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Original Article

Polymorphisms in checkpoint kinase 2 may contribute to lymph node metastasis from esophageal cancer

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Abstract: Esophageal cancer, which is commonly accompanied by lymph node metastasis, is among the deadliest of cancers and carries a grim prognosis. We investigated the association between genetic variation in checkpoint kinase 2 (*CHEK2*), which has been linked to metastasis in other cancers, and the risk of developing lymph node metastasis from esophageal cancer. *CHEK2*-122 G/C genotypes were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in 296 subjects with esophageal cancer (67 cases with and 229 cases without lymph node metastasis). The associations between *CHEK2* genotypes and the risk of lymph node metastasis from esophageal cancer were estimated by computing odds ratios (OR) and their 95% confidence intervals (CI). The *CHEK2* GG, GC, and CC genotype frequencies in patients with and without lymph node metastasis were 47.8%, 40.3%, and 11.9% and 31.0%, 50.7%, and 18.3% respectively, and were statistically significant (χ^2=6.591, P=0.037). Logistic regression analyses revealed that the *CHEK2*-122 GC genotype significantly reduced the risk of lymph node metastasis (adjusted OR=0.54, 95% *CI*=0.29-0.93, P=0.028) compared to the GG genotype. Subsequently, we propose that the *CHEK2*-122 G/C polymorphism may play a protective role in preventing lymph node metastasis from esophageal cancer, and may also provide insight toward determining patient prognosis without the use of surgery.

Keywords: Checkpoint kinase 2, esophageal cancer, lymphatic metastasis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, single nucleotide polymorphism

Introduction

Esophageal cancer is among the top four most deadly malignant tumors [1]. Lymph node metastasis is commonly used to determine prognosis; however, variation has been found between patients who were exposed to similar environments and had similar tumors, suggesting that individual genetic differences may also be correlated with the development of lymph node metastasis [2]. Specifically, genetic differences in the cell cycle checkpoint kinase 2 (*CHEK2*) gene may play a role in the development of metastasis. *CHEK2*, a cell cycle regulatory gene located on chromosome 22, can activate cell cycle checkpoints causing the cell division to pause [3]. Mutations in *CHEK2* have been shown to correlate with breast cancer development and the recurrence of bladder cancer [4, 5]. Furthermore, *CHEK2* polymorphisms have also been shown to correlate with glioblastoma prognosis [6], suggesting that the gene plays a role in the development and severity of metastasis.

Gu *et al.* genotyped CHEK2 rs738722, rs2236141 and rs2236142 single nucleotide polymorphisms (SNPs) in 380 esophageal cancer cases and 380 healthy controls in Jiangsu Province in Southeast China, their results did not support a significant association between CHEK2 SNPs and the risk of esophageal cancer, but functional variant CHEK2 at rs738722 and rs2236142 was reported to contribute to lymph node metastasis susceptibility [7]. Here, we performed a case-control study in Henan, a Central Province in China, aimed at providing a rationale for the treatment and prognosis of

Table 1. Participant profiles

Variable	Lymph node metastasis		t/χ^{2*}	p
	Yes (N=67)	No (N=229)		
Age (Mean ± SD, yr)	61.2±11.4	60.6±11.2	0.373	0.710
Gender (%)				
Male	48 (71.6)	154 (67.2)	0.462	0.497
Female	19 (28.4)	75 (32.8)		
Smoking status (%)				
Smokers	31 (46.3)	82 (35.8)	2.403	0.121
Nonsmokers	36 (53.7)	147 (64.2)		
Alcohol consumption History (%)				
Yes	25 (37.3)	73 (31.9)	0.692	0.406
No	42 (62.7)	156 (68.1)		

*a *t* value is given for age data analysis; a χ^2 value is given for all other variables.

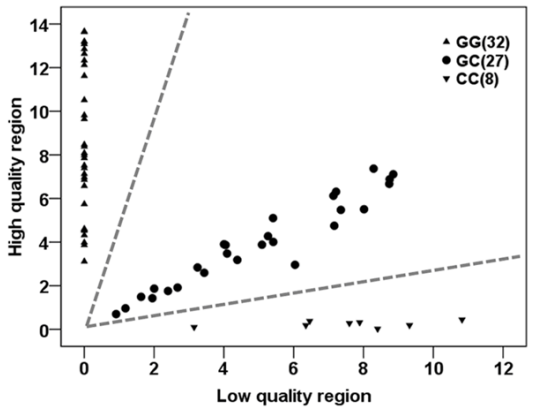

Figure 1. Genotyping of *CHEK2*-122 G/C (rs2236142) by MALDI-TOF-MS.

esophageal cancer by exploring the correlation between *CHEK2* SNPs at -122 G/C (rs2236142) and lymph node metastasis from esophageal cancer.

Methods

Study participants

The study included 296 patients with esophageal cancer. Each patient had received radical surgery for esophageal cancer in the First Affiliated Hospital of Zhengzhou University between 2010 and 2013 and was diagnosed with squamous cell carcinoma by postoperative pathological and histological examinations. Lymph node metastasis was confirmed by pathological examination of lymph nodes taken intraoperatively. Of the 296 patients, lymph node metastasis was discovered in 67. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and all participants signed the informed consent statements.

Specimen collection

Two mL fasting venous blood was drawn from the cubital vein and placed in an anticoagulant tube. Next, 3.8% sodium citrate was added according to a 9:1 ratio of blood to anticoagulant, and the mixture was centrifuged at 3000 rpm for 15 min. Then, DNA was extracted using a DNA extraction kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol, and standardized to a concentration of 30-50 ng/μL.

CHEK2 genotyping

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Sequenom, San Diego, CA) was used to detect genetic polymorphisms of *CHEK2* as the previous report [7]. Primers were synthesized by Sangon Biotech (Shanghai, China). One μL of DNA sample was mixed with 1.8 μL of triple distilled water, 0.5 μL 10x PCR buffer, 0.4 μL $MgCl_2$, 0.1 μL dNTP, 1 μL of each PCR primer, and 0.2 μL Taq enzymes (Qiagen, Venlo, Netherlands). PCR reaction conditions were as follows: 95°C for 2 min, 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, for a total of 45 cycles, followed by 5 min of 72°C and cooling to 25°C. After PCR amplification, remaining dNTPs were dephosphorylated by the addition of 1.53 μL triple distilled water, 0.17 μL SAP buffer, and 0.3 U alkaline phosphatase. Reaction conditions were as follows: 37°C for 40 min followed by 85°C for 5 min to inactivate the enzymes, and then cooling to 25°C. After dephosphorylation, single base primer extension for the determination of SNPs was performed using 0.619 μL triple distilled water, 0.2 μL 10× iPLEX buffer, 0.2 μL termination mixture, 0.041 μL iPLEX enzyme, and 0.94 μL of each extension primer. Reaction conditions were: 94°C for 30 s, 94°C for 5 s, 52°C for 5 s, and 80°C for 5 s for 5 internal cycles, then 72°C for 3 min for a total of 40 external cycles, followed by cooling to 25°C. Finally, 6 mg of ion-exchange resin for desalting was added to the termination mixture, followed by 25 μL of triple distilled water for suspension.

Table 2. Genotype and allele frequency among study participants [N (%)]

Lymph node metastasis	N	Genotype frequency			Allele frequency	
		GG	GC	CC	G	C
Yes	67	32 (47.8)	27 (40.3)	8 (11.9)	91 (67.9)	43 (32.1)
No	229	71 (31.0)	116 (50.7)	42 (18.3)	258 (56.3)	200 (43.7)
χ^2			6.591		5.743	
p			0.037		0.017	

A MassARRAY Nanodispenser was used to drip a sample of the final product onto a spectroCHIP and a MALDI-TOF mass spectrometer was used to analyze the samples (all equipment was purchased from Sequenom, San Diego, CA, and operated according to the manufacturer's instructions). Genotyping results were analyzed using MassARRAY RT and MassARRAY Typer 4.0 software. Ten per cent of the samples were randomly selected for repeat detection to verify accuracy.

Statistical methods

SPSS 17 (IBM, Bethesda, MD) was used to perform statistical analysis. A Student's t-test was used to compare the ages of patients with lymph node metastasis to patients without lymph node metastasis, and a chi-square test was used to compare sex, smoking habits, drinking habits, and genotype and allele frequencies in the two groups of patients. The correlation between polymorphisms, esophageal cancer, and the risk of lymph node metastasis was determined using the odds ratio (OR) and 95% confidence interval (CI) obtained from logistic regression analysis. All statistical tests were two-sided, where P<0.05 was considered statistically significant.

Results

Participant profiles

There were no statistically significant differences between patients with lymph node metastasis and patients without lymph node metastasis with regards to age, sex, or history of smoking or alcohol consumption (**Table 1**).

CHEK 2-122 G/C

Genotypic analysis of *CHEK2* indicated that there were 3 different genotypes present at *CHEK2*-122 G/C (**Figure 1**). Differences in genotypes and allele frequencies at *CHEK2*-122 G/C between patients with and without lymph node metastasis were statistically significant (P<0.05) (**Table 2**).

CHEK2 genotype is correlated with lymph node metastasis from esophageal cancer

Logistic regression analysis showed that after adjustment in the ages, sex, smoking and alcohol consumption, the risk of lymph node metastasis from esophageal cancer in patients with the GC genotype was 0.54 times (95% CI=0.29-0.93, P=0.028) that in patients with the GG genotype, while the risk of lymph node metastasis from esophageal cancer in patients with C alleles (GC and CC) was 0.51 times (95% CI=0.29-0.90, P=0.019) the risk for patients with the GG genotype (**Table 3**).

Discussion

In China, esophageal cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death with an estimated 259 235 new cases and 211 084 deaths in 2008. Henan, a central China, has among the highest incidence rates in the world (>100 per 100 000 population) [8]. Although diagnosis and treatment rates are improving, the prognosis for esophageal cancer patients remains quite poor [9]. Lymph node metastasis is a clinical indicator used to determine prognosis in these patients and is the primary determinant for postoperative adjuvant chemotherapy candidates [10].

We investigated the connection between polymorphisms of the *CHEK2* gene, which is likely involved in metastasis as previous report [7], and lymph node metastasis from esophageal cancer. Lymph node metastasis from esophageal cancer involves a series of discrete biological processes in which cancer cells invade the surrounding tissues and infiltrate the lymphatic vessels and lymph nodes. Studies have shown that *CHEK2* plays a role in the occurrence of tumors, and mutations in *CHEK2* may increase the risk of breast and colon cancer [11-13]. Through the detection of polymorphisms at 122 G/C of the *CHEK2* gene in 296 patients with esophageal cancer, we found that the differences in genotype and allele frequencies

Table 3. Genotype frequency and risk of lymph node metastasis from esophageal cancer

Genotype	Lymph node metastasis		OR (95% *CI*)	p	OR (95% CI)*	P*
	Yes (N=67)	No (N=229)				
GG	32 (47.8)	71 (31.0)	1.00		1.00	
GC	27 (40.3)	116 (50.7)	0.52 (0.29-0.93)	0.028	0.54 (0.30-0.97)	0.040
CC	8 (11.9)	42 (18.3)	0.42 (0.18-1.00)	0.051	0.44 (0.18-1.05)	0.064
GC+CC	35 (52.2)	158 (69.0)	0.49 (0.28-0.86)	0.012	0.51 (0.29-0.90)	0.019

*Adjusted for age, sex, smoking, and alcohol consumption.

between patients with and without lymph node metastasis were statistically significant. Namely, the rate of carrying alleles at 122 G/C (rs2236142G) in the promoter region of *CHEK2* in patients with lymph node metastasis was increased and could be responsible for increasing the risk of lymph node metastasis. These results imply the *CHEK2* locus as a biological marker of susceptibility to lymph node metastasis in patients with esophageal cancer and could help predict lymph node metastasis prior to surgery.

Disclosure of conflict of interest

None.

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