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Original Article

Association of MTHFR and TNF-α genes polymorphisms with susceptibility to Legg-Calve-Perthes disease in Iranian children: A case-control study

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ABSTRACT

Objective: The aim of this study was to assess the association of MTHFR and TNF-α genes polymorphisms with Legg-Calvé-Perthes' disease (LCPD) risk in the Iranian children.
Methods: A total of 45 children with LCPD and 55 healthy controls were recruited to the study. Genotyping was performed via the RFLP-PCR method and genetic risk was calculated by odds ratio (OR) with its corresponding 95% confidence interval (CI).
Results & conclusion: Our case-control study failed to determine any association of MTHFR (677C > T and 1298A > C) and TNF-α (−308G > A and −238G > A) polymorphisms with LCPD risk. More studies with larger sample size are warranted to validate our findings.

1. Introduction

Legg-Calvé-Perthes' disease (LCPD) also known as the children of avascular necrosis is a childhood osteonecrosis of the hip. The annual incidence of LCPD ranged from 0.4 per 100,000 to 29.0 per 100,000 children < 15 years of age.[1] There is an increased incidence of LCPD in the urban population as compared to the rural population and occurs five times more commonly in boys than in girls.[2] Although the extensive literature pertaining to LCPD, there is still a gap in our knowledge about its risk factors, etiology and preventive strategies.[1,3,4] LCPD is a multifactorial disorder and apparently both genetic and environmental factors have been implicated in its etiopathogenic mechanisms.[2] However, a few familial cases with autosomal dominant inheritance due to mutations in a single gene such as COL2A1 have been reported.[5,6] The most common risk factors for LCPD include positive family history, ethnicity, gender, low birth weight, abnormal birth presentation, and second hand smoke.[7]

LCPD is initiated by a disruption of blood flow to the capital femoral epiphysis. Several studies have been reported a significant relationship between hypercoagulability and osteonecrosis.[8] Thus, it is hypothesize that variations in genes related to the endothelial cell, blood vessel repair and regeneration may be involved in the LCPD susceptibility. The MTHFR 677C > T and 1298A > C polymorphisms are associated with a reduced the MTHFR enzyme activity in the folate metabolic and elevated serum homocysteine level or hyperhomocysteinemia. Homocysteine directly damages the vascular matrix because it affects the biosynthetic and biochemical functions of the vascular cell. In addition, the increase in TNF-α expression induces the production of ROS (reactive oxygen species), resulting in endothelial dysfunction in many pathophysiological conditions.[9] However, the association of MTHFR (677C > T and 1298A > C) and TNF-α (−308G > A and −238G > A) polymorphisms with LCPD risk was still inconclusive. Thus, to scrutinize the association of most common polymorphisms of TNF-α and MTHFR genes with susceptibility to LCPD in the Iranian children, we performed this case-control study.

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2. Materials and methods

2.1. Study subjects

This study was approved by the Institutional Review Board and written informed consent was acquired from parents of each participant. A total of 45 children diagnosed with LCPD between January 2013 and April 2017 were retrospectively included in this study. The diagnosis was performed based on the standard clinical criteria, ultrasonographic examination and radiographic signs. A total of 55 healthy children with no history of orthopedic disease were recruited from the general population. All participants were recruited from different region of Iran. The case and controls were age-, gender-, and ethnicity-matched.

2.2. Polymorphism selection and genotyping

Four SNPs including the 677C > T and 1298A > C in MTHFR gene, and −308G > A and −238G > A in the TNF-α gene were chosen for genotyping based on their important roles in human body. Genomic DNA was extracted from peripheral blood using the Roche kit (Mannheim, Germany) following the manufacturer's instructions. The working DNA solution was stored at −20 °C until analyzed. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was utilized for SNPs genotyping following a modifying a previously described protocol. The PCR amplification reaction was performed in a total volume of 25 μl containing approximately 100 ng of genomic DNA. Size analysis of the digested fragments was resolved after separation of the PCR products digested in a 3% electrophoresis gel or polyacrylamide gel electrophoresis (PAGE) stained with ethidium bromide (EtBr) and then visualized on a UV transilluminator.

2.2.1. MTHFR 677C > T and 1298A > C genotyping

The primer sets used for MTHFR polymorphisms were as follows: MTHFR 677C > T (rs1801133) polymorphism: forward, 5′-TGAAGG AGAAGGTGTCTGCGGGA-3’; reverse, 5′-AGGACGGTGCGGT GCGGTG AGAGTG-3’; and MTHFR 1298A > C polymorphism: forward, 5′-CTT CTACCTGAAGAGCAAGTC-3’; reverse, 5′- CATGTCCACAGCATGGAG-3’. The cycling condition for MTHFR 677C > T polymorphism was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 60 s, 53 °C for 45 s, 72 °C for 60 s, and 72 °C for 5 min. The cycling condition for MTHFR 1298A > C polymorphism was as follows: 94 °C for 5 min; 30 cycles of 95 °C for 1 min, 60 °C for 1 min; and 72 °C for 3 min. The MTHFR 677C > T and 1298A > C polymorphisms PCR products of were then digested with the Hinf I and MboII restriction enzyme as per the manufacturer's instructions, respectively. The amplified MTHFR 677C > T region yielded a 198 bp products which the product was identified as homozygous (CC); three fragments of 198, 175, and 23 bp were identified as heterozygous (CT); and two fragments of 175 and 23 bp were identified as homozygous (TT) genotype. For MTHFR 1298A > C polymorphism: the products detected by 176, 30, 28, and 22 bp (A allele), or 176, 30, 28, and 22 bp (C allele).

2.2.2. TNF-α −308G > A and −238G > A genotyping

The primer sets used were as follows: TNF-α −308G > A (rs1800629) polymorphism forward, 5′-TGGAGTATGTCATCTGCCA CTG-3’; reverse, 5′-GATGGATGGTAGGGACTTTGAG-3’; and TNF-α −238G > A (rs361525) polymorphism forward, 5′-GGGATTACAAAA CCTGGCTG-3’; reverse, 5′-GAGAAGCTTACTCCAAGGAC-3’. PCR condition for TNF-α −308G > A polymorphism was as follows: after an initial denaturation step at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and final elongation cycle at 72 °C for 5 min. The cycling condition for TNF-α −238G > A polymorphism was as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s; and 72 °C for 5 min. The TNF-α −308G > A and TNF-α −238G > A PCR product were digested with 10 units of NcoI and MspA1I restriction endonuclease (RE) for more than 1 h, respectively. For TNF-α −308G > A the digestion produced an uncut 222 bp fragment from the mutant allele (A allele), and two 130 and 20 bp fragments from the wild-type allele (G allele). TNF-α −238G > A the digestion produced the digestion produced an uncut 150 bp fragment from the mutant allele (A allele), and two 130 and 20 bp fragments from the wild-type allele (G allele).

2.3. Statistical analysis

All statistical analyses were performed with the SPSS 20.0 software (SPSS Inc. Chicago, IL, USA), and P-values of less than 0.05 were considered significant. Genotype and allele frequencies of were calculated with direct counts. Differences in the demographics and genotypes between cases and controls were compared by chi-squared (χ^2) test and Fisher's exact test when appropriate. Hardy–Weinberg equilibrium for the genotype frequencies in controls was calculated by a goodness-of-fit $\chi 2$ test. Odds ratios (OR) and 95% confidence interval (CI) were calculated to assess the relative risk.

3. Results

3.1. Characterization of study subjects

In the present study, we compared the genotype and allele distribution of MTHFR and TNF-α polymorphisms between LCPD patients and healthy subjects. As shown in Table 1, a total of 45 LCPD patients (male/female: 27/18; mean age: 8.52 ± 3.22 years) and 55 healthy subjects (male/female: 32/23; mean age: 9.22 ± 2.28 years) were included in this study. There was no significant difference in age and sex distribution between LCPD patients and controls (Table 1).

3.2. Association of MTHFR polymorphisms with LCPD

Table 2 displays the estimated ORs and distribution of MTHFR 677C > T and 1298AC polymorphisms genotypes and allelic frequencies in LCPD patients and control subjects. The genotype frequencies of 677C > T and 1298AC polymorphisms in MTHFR gene was in agreement with the HWE among healthy controls (P = 0.741 and p = 0.121, respectively). However, no significant differences of SNP genotype and allele distribution for MTHFR 677C > T and 1298A > C polymorphisms were observed between the LCPD patients and controls (p > 0.05). Among the LCPD patients, the MTHFR 677C > T mutant allele frequency was 23.3% compared with 20.9% among the 55 control subjects. For MTHFR 1298AC, the mutant allele frequency was 26.8% compared with 23.6% among the 55 control subjects.

3.3. Association of TNF-α polymorphisms with LCPD

Table 3 displays the estimated ORs and frequency of TNF-α −308G > A and −238G > A polymorphisms in LCPD patients and control group. The genotype frequencies of −308G > A and

Table 1
Characteristics of the children with LCPD patients and healthy subjects.

Variables	Cases (n = 45)	Control (n = 55)	P-value
Age (± SD)	8.52 ± 3.22	9.22 ± 2.28	> 0.05
Gender			
Male	27 (60.0)	32 (58.2)	> 0.05
Female	18 (40.0)	23 (41.8)	> 0.05
Site of LCPD			
Right	21 (46.7)		
Left	16 (35.5)		
Bilateral	8 (17.8)		

Table 2
Distribution of MTHFR 677C > T and 1298A > C genotypes and allelic frequencies in LCPD patients and control subjects.

Genotypes	Cases (n = 45)	Control (n = 55)	OR (95% CI)	P-value
MTHFR 677C > T				
Genotype				
CC	27 (60.0)	34 (61.8)	Ref.	
CT	15 (33.3)	19 (34.6)	0.947 (0.412–2.178)	0.899
TT	3 (6.7)	2 (3.6)	1.893 (0.302–11.852)	0.495
Allele				
C	69 (76.7)	87 (79.1)	0.869 (0.444–1.699)	0.681
T	21 (23.3)	23 (20.9)	0.946 (0.474–1.888)	0.874
Dominant (TT + CT vs. CC)			1.079 (0.482–2.420)	0.853
Recessive (TT vs. CT + CC)			1.893 (0.302–11.852)	0.495
MTHFR 1298A > C				
Genotype				
AA	23 (51.1)	30 (54.5)	Ref.	
AC	20 (44.5)	24 (43.7)	1.033 (0.467–2.285)	0.935
CC	2 (4.4)	1 (1.8)	2.512 (0.220–28.634)	0.458
Allele				
A	66 (73.3)	84 (76.4)	0.851 (0.448–1.617)	0.651
C	24 (26.7)	26 (23.6)	1.175 (0.618–2.232)	0.623
Dominant (TT + CT vs. CC)			1.148 (0.521–2.528)	0.732
Recessive (TT vs. CT + CC)			2.512 (0.220–28.634)	0.458

OR: Odds Ratio; CI: Confidence Interval.

−238G > A polymorphisms in TNF-α gene was in agreement with the HWE among healthy controls (P = 0.338 and p = 0.169, respectively). However, No evidence was found for a significant difference in genotype distribution of TNF-α −308G > A and −238G > A polymorphisms between the LCPD patients and controls (p > 0.05). Among the LCPD patients, the TNF-α −308G > A the mutant allele frequency was 23.3% compared with 24.6% among the 55 control subjects. For TNF-α −238G > A, the mutant allele frequency was 18.9% compared with 16.4% among the 55 control subjects.

4. Discussion

Because of the importance of coagulation process in the pathophysiology of osteonecrosis, it is suggested that a number of genetic polymorphisms associated with thrombophilia have been studied as potential genetic modifiers of osteonecrosis. Studies have shown that the process causing to cell death in the femoral head of patients with osteonecrosis of the femoral head includes an increased rate of apoptosis rather than purely the necrosis of bone cells.[10,11] However, currently available published data on the association of genetic factors involved in the coagulation process and the risk of LCPD not enough to conclusion. We therefore performed a case-control study to provide evidences on the association of MTHFR and TNF-α polymorphisms with LCPD susceptibility in the Iranian children. This is first study the association of MTHFR and TNF-α in 45 children with LCPD in the Iranian and also Asians population.

Elevated levels of homocysteine (Hcy) have been linked to osteonecrosis.[12] in addition, it was reported that the MTHFR polymorphisms implicated in a wide variety of human thromboembolic diseases.[13] MTHFR is a key regulatory enzyme in folate and homocysteine metabolism. MTHFR gene is localized chromosomal region 1p36.3, has 11 exons and 2.2 kb in length.[14,15] Two common functional polymorphisms in the MTHFR gene, MTHFR 677C > T and 1298AC, are significantly reduced activity of the MTHFR enzyme. In 2015, Srzentić et al. performed a case-control with 37 patients and 50 controls about MTHFR 677C > T and TNF-α −308G > A polymorphisms and LCPD susceptibility in a Serbian population. They have not observed a significant association between MTHFR 677C > T polymorphism and

Table 3
Distribution of TNF-α −308G > A and −238G > A genotypes and allelic frequencies in LCPD patients and control subjects.

Genotypes	Cases (n = 45)	Control (n = 55)	OR (95% CI)	P-value
TNF-α -308G > A				
Genotype				
GG	26 (57.8)	30 (54.5)	Ref.	
GA	17 (37.8)	23 (41.8)	0.845 (0.377–1.892)	0.682
AA	2 (4.4)	2 (3.6)	1.233 (0.167–9.115)	0.838
Allele				
G	69 (76.7)	83 (75.4)	1.069 (0.556–2.055)	0.842
A	21 (23.3)	27 (24.6)	0.936 (0.487–1.799)	0.842
Dominant (AA + GA vs. GG)			0.877 (0.396–1.941)	0.746
Recessive (AA vs. GA + GG)			1.233 (0.167–9.115)	0.838
TNF-α -238G > A				
Genotype				
GG	29 (64.4)	37 (67.2)	Ref.	
GA	15 (33.3)	18 (32.8)	1.028 (0.445–2.375)	0.949
AA	1 (2.2)	0 (0.0)	3.742 (0.149–94.097)	0.423
Allele				
G	73 (81.1)	92 (83.6)	0.840 (0.405–1.744)	0.640
A	17 (18.9)	18 (16.4)	1.190 (0.573–2.471)	0.640
Dominant (AA + GA vs. GG)			1.134 (0.494–2.602)	0.766
Recessive (AA vs. GA + GG)			3.742 (0.149–94.097)	0.423

OR: Odds Ratio; CI: Confidence Interval.

LCPD susceptibility. Similarly, our findings showed that the MTHFR 677C > T and 1298A > C polymorphisms were not associated with LCPD risk in the Iranian children. However, the frequency of mutant allele of MTHFR 677C > T polymorphism in the Iranian LCPD children (23.3%) was lower than the Serbian population (32%). Moreover, several epidemiological and case-control studies have found that MTHFR polymorphisms might not play a significant role in susceptibility to ONFH.[8,16]

TNF-α is a pro-inflammatory molecule that may play an important role in the development of the immune response. In addition, TNF-α as an intercellular communicating mediator, is involved in building of transient or long-lasting cell to cell structures.[17] The TNF-α gene is approximately 3 kb in length, contains four exons and is mapped to major histocompatibility complex III (MHC III) region on chromosome 6p21.3.[18] Within the promoter of TNF-α gene there are two common SNPs increasing the expression level of TNF-α, such as TNF-α −308G > A and TNF-α −238C > T. In this study, we have not found significant association between TNF-α −308G > A and TNF-α −238C > T polymorphisms and LCPD risk. Our result on TNF-α −308G > A polymorphism is consistent with the previous study performed by Srzentić et al., in a Serbian population with LCPD.[11] However, previous studies showed that TNF-α −308G > A and TNF-α −238C > T polymorphisms were significantly associated with increased risk of non-traumatic osteonecrosis of the femoral head (NONFH) and osteonecrosis of femoral head (ONFH).[19,20]

The present case-control study has a number of strengths and some limitations. To the best knowledge, this is the first case-control study to assess the association of MTHFR 1298A > C and TNF-α −238G > A polymorphisms with the risk of LCPD. In addition, this is the first case-control study on MTHFR and TNF-α polymorphisms association with LCPD among Iranian children and also Asians populations. However, in the present study the number of cases and controls is small and not sufficient enough to get a conclusive result. Small sample size is often accompanied with selection biases and lacks sufficient power to support or deny an association. In addition, in the present study, the serum level of homocysteine and TNF-α were not evaluated in LCPD patients.

5. Conclusion

Our findings indicate that MTHFR and TNF-α polymorphisms not statistically confer susceptibility to LCPD in the Iranian children. However, the present study and also the previous study results may be underpowered because the sample size of the LCPD patients and healthy subjects were small. In addition, the present study control group may not always be truly representative of the Iranian general population as Persian, because they are from different ethnicity including Azeri, Kurdish, Lurs and so on. Therefore, a methodologically preferable design with large samples is needed to avoid selection bias and to increase the statistical power.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jor.2018.08.042.

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