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Correlation between COX-2 gene polymorphism and susceptibility to nasopharyngeal carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate cyclooxygenase-2 (COX-2) gene polymorphism in peripheral blood cells of patients with nasopharyngeal carcinoma (NPC) and normal people, and to explore the correlation between polymorphism and the occurrence of NPC.

PATIENTS AND METHODS: The genotype and allele distributions of gene loci COX-2-899 (G/C) and COX-2-1195 (G/A) in peripheral blood of 56 normal people and 114 NPC patients were analyzed via reverse transcription-polymerase chain reaction (RT-PCR). The genetic equilibrium was detected by TaqMan genotyping technique. Meanwhile, the risk factors for NPC were analyzed via multivariate logistic regression analysis. Subsequently, the effects of risk factors, clinical features, and gene polymorphism of NPC on the prognosis and survival of patients were analyzed using univariate and multivariate COX regression analysis. Finally, the correlation of smoking, Epstein-Barr (EB) virus infection and COX-2-1195 gene polymorphism with NPC was explored *via* χ^2-test.

RESULTS: There was a significant difference in the genotype and allele distributions at COX-2-1195 (G/A) between the two groups ($p<0.05$). However, no significant differences were observed at COX-2-899 (G/C) between the two groups ($p>0.05$). According to the results of univariate and multivariate COX regression analysis, smoking and EB virus infection were risk factors for NPC ($p<0.05$). The χ2-test indicated that there was an evident gene polymorphism at COX-2-1195 in smokers and EB virus-infected people compared to non-smokers and non-infected people ($p<0.05$).

CONCLUSIONS: COX-2-1195 gene polymorphism is associated with susceptibility to NPC. Smoking and EB virus infection are major risk factors for NPC, both of which can affect COX-2-1195 gene polymorphism.

Key Words:
COX-2 polymorphism, Nasopharyngeal carcinoma (NPC), Risk factors, Smoking, EB virus.

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer caused by nasopharyngeal epithelial lesions, which shows distinct national and geographical distribution. The morbidity rate of NPC is the highest in Southeast Asia, medium in the Arctic and North African population, and relatively low in Western countries. For example, the morbidity rate of NPC is as high as 25-50/100,000 in southeastern China and Hong Kong[1,2]. Previous studies have demonstrated that in spite of unique geographical distribution pattern, the pathogenesis of NPC is complex. It is associated with viruses, smoking, drinking, environmental factors, carcinogens, genetic factors and history of ENT diseases[3]. Among the factors influencing the occurrence of NPC, viruses have a significant effect. Additionally, it is reported that Epstein-Barr (EB) virus significantly increases gene mutation rate and recombination rate of nasopharyngeal epithelial cells. Meanwhile, it blocks the expression of deoxyribonucleic acid (DNA) damage repair gene and activates immune-related genes (such as tumor necrosis factor gene), metabolic enzyme genes (glutathione transferase gene) and proto-oncogenes. Eventually, this may aggravate the genetic variation and malignant progression of epithelial cells[4]. Increasingly, several studies have proved that increased susceptibility to NPC is closely associated with genetic variation. This suggests that changes in gene polymorphism may be an important factor for the occurrence of NPC. Cyclooxygenase-2 (COX-2) is highly expressed in NPC, which is associated with tumor invasion and migration. However, the correlation between its gene polymorphism and occurrence of NPC in the Chinese population has not been fully elucidated. Therefore, COX-2 gene polymorphism was analyzed to provide relevant evidence for the research and clinical treatment of NPC.

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COX-2 is located on chromosome 1q31.1 and contains 10 exon sequences[5]. Its positive rate in NPC tissues is up to 75.58%, which is significantly higher than that of normal nasopharyngeal tissues. COX-2 promotes the migration and invasion of cancer cells by regulating the interaction between myeloid-derived immunosuppressive cells and NPC cells[6]. However, its inhibitor celecoxib suppresses the invasion and migration of NPC cells through the down regulation of the activity of matrix metalloproteinase-2 and -9[7]. These findings suggest that silencing COX-2 can improve the efficacy of chemoradiotherapy *in vitro* and *in vivo*. Currently, COX-2 gene polymorphism is closely associated with the occurrence of gastric cancer and liver cancer[8,9]. However, its correlation with NPC in Chinese population remains unclear. COX-2 has single nucleotide polymorphism at multiple loci, such as COX-2-899 (G/C), 1195 (G/A), 2765 (G/C), and 8473 (T/C).

In this study, polymorphism was explored at two adjacent loci COX-2-899 (G/C) and 1195 (G/A) in normal people and NPC patients to determine the gene polymorphism at two loci and its correlation with NPC.

Patients and Methods

Research Subjects

A total of 114 patients with new-onset NPC were selected as research subjects, including 73 males and 41 females aged 43-69 years old. Inclusion criteria: patients definitely diagnosed by pathology with complete follow-up data, those with informed consent, and those without chemoradiotherapy before the acquisition of peripheral blood mononuclear cells. Meanwhile, 56 healthy volunteers were randomly selected from the hospital as control group. This investigation was approved by the Ethics Committee of Weifang People's Hospital. Signed written informed consents were obtained from all participants before the study.

Methods

Main reagents: human peripheral blood lymphocyte separation medium (Haoyang, Tianjin, China), TRIzol cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA), RNA reverse transcription kit (Sigma-Aldrich, St. Louis, MO, USA), DNA polymerase and dNTP (Promega, Madison, WI, USA), and amplification primers (SBS): COX-2-899 forward and reverse primers, COX-2-1195 forward and reverse primers and internal reference glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) forward and reverse primers (Table I).

Preparation and Extraction of Total RNAs

A total of 2 mL of peripheral venous blood was collected from patients in the morning. Collected blood samples were stored in ethylenediaminetetraacetic acid (EDTA) anticoagulant tube, and were diluted at 1- or 2-fold with sterile phosphate-buffer saline (PBS), followed by density-gradient centrifugation. Subsequently, the middle white layer was obtained using Ficoll- human peripheral blood lymphocyte separation medium. After washing, the layer was centrifuged to obtain peripheral blood lymphocytes. After that, mononuclear cells were lysed on ice using TRIzol for 30 min, followed by centrifugation with chloroform and precipitation to extract the total RNAs. Finally, the concentration of total RNA was detected by a spectrophotometer.

Reverse Transcription

A 20 μL reverse transcription system was constructed. According to the experimental instructions, 1000 ng of RNA was added into the reaction system, followed by addition of diethyl pyrocarbonate (DEPC; Beyotime, Shanghai, China)-treated water until the total volume reached 20 μL. The reaction system was centrifuged and precipitated. Reverse transcription was performed as follows: 37°C for 15 min, and 85°C for 5 min. Finally, reverse transcription products were taken out after cooling to 4°C, and complementary deoxyribonucleic acids (cDNAs) were stored at −80°C for later use.

Table I. Primer sequences in RT-PCR

Target gene	Primer sequences		Cycle	T
COX-2-899	Forward primer (5'-3') Reverse primer (5'-3')	5'-ACCCGTGGAGCTCACATTAACTAT-3' 5'-ATACTGTTCTCCGTACCTTCACCC-3'	40	58
COX-2-1195	Forward primer (5'-3') Reverse primer (5'-3')	5'-CCGGTACATGGCCTATTAACGTGCATACGG-3' 5'-CGTTGCGCAATGCTGACCGATCA-3'	40	58
GAPDH	Forward primer (5'-3') Reverse primer (5'-3')	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' 5'- CATGTGGGCCATGAGGTCCACCAC-3'	40	58

PCR Amplification

According to the instructions of the amplification kit (Invitrogen, Carlsbad, CA, USA), the total amplification system (25 μL) was prepared as follows: 1 μL of DNA template, 1 μL of forward primers, 1 μL of reverse primers, 12 μL of 2× TapMaster Mix, and 10 μL of enzyme-free water. The mixture was vibrated and mixed evenly, centrifuged and precipitated. The specific PCR amplification procedure was as follows: 94°C for 2 min, 94°C for 40 s, 55°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. After electrophoresis at 72°C and 120 V for 30 min, the images were analyzed using a gel imager (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The χ^2-test was performed for enumeration data of patients' features. Hardy-Weinberg genetic equilibrium test was conducted for subjects. Meanwhile, the χ^2-test was adopted for the comparison of genotype and allele frequency distributions. Risk factors for NPC were analyzed using the multivariate logistic regression analysis. The effects of risk factors, clinical features and gene polymorphism of NPC on the prognosis and survival of patients were analyzed using univariate and multivariate COX regression analysis. The correlation of smoking, Epstein-Barr (EB) virus infection and COX-2-1195 gene polymorphism with NPC was detected *via* the χ^2-test. $p \leq 0.05$ was considered statistically significant.

Results

Clinical Features of Research Subjects

A total of 114 NPC patients were enrolled in this study, including 73 males and 41 females. They were aged between 43 and 69 years old, comprising 93 cases aged below 55 years old, and 21 cases aged 55-69 years old. All patients were pathologically diagnosed with NPC for the first time, with no chemoradiotherapy. There were 76 cases with body mass index (BMI) >24, 78 drinkers, 83 smokers, 31 cases of hypertension, 74 cases of EB virus infection, 28 cases of enlargement of lymph nodes and 21 cases of metastasis. In terms of clinical stage, there were 34 cases in stage I-II and 80 cases in stage III-IV. Among 56 healthy volunteers in control group, there were 33 cases with BMI >24, 25 drinkers, 27 smokers, 13 cases of hypertension, 17 cases of EB virus infection and 0 cases of enlargement of lymph nodes (Table II).

Hardy-Weinberg Genetic Equilibrium Test

To verify COX-2 genetic equilibrium in subjects, the χ^2-test was performed for COX-2-899/1195 genotype frequency. The results found that subjects in case group and control group met COX-2 genetic equilibrium ($p>0.05$) (Table III).

COX-2-899 and COX-2-1195 Genotypes and Alleles

After RT-PCR, genotype results of all subjects were analyzed. The χ^2-test indicated that there were significant differences in the genotype and allele distributions at COX-2-1195 between the two groups ($p<0.05$). However, no significant differences were observed at COX-2-899 between the two groups ($p>0.05$) (Table IV).

Logistic Regression Analysis of Risk Factors For NPC

According to the clinical features of subjects, gender, BMI, age, smoking, drinking and EB virus infection in both groups were analyzed and assigned (Table V). Subsequently, the influences of gender, BMI, age, smoking, drinking and EB virus infection on the occurrence of NPC were detected *via* multivariate logistic regression analysis. The results showed that smoking and EB virus infection were major risk factors for NPC [OR (95% CI) = 4.37 (1.33-6.18) and 10.01 (2.22-27.86), $p<0.05$] (Table VI).

Correlations Between COX-2-1195 Gene Polymorphism and Risk Factors of NPC Patients

The above analysis demonstrated that COX-2-1195 gene polymorphism was significantly associated with the occurrence of NPC. Therefore, the degree of correlation between COX-2-1195 gene polymorphism and the clinical features of patients was analyzed. As shown in Table VII, COX-2-1195 gene polymorphism was significantly associated with smoking ($p=0.001$) and EB virus infection ($p=0.001$). Combined with logistic regression analysis, these results suggested that smoking and EB virus infection were high-risk factors for NPC.

Table II. Clinical features.

	Case group		Control group		χ^2	*p*
	N	%	N	%		
Gender					1.722	0.19
Male	73	64.04	30	53.57		
Female	41	35.96	26	46.43		
BMI						
>24	76	66.67	33		0.977	0.32
≤24	38	33.33	23			
Age					0.994	0.32
43-55	93	81.58	42	75		
55-69	21	18.42	14	25		
Drinking					8.892	0.003
Yes	78	68.42	25	44.64		
No	36	31.58	31	55.36		
Smoking					9.945	0.002
Yes	83	72.81	27	48.21		
No	31	27.19	29	51.79		
Hypertension						
Yes	31	27.19	13	23.21	0.310	0.58
No	83	72.81	43	76.79		
EB virus infection						
Yes	74	64.91	17	30.36	18.026	<0.01
No	40	35.09	39	69.64		
Enlargement of lymph nodes						
Yes	28	24.56	–	–		
No	86	75.44	–	–		
Metastasis			–	–		
Yes	21	18.42	–	–		
No	93	81.58	–	–		
Clinical stage			–	–		
I-II	34	29.82	–	–		
III-IV	80	70.18	–	–		

Correlation Analysis of COX-2-1195 Gene Polymorphism with Smoking and EB Virus

The genotype and allele frequency of COX-2-1195 was detected *via* the χ^2-test in smokers and non-smokers, EB virus-infected and non-infected subjects. Results revealed that the genotype and allele frequencies of COX-2-1195 showed significant differences between smokers and non-smokers, as well as between EB virus-infected and non-infected subjects ($p>0.05$) (Tables VIII and IX). Combined with logistic regression analysis and univariate and multivariate regression analysis, the findings suggested that smoking and EB virus infection were risk factors for NPC, and could affect COX-2-1195 gene polymorphism as well.

Table III. Hardy-Weinberg genetic equilibrium test.

Gene	Genotype	Case group			Control group		
		N	χ^2	*p*	N	χ^2	*p*
COX-2-899	G/G	39			28		
	G/C	59	0.308	0.857	20	0.723	0.696
	C/C	16			8		
COX-2-1195	G/G	27			25		
	G/A	43	2.741	0.254	18	2.866	0.239
	A/A	44			13		

Table IV. COX-2-899/1195 genotypes and alleles.

		Case group		Control group					Case group		Control group			
Gene	Genotype	N	%	N	%	χ^2	P	Allele	N	%	N	%	χ^2	P
COX-2-899	G/G	39	32.21	28	50			G	137	60.09	76	67.86	1.94	0.16
	G/C	59	51.75	20	35.71	4.46	0.108	C	91	39.91	36	32.14		
	C/C	16	14.04	8	14.29									
COX-2-1195	G/G	27	23.68	25	44.64			G	97	42.54	68	60.71	9.928	0.002
	G/A	43	37.72	18	32.14	8.37	0.015	A	131	57.46	44	39.29		
	A/A	44	38.60	13	23.21									

Table V. Logistic regression analysis and variable assignment.

Variable	Assignment
Gender	Male=1, female=0
BMI	≤24=1, >24=0
Age	43-55=0, >55=1
Smoking	Yes=1, No=0
Drinking	Yes=1, No=0
EB virus infection	Yes=1, No=0

Table VI. Logistic regression analysis of risk factors.

Independent variable	β	S*	OR	95%CI	p
Smoking	1.73	0.49	4.37	1.33-6.18	0.029
EB virus infection	2.82	0.89	10.01	2.22-27.86	0.0032

Table VII. Univariate and multivariate COX regression analysis.

Variable	Type	OR (95% IC)	p
Univariate analysis (n=114)			
COX-2-1195 gene polymorphism	G/A	0.983 (0.526-1.835)	0.032
Gender	Male/Female	0.048 (0.023-0.651)	0.365
BMI	≤24/>24	0.054 (0.038-0.702)	0.291
Smoking	Yes/No	1.876 (0.812-4.394)	0.011
Drinking	Yes/No	2.157 (1.274-3.653)	0.0023
EB virus infection	Yes/No	1.884 (0.809-4.391)	0.0011
Enlargement of lymph nodes	Yes/No	1.058 (0.303-3.694)	0.052
Metastasis	Yes/No	1.456 (0.481-4.410)	0.067
Clinical stage	I-II/III-IV	2.050 (0.947-4.436)	0.031
Multivariate analysis (n=114)			
COX-2-1195 gene polymorphism	G/A	1.062 (0.561-2.009)	0.045
Smoking	Yes/No	0.885 (0.255-3.069)	0.001
Drinking	Yes/No	1.534 (0.508-4.636)	0.448
EB virus infection	Yes/No	1.961 (0.920-4.182)	0.001
Clinical stage	I-II/III-IV	1.052 (0.873-2.101)	0.062

Discussion

COX is a a rate-limiting enzyme in the synthesis of prostaglandin, including two subtypes of COX-1 and COX-2. COX-1 is constitutively expressed in many tissues, and it also plays an important role in tissue homeostasis. In contrast, COX-2 is an inducible enzyme that produces prostaglandin at the site of inflammation and wound healing[10]. It is noteworthy that COX-2 is highly expressed in various cancers, including lung cancer, breast cancer, ovarian cancer, colorectal cancer, and NPC[11-14]. Functionally, COX-2 is considered to play an important role in the initiation and progression of human diseases. Moreover, COX-2 increases the secretion of cytokines, stimulates the growth of tumor stem cells, resists DNA damage, and enhances the resistance of tumor cells to conventional chemotherapy and radiotherapy. Previous studies have demonstrated that COX-2 shows significant effects on apoptosis, tumor growth, angiogenesis, invasion, and metastasis. In tumor research, single nucleotide polymorphism of COX-2 is significantly associated with increased susceptibility to tumors. For example, polymorphism of COX-2-765 (G>C) in colorectal cancer and gastric cancer can increase the incidence rate of tumors[15]. Currently, it has been proved that the COX-2 gene rs5275 polymorphism is significantly associated with increased susceptibility to NPC[16]. However, the correlations of polymorphisms at COX-2-899 (G>C) and COX-2-1195 (G>A) with the susceptibility to NPC have not been fully elucidated. Therefore, RT-PCR amplification, restriction digestion, and gel electrophoresis were performed for the two loci in both groups. It was found that the genotype and allele distributions at COX-2-1195 showed significant differences between the two groups ($p<0.05$). However, no significant difference was observed at COX-2-899 between the two groups ($p>0.05$). In addition, COX-2 is an inducible cyclooxygenase that regulates angiogenesis and increases invasion and progression of tumor cells. Its polymorphism at multiple exons loci has been confirmed to be associated with tumor susceptibility. Compared to normal people, COX-2-1195 gene polymorphism in NPC patients increases the expression of tumor-associated proteins, inhibits cell senescence, and enhances cell division and proliferation. In future studies, the correlation between changes in COX-2-1195 gene and changes in tumor-associated proteins should be analyzed at the protein level to provide targets for the treatment of NPC.

Some studies[17-19] have demonstrated that internal and external chemical or physical factors, such as nicotine, alcohol, viral infection, diet, high fever and stress, can significantly increase intracellular oxidative stress, DNA damage, genetic variation, expression of tumor-associated proteins and incidence rate of tumors. NPC is a malignant nasopharyngeal tumor with strong geographical distribution characteristics. It has been found that NPC is closely related to smoking and EB virus infection.

Table VIII. Correlation analysis between COX-2-1195 gene polymorphism and smoking.

		Smoking		Non-smoking					Smoking		Non-smoking			
Gene	Genotype	N	%	N	%	χ^2	*P*	Allele	N	%	N	%	χ^2	*P*
COX-2-1195	G/G	27	24.55	27	45			G	90	40.91	71	59.17	10.382	0.001
	G/A	36	32.73	17	28.33	8.056	0.018	C	130	59.09	49	40.83		
	A/A	47	42.73	16	26.67									

Table IX. Correlation analysis between COX-2-1195 gene polymorphism and EB virus infection.

		EB virus		No EB virus					EB virus		No EB virus			
Gene	Genotype	N	%	N	%	χ^2	*P*	Allele	N	%	N	%	χ^2	*P*
COX-2-1195	G/G	23	25.27	30	37.97			G	68	37.36	84	53.16	8.652	0.003
	G/A	22	24.18	24	30.38	6.408	0.041	C	114	62.64	74	46.84		
	A/A	46	50.55	25	31.65									

Studies have shown that smoking and EB virus infection increase gene mutation and recombination, thereby enhancing cellular signal transduction, inactivating tumor suppressor genes, and inhibiting cell death pathways. Ultimately, this can lead to increased resistance to genotoxicity and malignant cell transformation[3,20,21]. In this study, risk factors for NPC in research subjects were analyzed *via* logistic regression analysis. It was found that smoking and EB virus infection were closely related to NPC ($p<0.05$). The correlations of clinical features of patients with COX-2-1195 (G/C) gene polymorphism were analyzed *via* univariate and multivariate COX regression analysis. The results indicated close correlations of smoking and EB virus infection with NPC ($p<0.05$). In addition, COX-2-1195 (G/C) genotype and allele frequencies were studied for smoking and EB virus infection to detect their association with COX-2-1195 (G/C) gene polymorphism. Results showed that COX-2-1195 (G/C) gene polymorphism increased significantly in smokers and EB virus-infected people compared to non-smokers and non-infected people ($p<0.05$). Therefore, it was speculated that smoking and EB virus infection could increase gene polymorphism and induce NPC through the regulation of gene mutation and recombination at COX-2-1195.

The present study indicated that COX-2-1195 had evident gene polymorphism in peripheral blood cells in NPC. However, no significant gene polymorphism was found at COX-2-899. Therefore, the correlations of COX-2 gene function and protein expression with the occurrence and progression of NPC were analyzed in view of the differences in genotype and allele frequencies. Furthermore, our findings might provide a new direction for searching for the mechanism of NPC.

Conclusions

We proved that COX-2-1195 gene polymorphism is associated with susceptibility to NPC. Smoking and EB virus infection are the major risk factors for NPC, both of which can affect COX-2-1195 gene polymorphism.

Conflict of Interests

The authors declared that they have no conflict of interests.

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