Genetic risk factors for venous thromboembolism among infertile men with Klinefelter syndrome

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ABSTRACT

Background: Klinefelter syndrome (KS) is one of the commonest sex chromosome disorders. Affected males become infertile and highly susceptible to several health problems, including vascular thromboembolism (VTE). The risk of VTE may be exacerbated by an underlying genetically inherited thrombophilia. In this study, we aimed to investigate the genotype and allele frequencies of common gene polymorphisms related to hereditary thrombophilia in infertile males with KS compared to normal, fertile men.

Methods: Eighty-five infertile males with KS and 75 healthy control males were included in this case-control study. Genetic testing was done using an extended thrombophilia gene panel by Multiplex PCR reverse hybridization method.

Results: There was an increased frequency of mutant alleles and heterozygous genotypes of FV Leiden, FV H1299R, Pro G20210A, MTHFR C677T and PAI-1 4G/5G thrombophilic gene polymorphisms in KS patients compared to the control group. It was shown that 10.7% of KS patients had the A3 haplotype of the EPCR gene in comparison to 5.3% of control patients. The A3/A3 genotype was found only in KS patients (7.1%). Carriers of more than one mutant allele in KS patients exceeded the control (p < 0.001).

Conclusion: A high prevalence of thrombophilic gene polymorphisms and the coexistence of different mutant alleles were evident in infertile KS males. These data highlight the importance of conducting further studies to understand the role of hereditary thrombophilia in predicting venous thrombosis in patients with Klinefelter syndrome.

Introduction

In 1942, Dr. H. Klinefelter et al. first described a set of symptoms that has come to be known as Klinefelter syndrome (KS). With an incidence of about 1 in 500–1000 newborns, it is the most frequently occurring sex chromosome disorder in males [1,2]. Klinefelter syndrome is the most common cause of male infertility and hypogonadism and represents almost 11% of all cases with azoospermia and 0.7% of all cases with oligozoospermia [3].

KS is the commonest sex-chromosome disorder caused by the non-disjunction of chromosome X and results in either a classic form, in which there is an extra copy of chromosome X (47, XXY), or a mosaic form [4,5].

Both morbidity and mortality are significantly increased in KS, This may be explained by the fact that KS patients are at an increased risk of developing serious medical problems that include the following: 1- Motor, cognitive, and behavioral dysfunction, 2-Tumors and cancer, 3- Vascular diseases, 4- Endocrinial, metabolic, and autoimmune diseases [6]. Other mechanisms could be attributed to altered gene dosage due to the presence of an extra X chromosome, or disturbed inactivation of some genes carried on the X chromosome [7,8]. Endocrinal disturbances and delayed testosterone treatment are also recognized as

Abbreviations: FV Leiden, Factor V leiden; PTH, Prothrombin; MTHFR, 5, 10-methylene tetrahydrofolate reductase; PAI-1, plasminogen activator inhibitor 1; EPCR, Endothelial protein C receptor; VTE, Venous thromboembolism; PROCR, Protein C receptor gene; APC, activated protein C; MAF, Minor Allele Frequency; SNP, Single Nucleotide Polymorphism; BMI, Body mass index; LDL, Low density lipoprotein; HDL, High density lipoprotein; FSH, Follicle stimulating hormone; LH, Luteinizing hormone

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In Table 1 we summarized some of these gene mutations that are described below:

- **FV Leiden (G1691A)** and **FV HR2 (H1299R)** gene polymorphisms have been previously reported as risk factors closely related to hereditary thrombophilia. Individuals carrying FV LeidenG1691A mutations are at high risk of thrombosis through its enhancement of activated protein C resistance [12].

- The **Pro20210A** mutation (Prothrombin) is the second most common cause of hereditary thrombophilia after the Factor V Leiden mutation.

- It has been established that individuals who have a Plasminogen Activator Inhibitor 1 (PAI-1) gene mutation have high levels of plasma PAI-1 that may cause impaired fibrinolysis. The 4G allele of PAI-1 gene polymorphism is considered to be a high risk factor for thromboembolic events such as deep vein thrombosis [13] and myocardial infarction, while it has been suggested that the protective role of the FXIII V34L variant against VTE is attributable to a high genotype/allele frequency of FXIII V34L polymorphism in healthy individuals who have altered FXIII activity and fibrin clot stability [16].

- MTHFR C677T and MTHFR A1298C variants of the methylene tetrahydrofolate reductase (MTHFR) gene have been widely studied as one of the risk factors for hereditary thrombophilia. MTHFR is an essential enzyme responsible for conversion of 5,10-methylenetetrahydrofolate to 5-methyl tetrahydrofolate and homocysteine production. It was established that hereditary thrombophilia is associated with mutations of the MTHFR C677T and A1298C variants of the MTHFR gene [14].

The **EPCR (Endothelial Protein C Receptor)** gene, also known as PROCR (protein C receptor gene), encodes a protein receptor (EPCR) that is crucial for the protein C activation involved in the blood coagulation cascade. Among its four identified haplotypes, the A3 haplotype is found to be associated with venous thrombosis [18].

Undeniably, the primary cause of an increased tendency to vascular thrombosis in KS patients is not clearly understood, but hereditary thrombophilia is considered one of the precipitating factors for VTE in men with KS. The aim of this study was to investigate the genotype and allele frequencies of common gene polymorphisms related to hereditary thrombophilia in infertile males with KS compared to healthy males.
Table 2: Clinical and laboratory characteristics of KS patients and control.

| Age (years)     | KS (n = 85) | Control (n = 75) | P value |
|-----------------|-------------|------------------|---------|
| BMI (kg/m²)     | 30.75 ± 6.93 | 30.25 ± 8.96     | 0.6901  |
| Diabetes mellitus| 27.23 ± 4.12 | 22.8 ± 2.61      | < 0.001*|
| Hypertension    | 5(5.9%)     | 0(0.0)           | 0.061   |
| History of previous thrombosis | 3(3.5%)     | 0(0.0)           | 0.248   |
| History of varicose veins | 6(7.1%)     | 2(2.7%)          | 0.284   |
| Fasting blood sugar (mg/dl) | 88.7 ± 4.5  | 85.6 ± 2.5       | < 0.001*|
| Systolic blood pressure (mmHg) | 135 ± 22    | 96 ± 20          | < 0.001*|
| Diastolic blood pressure (mmHg) | 88 ± 8.71   | 87.5 ± 11        | 0.749   |

BMI: Body mass index, LDL: Low density lipoprotein, HDL: High density lipoprotein, FSH: Follicle stimulating hormone, LH: Luteinizing hormone.

*Statistically significant at p < 0.05.

Materials and methods

Study subjects

The present study included 85 newly diagnosed, untreated, infertile males with KS, referred from the Andrology Outpatient Clinic, Faculty of Medicine, Alexandria University, Egypt, between February 2017 and April 2018. In addition, 75 age and ethnicity matched healthy males who had at least one normal child were also selected as a control group. History, clinical examination and laboratory investigations were done. Laboratory investigations included fasting blood sugar and serum lipid profile (LDL, HDL, Cholesterol and triglycerides) on Beckman Coulter analyzers, USA. Roche Elecys assays were used to assess serum hormone levels of FSH, LH, Total Testosterone (TT) and Estradiol by the electrochemiluminescence method on a Cobase 411 analyzer (Roche Diagnostics GmbH, Germany) with limits of detection (LOD) of < 0.100 mIU/ml, 0.100 mIU/mL, 0.025 ng/mL and 18.4 pmol/L respectively. Established reference ranges were as follows: FSH: 1.3-12.4 mIU/mL, LH:1.7–8.6 mIU/mL, TT: 2.49–8.36 pg/ml and Estradiol: 27.9–156.38 pmol/L.

Chromosome analysis from peripheral blood using trypsin and giemsa stain, according to Seabright with modifications (1971), was carried out on all participants to confirm the diagnosis and exclude other chromosome aberrations [19].

KS patients with a chromosomal abnormality other than 46,XXY or chromosomal mosaicism were excluded. For the control group, individuals with a history of thromboembolic events or hormonal disturbances were excluded and only men with a normal male karyotype (46,XY) were included in the study. The number of healthy men excluded from the control group based on presence of VTE events was not available.

Informed written consent was obtained from all participants. Approval of the study by the ethical committee of the Faculty of Medicine, University of Alexandria, Egypt was acquired. The study protocol was consistent with the guidelines of the Declaration of Helsinki, 1964.

Molecular genetic analysis

DNA extraction

3 ml blood samples were collected from all participants for DNA extraction using Quick-DNA Miniprep Kit (ZymoResearch, USA, Cat.No.3024) according to the manufacturer’s instructions. The purity and concentration of extracted DNA were measured by a NanoDrop TM 1000 spectrophotometer and stored at −20 °C for future use.

Genotyping

Genotyping was run for nine well-known thrombophilic gene polymorphisms using the Multiplex PCR reverse-hybridization method in combination with CVD Strip Assays, Vienna Lab Diagnostics GmbH * (REF. 4-360).

The gene polymorphisms studied were summarized in Table 1 according to data provided by the National Center for Biotechnology Information (NCBI).

DNA amplification was carried out on an Applied Biosystem Thermal Cycler 2720. PCR conditions were as follows: A Pre-PCR step was run at 94 °C for 2 min. followed by 35 cycles of 94 °C for 15 sec. then 58 °C for 30 sec., followed by 72 °C for 30 sec., then a final extension at 72 °C for 3 min. Finally, the amplification products were selectively hybridized to allele-specific oligonucleotide probes immobilized on a test strip. Mutations were detected by enzymatic color reaction visible to the naked eye and the interpretation of genotypes was determined according to the enclosed Collector TM sheet.

One of three genotypes for each polymorphism was obtained for every individual: normal, heterozygous, or homozygous. EPCR alleles were A1 (H1), A2 (H2), A3 (H3), and so the resulting genotypes could be: A1/A1, A1/A2, A1/A3, A2/A2, A2/A3, A3/A3.

Statistical analysis

Data were computerized and analyzed using an IBM SPSS software package version 20.0 (Armonk, NY:IBM Corp). Student t-test was used to compare the two groups for normally distributed quantitative variables. Chi-square and Fisher’s exact test were used for qualitative variables. Genotype and allele frequency were calculated by direct counting. Comparisons between groups were done using Chi-square test, Fisher’s exact test, or Monte Carlo, according to data obtained. The risk of association between different genotypes and hereditary thrombophilia in KS and control men was assessed by odds ratio and 95% Confidence Interval (OR (95% CI)). The significance of the results obtained was judged at the 5% level.

Results

Table 2 illustrates the clinical and laboratory characteristics of the KS patients in comparison to the control group. A highly significant difference was found between both groups regarding fasting blood sugar (P < 0.001), lipid profile (P < 0.001) and hormonal assay (P < 0.001).

None of the KS patients had a thromboembolic event at the time of the study. Chromosome analysis of clinically suspected KS cases was 47, XXY karyotype.

Table 3 and Table 4 illustrate the genotype and allele frequency in both KS cases and controls.

FV Leiden and FV HR2 Polymorphisms

Significant differences in heterozygous and normal homozygote genotypes between KS cases and control were noticed for both polymorphisms (P < 0.001), while mutant homozygous genotypes (AA for FV Leiden and GG for FV HR2 gene polymorphisms) were not found in KS males or controls. Mutant alleles of both polymorphisms were significantly higher in KS men than the control (P < 0.001).

Prothrombin G20210A Polymorphism

The frequency of GG and AG genotypes in KS patients was 83.5%
and 16.5% versus 89.3% and 10.7% in the control, while the AA genotype was not found in either group (P = 0.359). The allele frequency of MTHFR gene polymorphisms in patients with KS compared to control group. The frequency of mutant allele (T) frequency between both groups was statistically insignificantly elevated in KS men (P = 0.013). We also found a statistically significant difference in genotype and frequency in genotype and frequency in mutant allele (T) frequency in both groups compared to none in the control group (P < 0.001). Five KS patients (5.9%) had more than 5 mutant alleles in comparison to 43.5% of KS men, while 50.6% of KS men were carriers of 3–5 mutant alleles of the disease. The frequency of mutant alleles in comparison to none in the control group (P < 0.001). Five KS patients (5.9%) had more than 5 mutant alleles of the disease.

**Table 3**

| Genotype | Cases (n = 85) | Control (n = 75) | OR (95%C.I) | P value |
|----------|----------------|-----------------|--------------|---------|
| FV G1691A (Leiden) | | | | |
| GG* | 45(52.9%) | 64(85.3%) | Reference | < 0.001 |
| GA | 40(47.1%) | 11(14.7%) | 5.172(2.398–11.154) | |
| AA | 0(0%) | 0(0%) | – | |
| FV H1299R | | | | |
| AA* | 3(3.5%) | 45(60%) | Reference | < 0.001 |
| AG | 82(96.5%) | 30(40%) | 41.0(11.849–141.86) | |
| GG | 0(0%) | 0(0%) | – | |
| Pro G20210A | | | | |
| GG* | 71(83.5%) | 67(93%) | Reference | 0.359 |
| AG | 14(16.5%) | 8(10.7%) | 1.651(0.651–4.188) | |
| AA | 0(0%) | 0(0%) | – | |
| MTHFR C677T | | | | |
| CT | 49(57.6%) | 51(68%) | Reference | 0.178 |
| CC | 36(42.4%) | 24(32%) | 1.561(0.816–2.986) | |
| TT | 0(0%) | 0(0%) | – | |
| MTHFR A1298C | | | | |
| AA* | 42(49.4%) | 33(44%) | Reference | 0.494 |
| AC | 43(50.6%) | 42(56%) | 0.804(0.431–1.500) | |
| CC | 0(0%) | 0(0%) | – | |
| Factor XIII V34L | | | | |
| GG* | 51(60%) | 32(42.7%) | Reference | 0.035 |
| GT | 44(40%) | 42(56%) | 0.507(0.269–0.955) | |
| TT | 0(0%) | 1(1.3%) | – | |
| PAI-1 4G/5G | | | | |
| 5G/5G* | 15(17.6%) | 32(42.7%) | Reference | 0.013 |
| 4G/5G | 46(54.1%) | 38(50.7%) | 2.583(1.221–5.461) | |
| 4G/4G | 24(28.2%) | 5(6.7%) | 10.240(3.267–32.087) | |
| EPCR Haplotype | | | | |
| A1/A1 | 23(27.1%) | 22(29.3%) | Reference | – |
| A1/A2 | 26(30.6%) | 22(29.3%) | 1.130(0.500–2.554) | 0.768 |
| A1/A3 | 0(0%) | 0(0%) | – | |
| A2/A2 | 24(28.2%) | 23(30.7%) | 0.998(0.441–2.261) | 0.996 |
| A2/A3 | 6(7.1%) | 8(10.7%) | 0.717(0.214–4.204) | 0.590 |
| A3/A3 | 6(7.1%) | 0(0%) | – | |

*Normal homozygous genotype, OR: Odds ratio, CI: Confidence Interval, statistically significant at p < 0.05.

**Table 4**

| Allele frequency of the thrombophilia gene polymorphisms in patients with KS compared to control group. | Cases (n = 85) | Control (n = 75) | OR (95%C.I) | P value |
|-----------------------------------------------|----------------|-----------------|--------------|---------|
| FV G1691A (Leiden) | | | | |
| G | 130(0.765) | 139(0.927) | 3.888(1.913–7.899) | < 0.001 |
| A* | 40(0.23) | 11(0.07) | – | |
| FV H1299R | | | | |
| A | 88(0.518) | 120(0.80) | 3.727(2.259–6.148) | < 0.001 |
| G* | 82(0.48) | 30(0.20) | – | |
| Pro G20210A | | | | |
| G | 156(0.918) | 142(0.947) | 1.593(0.649–3.909) | 0.309 |
| A* | 14(0.053) | 8(0.053) | – | |
| MTHFR C677T | | | | |
| C | 134(0.788) | 126(0.84) | 1.410(0.796–2.496) | 0.238 |
| T* | 36(0.212) | 24(0.16) | – | |
| MTHFR A1298C | | | | |
| A | 127(0.747) | 108(0.72) | 0.871(0.529–1.431) | 0.585 |
| C* | 43(0.253) | 42(0.28) | – | |
| Factor XIII V34L | | | | |
| G | 136(0.80) | 106(0.707) | 0.602(0.36–1.007) | 0.053 |
| T* | 34(0.20) | 44(0.293) | – | |
| PAI-1 4G/5G | | | | |
| 5G | 76(0.447) | 102(0.68) | 2.628(1.664–4.152) | < 0.001 |
| 4G* | 94(0.553) | 48(0.32) | – | |
| EPCR Haplotype | | | | |
| A1 | 72(0.424) | 66(0.44) | Reference | 0.114 |
| A2 | 80(0.471) | 76(0.507) | 0.965(0.610–1.526) | |
| A3* | 18(0.106) | 8(0.053) | 2.063(0.841–5.059) | |

* Mutant allele (MAF: Minor allele frequency), OR: Odds ratio, CI: Confidence Interval, statistically significant at p < 0.05.

**EPCR polymorphism**

No significant difference was found between the groups regarding the genotype frequency of EPCR polymorphism. We found the A3/A3 genotype in 7.1% of KS men but absent in the control. Again, the frequency of the A3 allele was more common in KS men than in the control (0.166 versus 0.053) with odds ratio of 2.063 (95%CI 0.841 to 5.059), but this difference was statistically insignificant. (P = 0.114).

**Combined mutant alleles**

In Table 5, we analyzed the data obtained concerning the frequency of male carriers of coexisting mutant alleles of the different polymorphisms studied. It was found that 90.7% of control men had < 3 mutant alleles in comparison to 43.5% of KS men, while 90.6% of KS men were carriers of 3–5 mutant alleles as opposed to 9.3% of the control men (P < 0.001). Five KS patients (5.9%) had more than 5 mutant alleles in comparison to none in the control group (P < 0.001).

**Discussion**

The tendency towards thromboembolism is a serious complication related to KS. Some KS patients may suffer from VTE in the form of deep vein thrombosis or pulmonary embolism, or both [20] while others may complain of recurrent venous ulcers [21,22]. The primary cause of this increased risk of VTE in KS patients is not clearly understood [8] and so this has encouraged scientists to look for the cause and whether it is an outcome of an underlying genetic susceptibility, hormonal disturbances, or both.

In this study, screening for hereditary thrombophilia was done using a broad panel of six genes known to be established risk factors for thromboembolism and which, to the best of our knowledge, have never been assessed before in infertile men with KS.

Based on studies published previously, the relative risk for venous thrombosis in carriers of FV Leiden mutation is estimated to be three to eight fold [12] and two to five fold in carriers of Pro G20210A
mutation. In carriers of both Pro G20210A and factor V Leiden mutations, the risk may even rise to nine fold [13,23]. In the current study, a high percentage of KS men were carriers of FV Leiden (G1691A), FV HR2 (H1299R) and Pro G20210A gene mutations and this should alert us to the possibility of the occurrence of such thrombotic events. Unfortunately, we have no data on the follow up for such cases and a large prospective study is needed to confirm these findings.

In case of PAI-14G/5G gene polymorphism, KS carriers of the 4G/5G variant may have increased plasma levels of PAI-1 which, in the presence of an underlying hypercoagulable state associated with Klinefelter syndrome, could increase the risk of vascular thrombosis [24,25]. High plasma levels of PAI-1 may impair the fibrinolysis cascade provoked by the underlying hormonal imbalance and result in an increased tendency to vascular thromboembolism [15,26].

Regarding the FXIII V34L polymorphism, the minor allele frequency (T allele) was 0.29 in controls recruited from Egypt, which falls within the range (0.01–0.4) reported in different ethnic groups [27]. Here, a protective role for the T allele of FXIII V34L polymorphism against vascular thrombosis could be suggested (OR was 0.602), but even so, a detailed population study is needed to confirm this.

In addition, it has been reported that the H3 (A3) haplotype of the EPCR gene is a potential risk factor for venous thrombosis through its association with high levels of a soluble form of EPCR that results in inhibition of APC [28,29] while a protective role for the A1 haplotype has been suggested [30,31]. Indeed, in the present study we found a high frequency of the H3 haplotype in KS males compared to controls and theA3/A3 genotype was absent in healthy men. By literature review, it is the first time to assess the prevalence of EPCR haplotypes in infertile KS males. However, this difference was insignificant which could be attributed to gene-gene interactions, sample size, or both.

Furthermore, the coincidence of more than one mutant allele of different thrombophilic gene polymorphisms was more evident in infertile men with KS than the controls. In view of published reports regarding this issue, we assume that KS patients who carry the combined heterozygous genotypes of such genes are at high risk of developing thromboembolism, and this is probably caused by an interaction between genetic products that share the same biological pathway and which may result in impaired fibrinolysis or coagulation cascades, or both [32,33,34].

Biases that may result from the underlying genetic background were minimized in the present study by sample size in conjunction with matched ethnicity between patients and control, which strengthened the current work. The main limitation was the absence of VTE events in both groups, which rendered the sample of control men not representative of the general population and so the susceptibility of KS men to VTE could not be assessed.

### Conclusion

The results reached during this study make clear that heterozygote genotypes, compound genotypes, and mutant alleles of several key thrombophilic genes are found in high frequency along the genetic profile of infertile KS males. Further research is recommended on large cohorts in order to investigate the genotype-phenotype correlation in KS syndrome in an attempt to provide proper management and genetic counseling to high risk families.

### Declarations

#### Ethics approval and consent to participate

The study was approved by the ethics committee of the Faculty of Medicine, Alexandria University (IRB NO. 00007555- FWA NO.00018699). An informed written consent was obtained from all participants in the study according to the Declaration of Helsinki 1964 and its extended demands.

#### Consent for publication

Consent for publication was obtained from all authors to the Journal of Clinical and Translational Endocrinology.

#### Availability of data and material

All data generated or analyzed during this study are included in this published article.

#### Competing interests

The authors declare that they have no competing interests.

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### Appendix A. Supplementary data

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T.M. Hussein, et al.
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