymorphisms (SNPs) of *VEGFR2* that modulate angiogenesis have been associated with an increased risk of malignancy and higher cancer aggression (10-12). The rs2071559 polymorphism is located in the promoter region of *VEGFR2* and certain studies have found that this polymorphism affects mRNA and protein expression (13). However, little is known about the association between rs2071559 and the etiology, prognosis and tumor-node-metastasis (TNM) stage of NPC. The aim of the present study was to determine whether the rs2071559 polymorphism in the *VEGFR2* gene is associated with the risk of developing NPC and associated cancer invasiveness.

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Materials and methods

Study subjects. A total of 171 incident NPC cases were consecutively recruited from the First Affiliated Hospital

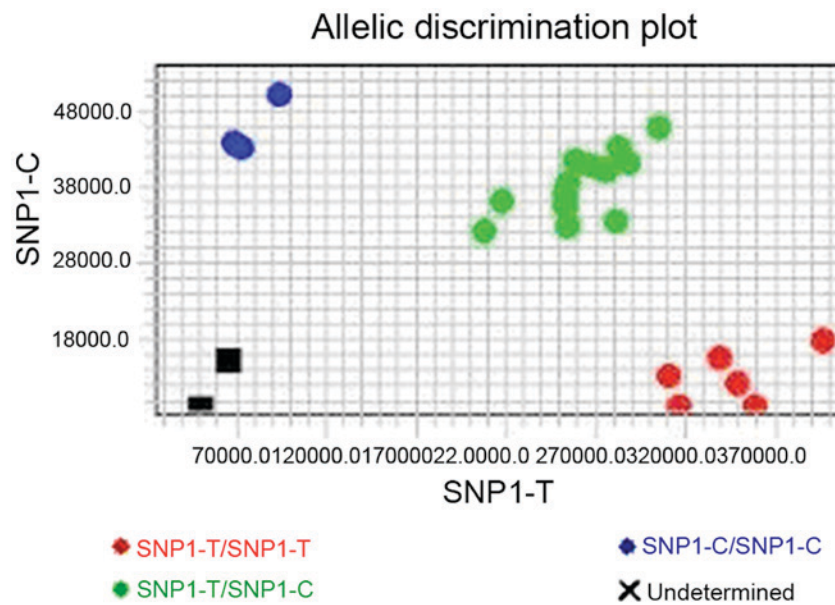


Figure 1. Allelic discrimination using TaqMan quantitative polymerase chain reaction. The blue points represent CC homozygous individuals. The green points represent TC heterozygous individuals. The red points represent TT homozygous individuals. The black points represent undetermined individuals. SNP, single nucleotide polymorphism.

of Guangxi Medical University of Nanning City (Nanning, China) between June 2009 and June 2010. All subjects were genetically unrelated and were from Nanning and the surrounding regions in China. The inclusion criteria were as follows: i) Newly diagnosed NPC; ii) no previous history of malignant tumors of other organs; iii) pathologically diagnosed as NPC. The histological type was confirmed by 2 independent pathologists according to the World Health Organization classification (14). TNM stage designation was performed according to the definitions of the 2009 edition of Union for International Cancer Control-American Joint Committee on Cancer clinical staging criteria (15). There was no ambiguity in the diagnosis. The control group was comprised of 184 unrelated healthy peripheral blood donors who visited the general health check-up division at the same hospital. Selection criteria for the control group included having no evidence of any personal or family history of cancer or other serious illness. The control group was comparable to the NPC cases with respect to age, sex and ethnicity. General information about the healthy controls was extracted from a standard questionnaire. Written informed consent was obtained from all subjects. All participants agreed to participate in the study and provided peripheral blood samples. The present study was approved by the Ethics Committee of Guangxi Medical University.

Genotype analysis. Genomic DNA was extracted from peripheral blood using a commercially available kit, according to the manufacturer's protocol (AxyPrep Blood Genomic DNA Miniprep kit; Axygen; Corning Incorporated, Corning, NY, USA). The rs2071559 polymorphism was detected using TaqMan probe-based polymerase chain reaction (PCR) and the PCR primers were designed by us based on the GenBank reference sequence and primer 5.0 (16). This method was carried out by PCR amplification using the primers: rs2071339 forward, 5'-GCTCTTAATCAGAAAACGCACTTG-3' and

reverse, 5'-GGCTAGGCAGGTCACTTCAAA-3'; the allele T TaqMan probe 5'-FAM-CAGTTCGCCAACATTCCC GCT-TAMRA-3'; and the allele C TaqMan probe 5'-HEX-AGT TCGCCAGCATTCCCGCT-TAMRA-3' in a 10 μ l reaction mixture containing ~80 ng genomic DNA, 0.45 μ l of each primer, and 0.25 μ l of each probe. Reactions were cycled with the following parameters: Preheat at 60°C for 1 min and then at 95°C for 10 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 31 sec, and finally, a final post-read for 1 min. Genotype analysis was automatically generated by Applied Biosystems® 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). We did not repeat the experiment, but validated it by direct sequencing.

Statistical analysis. The χ^2 test and Student's t-test were used to compare differences in sex and age distribution between nasopharyngeal carcinoma patients and the healthy control group. The Hardy-Weinberg equilibrium was verified by the calculation of genotype frequencies. Univariate and multivariate logistic regression analyses were used to estimate the correlation between rs2071559 polymorphism and NPC risk by computing the odds ratio (OR) and 95% confidence interval (CI), while accounting for confounding factors such as sex and age. In all statistical tests, $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with statistical analysis system software (SPSS 16.0; SPSS, Inc., Chicago, IL, USA).

Results

qPCR. qRT-PCR with TaqMan probes was used to identify the genotype of the rs2071559 polymorphism in patients with NPC and healthy controls (Fig. 1). The C and T alleles were each associated with a different fluorescent label. The fluorescence signal generated by homozygotes was small, while heterozygotes produce 2 different fluorescence signals. Although the

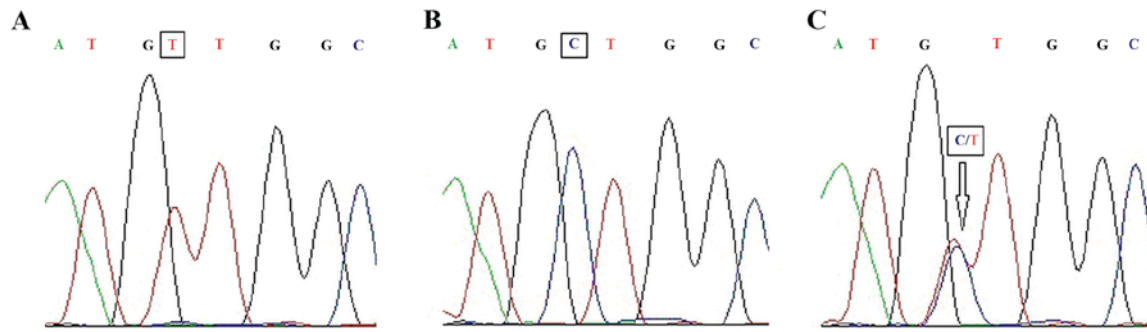


Figure 2. Sequences of 3 rs2071559 genotypes. (A) TT homozygote, (B) CC homozygote, and (C) TC heterozygote.

Table I. Clinical characteristics of patients with NPC and healthy controls.

Characteristics	Patients	Controls	P-value
Sex			0.061
Male	122	114	
Female	49	70	
Age, mean \pm SD	44.17 \pm 11.34	43.99 \pm 12.74	0.845
Histological type			
UCNT	112	-	-
DNKC	59	-	-
Tumor size			
T1	2	-	-
T2	40	-	-
T3	65	-	-
T4	64	-	-
Lymph node status			
N0	16	-	-
N1	41	-	-
N2	89	-	-
N3	25	-	-
Distant metastasis			
Yes	18	-	-
No	153	-	-

SD, standard deviation; UCNT, undifferentiated carcinoma of nasopharyngeal type; DNKC, differentiated non-keratinizing carcinomas; NPC, nasopharyngeal carcinoma.

amplification efficiency of each sample is not exactly the same, the genotypes cluster together.

Sequencing of rs2071559 genotypes. In order to verify the accuracy and reliability of the qPCR results, the present study sequenced each allele separately (Fig. 2). The 3 DNA sequences in Fig. 2 represent the TT, TC, and CC genotypes. The sequencing results validated the qPCR results.

rs2071559 polymorphism and NPC risk. The differences in age and sex between the 171 patients with NPC and 184 healthy controls were not statistically significant ($P=0.061$

and $P=0.845$, respectively; Table I). The genotype frequencies for the NPC cases and controls were in agreement with the Hardy-Weinberg equilibrium (0.055 for NPC and 0.388 for controls). The genotype or allele distribution of the rs2071559 polymorphism had no significant association with the risk of NPC following adjustment for age, sex and ethnicity by multivariate logistic regression analyses. The P-values were as follows: Co-dominant model, 0.994 and 0.200; dominant model, 0.736; recessive model, 0.253 and allele model, 0.394 (Table II).

rs2071559 polymorphism and the aggressiveness of NPC. Clinical characteristics of patients with NPC including tumor size, lymph node metastasis, distant metastasis, clinical stage and histological type of cancer were analyzed. The distribution of genotypes and alleles of the rs2071559 polymorphism for NPC patients with tumor characteristics is shown in Table III. Earlier lymph node status according to the TNM staging system was significantly associated with rs2071559 C allele and the related genotypes (OR 0.402, 95% CI 0.193-0.835, $P=0.016$; and OR 0.347, 95% CI 0.145-0.829, $P=0.024$, respectively; Table III). However, no significant correlations were identified between the genotype or allele distribution and the primary tumor size, distant metastasis, clinical stage or histological type (Table III).

Discussion

To the best of our knowledge, this is the first study targeting the association between the rs2071559 polymorphism and patients with NPC in a cohort from south China. The present study found that patients with NPC with the rs2071559 C allele and the related genotypes appeared to have lower risk of N3 lymph node metastases according to the TNM staging system, implying that the rs2071559 C allele may decrease the migratory ability and suppress the aggressiveness of NPC cells.

Bioinformatic analysis suggested that the rs2071559 polymorphism is located in the binding site for transcriptional factor E2F (involved in cell cycle regulation and interacts with Rb p107). The transition from the T allele to the C allele leads to the disappearance of the binding site for transcriptional factor E2F, which suppresses transcriptional activity and reduces the expression of *VEGFR2* (13). Consequently, An *et al* (17) found that the *VEGFR2* polymorphism was only associated with mRNA expression and had no association with protein

Table II. Comparison of NPC patients and controls by genotype frequencies of rs2071559 polymorphism.

Variable	Genotype	Control	Patient	Adjusted OR (95% CI) ^a	P-value
Co-dominant model	TT	66	65	1	
	CT	93	90	0.998 (0.633-1.573)	0.994
	CC	25	16	0.623 (0.302-1.286)	0.200
Dominant model	TT	66	65	1	
	CC+CT	118	106	0.919 (0.582-1.417)	0.736
Recessive model	TT+CT	159	155	1	
	CC	25	16	0.664 (0.325-1.283)	0.253
Allele model	T	225	220	1	
	C	143	122	0.873 (0.643-1.183)	0.394

^aAdjustment for age, sex and ethnicity. Logistic regression analysis was employed to assess the associations between genotypes and the risk of nasopharyngeal carcinoma. NPC, nasopharyngeal carcinoma; OR, odds ratio; CI, confidence interval.

Table III. Stratification analyses of the rs2071559 polymorphism with clinical characteristics of NPC patients.

Characteristics	Dominant model		Allele model	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (years)				0.233
≤40	1		1	
>40	1.240 (0.648-2.370)	0.618	1.365 (0.848-2.198)	
Sex				0.456
Male	1		1	
Female	0.752 (0.383-1.478)	0.486	0.801 (0.488-1.314)	
Nation				0.708
Han	1		1	
Minority	0.668 (0.340-1.311)	0.296	0.884 (0.540-1.448)	
Histological type				0.553
DNKC	1		1	
UCNT	0.764 (0.395-1.475)	0.508	0.850 (0.535-1.350)	
Tumor size				0.484
T1-3	1		1	
T4	1.283 (0.673-2.444)	0.516	1.198 (0.760-1.888)	
Lymph node status				0.016
N0-2	1		1	
N3	0.347 (0.145-0.829)	0.024	0.402 (0.193-0.835)	
Distant metastasis				0.997
M0	1		1	
M1	0.832 (0.180-3.840)	0.873	1.002 (0.328-3.059)	
Stage grouping				0.543
I-III	1		1	
IV	0.665 (0.289-1.529)	0.385	0.809 (0.438-1.495)	

P-values obtained by χ^2 test. NPC, nasopharyngeal carcinoma; OR, odds ratio; CI, confidence interval; DNKC, differentiated non-keratinizing carcinomas; UCNT, undifferentiated carcinoma of nasopharyngeal type.

expression or tumorigenesis. Zhang *et al* (18) demonstrated that the promoter variant rs2071559 T was associated with

reduced susceptibility to atherothrombotic stroke; however, it was inversely associated with the development of coronary

artery lesion in Kawasaki disease patients (19), implying that the T allele could increase neovascularization. In addition, as suggested by the data of Kariž and Petrovič (20), the rs2071559 CC genotype is a risk factor for myocardial infarction in Caucasians with type 2 diabetes mellitus.

Tumor growth and metastasis is dependent on the neovascularization of its surroundings. Depletion of the tumor blood supply inhibits growth and metastasis (21). Studies have demonstrated that the *VEGFR2* signaling pathway performs an important role in tumor neovascularization and could serve as a promising target for anti-tumor therapy. Duff *et al* (22) showed that *VEGFC* and *VEGFR2* were co-expressed and correlated with a number of metastatic lymph nodes. Another study has also shown that *VEGFA* could induce tumor neovascularization and lymph node lymphangiogenesis, and even promote lymphatic metastasis, through the *VEGFR2* pathway (23). **Together, these findings shed light on the findings of the present study. *VEGFR2* in patients with NPC with the rs2071559 T allele may exhibit higher transcriptional activity, subsequently increasing the migratory ability of NPC cells, in concert with certain other factors. The present study also found that patients with NPC with the rs2071559 C allele had decreased lymphatic metastasis, but no difference in NPC tumorigenesis. Additional studies are required to develop a detailed mechanism explaining these observations.**

There are also numerous studies that have found that *VEGFR2* affects the tumor response to radio-chemotherapy and other treatments. Nagy *et al* (24) found by multivariate analysis that only the expression of *VEGFR2*, and not patient age or tumor size, predicts the response of cervical carcinoma to radio-chemotherapy. Rydén *et al* (25) also found that tumor-specific expression of *VEGFR2* was associated with an impaired response to the adjuvant tamoxifen in breast cancer. However, certain studies have implied that a decrease in the expression of *VEGFR2* could increase the response of breast cancer to chemotherapy. Mele *et al* (26) found that *VEGFR2* expression in patients with breast cancer increased with treatment with either tamoxifen or epirubicin alone, but decreased in patients receiving tamoxifen plus epirubicin. Sorafenib, the first FDA-approved targeted agent for the treatment of advanced renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC), was initially identified as a Raf kinase inhibitor (27); however, it also inhibits *VEGFR2*. Therefore, Escudier *et al* (28) found that rs2071559 can predict sorafenib efficacy in patients with metastatic RCC, and Zheng *et al* (29) **demonstrated that rs2071559 was an independent factor in overall survival rate of patients with advanced HCC. These observations imply the potential synergism of such drugs. The risk of serious hematologic complications, including neutropenia, should be considered if anti-*VEGF* drugs are to be administered with chemotherapy (30). Assessing the maturation state of the microvessels, however, may allow improved characterization of the vasculature and could prove useful in guiding anti-angiogenic treatment (31,32).**

There are potential limitations of the present study. The *VEGFR2* gene is a polymorphic gene, and only rs2071559 was investigated. Other SNPs within *VEGFR2* gene may contribute to gene regulation (1,7,33). Thus, additional studies on other functional SNPs and haplotypes are required to delineate the genetic contribution of *VEGFR2* polymorphisms to NPC. Second, the sample size was relatively small. Additional

studies with larger populations are required to define the role of rs2071559 polymorphisms in the onset and course of NPC.

To conclude, the present results suggest that the rs2071559 polymorphism is associated with lymphatic metastasis of NPC, and may be a good prognostic indicator in patients with NPC. However, the precise molecular mechanism should be elucidated in further studies.

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