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Genetic Variants of RPL5 and RPL9 Genes among Saudi Patients Diagnosed with Thrombosis

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ABSTRACT

Background: Thrombosis directly affects the quality of life with increased mortality. The RPL5 (L5) gene on intron 6 on chromosome 1p22, rs6604026 is associated with multiple sclerosis risk, whereas RPL9 (L9) on 8 exons on chromosome 4p14 has been documented so far as being an essential involvement in the proliferation of protein synthesized cells mostly by gene products. **Objective:** The aim of this work was to assess genetic variants of RPL5 and RPL9 and thrombosis to characterize their role in the diagnosis of thrombosis among the Saudi population. **Methods:** The cross-sectional study involved 100 Saudi patients diagnosed with thrombosis (arterial or venous) in 50 healthy individuals as controls in the same age and sex groups. Primers were designed RPL5 and RPL9 for molecular analysis. The Sanger System ABI-3730xL (Hong Kong) automatic sequencing was used for DNA sequencing. Statistical analysis was performed using the Prism 5 and SPSS version-21 programs. **Results:** The male / female age ratio was 66.7 / 57.4, and the mean age was 61.2 years. Most of the patients were self-identifiable and without a previous history of thrombosis (61.0%). Most of the patients had just been diagnosed, that is, in the last five years (74.0%), about 43% of the patients underwent treatment using combination therapy (Aspirin and oral anticoagulants). New gene variants of RPL5 (5 SNPs) and RPL9 (9 SNPs) were detected in Saudi thrombotic patients. **Conclusion:** Mutations in RPL5 and RPL9 were reported in all thrombotic patients, represented by a new variant of the ribosomal protein gene and correlated with thrombosis in the Saudi population. These results may reflect an association between the ribosomal protein SNP gene and the incidence and progression of thrombosis in the Saudi population.

Keywords: Genetic variants, Ribosomal protein genes, RPL5, RPL9 Thrombosis, Saudi population.

1. BACKGROUND

Thrombosis has directly affected the quality of life with an increased rate of mortality (1). The risk of thrombosis Crohn's disease or ulcerative colitis patients is higher than those without the inflammatory intestinal disease (2-5). In the USA, more than 500,000 patients were diagnosed with deep venous thrombosis (DVT) each year (6, 7). The rate for DVT was about 3% in the first three months and more than 15% after thromboembolism (PTE). Risk factors for venous thromboembolism (VTE) are surgery, prolonged immobilization, tumor, obesity, oral contraception, tobacco, glucocorticoids, pregnancy, cardiac arrest, and idiopathic lung obstructive disease (1, 8, 9). When mucosal was damaged, an activity that increases the risk of thrombosis occurred. Many studies reported a wide variation in postoperative deep-vein thrombosis (DVT) tendencies (10, 11). Climate or racial factors have been assumed to be important in the genesis of this complication (12). Ribosomes are organelles that help facilitate the production of proteins composed of small subunits of the 40S as well as 60S formed of 4 RNAs, in which given or taken different 80 proteins structures. These proteins ribosomal are genes encoding of the 60S subunit placed in the cytoplasm, and their structure is stable which is known as ribonucleoprotein molecules (RNP) which is important to transport the nucleolus under ribosomes via non-ribosome-associated cytoplasm 5S rRNA (13). Although there was no correlation between the expression level and the severity of the disease, there was a variable expression of ribosome protein's gene after interacted with casein kinase II beta subunit in colorectal cancers compared with normal tissues. This gene is transcribed in intron 5 with the

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small nuclear RNA gene U21, which is typical for encoding ribosomal proteins, in which many pseudo genes of this gene are spread across the genome, and more rRNA arrangement for ribosomes developments required (14-19). Two transcript variants encoding the same protein, on the other hand, have been found in this gene (13, 14). RPL5 defects would be the cause of the Diamond-Blackfan anemia type 6 (DBA6) and maybe a manifestation of an intrinsic non-regenerative hypoplastic anemia in early infancy (13). RPL5 (L5) genes in intron 6 on chromosome 1p22, rs6604026 are associated with the risk of multiple sclerosis (15), while RPL9 (L9) in 8 exons on chromosome 4p14 has been documented to this extent Include the essential involved in cell proliferation of protein synthesis mostly with genes product (16). RPL9 is an element of the ribosome 60S subunit, where mRNA is translated into functional proteins. Studies revealed the presence of ribosomes in platelet that means the incidence of mutations in the RPL9 gene will affecting the translation process resulting in platelet dysfunction (17, 22). Different RPL9 gene polymorphisms had been contributed to various disease pathogenesis (18-20). A study conducted by Gan et al, (21) showed that ribosomal-related genes also including RPL31 and RPL9 may also be implicated in the pathogenesis of systemic vasculitis, which is presumed to be unrestricted genetics which also encourages its advancement of Takayasu arteritis by managing the pathways of a ribosome. Takayasu arteritis is an inflammatory process affecting the blood vessels described through systemic reduction with ventricular necrosis that is due to thrombus formation (21). The functions of both RPL9 and RPL5 through evolution have been highly conserved, which are led to greater biological discoveries in research, and its elevated expression is used as a diagnostic biomarker towards fast thrombosis recognition which may be supported by previous studies on the causal link between ribosomal protein (RPL5 and RPL9) and thromboembolism (22).

2. OBJECTIVE

The aim of this study was to assess the expression polymorphism of RPL5 and RPL9 (ribosomal protein genes) and thrombosis to characterize their role in thrombosis diagnosis among the Saudi population to determination, whether the levels of these two genes vary between the normal and the diseased.

3. METHODS

A cross-sectional study was involved 100 Saudi patients diagnosed with thrombosis (arterial or venous) against 50 healthy individuals as controls in the same age and sex groups. This study was conducted in Imam Abdulrahman Bin Faisal University after ethics clearance was obtained (IRB-2019-03-188). Peripheral blood samples from normal controls and thrombosis patients at King Fahad University Hospital, AL-Khobar was collected. The cases of thrombosis were both previously diagnosed as having either venous or arterial thromboembolism. The genes RPL5 and RPL9 were found in chromosome 1, chromosome 4, respectively. The design

of and primers for PCR was based on the references assembly Homo sapiens (RPL5) NC 000001.11 and (RPL9) NC 000004.12. RefSeq mRNA sequences NM 000969.5 and NM 000661.4 were used for RPL5 and RPL9 respectively for molecular analysis. Protein RefSeq sequences NP 000960.2 and NP 000652.2 for RPL5 and RPL9 were used to identify protein-level mutations. Genomic DNA was extracted from the entire blood of thrombosis cases and controls following standard protocols and used for the extraction of DNA (23). DNA concentrations were measured on the ND-1000 Nanodrop and purified. PCR was amplified, and DNA was isolated by agarose gel. RPL5 and RPL9 genes for direct DNA and independent PCR sequencing were also carried out to confirm each mutation identified and genomic DNA isolates were used as a PCR template. In the event of deletions and insertions, the PCR products were cloned against mutated and wild alleles. Blood samples were analyzed by the protocol, and the total RNA was isolated per the manual of the producer. To prevent possible DNA contamination and the enzyme; samples were treated and inactivated. For reverse transcription, were used for isolation of RNA in both patients and control subjects. The RPL5 and RPL9 were PCR-amplified by cDNA used specific primers to isolate PCR products and sequenced them as previously described (24). Standard curves were generated with PCR- amplified fragments of each target for each gene. Sanger automated sequencing ABI-3730xL system (Hong Kong) was used for DNA sequencing. The

Characteristics	Patients, n=100	Control, n=50	P. value
Sex			
Male	42(42%)	24(42%)	0.4916*
Female	58(58%)	26(58%)	
Age/ Mean \pm SD			
Total	61.2 \pm 1.3	67.6 \pm 3.2	0.0001**
Male	66.7 \pm 0.5	64.2 \pm 2.1	0.0001**
Female	57.4 \pm 1.1	59.5 \pm 0.8	0.0001**
Family history			
Yes	39 (39%)		
No	61(61%)		
Relative degree of family history, n=39			
Yes	18(46.2%)		
No	21(53.8%)		
Duration of thrombosis			
0-5 years	74(74%)		
5-10 years	23(23%)		
>10 years	3(3%)		
Treatment used			
Aspirin	28(28%)		
Oral anticoagulant	24(24%)		
Heparin (different types)	5(5%)		
Aspirin and oral anticoagulants	43(43%)		

Table 1. The characterization of normal controls and thrombosis patients at King Fahad University Hospital, Saudi Arabian. Fisher exact test* and T. test were used, P<0.05 was considered as significant**

chromatogram data FinchTV DNA analyzer was used to view the patient's DNA sequencing. After that, the sequence was entered into the Blast National Center of Biotechnology Information (NCBI) (<https://rb.gy/eec44k>) to compare the nucleotide sequences to the reference sequence in the database. Through the Alignment graphics, the variant details were identified and selected depending on 1000 Genomes phase 3. The amino acid sequences were identified and obtained from the GenBank. Then the protein sequence was entered into T-Coffee (<https://cutt.us/T-Coffee>) and Boxshade (<https://cutt.us/boxshade>) websites along with the reference protein sequence (<https://cutt.us/GeneBank>) in FASTA format to find the alignment of sequence matching. The statistical analysis was performed using the Prism 5 and SPSS version-21 programs. To measure the statistical differences between the cases and the controls, the independent sample T-test and the exact Fisher test were used. P-values below 0.05 are known to be clinically important. To assess patients and healthy Saudi people who participated in this study, Pearson correlation analysis and the p-value of genotypic features were measured.

4. RESULTS

Over one year, a total of 100 Saudi patients who were diagnosed with thrombosis were recruited. There was no significant correlation between the gender of both patients and control, while there were significant differences between the ages of both males and females (<0.05). The characterization of normal controls and thrombosis patients at King Fahad University Hospital, Saudi Arabian; Female DVT patients were represented (58%) of the study in comprising with males (42%) matching with the control. Patients who were exposed to DVT 0-5 years ago showed a higher number of patients which means, in the last 5 years the DVT patients were increased (74%). While other durations 5-10 years, and >10 years represented in low percentage (23%, 3% respectively). Regarding the family history (46.2%) of the patients had a family history of having DVT and the rest which represent (53.8%) do not have a family history. According to the treatment, patients who used Aspirin have represented 28% followed by oral anticoagulants (24%) and heparin (different types) about 5% of the patients, while patients received Aspirin and oral anticoagulant were 43% of the patients as seen in Table 1. The prevalence of thrombosis was showed that atherosclerosis (41%), Deep vein thrombosis (21%), and Superficial thrombophlebitis (19%) were the highest incidence when compared to other types of thrombosis (Figure 1). We sequenced the RPL5 gene in intron 6 and

db SNP b153 v 2	Nucleotide Change	Molecular Type	Nucleotide Alteration	Variant con- sequence
rs138979590	g.9329G>C g.9283G>C	Transcript	c.189+267G>C	Intron
rs558220259	g.9329A>G g.9283A>G	Transcript	c.189+288A>G	Intron
rs576892621	g.6984A>G g.9329A>G g.9283A>G	Transcript	None	Intron
rs182018447	g.9329G>A g.9283G>A c.189+387A>T	Transcript	c.189+387A>T	Intron
rs559377519	g.9329A>T g.9283A>T	Transcript	c.189+387A>T	Intron

Table 2. SNPs identified within RPL5 in the Saudi patients.

db SNP b153 v 2	Nucleotide Change	Molecular Type	Nucleotide Alteration	Variant con- sequence
rs191123038	g.5582 C>T g.5582 C>G g.5582 C>A	Transcript	None	Intron
rs370458857	g.5596 G>A	Transcript	None	Intron
rs567203778	g.5630 T>C	Transcript	None	Intron
rs201850421	g.5664 C>T	Transcript/ 60S RPL9	c.71C>T	Coding/Mis- sense
rs553047254	g.5670 T>C	Transcript	c.77 T>C c.77 T>A	Coding
	g.5670 T>A	60S RPL9	None	Missense
rs186201502	g.5691 C>T	Transcript	c.98C>T c.98 C>A	Coding
	g.5691 C>A	60S RPL9	None	Missense
	g.5707 C>T g.5707 C>G	Transcript	None	-
rs147466054	g.5707 C>A	60S RPL9	c.114C>T c.114C>G c.114C>A	Coding/ Missense
rs199892060	g.5728 C>T	Transcript	c.135 C>T	Coding
rs563851361	g.5738 G>C	Transcript	c.145G>C	

Table 3. SNPs identified within RPL9 in the Saudi patients

RPL9 gene in exon 8 from patients with unusual RPL5 and RPL9 gene expression patterns in PCR analysis. Five SNPs in the RPL5 gene were reported after sequencing (rs138979590, rs558220259, rs576892621, rs182018447, and rs559377519) and each patient SNP was identified according to the nucleotide change and alteration as thrombosis factor (Table 2) resulting in variant consequences among al RPL5 gene. Table 3 showed the multiple transcript variants of RPL9 gene among patients; rs191123038, rs370458857, rs567203778, rs201850421, rs553047254, rs186201502, rs147466054, rs199892060 and rs563851361, resulting in 3 different variant consequences including 20 codons (47.6%), 16 missenses (38.1%), 6 introns (14.3%). These identified RPL5 and RPL9 SNPs were found in Saudi patients. However, the normal samples were missed.

Figure 2 represented the ribosome protein L5. Positions sharing 88.0% amino acid identity are indicated in black. Positions sharing different 9.3% amino acids

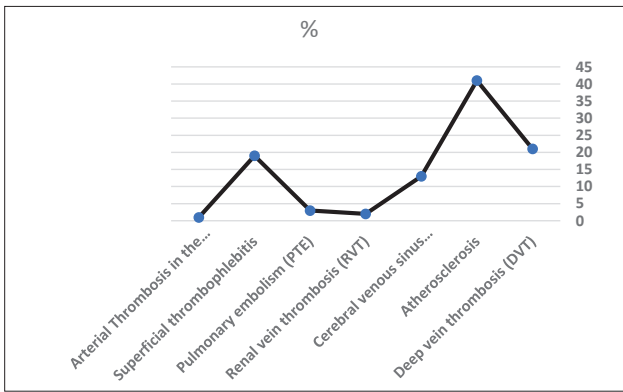


Figure 1. The thrombosis related incidence in Saudi patients The prevalence of Atherosclerosis among Saudi thrombotic patients was increased (41%), compared to other types, while the Arterial Thrombosis in the Eye is lowest one (1%).

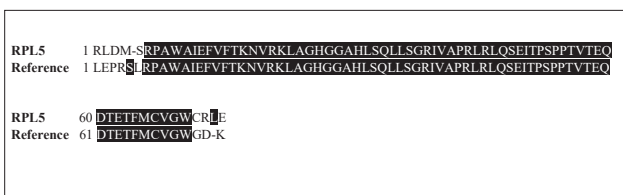


Figure 2. Multiple sequence alignment of RPL5-related sequences identified with BLAST. The alignment was done with ClustalW version 2.1, and shading was done with Boxshade version 3.21. RPL5, positions sharing 88.0% amino acid identity are indicated in black. Positions sharing different 9.3% amino acid are indicated in white, while 2.7% deletion amino acids indicated by (-).

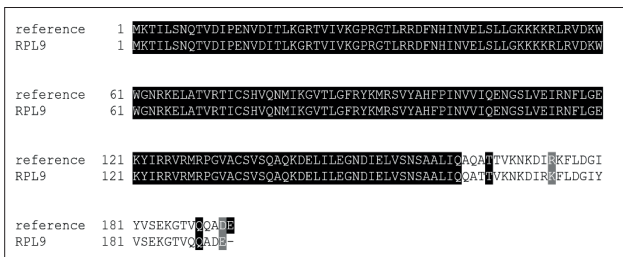


Figure 3. Multiple sequencing alignments using Boxshade program compared to the reference sequence of RPL9 gene. The region highlighted in black represents the conserved region of RPL9, Ribosome protein L9. Positions sharing 78.4% amino acid, the white region represents the mutant region (19.4%) while the region highlighted in gray represents conservatively substituted amino acids (1.5%). Positions deleted amino acid were indicated in (-) is about (0.70%)

are indicated in white, while 2.7% deletion amino acids are indicated by (-). RPL9, positions sharing 78.4% amino acid, the white region represents the mutant region (19.4%) while the region highlighted in gray represents conservatively substituted amino acids (1.5%). Positions deleted amino acids were indicated in (-) is about (0.70%) as shown in Figure 3. In figures 2 and 3, the normal gene reference sequence was used for comparison in both RPL5 and RPL9 (ID-gene: 6133, 6125 respectively).

5. DISCUSSION

Ribosomes are vital to life and generate all proteins needed to grow cells and sustain them. A mature ribo-

some in eukaryotes consists of four different ribosomal RNAs (25). In this study, we determined that the RPL5 gene a new SNP variant associated with thrombosis in the Saudi population. Our results indicated the presence of these variants in correlation with the occurrence of thrombosis in Saudi patients. This result agrees with that in a previous study, which identified that these genes were upregulated in patients with venous thromboembolism (22). Also, during the formation of platelets, previous studies have examined ribosomes. Particularly, it has been identified that RPS6 ribosomal protein was involved in circulating platelets, common hemostasis cellular activators, including thrombosis (17). Platelets facilitate the production of immune cells and enable the development of a cell surface leukocyte trap that leads to the spread of thrombosis (26, 27). There are few studies generally being on relationships between such mutations of the ribosomal protein and thrombosis. Therefore, we hypothesize that blood clots and RPL5 were related to platelets. Identified SNPs (Intron 6) may associate with thrombosis risk among Saudi patients. We did not observe any significant association with other SNPs, which may be due to a limited number of thrombosis cases in the selection of the study groups, which may reflect differences. Of note, for RPL5 SNPs, this variation indicates a clear association with this disease. Our results do not provide any evidence that these variants are associated with thrombosis, which also indicates differential effects across pathophysiological disease, as has been observed with some SNPs.

However, recent research suggests that the clear effects of RPL5 depletion on p53 induction can be attributed to reduced global translation and not decreasing stability at p53. Thus, in terms of biogenesis of ribosomal responses, the only Ribosome protein (RP) needed for p53 induction appears to always be RPL5, which impairs the 60S translation of ribosomal subunit to the same degree as other essential RPs (28). Certain RP transcripts change cellular levels as a function of growth, production, and tumors (29). Furthermore, it is known that many RPs have essential functions in many other cellular processes. In recent years, mutations in ribosomal proteins have been identified in patients with various diseases; these mutations may lead to anatomical anomalies in humans (30), Such as contribution to Diamond-Blackfan anemia (31). Because most studies thus far have been confined to tumor cells, it is unclear whether a decrease in the ribosome content owing to the translation p53 checkpoint loss or alternative p53 cell cycle activation is a mechanism for controlling the growth of cells and reducing concentrations of RPL5 (16).

Impairment of ribosome in the regulation of gene expression contributes to the stimulation of p53 and the interruption of cell cycle involved in thrombosis response results in the blocking of G1, and a novel block of G2/M when either subunit is disrupted (16). Thus, the p53 induction mechanisms in thrombosis patients may be essential for RPL5, more than localization. Saudi thrombosis patients were determined to have many variants of the RPL5 gene compared to the controls,

which indicated that the function of the RPL5 gene and its role in thrombosis may be important for splicing or translational control. In this study, by focusing on the coding intron, we may have missed potentially important regulatory non-coding regions. While RPL9 gene multiple sequencing alignment showed an amino acid substitution, insertion and deletion mutations resulted in different variants of the RPL9 gene. As a consequence of the SNPs, a noncoding region of RNA (intron) has been found, along with codons and missenses which occurred due to a single nucleotide and codon encoding for varied amino acids result. A study conducted by Baik *et al.* revealed that ribosomal proteins are not only involved in protein synthesis but also have a secondary function known as extra-ribosomal functions that help in the development of cancer (32). A specific single nucleotide polymorphism (rs563851361) was found in colorectal cancer (CRC) causing missenses mutation due to the exchange of arginine to glutamine, while in our study the same variant has been found in thrombosis patient, but the mutation occurred as results of exchange arginine to glycine (32). The previous study has shown that other conditions could be occurred due to a mutation in the RPL9 gene, such as Takayasu arteritis which is an inflammation of blood vessel that characterized by microvascular fibrosis and systemic limiting observed that is due to thrombus formation because of ribosomal protein-related genes along with RPS3A, RPL31, including RPL9 could endorse the developments of Takayasu arteritis (21). The current research demonstrated these variants in Saudi patients. Therefore, more studies with a large sample size and focusing on the different type of thrombosis are maybe more accurate to point out the correlation of their RPL5 and RPL9 gene variants with ribosome cascade regulating cell to patients with thrombosis

6. CONCLUSION

Different forms of RPL5 and RPL9 thrombosis-associated mutations were found, and a first distinction was made between these groups and patients with other mutations linked to clinical results and effects of treatment. The RPL5 variants rs138979590, rs558220259, rs576892621, rs182018447 and rs559377519 were related to thrombosis patients. Multiple transcript variants of RPL9 gene rs191123038, rs370458857, rs567203778, rs201850421, rs553047254, rs186201502, rs147466054, rs199892060, rs563851361 were considered among these patients, which resulting in 3 different variant consequences including 20 codons (47.6%), 16 missenses (38.1%), 6 introns (14.3%). Due to the discovery of different RPL5 and RPL9 mutations in Saudi patients, there was a strong association between ribosomal protein genes (RPL5 and RPL9) with the thrombosis incidence and development in the Saudi population, which may have helped to enhance the medical diagnosis of thrombosis. All patients with thromboembolism can carry out molecular analysis of RPL5 and RPL9 as public health concerns in representative samples of the Saudi population. The hypothesis that the variability of the individ-

ual is responsible for the heterogeneity of the RPL5 and RPL9 should be strengthened. Blood RPL5 and RPL95 can be tracked in patients with thromboembolism by estimating the prevalence in large countries such as Saudi Arabia, which can help authorities guide public health services in more effective ways and manage the health system.

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