Gene variants in pro-coagulant and anti-coagulant genes could be prognostic genetic markers of COVID-19 susceptibility.

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Abbreviations:

ATIII: Anti-thrombin III

PROS: Protein S

PROC: Protein C

SELP: Selectin P

SELE: Selectin E

THBD: Thrombomodulin

TFPI: Tissue factor pathway inhibitor

tPA: Tissue plasminogen activator

uPA: Urokinase plasminogen activator

VTE: Venous thrombo-embolism

Abstract:

Background: Present study aimed to identify DNA polymorphisms (variants) which can modulate the risk of COVID-19 infection progression to severe condition. TaqMan based SNP genotyping assay was performed for 11 single nucleotide polymorhisms (SNPs) in pro-coagulant and anti-coagulant genes.

Methodology: A total of 33 COVID-19 patients, including dead, severe and moderately infected individuals were compared to 35 healthy controls. Both alleles in the SNP were labeled with two different fluorescent dyes (FAM and VIC) during assay formulation. DNA of study subjects were mixed with SNP assay and TaqMan master mix on 96 well PCR plate according to manufacturer's protocol and RT-PCR was performed. Allelic discrimination assay gave clear results for presence of specific allele in each sample. Three SNPs were located in the pro-coagulant genes, another three involved in blood clot dissolution while rest five were in the genes encoding natural anti-coagulants. COVID-19 infected patients were further sub-divided into three groups, deceased (n=16), severe (n=10) and moderately infected (n=7).

Results: SNP genotyping showed significant difference between COVID-19 patients and controls in two SNPs, rs6133 in Selectin-P (SELP) and rs5361 in Selectin-E (SELE) gene. Also, rs2020921 and rs8176592, in clot dissolution genes, tissue Plasminogen activator (tPA) and tissue factor pathway inhibitor (TFPI) respectively showed significant genotypic and allelic difference in patients of COVID-19 compared to healthy controls. Further three SNPs rs2227589, rs757583846 and rs121918476 in natural anti-coagulant genes anti-thrombin III (ATIII), protein C (PROC) and protein S (PROS) respectively showed statistically significant difference between the study groups.

Conclusion: Our findings indicate that gene variants, those involved in coagulation and anti-coagulation may play a major role in determining individual susceptibility to COVID-19.

Introduction:

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly infectious disease, first reported in December 2019 in Wuhan, Hubei Province, China (Zhu et al. 2020). Since then COVID-19 has spread rapidly across the world becoming a global pandemic. The outbreak of COVID-19 infection has completed two years, and even now effective measures for prevention and treatment of SARS-CoV-2 remains limited. The morbidity caused by COVID-19 infection varies significantly and is known to be associated with age, sex and specific pre-existing conditions (Zhou et al. 2020, Jin J-M et al. 2020).

Infection severity and mortality is significantly higher in people with advanced age and comorbid conditions like cancer, diabetes, hypertension and other cardiovascular diseases (Garg et al. 2020). On the other hand, majority of COVID-19 infected individuals, especially younger population, recover within few days without any complications (Dong et al. 2020). Such variations in disease severity can be explained by genetic variability resulting in varying immune response amongst the effected individuals.

SARS-CoV-2 virus enters into the host cell via Angiotensin converting enzyme 2 (ACE2) and the transmembrane serine protease (TMPRSS2) of host for SARS-CoV-2 spike (S) protein priming (Hoffman et al. 2020). So far, many independent research groups have demonstrated polymorphisms in ACE2 (p.Arg514Gly) and TMPRSS2 (p.Val160Met) genes to be associated with differential genetic susceptibility to COVID-19 (Hou et al. 2020). Other ACE2 variants (rs233575 and rs2074192) have also been associated with hypertension and more severe outcomes of COVID-19 infection (Hamet et al. 2021). A recently done genome wide association (GWAS) study identified eight variants as susceptibility loci for COVID-19 mortality. These included genes related to cilia dysfunctions (DNAH7 and CLUAP1), cardiovascular diseases (DES and SPEG), thrombo-embolic disease (STXBP5), mitochondrial dysfunction (TOMM7), and innate immune system (WSB1) (Hu et al. 2021).

Hyper-immune response during COVID-19 infection leads to endothelial injury and activation of coagulation cascade (Ahmed et al. 2020). This leads to increased risk of thromb-embolic manifestations. Besides respiratory failure being the most common cause of death during COVID-19 infection, the progression of the disease is marked by disseminated intravascular coagulation (DIC) (Tang et al. 2020, Iba et al. 2020). As the infection progresses, it leads to coagulation abnormalities and significant elevation in coagulatory parameters; such as increase in prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, D-dimer and fibrin degradation products (FDP), along with consistent decline in anticoagulant levels during infection (Tang et al. 2020, Yu et al. 2020, Guan et al. 2020). Clinical relevance of coagulation system in COVID-19 is evident from higher incidences of venous thrombo-embolic events, stroke and acute coronary syndrome in infected patients even after receiving prophylactic heparin (Tang et al. 2020, Logiani et al. 2020). Most of the guidelines for COVID-19 treatment published till date recommend therapeutic use of anticoagulants such as low-molecular-weight heparin (LMWH) or un-fractionated heparin in patients who are suspected to develop macro-thrombi or pulmonary embolism (Cuker et al., ASH 2021a). However, intially these guidelines did not address the requirement of outpatient anti-coagulant prophylaxis (Moores et al. 2020, Chandra et al. 2021),

recent guidelines of American Society of Hematology (ASH) gave update on post-discharge thrombo-prophylaxis (Cuker et al. ASH 2021b).

In view of above findings, the presence of natural anti-coagulants in blood might be playing a significant role against progression of COVID-19 infection. Present study hypothesizes that inherited deficiency of natural anti-coagulants and clot dissoultion genes due to mutations, may be associated with increased risk of severe COVID-19 infection. Thus, we evaluated the possible association of eleven single nucleotide polymorphisms (SNPs) in nine genes including pro-coagulants and those reportedly involved in blood clot dissolution and synthesis of natural anti-coagulants in blood with COVID-19 susceptibility and severity.

Methodology:

Study Design

The study subjects were broadly classified into three study groups. Firstly, blood was collected from COVID-19 patients (diagnosis confirmed by RT-PCR) from Rajiv Gandhi Super Speciality hospital (RGSSH). Thirty three COVID-19 patients were further divided into three groups based on their infection severity, according to the guidelines laid by Ministry of health and family welfare, Government of India (https://www.mohfw.gov.in/pdf). These included 16 deceased, 10 severe and 7 moderately infected patients. Eleven common mutations in pro-coagulants, anti-coagulants and clot dissolution genes in these COVID-19 patients were compared with comparative number of healthy controls (n=35). Amongst control group, subjects with any history of thrombotic risk factors were not included in the study. All control subjects were young healthy males with average age of 34. 5 years (IQR=30.75–41.75).

The study parameters and design was approved by the Ethical Committee of Indian Council of Medical Research (ICMR), India. Written informed consent was obtained from all study participants before collection of their blood sample. All experimental protocols were conducted in accordance to the Strengthening of the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Basic clinical characteristic was noted for each study participant, including, age, sex and presence of co-morbidity. The study comprises of 12 males and 11 female patients. The age of patients ranges from 40 yrs to more than 60 yrs. Out of 33, twenty five patients were having co-morbid conditions.

DNA isolation and quantitation

For genotyping analysis, peripheral blood samples were collected in EDTA vacutainers. High molecular weight DNA was extracted using QIAampDNA isolation kit (Qiagen, Germany), using manufacturer's protocol. Quantitative analysis of genomic DNA was done using Multiscan

spectrophotometer (Thermo Fischer Scientific, USA). For qualitative analysis, 100ng DNA samples were loaded on 0.7% agarose gel containing Ethidium Bromide and run for 20 min and visualized under UV.

TaqMan based Genotyping

A total of eleven SNPs in nine genes were selected for genotyping in the present study (Table 1). Profiling was done using RT-PCR based TaqMan SNP genotyping assay (Thermo Fischer Scientific, USA). Detailed steps for preparation of 96 well reaction plates and RT-PCR cycle is elucidated in Figure 1.

Table 1: *Details of polymorphisms studied in clot dissolution and anti-coagulant genes*

S. No.	Gene	SNP	Polymorphism	SNP Type	SNP location and observed codons	Observed Amino acid	Function
1.	Selectin-P (SELP)	rs6136	G/T, Transversion Substitution	Intragenic	2328 ACT,CCT	T,P 756	Mediates platelet activation and de-granulation
2.	Selectin-P (SELP)	rs6133	A/C, Transversion Substitution	Intragenic	1980		
3.	Selectin-E (SELE)	rs5361	G/T, Transversion Substitution	Missense Mutation	602 AGT,CGT	S,R 149	Cell adhesion, leukocyte accumulation at the site of inflammation
4.	Tissue Plasminogen activator (tPA)	rs2020921	A/G, Transition Substitution	Silent Mutation	609	-	Catalyzes the conversion of the zymogen plasminogen to the active enzyme plasmin
5.	Urokinase Plasminogen activator (uPA)	rs2227564	C/T, Transition Substitution	Missense Mutation	566 CCG,CTG	P,L 124	Activates plasminogen and facilitates fibrinolysis
6.	Tissue factor pathway inhibitor (TFPI)	rs8176592	A/G, Transition Substitution	Intron	-	-	Inhibits coagulation factor Xa and VIIa
7.	Anti-thrombin III	rs2227589	C/T, Transition	Intron	-	-	Inhibits

	(ATIII)		Substitution				coagulation by neutralizing the enzymatic activity of thrombin
8	Anti-thrombin III (ATIII)	rs121909569	G/A, Transition Substitution	Missense Mutation	561 CCT,TCT	P,S 148	
9	Protein C (PROC)	rs757583846	C/T, Transition Substitution	Missense Mutation	186 CGG,TGG	R,W 57	Blocks activity of factor Va and factor VIIIa, thus inhibits coagulation
10.	Protein S (PROS)	rs121918476	G/A, Transition Substitution	Missense Mutation	2109	-	Protein S acts as a cofactor to activated protein C (APC) in the degradation of FVa and FVIIIa
11.	Thrombomodulin (THBD)	rs 16984852	C/A, Transversion Substitution	UTR 5	10	-	Acts as co-factor, Enhances thrombin-induced activation of protein C

Statistical analysis

Statistical computations were performed using GraphPad Prism 5. Data sets were compared using the $\chi 2$ test and Fisher's exact test. $p< 0.05$ was considered statistically significant. Odd's ratio (OR) with 95% confidence interval (CI) was calculated for all SNPs to compare COVID-19 vs control groups. Forests plots depicting OR (with 95% CI) and heterogeneity were generated using online bio-informatic tool Genyo (Marugan et al. 2017).

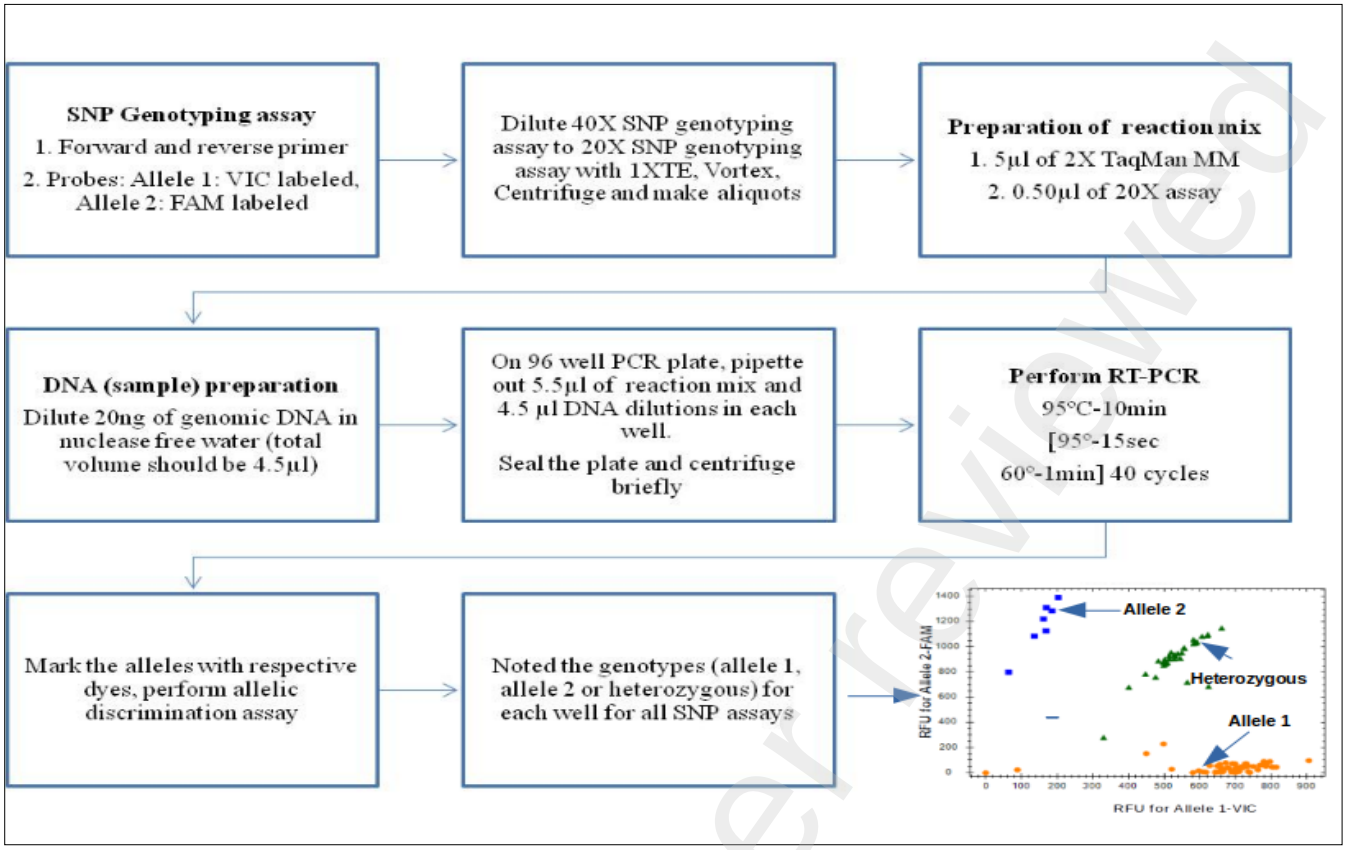

Figure 1: *Workflow of TaqMan SNP genotyping assay*

Results:

Basic characteristics of COVID-19 patients

Twenty two out of thirty three COVID-19 infected patients were males. Twenty five amongst them were suffering from one or more co-morbidities, such as type 2 diabetes mellitus, hypertension, cardiovascular disorder, bronchial asthma, cystic bronchiectasis, nephrectomy etc. These patients were of varying age group, however, 20 out of 33 were above the age of 50 years. Also, 18 out of 25 comorbid patients were above 50 years of age.

SNP genotyping

Genotyping was performed for common polymorphisms in seven genes using TaqMan technology. Three SNPs were studied in the glycoproteins involved in adhesion of platelets and leukocytes during the process of coagulation and inflammation - selectin-P (SELP) and selectin-E (SELE). Five SNPs were studied in four genes, Protein C (PROC), Protein S (PROS), Anti-thrombin III (ATIII) and Thrombomodulin (THBD), involved in synthesis and activation of natural anti-coagulants in the body; while other three genes, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and tissue factor pathway inhibitor (TFPI) are involved in the process of blood clot dissolution (Table 1).

To examine the potential impact of SNPs in these genes and their association with susceptibility and severity of COVID-19 infection, the genotypic and allelic frequency distribution of 11 SNPs were compared across the two study groups, COVID-19 patients and healthy controls. The distribution of three genotypes, allele 1, allele 2 and heterozygous as well as two allele types were compared using statistical tests (Chi Square and Fisher's exact test respectively), using Graph Pad (Prism 5.0) and the significance value was noted (as listed in Table 2).

Table 2: *Statistical analysis depicting genotypic and alleleic distribution significance amongst the two study groups across 11 SNPs.*

Study name	SNP	Chi square	Genotypic association p-value	Allelic association p-value	Statistically significant association
COVID-19 vs Control					
SELP (rs6136)	G/T	1.46	0.43	0.28	Non significant
SELP (rs6133)	A/C	3.25	0.19	**0.0002**	Significant
SELE (rs5361)	G/T	6.18	**0.04**	**0.004**	Significant
tPA (rs2020921)	A/G	7.59	**0.0058**	**0.0001**	Significant
uPA (rs2227564)	C/T	4.86	0.0878	0.099	Non significant
TFPI (rs 8176592)	A/G	29.97	**< 0.0001**	**0.01**	Significant
ATIII (rs121909569)	G/A	15.34	**0.0005**	0.688	Significant
ATIII (rs 2227589)	C/T	0.029	0.8645	1	Non significant
PROC (rs757583846)	C/T	25.9	**< 0.0001**	**0.009**	Significant
PROS (rs 121918476)	G/A	9.008	**0.0027**	**0.0001**	Significant
THBD (rs 16984852)	C/A	2.38	0.304	0.09	Non significant

COVID-19 vs healthy controls

Significant difference was observed amongst patients of COVID-19 and healthy controls in seven out of eleven SNPs under study including, SELP (rs6133) A/C, SELE (rs5361) G/T, tPA (rs2020921) A/G, TFPI (rs8176592)A/G, ATIII (rs121909569) G/A, PROC (rs757583846) C/T and PROS (rs121918476) G/A. Statistical significance was both at genotypic and allelic level except for ATIII (rs121909569) which showed significant difference only at genotypic level, and SELP (rs6133) and SELE (rs5361) which showed significant allelic difference only, when the two groups were compared (Table 2).

OR was found to be significant for SELP (rs6136 and rs6133), SELE (rs5361), tPA (rs2020921), ATIII (rs121909569) and PROC (rs757583846) (Figure 2). This indicates that these SNPs are significantly associated with COVID-19 (Figure 2).

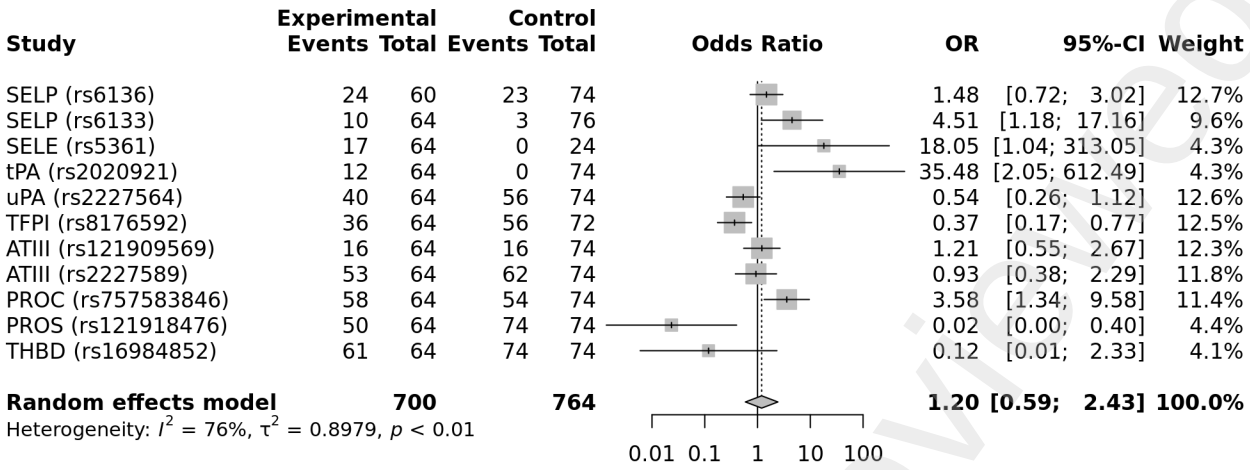

Figure 2: *Forest plot: Allelic distribution of 11 SNPs under study: comparison of COVID-19 patients and healthy controls*

Sub-group analysis was also done for the 11 SNPs under study, including 3 SNPs in pro-coagulant genes, 3 in clot-dissolution genes and 5 in anti-coagulant genes. Overall OR was significant for both pro-coagulant and anti-coagulant SNPs (Table 3).

Table 3: Sub-group analysis: Allele contrast model

Sub-group	Number of studies	Test of association			Test of heterogeneity		Publication bias
		OR	**95% CI**	**p-val**	**p-val**	**I^2**	**p-val (Egger's test)**
Overall	11	1.02	[0.7531; 1.3943]	0.87	0	0.756	0.57
Pro-coagulant	3	2.10	[1.1364; 3.8857]	0.01	0.1127	0.542	0.14
Clot-Disolution	3	0.51	[0.3068; 0.8569]	0.01	0.0096	0.784	0.15
Anti-Coagulant	5	1.22	[0.7464; 2.0022]	0.42	0.0053	0.728	0.15

While the COVID-19 patients and control group showed significant change in genotypic and allelic distribution of seven SNPs, there was no specific pattern of allelic frequency distribution amongst the deceased, severe and moderately infected COVID-19 patients. This could be attributed to low and uneven sample size amongst the patients' sub-groups in the present study. However, specific risk alleles could be recognized when the total COVID-19 patients were compared with the healthy controls (Table 4).

Table 4: Allele frequencies of 11 SNPs compared between controls and COVID-19 patients (deceased, severe and moderately infected) for identification of risk allele associated with the COVID-19 disease severity

Study name	SNP	Controls	Sub-groups of COVID-19 patients			COVID-19 Patients	Risk allele
			Deceased	Severe	Moderate		
SELP (rs6136)	G (Allele 1)	0.31	0.46	0.33	0.4	0.4	No risk allele
	T (Allele 2)	0.68	0.53	0.66	0.6	0.6	
SELP (rs6133)	A (Allele 1)	0.03	0.1	0.15	0.16	0.15	A
	C (Allele 2)	0.96	0.9	0.85	0.83	0.84	
SELE (rs5361)	G (Allele 1)	0	0.5	0	0.58	0.26	G
	T (Allele 2)	1	0.5	1	0.41	0.73	
tPA (rs2020921)	A (Allele 1)	0	0.13	0.1	0.42	0.18	A
	G (Allele 2)	1	0.86	0.9	0.57	0.81	
uPA (rs2227564)	C (Allele 1)	0.76	0.6	0.65	0.64	0.62	No risk allele
	T (Allele 2)	0.24	0.4	0.35	0.35	0.37	
TFPI (rs 8176592)	A (Allele 1)	0.78	0.6	0.6	0.42	0.56	G
	G (Allele 2)	0.22	0.4	0.4	0.57	0.43	
ATIII (rs121909569)	G (Allele 1)	0.22	0.33	0.1	0.28	0.25	No risk allele
	A(Allele 2)	0.78	0.66	0.9	0.71	0.75	
ATIII (rs 2227589)	C (Allele 1)	0.84	0.9	0.7	0.85	0.82	No risk allele
	T (Allele 2)	0.16	0.1	0.3	0.14	0.17	
PROC (rs757583846)	C (Allele 1)	0.73	0.86	1	0.85	0.90	C
	T (Allele 2)	0.27	0.13	0	0.14	0.09	
PROS (rs 121918476)	G (Allele 1)	1	0.73	1	0.57	0.78	A
	A (Allele 2)	0	0.26	0	0.42	0.21	
THBD (rs 16984852)	C (Allele 1)	1	0.93	0.95	1	0.95	No risk allele
	A (Allele 2)	0	0.06	0.05	0	0.04	

Discussion:

COVID-19 infection is frequently associated with thrombo-embolic events especially venous thrombosis. All COVID-19 patients admitted to hospital are recommended to receive pharmacologic thrombo-prophylaxis unless they have risk of bleeding. Chandra and co-workers recently reviewed and listed all on-going randomized control trials (RCTs) in patients with COVID-19 regarding timing, dosage, choice and duration of anti-coagulation along with current guidelines and recommendations on use of anti-coagulation in COVID-19 (Chandra et al. 2021). Most of these

RCTs are in phase 3 and phase 4 of clinical trials and results are very promising. VTE prophylaxis and treatment with anti-coagulants reduces mortality rate in patients hospitalized with COVID-19 (Vaughn et al. 2021).

Several Genome wide association analysis have been conducted by independent research groups to identify potential genetic risk factors which may lead to susceptibility to COVID-19. In one such study involving 1980 patients of COVID-19 from Italy and Spain, identified a 3p21.31 gene cluster (spanning genes *SLC6A20, LZTFL1, CCR9, FYCO1, CXCR6* and *XCR1*) as genetic susceptibility locus along with potential involvement of ABO blood group system (The Severe COVID-19 GWAS Group, 2020). Another genome wide association study (GWAS) from China, conducted on 885 severely infected COVID-19 patients and 546 mild or moderately infected patients highlighted two loci at 11q23.3 and 11q14.2, which confer susceptibility to COVID-19 severity (Li et al. 2021). Besides these, genetic association studies have been conducted so far to link SNPs with COVID-19 severity. GG genotype and G allele of ACE rs2285666 SNP has been significantly associated with 2 fold increase in SARS-CoV-2 infection risk (Mohlendick et al. 2021). A recent study on 750 SARS-CoV-2 patients also evaluated impact of polymorphsims in interferon lambda 3 and 4 (*IFNL3/4*) on resistance and susceptibility to COVID-19 and found that frequency of favourable genotypes was higher in survivors group compared to non-survivors. They also associated higher levels of low density lipoprotein (LDL), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP); and lower levels of hydroxyvitamin D and un-favourable genotypes (*IFNL3:* rs12979860 TT, rs8099917 GG, rs12980275 GG, and *IFNL4* rs368234815) with severity of COVID-19 infection (Rahimi et al. 2021).

Although the association of COVID-19 infection progression and coagulopathy is well established along with the significance of anti-coagulant therapy (Hanny et al. 2020, Leentjens et al. 2021), no study has reported signigicance of SNPs in pro-coagulant and anti-coagulant genes with COVID-19 susceptibility and severity. Thus, the present study focuses on the variants in pro-coagulant genes, clot dissolution and anti-coagulant genes for their association with COVID-19 susceptibility. Dimnished activity of anti-coagulants such as Protein S has been found in 65% of COVID-19 patients and was also associated with survival and disease severity (Stoichutoiu et al. 2020). Thus we hypothesize that the common mutations in natural anti-coagulants may play a role in thrombotic manifestations during COVID-19 infection and thus might be related to its pathogenesis and severity.

SNPs in genes associated with coagulation such as Fibrinogen (FG) Gamma, FG-Alpha, and F5 which mediate increases in D-dimer concentration and SNPs in ABO, CBS, CPS1 and MTHFR which mediate changes in homocysteine levels, could confer to greater risk of thrombotic

complications in COVID-19 patients (Abu-Farga et al. 2020). However, common mutations present in the coagulation factors such as pro-thrombin (F2, rs1799963), factor V leiden (F2, rs6025); and thrombosis pre-disposing gene, plasminogen activator inhibitor (PAI-1, s1799768) did not show any relation with severe novel coronavirus pneumonia (NCP) (Kiraz et al.2021). Interestingly, present study revealed seven common genetic variants located in pro-coagulant genes (SELP and SELE), clot dissolution gene (tPA) and natural anti-coagulant genes (ATIII, PROC, PROS) to be significantly associated with COVID-19 disease when the patients were compared with healthy controls. Combined effect of these mutations could produce a hyper-coagulable state during COVID-19 infection and might lead to severe infection. The results of the present study need to be validated in a larger cohort.

Conclusions:

Development of preventive and thrapeutic strategies to combat COVID-19 progression can only be developed by understanding the molecular and genetic basis of its prognosis. At present there are no definite guidelines for anti-coagulation therapy, its dosage and duration for the patients diagnosed with novel coronavirus. Although number of clinical trials and supportive studies are available which suggest thrombo-prophylaxis during COVID-19, its post discharge need, duration and dose remains a matter of debate! To the best of our knowledge, this is the first study which highlights the significance of SNPs in pro-coagulant genes and anti-coagulant genes associated with susceptibility and severity of COVID-19 infection.

Limitation of study

Availability of samples was the limitation of the present study. Our preliminary results need to be validated in a larger cohort.

Conflict of interest statement

The authors declare that there is no conflict of interest or financial disclosure related to this publication.

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