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Research on the correlation between the polymorphism of the back-2 gene and osteoarthritis

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Abstract. – OBJECTIVE: The objective of the present study was to investigate the correlation between polymorphisms of the back-2 gene and osteoarthritis.

PATIENTS AND METHODS: We enrolled 76 patients with osteoarthritis who were admitted to our hospital between February 2014 and February 2015 for treatment as the observation group, and 46 healthy subjects as the control group. The analysis of back-2 gene polymorphisms (rs28502) was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). mRNA expression of the different genotypes was measured with reverse transcriptase-polymerase chain reaction (RT-PCR), and the protein expression of back-2 of different genotypes was measured with enzyme-linked immunosorbent assay (ELISA) and Western blotting.

RESULTS: At locus 173 of the back-2 gene, there were a total of three genotypes, i.e. CC, CT, and TT. The frequencies of these genotypes in healthy subjects and osteoarthritis patients were 9.5%, 82.2%, 8.3% and 47.4%, 7.5%, 45.1%, respectively. There was a significant difference ($p<0.05$). However, the frequency of C/T in healthy older subjects and osteoarthritis patients was 50.6%, 49.4%, 51.15%, 48.85%, respectively, and there was no significant difference ($p>0.05$). RT-PCR showed no significant difference in mRNA expressions of the back-2 gene between the control group and observation group ($p>0.05$), although ELISA indicated that the protein expression of back-2 (12.3±0.36 μg/L) in osteoarthritis patients was significantly higher than in healthy subjects (1.52±0.18 μg/L) ($p<0.05$). Moreover, Western blotting analysis indicated that the protein expression of back-2 in osteoarthritis patients was significantly higher than in healthy subjects.

CONCLUSIONS: Genetic polymorphisms of back-2 are associated with the metabolic syndrome in older people, i.e. older people with the CC or TT genotypes may be at high risk for metabolic syndrome.

Key Words
Back-2, Gene polymorphism, Osteoarthritis, PCR-RFLP, Gene diversity.

Introduction

As a common orthopedic disease in the middle-aged and older population, osteoarthritis (OA) is characterized clinically by pain in bone and joints[1], decreased flexibility in action, and joint-friction in acute action[2]. Statistics revealed that the incidence of OA in China is about 3.42%, in which the middle-aged and older population account for 78.5% of those with OA[3]. With the advent of gray society[4], the aging population in China has been continuously increasing, which has contributed to the substantial increase in the middle-aged and older population with OA in China[5]. According to clinical studies[6] and statistics, OA is caused by complex factors, e.g. environmental factors such as dietary habits. Sagawa et al[7] that poor dietary habits such as insufficient intake of vitamins or excessive intake of meat can give rise to OA. Moreover, Scott et al[8] showed that genetic factors such as mutations may by closely associated with the onset of OA. Kumm et al[9] have also indicated that the back gene in humans is primarily involved in the metabolism of trace elements, such as phosphorus. In addition, Nguyen et al[10] indicated that the expression of relevant genes in the notch 1 signaling pathway in OA patients is significantly lower than in healthy subjects, while notch1 signaling can participate in the generation of osteoblasts and the regulation of calcium content in the cytoplasm. Qing et al[11] shown that the back gene primarily participates in bone metabolism-related processes. For example, mutation of the back gene can induce recessive genetic bone

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disease with clinical manifestations such as hyperphosphatemia and heterotopic ossification[12].

In this study, we investigated for the first time the correlation between the back gene and onset of OA, to provide theoretical and experimental evidence for the diagnosis and treatment of OA.

Patients and Methods

Patients

Seventy-six OA patients who were admitted to our hospital between February 2014 and February 2015 for treatment were enrolled as the observation group, in which there were 42 males and 34 females, with average age of 48.3±14.2 years. Simultaneously, we selected 46 healthy subjects as the control group, in which there were 24 males and 22 females, with average age of 47.2±12.3 years. This study was approved by the Ethics Committee of the 97th Hospital of the PLA. Signed written informed consents were obtained from all participants before the study.

Inclusion Criteria

1. Patients who were diagnosed with OA according to the diagnostic criteria of OA of the Clinical Diagnosis and Treatment of Common Diseases in Elderly; 2. Patients with no other diseases; 3. Patients aged over 30 years.

Exclusion Criteria

1. Patients with OA; 2. Patients with other relevant diseases such as heart disease; 3. Patients aged less than 30 years.

Reagents

Molecular reagents used in this study, such as ExTaq enzyme, dNTPs, 6×buffer, and genome extraction kit were from Axygen (Tewksbury, MA, USA); agarose and GODVIEW were from Solarbio Biotech Co., Ltd. (Beijing, China); the remaining reagents were from Sangon Biotech (Shanghai, China).

Equipment

Reverse transcriptase-polymerase chain reaction (RT-PCR) apparatus (ABI, Waltham, MA, USA); protein electrophoresis apparatus (Beijing Liuyi, Beijing, China), multifunction microplate reader (Bio-Rad, Hercules, CA, USA).

Table I. Primer sequences of the back-2 gene.

Name of primer	Sequence
back-2-F	GCCAGGGCCCCTCCTTCAA
back-2-R	TACCCTCAGACCCACGAGT

Genome Extraction

In this study, the genome extraction kit was from Axygen (Tewksbury, MA, USA), and the procedures were carried out according to the manufacturer's instructions.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

The primers used in this study were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. The primer sequences are shown in Table I. The acquired PCR products were connected and transferred to DH5α cells for PCR verification and sequencing according to the relevant procedures involving gel extraction and connecting with the T-vector as described in the Molecular Cloning Manual.

Test of Genetic Polymorphisms of Back-2 Through Restriction Enzyme Digestion

The back-2 gene was amplified using PCR, and SEP1 endonuclease was used to digest the PCR products at the loci predicted by the primers[13].

Sequencing

E. coli cells, in which the plasmid had been inserted as the template, were used to carry out colony PCR verification, and delivered to Sangon Biotech (Shanghai, China) for sequencing.

RNA Extraction

RNA was extracted from blood samples collected from patients in the observation group and control group. The weight of the extracted RNA was assayed.

RT-PCR

To investigate the differences in mRNA expression of back-2 in the different groups, we performed RT-PCR with complementary Deoxyribose Nucleic Acid (cDNA) that was obtained from the reverse transcription of RNA as the template. The primer sequences are shown in Table II.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was carried out as previously described[14].

Table II. Primers for RT-PCR.

Name of primer	Sequence
q back-2-F	TGCTAGCTGATCGATCGATCGTCG
q back-2-R	CGTAGCTGATCGATGCTAGCTAGC
GAPDH-F	TGCTAGGCTAGGACGCTAGCTAC
GAPDH-R	CTGGGCTAGATCGACGAGAGCTC

Immunohistochemistry

Immunohistochemistry was performed according to the Guideline for Molecular Biology Experiment.

Statistical Analysis

All data were analyzed using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA). All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD) Post Hoc Test. Studies on the correlation between the gene and differentiated thyroid carcinoma were performed using odds ratio (OR) and 95% confident interval. OR value was calculated by unconditional logistic regression and the results were calibrated by other factors, such as sex. p-values < 0.05 were considered statistically significant

Results

PCR Amplification and Restriction Enzyme Digestion of Back-2 of Different Genotypes

Using total DNA extracted from the different subjects as template, and back-2-F/R as primers, we amplified the full-length sequence of the back-2 gene using PCR. The results of electrophoresis are shown in Figure 1. The amplified length of the back-2 gene was about 500 bp. Pm11 was used to digest the acquired PCR product (Figure 2). After digestion with Pm11, there were three bands corresponding to the digested products of the back-2 gene obtained through amplification of genetic samples from healthy subjects as template. The lengths were 500 bp, 300 bp, and 200 bp, respectively. Only one (500 bp) or two bands (300 bp and 200 bp) were identified with PCR digestion of the product of the back-2 gene in samples from OA patients as template.

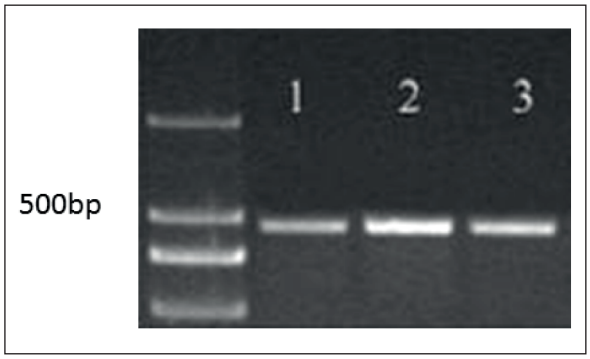

Figure 1. The results of electrophoresis of PCR products of the back-2 gene. 1: PCR amplification of the back-2 gene in healthy subjects; 2-3: PCR amplification of the back-2 gene in OA patients.

Sequencing Results of the PCR Product of Back-2 of Different Genotypes

Using the DNA from healthy subjects and OA patients, we performed PCR and obtained the back-2 gene that connected with T-19simple followed by sequencing. The results of sequencing

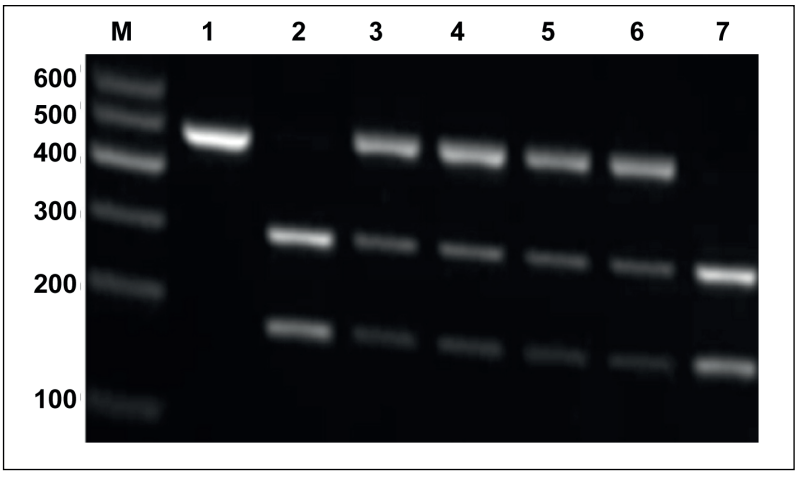

Figure 2. PCR digestion products of the back-2 gene in healthy subjects and OA patients. 1,2 and 7 represent the results of digestion with Pm11 of the amplified back-2 gene with DNA from OA patient as template; 3,4,5, and 6 represent the results of digestion with Pm11 of the amplified back-2 gene with the genome from OA patients as the template.

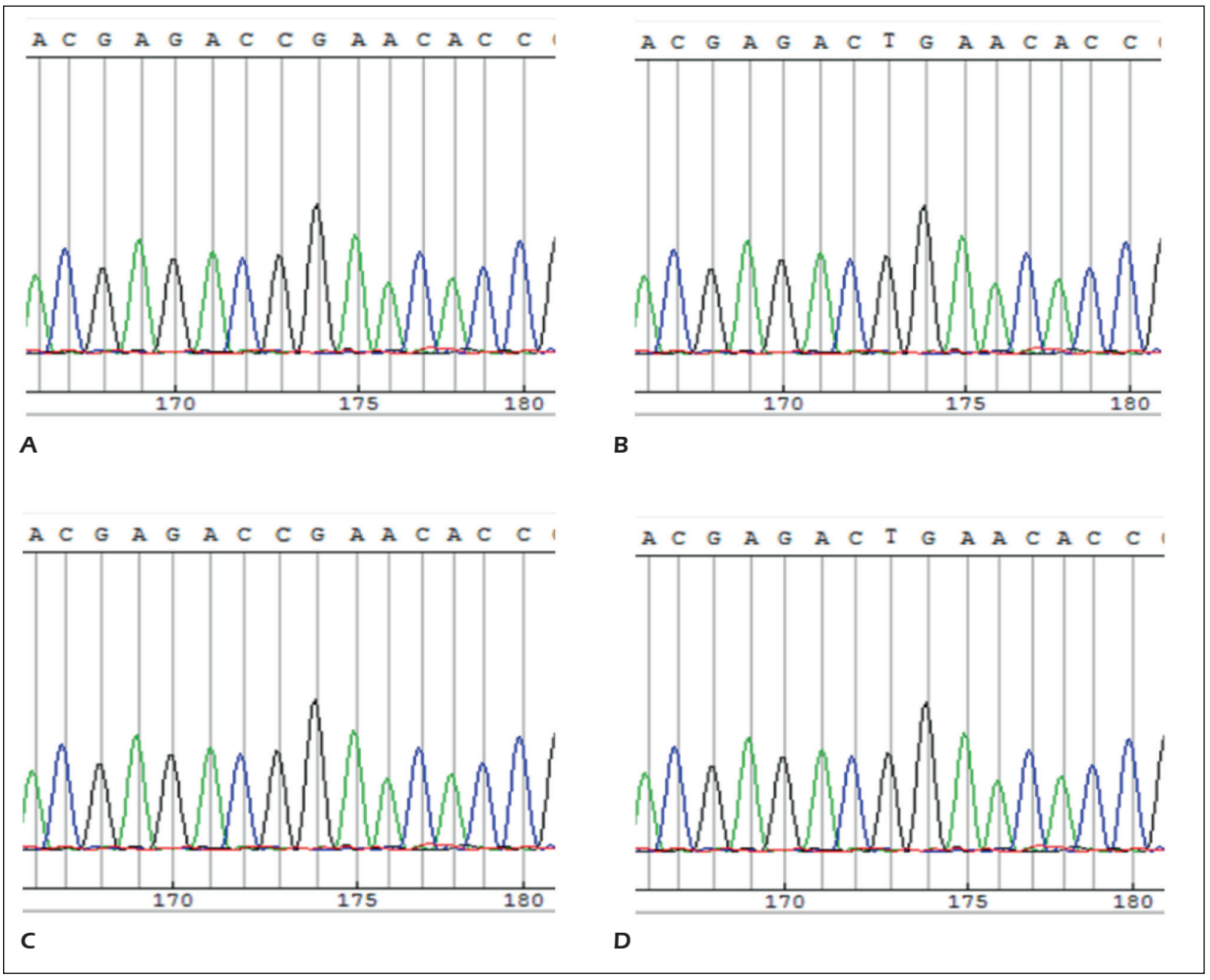

Figure 3. Sequencing results of the PCR product of back-2 in different genotypes. **A-B**, Sequencing results of the PCR product of back-2 in healthy subjects; **C-D**, Sequencing results of the PCR product of back-2 in OA patients.

are shown in Figure 3. The genotype of healthy subjects showed C/T at locus 173, while in OA patients there was TT or CC, indicating that there was a polymorphism at locus 173 in healthy subjects and OA patients.

Statistical Analysis of Alleles and Genotypes of The Observation Group and Control Group

Statistical analysis of the sequencing results of the healthy subjects and OA patients showed that the gene frequencies of C/T at locus 173 in subjects of the observation group were 50.6% and 49.4%, respectively, while the frequencies in OA patients were 51.15% and 48.85%, respectively (Table III). There was no statistically significant difference (p>0.05, Figure 3). The statistical analysis of gene frequencies of back-2 in healthy subjects and OA patients showed that there were three genotypes at locus 173 of the back-2 gene, i.e. CC, CT, and TT, and the genotype frequencies in healthy subjects and OA patients were 9.5%,

Table III. Statistical analysis of allele frequency in the control group and observation group.

Group	Cases (N)	Allele	Frequency (%)	χ^2	*p*
		T	C		
Control group	76	50.6	49.4	14.04	0.105
Observation group	46	48.85	51.15		

Table IV. Statistical analysis of allele frequency in the control group and observation group

Group	Cases (N)	Genotype frequency		(%)	χ^2	p
		CC	CT	TT		
Control group	76	9.5	82.2	8.3	16.054	0.000
Observation group	47.4	7.5	45.1	7.8		

82.2%, 8.3% and 47.4%, 7.5%, 45.1%, respectively (Table IV). The differences were statistically significant (p<0.05).

Results of RT-PCR of the Back-2 Gene of Different Genotypes

Using total RNA extracted from healthy subjects and OA patients, we carried out RT-PCR to measure mRNA expression of the back-2 gene in the different samples (Figure 4). There was no significant difference in mRNA expression of the back-2 gene between healthy subjects and OA patients (p>0.05).

Protein Expression of Back-2 of the Different Genotypes

Using total protein extracted from the cells from healthy subjects and OA patients, we assayed the protein expression of back-2 using ELISA (Figure 5). The protein expression of back-2 (12.3±0.36 μg/L) in OA patients was significantly higher than in healthy subjects (1.52±0.18 μg/L) (p<0.05), indicating that the occurrence of OA is associated with the protein expression of back-2.

Protein Expression of Back-2 of the Different Genotypes By Western Blotting

Using protein extracted from the cells from healthy subjects and OA patients, we assayed the protein expression of back-2 by Western blot (Figure 6). The protein expression of back-2 in OA patients was significantly higher than in healthy subjects (p<0.05). This observation was consistent with the results obtained with ELISA.

Discussion

The major clinical symptoms of OA include articular degeneration, and variations in articular cartilage. Synovial and cartilage structures can be gradually observed with the exacerbation of disease, which manifest as restricted joint motion and joint friction that are caused by long-term exercise. Statistics[15] have shown there are roughly 30 million Chinese patients suffering from arthri-

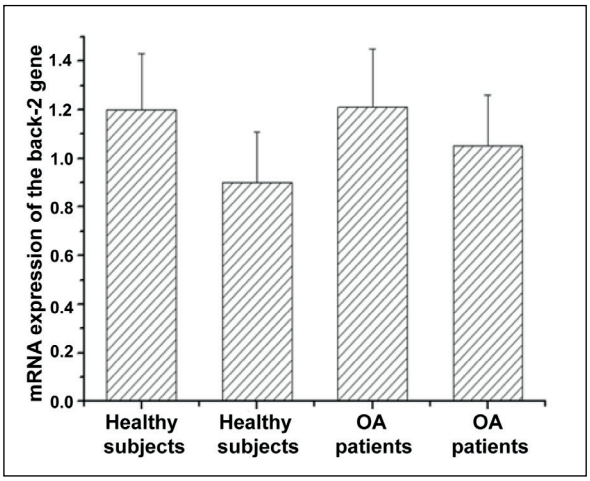

Figure 4. RT-PCR results of back-2 gene expression of the different genotypes.

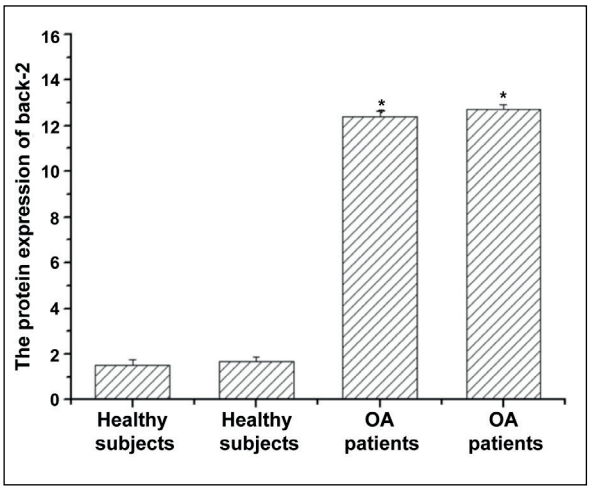

Figure 5. Protein expression of back-2 gene of the different genotypes. *Statistically significant difference between groups.

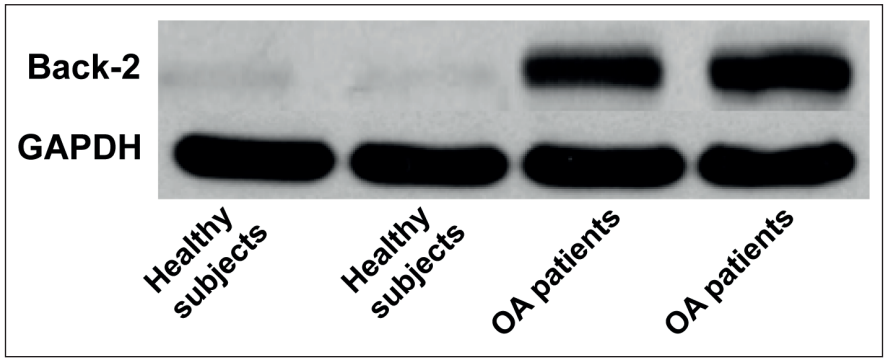

Figure 6. Protein expression of back-2 of the different genotypes using Western blot.

tis, and they are mostly in the middle-aged and older population. Therefore, increasing research on the pathogenesis and treatment of arthritis has become important for improving the efficacy of treatment. In recent years, with continuous discoveries and in-depth research on arthritis, researchers have found that arthritis is associated with various factors such as genetics, age, and sex[16]. With increasing age, gradual changes occur in the levels of hormones, and the rate of absorption of osteogenesis-related elements, such as calcium and phosphorus, decreases, which can lead to a reduction in bone density[17]. Research[18] has shown that inflammatory factor such as IL-1 in synovial cells and mononuclear macrophages, can largely promote the occurrence and exacerbation of arthritis. Further studies[19] have shown that chondral sclerosis induced by OA may be caused by the deficiency of osteogenesis-related elements in cartilage, such as calcium and phosphorus, although other studies have indicated that the back-2 gene is mainly involved in the cell transport and absorption of elements such as calcium and phosphorus. Experiments[20] have shown that in back-2 knockout animal cells, there was a significant decrease in the rate of absorption of calcium and phosphorus, although the rate of excretion was also significantly increased, indicating that proteins that are encoded by the back-2 gene participate in the cell absorption and excretion of calcium and phosphorus in rats. In this study, we found for the first time that there are a total of three genotypes at locus 173 of the back-2 gene, i.e., CC, CT, and TT. Comparisons of frequencies of these three genotypes between the healthy subjects and OA patients showed there was a significant difference ($p<0.05$). However, the comparison of the frequency of C/T in healthy older subjects and OA patients showed no significant difference ($p>0.05$). Measurement of the mRNA expression of the back-2 gene in healthy subjects and OA patients showed there was no significant difference, indicating that the different genotypes did not affect the transcription of the back-2 gene. However, ELISA and Western blotting analysis showed that there were significant differences in the protein levels of back-2 in healthy subjects and OA patients, suggesting that the different genotypes affected the function of the back-2 protein by affecting the translation of the back-2 gene, and its protein activity. However, we did not carry out in-depth analyses on the correlation between the different genotypes and the cell absorption of calcium and phosphorus. Therefore, these two important factors will addressed in future studies.

Conclusions

Genetic polymorphisms of back-2 are associated with the metabolic syndrome in older people, i.e. older people with the CC or TT genotypes may be at high risk for metabolic syndrome. Further studies were needed to explore the correlation between the different genotypes and the cell absorption of calcium and phosphorus in future.

Conflict of Interests:

The authors declared no conflict of interest.

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