Research Article

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Determination of molecular pathways and gene ontology of genes associated with Raynaud's phenomenon

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Abstract

Objectives: Raynaud's phenomenon (RP) is a disease that causes discoloration of the fingers. The purpose of this study is to identify the molecular pathways in which genes related to RP illness are involved, as well as uncover the biological processes and molecular functions connected with those genes via the use of gene ontology (GO) analysis.

Methods: Genes associated with RP in the MalaCards Human Diseases database were detected. Twenty genes obtained from the MalaCards Human Diseases database were included in the study for gene ontology analysis via the STRING database. Accordingly, possible interactions between 20 genes were determined through STRING and network enrichment was performed.

Results: A significant enrichment by gene ontology enrichment analysis was detected in a subset of genes involved in biological processes including cellular response to luteinizing hormone stimulus, negative regulation of fibrinolysis, negative regulation of smooth muscle cell apoptotic process, plasminogen activation, cellular response to follicle-stimulating hormone stimulus. The assay for molecular function determined enrichment of a subset of genes in chemoattractant activity, growth factor activity, heparin binding, sulfur compound binding, growth factor receptor binding. Through the use of KEGG pathways, we were able to identify many molecular processes that contribute to RP, including the AGE-RAGE signaling pathway in diabetic complications, complement and coagulation cascades, fluid shear stress, atherosclerosis.

Conclusions: Some individuals may have a genetic predisposition to the onset of Raynaud's phenomenon. Our data showed that it is associated with genes involved in vascular damage and fibrosis, especially in RP. Therefore, we can include RP disease in the group of vascular diseases.

Keywords: Raynaud's phenomenon; pathway; database; genes; gene ontology

Introduction

Raynaud's phenomenon (RP) is a disorder caused by reversible paroxysmal vasospasm of small-diameter arteries and cutaneous arterioles, characterized by paleness, cyanosis, and redness, especially in the 2/3 tips of the fingers, triggered by cold and emotional stress. RP often affects the digital arteries of the upper extremities and, less frequently, the digital arteries of the lower extremities. Its etiopathogenesis has not been fully determined [1]. Most general population surveys report RP prevalence between 3 and 5 %. Geographic location, population investigated, and case determination procedure determine the main RP prevalence: 2–20 % in females and 1–12 % in men [2].

The main forms include primary RP, which is benign, and secondary RP, which is linked to connective tissue illnesses such as systemic sclerosis. The term "Primary Raynaud's Phenomenon", also called Raynaud's disease, is generally used when no underlying pathology is detected. Sudden-onset attacks in primary RP are thought to be due to vascular hyperactivity [3]. The skin changes of necrosis, ulceration, and gangrene are uncommon in cases of idiopathic primary RP, which does not have a vascular or collagen tissue disease as a cause. Primary RP covers several entities, such as functional vasospastic disorder, inappropriate thermoregulatory response, and cold intolerance. The formation mechanism of primary RP is considered a local vasospastic response defect without any structural pathology in thermoregulatory vessels, while secondary RP is a pathology that can cause vascular organic changes [4]. Primary RP usually starts at an earlier age than secondary RP and often shows symmetrical involvement.

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The term "Secondary Raynaud's Phenomenon" is used for the form associated with irreversible structural vascular pathologies, which develop due to a wide variety of rheumatic, hematological, endocrinological, and vascular pathologies. A condition accompanied by vascular disease or collagen tissue disease is called secondary RP, or Raynaud's syndrome [5]. Secondary RP is often accompanied by many systemic autoimmune rheumatic diseases, including scleroderma, mixed connective tissue disease, systemic lupus erythematosus (SLE), dermatomyositis, Sjögren's syndrome, mixed and undifferentiated connective tissue disorders, and idiopathic inflammatory myopathies [1].

The pathogenesis of RP is likely to include both neurological and vascular consequences. In both cases, the pathophysiology differs slightly, as RP may be idiopathic or caused by a secondary cause. Primary RP is an isolated vasospastic syndrome with functional alterations; secondary RP, caused by a systemic disease, has numerous causes. Endothelial cell injury, asymmetry in the release of vasoactive chemicals, structural abnormalities, intravascular lesions that obstruct blood flow, and heightened vasoconstriction are all potential causes [6].

As a secondary manifestation of RP, vibration-induced white finger (VWF) is a key component of the hand-arm vibration syndrome. This vasospastic illness is a prescribed occupational ailment in most industrialized nations and affects workers who are exposed to vibration from handheld instruments for lengthy periods of time. It is thought that the physical properties of hand-transmitted vibration (HTV) determine both the beginning and the severity of VWF, alongside certain individual and environmental variables (such as smoking and cold exposure) [7]. Workers in the Scandinavian nations of North America, Europe, and Asia are more likely to get VWF as a result of vibration exposure than those in tropical or equatorial areas [8]. Epidemiological data links VWF to the intensity, duration, and frequency of vibration in professional users of several vibratory instruments [7]. Evidence from epidemiological research shows that VWF has been on the decline in recent decades, thanks to improvements in work organization, shorter daily exposure times, and instruments with built-in antivibration technologies [8].

Vasculopathy, inflammation, and fibrosis are the hallmarks of systemic sclerosis (SSc), a chronic inflammatory disorder. RP has a major influence on everyday life and affects more than 96 % of people with SSc. There is a wide range of digital vasculopathy in SSc patients, from temporary RP attacks to more permanent tissue damage, including digital ulcers and gangrene [9]. Dysregulated neuroendothelial regulatory systems may shed light on the pathophysiology of RP, particularly in cases where it is a subsequent complication of SSc. The main problem is that vasodilation and vasoconstriction are not balanced [10].

Genetic factors are increasingly thought to have a role in the etiopathogenesis of SSc, particularly as epidemiologic evidence for environmental causation is lacking in most instances. Research on other autoimmune illnesses, such as lupus, that has recently undergone genome-wide scanning may shed light on the genetics of SSc. Disease risk is elevated in certain groups due to MHC class II alleles; however, these alleles are more closely linked to certain autoantibody profiles. There is also evidence of a similar age of onset in different generations in multi-case families, as well as familial clustering of the primary RP. There is preliminary evidence that suggests monozygotic twins are more likely to share a diagnosis of primary RP than are dizygotic twins [11]. Due to its rarity, familial aggregation for SSc is now a substantial risk factor for the illness and strengthens the case for genetics in etiopathogenesis. Various genes located in the extracellular matrix, such as fibrillin-1, have been determined to contribute to a complex genetic disease [11]. The vasculopathy of scleroderma has been characterized using a number of different vascular disease indicators, all of which point to an active endothelium. Increased levels of plasma endothelin, soluble E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and evidence of activated platelets are all indicators of vascular inflammation [12].

Phosphodiesterases, often known as PDEs, are isoenzymes responsible for regulating the concentration of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) inside cells. cGMP, a key regulator of smooth muscle tone, is preferentially degraded by phosphodiesterase isoenzyme 5 (PDE5) [13]. Phosphodiesterases, in particular the cGMP-specific PDE5 isoenzyme, hydrolyze cGMP, allowing nitric oxide to vasodilate and prevent platelet activation. The selective inhibition of cGMP-specific PDE5 by sildenafil, tadalafil, and vardenafil elevates cGMP, which in turn enhances cGMP-dependent microvascular and macrovascular dilatation. Due to their impact on microvascular and macrovascular circulation, PDE5 inhibitors are being investigated in RP patients [14].

The use of PDE5 inhibitors has been considered a potential alternative to traditional vasodilator treatment for individuals with severe Raynaud's disease symptoms. It was found that after sildenafil and alprostadil treatment, the average capillary flow rate improved, the cumulative attack length shortened, and the average frequency of Raynaud attacks decreased. It was observed that the use of sildenafil reduced the frequency and intensity of Raynaud's attacks in a group of patients with systemic sclerosis and digital ulcers,

and 75 % of patients with digital ulcers resistant to standard treatment recovered completely [15].

SSc-related disorders are characterized by pathognomonic autoantibodies called anti- Centromere protein B (anti-CENP-B) and anti-Topoisomerase I (anti-TOPO-1). These autoantibodies hasten the senescence of vascular endothelial cells and functional impairment, which induce RP [16]. Ro52/tripartite motif-containing 21 (TRIM21) is an E3 ubiquitin ligase that regulates inflammation, apoptosis, and oxidative stress by ubiquitination. Anti-Ro52/TRIM21 antibodies (anti-Ro52) may be found in the serum of people with Sjögren's syndrome, polymyositis/dermatomyositis, SSc, and SLE. The prevalence of RP was observed to be considerably higher in anti-Ro52-positive individuals [17].

It is quite probable that many of the biological alterations identified in RP are secondary symptoms of the fundamental abnormality that is present. Although sympathetic nervous system hyperactivity has been proposed as a possible cause of nerve abnormalities and dysfunction, evidence suggests that the vessel wall may play a more significant role. Recognizing the genes responsible for RP, a prevalent but poorly understood illness with a strong genetic component, may lead to a better understanding of the disease's genesis and, ultimately, the development of more effective therapies. Vasodilator calcitonin gene-related peptide (CGRP) is encoded by two different genes in humans [calcitonin related polypeptide alpha (CALCA) and calcitonin related polypeptide beta (CALCB)] and comes in two different forms [alpha calcitonin gene related peptide (α-CGRP) and beta calcitonin gene related peptide (β-CGRP)]. A deficiency in the release of the vasodilatory calcitonin gene-related peptide in digital cutaneous neurons is seen in patients with RP [18]. By investigating the nitric oxide synthase 1 (NOS1) gene, which produces nitric oxide synthase (nNOS), it was discovered that nitric oxide (NO) derived from NOS regulates the reparative vasodilator response after cold treatment [19].

The endogenous vasoconstrictor endothelin is crucial to the development of RP. After cold exposure, plasma EDN1 and vWF concentrations in RP patients were significantly higher than in healthy individuals. Adrenoceptor alpha 2A (ADRA2A), adrenoceptor alpha 2B (ADRA2B), and adrenoceptor alpha 2C (ADRA2C) are the genes that code for the three different forms of α2-adrenoreceptors [20].

The human α2C-AR gene (ADRA2C) has a frequent genetic variation that causes the deletion of the four amino acids del322–325. Due to the crucial involvement of α2C-AR in cold-induced vasoconstriction, the del322–325 variation may reduce the effectiveness of this response. The α2C-AR is a significant modulator of cold-induced vasoconstriction, making it a promising therapeutic target for subtype-specific antagonists [21]. Overexpression of alpha α2A-adrenoreceptors has a unique function in the genesis of RP, in contrast to α2C-adrenoreceptors, which have long been assumed to be the principal source of the cold-induced vasospastic events typical of RP. Even in temperature-neutral settings, it was shown that hypersensitivity to catecholamine-induced vasospasms is caused by a signaling pathway that is encoded in the gene ADRA2A called α2A-adrenoreceptor signaling [20].

In this study, it was aimed to elucidate the molecular pathogenesis of the disease by determining the genetic mechanisms in the inflammation and hematopoiesis process associated with RP disease. In the study, molecular pathways involving RP-related genes were determined and their biological processes and molecular functions were revealed by gene ontology (GO) analysis.

Materials and methods

Selection of RP-related genes via the MalaCards Human Diseases database

Inspired by the structure and depth of the famous GeneCards human gene database, MalaCards is an integrated database of human illnesses and annotations (https://www.malacards.org/). In this study, 20 genes associated with RP were identified through the MalaCards Human Diseases database. These genes were determined as [centromere protein B (CENPB), endothelin 1 (EDN1), adrenoceptor alpha 2C (ADRA2C), apolipoprotein H (APOH), phosphodiesterase 5A (PDE5A), von willebrand factor (VWF), angiotensin II receptor type 1 (AGTR1), thrombomodulin (THBD), ADAM metallopeptidase with thrombospondin type 1 motif 13 (ADAMTS13), selectin E (SELE), regulator of chromosome condensation 1 (RCC1), phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha (PIK3C2A), platelet factor 4 (PF4), plasminogen activator, tissue type (PLAT), threonyl-TRNA synthetase 1 (TARS1), RNA binding region containing 3 (RNPC3), tripartite motif containing 21 (TRIM21), U2 small nuclear RNA auxiliary factor 1 (U2AF1), small nuclear ribonucleoprotein U1 subunit 70 (SNRNP70), C-reactive protein (CRP)].

Identifying RP-related pathways through the GeneCards Suite database

All annotated and predicted human genes are available in GeneCards, a searchable, integrated database (https://www.genecards.org/). Human biological pathways are described in detail in PathCards, a comprehensive database (https://pathcards.genecards.org/). In this study, RP-related pathways were identified by clustering them into SuperPaths based on gene content similarity in the GeneCards Suite database.

Gene ontology (GO) analysis of genes via the STRING database

Physical and functional protein-protein interactions are collected and integrated in the STRING database (https://string-db.org/). Gene-gene and network interactions were constructed using String v12.0 analysis. It was determined that combined scores greater than 0.4 were significant. It was determined that the connection between genes was substantial as a result of node-node interactions since the aggregate score was greater than 0.4 in all 18 genes.

Results

Using the GeneCards Suite database in conjunction with the MalaCards Human Diseases database, we found that RP is linked to the following biological processes: immune cytokine signaling; the PI3K-Akt signaling pathway; blood-brain barrier and immune cell transmigration (VCAM-1/CD106 signaling); development VEGF signaling via VEGFR2-generic cascades; the CCL18 signaling pathway; photodynamic therapy-induced NF-kB survival; interleukin-4 and interleukin-13 signaling; development leptin signaling via JAK/STAT and MAPK cascades; HIF1Alpha pathway; pluripotent stem cell differentiation pathway; Thromboxan A2 receptor signaling.

The GeneCards Suite database, which is more comprehensive than the MalaCards Human illnesses database, revealed that four pathways (disinhibition of SNARE formation, hemostasis, platelet activation, signaling and aggregation, platelet degranulation) respond to increased platelet cytosolic Ca^{2+} (Figure 1), while another 19 pathways (blood clotting cascade, blood coagulation signaling pathways, common pathway of fibrin clot formation, complement and coagulation cascades, defective F8 binding to von Willebrand factor, defective F8 cleavage by thrombin, defective F8 sulfation at Y1699, defective F9 activation, defective F9 secretion, defective factor IX causes hemophilia B, defective factor VIII causes hemophilia A, defective factor XII causes hereditary angioedema, defective gamma-carboxylation of F9, defects of contact activation system (CAS) and kallikrein/kinin system (KKS), extrinsic pathway of fibrin clot formation, extrinsic prothrombin activation pathway, formation of fibrin clot (clotting cascade), intrinsic pathway of fibrin clot formation, thrombin/protease-activated receptor (PAR) pathway) are involved in illnesses of hemostasis (Figure 2).

Twenty genes obtained from the MalaCards Human Diseases database were included in the study for gene ontology analysis via the STRING database. Accordingly, possible interactions between 18 (CENPB, EDN1, ADRA2C, APOH, PDE5A, VWF, AGTR1, THBD, ADAMTS13, SELE, PIK3C2A, PF4, PLAT, RNPC3, TRIM21, U2AF1, SNRNP70, CRP) genes were determined through STRING and network enrichment was performed. The coefficient of local clustering, on average, is 0.866. The p-value for the enrichment of PPIs is less than 1.0e-16 (Figure 3).

A collection of genes, including those implicated in cellular response to luteinizing hormone stimulation (GO:0071373), negative regulation of fibrinolysis (GO:0051918), negative regulation of smooth muscle cell apoptotic process (GO:0034392), plasminogen activation (GO:0031639), and cellular response to follicle-stimulating hormone stimulus (GO:0071372), were significantly enriched by gene ontology enrichment analysis. Molecular mechanisms associated with RP, including the AGE-RAGE signaling pathway in diabetic complications (hsa04933), complement and coagulation cascades (hsa04610), fluid shear stress, and atherosclerosis (hsa05418), were determined via KEGG pathways.

Data obtained from the Reactome pathway, we found that RP, formation of fibrin clot (clotting cascade) (HSA-140877) (false discovery rate: 0.0114) and hemostasis (HSA-109582) (false discovery rate: 0.00018) can play an important role on RP. Using the genomic association method, the Cytoscape plugin selected the five highest-scoring genes (VWF, ADAMTS13, PLAT, PF4, THBD) from the network (Table 1). According to our results, investigation of VWF, ADAMTS13, PLAT, PF4, and THBD genes at the protein level may play a role in elucidating the pathophysiology of RP at the molecular level.

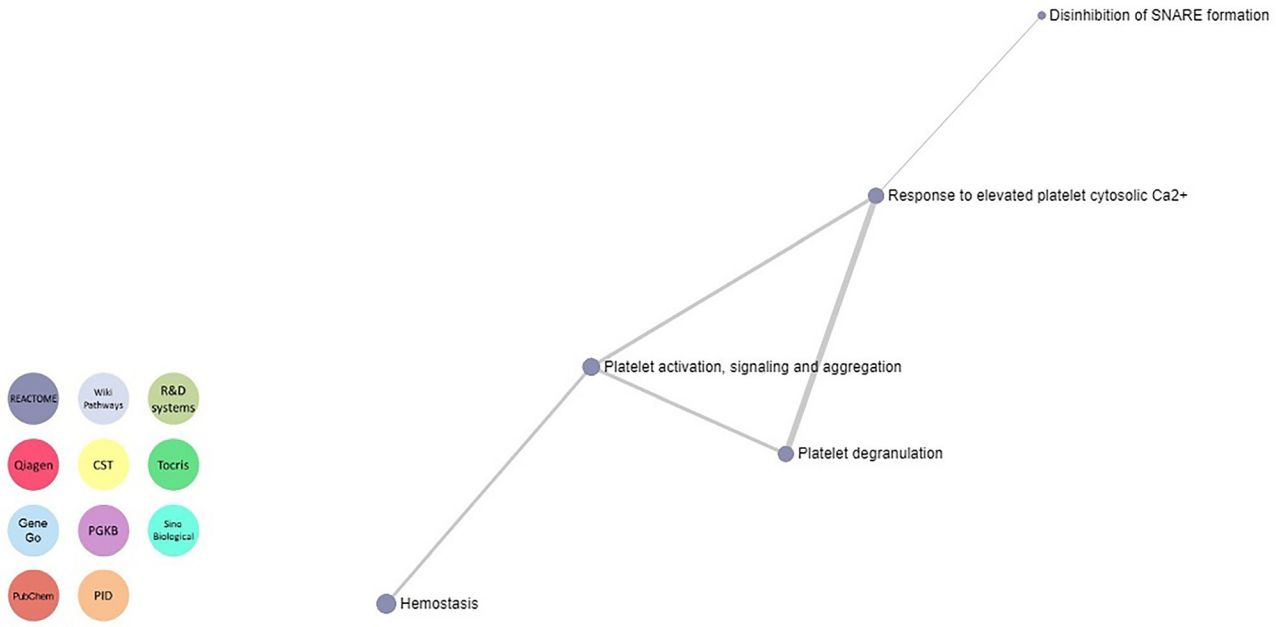

Figure 1: Pathway network for response to elevated platelet cytosolic Ca^{2+} (https://pathcards.genecards.org/).

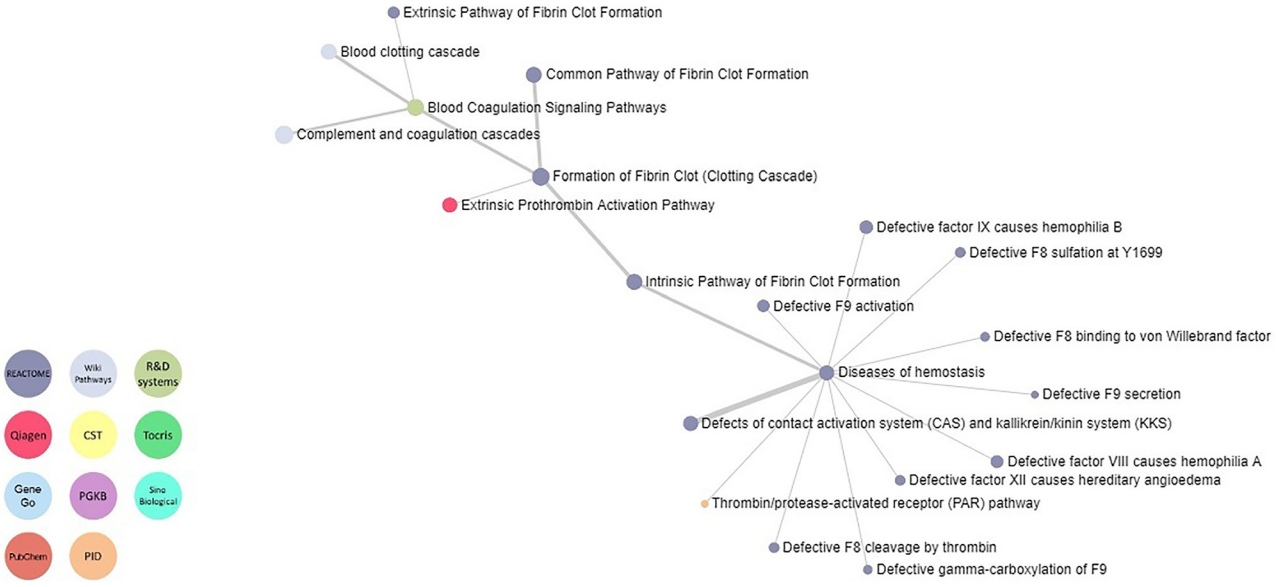

Figure 2: Pathway network for diseases of hemostasis (https://pathcards.genecards.org/).

Discussion

Primary and secondary RP are the two categories of RP. Patients with primary RP often report moderate-to-severe symptoms but no long-term complications or ischemic damage. A family history of familiar recurrence in first-degree relatives is present in around 25 % of PRPs, according to estimates. The median onset of primary RP occurs at age 14, with only 27 % of instances appearing in those older than 40. Systemic autoimmune disorders, such as Ssc or mixed connective tissue disease, are the most common causes of secondary RP. Ischemia may also cause RP to present as a solitary, acute, or subacute illness, with symptoms including fever, discomfort, and tingling or numbness in the fingers [22].

Patients with SSc have been shown to develop agonistic autoantibodies (Aabs) directed against the angiotensin II

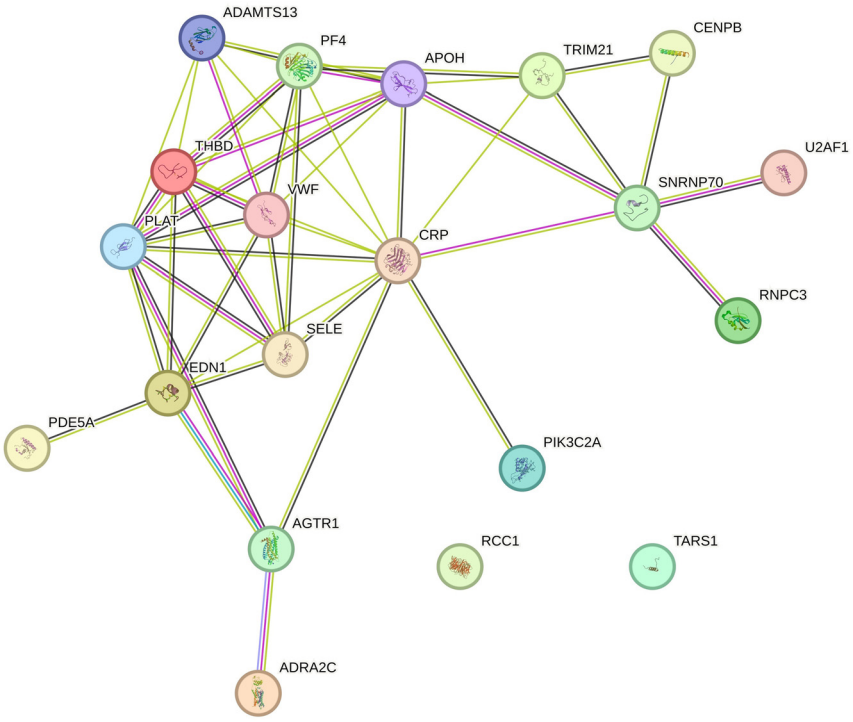

Figure 3: Detection of interactions between 18 different genes expressed in patients with Raynaud's phenomenon (String v12.0) (https://string-db.org/).

Table 1: Five highly interconnected genes were predicted by cytoscape based on score values.

Node 1	Node 2	Node 1 accession	Node 2 accession	Score
VWF	ADAMTS13	ENSP00000261405	ENSP00000360997	0.997
VWF	PLAT	ENSP00000261405	ENSP00000220809	0.934
VWF	PF4	ENSP00000261405	ENSP00000296029	0.932
THBD	PLAT	ENSP00000366307	ENSP00000220809	0.913

receptor type 1 (AGTR1) and the endothelin A receptor (ETAR). Recently discovered SSc sera include agonistic Aabs against AGTR1 and ETAR, which may contribute to the disease's pathophysiology. Aab-induced immune cell activation via the angiotensin II type 1 receptor (AT1R) and ETAR may play a role in the pathogenesis or onset of SSc due to decreased receptor expression in patients, inflammatory and profibrotic effects *in vitro*, and clinical associations [23].

E-selectin upregulation in the skin of SSc patients has been used as evidence for endothelial cell activation. E-selectin is involved in adhesion with lymphocytes, monocytes, and neutrophils, and its expression is exclusive to activated endothelium cells. Endothelial cells migrate to perivascular regions, and E-selectin plays a role in this process. Even in the earliest stages of SSc, when the only noticeable symptoms are RP and aberrant capillaroscopic findings, e-selectin expression may be seen in the salivary glands [24]. Brevetti et al. discovered that individuals with secondary RP had considerably greater plasma levels of soluble forms of ICAM-1, VCAM-1, E-selectin, and VWF compared to those with main RP and controls. Among those diagnosed with secondary RP, soluble forms of ICAM-1, E-selectin, and VWF were shown to have the greatest associations [25]. It is partly caused by inflammatory alterations linked to elevated fibrinogen and CRP in RP patients. Fibrin clots that are formed in the presence of CRP have been shown to be thicker and more resistant to lysis [26]. In line with our results, VWF, ADAMTS13, PLAT, PF4, and THBD genes have high interaction scores in the cytoscape molecular interaction network.

When compared with the literature, in parallel with our study, it was observed that Raynaud's patients exhibited high levels of von Willebrand factor activity and fVIII/vWf antigen. It has been demonstrated that in individuals with Raynaud's phenomenon, elevated VWF levels may be indicative of vascular damage [27]. There may be greater endothelial damage in males with Raynaud's owing to subclinical atherosclerosis or higher thrombotic potential, as shown by the positive correlation between VWF and RP in this population [28]. Therefore, it can be argued that it can be used as a biomarker for endothelial damage with further research on VWF gene expression levels in men with RP.

The cleavage of von Willebrand factor requires the zinc-containing metalloprotease ADAMTS13, which is generated by endothelial cells and megakaryocytes. ADAMTS13 is also known as a disintegrin and metalloproteinase with

thrombospondin type 1 motives [29]. In our study, VWF and ADAMTS13 genes were determined to be the most interconnected genes (score: 0.997). Therefore, it can be concluded that VWF and ADAMTS13 genes have a significant effect on the pathogenesis of RP since the deficiency of ADAMTS13, the von Willebrand factor degrading protease, causes endothelial damage, followed by vascular damage and fibrosis. Fibrinolysis is physiologically activated by tissue-type plasminogen activator, a serine proteinase secreted by endothelial cells [30]. Patients with RP who subsequently develop SSc or other connective tissue illnesses have been shown to have elevated levels of PLAT, one of the indicators of endothelium damage [31]. These findings suggest that the PLAT gene can be used as a biomarker in the diagnosis of endothelial damage of RP in relation to our study.

Activated platelets deposit a large amount of a protein called PF4 onto the endothelium. Patients suffering from chronic inflammation have been shown to have elevated amounts of circulating PF4 [32]. RP is frequently seen in systemic sclerosis, which is a chronic autoimmune connective tissue disease. PF4, an antiangiogenic factor, is dysregulated in SSc [33]. Patients with extensive scleroderma-associated RP had a higher PF4 level than patients with primary RP and controls [34]. We can conclude that endothelial cell damage caused by platelet aggregation in RP may be related to PF4 levels.

Thrombomodulin is a glycoprotein that is found on the surface of the vascular endothelium. It acts as a receptor for protein C, protein S, and α-thrombin. Cleavage fragments of thrombomodulin are released into the blood after vascular endothelial injury. During the course of the research, VWF and soluble thrombomodulin levels were analyzed. Increased circulating VWF concentrations may be a reflection of endothelial injury, and it has been shown in the past that VWF levels are elevated in patients with primary RP and SSc. Although soluble thrombomodulin is also a measure of endothelial injury, it was not shown to be elevated in the serum of SSc patients [35]. The elevated soluble thrombomodulin concentrations in individuals with vasculitis imply that THBD may be viewed as a particular biomarker of vascular endothelial damage in RP.

In this study, the association of endothelial and platelet activation and damage with expression levels was investigated. In our study, the genes whose expression levels were determined can be used as biomarkers showing the progression of the disease by investigating the relationship between the development process of Raynaud's phenomenon and endothelial damage and platelet aggregation. As a result, these genes identified as biomarkers will provide an understanding of the cellular and molecular mechanisms of the pathological changes in RP in the diagnosis and differentiation of primary and secondary RP.

We believe that the identification of the molecular pathways that contribute to the development of RP may be helpful in elucidating the clinical significance of altered fibrin clot properties in the context of thrombotic risk and identifying the functions of the genes that lead to the prothrombotic clot phenotype associated with advanced endothelial damage and inflammatory conditions. It is feasible that the activities of the genes that are engaged in these molecular pathways may be uncovered, that the development of RP illness can be prevented, and that more therapeutic options can be developed.

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