

# The relation of PROZ1 polymorphism and risk of early fetal loss in Iraqi patient's diagnostic with Antiphospholipid Syndrome

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## ABSTRACT

**Background:** Low level of protein Z associated with genetic background is being fundamental as it is interfering with PZ gene polymorphism and it can be combined with highly incidence of thrombosis. The purpose of this study is to analyse the prevalence and clinical significance for the PROZ (PZ) polymorphism in Iraqi patient's diagnostic with antiphospholipid syndrome (APS), one from three main kinds of thrombophilia that is distinguished through the accumulation of antiphospholipid (aPL) antibodies (Abs).

**Results:** The target fragment 320 bp of PROZ gene (spanning PZ intron F region) was amplified in 65 samples including 32 women diagnostics with antiphospholipid syndrome and 33 controls. The G/A222 SNP which was previously known rs3024735 was detected in intronic sequences of the PROZ gene. G/A222 SNP was only localized in two groups of the investigated PROZ gene samples in comparison with their corresponding referring DNA sequences. The distribution of PROZ genotypes within the patients and healthy group of study, revealed no significant differences (P value = <0.81), in the mutant G/A and G/G normal genotypes. The incidence of the A allele was nearly similar between the control (0.24%) and APS group (0.26%) (P value = <0.84).

**Conclusion:** there is no association between the rs3024735 SNP on PZ intron F region amplicon and the risk of disease incidence in Iraqi patients previously diagnostic with APS and pregnancy morbidities

**Keywords:** PROZ1, polymorphism, rs3024735 SNP, Iraqi patient's, and Antiphospholipid Syndrome.

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## INTRODUCTION

The term thrombophilia was initially used by Egeberg (1965), Once a family with predisposition to thromboses showed antithrombin III deficiency (Egeberg, 1965, Palomo et al., 2009). This term was extended to clarify a propensity state which leads to the case of thrombosis. Research described three main kinds of thrombophilias that include inherited, acquired and mixed (Harris and Pierangeli, 2004). Acquired thrombophilia is the Antiphospholipid syndrome (APS) that is distinguished through the accumulation of antiphospholipid (aPL) antibodies (Abs), arterial thrombosis and/or recurrent venous, fetal loss and in some cases autoimmune thrombocytopenia (Palomo et al., 2009).

APS or sometimes antiphospholipid antibody syndrome APLS, is a case of an autoimmune, hypercoagulable state which is caused by antiphospholipid antibodies. This syndrome prompt blood clotting (thrombosis) in blood vessels and related with pregnancy complications such as abortion and the birth of a dead fetus (Di Prima et al., 2011, Greaves et al., 2000). Research showed that low levels of protein Z (PZ), Vitamin K-dependent protein, is can be combined with highly incidence of thrombosis. Gradual increasing of PZ levels are being discovered to be closely associated with gestational age, particularly within the trimester of pregnancy, reach up to 20%. The increasing may be due to the mechanisms of compensatory that resulted from increased concentration of factor FXa (Erez et al., 2007), then decline up to 30%, below the level in the first trimester. Studies revealed that PZ levels becomes attenuated in patients suffering from abnormal pregnancy outcomes, involving lower birthweight baby, high blood pressure, and diabetes.

Protein Z was initially discovered in bovine plasma, human protein Z was described and purified by Broze

and Miletich in 1984 (Broze and Miletich, 1984). It is a factor dependent on vitamin K, which is homologous to other factors dependent on vitamin K such as VII, IX, X factors as well as C and S proteins (Højrup et al., 1985), whilst no enzymatic activity is present in this protein. However, PZ work as a cofactor for factor Xa inhibition via protease inhibitor PZ-dependent serpin (ZPI) (Almawi et al., 2013). Moreover, PZ deficiency was found to be associated with a procoagulant condition, illustrated by highly concentration of FXa and thrombin, and it is associated with multiple types of thrombotic disorders. In terms of genetic background, studies reported that PZ deficiency has been considered as constitutional, and some of PROZ (PZ gene) allelic variants (polymorphisms) are connected with some particular adverse complications within pregnancy (recurrent miscarriage, stillbirth, preeclampsia, intrauterine growth restriction, and placental abruption) (Almawi et al., 2013, El-Hamid and El-Khayat, 2011). The differential pattern of linkage disequilibrium between the variants upregulate PZ production, and disease association, suggest that the low level in PZ is to somewhat, constitutional (Miletich and Broze, 1987, Almawi et al., 2013). However the effective sides of those variants have not been extensively evaluated, some have been recognized to affect plasma PZ levels, namely G79A, A13G, and G42A; the lowest plasma PZ levels have been detected in the variants A/A (G42A) and G/G (A13G) and A/A (G79A) homozygous genotypes (Lichy et al., 2004, El-Hamid and El-Khayat, 2011).

The Aim of this study is to analyse the prevalence and clinical significance for the PROZ (PZ) polymorphism in Iraqi patient's diagnostic with antiphospholipid syndrome (APS) using Single-strand conformation polymorphism (SSCP) technique, as it has significant

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advantages over many other accurate analysis nucleic acid techniques for the allelic sequence variation.

### METHODS

#### Samples collection

Blood samples were collected from 32 women diagnostic with antiphospholipid syndrome and early fetal lost. Patients' samples were recruited from Hilla Hospital for Obstetrics and Gynecology in the period between 2018-2019. The plane and sampling protocol were reviewed and approved by university of Babylon/ College of Science along with Hilla Hospital for Obstetrics and Gynecology ethical committee. Patients filled out questionnaire forms regarding to previous events of arterial thrombosis, history and number of abortions, any immune disease, any infection with Toxoplasma parasite or Cytomegaly virus, Arthritis, Diabetes, and family history with antiphospholipid syndrome. Control samples were collected from 33 women have no clinical evidence or medical history of previous morbidity or thrombosis during pregnancy.

#### Sample preparation

Samples with 5 ml of blood were divided into two groups. For the first group 2 ml of blood was collected in tubes containing EDTA, then stored under freezing until the samples being ready for gDNA extraction. For the second group 3 ml of blood were centrifuged at 6000 rpm for 15 minutes. The upper layer including the serum was examined for antiphospholipid test and Turch test (that included Toxoplasma, rubella, herpes test, and Cytomegaly virus test).

#### Genomic DNA extraction without previous cell lysis (straight phenol - SP)

The blood samples were drawn with no anti-coagulants. Aliquots of 500 µl of blood were added to a 1.5 ml Eppendorf tubes with 500 µl of phenol (adjusted with Tris-HCl, pH 8.0) and mixed vigorously by inversion. Subsequently, samples were kept at room temperature for various periods of time until further processing. After centrifugation at maximum speed in a microfuge (14 000 g for 5 min), the aqueous phase was separated and subsequently extracted with phenol chloroform-isoamyl alcohol (25:24:1) as well as chloroform. Approximately 300 µl of the last aqueous phase was precipitated with two volumes of ethanol 96 % and centrifuged at room temperature. The supernatant was discarded and the precipitant was rinsed with 70 % ethanol, dried and resuspended with 100 µl of sterile deionized and distilled water (Albariño and Romanowski, 1994).

#### Polymerase Chain Reaction (PCR) (Amplification of PZ intron F region containing rs17882561 site)

After gDNA extraction PCR amplifications were performed by using the forward primer 5-TAACACCATAGACAGAGTCCGATATTCGC-3' and reverse primer 5-ATGAACTCG GCATTAGAACATGGTTGGA-3', that selects for the sequence (sequences flanking PZ intron F region including rs17882561 site). PCR mixture was set in a final volume of 25 µL for each sample which consisting of 2 µL genomic DNA, 10 µL universal master mix (0.1 units/µL Taq DNA Polymerase), 0.5 µL MgCl<sub>2</sub>, 1 µL for each forward and reverse primer along with 11.5 µL di deionised distilled water (ddw). The PCR condition was set as follows: an initial heating at 94°C for 5 minutes,

followed by 35 cycles in program including: denaturation 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72 for 5 minutes. After amplification, The PCR product were visualized on 1.6 % agarose gel electrophoresis gels using 3µl of 1 Kb Hyper Ladder I (Bioline H1K5- 1006).

#### Single Strand Conformation Polymorphism (SSCP)

##### Analysis by Nondenaturing PAGE

The Single Strand Conformation Polymorphism technique of amplified gene fragments is carried out following the procedure adapted by Byun (2009) (Byun et al., 2009), via Bio-Rad Protein II xi Cell for vertical gel electrophoresis unit (Bio-Rad laboratories). About 2.5 µl of amplified DNA and 2.5 µl of a formamide dye (9.5 ml formamide, 100 ml of 1 molar NaOH, and 5 mg of bromophenol blue) is placed in PCR tube and heated for 5 minutes at 95°C in water bath. The samples are immediately placed in ice-cooled box after denaturation for 10 minutes, then loaded on a nondenaturing 8 % acrylamide gel (16.1 ml of 30% acrylamide, 12.3 ml of 5x TBE buffer, 38.7 ml of N, N,N',N'-tetramethylethylenediamine (TEMED), 769 µl of 10% Ammonium persulphate (APS), and 32.3 ml distilled water) (Orita et al., 1989). Electrophoresis is conducted for 5 hours in a 0.5 X Tris borate (pH 8.3)-EDTA buffer at 100 volts. After completion, glass plates are detached from the assembly and silver staining is added to the gel to imagine SSCP band patterns via three steps of staining adapted by Byun (2009). Immediately, when the staining completed, the gel is gently migrated on the transparency and visualized by digital camera (Nikon, Japan).

##### Statistical analysis

The genetic association analysis was calculated by using SPSS software version 26 to analyses allele frequency and genotyping for both patient and control groups.

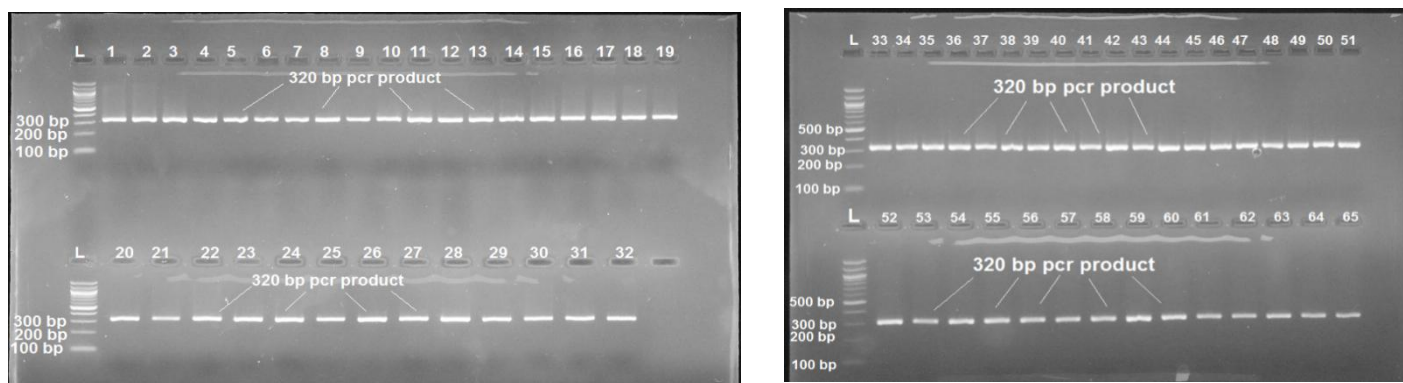
### RESULTS AND DISCUSSION

#### Genomic DNA amplification by PCR

The target fragment of *PROZ* gene (spanning PZ intron F region) was amplified in both patients and controls samples. The bands size is 320 bp when measured using 1Kb ladder (see figure 1).

Alignment of PCR product for all the sequenced samples were analysed by using BioEdit Sequence Alignment Editor Software (Version 7.1) with the corresponding reference database sequences. PCR product of sequenced samples showed 99% similarity with the intended Homo sapiens reference target sequences in NCBI BLAST website. The PCR fragments are in the same size of bands obtained by PCR for protein Z gene (spanning PZ intron F region) in Egyptian study by Eissa et al. (2018), that investigated polymorphism in *PROZ* gene locus (rs17882561) in patients with antiphospholipid (Eissa et al., 2018).

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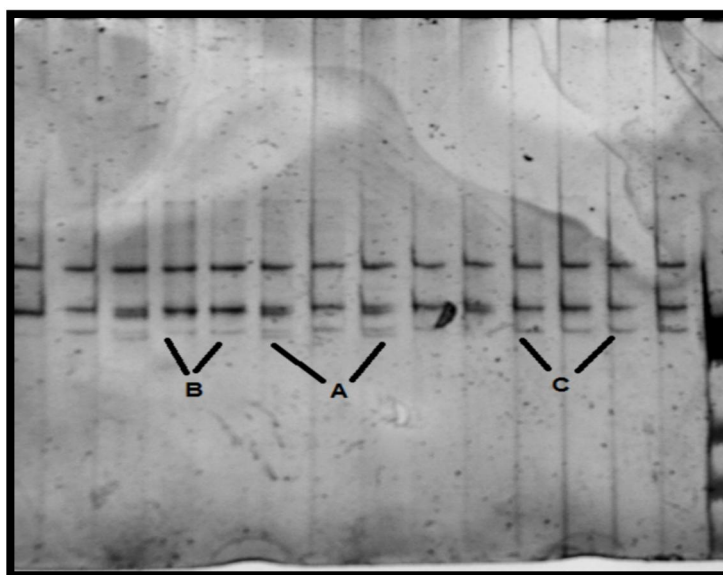


**Figure 1.** Agarose gel electrophoresis photo shows PCR products of PZ intron F region amplicon for genomic DNA of samples (1-32 and 33- 65 represent patients and control samples respectively). Samples run in 1.6 % Agarose gel at 100 volts for 50 minutes. The size of the bands is approximately

**PCR-SSCP Analysis**

SSCP results of 65 samples including patients and control show different bands pattern on PZ intron F region amplicon. The figure 2 in the electropherogram showed two different band sizes of A pattern, while each B and C

pattern appeared single band with different sizes. Through this locus, six samples were analyzed and sequenced, 2 samples for each type of patterns of the amplified *PROZ* sequences in the chromosome 13 of Homo sapiens.



**Figure 2.** Polyacrylamide gel photo of SSCP results show three electrophoretic patterns (A, B, and C) on PZ intron F region in both patients and control samples. Samples run in 8% of acrylamide 100 volts for 5 hours at room temperature.

Results of 320 bp alignment for these six samples observed one SNP in the specific locus that was only localized in two groups of the investigated *PROZ* gene in

respect with corresponding referring DNA sequences (Figure 3).

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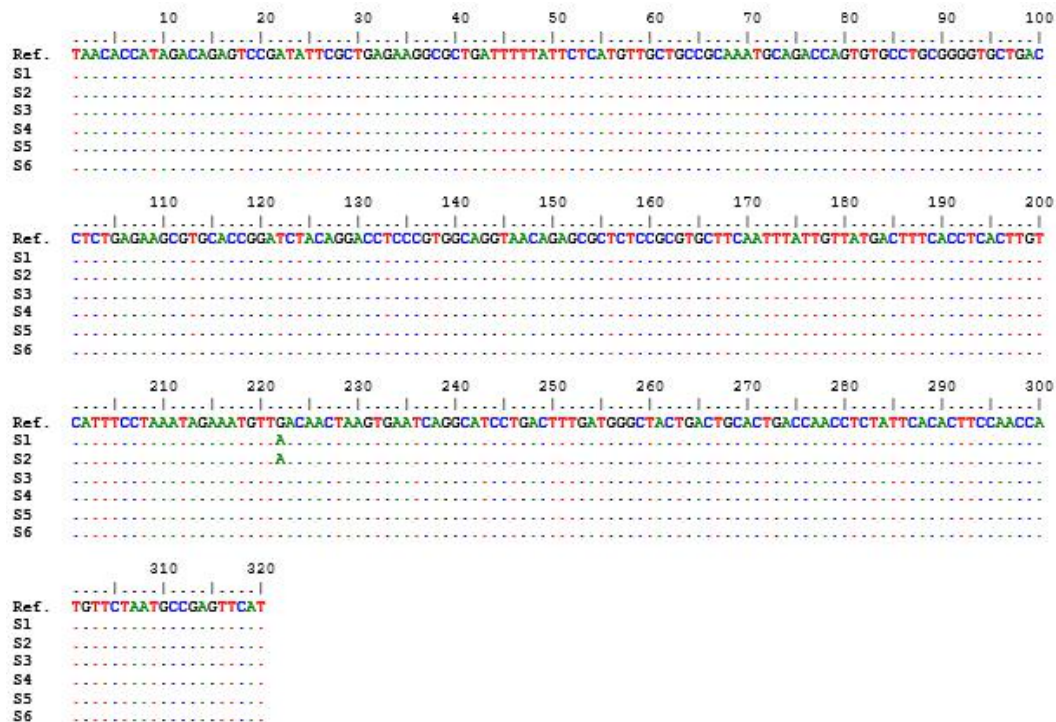


Figure 3. DNA sequences alignment of six 320 bp samples in the specific locus through *PROZ* gene in respect with corresponding DNA reference. Single nucleotide variant was highlighted by its position in the amplicon. Abbreviation of “ref” indicate sequence referring to the NCBI and “S1-S6” indicating 1 to 6 samples.

This SNP had taken a selective distribution amongst the analyzed samples; in which G222A was detected in heterozygous status (mutant heterozygous status), only in S1 (S1= sample 1) and S2 genetic groups, while S3,S4,S5 as well as S6 genetic groups, had exerted a normal homozygous status (G/G) (Figure 4). The distribution of *PROZ* genotypes within the amplicon of

the patients and controls, revealed non-significant statistical differences (P value = <0.81), in the mutant G/A and normal G/G genotypes. The incidence of the A allele in the control group (0.24%) is nearly similar in comparison to the APS group (0.26%) (P value <0.84, Odd ratio 1.08).

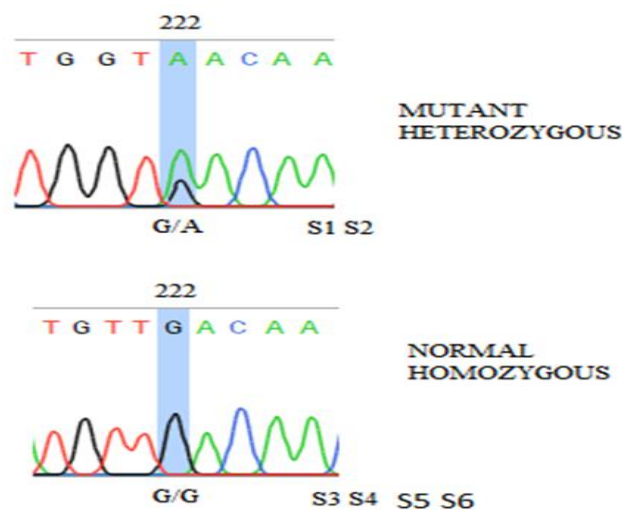


Figure 4. The pattern of the observed substitution mutation within the DNA chromatogram of the targeted 320 bp amplicons within the *PROZ* gene. The observed substitution mutation was highlighted according to their positions in the PCR products. S1 – S6 refer to the studied no. 1 to no. 6 samples. The symbol “>” refers to the mutation event.

The existence of the identified SNP was analysed based on *PROZ* dbSNP database within chromosome no. 13 (GenBank Acc. no. NC\_000013.11). By reviewing the

dbSNP website, it was found that the SNP was previously known, namely rs3024735. However, this SNP was detected in intronic sequences of the *PROZ* gene.

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To summarize all the results obtained from the sequenced 320 bp fragments, the exact position of the

given variation was described in the NCBI reference sequences (Table 1).

**Table 1. The pattern of the observed G222A SNP in the 320 bp amplicons of PROZ DNA sequences in comparison with their corresponding NCBI referring sequences (GenBank acc. no. NC\_000013.11). The symbol "S" refers to the sample number.**

Sample No.	Native Allele	Allele	Position in the PCR fragment	Zygoty status	Position in the reference genome	Variant summary
S1 and S2	G	A	222	Heterozygous	113165199	g.113165199G>A (rs3024735)

As far as our knowledge goes, this is the first work to be carried out for exploring the association between the polymorphism of the PROZ gene target sequence and its prevalence and clinical significance in APS Iraqi patients. No significant differences have been detected for SNP between the patients and controls, so there is no association between the rs3024735 SNP on PZ intron F region amplicon and the risk of disease incidence in Iraqi patients previously diagnostic with APS and pregnancy morbidities. Comparing with other studies that investigated the risk of G79A and adverse outcome of pregnancy, inconsistencies conclusions had been achieved and that might be due to the variety in the genetic background and other demographic features of the studied populations. Egyptian study, reported the same conclusion that polymorphism in PZ intron F region G79A had no association with increased risk of thrombosis in APS (Eissa et al., 2018). However, Bahraini study documented the association between G79A minor allele and an increased risk of adverse pregnancy outcomes in 287 samples from women with idiopathic pregnancy loss along with 308 control (Al-Shaikh et al., 2013). Dossenbach-Glaninger (2008) hypothesized that thrombosis associated with early fetal loss could be explained as a polygenetic disorder dependent on the presence of more than one coagulant risk factors, thus PZ intron F G79A polymorphism may exhibit a protective role in the development of recurrent early miscarriage during the period between the 8th and 12th weeks of gestation (Dossenbach-Glaninger et al., 2008). Moreover, Greek study in 2009 revealed that however there was similar frequency of the 79A allele detected between small number of cases and control samples, PZ levels were significantly lower in the 79A allele carriers (Effraimidou et al., 2009).

### CONCLUSIONS

No significant differences have been detected for SNP between the patients and controls, so there is no association between the rs3024735 SNP on PZ intron F region amplicon and the risk of disease incidence in Iraqi patients previously diagnostic with APS and pregnancy morbidities.

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