DETECTION OF PROTHROMBIN GENE POLYMORPHISM AMONG SUDANESE WOMEN WITH RECURRENT SPONTANEOUS ABORTION

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ABSTRACT

Background: Recurrent spontaneous abortion (RSA) has traditionally been defined by two or more consecutive pregnancy losses before 20 weeks' gestation. RSA has been estimated to occur in approximately 1% of all couples. The World Health Organization (WHO) defines it as expulsion or extraction of an embryo or fetus weighting 500 g or less.

Methods: This was a descriptive analytical case-control hospital-based study conducted at the research laboratory of the national center of neurological sciences (NCNS), Khartoum, Sudan during the period April to September, 2021. The study aimed to detect prothrombin gene polymorphisms among Sudanese women with recurrent spontaneous abortion. Women with more than three recurrent abortions were included in this study as cases and those healthy women with no history of abortion at reproductive age were included as controls. From each subjects 3 ml of venous blood was collected in sterile containers with Ethylene Diamine Tetra-acetic Acid (EDTA) DNA was isolated from venous blood samples by the standard phenol chloroform extraction method. The factor II gene was amplified using conventional PCR. PCR products were sent for sequencing to Macro gene Europe Laboratory.

Results: In the present study 345bp of factor II gene was detected with gel electrophoresis after PCR. The PCR result shows that about 41 of cases were positive and only 3 were negative. For the control group the PCR result revealed that 36 were negative and 14 were positive. When compared between case and control there was great statistically significant (P=0.001, Chi= 40.962)

The sequencing results were analyzed using different bioinformatics soft-wares and tools. When the cases were compared with the normal reference one single Base Exchange was found G to A (G20210A). While when the controls were compared with normal reference, no any single base exchange was found among the all control groups, the mutation was confirmed by the Mutation taster program.

Conclusion:

The result of Prothrombin gene mutation showed (P=0.001) significant variations among women with RSA case group (93.2%) compared to controls group (28.0%) and might increase risk for recurrent spontaneous abortion development. The sequencing results analysis confirmed the present of G to A (G20210A) mutation among Sudanese women with recurrent spontaneous abortion.

Keywords: Prothrombin gene, mutation G20210A, PCR, sequencing, Macro gene.

Introduction

Recurrent spontaneous abortion (RSA) has traditionally been defined by two or more consecutive pregnancy losses before 20 weeks' gestation. RSA has been estimated to occur in approximately 1% of all couples [1]. The World Health Organization (WHO) defines it as expulsion or extraction of an embryo or fetus weighting 500 g or less [2].

Recurrent spontaneous abortion causes by various genetic and non-genetic factors, which include uterine and cervical anatomic abnormalities, ovarian dysfunction, endocrine problems, immunologic abnormalities, chromosome abnormalities and thrombophilia [3]. However, its cause remains unknown in 50% to 60% of cases, thereby making it a frustrating condition for both physicians and patients. [4] A normal pregnancy is dependent on sufficient placental circulation and fetal vasculature. Abnormalities of placental vasculature may cause several gestational complications like pregnancy loss, intrauterine fetal death, intrauterine growth restriction and preeclampsia [5].

Prothrombin (factor II) is a blood protein that is desired for the blood to clot and It is a vitamin K-dependent protein which is synthesized in the liver and circulates with a half-life of approximately three to five days. Blood clots are collected of a combination of blood platelets and a meshwork of the blood clotting protein fibrin. Prothrombin is needed to form fibrin [6].

The human prothrombin gene expansion 21 kb on chromosome 11p11-q12 and composed of 14 exons and 13 introns, which consider for 90 percent of the sequence. Prothrombin gene (G20210A) mutation is connected with raised risk of thrombosis and it is the most identifiable risk factor for venous thrombosis and is in fact the second most common genetic defect for inherited thrombosis, with Factor V Leiden being the most common [7].

The mutation leads to an increased amount of thrombin circulating in the person's blood stream. The exact mechanism by which the prothrombin gene mutation results in a thrombophilic state is unclear. It is thought that the increased amount of circulating prothrombin provides a springboard upon which the clotting cascade can get started and that, in some circumstances, it may run out of control because of that springboard potential. [8,9].

Material and methods

This study will be case-control hospital base study design, conducted at the research laboratory of the national center of neurological sciences (NCNS), Khartoum, Sudan during the period April toSeptember2021. All patients attending obstetrics and gynecology unit at Ibrahim Malik teaching hospital and diagnosed with unexplained recurrent spontaneous abortion during the aforementioned period were included as cases until the sample reached (44). In addition to that, apparently healthy (50) women with no history of abortion and without any other risk factor related to abortion were selected as control group.

From each subjects 3 ml of venous blood was withdrawn with minimal stasis from the ante-cubital 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 identification number and stored at -20°C for molecular analysis. The participants were interviewed with questionnaires; the questions were about demographic data and clinical information along with other data required in the study**.**

The DNA was isolated from peripheral blood leukocyte by standard phenol chloroform extraction method. Primers were designed by using Prime3 software. The forward primer for FII 20210was designed as "5- F: TCTAGA AAC AGT TGC CCT GGC3"and reverse as "5- ATA GCA CTG GGA GCA TTG AAGC-3" with product size of 345bp fragment. Then the PCR was done by specific protocol; 14 ul of ddH$_2$O was placed in PCR tube, then 4 ul of master mix, 1 ul of forward primer, 1 ul of reverse primer and 2 ul of DNA sample was added then vortex. The PCR tube containing this mixture was place in commercial thermal cycler (Swift™MaxPro SWT-MXP-BLC-4) at following condition: Denaturation temperature 94°C for 30 secs, annealing temperature at 63°C for 30 sec and extension temperature at 72°C for 30 sc, the final elongation was adjusted for 10 minutes at 72 °C. PCR reaction was set at 35 cycles.

The PCR amplification product was separated on agarose gel (0.7g of agarose gel + 28 ml of DW + 7 ml T.E buffer, this mixture was placed in microwave for 1 minute and then 1 ul of Ethidium Bromide was added and mixed then poured on Gel-Electrophoresis Try and left to dry). After it dried the PCR amplification products and 100-base-pair DNA ladder were filled in the wells, then the running buffer was poured and ran at 150V for 16 minutes and the results were trans-illuminated with UV light and visualized through gel documented system. PCR products were sent for sequencing to Macro gene Europe Laboratory.

SPSS version 23 statistical software (SPSS Inc., USA) was used for statistical analysis. Data were expressed as means with standard deviations. The sequencing results were analyzed using different bioinformatics soft-wares and tools.

Ethical clearance for this study was obtained from ethical review committee, Faculty of Medical laboratory, University of Medical Science and Technology the participants was fully informed about the advantages and disadvantages before participation in the research (verbal informed consent).

Result

The epidemiological study

Current study includes 94 participants; 44 as a case group and 50 health women as control group. Demographic characteristics of case group about two third their age group between (25-34) 36.4% and (35- 40) 34.1%, while two third had a low and high educational level 36.4%, 36.4% respectfully. The average weight was (m=59.75, SD=15.25) for the control group, half of participant were aged between (18-24) 50%, one third was aged between (25-34) 38%. (Table1, 2) (Figer1)

Distribution of case group according to miscarriage related variables; 61.4% were incomplete miscarriage, more than 3 months was the duration between miscarriage reported by 75% of participants, about 60% received no treatment, the most prominent diagnosis was reported was Unexplained for all cases 100%. (Table 3)

Table 1.

Distribution of case group according to demographic variables (n=44)

Demographic variables		Frequency	Percent
Age	18-24	6	13.6
	25-34	16	36.4
	35-40	15	34.1
	>40	7	15.9
	Total	44	100.0
Educational level	Low	16	36.4
	Moderate	12	27.3
	High	16	36.4
	Total	44	100.0
Weight		M=59.75 SD=15.25	

Table 2.

Distribution of control group according to age variables (n=50)

Age		Frequency	Percent
Control	18-24	25	50.0
	25-34	19	38.0
	35-40	3	6.0
	>40	3	6.0
	Total	50	100.0

Table 3.

Distribution of case group according to Miscarriage related variables (n=44)

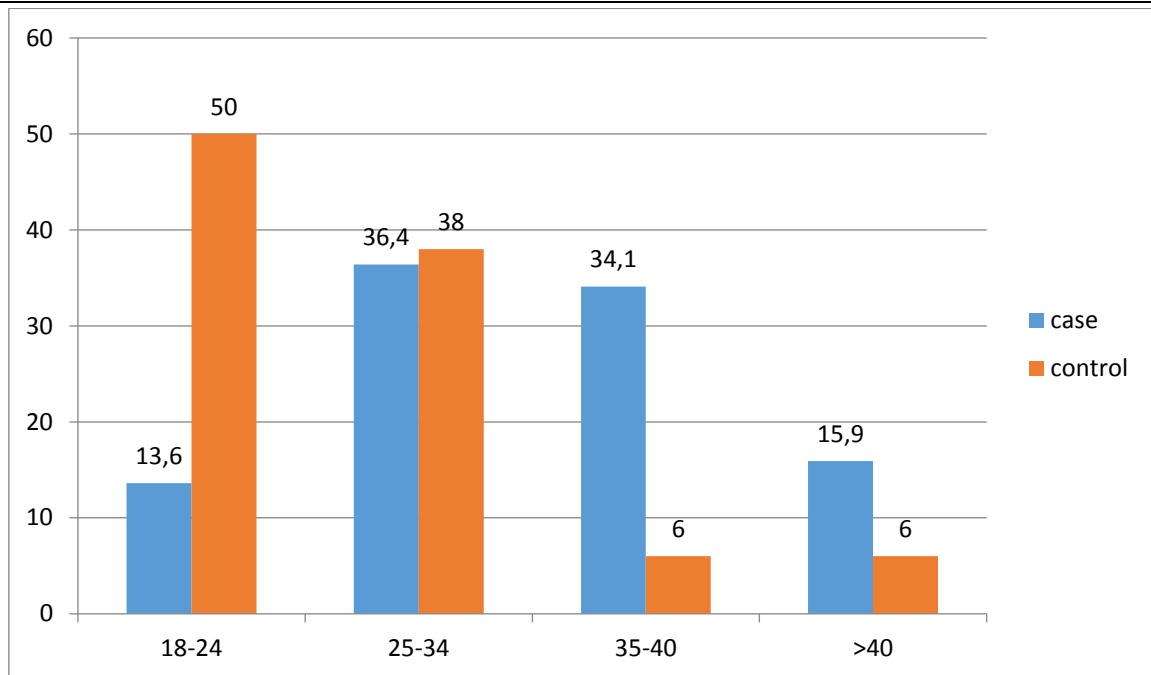| Miscarriage		Frequency	Percent
Miscarriage outcome	Incomplete	27	61.4
	Completes	17	38.6
	Total	44	100.0
Duration between miscarriage	1-3m	11	25.0
	>3m	33	75.0
	Total	44	100.0
Treatment	Cure settling	3	6.8
	Medication	15	34.1
	No	26	59.1
	Total	44	100.0
Miscarriagediagnosis	Unexplained	44	100.0
No ofmiscarriage		M=2.98 SD=1.4	

Figure 1. Distribution of case and control group according to age variables.

Molecular study

In the present study 345bp of factor II gene was detected with gel electrophoresis after PCR (Figure 2). The PCR result shows that about 41 of cases were positive and only 3 were negative. For the control group the PCR result revealed that 36 were negative and 14 were positive (Table 4). When compared between case and control there was great statistically significant (P=0.001, Chi= 40.962) (Table 4)

Sequencing

The sequencing results were analyzed using different bioinformatics soft-wares and tools. The obtained sequences aligned using BioEdit-ClustalW software with a normal sequence from GenBank gene (accession number NC_000011.10 in NCBI).

When the cases were compared with the normal reference one single Base Exchange was found G to A (G20210A). While when the controls were compared with normal reference, no any single base exchange was found among the all control groups (Figure 4, 5)

Mutation Taster

Mutation taster was used to confirm the mutation which revealed; G>A Base Exchange, disease causing polymorphism was predicted, amino acid sequence was changed, protein features might be affected and splice site also was changed, alteration location was at chromosome 11, alteration type was single base exchange, cDNA changes position was 1518, G>A regulatory features was histone, histone 3 Lysine 27 Tri-Methylation (H3K36mes3) (Figure 6)

Table 4

Distribution of PCR result and Cross tabulation in the cases and control group

		Group		Total
		Case	Control	
Result	**Positive**	41	14	55
	Negative	3	36	39
Total		44	50	94
***P*= 0.001**		**Chisquare = 40.962**		

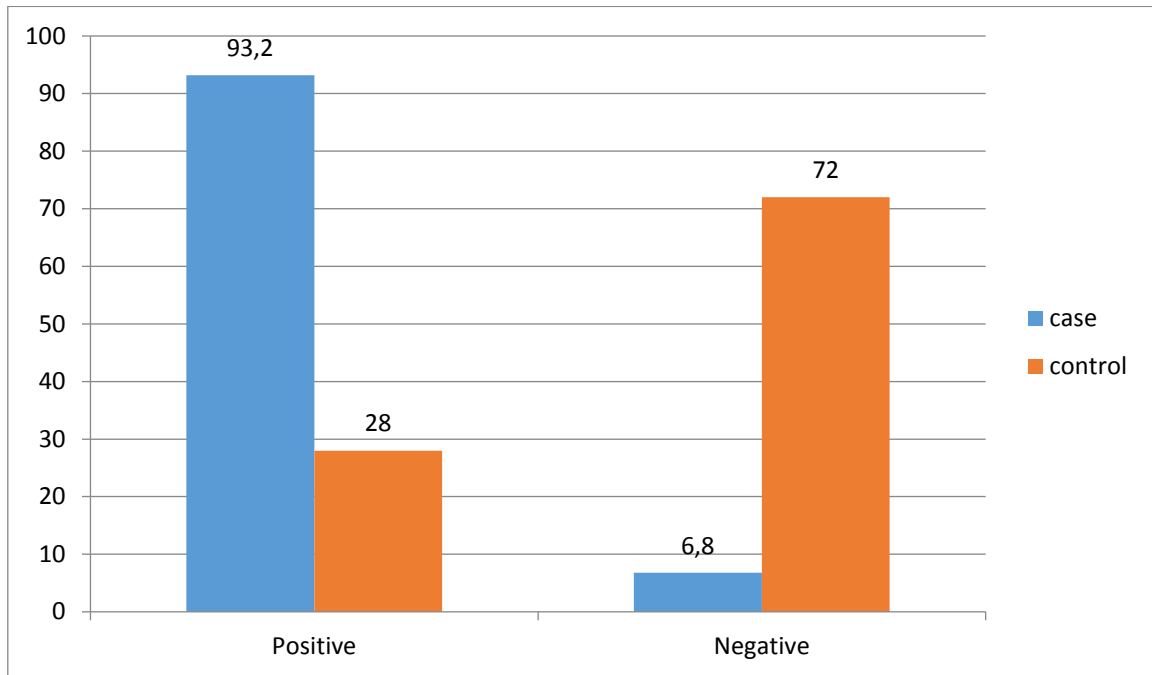*Figure 2: 345 bp of factor II gene detected with gel electrophoresis*

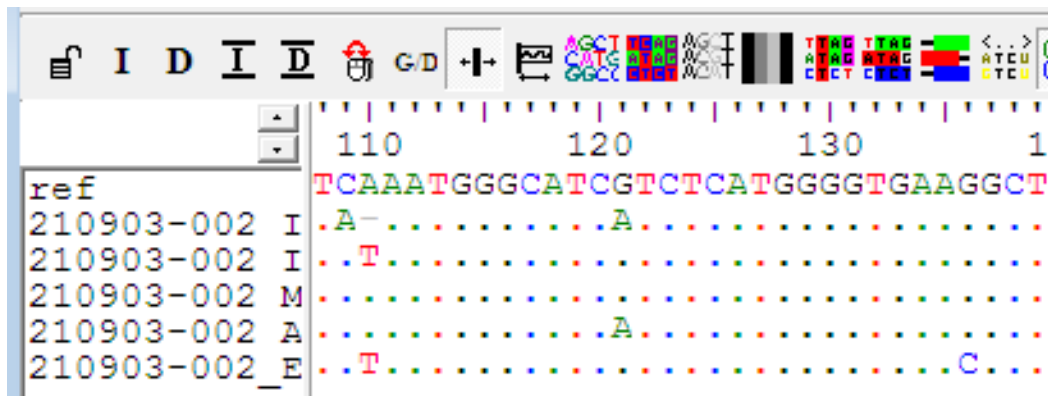*Figure 3. Distribution of case and control group according to PCR result*

Figure 4:
Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of II gene.
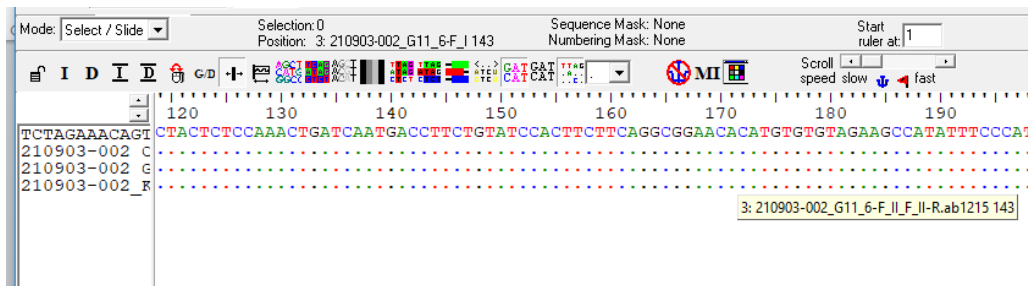

Figure 5:
Multiple sequence alignment using Bio-Edit clustal W for control group with reference gene sequence of II gene.

Figure 6: result of G>A singles Base Exchange tested in mutation taster application.
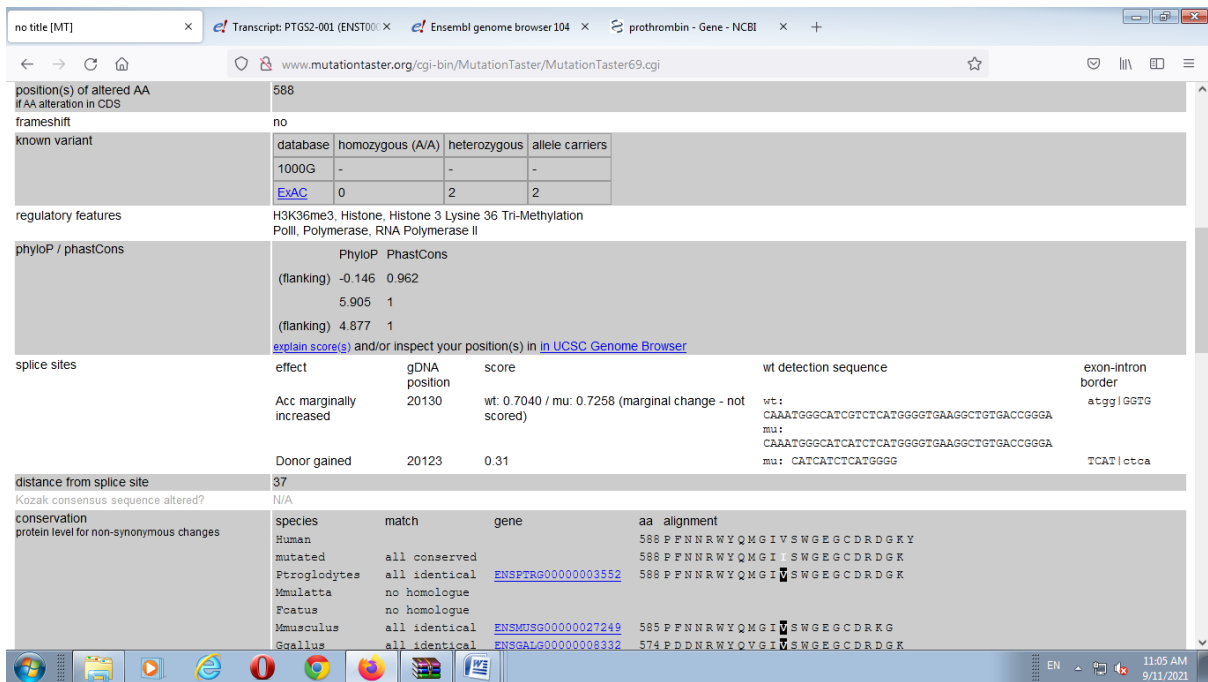

Table 5

Cross tabulation of PCR gene mutation finding according to age group distribution

			Case		Total	Control		Total
			Positive	Negative		Positive	Negative	
Age	18-24	Count	6	0	6	9	16	25
		% within Age	100.0%	0.0%	100.0%	36.0%	64.0%	100.0%
	25-34	Count	15	1	16	3	16	19
		% within Age	93.8%	6.2%	100.0%	15.8%	84.2%	100.0%
	35-40	Count	14	1	15	1	2	3
		% within Age	93.3%	6.7%	100.0%	33.3%	66.7%	100.0%
	>40	Count	6	1	7	1	2	3
		% within Age	85.7%	14.3%	100.0%	33.3%	66.7%	100.0%
Total		Count	41	3	44	14	36	50
		% within Age	93.2%	6.8%	100.0%	28.0%	72.0%	100.0%
Chi square			0.786			0.405		

Table 6

Association between of PCR gene mutation finding and number of miscarriage

PCR gene mutation finding	N	Mean	Std. Deviation	P value
Positive	41	3.05	1.431	
Negative	3	2.00	.000	0.216
Total	44	2.98	1.406	

Table 7.

Association between weight and number of miscarriage

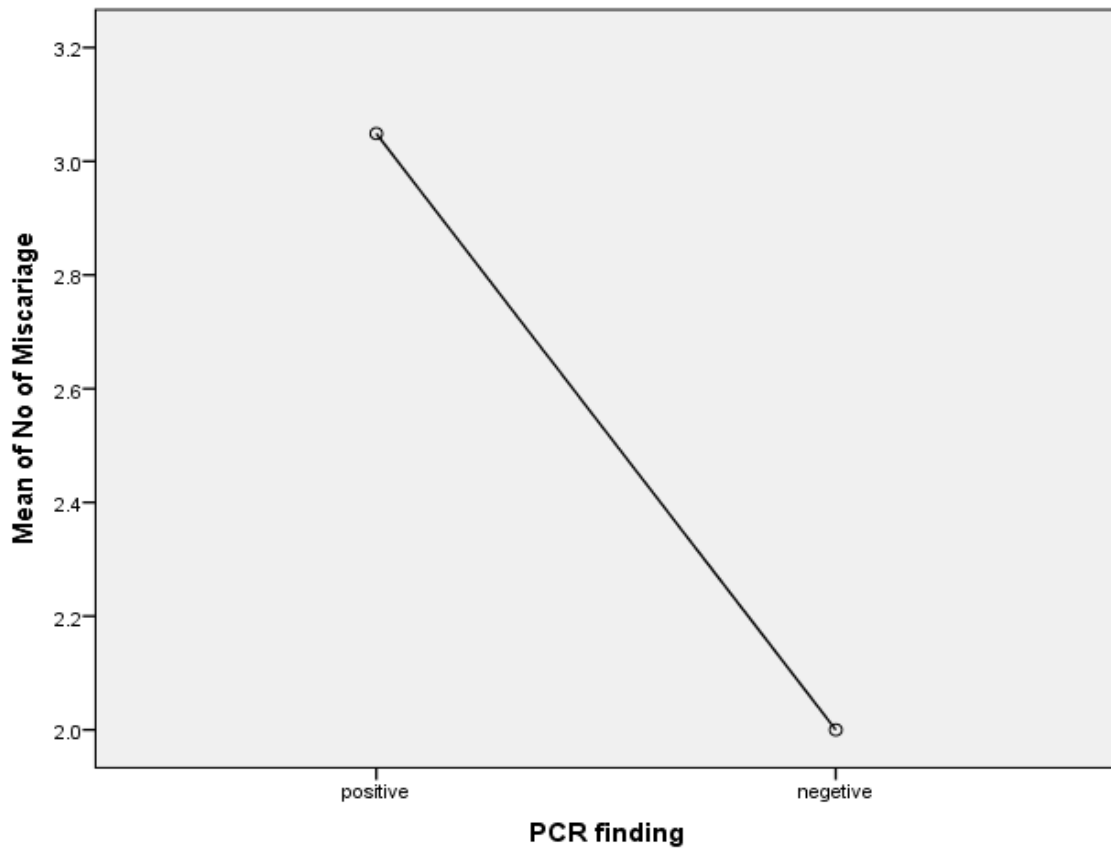| Correlations		Weight	No of miscarriage
Weight	Pearson Correlation	1	-.189-
	Sig. (2-tailed)		.220
	N	44	44
No of miscarriage	Pearson Correlation	-.189-	1
	Sig. (2-tailed)	.220	
	N	44	44

Figure7: Association between of PCR gene mutation finding and number of miscarriage

Discussion

Recurrent spontaneous abortion is the most common complication of pregnancy. Exploring the relation between Prothrombin gene mutations with recurrent miscarriages is a challenge.

The result of this study found that; the presence of Prothrombin allele was higher among cases compared to the controls group (P 0.001), the mutation among cases group about 93.2% while about 28.0% among controls group. This finding was agreed with study performed by SalimSehirali et al which reported; FII mutation significantly higher in Turkish women with RSA.[10].Also another study concluded that; Recurrent spontaneous abortion among Saudi pregnant women was strongly associated with thrombophilic mutation related to FV and FII[11].And disagree with study done by

Reza Behjati, et al which found; frequency of FII mutation in patients with infertility and RSA was not statistically higher than control group (P>0.05)[12].

In the present study the sequencing results analysis revealed that; when the cases were compared with the normal reference one single Base Exchange was found G to A (G20210A) and the mutation was confirmed by the mutation taster. Also our result found there was significant association between the Prothrombin G20210A mutations and repeated of spontaneous abortion among Sudanese women. This result was differed from another study done by AsaadMohammed, et al they reported; no significant association between cases carriage any this mutation (Prothrombin G20210A) and risk with recurrent pregnancy miscarriage in Sudanese women (P= 0.091) [13].

In addition to that Fabio L. Lino et al. said; among 112 Brazilian women with RSAs and 98 health control women observed that no association between the occurrence of RSA and the gene polymorphisms of Prothrombin (G20210A)(P>0.05)[14] . another study done by Majid, *et.al.,* among 80 Iranian women with recurrent pregnancy loss no Prothrombin mutations in cases and controls was found and concluded these data did not confirm that Prothrombin gene G20210 mutation might play a role in recurrent pregnancy loss in Iranian women (p=1)[15]. whileGoncaImirYenicesu found the frequency of heterozygous mutation for Prothrombin G20210A was 7% in RPL women, however there was no heterozygous mutation in control group (P < 0.05) [16].

Mutations in the Prothrombin gene (FII, G20210A) were considered as one of the risk factors for hereditary thrombophilia and can lead to possible miscarriagesact either in the first half of pregnancy or later in pregnancy [17,18].

Conclusion

The result of Prothrombin gene mutation showed (*P*=0.001) significant variations among women with RSA case group (93.2%) compared to controls group (28.0%) and might increase risk for recurrent spontaneous abortion development.

The sequencing results analysis confirmed the present of G to A (G20210A) mutation among Sudanese women with recurrent spontaneous abortion.

References

1. Lee RM, Silver RM. Recurrent pregnancy loss: Summery and clinical recommendations. SeminRepord Med 2000; 18: 433-440.

2. Regan L, Rai R. Epidemiology and the medical causes of miscarriage. Baillieres Best Pract Res ClinObstetGynaecol 2000;14:839-54.

3. Carrington B, Sacks G, Regan L. Recurrent miscarriage: pathophysiology and outcome. CurrOpinObstetGynecol2005;17: 591-59

4. McNamee K., Dawood F., Farquharson R. Recurrent miscarriage and thrombophilia: an update. CurrOpinObstet Gynecol. 2012; 24(4):229-234.

5. Reynolds LP, Borowicz PP, Caton JS, et al. Developmental programming: the concept, large animal models, and the key role of uteroplacental vascular development. J AnimSci 2010;88:E61-72.

6. Elizabeth A. Varga, MS and Stephan Moll. Prothrombin 20210 Mutation. American heart association. 2013; 110: e15-e18.

7. Salwa K. and Joseph D. D. Hereditary thrombophilia. Thromb J 2006; 4: 15.

8. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3' untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88:3698-3703

9. Yenicesu G. I., Cetin M., Ozdemir O., Cetin A., Ozen F., Yenicesu C., et al. A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss. Am J ReprodImmunol 2010; 63: 126-36.

10. Salim Sehirali, M. Murat Inal, Yusuf Yildirim Zuhal Balim, Buket Kosova, Tulin Karamizrak Muzaffer Sanci, Nejat Topcuoglu, Sivekar Tinar: Prothrombin G20210A mutation in cases with recurrent miscarriage: a study of the mediterranean population, Arch Gynecol Obstet 2005; 273: 170–173.

11. Salamat N., Saleem M., Ahmed T. Lupus coagulant and anticardiolipin antibodies in patients with recurrent fetal loss: A case control Study. Ann Saudi Med 2000; 20(5- 6):450-3.

12. Reza Behjati, Mohammad Hossein Modarressi, Mahmood Jeddi-Tehrani, Pouneh Dokoohaki, Jamileh Ghasemi, Amir Hassan Zarnani, Mahmoud Aarabi, Toktam Memariani, Marefat Ghaffari, Mehdi A. Akhondi: Thrombophilic mutations in Iranian patients with infertility and recurrent spontaneous abortion, Ann Hematology 2006; 85: 268–271.

13. Assad Mohammed Babker, Fath Elrahaman Gameel: molecular characterization of prothrombin G20201gene mutation in pregnant Sudanese women with spontaneous recurrent abortion. Rawal Medical Journal 2015;409(2):207-209

14. Fabio L. Lino, E´velyn Traina, Jose´ Augusto Barreto, Antonio Fernandes Moron and Rosiane Mattar: Thrombophilic Mutations and Polymorphisms, Alone or in Combination, and Recurrent Spontaneous Abortion, Clinical and Applied Thrombosis/Hemostasis 2015, Vol. 21(4) 365-372.

15. Majid Ardestani, Hossein Nodushan, Abbas Aflatoonian, Nasrin Ghasemi, Mohammad Hasan Sheikhha: Case control study of the factor V Leiden and factor II G20210A mutation frequency in women with recurrent pregnancy loss,Iran J Reprod Med 2013; 11 (1): 61-64.

16. GoncaImir Yenicesu, Meral Cetin, Ozturk Ozdemir, Ali Cetin1, Filiz Ozen, Cem Yenicesu, Caglar Yildiz, Nadir Kocak.: A Prospective Case–Control Study Analyzes 12 Thrombophilic Gene Mutations in Turkish Couples with Recurrent Pregnancy Loss, American Journal of reproductive immunology 2010; 63: 126-136

17. Rai R, Regan L – Recurrent miscarriage. Lancet 2006; 368:601-611.

18. Martinelli I, Taioli E, Cetin I, et al. Mutations in coagulation factors inwomen with unexplained late fetal loss. N Engl J Med 2000; 343:1015-1018