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THE INCIDENCE OF F8 GENE MUTATION IN SUDANESE PATIENTS AT RISK OF BOTH VENOUS AND ARTERIAL THROMBOSIS UNDER PROLONG USE OF WARFARIN TREATMENT

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ABSTRACT

Warfarin is a narrow therapeutic index agent. Complications from warfarin therapy occur even with proper monitoring . According to direct observations and very interesting findings in other research studies there is a need to detect the possibility of F8 gene mutation incidence that lead to an over expression of factor VIII protein which is essential for the formation of blood clots. The aim of this study is to investigate if there is a variation in F8 gene promoter region among Sudanese patients under warfarin therapy. This investigation was done by using Polymerase chain reaction -Restriction Fragment Length Polymorphism (PCR-RFLP) technique. The second aim is to find a possible association with the prolonged use of warfarin therapy and variation in F8 gene promoter region , to find a possible association between prolonged warfarin therapy and the incidence of thrombosis. Finally to detect if males are at higher risk of treatment failure compared to females. The investigation of F8 gene promoter region shows that most of the mutated samples belonged to males while only one female had a mutation . Six different samples were found to be mutant and two of them refer to fragment 1F from < -64 - 279 > , 3.1% of males had the genotype C-C which is the homozygous mutant type , while four refer to fragment AR from < 361 - 600> , 4.6% males had the homozygous mutant allele C-C while only 1.5% female .

There was a significant relation between the prolonged use of warfarin therapy and the elevation of the severity level.

KEYWORDS: F8 gene**, INR:** International Normalized Ratio, promoter region, **PT:** Prothrombin Time, **MVR:** Mechanical Heart Valves Replacement, **PCR:** Polymerase chain reaction.

INTRODUCTION

Warfarin is an effective therapy for both the treatment and prevention of thromboembolic events. Warfarin (Coumadin) is the most frequently prescribed oral anticoagulant, the fourth most prescribed cardiovascular agent and the overall eleventh most prescribed drug in the United States,with annual sales of approximately $500 million.[1]

The appropriate dose of warfarin is difficult to establish because it can vary among patients, and the consequences of taking an incorrect dose can be catastrophic. Because incorrect doses contribute to a high rate of adverse effects, there is interest in developing improved strategies for determining the appropriate dose.[2]

Warfarin as a point of interest

It is prescribed to more than 1 million patients in the United States annually. Because warfarin has a narrow therapeutic range and may increase the risk of bleeding events, therapy is individualized by monitoring the prothrombin time international normalized ratio (INR), a measure of anticoagulation status. The management of warfarin therapy is challenging, because of variability in patient response due to a multitude of factors including drug, diet, and disease-state interactions. In addition, genetic variation of patient may lead to significant differences in patient response to warfarin.[3] The anticoagulant and antithrombotic activity of warfarin depends on the clearance of functional clotting factors from the systemic circulation once the drug is administered.[4]

The association between venous thrombosis and factor VIII level

High Levels of factor VIII (FVIII) are associated with the risk of venous thrombosis.[5] In a previous study done by (Kathleen E. et, al. 2001) tissue factor–induced whole-blood coagulation in patients on warfarin therapy with similar international normalized ratios (INRs) were investigated. A significant correlation was identified between increasing factor

VIII levels and years on warfarin therapy (*r*_0.78, *P*_0.01), suggesting a possible factor VIII compensatory mechanism.[6]

Venous thromboemboembolism and factor VIII plasma level

Modern thrombophilia testing fails to identify any underlying prothrombotic tendency in a significant number of patients presenting with objectively confirmed venous thromboembo embolism (VTE). This observation has led to a search for other novel inherited or acquired human thrombophilias. Although a number of putative mechanisms have been described, the evidence behind many of these candidates remains weak. In contrast, an increasing body of work supports the hypothesis that increased plasma factor VIII (FVIII) levels may be important in this context.[7] Amplification of the F8 gene copy number seems to enhance factor VIII activity and was associated with VTE.[8]

F8 gene location

The *F8* gene is located on the long (q) arm of the X chromosome at position 28. More precisely, the *F8* gene is located from base pair 154,064,062 to base pair 154,255,350 on the X chromosome.[9] The location of this gene in chromosome X could give us a good explanation about the level of risk between males and females. Modern thrombophilia testing fails to identify any underlying prothrombotic tendency in a significant number of patients presenting with objectively confirmed venous thromboemboembolism (VTE). This observation has led to a search for other novel inherited or acquired human thrombophilias. An association between elevated plasma FVIII levels and VTE was first described in the Leiden Thrombophilia Study (LETS). Subsequently, these conclusions have been supported by an increasing number of independent case-control studies. Cumulatively, these studies have clearly demonstrated that high FVIII levels constitute a prevalent, dose-dependent risk factor for VTE. Furthermore, more recent studies have shown that the risk of recurrent venous thrombosis is also significantly increased in patients with high FVIII levels.[10]

The relation between elevated factor VIII levels and years on warfarin therapy

Kathleen E. et al[6] identified a significant correlation between increasing factor VIII levels and years on warfarin therapy (*r*_0.78, *P*_0.01), suggesting a possible factor VIII compensatory mechanism. This result suggest that control of anticoagulation in patients to a set INR therapeutic range may be less secure than anticipated. Patients with similar INRs show significant individual variability in their tissue factor coagulation response, suggesting different risks to anticoagulation when confronted with underlying vascular anomalies.

A strong positive correlation for all subjects was detected between years on warfarin therapy and FVIII levels (*P*_0.01, *r*_0.78). The longer an individual was on warfarin therapy, the higher his FVIII level was, suggestive of a compensatory mechanism. Interestingly, von Willebrand factor showed no corresponding correlation (*P*_0.70, *r*__0.18). Prothrombin, FV, FVII, FIX, FX, antithrombin III, fibrinogen, and platelets also did not show a correlation at this sample size. No correlation was detected between the circulating carrier of FVIII, von Willebrand factor, and years on warfarin therapy. This increase in FVIII levels over the course of warfarin therapy suggests the possibility that a compensatory pathway partially corrects for the vitamin K–deficient state that exists in these subjects.[6] In other hand another study by Musial J, et al 2010, find that increased FVIII activity in patients with VTE and AF does not change significantly during anticoagulation.[11] This different results of different studies gives as a serious question about the relation between elevated F8 level and possible mutation in F8 gene and, wither prolonged warfarin therapy has a role on this suggested mutation. In this study the promoter region of F8 gene was placed under focus depending on its function of Factor VIII expression and protein production. Two sites in the promoter region in Exon 1 of F8 gene < -64 - 279 > and < 361 to -600> were investigated using *Hha1* restriction enzyme. This study chasing on any sign of different in the sequence of F8 gene promoter suggested sites that could be a sign of mutation.

MATERIALS AND METHOD

This study was conducted at Ahmed Gasim Hospital, Khartoum North (Bahri) a centralized hospital for Cardiovascular diseases in Sudan. A total of 105 individuals has been involved in this study. Sixty five patients and forty normal control healthy individuals were selected. The Study population were adult patients above 17 years old. DNA isolation, DNA Quantification, DNA Amplification and PCR products digestion analysis with the agarose Gel electrophoreses were all used for the molecular typing of the samples. While the Statistical Package for Social Science (SPSS) ‘version 20’ was used for analysis of demographic and clinical characteristics. Haploview bioinformatics software ‘version 4.2’ was used for analysis and visualization of LD and haplotypes.

Ethical Consideration

The ethical consideration was obtained from Sudanese’s Medical Ethics Committee guidelines. The data records and sample collection were under the agreement of hospital

direction and patients them self's. The approval of the study was obtained from Ahfad University for Women and Federal Ministry of Health.

DNA isolation

procedure of DNA extraction

Genomic DNA was extracted from the buffy coat using Qiagen kits. In 1.5 microcentrifuge tube 20µl of Qiagen proteas (proteines K) was added, then 200 µl of buffy coat. After that 200 µl of AL buffer (lyses buffer) was mixed by bulse overtaxing (VM- 10 vortex WiseMix®) for 10 sec. Incubated for 56 ºC for 10 min. Then it was centrifuged to remove drops from the inside of the lid using Centrifuge 5424 Eppendorf ®, then 200 µl of ethanol was added to the mixture after that it was mixed again by bulse centrifugation. Then the mixture was carefully applied to the QiAamp mini spin column in 2ml collection tube and avoid wetting the rim then the cap was closed and then centrifuged at 8000 rpm for 1 min. The filtered was discharged , then 500 µl of AW1 washing buffer was added to QiAamp spin column and centrifuged at 8000 rpm for 1 min the filtered again was discharged . Five hundred µl of buffer AW2 (washing buffer 2) was added then it was centrifuged for 14000 rpm for 3 min then the filtered was discharged after that the QiAamp mini spin column was placed in a new 2ml collecting tube. Two hundred µl of AE elution buffer was added then it was incubated at room temp for 5 min, then it centrifuged at 8000 rpm for 1 min . Then DNA was transfer to 1.5 ml appandrof tube.

DNA Quanitifecation

Extracted Genomic DNA was quantified spectrophotometrically by diluting 15 µl from DNA + 435 µl of DW(Distell water). The mixture was vortexed for 10 sec for the hemogenesety of DNA then the DNA was measured at 260 nm and protein at 280 nm in Biophotometer (eppendorf Biophotometer plus). The devise was adjusted to zero with DW before measuring the DNA. The ratio of DNA was ranged between 1.6 to 2.0. The study required a minimum of 90 ng of genomic DNA. The samples were then aliquoted into two 0.5 separate PCR tube stored at-20 and -80◦C and assayed in batch.

Molecular typing

DNA Amplification and PCR products digestion analysis

The promoter region of F8 gene was amplified by polymerase chain reaction (PCR) using specific primer sequences.

Primer sequence

There were two primers utilized in this study related to the promoter region in Exon 1 of F8 gene. The first primer site from < -64 to - 279 > 1F primer and the second primer site from < 361 to -600> AR primer. The two primers were designed by primer3 software version 0.4.0.

PCR protocol

The samples were loaded to thermocycler (Fliex Cycler) to a newly adjusted temperature profile after some modification on the typical PCR protocol as shown below in table :1 .

Table: 1- PCR protocol

Cycle NO.	Temp.	Time
1 cycle	95°C	3 minutes
30 cycles	95°C	45 seconds
	63°C	13 seconds
	72°C	30 seconds
1 cycle	72°C	10 minutes (to finish replication on all templates)
1 cycle	4-10°C	indefinite period (storing the sample prior to further analysis)

Agarose Gel electrophoreses

Gel preparation

The gel was prepared by adding 3g of agarose to 100ml 1X TBE buffer from (Sigma®), then it was boiled for 2min then it was kept to cooled to 50 ◦C. Then 5 µl of Ethidum bromide from (sigma®) was added to the gel and it was poured into the tray containing combs then the gel was lived to solidified. After the gel was solidified the combs were removed.

DNA sample loading

The gel was placed into the electrophoreses tank and covered by 1X TBE. Then 10 µl from the DNA was loaded in to the gel wells. Then 3 µl of DNA marker was loaded into the 1st well for comparison and the negative control was included.

DNA visualization

The gel was placed in the gel documentation system and the result was photographed.

PCR products digestion

Aliquots of each PCR product will be digested with restriction enzyme (*Hha1*) at 37ºC for 1 hour. The DNA fragments was electrophoresed on a 3% agarose gel prepared by agarose powder of (vivantis®). An undigested PCR product, DNA 50 bp DNA marker (intron®) and/or 100 bp DNA marker (vivantis®) were loaded to the gel. Then bands were detected by gel documentation system and photographed.

RESULT INTERPRETATION

Table: 2 - shows the interpretations of the result.

Table: 2- Result interpretation

Primer	Wild type	Heterozygous (mutant)	Homozygous (mutant)
1F	125/40/12 bp	177/125/40/12 bp	177 bp
AR	71/20/19/2bp	112/71/20/19/2 bp	112 bp

RESULT AND DISCUSSION

Genetic variability among patients plays an important role in determining the dose of warfarin that should be used when oral anticoagulation is initiated, but practical methods of using genetic information have not been evaluated in a diverse and large population.[12] Investigation of possible variation in F8 gene promoter region was the first aim of this study, because the incidence of mutation in this region could lead to an over expression of factor VIII protein which is essential for the formation of blood clots. Therefore tow sites in the promoter region in exon1 of F8 gene were investigated using *Hha1* restriction enzyme. The first site was from < - 64 - 279 > amplified by primer1(1F) and the second site was from < 361 to -600> amplified by primer2 (AR). In the whole sample size of 65 patients and 40 control individuals which participated in this study only 2 samples in the site of primer1(1F) were detected as mutant due to the presence of undigested or uncut PCR product under the UV light of gel electrophoresis system as shown in figure 1.

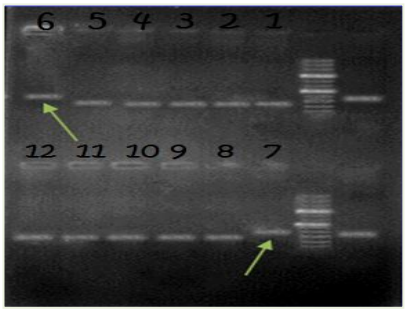

Figure 1-Fragment 1F:
This figure shows restriction enzyme digestion of fragment 1F in exon1. The samples on the upper row (from left to right) were 6,5,4,3,2,and 1. Line one from the right contains the PCR product . Line two contains 50 bp ladder . The samples in the second raw from (left to right)were 12 , 11 , 10 ,9,8 and 7 . The arrows point out the mutant samples (A6-A7) .

Homozygous mutated sample in size of 177 bp which is equal to the size of the typical PCR product, as the rest of the samples were wild type. Those mutated samples belonged to male patients. *Hha1* restriction enzyme digests those products to three bands and their sizes were 125/40/12 bp. Only the band of 125bp was visible but the other 2 bands were invisible. There weren't any mutated or undigested samples on the controls. Furthermore those samples were analyzed utilizing the second primer(AR). At the (AR)primer site, four different sample were detected as homozygous mutated samples due to the presence of one single band . Those samples were 15,50,41and 42 and "three of them were males and only one was a female ". They were digested in size differ than the size of the wild type, the exact size of those bands were unknown but it ranged between (50- 69) as shown in figure 2 & 3. Wild type were digested to three bands sized 71/20/19/2bp , actually only the first band was visible but the other bands were difficult to be detected in the agarose gel, in addition to that the typical PCR product size was 112 bp. Also the control samples overall were not mutated and they were detected as wild type. This result shows a detected variation in the promoter region of F8 gene.

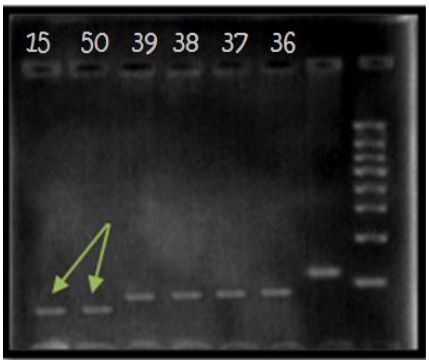

Figure 2- Fragment AR - a:
This figure shows *Hha1* restriction enzyme digestion of fragment AR in exon1 . The samples (from left to right) were 15,50,39,38,37and 36. Line one from the right contains 100 bp ladder. Line two contains the PCR product. The arrows point out the mutant samples (15-50).

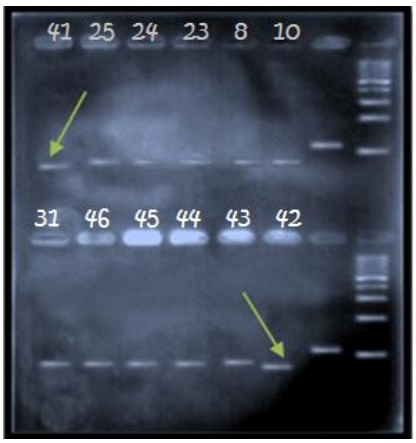

Figure 3-Fragment AR - b:
This figure shows Hha1 restriction enzyme digestion of fragments AR in exon1.The samples on the upper row (from left to right) were 41,25, 24,23,8, and 10. Line one from the right contains 100 bp ladder. Line two contains the PCR product. The samples in the second raw from (left to right) were 31 , 46 , 45 ,44,43 and 42 . The arrows point out the mutant samples (41- 42).

It is very important to investigate the correlation between the severity level of the patients and the dose consumed by them. In this correlation 100% of patients using a dose ranged from 10.5 -13 mg/day were in severe level . This indicates that this correlation is completely significant and that is proved by the *P-value* that was found to be 0.0001 [(*)] as in figure 4. It is important to mention that high dose is one of the criteria's of increasing the severity level. In previous studies they clearly demonstrated that high FVIII levels constitute a prevalent, dose-dependent risk factor for VTE. Furthermore, more recent

studies have shown that the risk of recurrent venous thrombosis is also significantly increased in patients with high FVIII levels.[10]

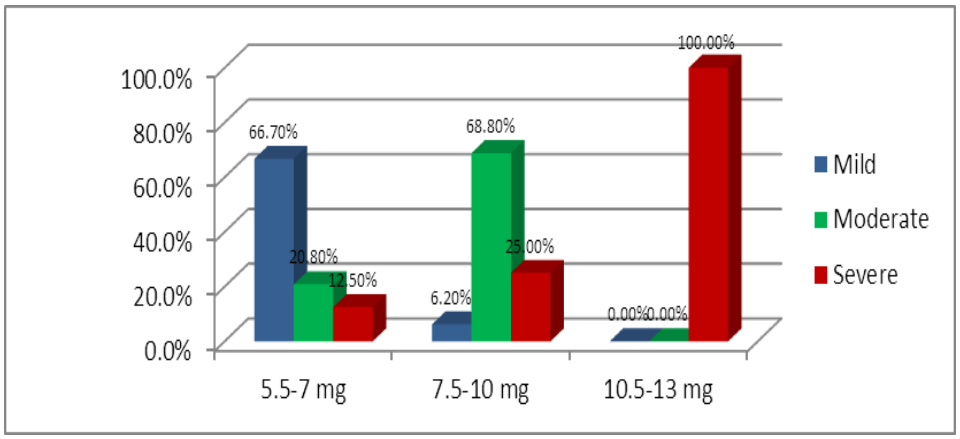

Figure- 4: The Association between patient severity and dose.

It is important to explain the relation between the duration of warfarin therapy and Severity levels. The result indicates that even though the patients at the first level of therapy had a very severe condition " According to different studies , patients in the first duration of therapy are difficult to monitor and it's also hard to give them an appropriate dose", Richard and colleagues mentioned that even careful monitoring to keep the prothrombin time at 1.5 – to 2.5 fold longer than normal values does not prevent complications in about twenty percent of the patients especially on those initiating therapy (13). The mild and moderate levels gradually decreased throughout eighteen years of therapy that ranged from (2-5y), (6-9y), (10-14y) and (15-20y). On the other hand, the severe level went through an equilibrium (20%) throughout the last fourteen years as shown in figure 5. The association was significant as the *P-value* was 0.046(*).

The result demonstrate the incidence of thrombosis and the duration of therapy. According to Pieter.W. and colleagues, high levels of factor VIII are a risk factor for thrombosis, with a greater impact on venous than on arterial thrombosis.[14] Kathleen.E. and colleagues Found that increase in plasma levels of FVIII was correlated with the length of time an individual was on warfarin therapy (*P*_0.01).[6] In this study the highest percent of thrombosis was found in patients with duration of therapy ranging from one month to one year(42.9%) . This is also related to what was stated before. On the other hand 28.6 % of patients with an incidence of thrombosis were in a duration of therapy ranging from 6-9 years and 15-20 years. Usually patients in therapy duration from 2-5 years are generally stable and easy to monitor. Although

it is important to mansion that the highest percent of incidence of thrombosis was found in patients originating from the Northern state (42.9%). Furthermore, a good observation about the association between the patients' tribes and their incidence of thrombosis was present. About 71.4% of patients related to Al-Ja,aliyin tribe had an incidence of thrombosis. Although 14.3% of Al-Mahas and Al-Manaseer tribes had an incidence of thrombosis as shown in figure 6. In fact, according to the geographical distribution of tribes in sudan, "these tribes are found in the Noerthern state" and they all come from an Arab origin. This correlation isn't significant according to the *P-value = 0.858* but it does give us a good explanation about a serious relation between patients that originated from the Northern state and the possibility of an inherited genetic variation amongst them. To prove what was mentioned, 4 from 6 mutated samples in this study were belonged to patients from Al-Mahas tribe while the other two samples belonged to patients from Al-Ja, aliyin tribe.

One of the important aims of this study is to investigate if there are possible differences between males and females such as if males are at a higher risk of treatment failure and /or thrombus formation than females. There was a comparison between males and females in relation with severity, incidence of thrombosis, high range of dose and patient with other conditions. About 16.9% of females had a higher percent of severity than males which had 13.8% of severity. Once again, females had a higher percent of incidence of thrombosis (6.2%) than males which had an incidence of thrombosis by 4.6%. On the other hand, males had a higher percent (7.7%) of the highest dose ranging from (10.5-13) mg/day rather than females who had 6.2% of this high dose. Finally, the females with other clinical conditions were at a higher percent (7.7%) rather than males which were (6.2%) with other clinical conditions. This result gives us an indication that males need a higher dose than females while females are at a higher risk than males to have a thrombus formation that leads to a higher severity level. This conclusion is harmonized with studies done by Keightley.AM. ,Balleisen.l. and colleagues, they found that factor VIII levels are influenced by sex (higher in women than men) and race (higher in blacks than whites).[15-16] Most likely, high factor VIII levels are the result of a combination of genetic and acquired factors.[14]

According to the result of PCR digesting analysis, an association between the genotype (primers) and the phenotypes was established. Two genotypes were used in this study and they are 1Fprimer genotype and AR primer genotype. The phenotypes were gender and the INR level of the patients. According to the association between the genotype of primer 1F

and the gender as a phenotype, 3.1% of males had the genotype C-C which is the homozygous mutant type, while 36.9% of them were not mutant " wild type " . On the other hand, females did not have any mutation in this sight of the gene while 60% got the wild type of the gene. This association was found to be significant according to the *P-value* which was = 0.059 $^{(*)}$. In relation to the INR level of those patients, which is divided to below and above 2. In patients with INR level below 2, 3.1% had got the homozygous mutant genotype C-C whereas none of the patients with the INR level above 2 had a mutant genotype. Furthermore, 89.2% of patients with the INR level below 2 got the wild genotype while 7.7% of them had an INR level above 2 and were normal. This association was found to be insignificant related to the *P-value* that was = 0.678. We should note that any INR below 2 might be a sign of a thrombus formation. On treatment analysis of the primary prevention trials by Hirsh J. et, al in atrial fibrillation found that a disproportionate number of thromboembolic and bleeding events occurred when the PT ratio was outside the therapeutic range. Subgroup analyses of other cohort studies also have shown a sharp increase in the risk of thromboembolism when the INR fell to <2.0 .[17] The second genotype (AR primer) which is designed by primer 3 software was in relation with the gender as the first phenotype . The highest percent of patients with mutant allele was found in males and 4.6% of them had the homozygous mutant allele C-C while only 1.5% of females had the mutant allele. In fact, those patients were three males and one female. Although 58.5% of females and 35.4% of males were normal with the wild genotype G-G, this association was found to be significant as the *P-value* was = 0.0240 $^{(*)}$. The second phenotype which is the INR level was found to be insignificant in relation with the genotype of the AR primer according to the *P-Value* that was = 0.551. 6.2% of patients with INR below 2 had the homozygous mutant genotype C-C while none of them with INR above 2 had the mutant genotype. The rest of the patients were normal with the wild allele. It is very important to compare this mutation in patients and normal controls. This comparison was done using Haploview analysis and visualization program. According to the Haploview program the association allele in AR primer genotype was G and the *P-value* was found to be 0.0237$^{(*)}$ which shows that this relation was significant. On the other hand the case/control ratio was: 80:0 / 122:8. The Min genotype was found to be 75%. Furthermore, the association allele in 1F primer genotype was G and the *P-value* of this correlation = 0.1132 was not significant, while the Min genotype was found to be 75%. The case/control ratio was found to be:80:0 /162:4 . In fact, the control samples were all normal and wild type.

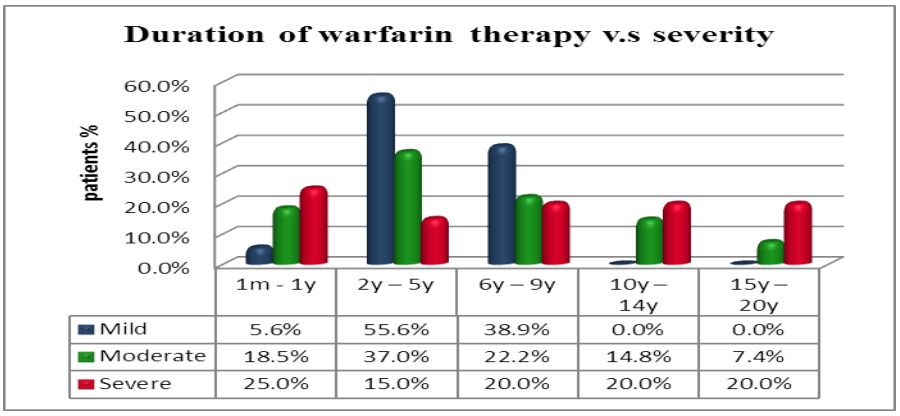

Figure -5: Association between the duration of warfarin therapy and Severity levels.

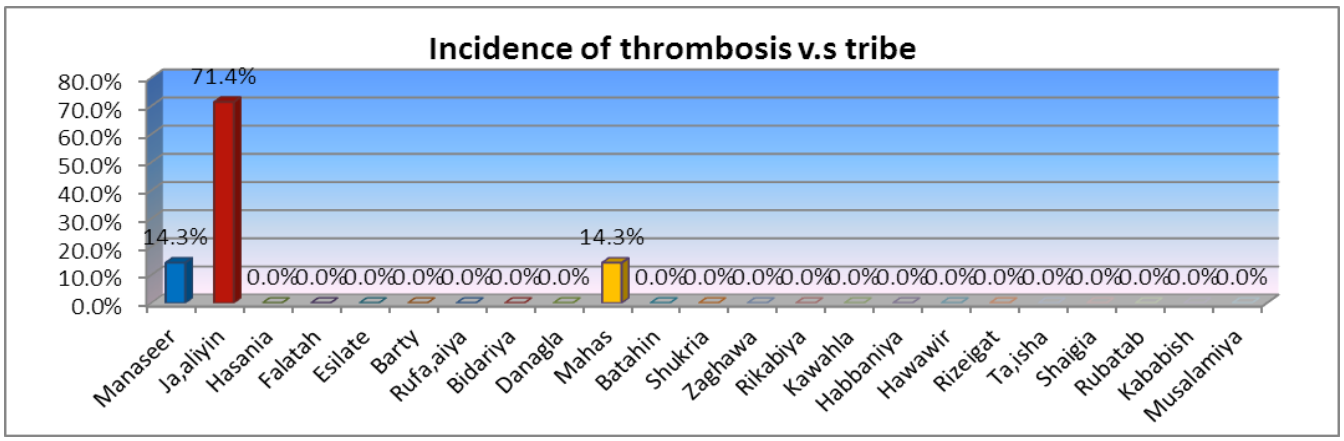

Figure -6: Incidence of thrombosis in relation to patient tribe.

CONCLUSION

A real variation was found at the promoter region of F8 gene. six different samples were found to be mutant and two of them refer to fragment 1F while four refer to fragment AR. Most of the mutated samples belonged to males wile only one female had a mutation. There was a remarkable finding about patients originating from Algezira and the Northern states and it is that there might be a possibility of an inherited genetic variation amongst them. The highest percent of thrombus formation (42.9%), the highest range of warfarin dose (10.5-13) mg/day and the highest severity level was found in patients originating from the Northern state. There was a significant relation between the prolonged use of warfarin therapy and the elevation of the severity level, but there was no significant correlation between the prolonged use of warfarin and the incidence of thrombosis. Comparing males and females, females had the highest percent of severity level (16.9%). They also had a higher percent of incidence of thrombosis than males (6.2%). Males needed a much higher dose than females and so, they maybe at a higher risk of treatment failure. Finally, there was no mutation present in control sample. The case/control ratio of AR fragment was: 80:0 / 122:8 and the case/control ratio of fragment 1F was found to be:80:0 /162:4.

RECOMMENDATION

A family base study is recommended to detect the possibility of inherited factors. Although an open end study is recommended to investigate and examine the effect of the prolonged use of warfarin in a more developed way. The Northern and Algezira state should be kept under focus. Furthermore Scanning the promoter region of factor 8 gene as a whole is favored. Finally the Practical methods of using the genetic information of patients should be evaluated.

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