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Detection of factor V G1691A, Prothrombin G20210A and Methylene tetra hydro folate reductase deficiency C677T Gene mutations among Sudanese Women with recurrent spontaneous abortion

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***Abstract*---**The current study is a prospective analytical case control study designed to investigate the relationship between Factor V Leiden G1691A, methylenetetrahydrofolate reductase (MTHFR) C677T and to the prothrombin G20210A mutation variant and adverse pregnancy outcomes. Material and Method: The study included hundred Sudanese women who experienced three or more of the adverse pregnancy loss as case group during their reproductive in the Omdurman Maternity Hospital (Sudan) these compared with ninety-four control group healthy women with at least more than two normal pregnancies and without any history of adverse pregnancy outcome or recurrent miscarriages. The study group data collected using structure questionnaire which was used to collect information about age, parity, medical and obstetric history, smoking, family medical and obstetric history, residency and relative marriage. Blood samples were collected from participants and total genomic DNA was isolated from blood leukocytes and the frequency of these gene mutations in the patients and controls were determined using PCR-restriction fragment length. Results the mutation was detected in 8 out of 100 cases (8.0%) and in 6 out of 94 controls (6.4%) (P- Value > 0.05). In study subjects, the general mutation prevalence of the Prothrombin gene was 3% among cases with (P- Value > 0.05). And there was no

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mutant gene detected among the control group. The frequency of Heterozygous A/C MTHFR gene was 3.0% in cases with (P- Value > 0.05), there was no mutant gene detected among the controls group. Results showed no significant variations in factor V Leiden, prothrombin G20210A and MTHFR C677T gene mutation distribution among women with RSA and controls. Prothrombin time (PT) and partial thromboplastin time (PTT) in women RSA in this study were not affected significantly (P > 0.05 and P > 0.05) respectively. In conclusion the study observed that FV Leiden, FII G20210A mutation and MTHFR C677T do not associated with recurrent spontaneous abortion.

***Keywords*---**factor V G1691A, Prothrombin G20210A, Methylene tetra hydro folate, Gene mutations, abortion.

Introduction

Spontaneous abortion and miscarriage are synonymous terms. In the medical literature, spontaneous abortion is most often used, while in clinical practice and among the general population miscarriage is the preferred term [1]. Spontaneous abortion or miscarriage is defined as the involuntary end of pregnancy before 20 weeks of gestation. Recurrent pregnancy loss (RPL), also known as recurrent miscarriages, is defined by the consecutive loss of two or more pregnancies with the same partner and having no more than one living child [2]. The exact frequency of miscarriages is, however, unknown as miscarriages frequently occur before the woman is aware of her pregnancy. There are numerous factors that may cause (RPL), but the underlying problem often remains undetected. Although much work has been done to identify the underlying mechanisms, the cause of miscarriage can be identified in only ~50% of cases. The known causes of RSA include chromosomal and metabolic abnormalities, uterine anomalies, and immunologic factors. RPL is estimated to occur in 2%–4% of reproductive-age couples [3]. The pregnancy associated with hypercoagulability sets a foundation for hemostatic abnormalities during pregnancy and may be associated with pregnancy complications Thrombophilia is considered still a debated problem that may be common in women with unexplained recurrent pregnancy loss, with prevalence as high as 65% in selected populations [4].The thrombophilia are a number of prothrombotic factors, which can either be inherited or acquired. The influence of thrombophilia in pregnancy is a popular research topic in recurrent miscarriage [5]. The inherited thrombophilia includes activated protein C resistance 95% due to factor V Leiden (FVL) mutation], protein S deficiency, protein C deficiency, antithrombin III deficiency, FII (prothrombin) mutation and hyperhomocysteinemia .Factor V Leiden (FVL) and prothrombin (G20210A) mutations are the most common causes have been implicated as risk factors of hereditary thrombophilia which in turn can result in placentation [6]. This study aimed at testing the association between FVL, FII, MTHFR and Recurrent Spontaneous Abortion in Sudanese women.

Materials and Methods

The study included hundred Sudanese women who experienced three or more of the adverse pregnancy losses as case group during their reproductive in the Omdurman Maternity Hospital (Sudan) these compared with ninety-four control group healthy women with at least more than two normal pregnancies and without any history of adverse pregnancy outcome or recurrent miscarriages. The study group data collected using structure questionnaire which was used to collect information about age, parity, medical and obstetric history, smoking, family medical and obstetric history, residency and relative marriage. Blood samples were collected from participants and total genomic DNA was isolated from blood leukocytes and the frequency of these gene mutations in the patients and controls were determined using PCR-restriction fragment length polymorphism participants and total genomic DNA was isolated from blood leukocytes and the frequency of these gene mutations in the patients and controls was determined using PCR-restriction fragment length polymorphism. DNA was extracted from the blood samples using Master pure DNA purification kit for blood GF-1 Blood DNA Extraction Kit, 50 PREPS (cat. No. GF-BD-050, Vivantis Technologies Sdn. Bhd., Malaysia). FV Leiden G1691A, MTHFR C677T and FII. a 345-bp genomic DNA fragment encompassing a part of the prothrombin gene that contains the mutation was amplified by PCR using specific primers Forward (5'TCT AGA AAC AGT TGC CTG GC-3') and Reverse primer (5'ATA GCA CTG GGA GCA TTG AAG C-3). And 267-basepair (bp) segment of the factor V gene was amplified using specific forward primer (5'TCA GGC AGG AAC AAC ACC AT-3') and reverse primer 5'GGT TAC TTC AAG GAC AAA ATA CCT GTA AAG CT3. MTHFR gene by using the site-specific primers Forward (5' TGA AGG AGA AGG TGT CTG CGG GA-3') and Reverse primers: 5'AGG ACG GTG CGG TGA GAG AGT G -3'. The reaction program was as follows: Denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds, 15 for 35 cycles and 72°C for 5 minutes. A master mix was prepared by adding Nuclease free water, 10x buffer, dNT P, tow primers l2, Taq DNA polymerase and DNA, the mixture was loaded into thermocycler according to the specific Temperature profile. The working solution of 1X TBE was prepared from the stock solution (1 L) which contained the following: 89 mM Tris base (108 gm), 89 mM boric acid (55 gm) 40 ml of 0.5M EDTA, adjust pH to 8.0.1.5% agarose was prepared from 1x TBE, and 5µl PCR products were loaded by mixing PCR products with 1µl loading dye, run on the gel for 30 mins and visualized on UV transilluminator. Factor V digested with 10 µl of DNA restriction enzyme MnI1 at 37°Cfor 18 h, subjected to 2% low melting point agarose and Prothrombin product (10 µL) was digested with 20 U of Hind III, at 37°C for 16 h, and loaded into 2% low melting point agarose gel, electrophoresed at 90 volts for 60 mins. MTHFC677T was digested by enzyme (Hindfl) by Added 10 µl mixtures to the 10 µl MTHFR products, a quick spinning is needed, 5- Incubated at 37 ºC 18 hours, and the reaction was stopped with 4 µl prom phenol blue dye, then 18 µl digested products was loaded into 2% agarose. Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages where appropriate [7,8] . The odds ratio (OR) and the 95% confidence interval (95%CI) were calculated for the presence of mutation between cases and controls and analyzed by SPSS program (version: 17.0). Data were analyzed using the Chi-square test to compare the

prevalence of MTHFR mutation between patients and controls (The test was considered significant when p-value <0.05)

Results

The participants included 194 women subjects. Out of them, 100 had a history of 3 or more events of recurrent fetal loss (abortion, miscarriage or still birth). Their mean age± SD was 25 ± 4. And 94 women were healthy the mean age of was 30 ± 4.Factor V Leiden mutation distribution showed higher prevalence among cases group than controls group. The mutation was detected in 8 out of cases (8.0%) and in 6 out of 94 controls (6.4%) P- Value =0.66, Odds Ratio=1.28, 95% CI (0.42 to 3.84) The prevalence of heterozygous FVL mutation in RM Women was found to be 8 % but in control was found to be 6.4%. Normal homozygous (G/G) among cases show 92% but in controls show 93.6%. Alleles G allele occurred with a frequency of 96. % Among cases and 96.8% in controls while mutant allele (A) was seen only in 4 % of the cases. Frequency of mutant allele (A) was 3.2 % and G allele occurred with a frequency of 96.8 % among controls. According to this result there is statistically insignificant between the cases and controls group (Table I&Fig1). Prevalence of the Prothrombin gene was 3% among cases with P- Value =0.091.but no mutant gene detected among control group. According to the genotyping in cases showed (Heterozygotes, 3.0%; Homozygotes, 97.0%), Alleles G (98.5%) and Alleles A (1.5%) while in controls group show Normal homozygous G/G (100%) and Alleles G (Alleles G). No significant association between cases carriage any of this mutation and risk with recurrent pregnancy miscarriage (Table 2&Fig2). Frequency of Heterozygous C/T MTHFR gene was 3.0% in cases with P- Value =0.091, there was no mutant gene was detected among the controls group. Normal homozygous gene was 97.0% in cases and 100% show in controls. The frequency of Alleles C was 98.5% in cases and 100% in controls while Alleles T was 1.5%. There was no significant association between cases carriage any of this mutation and risk of recurrent miscarriage (Table 3&Fig3). The cases group in our study was divided into subgroups based on time of recurrent abortion from second to eight times of repeated miscarriage. Our data indicates that factor V was most frequent with recurrent miscarriage, repeated in women with three time of repeated miscarriage by (37.65) fallow by women with four time of repeated miscarriage by(50%) and found in women with five time of repeated miscarriage by(12.5%) Prothrombin was found only among those women with three time of recurrent miscarriage with 100% and MTHFR present in three, four and five times of recurrent miscarriage women with equal percentage 33.3% foe each (Table 4)

Table 1
Frequency of factor V (Leiden) mutation among cases of recurrentpregnancy loss compared to controls

Genotype	Patients N (%)	Controls N (%)	P-value	OR (95%CI)
Heterozygous G/A	8(8.0)	6(6.4)	0.66	1.28(0.42 to 3.84)
Normal homozygousG/G	92(92.0)	88(93.6)		

Alleles G	192(96.0)	182(96.8)	0.67	0.76(0.27 to 2.33)
Alleles A	8(4.0)	6(3.2)		

Table 2
Frequency of Prothrombin mutation among cases of recurrent pregnancyloss compared to controls

Genotype	Patients N (%)	Controls N (%)	P-value	OR (95%CI)
Heterozygous G/A	3(3.0)	0	0.091	0
Normal homozygous G/G	97(97.0)	94(100)		
Alleles G	194(98.5)	188(100)	0.089	0
Alleles A	3(1.5)	0		

Table 3
Frequency of MTHFR mutation among cases of recurrent pregnancy losscompared to controls

Genotype	Patients N (%)	Controls N (%)	P-value	OR (95%CI)
Heterozygous C/T	3(3.0)	0	0.091	0
Normal homozygous C/C	97(97.0)	94(100)		
Alleles T	3(1.5)	0	0.089	0
Alleles C	194(98.5)	188(100)		

Table 4
Frequency of factor V (Leiden), Prothrombin and MTHFR related totimes of recurrent pregnancy loss

Times of recurrent abortion	Factor V		Prothrombin		MTHFR	
	Positive	Negative	Positive	Negative	Positive	Negative
Twice	0	8(8.8)	0	8(8.3)	0	8(8.3)
Three times	3(37.5)	57(62.6)	3(100)	57(59.4)	1(33.3)	59(61.5)
Four times	4(50.0)	16(17.6)	0	20(20.8)	1(33.3)	19(19.8)
Five times	1(12.5)	6(6.6)	0	7(7.3)	1(33.3)	6(6.2)
Six times	0	1(1.1)	0	1(1.0)	0	1(1.0)
Seven times	0	1(1.1)	0	1(1.0)	0	1(1.0)
Eight times	0	2(2.2)	0	2(2.1)	0	2(2.1)

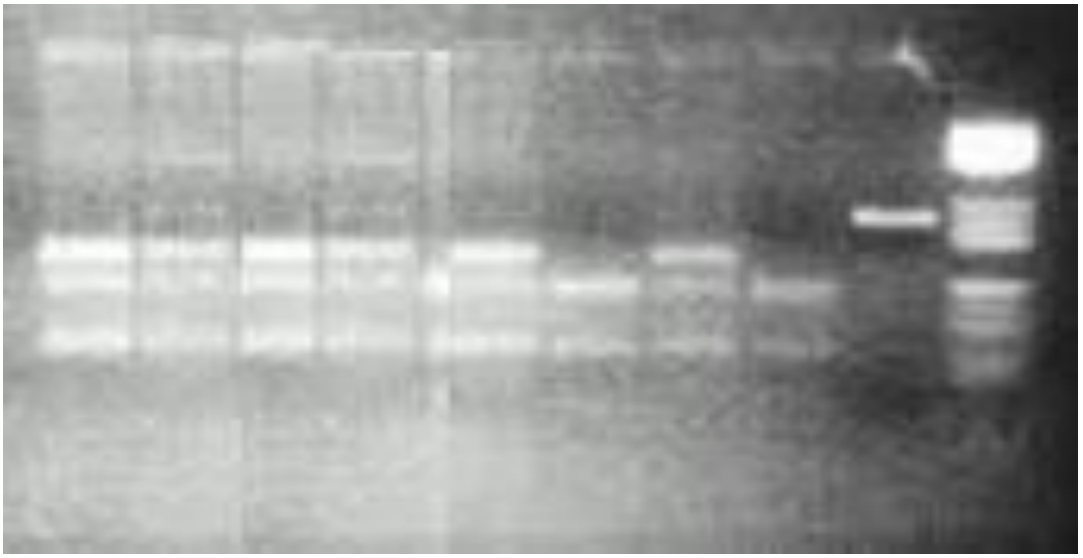

Fig 1: PCR amplification of FVL gene mutation

Digestion of factor v gene with MnI1 enzyme on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide , Lane 1 molecular weight marker 50 bp, lane2 undigested PCR products lane 3 and 5 were hetrozygous mutant (AG), Lane 4,6,7 and8 ,9 and 10 were Wild typ (AA), The 267 bp DNA products digested with MnI1.

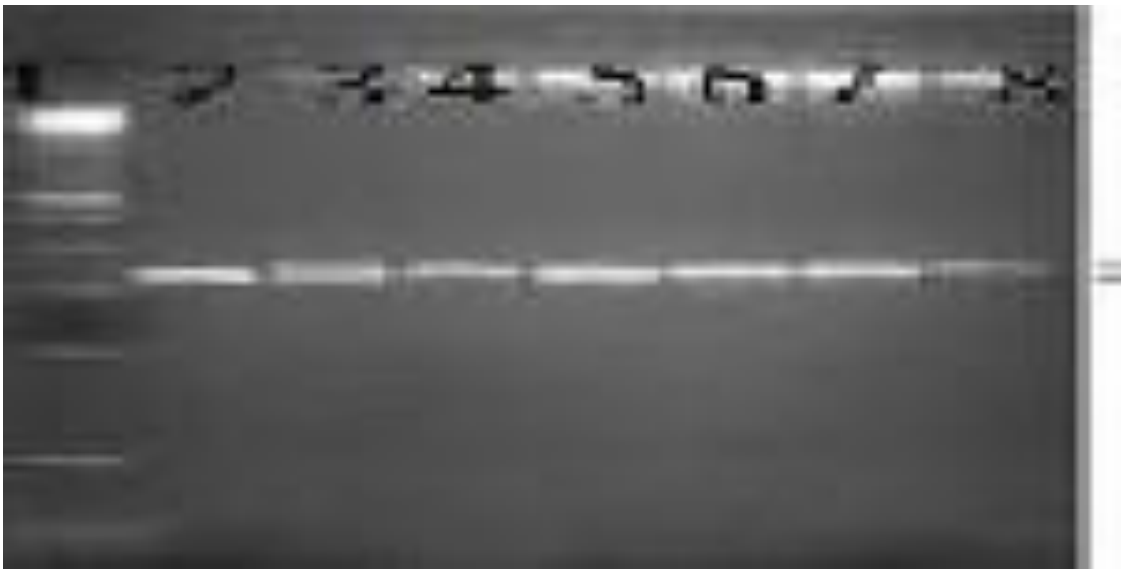

Fig 2: PCR amplification of Prothrombin gene mutation

Digestion of prothrombin gene with Hind III on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide , Lane 1 molecular weight marker 100 bp,

lane 2 (322 bp), mutant(AA), control, lane 3 and 5 were hetrozygous mutant (GA), Lane 4,6 and 7 were Wild type (GG), lane 8 undigested(345 bp)

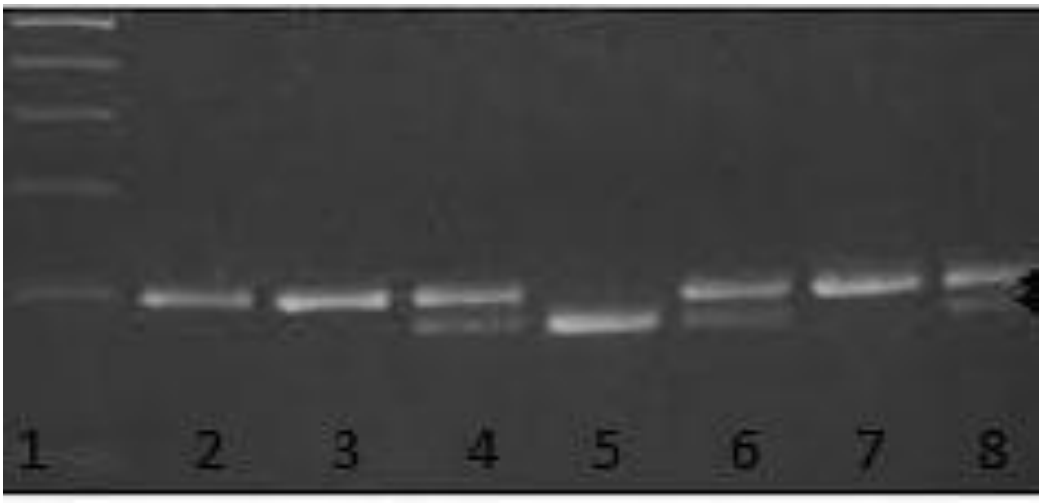

Fig 3: PCR amplification of MTHFR gene mutation

Digestion of MTHFR gene with Hindf1 on 2% agarose gel disolved in 1X TBE buffer, stanied with ethidium bromide, Lane 1 molecular weight marker 100 bp, lane 2 undigested(198 bp), lane 3 wild type (CC), Lane 4,6 and 8 were hetrozygous mutant(CT), lane 5 was Control homozygous mutant(TT)

Discussion

Pregnancy is a complicated physiological process that might lead to negative outcomes and could threaten the women's life or the fetus. Miscarriage is a common occurrence in the life cycle of the woman. Exactly how common this experience is not known exactly. Current literature suggests that the cause of RM is only identifiable in up to 40%-50% of cases. Improvement of pregnancy outcome is considered as an important area of action for those concerned with the improvement of women's health and pregnancy outcome. Exploring the relation between blood coagulation mutations with recurrent miscarriages is a challenge [9]. The prevalence of Factor V Leiden mutation was tested and calculated in both case and control groups. The presence of Factor V Leiden mutation was slightly higher among cases group compared to the controls group. The prevalence of the mutation among cases group was 8% while it was found to be 6% among controls group. Factor V Leiden mutation, involved in the etiology of poor pregnancy outcomes, and has been proposed as one of the leading factors that is associated with poor pregnancy outcomes. We did not find a strong association with factor V Leiden gene polymorphism and recurrent spontaneous abortion. Result of the present was in agreement with findings from the several large meta-analysis studies that have explored to determine any significant association between the factor V Leiden mutation and the presence of recurrent miscarriage during pregnancy. This result is agreement with study performed by Samieh, *et.al.,* [10] , among Iranian women in which the frequency of factor V Leiden gene mutation in total, (8.6%) of cases and 2(1%) of controls showed the factor V

Leiden mutation and the incidence of factor V Leiden was typically higher in preeclamptic women than control group, concluded that pregnant women with factor V Leiden mutation were prone for preeclampsia syndrome during pregnancy, but this risk factor was not correlated to pregnancy complications in the studied women. Extended to our study, the prothrombin G20210A mutation our result revealed that the mutation not common among recurrent spontaneous aborted Sudanese women they were found in 3 out of 100 women with frequency 3% and did not found any mutated gene among control group. We found there was no significant association between the prothrombin G20210A mutation and repeated of spontaneous abortion among Sudanese women. . This finding was agreeing with study performed by Robert, *et.al.,* [11], were found there was a total of 157 (3.8%) women had the prothrombin gene mutation and concluded that there was no association between the prothrombin G20210A mutation and pregnancy loss. Also the result is complete agreement with the one performed by Reaza , *et.al.,* [12] where were found no significant difference was observed in the frequencies of FII mutations between the patients and controls , were found that the frequency of prothrombin was 3.2% in cases and 0.0%, in the control group . In another study done by Majid, *et.al.,* [13], among 80 Iranian women with recurrent pregnancy loss no factor II mutation in cases and controls was found and concluded these data did not confirm that factor II prothrombin gene G20210 mutation might play a role in recurrent pregnancy loss in Iranian women. This result was disagreement with findings from the several large meta- analysis studies that have explored to determine any significant association between the prothrombin gene G20210 and the presence of recurrent pregnancy loss. In addition, we extended our study to the Methylene tetrahydrofolate reductase C677T gene mutation which suspected have role and associated with inherited thrombophilia's among Sudanese women with recurrent miscarriage. The prevalence of MTHFR C677T variant among women with recurrent miscarriage is still a matter of controversy. In our study we found that they did not found significant between this gene and recurrent. Also our study was disagreement with study done by Foka , *et.al.*, [14] the study was to investigate the relationship between recurrent miscarriages and factor V Leiden, prothrombin G20210A and C677T methylene tetrahydrofolate reductase (MTHFR) mutations was determined in a consecutive series of 80 recurrent miscarriage patients and 100 controls and suggest that that the presence of factor V Leiden and prothrombin G20210A polymorphism, but not MTHFR C677T homozygosity, could be additional risk factors for recurrent miscarriages. Furthermore, it was suggested that the prevalence of factor V Leiden and prothrombin G20210A mutations is more prominent in second trimester, primary fetal losses and it is independent of the existence of additional pathology predisposing to recurrent fetal losses.

Conclusion

The result of Factor V Leiden mutation showed no significant variations among women with RSA case group (8.0%) compared to controls group (6.4%) and didn't increased risk for recurrent spontaneous abortion development. No significant variations in MTHFR C677T genotype distribution among women who suffered from RSA (3.0%), and there was no mutant gene was detected among the controls group. FII G20210A mutation (3.0%), and there was no mutant gene was detected among the controls group and there was no significant association between cases

carriage any of this mutation and risk with recurrent pregnancy loss.

Recommendations

Further studies on large Sudanese women population with recurrent spontaneous abortion are needed to classify all DNA thrombophilia mutations by using more sensitive and accurate molecular methods in studying potential affected DNA Mutations regions such as real time PCR technique. As findings of the current study seems to indicate limited importance of genetic factors and a pronounced special concern should be paid for couples with recurrent miscarriage should be tested for immunologic, infections and other physical abnormalities in women reproductive system to find more risk factor among these women. More prospective studies are required to explain the relationship between thrombophilia especially factor XIII, XI and recurrent spontaneous abortion because it showed more frequency among these women

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