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Investigation of the association of the *RAN* (rs14035) and *XPO5* (rs11077) polymorphisms with venous thromboembolism

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Running head: *RAN* and *XPO5* genes and venous thromboembolism

Abstract

Introduction: Venous thromboembolism (VTE) is the third most common hemostatic disease worldwide. Studies have reported a role for microRNA (miRNA) in the homeostasis and development of VTE. The ras-related nuclear protein (*RAN*) and exportin 5 (*XPO5*) genes are involved in miRNA biogenesis, as both regulate the transport of pre-miRNA from the nucleus to the cytoplasm. Therefore, the aim of the current study is to examine the association between *RAN* (rs14035) and *XPO5* (rs11077) single nucleotide polymorphisms (SNPs) and VTE.

Methods: The study sample consisted of 300 subjects (150 patients and 150 age and sex matched controls). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and tetra-primer amplification refractory mutation system (T-ARMS) techniques were used to genotype rs14035 and rs11077, respectively.

Results: The results showed that there was a significant association between the *XPO5* rs11077 and the risk of VTE ($P < 0.05$). Subjects with AC (OR: 2.08, CI:1.26–3.44) and CC (OR: 1.77, CI: 0.88–3.55) genotypes were at increased risk of the developing VTE. Regarding *RAN* gene, no association was found between rs14035 and VTE ($P < 0.05$). In addition, no associations were found between *XPO5* rs11077 and *RAN* rs14035 genotypes with blood cell parameters ($P < 0.05$). As for the demographic characteristics, the results indicated a strong association between family history and body mass index (BMI) with the risk of VTE ($P < 0.01$).

Conclusion: The *XPO5* rs11077, BMI and family history might contribute to the development of VTE in Jordan.

Keywords: MicroRNAs; Venous Thromboembolism; Risk, Genes, Single nucleotide polymorphism.

INTRODUCTION

Venous thromboembolism (VTE) is a condition in which blood clots deposited on the walls of deep veins, particularly in the legs, causing deep vein thrombosis [1, 2]. The clots might transfer in the circulation to the lung, resulting in pulmonary embolism [3]. VTE ranks as the third most common hemostatic disease worldwide, with an incidence of 1–2 per 1000 individuals per year [4, 5]. Symptoms of VTE include calf pain, tenderness, redness, foot pain and leg swelling [6].

The influence of acquired factors causing VTE, such as age, gender, cancer, surgeries, medications, obesity, smoking, and physical inactivity has been widely investigated [7]. For example, it was found that age and body mass index (BMI) play an important role in the incidence of thrombosis, especially among women [8, 9]. Studies have shown that smoking and physical inactivity are strongly associated with VTE and other cardiovascular diseases [10, 11]. Finally, blood pressure and a history of surgery have a role in the development of VTE [12, 13]. The development of VTE is also attributed genetic factors that modulate the body's anticoagulation system [14]. For example, deficiency of anti-thrombin, protein C or S, resistance to activated protein C (APC-resistance), mutations in factor V Leiden, and genetic variants in prothrombin have been shown to increase the risk of VTE [15–18].

Studies have indicated a role for microRNAs (miRNAs) in the hemostasis and thrombosis development [19, 20]. The miRNAs are short types of non-coding RNA species (22–24 nucleotides) involved in the post-transcriptional regulation of gene expression [21]. A previous investigation reported low levels of miRNA-145 in VTE patients [22]. The miRNA-145 mediates its antithrombic

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effects by regulating tissue factor level/activity and the subsequent thrombogenesis [22]. In addition, mutations in miRNA biosynthesis genes have been shown to increase platelet receptors, as well as platelets reactivity [19, 23]. Another study demonstrated the role of miRNA biogenesis genes in ischemic stroke risk and prognosis [24]. Thus, mutations in miRNA biogenesis genes could increase the risk of VTE. The biogenesis of miRNA is accomplished by several key proteins [25]. The miRNAs are initially transcribed as a precursor molecule of 100–1000 nucleotides long with a stem-loop structure that is processed by a complex enzymatic structure in the nucleus to produce pre-miRNA. Then, the ras-related nuclear protein (RAN) GTPase and exportin 5 (XPO5) mediate the transport of pre-miRNA from the nucleus to the cytoplasm [26].

Among the genetic variations of *RAN* gene is the rs14035 single nucleotide polymorphism (SNP) [27]. The rs14035 SNP has been shown to play a role in the development of colorectal and hepatocellular cancers [28, 29]. In the *XPO5* gene, the rs11077 SNP has been shown to be associated with the risk of developing non-small-cell lung cancer, idiopathic primary ovarian insufficiency, and breast cancer [30–32]. *XPO5* rs11077 and *RAN* rs14035 have both been shown to be associated with VTE among Koreans [33]. Individuals with T allele of rs14035 SNP have a lower risk of VTE and individuals with the C allele of rs11077 SNP have an increased risk of VTE. In addition, the *RAN* rs14035 SNP has been shown to be associated with mortality among patients with large artery disease and ischemic stroke [24]. Among the effects of the rs14035 SNP on cellular activity is the accumulation of precursor miRNAs in the nucleus, leading to decreased miRNA levels in the cytoplasm and the subsequent mis regulation of gene expression [34]. Therefore, the aim of the current study is to investigate the association of *XPO5* rs11077 and *RAN* rs14035 with VTE among Jordanians.

MATERIALS AND METHODS

STUDY SUBJECTS

This study was a case control in design that included a total of 300 adult subjects recruited from Royal Medical Services hospitals in Jordan between March 2021 and February 2022. The VTE group consisted of 150 patients with VTE, and the control group consisted of 150 individuals without VTE. The VTE group included symptomatic patients were diagnosed according to the 2014 guidelines of European Society of Cardiology [35]. Exclusion criteria were VTE patients with systemic disease and patients whose diagnosis was not confirmed by neuroimaging or radiological methods [36, 37]. The control group was drawn from subjects visiting the Royal Medical Services hospitals and blood donors, who had no medical history of VTE, and who were negative for D-dimer test. The demographic data collected in this study consisted of age, gender, body mass index (BMI), hypertension, and family history of VTE. BMI was calculated using the participant's height and weight (BMI = kg/m²) [38, 39]. Hypertension was defined as high baseline blood pressure (≥ systolic blood pressure 140 mmHg or diastolic blood pressure ≥ 90 mmHg) or a patient who is taking anti-hypertensive medication [40].

ETHICAL APPROVAL

Informed consent was obtained from all participants according to the Institutional Review Board of Jordan University of Science and Technology (ethical approval identification number: 3-131-2020).

BLOOD COLLECTION

Two blood samples of 4 mL each, one in an ethylenediaminetetraacetic acid (EDTA) tube and one in a citrate tube, were obtained from each participant. The EDTA sample was used to measure complete blood count (CBC) parameters and to extract DNA. The citrate sample was used for serum preparation and D-dimer testing.

DNA EXTRACTION

DNA was extracted from whole blood samples using the Quick-DNA™ Miniprep Kit (Zymo Research- USA, catalog number: D3024). The extraction was done according to the manufacturer's instructions. The quality and quantity of extracted DNA were analyzed using a nanodrop spectrophotometer obtained from ThermoFisher Scientific (USA).

MOLECULAR ANALYSIS

The *XPO5* rs11077 A>C SNP was genotyped using T-ARMS PCR technique, whereas the *RAN*

rs14035 C>T SNP was genotyped using PCR-RFLP technique. For each of the SNPs, the PCR reaction volume was 20 μL, containing ready-to-use master mix (Promega, USA), 0.1 μM of each primer, and 20 ng of template DNA. The used primers for rs11077 [41] were outer forward: 5′ CAACTACT TGTGCCAGAGTTTCTCTTGG3', outer reverse: 5′ TGGTCTGTATTATCCTTGGATGACAACG3', allele A specific inner forward: 5' AGTACCTCCA AGGACCAGGGCTGAGA3', and allele C specific inner reverse: 5' CTCTAAAGGGGATGTTAGCA CTAAAGATTG3'. The PCR work protocol for rs11077 was denaturation at 95°C for 5 minutes, 30 cycles: 95°C/45s, 65°C/45s, and 72°C/30s, and a final extension of 72°C for 5 minutes. For rs14035 C > T SNP, the primers used were forward: 5′GAAGCACTTGCTCAAAATCTGTGAC3' and reverse: 5′ TGCCATCCACTGATGTTCCATC3' [24]. The PCR work protocol for rs14035 was denaturation at 95°C for 5 minutes, 30 cycles: 95°C/30s, 56°C/30s, and 72°C/30s, and a final extension of 72°C for 5 minutes.

PCR products were analyzed by loading 5 μl of the PCR products into 3% agarose gel electrophoresis at 120 volts for 90 minutes. The amplified fragment of rs14035 was 152 bp. Restriction of this fragment using *BslI* enzyme (Biolabs company, USA) produces 127 bp and 25 bp fragments in presence of the C allele. In the presence of the T allele, the amplified fragment stays intact (152 bp) after exposure to the enzyme. Visualization of amplified/restricted DNA fragments was achieved using ethidium bromide staining [42].

COMPLETE BLOOD COUNT (CBC)

Blood indices were measured using an automated hematology analyzer (XP-300, Sysmex) at the Royal Medical Services hospitals.

D-DIMER TEST

The citrated plasma samples were assayed for the D-dimer test using commercially available kit (DIALAB® Produktion, Neudorf, Austria). Plasma samples were mixed with latex reagent by adding 20 μL of the sample with one drop of reagent. Subsequent steps were performed according to the instructions provided in the kit. The results (whether positive or negative) were examined under a strong light source exactly 3 minutes after the reaction.

STATISTICAL ANALYSIS

Genotypes and alleles frequencies and their agreement with Hardy-Weinberg equilibrium were carried out by SNPStats software [43]. For descriptive analysis, GraphPad Prism software (version 5, San Diego, California, USA) was used. For categorical variables, data were presented as frequencies/ percentages (%) and compared between study groups using the Chi-square test. For continuous variables, data were presented as means and standard deviations (SDs) and compared between groups using Student t-test or analysis of variance (ANOVA) test. The results were considered statistically significant at $p < 0.05$.

RESULTS

The demographics of the study participants are listed in Table 1. Both the VTE group and the control group were match in age and sex. The participants were all from the northern Jordan, therefore both groups share same geographical area. There were no statistically significant differences between patients and controls regarding tobacco use ($P < 0.05$) and hypertension ($P < 0.05$). The two groups showed significant differences in terms of BMI and family history of VTE. BMI was higher in the VTE group than the control group ($p < 0.05$). Family history of VTE was higher in the VTE group than in the control group ($P < 0.001$). In the D-dimer test, all subjects with VTE were found to be positive, while those in the control group were found to be negative.

The PCR-RFLP procedure was used for genotyping the rs14035 SNP. Table 2 shows the distribution of genotypes and alleles for the rs14035 SNP among the study groups. The CC genotype was present in 46% of the patients and 39.3% of the controls, while the heterozygous genotype was found in 46% of the patients and 52% of the controls. The TT genotype was found in 8.1% of patients and 8.7% of controls. The distribution of genotypes corresponded to Hardy-Weinberg equilibrium ($P < 0.05$). No significant association was found between rs14035 and the prevalence of VTE ($P < 0.05$).

The rs11077 SNP was genotyped using the T-ARMS PCR technique. Table 3 shows the distribution of genotypes and alleles of rs11077 SNP among the study groups. The AA genotype was present in 45.3% of the VTE patients and 29.3% of the control group. The heterozygous AC genotype was found in 40.7% of the VTE patients

and 54.7% of the control group. The CC genotype was found in 14% of VTE patients and 16% of the control group. The distribution of genotypes corresponded with Hardy-Weinberg equilibrium ($P < 0.05$). A significant association was found between rs11077 and VTE. The AA genotype was found to be associated with an increased risk of VTE ($P = 0.14$). In addition, the A allele was found to be enriched in the patient group compared to the control group ($P = 0.02$).

Analysis of the haplotypes of rs14035 and rs11077 SNPs is shown in Table 4. The CA haplotype was common in both the control group (45.3%) and the patient group (41.2%), while the TC haplotype was rare in both the control group (14.3%) and the patient group (15.5%). None of the haplotypes was significantly associated with VTE ($P < 0.05$, Table 4).

Association analysis between SNPs (rs14035 and rs11077) and CBC parameters was examined using the ANOVA test. Table 5 represents the results for *RAN* rs14035 SNP, while Table 6 shows the *XPO5* rs11077 SNP results. No associations were found between *XPO5* rs11077 and *RAN* rs14035 genotypes with all examined blood cell parameters ($P < 0.05$).

Table 1

The demographic characteristics of the participants.

Variables*	Category	Controls n (%)	Patients n (%)	P-value
Mean age (year), mean ± SD		44.8 ± 12.2	47.8 ± 15.8	0.062
Mean BMI (kg/m²), mean ± SD		29.6 ± 5.4	28.3 ± 5.4	0.0
Gender	Male	74 (49%)	75 (50%)	0.908
	Female	76 (51%)	75 (50%)	
Smoking	Yes	54 (36%)	45 (30%)	0.269
	No	96 (64%)	105 (70%)	
Hypertension (mmHg)	Yes	53 (35.3%)	63 (42%)	0.235
	No	97 (64.7%)	87 (58%)	
Positive family history of VTE	Yes	14 (9.3%)	66 (44%)	< 0.001
	No	136 (90.7%)	84 (56%)	

* BMI: body mass index, SD: standard deviation, VTE: venous thromboembolism.

Table 2

Genotypes and alleles frequency of rs14035 in participants

rs14035	Control	Patients	OR (95% CI)	P-value
Genotypes				
CC	59 (39.3)	69 (46)	1.00	0.57
CT	78 (52)	69 (46)	1.29 (0.80–2.08)	
TT	13 (8.7)	12 (8.1)	1.25 (0.53–2.95)	
Alleles				
C	196 (65.3)	207 (69)		0.76
T	104 (34.7)	93 (31)		

OR: Odd Ratio, CI: Confidence Interval,

Table 3

The frequency and association of genotypes and alleles of rs11077 and study groups.

rs11077	Control	Patients	OR (95% CI)	P-value
Genotypes				
AA	68 (45.3)	44 (29.3)	1.00	0.014
AC	61 (40.7)	82 (54.7)	2.08 (1.26–3.44)	
CC	21 (14)	24 (16)	1.77 (0.88–3.55)	
Alleles				
A	197 (65.6)	170 (56.6)		0.02
C	103 (34.4)	130 (43.4)		

Table 4

Haplotype analysis of rs14035 and rs11077 with VTE.

Haplotype	Control %	Patients %	Cumulative frequency	OR (95% CI)	P-value
CA	45.4	41.2	43.2	1.00	–
CC	20.0	27.8	67.2	1.6 (0.95–2.67)	0.076
TA	20.2	15.5	85.2	0.85 (0.48–1.52)	0.59
TC	14.4	15.5	100	1.15 (0.68–1.93)	0.6

Table 5

Correlation of rs14035 with complete blood count (CBC) parameters

Parameter* Mean ± SD	CC	CT	TT	p-value
WBC (10*9/L)	9.66 ± 2.38	8.63 ± 2.47	9.39 ± 5.23	0.19
RBC (10*12/L)	4.75 ± 0.81	4.56 ± 0.80	4.59 ± 0.82	0.13
Hemoglobin ((g/dl)	12.80 ± 2.35	12.78 ± 2.32	12.53 ± 2.31	0.86
Hematocrit (%)	38.88 ± 6.85	39.27 ± 6.76	38.00 ± 6.57	0.66
Platelet (10*9/L)	269.3 ± 90.13	248.0 ± 88.05	206.8 ± 88.00	0.39
RDW (fL)	14.57 ± 2.30	15.13 ± 2.44	14.90 ± 1.61	0.14
PDW (fL)	11.58 ± 2.64	11.49 ± 2.40	11.80 ± 2.99	0.85
MPV	9.12 ± 1.76	9.25 ± 1.13	9.61 ± 1.86	0.36

* WBC: white blood cells, RBC: red blood cells, RDW: red cell distribution width, PDW: platelet distribution width, MPV: mean platelet volume.

Table 6

Correlation of rs11077 with CBC parameters

Parameter Mean ± SD	AA	AC Mean ± SD	CC Mean ± SD	P-value
WBC (10*9/L)	9.64 ± 5.37	8.78 ± 2.70	8.32 ± 2.41	0.09
RBC (10*12/L)	4.61 ± 0.77	4.63 ± 0.83	4.76 ± 0.82	0.56
Hemoglobin ((g/dl)	12.65 ± 2.47	12.88 ± 2.25	12.74 ± 2.14	0.73
Hematocrit (%)	38.28 ± 7.01	39.55 ± 6.72	39.00 ± 6.13	0.33
Platelet (10*9/L)	264.3 ± 88.77	264.0 ± 90.79	260.8 ± 83.17	0.97
RDW (fL)	14.76 ± 2.74	14.89 ± 1.81	15.05 ± 2.69	0.77
PDW (fL)	11.41 ± 2.57	11.44 ± 2.58	12.36 ± 2.25	0.07
MPV	9.20 ± 1.57	9.18 ± 1.77	9.44 ± 1.68	0.64

*WBC: white blood cells, RBC: red blood cells, RDW: red cell distribution width, PDW: platelet distribution width, MPV: mean platelet volume.

DISCUSSION

The mechanism of circulatory hemostasis is highly conserved across animal kingdom [44]. This includes maintaining the fluid status of the blood, repairing damaged vessels, and removing blood clots [44]. Molecules, such as miRNA, play an important role in regulating many genes, including those responsible for circulatory hemostasis [19, 45–47]. Defective miRNA biogenesis genes have been associated with several cardiovascular conditions including venous thromboembolism [48–51]. Therefore, in the current study, the association between *RAN* (rs14035) and *XPO5* (rs11077) polymorphisms and VTE was examined. According to the results, *XPO5* (rs11077) A < C polymorphism was found to be associated with VTE among Jordanians. The C allele was more frequently in the VTE group than in the control group. This finding is consistent with a study from Korea that showed a strong association between rs11077 and VTE [33]. The *XPO5* gene codes for exportin-5 protein. During miRNA biogenesis, pre-miRNA binds to exportin5 as they are transported from the nucleus to the cytoplasm [26]. This binding is important to stabilizing miRNAs and protecting them from cellular degradation [26].

Previous studies have implicated the *XPO5* (rs11077) polymorphism in the pathology of several diseases. For example, *XPO5* (rs11077) has been found to be associated with non-small cell lung carcinoma, response to chemotherapy, breast cancer, idiopathic primary ovarian insufficiency, and increased life span in patients with esophageal squamous cell carcinoma [27, 29–32]. These data illustrated the important role of *XPO5* rs11077 polymorphism in normal and disease conditions.

The results showed no association between *RAN* (rs14035) polymorphism and VTE. A previous study from Korea reported a role for this SNP in the development of VTE [33]. The clinical significance of *RAN* (rs14035) polymorphism has been reported in several previous studies. For example, rs14035 has been found to be associated with colorectal cancer, hepatocellular carcinoma, and post-stroke mortality [24, 28, 29]. Thus, the clinical significance of *RAN* polymorphisms might be modulated by population's genetic background and associated environmental factors [52].

The current study showed a significant association between VTE and the family history of this condition. Similarly, a study by Eikelboom and Weitz showed that family history was a strong risk factor for VTE [53]. Also, Zoller *et al.* illustrated the role of family history in predicting VTE among people of Sweden [54]. With regard to BMI, a study by Yang *et al.* showed that obese people are at a higher risk of developing VTE [55]. Also, a study by Eichinger *et al.*, showed that obesity increases the risk of secondary VTE [56].

Previous studies confirm the independent and significant role of smoking in the pathogenesis of VTE. For example, a study done by Severinsen *et al.*, who showed the major role of smoking in VTE and other cardiovascular diseases [57]. In addition, a study by Cheng *et al.*, revealed that smoking proportionally increases the risk of VTE independent of other cardiovascular diseases [58]. In contrast, this study revealed that there is no significant association between VTE and smoking. This finding may be due to the high percentage (about 36%) of smokers among the sample. In addition, the control group was mainly recruited from the blood bank, and it is known that smoking is common among the population of blood donors [59]. With respect to hypertension, it is suggested to be a strong risk factor among all cardiovascular diseases including VTE [60, 61]. In this study, there was no significant relationship between hypertension and VTE risk. This discrepancy may be due to the high incidence of hypertension among Jordanians, and about a third of Jordanian adults suffer from high blood pressure [62].

As for the cell blood count parameters, the study did not find any significant differences in all these parameters when different genotypes of studied polymorphisms were considered. The role of miRNA in hematopoiesis is well-documented [63, 64]. More studies are needed to confirm the present findings.

A limitation of the current study is the relatively small sample size. In addition, the study did not examine the association of studies SNPs with other risk factors of VTE such as factor V Leiden, protein C and protein S. Moreover, the study was conducted in northern part of Jordan. Therefore, more studies that consider such limitations are needed to confirm the study findings.

CONCLUSION

The *XPO5* rs11077, BMI and family history might contribute to the development of VTE in Jordan. These findings can be used in interventions focused on preventing VTE in Jordan.

Introducere: Trombembolismul venos (VTE) este a treia patologie a coagulării în întreaga lume. Studiile anterioare au raportat un rol al microARN-urilor în dezvoltarea VTE. Genele RAN şi exoportina 5 sunt implicate în procesele de biosinteză ale mi-ARN-urilor şi transportul acestora. Scopul studiului a fost de a evalua două polimorfisme genetice RAN (rs14035) şi XPO5 (rs11077) la pacienţii cu VTE.

Metode: Au fost incluşi 300 de pacienţi (150 cu VTE şi 150 de martori sănătoşi). A fost folosită tehnica T-ARMS şi PCR-RFLP pentru a evalua genotipurile rs14035 şi rs11077.

Rezultate: S-a observat o asociere semnificativ statistic crescută între XPO5 rs11077 şi riscul VTE ($p < 0.05$). Pacienţii cu genotipurile AC (OR: 2.08, CI:1.26–3.44) şi CC (OR: 1.77, CI: 0.88–3.55) au avut un risc mai mare de a dezvolta VTE.

Referitor la gena RAN nu s-au observant asocieri cu VTE. Totodată nu s-au observant asocieri între genotipuri şi parametrii hemogramei. S-a observat o asociere semnificativă între istoricul familial, BMI şi riscul de a dezvolta VTE ($p < 0.01$).

Concluzii: Polimorfismul XPO5 rs11077, BMI şi istoricul familial contribuie la dezvoltarea VTE la pacienţii din Iordania.

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Declaration of Interest: Authors have nothing to declare.

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