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Detection of Mthfr (C667t) and Mthfd (G1958a) Polymorphisms Among Sudanese Women with The Recurrent Miscarriages

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Abstract

Recurrent pregnancy loss affects 1 to 5% of women trying to conceive, I t has a significant impact at individual and social level. case-control study conducted at the research laboratory of the national center of neurological sciences, Khartoum, Sudan. All patients attending Ibrahim Malik teaching hospital and diagnosed with recurrent spontaneous abortion were included as case and healthy women at reproductive age were included as controls. The DNA was isolated by standard phenol chloroform extraction method, The PCR was done by using commercial thermal cycler machine with specific protocol. PCR products were sent for sequencing to Macro gene Europe Laboratory. 494 bp of MTHFR(C677T) and 174 bp of MTHFD (rs2236225) (G19581A) alleles were detected after PCR. The PCR result of MTHFR(C677T) shows; 40 (90%) for MTHFR (C677T) allele was positive in the cases and only12 (24 %) were positive for the control group. For MTHFD (G19581A) allele 42 (95%) were positive in the case group and 14 (28%) were positive in the control. When compared between case and control there was highly significant differences. The sequencing results for MTHFR C677; single Base Exchange was found C>T in the case groups, when used the mutation taster software the mutation was predicted. For MTHFD (G1958A) in the case group tow single Base Exchange were found T>A and G>A, the mutation taster software was predicted T>A mutation. According to this result the MTHF polymorphisms might become one of the main causes of unexplained diagnosis women with recurrent spontaneous abortion.

Keywords: Recurrent pregnancy loss, DNA, gene, mutation and polymorphism.

Introduction

Recurrent pregnancy loss (RPL) generally affects 1 to 5% of women trying to conceive. Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses before the 20th week of gestation [1,2]. The loss of pregnancy at any stage can be a traumatic experience and particular sensitivity is required in assessing and counseling couples with recurrent pregnancy loss. They are more susceptible to experiencing higher levels of depression, anxiety, and feeling guilty than the general population. Therefore, these couples may benefit from regular contact and reassurance [3].

Although RPL has a significant impact at the individual and social level, there are insufficient screening methods for risks of recurrent pregnancy loss. Previous reproductive history is an independent predictor of future pregnancy outcomes. The risk of a further miscarriage increases after each successive pregnancy loss,

reaching approximately 40.

Material and method

This was a case-control study conducted at the research laboratory of the National Centre of Neurological Sciences (NCNS), Khartoum, Sudan during the period April to September 2021 to detect the possible MTHF gene polymorphisms among women with RSA.

All patients attending the obstetrics and gynaecology unit at Ibrahim Malik teaching hospital and diagnosed with recurrent spontaneous abortion during the aforementioned period were included. Women with more than three recurrent abortions were included in this study as cases and those healthy women of reproductive age were included as controls. Women with chromosomal abnormalities, uterine anomalies, genital infections, and endocrinological disorders were excluded. The data was collected by using well stricture questionnaire. Ethical clearance for this study was obtained from the ethical review committee, Faculty of Medi Laboratory University of Medical Science and Technology, the participants were fully informed about the advantages and disadvantages before participation in the research (verbal informed consent). From each participant, 3 ml of venous blood was withdrawn with minimal stasis from the antecubital vein using a dry sterile disposable syringe and needle. Blood samples were dispensed into sterile containers with Ethylene Diamine Tetra-acetic Acid (EDTA). They were labeled with the subject's age, sex, and identification number and stored at -20°C for molecular analysis.

The DNA was isolated from peripheral blood leukocytes by standard phenol-chloroform extraction method. Primers were designed by using Prime3 software. The forward primer for MTHFR 677 was designed as "5-GGTCAGAAGCATATCAGTCATGAG -3" and reverse as "5-CTGGGAAGAACTCAGCGAACTCAG -3" with a product size of 494bp fragment. And MTHFD (rs2236225) forward primer "5-CTCCCAAAGTGCTGGGAGTA -3" and reverse as "5- CCTTATGAAGATACAGGCTAGTTGA -3" with a product size of 174bp fragment. The PCR was done by using a commercial thermal cycler machine (Swift™ MaxPro SWT-MXP-BLC-4) with h specific protocol according to the o annealing temperature of the primers. The PCR amplification product was separated on agarose gel and visualized through a gel-documented system. PCR products were sent for sequencing to Macro Gene Europe Laboratory.

Results

In the present study, 44 (46.8%) were selected as cases and 50 (53.2%) were selected as a control group (Table 1). The most affected age group was 25-34 years 16 (36.4%), followed by 35-40 years 15 (34.1%) (Table 2). The frequency of the abortion number in the case group; most of them had an abortion three times with a frequency of about 77.3 %, four times at about 9.1%, five times at 2.3%, six times at 6.8%, and nine times about 2.3% (Table 3). The frequency of their gestational age is; 11-14 weeks about 47.7%, 7-10 weeks about 34.1% and 6 weeks 18 % (Table 3).

Table 1: Distribution of study participants according to study group

	Frequency	Percentage	Valid percent	Cumulative Percent
Cases	*44*	*46.8*	*46.8*	*46.8*
Controls	*50*	*53.2*	*53.2*	*100*
Total	*94*	*100*	*100*	

Table 2: Basic characteristics of the study population

Parameters	No (%)	P value
Sex		
Case	*44 (46.8%)*	*0.000*
Control	*50 (53.2%)*	
Age		
18-24	*6 (13.6%)*	
25-34	*16 (36.4%)*	*0.000*
35-40	*15 (34.1%)*	
>40	*7 (15.9%)*	

Table 3. Distribution of variables

Variables		Frequency	Percent
Number of abortion	3	34	77.3
	4	4	9.1
	5	1	2.3
	6	3	6.8
	9	1	2.3
	Total	44	100.0
Gestational age	6 weeks	8	18.2
	7-10 weeks	15	34.1
	11-14 weeks	21	47.7
	Total	44	100.0
Durationb\t mis	1-3 month	13	29.5
	>3 months	30	68.2
	>10years	1	2.3
	Total	44	100.0
Folic acid	Yes	44	100.0
Smoking	Yes	1	2.3
	No	43	97.7
	Total	44	100.0
Genetic disease f	Yes	8	18.2
	No	36	81.8
	Total	44	100.0
Treatment	Yes	1	2.3
	Antibiotics	1	2.3
	Heparin	1	2.3
	Thyroxin	1	2.3
	Vitamin	1	2.3
	No	39	88.6
	Total	44	100.0
Diagnosis rpb	Unexplained	42	95.5
	enl uterus	2	4.5
	Total	44	100.0
How long they have been trying for pregnancy	< 2 years	21	47.7
	3 years	1	2.3
	5 years	11	25.0
	> 5 years	11	25.0
	Total	44	100.0
The outcome of the pregnancies	Miscarriage	44	100.0
If the outcomemiscarriage	Only miscarriage	20	45.5
	Miscarriage b\w term andpreterm	24	54.5
	Total	44	100.0
They were plannedpregnancy	Yes	1	2.3
	No	43	97.7
	Total	44	100.0
Genetic disease male side	Yes	7	15.9
	No	37	84.1
	Total	44	100.0

Molecular study

In the present study, 494 bp of MTHFR(C677T) and 174 bp of MTHFD (rs2236225) (G19581A) alleles were detected with gel electrophoresis after PCR (Figures 1 and 2). The PCR result shows that about 40 (90%) of the MTHFR (C677T) allele were positive in the cases and only 12 (24%) were positive for

the control group (Table 4). For MTHFD (G19581A) allele, 42 (95%) were positive in the case group and about 14 (28%) were positive in the control (Table 4). (Figures 3, 4,5and 6)

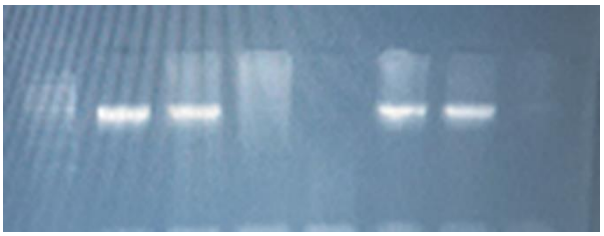

Fig1: 1494 bp of MTHFR (C677T) gene detected with gel electrophoresis.

Fig2: 174 bp of MTHFD (G19581A) gene detected with gel electrophoresis

Table 4. Distribution of MTHFR polymorphisms results among the study population

			Frequency	Percent
MTHFR 677	***Case***	*Positive*	*40*	*90.9*
		Negative	*4*	*9.1*
		Total	*44*	*100.0*
	Control	*Positive*	*12*	*24.0*
		Negative	*38*	*76.0*
		Total	*50*	*100.0*
MTHFD*(G19581A)*	***Case***	*Positive*	*42*	*95.5*
		Negative	*2*	*4.5*
		Total	*44*	*100.0*
	Control	*Positive*	*14*	*28.0*
		Negative	*36*	*72.0*
		Total	*50*	*100.0*

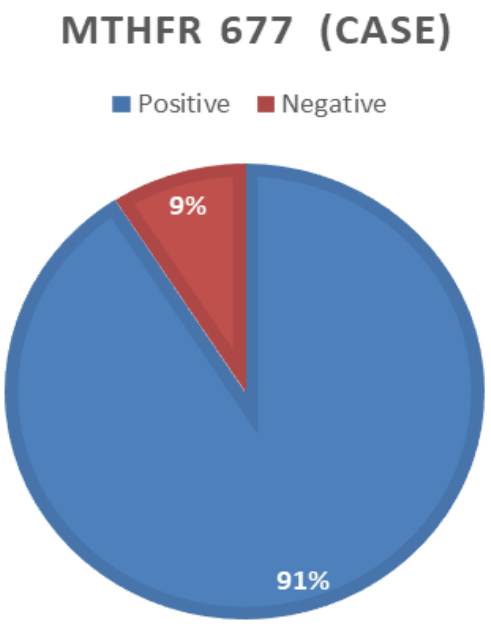

Fig (3): Distribution of MTHFR (C677T) polymorphisms among cases group

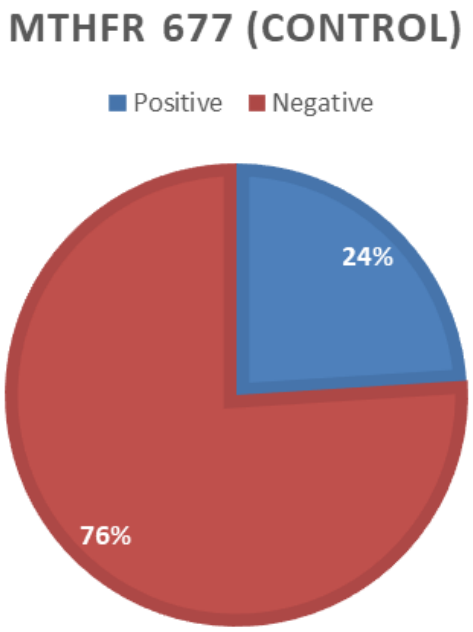

Fig (4): Distribution of MTHFR (C677T) polymorphisms among the control group

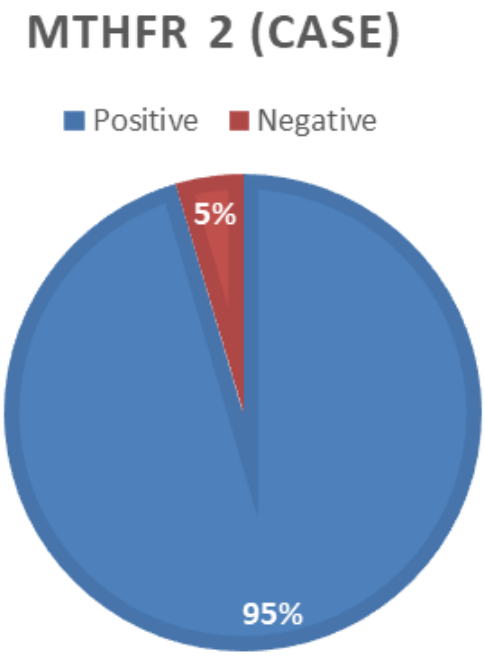

Fig (5): Distribution of MTHF (G19581A)) polymorphisms among cases group

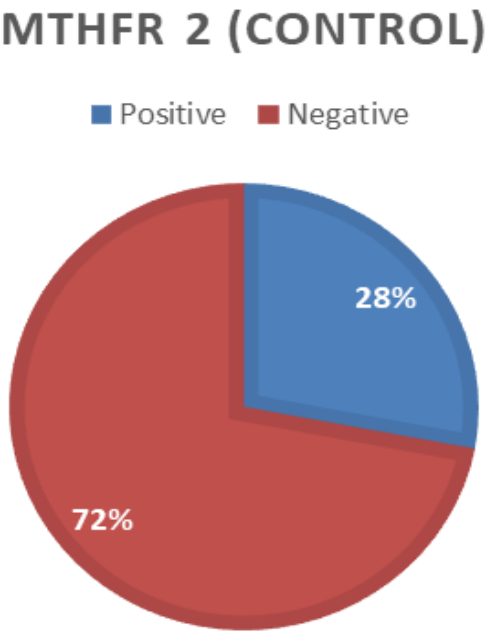

Fig (6): Distribution of MTHF (G19581A)) polymorphisms among the control group

Table 5. Association between MTHFR polymorphism and study population

Variables		Study population		Total	Chi squire	P. value	Odd Ratio
		Case	Control				
MTHFR C677T	*Positive*	*40 (76.9%)*	*2(23.1%)*	*52(100.0%)*	*42.4*	*0.000*	*31.7*
	Negative	*4 (9.5%)*	*38 (90.5%)*	*42 (100.0%)*			
	Total	*44(46.8%)*	*50 (53.2%)*	*94 (100.0%)*			
MTHFR ***(G19581A)***	*Positive*	*42 (75.0%)*	*14 (25.0%)*	*56 (100.0%)*	*44.2*	*0.000*	*54.0*
	Negative	*2 (5.3%)*	*36 (94.7%)*	*38 (100.0%)*			
	Total	*44 (46.8%)*	*50 (53.2%)*	*94 (100.0%)*			

Sequencing result

The sequencing results were analyzed using different bioinformatics soft-wares and tools for MTHFR C677T. The obtained sequences aligned using BioEdit-ClustalW software with a normal sequence from the GenBank gene (accession number NC_000001.11in NCBI).

When the cases were compared with the normal reference the single Base Exchange was found C>T While when the controls were compared with the normal reference, no single base exchange was found among all control groups (Fig. 7 and 8). when used the mutation taster software the mutation was predicted (Fig. 9).

For MTHFD (G1958A) sequences were aligned using BioEdit-ClustalW software with a normal sequence from the GenBank gene (accession number NC_0000014.91in NCBI). When the cases were compared with the normal reference two single Base Exchanges were found T>A and G>A While when the controls were compared with the normal reference, no single base exchange was found among the all-control groups (figure 10,11) and the mutation taster software was predicted T>A mutation (Fig. 12).

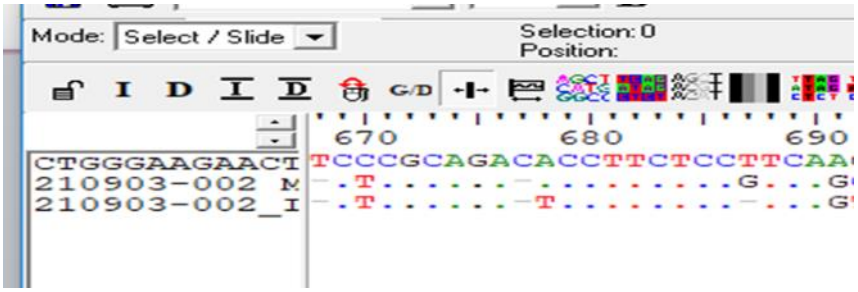

Figure 7: Multiple sequence alignment using Bio-Edit ClustalW for cases group with the reference gene sequence of MTHFR C677T gene

Figure 8: Multiple sequence alignment using Bio-Edit clustal W for control group with reference gene sequenceof MTHFR C677T gene

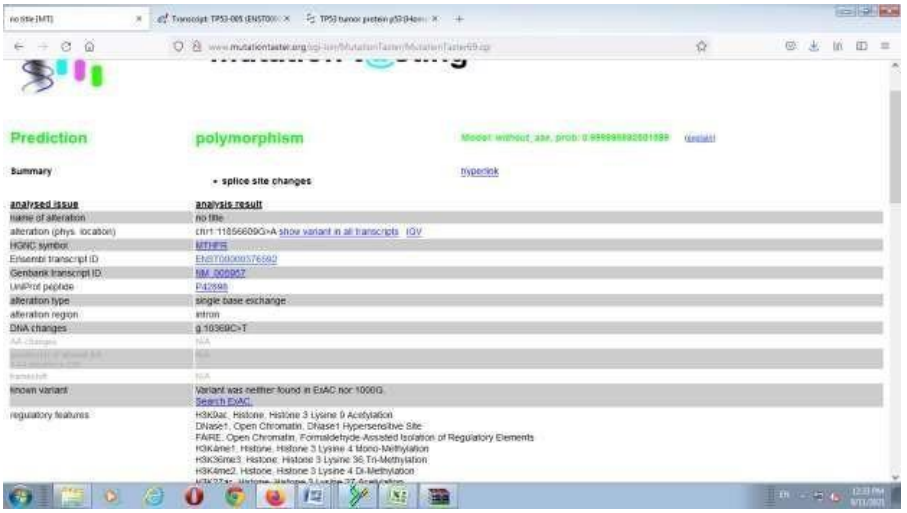

Figure 9: C>T singles Base Exchange tested in mutation taster software.

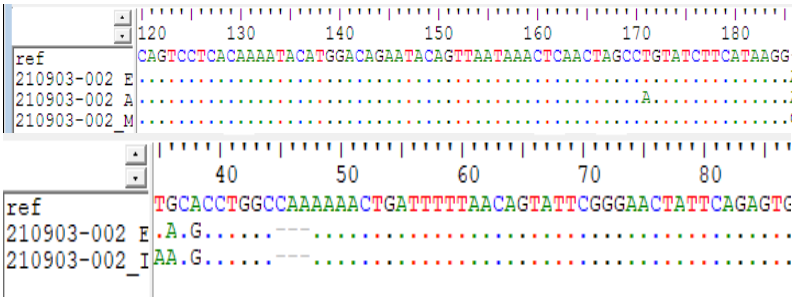

Figure 10: Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of MTHFD (G19581A) gene

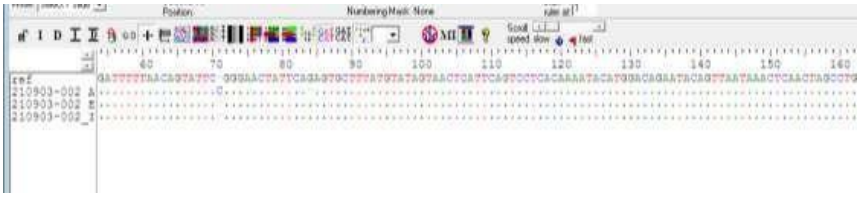

Figure 11: Multiple sequence alignment using Bio-Edit clustal W for control group with reference genesequence of MTHFD (G19581A) gene

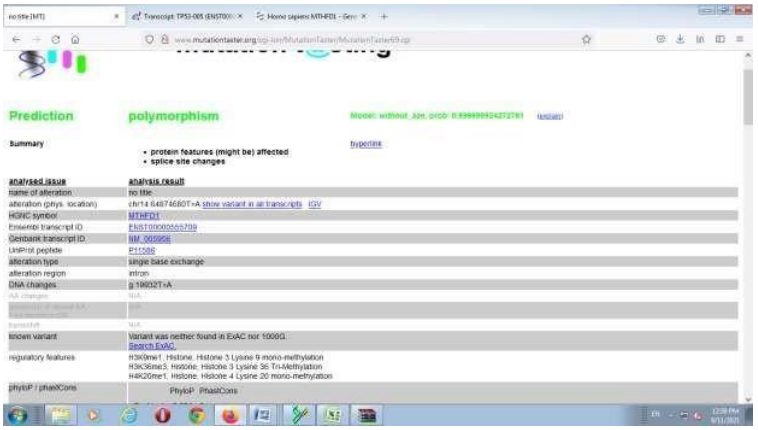

Figure 12: T>A singles Base Exchange tested in mutation taster software.

Discussion

several studies have evaluated whether a correlation between the C677T and A1298C polymorphisms of the methylenetetrahydrofolate reeducates (MTHFR) gene and a higher risk of recurrent pregnancy loss (RPL), only a few studies that evaluated the correlation between the MTHFD (rs2236225) (G1958A) and recurrent miscarriage. This study aimed to detect the MTHFR (C667T) and MTHFD (G1958A) polymorphisms among Sudanese women with the recurrent miscarriages

In the present study 44 women were selected as cases and 50 apparently healthy women were selected as a control group. The most affected age group was 25-34 years 16 (36.4%), followed by 35-40 years 15 (34.1%). The frequency of abortion number in the case group; most of them had an abortion three times with a frequency of about 77.3 %, the frequency of their gestational age is; 11-14 weeks about 47.7%, 7-10 weeks about 34.1% and 6 weeks 18 %, 97%of theses pregnancies were unplanned, this highlight lack of pre-pregnancy counseling for these women generally this big issue in Sudan but at least it should be provided for a high-risk group like women with RPL.

The PCR result shows that about 40 (90%) of the MTHFR (C677T) allele was positive in the cases and only 12 (24 %) were positive or the control group. For MTHFD (G1958A) allele, 42 (95%) were positive in the case group and about 14 (28%) were positive in the control. When compared between case and control for the two alleles there were highly significant differences.

The sequencing results revealed; when the cases were compared with the normal reference the single Base Exchange was found C>T While when the controls were compared with the normal reference, no single base exchange was found among the all-control groups, in addition, that when used the mutation taster software the mutation was predicted.

Park et al reported; MTHFR catalyzes the irreversible conversion of 5,10-methy-lentethrahydrofolate to 5-methyltetrahydrofolates (CH3-THF). The normal activity of MTHFR aids to maintain folate and methionine in the bloodstream at constant levels, preventing Hcy accumulation. Polymorphisms in the gene encoding MTHFR may lower its enzymatic activity. The variant C677T leads to a substitution of cytosine into thymine at position 677 within exon 4 of the MTHFR gene. This genetic variant leads to an amino acidic substitution in position 222 [6]

On the other hand, Domenico et al said; the C677T variant of the MTHFR gene does not appear to influence the predisposition to miscarriage in the first or second trimester of pregnancy. Thus, it may not be favorable to analyze them for diagnostic aims. However, daily intake of folic acid remains an important therapeutic practice for pregnant women in order to reduce the risk of congenital defects among other complications [8]. This study finding disagree with the study by Al-Achkar et al; 206 women with URPL versus healthy women showed an increased risk for RPL in individuals carrying the MTHFR 677T allele and the homozygous genotype 677TT. Also, the other study which used a larger samples size showed that; the frequency of the T allele of the MTHFR 677 locus in the URPL group was also statistically significantly higher than that in the control group, indicating that the MTHFR 677T allele is a risk factor for URPL, which is consistent with the results of the study by Al-Achkar et al [9].

Mtiraoui et al; analyzed genetic polymorphisms in women with RPL and found that the risk of developing URPL in women carrying the homozygous mutant genotype (CC) was increased. Klai et al.; also found that women with the mutated gene have an increased risk of miscarriage and other pregnancy complications [10,11].

For MTHFD (G1958A) gene sequences results showed; when the cases were compared with the normal reference two single Base Exchange were found T>A and G>A, while when the controls were compared with the normal reference, no single base exchange was found among the all-control groups and the mutation taster software was predicted T>A mutation.

Parle-McDermott et al reported; a polymorphism [1958G→A (R653Q, dbSNP rs2236225] within the gene encoding the trifunctional enzyme MTHFD1 (5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methyltetrahydrofolate cyclohydrolase, 10-formyltetrahydrofolate synthetase) is a maternal risk for severe placental abruption, a maternal risk for having a pregnancy affected by a neural tube defect (NTD) and is possibly involved in fetal in viability [12].

in addition, Anne Parle-McDermott et al said; identified the MTHFD1 1958AA genotype as an independent maternal risk factor for unexplained pregnancy loss during the second trimester of pregnancy [13].

Conclusion

In conclusion, our research result showed a highly significant association between the MTHFR C667T, MTHFD G 1958 A polymorphisms, and RSA in Sudanese women, according to this result the MTHF polymorphisms might become one of the main causes of unexplained diagnosis in women with recurrent spontaneous abortion.

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