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The Prevalence of Inherited Thrombophilic Polymorphisms in Saudi Females with Recurrent Pregnancy Loss Confirmed using Different Screening Protocols of PCR

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Abstract

Inherited thrombophilia has recently been identified as a major cause of thrombembolism, but it may also contribute to adverse pregnancy outcomes and recurrent pregnancy loss. Three gene mutations namely leiden (FV G1691A), prothrombin (FII G20210A), and methylenetetrahydrofolate reductase (MTHFR C677T) are the most common types of hereditary thrombophilias in women with RPL, which in turn can result in placentation. These are usually undiagnosed, because most carriers are asymptomatic. The aim of this study was to determine the association of specific inherited thrombophilias and recurrent pregnant loss (RPL) among Saudi women. The study included 142 females were 72 had a history of 2 or more events of fetal loss in any of the 3 trimesters of pregnancy. The remaining 70 were clinically healthy women with a good obstetric history and have designated as a control group. Detection of inherited thrombophilia genes mutations was confirmed using different PCR screening protocols. The frequencies of FV & FII mutations related to the pregnancy loss stages showed that FV mutation ratio was similar among cases with early or late stage pregnancy loss (25% - 26%) but significantly higher than that of controls (1.4%). On the other hand FII mutation ratio was high among cases with late pregnancy loss (50%) followed by early pregnancy loss (38%) and was significant higher than that of controls (1.4%). MTHFR C677T mutation was more common in group of women with fetal loss in first trimester compared to the controls. We have reported that the combinations of two or more thrombophilic polymorphism risk factors were observed in 10.8% healthy Saudi women with unexplained RPL while no more than one risk factor was observed in any of the controls. We concluded that there is a strong association between the combined inherited thrombophilic mutations related to FV G1691A, FII G20210A, and MTHFR C677T genes among Saudi women. Our data confirm the hypothesis that inherited thrombophilia is indeed a significant abnormality in the RPL subjects.

Keywords: Recurrent pregnancy loss; Thrombophilias; Prothrombin; Leiden; Methylenetetrahydrofolate reductase

Introduction

Recurrent pregnancy loss is a common health problem among Saudi women at the reproductive age. It is a heterogeneous condition with three or more successive losses affecting 1-2% and two or more successive losses affecting up to 5% of women. Recently, thrombophilias have been suggested as a possible cause of RPL [1]. Although thrombophilia is considered controversial, a common cause in women with unexplained recurrent pregnancy loss, with prevalence as high as 65% in selected populations [2,3]. The guideline of American College of Obstetricians and Gynecologists in Sept. 2013 records that the inherited thrombophilias in pregnancy are protein C deficiency, protein S deficiency, antithrombin deficiency, FV G1691 A, prothrombin G20210A and MTHFR C677T. On the other hand, FV G1691 A, prothrombin G20210A and MTHFR C677T are the most common causes have been implicated as risk factors of hereditary thrombophilias in women with RPL which in turn can result in placentation [4].

The three most common genetic thrombophilias known to predispose to venous thrombosis are: FV (factor V Leiden), methylenetetrahydrofolate reductase mutation (MTHFR, C677T) [5,6], and FII (prothrombin G20210) (Figure 1) [7]. In FV, arginine is substituted by glutamine at amino acid residue 506 in coagulation FV [8]. Due to this substitution, FV becomes resistant to degradation by activated protein C, increasing the risk of venous thromboembolism 3–5-fold in heterozygous individuals [9]. In FII G20210A, a G to A transition at position 20210 of the 3′ untranslated region of the FII gene has been found to be associated with increased prothrombin levels and a 3-fold increased risk for venous thrombosis in heterozygotes [10]. The homozygous state for the C to T transition at position 677 of MTHFR gene is associated with hyperhomocysteinaemia which predisposes to thrombosis [11]. The FV mutation is the most studied inherited thrombophilia in relation to pregnancy complications. FV is a genetic disorder was described as an underlying cause of APC (Anti Protein C) resistance. This mutation involves a guanine to adenine substitution (FV Arg506Gln) (G-to-A) at nucleotide 1691 in exon 10, which results in synthesis of a defective Leiden molecule (FV), resistant to cleavage by APC, [8-11].

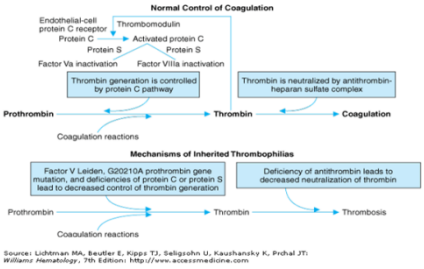

Figure 1: Diagram of normal control of coagulation.

Our data accumulated over the past five years suggest a possible association between thrombophilia and fetal loss in Saudi women. We have reported that FII (prothrombin) and FV mutations, may be important risk factors for neonatal stroke in Saudi newborns [12], We recommend Leiden G1691A and prothrombin G20210A mutations are important and must be included in the routine analysis of Saudi fetuses and pregnant women [12]. While recently we have reported an increase in the prevalence of FV G1691A Leiden and FII G20210A mutations in Saudi women with pregnancy loss using a multiplex allele-specific PCR amplification method [13]. The roles of the combined thrombophilic polymorphism (TP), in Saudi pregnancy loss have not yet been confirmed. We therefore aimed to investigate the prevalence of the three most common combined hereditary TP (FV, TL-MTHFR C677T, and FII G20210A) in Saudi women with pregnancy loss without apparent mechanical, anatomical, endocrinological or immunological causes. We have confirmed our results using different genetic screening PCR protocols. We validated our outcomes from multiplex allele-specific PCR amplification and RFLP-PCR with real time PCR (RT-PCR).

Materials and Methods

After obtaining informed consent, Genomic DNA samples of 142 Saudi women, who recruited and followed at Military Hospitals in Saudi Arabias under human ethical approval, were screened from Dec, 2009 to Dec, 2010. Seventy of them were healthy subject females were selected as control group with negative personal and negative family history for pregnancy loss and also with negative history to any thromboebolic diseases or they have at least one previous uneventful pregnancy. While other 72 females having history of recurrent pregnancy loss 2 times or more of abortions, miscarriages, intrauterine fetal death (IUFD) or still birth associated with placental vessel thrombosis or placental infarction on histopathology examination. Exclusion criteria included cases diagnosed with genetic/chromosomal aberrations, anatomic anomalies, and or endocrine/hormonal abnormalities.

PCR methods

Genomic DNA was isolated from the peripheral blood samples according to a standard protocol (Qiagen- Germany). DNA pellet was dissolved in 50 µl of TE buffer (~30 ng/ µl DNA concentration) and stored at -20°C until the genotype analysis was performed.

Polymerase chain reaction (PCR) amplification of DNA samples was performed using specific primers [12,13]. The amplified products were then electrophoresed in 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Primers were designed by using a web-based PRIMER 3.0 program (http://workbench.sdsc.edu). We used the "BLAST" program at http://www.ncbi.nlm.nih.gov/blast to check for the specificity of the primers. PCR product variants were then digested by specific restriction enzymes. A 208 bp genomic DNA fragment of FV around nucleotide 1691 was amplified by PCR and digested with Mnl-I [14]. A 253 bp DNA fragment of the 3'-untranslated region of the prothrombin gene that includes the nucleotide 20210 was amplified with the primer 5'-CAACCGCTGGTATCAAATGG-3' and a mutagenic primer as described by Poort et al. [15]. The amplified fragment was digested with Hind III. Analysis of the MTHFR 677C_T substitution was performed by amplification of a 198 bp DNA fragment and followed by Hinf I digestion, as described by De Franchis et al. [16]. Digested fragments were electrophoreses on 10% polyacrylamide gels and visualized by ethidium bromide staining (Figure 2).

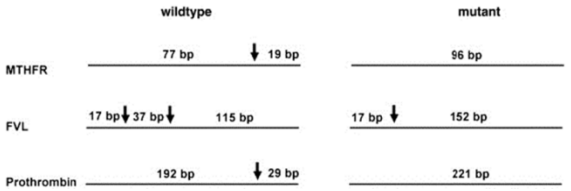

Figure 2: Schematic representation of Mnll digestion profiles of wild type and mutant alleles of MTHFR, FV and prothrombin amplicons. Fragments are not scaled.

Validation

Samples for FV, FII G20210A and MTHFR C677T genotypes were reanalyzed, and positive findings were confirmed specific probes in real time PCR [17] using software version: 4.4.

Data were analyzed using the SPSS program. Power calculations were performed to obtain the probability that differences in gene frequencies were statistically significant. P values >0.05 were considered significant, <0.01 highly significant, and >0.0001 extremely significant. Odds ratios were also calculated for the samples.

Result

The participants included 142 women subjects. Out of them, 72 had a history of 2 or more events of recurrent fetal loss (abortion, miscarriage or still birth). Their mean age ± SD was 25.13 ± 4.58.

The mean age of healthy women was 34.4 ± 4.88. 72.22% cases were early aborted, 8.33% were late aborted, and 22.22% were early and late aborted.

We recorded that the abortion cases with early or late or combined stages having FII (GA) heterozygous mutation were higher than those having FV (GA) heterozygous mutation and MTHFR C677T (28% vs. 16% vs. 8%, respectively) (Figure 3), but with no statistical significance (P=0.9). This denotes that the effect of the mutation is not confined to certain time of pregnancy i.e. whatever is the time of pregnancy, these mutations can have a detrimental effect particularly in early time of pregnancy <20 weeks gestation.

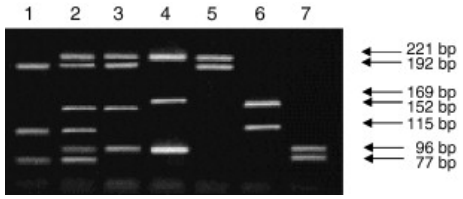

Figure 3: Multiplex (PCR–RFLP) analysis for MTHFR C677T, prothrombin G20210A and factor V Leiden. Lane 1: A prothrombin G20210A, factor V and MTHFR C677T wild-type allele. Lane 2: A prothrombin G20210A heterozygote, factor V Leiden and MTHFR C677T heterozygote allele. Lane 3: A prothrombin G20210 heterozygote, factor V Leiden homozygote and MTHFR C677T homozygote allele. Lane 4: Undigested wild-type 221-bp (prothrombin), 169-bp (factor V) and 96-bp (MTHFR) amplicons. Lane 5: A prothrombin G20210 heterozygote sample. Lane 6: A factor V Leiden heterozygote sample. Lane 7: A MTHFR C677T heterozygote sample.

Statistically, Melt data of RT-PCR confirmed the results of multiplex allele-specific PCR amplification and restriction fragment length polymorphism method (RFLP-PCR). There is a significant frequency of combined thrombophilic polymorphisms of FV G1691A, FII G20210A and TL-MTHFR C677T compared to controls P<0.01. We have reported that the combinations of two or more TP risk factors were observed in 10.8% healthy Saudi women with unexplained RPL while no more than one risk factor was observed in any of the controls, thereby confirming the hypothesis that thrombophilia is indeed a significant abnormality in the RPL subjects (Table 1).

Polymorphism	Patients (72)		Controls (70)		P
	No.	%	No.	%	
FV G1691A*	17	23.6	1	1.38	0.001
FII G20210A*	22	30.5	1	1.38	0.005
TL-MTHFR C677T*	13	18	7	10	0.12
Combined	8	10.8	0	0	0.01
P: probability P<0. 001 high significant					

Table 1: Throbophilic polymorphism in women with pregnancy Loss and controls.

For FV, there is statistically significant lower frequency of homozygous normal genotype (GG) among cases compared to that of controls [69.44% vs. 97.14%, P<0.001]. On the other hand, they disclosed a significant higher frequency of the heterozygous mutant form (GA) than that of controls [20.83% vs. 2.86%, P<0.001]. Thus, the total carriage rate of FV, a mutation represented in both heterozygote form (GA) and homozygote (AA), mutant genotype frequencies was significantly higher among cases versus controls [23.6% vs. 1.38%, P>0.001]. Regarding allelic frequencies, the mutant A allele was significantly higher in cases than in controls [13.19% vs. 0.71%, P>0.0001]; the reverse was noted with the wild type allele G which was significantly lower among cases compared to that of controls (85.81% vs. 99.29%) (Figure 4).

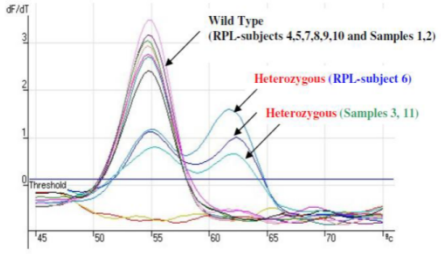

Figure 4: Identification of Factor V Leiden (Melt data).

For prothrombin, there is a statistically significant lower frequency of homozygous normal genotype (GG) among cases compared to that of controls [61.1% vs. 95.5%, P<0.005] (Figure 5).

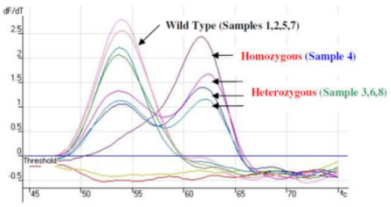

Figure 5: Identification of Prothrombin G20210A (Melt data). Samples 1 and 2 were negative for FII (Wild Type), samples 3, 6 and 8 were positive controls for FII (Heterozygous), sample 4 was positive for FII (Homozygous) and samples 5 and 7 were negative for FII (Wild Type).

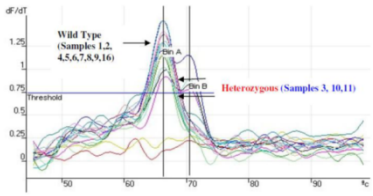

Figure 6: Identification of MTHFR C677T (Melt data). Samples 1 and 2 were controls for MTHFR C677T (Wild Type), sample 3 was positive control for MTHFR C677T (Heterozygous), samples 4,5,6,7,8,9, 16 were wild Type for MTHFR C677T and samples 10 and 11 were Positive for MTHFR C677T (Heterozygous).

On the other hand they showed a significant higher frequency of the heterozygous mutant form (GA) than that of controls [34.72% vs. 1.43%, OR= 36.7, 95% CI = 4.8-80.39, P<0.001]; thus, both heterozygote form (GA) and homozygote (AA) mutant genotype frequencies was significantly higher among cases versus controls [30.5% vs. 1.38%, P>0.005]. Regarding allelic frequencies, the mutant A allele was significantly higher in cases than that in controls [19.14% vs. 1.38% , P>0.001]; the reverse was noted with the wild type allele G

which was statistically significant lower among cases compared to controls (79.86% vs. 99.29%) (Figure 5). We have reported that methylenetetrahydrofolate reductase MTHFR C677T gene mutations were not individually related with recurrent pregnancy loss (Figure 6).

Discussion

Combined thrombophilia (i.e. inherited thrombophilia associated with acquired thrombophilia or more than one inherited thrombophilic defect) has been identified as a cause of RPL, but its real frequency is not clear. Several studies in the last years identified combined genetics thrombophilic defects in women with RPL, both early RPL and late RPL [18,19].

Our findings were in agreement with those reported by Kovacheva et al. [20] that genetic thrombophilic defects are common in women with RFL. On the other hand they reported the association with late fetal loss [20]. Published studies confirmed that thrombophilic polymorphisms are common in women with fetal loss without apparent cause and are associated with late pregnancy wastage; they confirmed that combinations of TP increase the risk for fetal loss [21]. Recent studies have been observed strong association of thrombophilia with unexplained recurrent pregnancy loss among Malaysian [22]. Recent study in Turkish women observed that FII mutation was significantly higher with RPL compared to controls (10.9% vs. 2.04%, P<0.05), [23]. Otherwise, other study has reported that Turkish women with RPL have no significant in FV or FII mutations compared to controls (7.9% vs. 7%, P=0.780) and (1.7 vs. 1.6%, P=0.931) respectively, while the presence both FV and FII mutations are not significantly different among cases compared to controls (9.6% vs. 8.6%, P=0.756) [24]. An Egyptian study reported that there were high statistically significant increases in the number of RPL cases with FV, prothrombin, and methylenetetrahydrofolate reductase gene mutations compared with normal control. The percentages of cases with heterozygous and homozygous mutations in FV gene (heterozygous 60% and homozygous 10%), FII gene (heterozygous 35% and homozygous 30%), and MTHFR gene (heterozygous 45% and homozygous 25%), were significantly different when compared with normal controls (p<0.001 for all) [25]. Another study among Egyptian reported cases which showed a significantly higher frequency of FV GA (OR=21·38, P<0·0001) and FII GA (OR=36·7, P<0·0001) genotypes. Cases with two or more risk factors had significant higher frequency of both mutant genotypes, while no significant difference could be elicited related to primary or secondary infertility, number of fetal losses, or phase of pregnancy loss. The authors confirmed that the screening for thrombophilic mutations may help in the prevention of unexplained RPL [26]. Settin et al. [27] have concluded that mutations related to the MTHFR gene are increased but not statistically significant in Egyptian women with unexplained pregnancy loss. Also, they observed that there was increased frequency among cases with pregnancy loss >4 times compared with those with ≤4 times but with no statistical significance [27].

In contradiction to our study; Kobashi et al. [28] didn't find FV and FII mutations either with RPL or controls in Japanese women. In Chinese women, FV and FII gene mutations may be rare and have no significance in URESA (unexplained recurrent early spontaneous abortion) [29].

Other studies carried out among Arab mediterranean countries have revealed also a relatively high mutation rate of FV like that done by Zammiti et al. [30] among Tunisian women with RPL who reported FV mutation of 19.4% of patients (4.3% in the homozygous state) and in 5.5% of controls. Some other studies showed uneventful role of FII unlike FV, FII was not found to be a risk factor for gestational VTE or RFL. This was showed in Tunisian patients with > or =3 consecutive early, late, or early-late recurrent pregnancy losses, together with age-matched controls. FV (20.54% vs. 6.06%), but not FII mutation (2.74% vs. 4.04%) was significantly higher in patients versus controls [31].

Among Chicago American women Goodman et al. [32] found a significant increased frequency of FV and FII gene mutation among women with history of RPL (p<0.0001). On the other hand Coulam et al. [33] studied Chicago's American women and found no differences in the frequency of specific gene mutations of FV or FII. However, the prevalence of homozygous mutations and total gene mutations among patients with recurrent miscarriage was significantly higher than among controls. Co-inherited mutations were found in 68% of women with a history of RPL contrasted to 21% of control women. Finally their conclusion instated that inherited thrombophilias are associated with recurrent miscarriage. This association is manifest by total number of mutations rather than specific genes involved. Another study reported a prevalence of FV mutations in patients compared to controls in American Brigham's women (8.0% vs. 3.7%, OR= 2.3, 95% CI= 1.0 -5.2; P=0.05) [34].

Based on the previous studies, we observed the contradiction among different studies for the same population. Discussions of genetic differences between major human populations have long been dominated under a fact that such differences account for only a small fraction of variance in allele frequencies, but nonetheless, this is widely understood to reflect the increased discriminatory power of allele statistics [35], and for that reason we confirmed our results using different screening of PCR. The results of real time PCR confirm that the combinations of TP increase the risk for fetal loss. We found strong association of thrombophilia with unexplained recurrent pregnancy loss in Saudi women.

Witherspoon et al. [35] concluded that the sample size of alleles differences among populations to study race correlated to geographic the originality should be around hundreds of samples. The fact that, given enough genetic data, individuals can be correctly assigned to their populations of origin is compatible with the observation that most human genetic variation is found within populations, not between them. It is also compatible with their finding that, even when the most distinct populations are considered and hundreds of loci are used; individuals are frequently more similar to members of other populations than to members of their own population [36]. Therefore, the sample size among differences account for only a small fraction of variance in allele frequencies between individuals of the same population is given under the fundamentals of the program of statistical analysis were used [35]. Our findings were in agreement with those reported by Witherspoon et al. [35]. In our study, the statistical power based on T test and confident intervals indicate that 142 samples are enough for power of significance for 0.05 when the effect size is 0.1. Our design was in agreement with the studies of references number from 24 to 28, and 31 to 34 which their sample sizes were 102, 40, 72, 70, 83 and 146, 150, and 113 respectively.

Conclusion

By identifying heritable thrombophilia, women might potentially prevent miscarriages, as well as serious maternal and neonatal complications. We recommend Saudi Females with RPL should be

supported by thrombophilia screening. This approach may be helpful to fight this major health problem that involves up to 10% of women of reproductive age by an appropriate antithrombotic treatment.

This study confirmed our result of previous studies that molecular screening for ledien and prothrombin mutations are important and must be included to the routine analysis of Saudi fetal and pregnant women. This may help in early therapeutic protocol and reduce the recurrent abortions.

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