STUDY ON IDENTIFYING THE C677T POLYMORPHISM OF THE MTHFR GENE BY THE PCR-RFLP TECHNIQUE IN PATIENTS WITH ABNORMAL SEMEN ANALYSES

Ha Thi Minh Thi, Nguyen Thi Nguyet Minh
Dept. Medical Genetics, Hue University of Medicine and Pharmacy

Abstract:
Background: The C677T polymorphism of the MTHFR gene is a risk factor of many diseases, including male infertility. This study is aimed at: (1) Improving a PCR-RFLP process using primers designed by the authors to identify the C677T polymorphism of the MTHFR gene; (2) Evaluating the frequencies of the C677T polymorphisms of the MTHFR gene in a volunteer group and in a group of males with abnormal semen analyses. **Materials and method:** DNA samples were extracted from the peripheral blood of 60 volunteers and 30 patients with abnormal semen analyses. Primers were designed using FastPCR software to improve the PCR-RFLP technique. The results of polymorphism were confirmed by DNA sequencing techniques. The prevalence of the C677T polymorphism of the MTHFR gene was evaluated. **Results:** We successfully designed primers to amplify a fragment of the MTHFR gene, including the C677T polymorphism and an obligatory restriction site of *Hin*fI (as an internal control). The MTHFR genotype frequencies in the volunteer group were: 71.67% (677CC); 25% (677CT); and 3.33% (677TT). These same genotype frequencies in patients with abnormal semen analyses were 66.67%; 30% and 3.33% respectively. In azoospermia patients, they were 53.34%; 40% and 6.66%; and oligospermia the frequencies were 80%; 20% and 0%. **Conclusion:** We successfully optimized the PCR-RFLP technique to identify the C677T polymorphism of the MTHFR gene. We evaluated the frequencies of the C677T s of the MTHFR gene in the volunteer group and a group of males with abnormal semen analyses.
Keywords: MTHFR gene C677T polymorphism, male infertility, PCR-RFLP

1. BACKGROUND

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in the folate metabolism process – an essential component for many functions of cells. This enzyme changes 5.10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the bioactive form. This process turns homocysteine into methionine; therefore methionine is used for the synthesis of proteins and fundamental compounds for the body as well [9].

In 1995, Frosst first discovered a genetic variation with the nucleotide C (cytosine) changing into T (thymine) at position 677, called the C667T polymorphism of the MTHFR gene in patients with vascular diseases [5]. Thence, this polymorphism was considered as a high risk factor in various diseases such as male infertility, recurrent miscarriage and colon cancer [3],[6]. With the purpose of facilitating study in the role of the C667T polymorphism of the MTHFR

gene in related diseases, a basic study aiming to establish a procedure about molecular biology techniques which help to identify this polymorphism is extremely necessary. C changing into T at nucleotide 677th of the MTHFR gene creates a new recognition site (GAGTC) for the *Hin*fI enzyme in this gene. Thus, using the PCR technique to amplify the MTHFR gene containing position 677, then digesting PCR products by *Hin*fI enzyme (PCR-RFLP technique) is the best selected molecular biology procedure in identifying the C667T polymorphism.

The C667T polymorphism of the MTHFR gene changes an amino acid at position 226 in each corresponding polypeptide chain, from alanine into valine. This change reduces methylenetetrahydrofolate reductase activity by approximately 70-80% in people with the 677TT homozygous genotype and 30-35% in people with the 677TT heterozygous genotype, resulting in a disorder of folate distribution in the cell [4]. Folate has a function as an antioxidant; therefore low folate levels in semen increases the risk of the destruction of sperm DNA. Additionally, lack of folate increases the chance of DNA breaking due to uracyl misinsertion instead of thymine [8]. Otherwise, the disorder in folate metabolism reduces the methyl donor, which is indispensable for nucleic acid methylation, and hence affects spermatogenesis [4],[7].

Consequently, in terms of mechanisms, the C667T polymorphism of the MTHFR gene is considered as a genetic marker in screening patients who have a decline in sperm count. Bezold (German 2001) was the first scientist who studied the C667T polymorphism of the MTHFR gene in male infertility and found a significant correlation between male fertility and the 677TT homozygous genotype. However, the studies of many researchers since then have been contradictory. Studies in Africa, India, and Southeast Asia show that the C667T polymorphism of the MTHFR gene is related to abnormal spermatogenesis, while most studies in Europe such as Italy, Holland have found no relationship [7]. Nevertheless, while doing a meta-analysis study, Tuttle Mann realized that there was a significant correlation between the C677T polymorphism and male infertility due to abnormal semen analysis [9].

In Vietnam, there has been no research of the C667T polymorphism of the MTHFR gene in groups of normal or abnormal people yet. Therefore, this study is very necessary and makes a significant contribution to the literature..

This study is aimed at:

1. Improving the PCR-RFLP process with primers designed to identify the C677T polymorphism of the MTHFR gene.

2. Evaluating the frequencies of the C677T polymorphisms of the MTHFR gene in a volunteer group and a patient group with abnormal semen analyses.

2. POPULATION AND METHOD

2.1. Population

- *Volunteer group:* 60 volunteers, including 30 males and 30 females who received a general examination at Hue University of Medicine and Pharmacy hospital and had no disease and already had children. Peripheral blood (1 ml) was taken from each person with anticoagulants EDTA in order to extract DNA.

- *Patient group with abnormal semen analyses*: 30 husbands with abnormal semen analyses who were members of infertile couples diagnosed by the WHO standard.

2.2. Method

2.2.1. DNA extraction: From 300 µl of peripheral blood according to standard protocol of mD_X InstaGene Genomic DNA kit (Cat. No. 7326028, Bio-Rad).

2.2.2. Perform PCR process to amplify a fragment of MTHFR gene containing nucleotide 677

- Primers were designed by FastPCR 5.3 software:

+ The MTHFR gene sequence (GB: AY338232.1) were found on GenBank. The 1020bp gene fragment containing the C677T polymorphism (from nucleotide 8221 to 9240) was used to design primers.

+ Sequences of selected fragments were imported to FastPCR software and the following command was entered: **–lpdl–350 –rpd550–1020**. The basic parameters were imported to design primers. After running the FastPRC program, the software displayed sequences of optimal primer pairs, and we then chose a primer pair: forward primer: 5' TCATGAGCCCAGCCACTCAC 3' and a reverse primer: 5' CAGCGAACTCAGCACTCCAC 3'

+ These sequences were sent to IDT company to order synthetic primers.

- PCR reaction components: 12.5µl GoTaq Green Master Mix 2 × (Promega); 1 µl forward primer (10 pmol/µl); 1 µl reverse primer (10 pmol/µl); 1 µl DNA template; 9.5 µl distilled water.

- Thermal cycling conditions: Denaturation at 95°C for 5 minutes was followed by 35 cycles at 95°C for 1 minute 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. A final extension of 8 minutes at 72°C.

- The reaction was performed by an Applied Biosystems 2720 thermal cycling machine at the Department of Medical Genetics, Hue University of Medicine and Pharmacy.

- PCR product was electrophoresed on 0.8% agarose gel, at 80V. The results were read under ultraviolet light after being dyed with ethidium bromide.

2.2.3. Digestion of PCR product by HinfI restriction enzyme

The reaction components: 11.5 µl PCR product, 2µl *Hin*fI enzyme, 1.5 µl buffer. The mixture was incubated overnight, at 37°C in a water bath. The digested products were separated by electrophoresis on 1.5% agarose gel, at 80V. We read the results under ultraviolet light after dyeing with ethidium bromide.

2.2.4. How to evaluate genotype

Table 2.1: Identifying genotype based on the size of digested products

Genotype	PCR product size	DNA fragment size after digesting PCR product by *Hin*fI enzyme
677CC	465	83; 382bp
677TT	465	83; 165; 217bp
677CT	465	83; 165; 217; 382bp

2.2.5. Verifying PCR-RFLP process and confirming the genotypes

We picked four PCR products (from two samples of 677CC, one sample of 677CT and one sample of 677TT) and sent them to another laboratory to identify the DNA sequence. The PCR product sequence was compared with the MTHFR gene sequence by the BLAST program to verify the specificity of the used primer pair. We compared the polymorphic results identified

due to the digestion of PCR product by the *Hin*fI enzyme with the results identified due to the sequencing.

3. RESULTS

3.1. The result of improving the PCR-RFLP technique

3.1.1. The result of performing PCR reaction with the designed primers

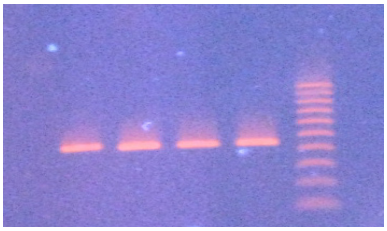

Fig. 3.1: Image of electrophoresed PCR product
Lanes 1 – 4: PCR product; lane M: standard 100 bp ladder. The bands of PCR product were clear and accored to the expected size (465 bp).

3.1.2. The result of performing the digestion of PCR product by HinfI enzyme

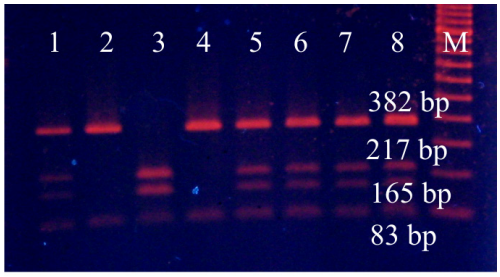

Fig. 3.2. Image of electrophoresed PCR product after digestion by HinfI enzyme

Lanes 1, 5, 6, 7, 8 have 4 bands with the size 83bp, 165bp, 217bp, 382 bp: genotype CT. Lanes 2, 4 have 2 bands with the size 83bp, 382 bp: genotype CC. Lane 3 has 3 bands with the size 83bp, 165bp, 217 bp: genotype TT. Digestion product by *HinfI* enzyme displayed clear bands, right size.

3.1.3. The result of verifying PCR-RFLP process and confirming C677T polymorphisms

The result of sequencing showed that the identifying C677T polymorphisms by PCR-RFLP process in 4 verified samples was correct.

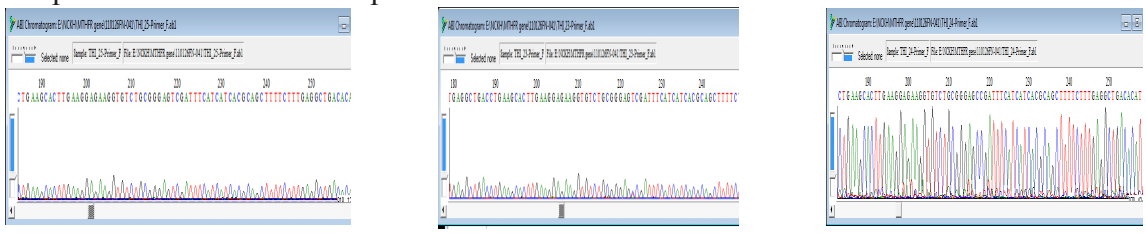

Fig. 3.3: Citation of the sequence of 677CC homozygous, 677CT heterozygous, 677TT homozygous samples

Comment: 677CC homozygous sample has only a nucleotide C peak (blue) at the corresponding position (arrow), and 677TT homozygous sample has only a nucleotide T peak (red), while 677CT heterozygous has two nucleotide C and T peaks (one blue peak and one red peak have the same height) at the same corresponding position.

3.2. The prevalence of polymorphisms

Table 3.1. The distribution of polymorphisms of volunteer group and Hardy-Weinberg equilibrium analysis

Geno-type	Observed frequency (%)	Expected frequency according to H-W (%)	C allele (%)	T allele (%)	p
677CC	43 (71.7)	42.5 (70.84)	86	0	0.631
677CT	15 (25.00)	15.99 (26.65)	15	15	
677TT	2 (3.33)	1.5 (2.51)	0	4	
Total	60 (100.00)	60 (100.00)	101 (84.17)	19 (15.83)	
			120 (100.00)		

Comment: The frequency of the 677TT homozygous genotype was very low (3.33%) as compared with 71.67% of the 677CC homozygous genotype. The distribution of CC, TT and CT genotypes in our research was in Hardy-Weinberg equilibrium (p > 0.05). The prevalence of the T allele only was 15.83%.

Table 3.2: The distribution of polymorphisms in the male volunteer group and the group of men with abnormal semen analyses

Genotype	Male volunteer		Abnormal semen analyses		p
	n	*%*	*n*	*%*	
677CC	22	73.34	20	66.67	> 0.05
677CT	7	23.33	9	30	
677TT	1	3.33	1	3.33	
Total	30	100	30		

Comment: There was no significant statistical difference in the C677T polymorphism frequencies between the male volunteer group and the group with abnormal semen analyses.

Table 3.3. The distribution of polymorphisms by the level of abnormal semen analysis

Genotype	Azoospermia		Oligospermia		p
	n	*%*	*n*	*%*	
677CC	8	53.34	12	80	> 0,05
677CT	6	40	3	20	
677TT	1	6.66	0	0	
Total	15	100	15	100	

Comment: Among 15 azoospermic people there were 7 people having the T allele (6 people with the 677CT heterozygous genotype and 1 person with the 677TT homozygous genotype), while among 15 oligospermic people there were only 3 people with the T allele (all of them have the 677CT heterozygous genotype, while no one had the 677TT homozygous genotype). However, there was no significant statistical difference in the frequencies of polymorphisms between two groups.

4. DISCUSSION

4.1. Optimizing the PCR-RFLP technique with own-designed primers to amplify the MTHFR gene fragment containing the 677th nucleotide

4.1.1. Designing primers

We used a 1020bp sequence containing the C677T polymorphism (from the 8221st nucleotide to the 9240th nucleotide) of the MTHFR gene which was provided by GenkBank for designing primers. In this sequence, the 677th position (this ordinal number just means on coding region – the exons) corresponds to the 8747th position of a completed gene. Thus, in the 677T polymorphism, the recognition site (it is also the restriction site) for the *Hin*fI enzyme includes 5 nucleotides GAGTC (from 8744 to 8748). This 1020bp sequence also contains two other recognition sites for the *Hin*fI enzyme: GACTC (from 8579 to 8583) and GATTC (from 9112 to 9116).

In this research, we found the GACTC recognition site (from 8579 to 8583) is rather near the C677T polymorphism (from 8744 to 8748), so we utilised this feature to design the

primer pair, creating a PCR product containing those two recognition sites. The GACTC recognition site exists in everybody, which means that PCR products are always digested in this site by the *Hin*fI enzyme. Thus it helped us have an internal control for the digestion reaction later. In order to create PCR products containing those two recognition sites for the *Hin*fI enzyme, we designed a forward primer located in the region from the 1st to 350th nucleotide, and a reverse primer located in 550th to 1020th nucleotide position of the selected 1020bp sequence. Thus, we used the following command: **"–lpdl–350 –rpd550–1020"**. All the data used in "PCR Primer or Probe Design Options" of FastPCR software is referenced from the basic standard of PCR primers.

After running the FastPCR software, we obtained some suggested primers, but we just chose the over 90% quality primer pairs. Simultaneously, the matched PCR products needed to satisfy this condition: the difference of size between the digestion products of the *Hin*fI enzyme (digestion of PCR products) had to be large enough (at least 50bp) in order for these products to be easily separated by electrophoresis on agarose gel. We selected the primer pair with the sequences shown in the method section (2.2). This primer pair amplified the DNA fragment of 465bp from the 8497th to the 8962nd position of the MTHFR gene.

The *Hin*fI enzyme recognizes and cuts at specific positions which have the 5'-GANTC-3' sequence of the DNA fragment. PCR products amplified from our own-designed primers always contain a recognition site of *Hin*fI – GACTC (from 8579 to 8583), and we called this the obligatory restriction site. This position will devide the PCR product into two fragments of 83bp and 382bp.

- If the PCR product was amplified from the 677T allele, there will be a second restriction site (GAGTC), and at this position the 382bp fragment will be divided into two fragments of 165bp and 217bp.

- If the PCR product was amplified from the 677C allele, there will not be the second restriction site (because the matched sequence is GAGCC, which is not a recognition site for *Hin*fI enzyme).

Therefore, the sizes of the products after digestion of the PCR product by the *Hin*fI enzyme will be clearly different between the 677CC, 677TT and 677CT genotypes, as shown in table 2.1. The difference between the sizes (83bp, 165bp, 217bp and 382bp) is large enough (more than 50bp) to be separated by electrophoresis on agarose gel.

The existance of an obligatory restriction site for enzyme *Hin*fI in PCR products serves as an internal control, which helps us confirm the activity of enzyme. If there is not a 83bp product, we know that the *Hin*fI enzyme is inactive. Obviously, designing the primer pair for amplifying DNA fragments with an obligatory restriction site beside the site of C677T polymorphism demonstrated the preeminence of this primer pair as compared with others that have no obligatory restriction site. It helped us exclude the incorrect polymorphisms (misindentified as the 677CC homozygous genotype in the case of inactive enzyme).

4.1.2. Optimizing the PCR-RFLP technique

Based on the data provided from the synthesized primer sequences, we selected the annealing temperature for PCR as 60°C. Figure 3.1 shows the obtained PCR products with clear bands with the size of 465bp, no nonspecific bands, an absence of primer dimers.

The results of sequencing PCR products of four selected samples also confirmed that those products were the expected fragments of the MTHFR gene. Thus, the PCR process used to amplify the desired fragment of the MTHFR gene conformed to the standard and the obtained PCR products could be used for subsequent molecular analysis.

Figure 3.2 shows that lanes 1, 5, 6, 7, 8 including 4 bands with sizes of 83bp, 165bp, 217bp and 382bp correspond with the 677CT heterozygous genotype. Lanes 2 and 4 including 2 bands with sizes of 83bp and 382 bp correspond with the 677CC homozygous genotype. Lane 3 includes 3 bands with sizes of 83bp, 165bp and 217bp corresponding with the 677TT homozygous genotype.

The sequencing of PCR products confirmed the 677CC, 677CT and 6677TT genotypes identified by the PCR-RFLP technique (Figure 3.3).

4.2. Evaluating the frequencies of the C677T polymorphisms of the MTHFR gene

4.2.1. In the volunteer group

The results of our study in Table 3.1 show that the frequency of the 677CC homozygous genotype at the 677th position of the MTHFR gene was the highest at 71.67%. The frequency of the heterozygous 677CT genotype was 25% and the least frequent was 677TT at 3.33%. The distribution of genotypes was in Hardy-Weinberg equilibrium ($p = 0.631$). The frequencies of the 677C allele and 677T were respectively 84.17% and 15.83%.

The study of Unfried (2002) with 74 normal women showed the frequencies of 677CC, 677CT and 677TT genotypes were respectively 62.2%; 32.4% and 5.4%. The frequency of 677T alelle was 21,6% [10]. There were no significant statistical difference in the frequencies between the Unfried's study and ours ($p = 0.2981 > 0.05$).

Bagheri's study (2010) on 216 normal people showed that the frequencies of 677CC, 677CT and 677TT genotypes were respectively 54.6%, 38% and 7.41%, and the frequency of 677T allele was 26% [1]. Thus, the frequency of the 677T allele of our study was lower than in this study ($p = 0.0283 < 0.05$). Bagheri quoted the frequencies of the 677T and 677C alleles in many countries, and the highest frequence of allele T was 58.69% (in Mexico), while the lowest was 4.5% (in Sri Lanka). Besides, this frequency was rather high in some countries such as Italy (44%), Korea (40.3%) and Japan (36.9%); and rather low in Kenya (4.9 %) and Indonesia (8.1 %). In some countries, this frequency was equivalent to our study, such as in African American groups (14%, $n = 496$; $p = 0.6862$) and in Yemen (17.4%, $n = 46$; $p = 0.9056$) and Britain (18.6%, $n = 94$; $p = 0.6384$).

4.2.2. In patients group with abnormal semen analyses

Table 3.2 showed the frequencies of the 677CC, 677CT and 677TT genotypes in the abnormal semen analysis group were 66.67%; 30% and 3.33%. This distribution showed no differences from the male volunteers group (73.34%, 23.33% and 3.33%). Analyzing the abnormal semen analysis group, we found that among 15 people who were azoospermic, there were 7 people having the T allele (6 people with the 677CT heterozygous genotype and 1 person with the 677TT homozygous genotype, the frequencies were 40% and 6.66%) as compared with 3 people having T allele (all of them had the heterozygous genotype, the frequency was 20%) out of 15 people who were oligospermic. However, our sample size was small so that we did not find significant statistical differences between the two groups. In the future, we shall continue the study with

bigger sample sizes in order to draw more accurate conclusions about the relationship between C677T and male infertility.

In summary, with this research we successfully optimized the PCR-RFLP technique to identify C677T polymorphisms of the MTHFR gene. We identified the frequencies of C677T polymorphisms in a healthy group and in a patients group with abnormal semen analyses. We hope that these results provide a database which plays as a part in futher research on high risk factors in pathological conditions related to this polymorphism of the MTHFR gene.

5. CONCLUSION

From our research, we drew the following conclusions:

5.1. We successfully improved the PCR-RFLP process with our own-designed primers. In particular, these primers were able to amplify PCR products containing an obligatory restriction site of the *Hin*fI enzyme as an internal control for the subsequent digestion reaction in the PCR-RFLP process.

5.2. We identified the frequencies of polymorphisms at the 677th position of the MTHFR gene in 60 volunteers: the 677CC homozygous genotype dominated with 71.67%, the 677CT heterozygous genotype was next most common at 25% and the 677TT homozygous genotype occupied 3.33%. These corresponding frequencies in azoospermic patients were respectively 53.34%; 40% and 6.66%, and in oligospermic patients were respectively 80%, 20% and 0%.

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DETECTING RAPIDLY DRUG RESISTANT AND ULTIDRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* BY USING MICROSCOPIC-OBSERVATION DRUG-SUSCEPTIBILITY (MODS) ASSAY

Ngo Viet Quynh Tram[1], Nguyen Thi Chau Anh[1], Le Nu Xuan Thanh[1], Nguyen Hoang Bach[1], Huynh Hai Duong[1], Le Xuan Cuong[1], Piero Cappuccinelli[2]

(1) Dep. Microbiology, Hue University of Medicine and Pharmacy

(2) Centre for Carlo Ubani, Hue University of Medicine and Pharmacy

Abstract:

Early detection of drug resistance is the key point in effective control of drug resistant tuberculosis. Culture and drug susceptibility testing using conventional methods are time-consuming while automated methods tend to be expensive, limiting their use in resource constrained setting. Hence, there is need for a rapid, reliable and cost effective test for detection of drug resistance. MODS assay has the advantage of simultaneous detection and DST of M.tuberculosis. The objective of this study was to use MODS assay for rapidly detecting drug resistance and multidrug resistance. Culturing and susceptibility testing was conducted by MODS assay directly for 252 sputum samples. M.tuberculosis was detected in 153 samples (60.7%) and 46 (30.1%) were antibiotic resistant. One-drug resistance was present in 30 strains (19.6%): 18 for RIF, 6 for INH and 3 for STR and EBM. Multidrug resistant M.tuberculosis as defined by WHO (resistant to RIF and INH) was observed in 8.5%. There were an additional 9.2% of strains showing resistance to two or more drugs.

1. BACKGROUND

Tuberculosis remains a major cause of morbidity and mortality in many countries and a significant public health problem worldwide. The growing problem of drug resistance in *Mtb* is accompanied by increasing demand for quick, cheap, and easy techniques to detect resistance [12]. Several methods to detect drug resistance are available, but none clearly satisfies the demand for quick, cheap and easy testing. Traditional agar-based methods can take months for results. Commercial drug susceptibility testing (DST) with liquid culture decreases turnaround times but requires expensive equipment. Molecular detection of gene mutations associated with drug resistance has also been developed, with variable sensitivity reported especially for in-house methods [8]. MODS assay has the advantage of simultaneous detection and DST of *Mtb* [7]. The greater sensitivity and speed of detection of MODS culture in comparison to gold standards were predicted on the basis of previous studies [1],[10]. The objective of study is to sue MODS assay for rapidly detecting drug resistant and multidrug resistant *Mtb*.

2. METHODS

- 255 sputum specimens of patients suspected or confirmed of having tuberculosis at the Hue University Hospital, Hue Central