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Research paper

The effect of *methylenetetrahydrofolate reductase* polymorphisms on susceptibility to human papilloma virus infection and cervical cancer

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ABSTRACT

Cervical cancer is the third most common cancer among women worldwide. Several factors lead to cervical cancer, among which human papilloma virus (HPV) infection has a prominent role. Methylenetetrahydrofolate reductase (MTHFR) is crucial in folate metabolic pathway and plays an important role in DNA synthesis and DNA methylation. *MTHFR* gene polymorphisms, including C677T and A1298C, lead to reduced enzyme activity. This case-control study aims to illustrate the association between *MTHFR* gene polymorphisms and the risk of cervical cancer.

This study was conducted on 196 samples, which included 96 cervical biopsy samples compared to 100 Pap smear samples of normal healthy women without HPV infection. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for the *MTHFR* polymorphism detection, followed by fluorescent amplification-based specific hybridization PCR method to detect HPV16 and HPV18.

The results show that the *MTHFR* 677TT genotype plays a protective role in cervical cancer ($P = 0.0030$) (OR = 0.21, 95% confidence interval [CI]: 0.07–0.59). Furthermore, there was a strong significant association between *MTHFR* 1298CC genotype and the risk of cervical cancer (OR = 10.69; 95% CI: 4.28–26.71, $P = 0.0001$).

It can be concluded that A1298C polymorphism is a genetic risk factor for cervical cancer in the assessed Iranian population group. It seems that *MTHFR* 1298CC genotype is more susceptible to HPV 16 infection. Combination analysis of *MTHFR* C677T and A1298C polymorphisms revealed that combined *MTHFR* 677CC and 1298CC are strongly associated with a risk of cervical cancer.

1. Introduction

Nowadays, cervical cancer is considered a preventable cancer owing to the fact that cervical cancer starts from cells with precancerous changes and it takes several years to develop cancer (Canavan and Doshi, 2000). However, it is still the second most common cancer among Iranian women aged 15–45 years (Mortazavi et al., 2002). Unfortunately, accurate data does not exist about the incidence and mortality of this cancer in Iran (Behtash and Karimizarchi, 2007). Several factors appear to cause this cancer, such as the use of contraceptive pills for >5 years, multiple sexual partners, early start of sexual life, multiple pregnancies, diet, smoking, and infection with human papilloma viruses (HPV), among which HPV plays a prominent role (Luhn et al., 2013). HPV has been proven to play a particular role of cervical cancer worldwide. Approximately 70% of cervical cancer cases are known to be engendered by HPV high-risk types 16 and 18 (Kreimer et al., 2005). HPV leads to the development of cervical cancer by inactivating tumor suppressors such as P53 and retinoblastoma. Although HPV infection is the main cause of cervical cancer, only a minority of patients develop cervical cancer cells (Gillison et al., 2014). The metabolism of folate is essential for proper cellular function. Within the folate pathway, one of the most vital enzymes is methylenetetrahydrofolate reductase (MTHFR) (Tomita et al., 2013). The *MTHFR* gene encodes a key regulatory enzyme catalyzes the reduction of 5, 10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF). 5,10-methylene-THF is essential for conversion of uridilate (dUMP) to thymidilate (dTMP). In the second pathway, 5-methyl-THF plays as a methyl donor in the remethylation of homocysteine to methionine, and then is converted to *S*-adenosylmethionine (SAM). SAM is involved in methylation of cytosines in DNA and regulation of gene transcription. Several polymorphisms are reported within the *MTHFR* gene, including C677T and A1298C, which are the most frequent ones. *MTHFR* gene polymorphisms are involved in increased or reduced risk of various cancers by two mechanisms which are opposed to each other. *MTHFR* gene polymorphisms are associated with reduced enzyme activity. Low activity of MTHFR enzyme and folate deficiency decrease the amount of 5-methyl-THF for DNA methylation and uracil misincorporation into DNA (Blount et al., 1997; Duthie et al., 2002).

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These events cause abnormal gene expression, increased DNA damage, decreased DNA repair and consequently increased risk of carcinogenesis. On the other hand, reduced MTHFR activity increases the availability of 5, 10-methylenetetrahydrofolate which in turn increases the synthesis of dTMP from dUMP, decreases the incorporation of uracil into DNA, and protects against carcinogenesis (Thompson et al., 2001; Goyette et al., 1996). A transition from C-to-T at position 677 in exon 4, is conducive to the substitution of alanine for valine that affects the catalytic domain of the enzyme, leading to reduced enzyme activity (Frosst et al., 1995).

Another common polymorphism results in the substitution of alanine with cytosine at position 1298 bp, and glutamine will convert to alanine at amino acid 429. This nucleotide change takes place in exon 7 in the area of gene regulation (van der Put et al., 1998; Goyette et al., 1995). Previous studies have suggested that polymorphic variants in *MTHFR* gene may be correlated with hypomethylation, allowing for various cancers (Kang et al., 1991). Several studies have been conducted to examine the association between *MTHFR* polymorphism and cervical cancer; however, different results have been revealed in different populations. This is the first study performed in an Iranian population that shows the role of polymorphism *MTHFR* in developing cervical cancer. It is predicted that polymorphism in *MTHFR* gene could make the host body more susceptible to viral HPV infection, and hence increase chances of developing cervical cancer.

2. Materials and methods

2.1. Sample collection

This case-control study was approved by a local ethics committee and all samples were provided by the patients' agreement. All samples were obtained from the Pathology Department of Mirzakoochakkhan Gynecology Hospital in Tehran, Iran, during 2012–2014. The inclusion criteria were the age range 20–47 years, non-smoking, no contraceptive pills for more than five years, no previous treatment with folic acid and vitamin B12 pills, and no immune system disorders. Control group consisted of Pap smear samples ($n = 100$) of women with negative cytological smears attending a gynecology clinic for regular examinations, and without HPV infection in 36 genotypes (aged 20–47 years, mean 33.37 ± 6.4). Patient group samples included 96 paraffin-embedded cervical biopsies with a histological diagnosis of high-grade cervical intraepithelial neoplasia (CIN) III, and invasive cervical cancer (ICC) (aged 20–47 years, mean 35.09 ± 7.04). The pathologist initially reviewed all the retrieved slides associated with each sample, then the exact area of cervical tissue that was composed of cells with high grade CIN and invasive cancer was selected for cutting into 10-μm sections.

2.2. DNA extraction

All steps of DNA extraction were done under non-contamination protocols. In order to extract DNA from paraffin-embedded blocks of cervical tissue, deparaffinization of the samples was carried out according to standard methods (Chan et al., 2001). Genomic DNA was extracted from cervical tissues and smear samples by High Pure PCR Template Preparation Kit (Roche Molecular Biochemical, Mannheim, Germany), according to the manufacturer's instructions. The purity and the concentration of the extracted DNA were determined by DeNovix DS-11 Spectrophotometer (DeNovix Technologies, Wilmington, Delaware, USA). DNA samples were stored at −20 °C.

2.3. HPV detection and genotyping

By using HPV Direct Flow CHIP Kit PCR amplification, followed by reverse-dot blot hybridization, based on DNA-Flow Technology e-BRID System (MASTER DIAGNÓSTICA, 18,016 Granada, Spain) for selecting control group samples in terms of having negative test results in screening of 36 HPV genotypes (high-risk HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82; and low-risk HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81, 84, and 89), samples were amplified directly. Fluorescent amplification-based specific hybridization PCR (FLASH-PCR) Detection Kit (fluorescence end-point detection) was used for HPV 16 and HPV 18 detection in both case and control groups, according to the manufacturer's recommendations (DNA-technology, Moscow, Russia). PCR reactions were optimized with 10 μl of PCR buffer and 75 ng of genomic DNA.

The PCR conditions for both genotypes were as follows: 5 min at 95 °C for one cycle, and 40 s for 45 cycles at 93 °C, for 40 s at 64 °C, for 20 s at 72 °C, and final extension for 5 min at 72 °C, followed by one cycle for 40 s at 4 °C. Positive and negative controls were used from samples that had been previously tested. PCR product tubes were transferred to a Fluorescent Detector (FD)-12 (DNA Technology, Russia) for detection and interpretation of each tube followed by reading and analyzing with Gene V4 software. According to the protocol, the threshold values are 1.75–2.10 for a specific product and 2.50 for internal control (DNA-technology, Moscow, Russia). DNA probes were used for detection of PCR products of a specific sequence and internal control samples were labeled with fluorescent FAM and HEX labels, respectively.

2.4. MTHFR polymorphisms C677T and A1298C

The presence of the *MTHFR* C677T and A1298C polymorphisms were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR reactions were performed using the following primer pairs for C677T mutation: F: 5′-TGAAGGAGAAGGTGTCTGCGGG-3′; and R: 5′-AGGACGGTGCGGTGAGAGT-3′ (Abbate et al., 1998), and primers for A1298C polymorphism were amplified with the forward primer 5′-AAGGAGGAGCTGCTGAAGATG-3′ and the reverse primer 5′-CTTTGCCATGTCCACAGCATG-3′ (Ebrahimzadeh-Vesal et al., 2014).

The PCR amplification for both reactions was performed in a total volume of 25 μl containing 16.25 μl DDW, 2.5 μl 10× PCR buffer, 2 μl $MgCl_2$ (25 mM), 1 μl dNTP (2.5 mM), 0.5 μl Taq DNA polymerase, 1 μl of each primer and 50 ng (0.75 μl) DNA. The PCR conditions for both polymorphisms were as follows: an initial denaturation step at 94 °C for 5 min was followed by 30 cycles at 94 °C for 40 s, 67 °C for 40 s, and 72 °C for 40 s, with a final extension at 72 °C for 5 min.

The amplified fragment of 198 bp was digested with HinfI enzyme. Restriction digestion with HinfI (Fermentas, Germany) could differentiate *MTHFR* C677T polymorphism from normal sequence. Digestion of the PCR products was performed at 37 °C for 2 h. Separation of fragments was done by electrophoresis on 3% agarose gel stained with ethidium bromide. Presence or absence of different fragments was visualized under UV transilluminator. The presence of T allele at nucleotide 677 of the *MTHFR* gene produced a restriction site for the HinfI enzyme. Individuals homozygous for the T allele showed two bands of 175 and 23 bp. Individuals homozygous for the C allele showed a single band of 198 bp. Those heterozygous for both the C and T alleles showed three bands of 198, 175 and 23 bp.

The 237 bp PCR product was digested with MboII enzyme (Fermentas, Germany) at 37 °C for 2 h. Following digestion, the samples were evaluated in a 3% agarose gel stained with ethidium bromide. Digested products were separated by electrophoresis on agarose gel. Wild-type genotype (1298AA) resulted in 182, 28, and 27 bp fragments, and mutant genotype (1298CC) resulted in 210 and 27 bp fragments, following restriction enzyme digestion. Due to the small size of 28, 27 and 23 bp, they were not visible on agarose gel.

2.5. Statistical analysis

Descriptive statistics were used to describe the study population based on the case-control status.