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Original Article

Correlation between cytotoxic T lymphocyte-associated antigen-4 -1661A/G and -1772T/C gene polymorphisms and gastric cancer

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Abstract: Objective: The goal of this study was to investigate possible correlation between the single nucleotide polymorphisms of -1661 and -1722 loci in the promoter region of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) gene and the incidence of gastric cancer in Inner Mongolia. Methods: Polymerase chain reaction-restricted fragment length polymorphism analysis was used to detect the distribution of gene polymorphisms of -1661 and -1722 loci in CTLA-4 promoter region between the gastric cancer patients (n = 116) and the healthy controls (n = 138), and then to analyze the relationship between gene polymorphisms and the risk of gastric cancer. Results: The distribution of CTLA-4 -1661A/G and -1722T/C gene polymorphisms in the control group satisfied the Hardy-Weinberg equilibrium law (P > 0.05) as well as the genetic linkage equilibrium rule, thus had a good representation of the population. In comparison with the healthy control group, the distribution frequency of AA genotype at -1661 locus of CTLA-4 promoter region in the gastric cancer group was significantly decreased (P < 0.05); the frequency of AG and GG genotypes were markedly increased (P < 0.05), and the risk of having gastric cancer in individuals with GG genotype was 5.710 times higher than that in individuals with AA genotype (95% CI: 2.011~16.219). Furthermore, the frequencies of TT and TC genotypes at CTLA-4 -1722 locus in the gastric cancer group were notably increased (P < 0.05), and the risk of gastric cancer in individuals with TT and TC genotypes were 9.670 times (95% CI: 2.151~43.465) and 8.595 times (95% CI: 1.917~38.538) higher than those with CC genotype, respectively. Conclusion: The gene polymorphisms of -1661 and -1722 loci in the CTLA-4 promoter region are associated with increased genetic susceptibility to gastric cancer.

Keywords: Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), single nucleotide polymorphisms (SNPs), gastric cancer

Introduction

Gastric cancer, a malignant tumor with high incidence around the world, occurs and develops as a probable result of interaction between genetic and environmental factors. Genetic susceptibility to cancer plays a crucial role in the tumorigenesis, which is mainly affected by polymorphisms of tumor-related genes [1, 2]. Tumor cells are capable of regulating transcriptional and translational levels of oncogenes and anti-oncogenes through multiple pathways, and thus affecting biological behaviors of tumors [3, 4]. Single nucleotide polymorphisms (SNPs) are genetic variation, which refer to the polymorphisms of DNA sequence caused by the mutation of a single nucleotide. SNPs alter DNA sequences by base transition, transversion, insertion, and deletion, etc., thereby affecting gene transcription and protein expression processes [5].

The immune system provides immune surveillance and killing. The activation of T cells is an important mechanism for the body to resist tumors. However, tumor cells could obtain immune escape by inducing immunosuppression and suppressing immune cells [6]. Currently, a growing number of studies have been focused on the relationship between cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and tumor biological behaviors [7].

CTLA-4, also known as CD152, is a leukocyte differentiation antigen as well as a transmembrane receptor of T cells, which belongs to the inhibitory molecule of immunoglobulin superfamily [8]. Both CTLA-4 and CD28 bind to the B7 ligand on antigen-presenting cells. After binding with B7 ligand, CTLA-4 blocks T cells activation and induces T cells anergy to participate in the negative modulation of immune response [9]. Therefore, blocking the immune checkpoint of B7/CTLA-4 and inhibiting CTLA-4 could enhance the sustained activation of T cells, strengthen the ability of activated specific T cells, and produce stronger anti-tumor effects [10].

Studies have shown that the CTLA-4 gene has various SNP sites, and its polymorphisms are connected with genetic susceptibility to gastric cancer, colorectal cancer, bladder cancer, and other tumors [11]. This study aimed to explore the distribution of gene polymorphisms of -1661A/G and -1722T/C sites in CTLA-4 promoter region in the population of Inner Mongolia, and the correlation between CTLA-4 gene polymorphisms and gastric cancer as well as provide a theoretical basis at genetic level for the pathogenesis, early diagnosis, prevention and targeted therapy of gastric cancer.

Materials and methods

Clinical data

A total of 116 patients with gastric cancer who were diagnosed and treated in Inner Mongolia Baogang Hospital from April 2017 to April 2018 were included in the gastric cancer group, including 82 males and 34 females, aged from 26 to 82 years, with an average age of 51.4 ± 2.5 years. All patients with gastric cancer were diagnosed by postoperative pathology, and were treated with no radiotherapy or chemotherapy before surgery. In the control group, 138 healthy subjects who received a physical examination during the same period were selected, including 93 males and 45 females, aged from 31 to 87 years, with an average age of 53.9 ± 1.6 years. Two milliliters of fasting venous blood were collected from each subject. All subjects signed informed consent and this research project was reviewed and approved by the Ethics Committee of Inner Mongolia Baogang Hospital.

Instruments and methods

DNA extraction: In this experiment, genomic DNA was extracted by phenol-chloroform method. The specific operation steps were as follows: two milliliters of fasting venous blood were collected from each subject and transferred to the tubes with EDTA. Then DNA lysate solution and proteinase K were used for lysis and digestion, respectively. The mixtures of Tris-saturated phenol, chloroform, and isoamyl alcohol were used to continuously extract DNA. After dilution, the extracted DNA was centrifuged for later use.

Polymerase chain reaction-restricted fragment length polymorphism: PCR-RFLP analysis was used to detect gene polymorphisms at the -1661 and -1722 loci in CTLA-4 promoter region. The primers were synthesized by Shanghai Shenggong Bioengineering Technology Service Co., Ltd. The sequence of forward primer was 5'-CTAAGAGCATCCGCTTGCACCT-3' and the sequence of reverse primer was 5'-TTGGTGTGATGCACAGAAGCCTTTT-3'. The corresponding product was a 486 bp gene fragment in the promoter region of CTLA-4 comprising the -1661 and -1722 locus. PCR amplification reaction system of 10 uL was under the following conditions: initial denaturation at 94°C for 3 minutes, 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes. The amplified products were incubated at 37°C for 4 hours with restriction enzymes of MseI and Bbv1 separately. The genotyping results were determined according to the electrophoresis bands after the digested products were separated by 2% agarose gel electrophoresis.

Genotyping: the length of amplified fragment was 486 bp. After the restriction endonuclease MseI treatment, subjects with GG genotype appeared a fluorescence band at 486 bp, the AG genotype showed fluorescence bands at 486 bp, 347 bp and 139 bp, while the AA genotype at 347 bp and 139 bp. When the amplified fragment was digested by restriction enzyme Bbv1, subjects with TT genotype were indicated by the fluorescence band at 486 bp, the CC genotype was found at 257 bp and 229 bp, and

Table 1. Overall composition of gastric cancer and control subjects

Related factors	Gastric cancer group (n = 116)	Control group (n = 138)	χ^2	P
Gender			0.320	0.572
Male	82 (70.7%)	93 (67.4%)		
Female	34 (29.3%)	45 (32.6%)		
Age (year)			2.729	0.157
Range	26~82	31~87		
Average	51.4 ± 2.5	53.9±1.6		
Smoking			1.252	0.263
Smoking	15 (37.5)	16 (40.0)		
Non-smoking	11 (27.5)	10 (25.0)		
Drinking			0.084	0.772
Drinking	50 (43.1%)	57 (41.3%)		
Non-drinking	66 (56.9%)	81 (58.7%)		

Table 2. Hardy-Weinberg equilibrium test of -1661A/G and -1722T/C in the control group

Genotypes	Theoretical value	Actual value	χ^2	P
-1661A/G			0.310	0.856
AA	96	92		
AG	38	41		
GG	4	5		
-1722T/C			0.629	0.730
TT	50	56		
TC	66	63		
CC	22	19		

the TC genotype showed fluorescence bands at 486 bp, 257 bp and 229 bp.

Statistical methods

The data were analyzed with the use of statistical software SPSS version 17.0. All measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± sd). The χ^2 test was utilized to analyze the general information of all subjects and compare the distribution of genotypes and alleles between groups. Besides, the χ^2 test was used to detect whether the genotype distribution of -1661 and -1772 sites in the control group satisfied the Hardy-Weinberg equilibrium law. The logistic regression analysis was used to calculate the interaction between gene polymorphisms and the risk of gastric carcinoma, and the relative risk is represented by the OR value and 95% confidence interval (95% CI). $P < 0.05$ was considered statistically significant.

Results

General information

Clinical data of 116 patients with gastric cancer and 138 healthy controls were collected and analyzed by retrospective case-control study. There were 82 males and 34 females in the gastric cancer group, aged 26-82 years, with an average age of 51.4 ± 2.5 years. Additionally, there were 93 males and 45 females in the control group, aged 31-87 years, with an average age of 53.9 ± 1.6 years. In the analysis of smoking status, there were 28 cases of smoking and 88 cases of non-smoking in the gastric cancer group while 42 cases of smoking and 96 cases of non-smoking in the control group. As for the drinking condition, there were 50 drinkers and 66 non-drinkers in the gastric cancer patients while 57 drinkers and 81 non-drinkers in the healthy subjects. Baseline data of subjects in the gastric cancer group and the control group were equally comparable, and the differences were not statistically significant (all $P > 0.05$), as shown in **Table 1**.

Hardy-Weinberg equilibrium test in the control group

By searching the NCBI-SNP database, the frequency of CTLA-4 -1661A/G (rs4553808) allele in East Asian population was A = 0.834, G = 0.166, and the frequency of CTLA-4 -1722T/C (rs733618) allele was C = 0.400, T = 0.600. The theoretical values of genotypes at the -1661 locus in the control group were calculated as AA = 138 * 0.834 * 0.834 = 96, AG = 138 * 0.834 * 0.166 * 2 = 38, GG = 138 * 0.166 * 0.166 = 4. The theoretical values of genotypes at the -1722 locus in the control group were TT = 138 * 0.600 * 0.600 = 50, TC = 138 * 0.600 * 0.400 * 2 = 66, CC = 138 * 0.400 * 0.400 = 22. The differences between theoretical value and actual value were compared by χ^2 test. The results showed that distribution of CTLA-4 -1661A/G and -1722T/C gene polymorphisms in the control group met the Hardy-Weinberg equilibrium law ($P > 0.05$, the difference was not statistically significant). These data also indicate that subjects in the control group satisfied the genetic linkage equilibrium rule and had a good representativeness of population as shown in **Table 2**.

Table 3. Distribution of genotype and allele at CTLA-4 -1661A/G locus

Genotypes	Gastric cancer group (n = 116)	Control group (n = 138)	χ^2	P
-1661A/G			13.261	0.001
AA	58 (50.0%)	92 (66.7%)		
AG	40 (34.5%)	41 (29.7%)		
GG	18 (15.5%)	5 (3.6%)		
Allele			13.709	< 0.001
A	156 (67.2%)	225 (81.5%)		
G	76 (32.8%)	51 (18.5%)		

Table 4. Distribution of genotype and allele at CTLA-4 -1722T/C locus

Genotypes	Gastric cancer group (n = 116)	Control group (n = 138)	χ^2	P
-1722T/C			12.257	0.002
TT	57 (49.1%)	56 (40.6%)		
TC	57 (49.1%)	63 (45.7%)		
CC	2 (1.8%)	19 (13.7%)		
Allele			6.158	0.013
T	171 (73.7%)	175 (63.4%)		
C	61 (26.3%)	101 (36.6%)		

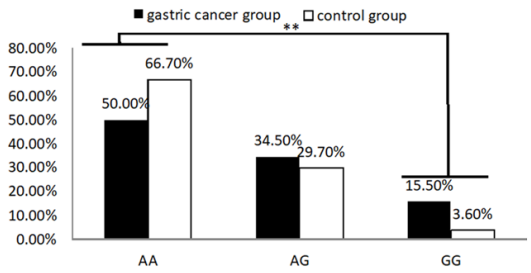

Figure 1. Distribution frequency of AA, AG and GG genotypes at the CTLA-4 -1661A/G locus. With AA genotype as reference, the distribution frequency of GG genotype in the gastric cancer group was higher than that in the control group, $^{**}P < 0.01$.

Distribution of genotype and allele at CTLA-4 -1661A/G locus

The genotypes of CTLA-4 -1661A/G locus included AA, AG and GG types. There were 58 (50.0%) cases of AA genotype, 40 (34.5%) cases of AG genotype and 18 (15.5%) cases of GG genotype in the gastric cancer group, while there were 92 (66.7%) cases of AA genotype, 41 (29.7%) cases of AG genotype and 5 (3.6%) cases of GG genotype in the group of healthy control. Frequency of allele A was 156 (67.2%) in the gastric cancer group and was 225 (81.5%) in the control group, and frequencies of allele G were 76 (32.8%) and 51 (18.5%) in both groups, respectively. The difference of genotype distribution at -1661 locus between the gastric cancer group and the control group was statistically significant ($\chi^2 = 13.261$, $P < 0.05$). In addition, the distribution of A/G alleles in the above two groups showed statistically significant differences ($\chi^2 = 13.709$, $P < 0.05$) as shown in **Table 3**.

Distribution of genotype and allele at CTLA-4 -1722T/C locus

The genotypes of CTLA-4 -1722T/C locus included TT, TC and CC types. There were 57 (49.1%) cases of TT genotype, 57 (49.1%) cases of TC genotype and 2 (1.8%) cases of CC genotype in the gastric cancer group, while there were 56 (40.6%) cases of TT genotype, 63 (45.7%) cases of TC genotype and 19 (13.7%) cases of CC genotype in the group of healthy control. Frequency of allele T was 171 (73.7%) in the gastric cancer group and was 175 (63.4%) in the control group, and frequencies of allele C were 61 (26.3%) and 101 (36.6%), respectively. The difference of genotype distribution at -1722 locus between the gastric cancer group and the control group was statistically significant ($\chi^2 = 12.257$, $P < 0.05$). Moreover, the distribution of T/C alleles in the above two groups indicated statistically significant differences ($\chi^2 = 6.158$, $P < 0.05$) as shown in **Table 4**.

Relationship between gene polymorphisms of CTLA-4 -1661A/G locus and gastric cancer

The data of genotype and allele distribution at CTLA-4 -1661A/G locus in the healthy subjects and the gastric cancer patients were included in the logistic regression analysis, and AA genotype was regarded as the corresponding reference. The results suggested that the distribution frequency of GG genotype in the gastric cancer group was much higher than that in the control group ($P < 0.05$), as shown in **Figure 1**. After analysis, it was found that the GG genotype at -1661A/G locus was closely related to gastric cancer, and the risk increased by 5.710 times (95% CI: 2.011~16.219, $P < 0.05$). Compared with the allele A, the allele G notably increased the risk of gastric cancer with an OR value of 2.149 (95% CI: 1.427~3.237, $P < 0.05$) as shown in **Table 5**.

Table 5. Relationship between gene polymorphisms of CTLA-4 -1661A/G locus and gastric cancer

Genotypes	Gastric cancer group (n = 116)	Control group (n = 138)	OR (95% CI)	χ^2	P
-1661A/G					
AA	58 (50.0%)	92 (66.7%)			
AG	40 (34.5%)	41 (29.7%)	1.548 (0.897, 2.671)	2.460	0.117
GG	18 (15.5%)	5 (3.6%)	5.710 (2.011, 16.219)	10.701	0.001
Allele					
A	156 (67.2%)	225 (81.5%)			
G	76 (32.8%)	51 (18.5%)	2.149 (1.427, 3.237)	13.422	< 0.001

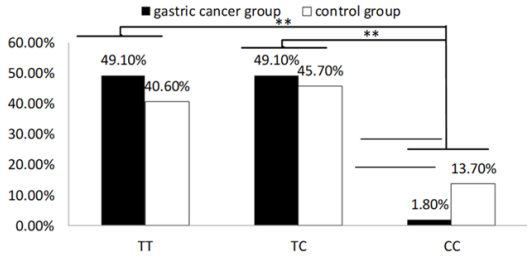

Figure 2. Distribution frequency of TT, TC and CC at the CTLA-4-1772T/C locus. With CC genotype as reference, the distribution frequencies of TT and TC genotype in the gastric cancer group were higher than that in the control group, $^{**}P < 0.01$.

Relationship between gene polymorphisms of CTLA-4-1772T/C locus and gastric cancer

The data of genotype and allele distribution at CTLA-4-1772T/C locus in the healthy subjects and the gastric cancer patients were included in the logistic regression analysis, and CC genotype was regarded as the corresponding reference. The results suggested that the distribution frequencies of TT and TC genotypes in the gastric cancer group were higher than that in the control group ($P < 0.05$), as shown in **Figure 2**. In comparison with the CC genotype, subjects carrying TT and TC genotypes of CTLA-4 -1722 locus increased the risk of gastric cancer to 9.670 times (95% CI: 2.151~43.465) and 8.595 times (95% CI: 1.917~38.538), respectively, (both $P < 0.05$). Compared with allele C, the allele T markedly increased the risk of gastric cancer with an OR value of 1.618 (95% CI: 1.105~2.369, $P < 0.05$) as shown in **Table 6**.

Discussion

The generation of malignant tumors is essentially a malignant process in which normal cells lose the ability to regulate proliferation and growth at the genetic level under various carcinogenic factors, resulting in constant proliferation. The occurrence and development of gastric cancer, a common malignant tumor of the digestive system, involve multiple genes, multiple links and multiple factors including environmental and genetic factors [12-14].

CTLA-4 is an immunoregulatory molecule expressed on the appearance of activated $CD4^+$ and $CD8^+$ T cells. As a receptor of B7 molecule, activated CTLA-4 could reduce the function of T cells and inhibit T cells signal transduction, and thus participate in the negative modulation of immune response. When expression of CTLA-4 on the surface of T cells is increased, more inhibitory second signaling molecules will be generated, thereby down-regulating the activation of T cell and reducing body's ability to resist tumors [15-17].

Here, a case-control study was conducted on the distribution of CTLA-4 -1661A/G and -17222T/C gene polymorphisms between the gastric cancer patients and the healthy subjects. Compared with healthy individuals, the distribution frequency of AA genotype at the -1661 locus in the promoter region of CTLA-4 gene was significantly decreased in the gastric cancer patients, while the frequencies of AG and GG genotypes were dramatically increased. The individuals carrying GG genotype increased the risk of gastric cancer to 5.710 times compared with AA genotype (95% CI: 2.011~16.219). Compared with allele A, allele G notably increased the risk of gastric cancer with an OR value of 2.149 (95% CI: 1.427~3.237). In addition, the frequencies of TT and TC genotypes at CTLA-4 -1722 locus were significantly increased. The risk of gastric cancer in individuals with TT and TC genotypes increased by 9.670 (95% CI: 2.151-43.465) and 8.595 times (95% CI:

Table 6. Relationship between gene polymorphisms of CTLA-4-1772T/C locus and gastric cancer

Genotypes	Gastric cancer group (n = 116)	Control group (n = 138)	OR (95% CI)	χ^2	P
-1722T/C					
TT	57 (49.1%)	56 (40.6%)	9.670 (2.151, 43.465)	8.755	0.003
TC	57 (49.1%)	63 (45.7%)	8.595 (1.917, 38.538)	7.896	0.005
CC	2 (1.7%)	19 (13.7%)			
Allele					
T	171 (73.7%)	175 (63.4%)	1.618 (1.105, 2.369)	6.115	0.013
C	61 (26.3%)	101 (36.6%)			

1.917~38.538), respectively. Compared with allele C, allele T markedly rose the risk of gastric cancer with an OR value of 1.618 (95% CI: 1.105~2.369).

This study suggests that the gene polymorphisms of CTLA-4 -1661A/G allele are associated with susceptibility to gastric carcinoma, and the threat of gastric cancer is elevated in individuals carrying AG and GG genotypes. Furthermore, the gene polymorphisms of CTLA-4 -1722T/C allele also lead to higher susceptibility of gastric carcinoma, and individuals with TT and TC genotypes have an elevated risk of gastric cancer. This study is basically in agreement with the analysis results at home and abroad [18-21].

The generation and development of gastric cancer are the consequence of interaction of multiple genes. In this study, gene polymorphisms at -1661 and -1722 sites in the CTLA-4 promoter region were found to be connected with the genetic susceptibility to gastric cancer. However, it is inconclusive whether there is a synergistic effect between the two genetic sites, which needs further researches. Furthermore, the role and effect of SNPs in the pathogenesis and treatment of gastric carcinoma remain to be further clarified.

In conclusion, this study suggests that the mutations at the loci of CTLA-4 -1661 and -1722 may be the possible mechanism of immune escape in gastric cancer, and provides new ideas for targeted therapy and individualized treatment of gastric cancer patients.

Disclosure of conflict of interest

None.

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