Association of *MTHFR 677C>T, 1298A>C* and *MTR 2756A>G* Polymorphisms with Risk of Retinoblastoma

Asociace polymorfizmů *MTHFR 677C>T, 1298A>C a MTR 2756A>G* s rizikem rozvoje retinoblastomu

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Summary

Background: The MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms have been investigated in several different cancer types. However, the role of these polymorphisms in the development of retinoblastoma remains unclear. Here, we have evaluated the association of the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms with the risk of retinoblastoma in Iranian children. ***Methods:*** The MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms in 66 patients with retinoblastoma and 99 age- and gender-matched healthy controls were detected on the ABI PRISMs 7500 Real-Time PCR System. The association between these polymorphisms and the risk of retinoblastoma was analysed by an odds ratio with a 95% confidence interval. ***Results:*** Our results showed a significant association between the MTR 2756A>G polymorphism and the risk of retinoblastoma. In the MTR 2756A>G polymorphism, the AG (39.4%) and GG (9.1%) genotype frequencies in the cases were found to be higher in comparison with the controls, showing a significant difference ($p < 0.05$). However, no significant difference was observed in the allelic or genotypic frequencies for both the MTHFR 677C>T and 1298A>C polymorphisms in the retinoblastoma patients of the controls ($p > 0.05$). ***Conclusions:*** Our results suggested that the MTR 2756A>G polymorphism might be associated with an increased risk of retinoblastoma in Iranian children. However, the results show that the MTHFR 677C>T and 1298A>C polymorphisms are not significantly associated with an increased risk of retinoblastoma.

Key words

retinoblastoma – childhood – *MTHFR* gene – *MTR* gene – single-nucleotide polymorphism

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Souhrn

Úvod: U různých typů nádorových onemocnění byly studovány polymorfizmy MTHFR 677C>T, 1298A>C a MTR 2756A>G. Role těchto polymorfizmů v rozvoji retinoblastomu však zůstává nejasná. V této studie jsme hodnotili asociaci polymorfizmů MTHFR 677C>T, 1298A>C a MTR 2756A>G s rizikem rozvojem retinoblastomu u dětí v Íránu. ***Metody:*** Pomocí Real-Time PCR systému ABI PRISM 7500 byly zachyceny polymorfizmy MTHFR 677C>T, 1298A>C a MTR 2756A>G u 66 pacientů s retinoblastomem a 99 zdravých kontrolních subjektů odpovídajícího věku a pohlaví. Asociace mezi těmito polymorfizmy a rizikem rozvoje retinoblastomu byla analyzována pomocí poměru šancí s 95% intervalem spolehlivosti. ***Výsledky:*** Naše výsledky ukázaly významnou asociaci mezi polymorfizmem MTR 2756A>G a rizikem rozvojem retinoblastomu. V případě polymorfizmu MTR 2756A>G byly četnosti výskytu genotypu AG (39,4 %) a GG (9,1 %) statisticky významně vyšší v porovnání s kontrolním vzorkem ($p < 0,05$). U polymorfizmů MTHFR 677C>T a 1298A>C však nebyly pozorovány žádné významné rozdíly ve frekvenci alel nebo genotypové frekvenci mezi pacienty s retinoblastomem a kontrolními subjekty ($p > 0,05$). ***Závěry:*** Naše výsledky naznačují možnou asociaci polymorfizmu MTR 2756A>G se zvýšeným rizikem rozvoje retinoblastomu u dětí v Íránu. Výsledky však také ukázaly, že polymorfizmy MTHFR 677C>T a 1298A>C nejsou se zvýšeným rizikem rozvoje retinoblastomu významně spojeny.

Klíčová slova

retinoblastom – dětství – gen *MTHFR* – gen *MTR* – jednonukleotidový polymorfizmus

Introduction

Retinoblastoma is the most common intraocular tumour in children, representing approximately 3% of all childhood cancers between the ages of 0–14 years [1,2]. It is responsible for approximately 3,000 cancer-related childhood deaths worldwide each year [3]. Although retinoblastoma can occur at any age, 95% of the cases are presented in children under the age of 5. Its incidence is approximately 1 in 15,000–28,000 live births and represents 2–4% of all paediatric malignancies [4]. Worldwide, it is estimated that there are approximately 5,000–8,000 new cases diagnosed each year [5]. However, it is an example of successful cancer treatment with the overall survival exceeding 95% in developed countries [6].

The data suggest that retinoblastoma is not a homogeneous tumour on the genomic level, and that inter-tumour heterogeneity exists between retinoblastoma tumours [7,8]. The majority of heritable retinoblastoma cases (75% of heritable patients) have no affected parents and are therefore called non-familial heritable patients with *de novo* mutation. Classically, retinoblastoma results from biallelic loss of the retinoblastoma susceptibility gene (*RB1*). However, with the continued development of medical genetics, an increasing number of studies have focused on the identification of RB genetic biomarkers such as gene polymorphisms, oncogenes (*KIF14, MDM4, E2F3, MYCN* and *DEK*), potential tumour suppressors (NGFR and CDH11), microRNA and long non-coding RNA [7,9]. Among them, the C677T and A1298C polymorphisms in the *MTHFR* gene and the 2756A>G polymorphism at the *MTR* gene have been assessed as potential candidates. MTHFR is an enzyme that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methytetrahydrofolate, the carbon donor for the remethylation of homocysteine to methionine [10,11]. Moreover, the MTR product, a vitamin B_{12}-dependent enzyme, plays a crucial role in the folate metabolic network [12].

The *MTHFR* gene is localised on chromosome 1 at 1p36.6, which includes 11 exons spanning 2.2 kb [13]. Two polymorphisms, 677C>T (exon 4) and 1298A>C (in exon 7) in the *MTHFR* gene have been shown to have reduced MTHFR activity. The C677T polymorphism is a C to T transition at base pair 677 resulting in an alanine to valine substitution and the A1298C polymorphism is an A to C transition at base pair 1298 leading to a glutamate to alanine substitution [14,15]. Moreover, the *MTR* gene is mapped to chromosome band 1q43, close to the telomeric region of the long arm and the MTR 2756A>G polymorphism is an extensively investigated polymorphism. These polymorphisms have been increasingly attracting attention in different cancer types. In recent years, a few studies have explored the possible association of the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms with the risk of retinoblastoma and the results of these published studies remain inconsistent and inconclusive [16,17]. Therefore, the purpose of the present study was to analyse the association of the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms with the risk of retinoblastoma in Iranian children.

Materials and Methods

Study Population

The study procedures were approved by the Ethics Committee for Human Research of Shahid Sadoughi University of Medical Sciences and Bam University of Medical Sciences. Moreover, informed consent had been obtained from all the study subjects. A total of 66 children diagnosed with retinoblastoma (ranging between 1 month and 6 years of age) who were referred to the different clinics between 2013 and 2017 were included in this study. Ninety-nine age-, gender- and ethnicity-matched unrelated healthy children who visited the clinic, with no family history of cancer, originating from the same geographical regions were included.

SNPs Genotyping

Genomic DNA was isolated from the donated venous blood samples using a commercial DNA extraction kit according to the manufacturer's instructions and was stored at −20°C in a dedicated area that was only used for polymerase chain reaction (PCR). The MTHFR 677C>T (rs1801133), 1298A>C (rs1801131) and MTR 2756A>G (rs1805087) polymorphisms were detected on the ABI PRISMs 7500 Real-Time PCR System (PE Applied

Biosystems, Foster City, CA, USA). The primers, probes and reaction conditions were obtained on the basis of published data and synthesised by Applied Biosystems. Briefly, the assay was performed under universal conditions, and PCR reactions were carried out in a final volume of 5 mL containing 500 nM of each primer, 200 nM each probe, 25 mM each dNTP, 1 M Tris-HCL (pH 8.3), 1 M $MgCl_2$, 300 mM KCl, 100% glycerol, ROX reference dye, 1 U TaqMan Universal PCR Master Mix (Applied Biosystems) and 30 ng genomic DNA. The following thermal cycling condition was used: 60°C for 1 min and 95°C for 10 min (Taq activation), 50 cycles at 92°C for 15 s (denature), and at 60°C for 1 min (anneal/extend). After the PCR reaction, the plates were scanned by an allelic discrimination on the 7500 Real-Time PCR system using SDS 2.4 software for allelic discrimination (Applied Biosystems) to determine the genotypes by allelic discrimination. The genotyping accuracy was assessed by repeating 10% of the samples, which were selected randomly from both cases and healthy subjects, and a concordance of 100% was observed for both polymorphisms for all samples.

Statistical Analysis

The statistical analysis was performed on the software package SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 20.0 analysis was conducted to establish whether the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms are associated with retinoblastoma in patients. Pearson's chi-square ($\chi 2$) test was performed to assess the genotype frequency distribution for the different subject groups. The Hardy-Weinberg equilibrium (HWE) of the genotype distribution among the controls was tested by a goodness-of-fit $\chi 2$ test. The significance of the results was accepted if the p-value was less than 0.05. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate the strength of the associations of the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms with retinoblastoma.

Results

A total of 165 samples were included in this case-control study, including 66 childhood retinoblastoma cases and 99 healthy subjects. The characteristics of the retinoblastoma cases were summarised in Tab. 1. No significant differences were found between the retinoblastoma cases and controls in age and gender ($p > 0.05$). The distribution of the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms in the cases and controls are presented in Tab. 2. All the allelic and genotypic frequencies in the controls were in agreement with the HWE equilibrium ($p = 0.346$, $p = 0.613$ and $p = 0.239$, respectively), and the minor allele frequencies in the control group were 0.384, 0.613 and 0.163, respectively.

The frequencies of the MTHFR 677C>T genotypes for wild homozygotes (CC), heterozygotes (CT), and mutant homozygotes (TT) of the retinoblastoma cases were 36.7, 50.0, and 13.3%, respectively, whereas the healthy control groups showed 35.4, 52.5, and 12.1%, respectively. In the MTHFR 1298A>C polymorphism the frequency of AA, AC and CC was 59.1, 31.8 and 9.1%, respectively, in the cases and 57.6, 35.3 and 7.1%, respectively, in the controls. However, no significant risk of retinoblastoma was found to relate to the mutant genotypes of the MTHFR 677C>T and 1298A>C polymorphisms (Tab. 2). The allele frequency of both the alleles in the MTHFR 677C>T and 1298A>C polymorphisms among the cases and controls is given in Tab. 2. The values of the ORs with 95% CI for the MTHFR 677C>T and 1298A>C polymorphisms are presented in Tab. 2. Although the mutant homozygote and heterozygote genotype frequencies of both the polymorphisms in the cases were higher in comparison with the controls, the polymorphisms did not show a significant association between the cases and controls (Tab. 2).

In the MTR 2756A>G polymorphism, the AG (39.4%) and GG (9.1%) genotype frequencies in the cases were found to be higher in comparison with the controls, showing a significant difference ($p < 0.05$). However, the prevalence of the MTR 2756G allele was not significantly higher in the cases (0.132) (Tab. 2). Moreover, the values of the ORs with 95% CI were as follows: dominant model (AG/GG, OR 2.065, 95% CI 1.085–3.927, $p = 0.027$) and recessive model (AA/AG, OR 0.102, 95% CI 0.012–0.868, $p = 0.037$) (Tab. 2).

Tab. 1. Clinical characteristics of retinoblastoma patients.

Variables	Frequency (%)
Gender	
male	39 (59.1)
female	27 (40.9)
Age at diagnosis (months)	
< 24	45 (68.2)
> 24	21 (31.8)
Family history of retinoblastoma	
yes	11 (16.7)
no	55 (83.3)
Laterality	
unilateral	48 (72.7)
bilateral	18 (27.3)
Tumor aggression	
low	26 (39.4)
high	37 (60.6)

Discussion

Retinoblastoma is an embryonic malignant neoplasm of retinal origin [2,18]. Retinoblastoma tumours can be hereditary or spontaneous and they display a high degree of genetic heterogeneity [19]. Mutations in the *RB1* gene have been implicated to play a role in the heritable genetic form of this disease [7]. According to the statistics, about 40% of all retinoblastoma patients have a germline mutation in the *RB1* gene, although only less than 7% have a positive family history [20,21]. The risk of retinoblastoma is substantially increased when the subject has parents who have had retinoblastoma or are of an advanced parental age, as well as subjects who were conceived by *in vitro* fertilisation [22,23].

Tab. 2. Association of MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms with retinoblastoma risk.

Polymorphisms	Cases (n = 66)	Controls (n = 99)	Crude OR (95% CI)	P-value[a]
MTHFR 677C>T				
Genotype				
CC	24 (36.7)	35 (35.4)	Ref.	
CT	33 (50.0)	52 (52.5)	0.913 (0.488–1.707)	0.775
TT	9 (13.3)	12 (12.1)	1.165 (0.461–2.945)	0.747
Allele				
C	81 (66.4)	122 (61.6)	Ref.	
T	51 (41.8)	76 (38.4)	1.153 (0.728–1.826)	0.544
Genetic Mode				
dominant	42 (63.6)	64 (64.6)	0.976 (0.510–1.868)	0.942
recessive	57 (86.4)	87 (87.9)	0.858 (0.340–2.169)	0.747
MTHFR 1298A>C				
Genotype				
AA	39 (59.1)	57 (57.6)	Ref.	
AC	21 (31.8)	35 (35.3)	0.813 (0.416–1.586)	0.543
CC	6 (9.1)	7 (7.1)	1.337 (0.428–4.172)	0.617
Allele				
A	99 (81.1)	149 (75.2)	Ref.	
C	33 (18.9)	49 (24.8)	1.127 (0.675–1.884)	0.647
Genetic Mode				
dominant	27 (40.1)	42 (42.4)	0.940 (0.499–1.768)	0.847
recessive	60 (90.9)	92 (92.9)	0.761 (0.244–2.374)	0.638
MTR 2756A>G				
Genotype				
AA	34 (51.5)	68 (68.6)	Ref.	
AG	26 (39.4)	30 (30.3)	1.495 (0.777–2.875)	0.228
GG	6 (9.1)	1 (1.1)	9.800 (1.152–83.398)	0.037
Allele				
A	94 (77.1)	166 (83.8)	Ref.	
G	28 (22.9)	32 (16.2)	1.545 (0.877–2.724)	0.132
Genetic Mode				
dominant	32 (48.5)	31 (31.3)	2.065 (1.085–3.927)	0.027
recessive	60 (90.9)	98 (98.9)	0.102 (0.012–0.868)	0.037

[a]two-sided χ^2 test for the frequency distributions of genotype between cases and controls
OR – odds ratios, CI – confidence intervals

The genetic variants at the *MTHFR* gene have been implicated as risk factors for several types of tumours [24]. However, reports on the association of polymorphisms at the *MTHFR* gene with susceptibility of retinoblastoma are inconclusive. In the present study we evaluated the association of the MTHFR 677C>T and 1298A>C polymorphisms with the risk of retinoblastoma in central and southern Iranian children. Our results showed that the MTHFR 677C>T and 1298A>C polymorphisms were not significantly associated with an increased risk of retinoblastoma in the Iranian population. Similarly, de Lima et al. have investigated the roles of MTHFR 677C>T and 1298 A>C, MTR 2756A>G, RFC 80A>G and TYMS in retinoblastoma susceptibility in a population from north-east Brazil. They found that the MTHFR 677C>T and 1298A>C, RFC 80A>G and TYMS polymorphisms are not significantly associated with an increased risk of retinoblastoma [17]. Moreover, Bisht et al. have found a greater frequency of a mutant heterozygous genotype for both the MTHFR 677C>T and 1298A>C polymorphisms in retinoblastoma patients than in the healthy controls. Therefore, they have found a strong association of the MTHFR 677C>T and 1298A>C polymorphisms with retinoblastoma pathogenesis in an Indian population. They have suggested a possible and important role of a one-carbon metabolism pathway, methylation regulation, and an increased risk of retinoblastoma initiation [25]. Inconsistent with our results, the previous study in the Iranian population demonstrated that MTHFR 677C>T is associated with retinoblastoma. In 2016, Soleimani et al. conducted a case-control study of 96 patients with retinoblastoma and 204 healthy controls to investigate the association of the MTHFR 677C>T and 1298A>C polymorphisms with susceptibility of retinoblastoma in a northern population from Iran. Their results showed that the MTHFR 677C>T polymorphism was associated with the risk of retinoblastoma in the Iranian population. Moreover, they have reported that the T allele had a protective effect on the susceptibility of retinoblastoma [16]. Iran is a multi-national

or multi-racial community with different ethnic groups, including Persians, Azeri, Kurds, Gilakis, Mazandaranis, Lurs, Tats, Talysh, Turkmen, Arabs and Baloch [26]. Thus, the differences between our results and the previous study might be describing a distinct pattern of *MTHFR* gene polymorphisms in different ethnic groups in Iran. Moreover, other genetic and environmental factors may modify the effect of MTHFR polymorphisms in Iranian ethnic groups.

The *MTR* gene encodes a protein containing 1,265 amino acids (140.5 kDa), which plays a key role in DNA repair, maintaining adequate intracellular folate, methionine and normal homocysteine concentrations [18]. The *MTR* gene contains a common polymorphism at nucleotide 2756 (MTR 2756A>G), which promotes the substitution of aspartic acid with glycine residue, affecting the enzyme activity and inducing hyperhomocysteinemia and DNA hypomethylation [17,27]. Plenty of studies have found that *MTR* A2756G polymorphism has been associated with different types of cancers [12,27]. In this case-control study, the association between MTR 2756A>G polymorphism and risk of retinoblastoma was also investigated. Our results showed that the MTR 2756A>G polymorphism is significantly associated with an increased risk of retinoblastoma. Similarly, Akbari et al. have found a significant association between the MTR 2756A>G polymorphism and the risk of retinoblastoma in the Iranian patients [18]. Moreover, de Lima et al. have showed that this polymorphism is associated with an increased risk of retinoblastoma in the Brazilian population [17]. These findings suggested that the MTR 2756A>G polymorphism may be associated with the development of retinoblastoma, possibly by reducing S-adenosyl-methionine (SAM) levels and causing DNA hypomethylation.

In summary, the current study results showed that the MTHFR 677C>T and 1298A>C polymorphisms may not be associated with an increased risk of retinoblastoma in Iranian children. However, we have found that the MTR 2756A>G polymorphism is significantly associated with an increased risk of retinoblastoma in Iranian children. Moreover, the main limitation of this study is the small population size being analysed for the MTHFR 677C>T, 1298A>C and MTR 2756A>G polymorphisms. Therefore, to validate these associations and our findings further, large and well-designed epidemiological studies are warranted.

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