Original Article
Klotho gene polymorphisms are related to colorectal cancer susceptibility

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Abstract: Aim: The purpose of this study was to investigate the relationship of *Klotho* gene G-395A and C1818T polymorphisms with colorectal cancer (CRC) susceptibility. Methods: 125 CRC patients and 125 controls were enrolled in the study. G-395A and C1818T polymorphisms were genotyped with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology. Haploview software was utilized to conduct linkage disequilibrium and haplotype analysis. Odds ratio (OR) and 95% confidence interval (95% CI) were used to analyze the correlation of genotypes and haplotypes with CRC susceptibility. Results: AA and GA genotypes of G-395A polymorphisms were related with CRC risk (AA: OR = 4.161, 95% CI = 1.437-12.053; GA: OR = 1.958, 95% CI = 1.133-3.385). The frequency of A allele was much higher in case group, compared with controls (31.2% vs.17.6%) and the value of OR AND 95% CI suggested that A allele served as a risk factor for CRC (OR = 2.123, 95% CI = 1.393-3.236). Haplotypes analysis indicated that A-C and A-T haplotypes were significantly associated with risk of CRC (OR = 1.822, 95% CI = 1.124-2.954; OR = 2.877, 95% CI = 1.340-6.176). Conclusion: G-395A polymorphism of *Klotho* gene could increase the risk of CRC.

Keywords: *Klotho,* polymorphisms, colorectal cancer

Introduction

Colorectal cancer (CRC), a general term for colon and rectal cancer, is one of the most common malignant tumors of digestive tract [1]. It has been reported that CRC is the fourth most common cancer for men and the third most common cancer for women all over the world [2]. Its incidence shows a trend of rapid rising recently, especially in developing countries. Meanwhile, the exact underlying molecular mechanism about the development of CRC is still unclear, so the mortality rate of patients with CRC is high. Currently, with the development of molecular biochemistry, many studies found that the development of CRC was associated with multiple genes and mutations, such as *Bcl-xL* [3], *TIMP-1* and *SMAD3* [4], *MGMT* [5], *LYTK1* [6]. Therefore, we attempted to explore the role of *Klotho* polymorphisms in the pathogenesis of CRC and investigated if the gene could help in the diagnosis of high-risk population for CRC, which is vital for improving early

diagnosis and timely treatment of CRC patients [7, 8].

Found in 1997, *Klotho* gene is related to aging process [9, 10] and starts to attract people's attention on its influence on tumors in recent years [11, 12]. Multiple researches successively reported the relevance of *Klotho* gene to tumors like breast cancer, lung cancer, pancreatic cancer and colon cancer [12-15]. In addition, more than 10 mutations or single nucleotide polymorphisms (SNPs) have been reported in human *Klotho* gene. Polymorphisms of G-395A in the promoter region and C1818T in exon 4 have been reported to be associated with many physiological processes. For example, Shimoyama, et al found that *Klotho* gene SNPs G-395A and C1818T were associated with lipid metabolism in men, and glucose metabolism, bone mineral density and systolic blood pressure in women for Japanese [16]. But studies on the correlation of *Klotho* gene with CRC are few, so we determined to explore the

Table 1. Genotypes and alleles distribution of G-395A and C1818T polymorphisms

Genotype/ allele	Cases (n = 125%)	Controls (n = 125%)	χ^2	P value	OR (95% CI)
G-395A					
GG	62 (49.6)	86 (68.8)	-	-	1
GA	48 (38.4)	34 (27.2)	5.858	0.016	1.958 (1.133-3.385)
AA	15 (12.0)	5 (4.0)	7.779	0.005	4.161 (1.437-12.053)
G	172 (68.8)	206 (82.4)	-	-	1
A	78 (31.2)	44 (17.6)	12.534	0.000	2.123 (1.393-3.236)
C1818T					
CC	75 (60.0)	71 (56.8)	-	-	1
CT	43 (34.4)	48 (38.4)	0.380	0.538	0.848 (0.502-1.432)
TT	7 (5.6)	6 (4.8)	0.029	0.864	1.104 (0.354-3.445)
C	193 (77.2)	190 (76.0)	-	-	1
T	57 (22.8)	60 (24.0)	0.100	0.751	0.935 (0.618-1.415)

According to literature, primers were synthesized by Shanghai SANGON Biological Engineering Technology Service Co. Ltd. PCR primers for amplification of the *Klotho* SNPs (G-359A, C1818T) were: G-395A forward 5'-CGT GGA CGC TCA GGT TCA TTC-3' and reverse 5'-TCC CTC TAG GAT TTC GGC CAG T-3' C1818T forward 5'-CCC AGA TCG CTT TAC TCC AG-3' and reverse 5'-CAC TGG GGT GAT GTT GAC AC-3'. Every PCR reaction mixture was 20 µL, including 0.3 µl of each primer (10 µmol/L), 1.5 mmol/L of MgCl$_2$, 0.8 mmol/L of dNTP, 0.5 U of REDTaq DNA polymerase, 1 µL of genome DNA (80 µg/µl) and 15.7 µl of ddH$_2$O. PTC 200 Therma cycler was used. PCR procedure was as follows: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing for 45 s and extension at 72°C for 45 s, continuation at 72°C for 10 min after all cycles, and finally at 10°C. PCR products of G-395A and C1818T were digested by Hpy188III and Nsi endonucleases respectively. The digestion products were separated through 2% agarose gel electrophoresis with ethidium bromide staining.

relationship between risk of CRC and *Klotho* gene polymorphism (G-395A and C1818T).

Materials and methods

Objects of study

125 patients from the military general hospital of Beijing PLA, with a median age of 47 (22-65 years old), were collected in the study using case-control design. 125 controls were 25-63 years old with a median age of 51. Patients with CRC were diagnosed by histopathology. All patients were not related by blood and did not experience either radiotherapy or chemotherapy. Samples were collected in accordance with the national ethics standards for human genome study, and written informed consent from objects of study was obtained.

DNA extraction

5 mL venous blood from elbow was collected from every participator and performed anticoagulant through Ethylene Diamine Tetraacetic Acid (EDTA). DNA was extracted through phenol-chloroform method (using blood genomic DNA Extraction Kit by Shanghai Huashun Biological Engineering Co Ltd.). Genomic DNA was stored at -80°C until utilized.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Klotho gene polymorphism was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology.

Statistical analysis

The genotype distribution of control group was checked by Hardy-Weinberg equilibrium (HWE) using PLINK1.07 software (statistically significant difference with *P* < 0.05). The association between risk of CRC and *Klotho* polymorphisms was tested with odds ratio (OR) and 95% confidence interval (CI), calculated by χ^2 test. Haplotypes analysis was conducted with Haploview software. All the analses were conducted in PASW Statistics 18 software. *P*-value less than 0.05 was considered as significant level.

Results

Essential features of study objects

There are 125 CRC patients and 125 healthy individuals in the study. No significant differ-

Table 2. Haplotype analysis of G-395A and C1818T polymorphisms

Haplotype site1-site2	Cases (2n = 250)	Controls (2n = 250)	χ^2	P value	OR (95% CI)
G-C	141	156	-	-	1
G-T	31	50	2.174	0.140	0.686 (0.415-1.134)
A-C	56	34	6.011	0.014	1.822 (1.124-2.954)
A-T	26	10	7.866	0.005	2.877 (1.340-6.176)

ences were found between the two groups neither in age nor gender. The genotypes distributions of G-395A and C1818T polymorphisms of controls were accorded with HWE ($P > 0.05$). All samples had good representative.

Correlation analysis between Klotho polymorphims and CRC risk

The analysis results showed that the frequencies of AA and GA genotypes in G-395A site were significantly higher in CRC patients compared to controls ($P = 0.016$, $P = 0.005$, respectively). The result of OR and 95% CI indicated that persons with AA and GA genotype are more likely to get CRC (AA: OR = 4.161, 95% CI = 1.437-12.053; GA: OR = 1.958, 95% CI = 1.133-3.385). Meanwhile, we found that A allele was also a risk factor for CRC (OR = 2.123, 95% CI = 1.393-3.236). However, the genotypes and alleles distribution C1818T polymorphism had no significant difference between two groups ($P > 0.05$) (**Table 1**).

Analysis of haplotype frequency of Klotho gene

Linkage disequilibrium analysis was performed on G-395A and C1818T sites of *Klotho* gene using HaploView software (**Table 2**). The analysis showed that G-395A and C1818T loci of *Klotho* gene formed 4 haplotypes. The correlation analysis suggested that A-C and A-T haplotypes could increase the risk for CRC (OR = 1.822, 95% CI = 1.124-2.954; OR = 2.877, 95% CI = 1.340-6.176). But other haplotypes showed no significant correlation with CRC susceptibility.

Discussion

CRC, developed in colon or rectum, is one of the most common malignant tumors, the morbidity of which is rising year by year. As its pathogenesis is extremely complex [17], deeper studies are required to improve the identification and treatment of patients with CRC. In recent years, many researchers focused on exploring new biomarkers that were used in early diagnosis and treatment of tumors. Lots of biomarkers have been found and *Klotho* gene is one of them.

The human *Klotho* gene located at chromosome 13q12, is composed of five exons and ranges over 50 kb. Found in 1997, *Klotho* is regarded as one anti-aging gene by earliest studies [18-20]. Recently, researches on the functions of *Klotho* gene have found its close correlation with malignant tumors and possible functions varied in different tumors. Lee et al. reported that epigenetic silencing of *klotho* may occur during the late phase of cervical tumorigenesis, and consequent functional loss of *Klotho* may led to aberrant activation of the canonical Wnt pathway in cervical carcinoma [21]. Besides, *klotho* could inhibit multiple growth factor signaling pathways and serve as an endogenous anti-EMT factor in mice [22]. Meanwhile *klotho* plays an anti-oncogene in human lung cancer cell line A549 by inhibiting growth and promoting apoptosis [23].

However, there were few reports about the *klotho* gene and CRC. In this paper, a case-control involved 125 patients and 125 healthy persons was performed to analyze the correlation of *Klotho* polymorphisms (G-395A and C1818T) with CRC susceptibility. HWE test on controls showed that study population were representative. From the analysis, we found that AA and GA genotypes of G-395A served as risk factors for CRC and C1818T polymorphism showed no effects on the pathogenesis of CRC, which was consistent with the researches coronary heart disease, but not with vasospastic angina [24, 25]. In addition, the haplotype analysis indicated that A-C and A-T haplotypes were both significantly associated with CRC risk.

In conclusion, G-395A polymorphism in human *Klotho* gene was related with CRC risk. Since the precise function mechanism is still not clear, so further studies are required to clarify the issue.

Disclosure of conflict of interest

None.

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