RESEARCH ARTICLE

Association of Factor V Leiden *G1691A* and Prothrombin gene *G20210A* mutations with adverse pregnancy outcomes

Sidra Asad Ali,[1] Bushra Moiz,[2] Lumaan Sheikh[3]

Abstract

Objective: To determine the association of Factor V Leiden / prothrombin gene mutation in Pakistani women with adverse pregnancy outcomes.

Method: The prospective study was conducted at the Aga Khan University Hospital, Karachi, from January 1 to December 31, 2016, and comprised females > 40 years having history of two or more foetal losses with no apparent aetiology. Restriction fragment length polymorphism- Polymerase chain reaction was performed using MnlI and HindIII restriction enzymes for factor V Leiden G1691A and prothrombin gene mutation G20210A. Females with two or more consecutive normal pregnancies were enrolled as the control group. Data was analysed using SPSS 19.

Results: Of the 172 participants with a mean age of 29.3±5.9 years (range: 19-38 years). 86(50%) each were healthy controls and those with recurrent pregnancy loss. There were 238 livebirths among the controls compared to 13 in the other group. Factor V Leiden *G1691A* was identified in 2(2.3%) women, and prothrombin gene mutation *G20210A* in 1(1.2%) woman in the patient group, while no mutation was identified in the control group.

Conclusion: The prevalence of Factor V Leiden / prothrombin gene mutation in women with recurrent pregnancy loss was found to be very low.

Keywords: Abortion, Pregnancy, Mutation, Thrombophilia. (JPMA 71: 1780; 2021)

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Introduction

In a normal pregnancy, there is altered haemostasis and a predilection for hypercoagulability. Elevated clotting factors, including fibrinogen, VII, VIII, X, and von Willebrand factor, decreased natural anticoagulants, like protein S, and increased fibrinolytic inhibitors with subsequent activated protein C resistance (APCR) are key contributors to this hypercoagulable state.[1] Compared to non-pregnant females, the risk of thrombosis is 4-6 times higher in pregnant females.[2] It has been observed that venous thromboembolism (VTE) is a complication in 1.72 pregnancies per 1000 births, with a mortality of 1.1 in 100,000 births.[3] Approximately half of these women have underlying thrombophilia.[4] Inherited thrombophilia is a genetic predisposition towards thrombosis due to the deficiency of one or more natural anticoagulants, like anti-thrombin III, protein S and/or C, or secondary to prothrombin gene mutation (PGM) or Factor V Leiden (FVL) mutations. FVL is a missense point mutation in the Factor V gene G1691A that results in abnormal Factor V protein resistant to inactivation by protein C, leading to unregulated coagulation. PGM is a precursor to thrombin that plays a key role in coagulation. Point mutation in the 3'-untranslated region of PGM gene G20210A also increases the possibility of thrombosis. While VTE risk is increased to 0.5-3.1% in a pregnant woman who happens to be an FVL heterozygote, it increases further to 2.2-14% if she is homozygous for FVL.[5] Similarly, thrombotic risk during pregnancy is 0.4-2.6% in a PGM heterozygote, but increases to 2-4% with PGM homozygosity.[5]

In contrast to VTE, the association of inherited thrombophilia with adverse pregnancy outcomes (APOs) is debatable. For example, a case-control study examining 5000 women found a high association of FVL with stillbirths, but not with foetal losses.[6] In contrast, an association of foetal losses >10 weeks with maternal thrombophilia has also been reported.[7] Similarly, a meta-analysis in 2009 found no or weak association between FVL/PGM and foetal growth restriction (FGR).[8] Moreover, there is no substantial evidence of an association of inherited thrombophilia with either preeclampsia[9] or placental abruption.[10]

The frequency of FVL and PGM in women with recurrent pregnancy loss (RPL) in Asian countries have been studied extensively (Table-1). While a low prevalence of 1.0% for PGM[11] and 1.3% for FVL[12] has been reported in healthy individuals in northern Pakistan, this sixth most populous country does not have a national registry database for thromboembolic diseases. Results were variable with two studies reporting higher frequencies of 12% and 19% of

[1]Patel Hospital, Karachi, [2]Department of Pathology and Laboratory Medicine, [3]Department of Obstetrics and Gynecology, Aga Khan University, Karachi, Pakistan.

Correspondence: Bushra Moiz. Email: bushra.moiz@aku.edu

APCR[13,14] and another reporting a lower frequency of 5.4% for FVL mutation[15] in women with foetal losses. No study has ever reported PGM in women with RPL. The current study was planned to investigate the association of RPL with FVL and PGM mutations in the local setting.

Patients and Methods

The prospective study was conducted at the Aga Khan University Hospital (AKUH), Karachi, from January 1 to December 31, 2016.

After approval from the institutional ethics review committee, the sample size was calculated at 95% confidence interval (CI) and 80% power with an FVL incidence of 5.4% in women with RPL in Pakistan.[15] RPL was defined as two or more foetal losses in first or second trimester or both, while APO included preeclampsia,[16] placental abruption,[17] FGR[18] and stillbirth[18] besides RPL. Patients with RPL in the cases group with or without APO were recruited from the obstetric clinics dealing with high-risk pregnancies. Women with normal pregnancy in the control group were enrolled when they visited laboratory for oral glucose tolerance test (OGTT). All women were enrolled after taking written informed consent. Those included were pregnant women aged >40 years with no apparent aetiology. Those with pelvic/anatomical pathology, endocrine dysfunction, hypertension, diabetes, autoimmune diseases, chromosomal disorders and liver dysfunction were excluded. A cut-off at 40 years was kept as the incidence of RPL increases to 50% after this maternal age.[19]

Clinical details were obtained through medical charts' review while thrombophilia screening results were obtained from the laboratory database. At the institution's clinical laboratories, protein C (chromogenic assay), anti-thrombin III (chromogenic assay), protein S (clotting-based assay) and lupus anticoagulants (Russel Viper Venom test) were assayed on Sysmex CS-2000i (Sysmex®, Singapore), while anticardiolipin antibodies (aCL) were performed through enzyme-linked immunosorbent assay (ELISA) (ETI max 3000, Siemens®, Italy).

For deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) studies for mutational analysis, 5ml sodium ethylene di-amine tetra acetate peripheral blood samples were collected from each participant and it was refrigerated till further analysis.[18] DNA was isolated using Wizard® Genomic DNA Purification kit (Promega USA Cat No: A1125) and Restriction fragment length polymorphism (RFLP)-PCR was performed using previously established protocols with restriction enzymes MnlI and HindIII for FVL[20] and PGM[21] respectively. According to the size of bands, the subjects were classified as wild (345bp), heterozygote (345,322,23bp) or homozygote (322,23bp) for PGM, and wild (37,82,104bp), heterozygous (37,82,104,141bp) and homozygous (82,141bp) for an FVL mutation.

Data was analysed using SPSS 19, and normality of data was checked through Shapiro Wilk's test. Gene counting was done by dividing each type of allele with the total number of alleles in the sample. Probable allelic frequency of FVL and PGM was computed through Hardy Weinberg equilibrium using chi-square test.

Results

Of the 172 participants with a mean age of 29.3±5.9 years (range: 19-38 years), 86(50%) each were healthy controls and RPL cases. There were 238 livebirths among the controls compared to 13 in the other group. There was no family or personal history of VTE among the subjects. In the RPL group, 23(26.7%) patients had one or more additional APOs, like severe preeclampsia 12(13.9%), FGR 8(9.3%), placental abruption 2(2.3%) and stillbirth 2(2.3%).

Thrombophilia screening was routinely advised to all patients with APOs, but results were available for 57(66.2%) subjects, as 29(33.7%) patients refused testing

Table-1: Frequency of FVL and PGM with RPL in Asian countries.

Country	Year	Sample size	FVL N(%)	p-value	PGM N(%)	p-value	Reference
India	2015	587	20 (3.4)	0.2	Not done	-	31
India	2013	107	9 (8.4)	0.05	0(0)	-	32
Iran	2013	80	02 (2.5)	0.400	0(0)	-	33
Iran	2006	65	13 (20)	<0.001*	3 (4.6)	1.000	34
Pakistan	2015	56	3 (5.4)	0.001*	Not done	-	15
Saudi Arabia	2016	98	15(15.5)	<0.05*	6(6)	<0.05*	35
Saudi Arabia	2016	59	0	-	Not done	-	36
Turkey	2018	2660	213 (8)	-	177 (6.6)	-	37

*Statistically significant result for cases vs. controls
FVL: Factor V Leiden; PGM: Prothrombin gene mutation; RPL: Recurrent pregnancy loss.

Table-2: Comparison of thrombophilia screening results in patients having recurrent pregnancy losses (RPLs) with or without additional adverse pregnancy outcome (n=57). All values are mean ± standard deviation (SD).

Thrombophilia		Inherited		Acquired		
Laboratory parameters	Protein S %	Protein C %	Anti-thrombin III %	aCL IgG GPL-U/ml	aCL IgM MPL-U /ml	Lupus anticoagulant ratio
Reference range	56-121	70-140	74-126	<10	<5	0.8-1.2
RPL** only (n=34)	92.5±18.1	94.8±11.1	95.2±10.7	12.4±4.5	9.7±4.1	0.6±0.7
RPL with other APO† (n=23)	93.6±9.3	98.7±20.3	94.3±11.9	13.4±3.3	10.1±3.7	0.6±0.2
p-value	0.800	0.488	0.764	0.382	0.723	0.908
Total (N=57)	92.9±15.5	96.1±14.8	94.9±11.0	12.7±4.1	9.9±3.9	0.6±0.6

aCL: Anti-cardiolipin antibodies; APO: Adverse pregnancy outcome; Ig: Imuunoglobulin.

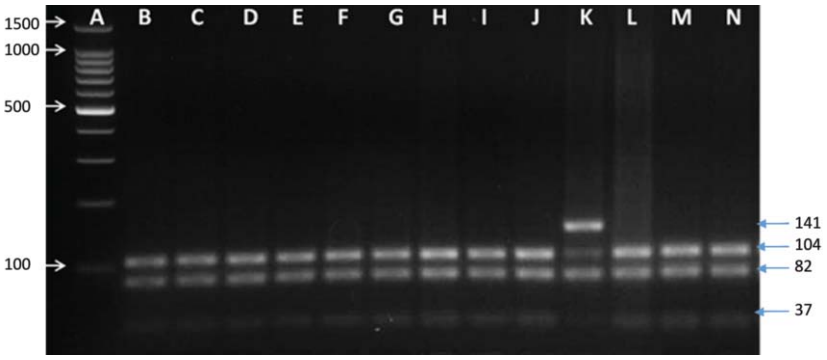

Figure-1: Results of Restriction fragment length polymorphism- Polymerase chain reaction (RFLP-PCR) of Factor V Leiden (FVL) with MnlI. Lane A: 100bp size marker; lane B-J and L-N are wild type; lane K shows 141,104, 82 and 37bp, indicating heterozygous factor V G1691A.

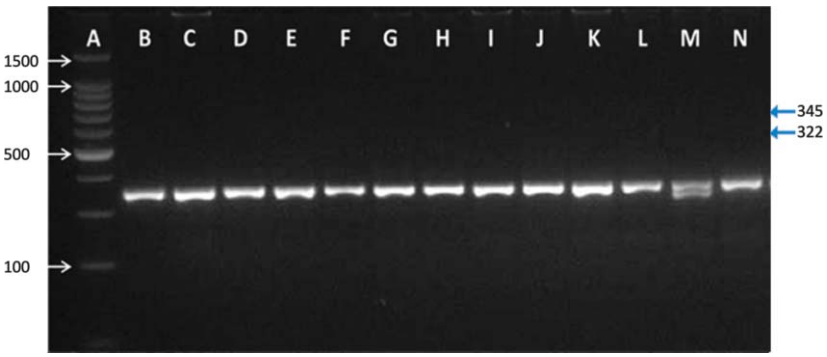

Figure-2: Results of Restriction fragment length polymorphism- Polymerase chain reaction (RFLP-PCR) of Prothrombin gene mutation (PGM) with HindIII. Lane A: 100bp size marker; lanes B-L and N are wild types, and lane M shows 345bp and 322bp, indicating heterozygous prothrombin G20210A.

Table-3: Maximum likelihood estimates of allelic frequency for FVL and PGM in participants (n=172) where G was the wild allele and A was the mutated allele.

Alleles	FVL	PGM
G	0.9940	0.9970
A	0.0060	0.0030
A/A*	0.0036	0.0009
G/A**	1.1930	0.5982
G/G†	98.8036	99.4009

A/A* is homozygous, and G/A** is heterozygous for FVL and PGM while G/G† represents normal population. FVL: Factor V Leiden; PGM: Prothrombin gene mutation.

because of cost-related issues. Protein S deficiency was seen in 2(2.3%) cases, while lupus anticoagulant, APCR and Pro Global C clotting assay were not identified in any case. There were 14(24.5%), 28(49%) and 5(8.8%) patients who were aCL-positive respectively for both immunoglobulin-G (IgG) / IgM, IgM-only and IgG-only (Table-2).

RFLP-PCR of FVL G1691A with MnlI gene showed 2(2.3%) patients having no livebirths and more than two first-trimester losses as heterozygotes for FVL mutation (Figure-1).

In parallel to FVL, no patient was homozygous for PGM mutation (Figure-2). Only 1(1.2%) patient was identified as heterozygous for PGM G20210A (Table-3). This patient was a 35-year-old woman with no livebirth and had recurrent first-trimester pregnancy losses. Her inherited thrombophilia screening was negative, but showed aCL positivity.

Discussion

A significant finding of the current study was the presence of aCL antibodies in 82% of the tested patients. This may be the underlying reason for recurrent foetal losses for majority of patients though there was no personal or family history of VTE.

Thrombophilia may result in foetal losses either due to thrombosis of placental vessels with placental insufficiency[22] or because of invasion of maternal vessels by syncytiotrophoblast causing micro-thrombi at implantation site.[23] However, this hypothesis was challenged because RPL was not significantly high in women who had VTE.[24] This raised the possibility of thrombophilia as a contributory rather than the sole factor in RPL genesis. Association of FVL and PGM was

particularly reported with late pregnancy complications, like preeclampsia, second-trimester losses, intra-uterine growth restriction (IUGR) and placental abruption.[24] However, these conclusions were driven by small and weak retrospective data and the included studies were designed primarily to assess VTE risk rather than APO. To avoid bias of retrospective studies, Rodger et al. in 2010 analysed data from 7 prospective studies comprising more than 16,000 women, and observed a small but absolute risk of pregnancy losses with FVL.[25] The same study reported a pooled odds ratio (OR) estimate of 1.13 and wide 95% confidence intervals (CIs) of 0.64-2.01) for foetal losses due to PGM, including only 4 studies with an insufficient sample size of 9225.[25] An updated meta-analysis by the same investigator found a similar small risk for foetal losses with FVL, but not with PGM.[26] However, these studies were ethnically-biased because 77% of the included women were white Caucasians. FVL and PGM demonstrate an uneven geographical distribution and are more common in Europe than in Asia.[27,28] Reports from Asian countries are more variable and are limited by small sample sizes, heterogeneous populations and in the definition of RPL to derive any meaningful data. Indeed, the cost-benefit analysis did not favour FVL/PGM testing in women with RPL in a report from India.[29] Moreover, there are no benefits of anticoagulating women with inherited thrombophilia for preventing RPL, as observed in a recent meta-analysis.[30]

The current study is the first local study to determine the association of FVL/PGM with RPL, and may serve as a database for future work for larger studies in the country. The study's limitations are the small sample size, being single-centre and having incomplete thrombophilia workup. Large systematic studies are recommended in the local setting to better understand the role of these mutations in pregnancy losses.

Conclusion

There was no association between FVL/PGM mutations and RPL in Pakistani women. Thrombophilia workup for FVL/PGM is expensive and its need in a developing country should be rationalised.

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