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Association of *MTHFR* C677T with Idiopathic Recurrent Pregnancy Loss in Anhui Province of China

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ABSTRACT The *MTHFR* C677T has been reported to be involved in Recurrent Pregnancy Loss (RPL) while prevalence and aetiological differences exist among various regions and ethnic groups. So, far no data is available on genetic relationship between polymorphisms in *MTHFR* and RPL population of Han ethnicity in Anhui province of the China. To study the association of *MTHFR* C677T with RPL in Anhui's population, the researchers used a swift enzymatic color-reaction-based biochip and asymmetric multiplex PCR. A total of 538 (201 RPL and 337 controls) unrelated Han Chinese women from Anhui province were recruited for the current study. However, no significant differences were noted in frequencies of C677T genotypes; CC (29.4%), CT (50.1%) and TT (20.5%) and CC (23.9%), CT (55.7%), and TT (20.4%) among RPL and controls, respectively. Furthermore, stratified analysis based on age failed to find association of *MTHFR* C677T with RPL. Subsequently, comprehensive review of recently published similar studies displayed heterogeneity of *MTHFR* C677T with RPL. Therefore, It is concluded that variants in *MTHFR* other than C667T may have association with RPL in Han Chinese women from Anhui province.

INTRODUCTION

Recurrent pregnancy loss (RPL) is defined as two or more consecutive pregnancy losses within 20 weeks of the gestation (Medicine 2013). So far the aetiology of RPL is not well studied; however, several exogenous or endogenous contributing factors have been identified, which include nutritional/environmental elements, infections, genetic, placental, endocrine and immunologic abnormalities (Peres Wingeyer et al. 2018). Altogether, these factors contribute about 50 percent to the aetiology of RPL (Coulam et al. 1997; Park et al. 2011).

The genetic and non-genetic thrombophilia has been associated with various obstetric problems such as stillbirth, placental abruption, defective foetal growth and RPL (Farahmand et al. 2016; Simcox et al. 2015; Tranquilli et al. 2010). The variants in several genes have been associated with pregnancy loss and foetal abnormalities (Pereza et al. 2017; Toufaily et al. 2018). The exact role of *MTHFR* in maintenance of pregnancy is yet to be explored, however it's variants are shown to be associated with RPL (Zhang et al. 2019). Few studies have reported the positive association of C677T in women with RPL in Syrian and Chinese population (Al-Achkar et al. 2017; Cao et al. 2013; Cao et al. 2014), but still a firm conclusion is yet to be finalized (Hwang et al. 2017; Zhang et al. 2018). Recent technologi-

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cal advancement made it easy to diagnose genetic causes of certain disorder(s), the choice of diagnostic techniques depends on its efficiency and efficacy. Thus Target-enriched multiplex polymerase chain reaction (Tem-PCR) has high sensitivity, reproducibility and automaticity to increase accuracy, duplicability and it also reduces the time span and makes an ease to process a huge number of samples at the same time (Xue et al. 2016).

Objective of the Study

The objective of the study was to find out the association of the *MTHFR* C677T with recurrent pregnancy loss in women recruited from Anhui, China.

The researchers employed an enzymatic color reaction based biochip detection method (Tem-PCR). The *MTHFR* C677T polymorphism hasn't been studied in recurrent pregnancy losses in Han Chinese population of Anhui province of China. For current study the researchers purely identified and recruited idiopathic recurrent pregnancy loss cases.

MATERIAL AND METHODS

Subjects

A total of 538 (201 RPL and 337 controls) unrelated Han Chinese women were recruited and studied at Parental and Child Health Hospital Hefei, Anhui, P.R China. Women with two or more idiopathic consecutive loss of pregnancies within the 20 weeks of the pregnancy were considered RPL (Recurrent Pregnancy Loss), while women with single normal live birth were recruited as healthy controls of the study. Women with known reason(s) of miscarriage, including an anatomic abnormality, a chromosomal anomaly, and an autoimmune response (including systemic lupus erythematosus), an autoimmune reaction, or endocrinological problems (such as hypothyroidism, hyperprolactinemia or diabetes) were excluded from the study. The project was explained verbally in detail and written informed consents were taken from all the participants before recruiting for the study. Current study was approved by the Ethical Review Committee of Maternal and Child Health Hospital Hefei and Anhui Medical University, Anhui, PR China. All the sampling and experimental procedures were performed by strictly following the guidelines and comparable standards of the Helsinki declaration 1964 and its latest amendments.

DNA Extraction and Primers Modification

From all the participants, a 5 mL of venous blood sample was collected in EDTA tubes and genomic DNA was isolated by using the DNeasy Blood and Tissue Kits (QIAGEN, Hilden, Germany). Quantity and purity of the extracted DNA samples were determined by ultraviolet spectrometry (ActGene Inc, Taipei, Taiwan) and kept at -20 °C until use.

The *MTHFR* C677T (rs1801133) polymorphism was genotyped using a commercially available hybridization and enzymatic kit (BST06013, BaiO Technology Co, Ltd, Shanghai, China). The reverse primer was labeled with a biotin at the 5′end and probes were modified by amine group.

Biochip Preparation, M-PCR and Hybridization

Probes were immersed in TE buffer with pH 7.6 and mixed with Spotting buffer. The probes were covalently attached to the amine group on 5¹end onto the surface of aldehyde coated slides. Positive and negative controls were reproduced in triplicates to set an off the cut value. The 22 ul m-PCR was performed containing 2.5 U of Taq DNA polymerase with concentration of forward, reverse primers and dNTPs was 0.2 uM, 0.6 uM and 40 uM respectively. Two set of the primers were used to amplify *MTHFR* C677T [F]: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and [R]: 5'-AGG ACG GTG CGG TGA GAG TG -3'. The Tem-PCR was performed according to the following conditions: 50°C for 5 min, pre-denaturation at 94°C for 5min, followed by 35 cycles of denaturing at 94°C for 25s, annealing at 56°C for 25s, and extension at 72°C for 25 s, and a final elongation at 72°C for 5 min. Biochip reaction was performed as explained previously by Xue et al. (2016). The amplified products were subsequently dispensed into a hybridization reaction chamber to hybridize. TYPE 2.0 software (BaiO Tech-

nology Co, Ltd, Shanghai, China) was used to analyze the arrays according to the instructions of the manufacturer.

Statistical Analyses

Data were processed using SPSS software packages version 20.0 (IBM SPSS Inc., Armonk, NY, USA). Both groups were compared for genotypic and allelic frequencies. The significance of the differences in genotypic distribution was analyzed using a Chi-square test (χ^2), P <0.05 was considered as significant. Odds ratio (OR) and 95% confidence intervals (CIs) were inspected to assess the relationship between genotypes and RPL.

RESULTS

A total of 538 (201 RPL and 337 controls) unrelated Han Chinese women were inquired about their age, previous history of miscarriages and number of live births. Interestingly, individuals in RPL group were significantly younger than the control group (p<0.001). Moreover, significant difference was found in the number of miscarriages (median 3) and live birth (median 2) among both groups (p<0.001) (Table 1). DNA samples from both groups were genotyped for C677T (rs1801133) and genotypic frequencies were analyzed for association with PRL. Physical arrangement of probes on the chip was first determined to check the efficiency. 0,1,2 and 3 indicating position of positive control, wild-type, mutant, and negative control , respectively (Figs. 1 A and B). Next, the genotypic frequencies were calculated among RPL and control groups and results were presented as genotype CC, CT, TT, respectively (Table 2). Frequency analysis was performed for each genotype in both groups. RPL group showed CC (29.4%), CT (50.1%) and TT (20.5%), while control group had CC (23.9%), CT (55.7%), and TT (20.4%) respectively. Similarly, student *t*-test analysis revealed no significant difference between genotypes in both groups. The frequency of allele T 48.26 percent (194/402) was slightly higher in the RPL group than in the control group 45.55 percent (307/674) (P=0.441, OR = 1.115, 95% CI 0.871 < 1.428), even after adjusting for age no significant difference was seen (Table 2). Subsequently, the researchers performed stratified analysis of *MTHFR* C677T on the basis of age difference between RPL and controls, no significant association was found between RPL and controls (Table 3). Thus, the results of this study showed a distribution of *MTHFR* genotype followed Hardy-Weinberg equilibrium, which revealed that no selection happened among genotypes.

Table 1: Basic information of RPL patients and controls

Clinical features	*RPL patients group n=201*	*Control group n=337*	*P value*
Age (Y)	29.6±5.2	34.2±4.8	<0.001
Previous miscarriage, median	3	0	<0.001
Number of live Births, median	0	2	<0.001

RPL, recurrent pregnancy loss. Y, years.

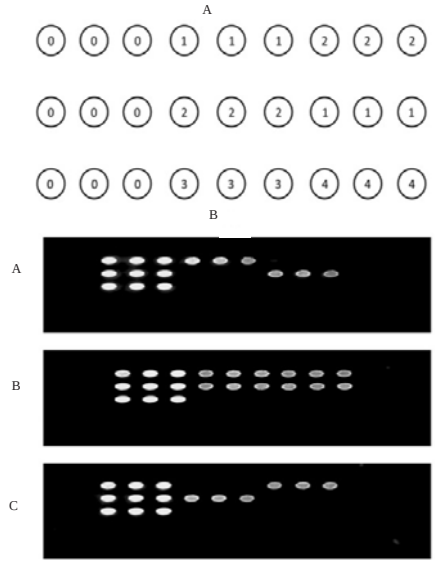

Fig. 1. (A) The physical arrangement of probes on the chip figure 0,1,2,3 that indicate position of positive control, wild-type, mutant, negative control and blank, respectively.
Fig. 1. (B) Results of the *MTHFR* C677T polymorphism a, b, c show genotype CC, CT, TT, respectively.

Table 2: Occurrence of C677T genotypes in RPL and controls

Genotype	*RPL*	*Control*	*Unadjusted OR*		*Adjusted OR*	
T T	69 (20.5)	41 (20.4)	1.23 (0.73 - 2.06)	0.441	1.23 (0.70 - 2.16)	0.476
C T	169 (50.1)	112 (55.7)	1.37 (0.90 - 2.08)	0.144	1.37 (0.87 - 2.15)	0.181
CC	99 (29.4)	48 (23.9)	Reference			

Data presented as N (%); OR indicates odds ratio and 95% CI; P<0.05 considered significant.

Table 3: Distribution of C677T genotypes based on age

Age	*Genotype*	*RPL*	*Controls*	*OR (95% CI)*	*p value*
<30	C/C	30	25	Ref	Ref
	C/T	76	43	1.43 (0.7693-2.820)	0.2414
	T/T	24	20	1.00 (0.4510-2.217)	1.00
>30	C/C	18	74	Ref	Ref
	C/T	36	126	1.175 (0.6227-2.216)	0.6189
	T/T	18	49	1.510 (0.7158-3.186)	0.2774

DISCUSSION

A number of factors are needed to achieve a successful pregnancy, such as maternal immunity, maternal physiological health, foetal vasculature and homeostasis (Ewington et al. 2019). Physiological changes as well as inadequate intake of vitamins and folic acid may affect the activities of essential enzymes contributing in single carbon folate-metabolism pathway (Bahous et al. 2018; Kaur et al. 2018). MTHFR is a principal enzyme of the folate and homocysteine metabolic pathway and any variation in it can cause serious problems such as RLP (Bahous et al. 2018). Hence, polymorphisms in the gene coding MTHFR enzyme are the essential biomarkers to estimate the risk of RPL, and its levels and activity in blood can be affected by C677T (Hwang et al. 2017).

Based on the previous knowledge and contradicting results in various populations, the researchers investigated *MTHFR* C677T in Han Chinese women suffering from idiopathic RPL. Women with no genetic and cytogenetic or other unknown non-genetic causes were chosen. To the best of the researchers' knowledge, this is the first study on the RPL patients from the Anhui province of China to investigate *MTHFR* polymorphism. Besides having reasonable statistical power of sample size, the current study could not find the association of either homozygous mutant or heterozygous mutant alleles with RPL. Furthermore, the researchers' stratified analysis of *MTHFR* polymorphism on the bases of age was also failed to find significant association. Thus, the researchers believe that C677T has very less role in pregnancy maintenance. However, two studies from neighbouring provinces (Jiangsu and Zhejiang) showed a significant genotypic differences among RPL and controls (Cao et al. 2014; Luo et al. 2015). These studies used large sample size which could give statistical difference between patients and control groups, comparatively less sample size could be a limitation of this study. However, a Chinese meta-analysis showed the relationship of *MTHFR* C677T and A1298C polymorphisms and RPL risk collectively or solely (Yang et al. 2016). Current control participants might have not declared correct information about their history of miscarriage, which could be a least expected reason for similarity in statistical scores of both groups. Furthermore, review of literature from recently several published studies, only a few studies showed positive association of *MTHFR* with RPL further indicating the heterogeneity of disorder. Interestingly, most of the studies showed positive association of C667T with Indians and European RPL patients which indicate that it is ethnically limited and yet vast differences existed between various ethnicity (Table S1).

CONCLUSION

The present study attempted to find whether C677T has association with RPL in Han Chinese women from Anhui Province of China.

Analyses of data revealed no significant difference among the genotypes in patients and control thus no association with pregnancy loss. Hence, it is concluded that the other variants of *MTHFR* or other genes might have role in aetiology of RPL. This study's findings contribute to existing data relevant to genetics causes of RPL in Chinese population.

RECOMMENDATIONS

Based on our findings we recommend a large scale study on Chinese RPL population for studying association of C677T and other *MTHFR* polymorphisms. Furthermore, a comprehensive meta-analysis should be performed to find out the genotypic differences among various ethnic groups and populations.

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Table S1: Literature review of *MTHFR* C677T and A1288T in RPL patients

Genotype		*RPL size*	*Control size*	*P value*	*Ethnicity*	*Findings*	*Reference*
C677T	*A1288T*						
Yes	N/A	41	18	0.95	Israel	NS	(Settin et al. 2011)
Yes	N/A: not available	173	67	0.194	England	NS	(Holmes et al 1999)
Yes	Yes	80	125	0.09,0.351	Sweden	★, NS	(Zetterberg et al. 2002)
Yes		133	74	0.03	European	★	(Unfried et al. 2002)
Yes	N/A	85	76	NS	Japan	NS	(Makino et al. 2004)
Yes	N/A	104	120	0.90	South Indian	NS	(Vettriselvi et al. 2008)
Yes	N/A	84	80	0.03	North India	★	(Mukhopadhyay et al. 2009)
Yes	N/A	140	140	0.04	India	★	(Govindaiah et al. 2009)
Yes	Yes	70	136	0.05,0.06	Egyptian	★, NS	(Settin et al. 2011)
Yes	N/A	106	140	0.003	North India	★	(Nair et al. 2012)
N/A	Yes	129	202	0.006	Indian (Banaras)	☆	(Nair et al. 2013)
Yes	Yes	204	116	0.434,0.155	Persia	NS	(Yousefian et al. 2014)
Yes	Yes	481	2559	0.16,0.17	Caucasian	NS	(Hubacek et al. 2015)
Yes	Yes	100	106	0.0003,0.014	Syria	★ ☆	(Al-Achkar et al. 2017)
Yes	Yes	302	315	0.142, 0.517	Korean	NS: no significant	(Hwang et al. 2017)
Yes	N/A	70	70	0.033	Nepal	★	(Sah et al. 2018)
Yes	Yes	380	387	0.45, 0.46	Italy	NS	(Dell'Edera et al. 2018)
Yes	Yes	222	988	0.023	Australia	C677T significant	(Kos et al. 2018)

★; C677T significant, ☆; A1288T significant, NS; no significant, N/A; not available