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Factor VIII Intron 22 Inversion in Severe Hemophilia A Patients in Palestine

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Dedication

I would like to dedicate my work to my parents, sisters and brothers for their encouragement. To my beloved wife, son and daughter; Rania, Jad and Jwana for their patience and whose continuous efforts and help made it possible, with love and respect.

Acknowledgment

There are many people to whom I am very grateful for their contributions towards this thesis and made it possible. First of all, I would like to thank Dr. Nael S. Abu Hasan and Dr. Fekri Samarah for giving me the opportunity to work under their supervision, and their readiness to read, evaluate and improve this thesis. I would like to thank the Palestinian Society for Bleeding and Disorders for their support and valued efforts in data collection of concerned patients. Special thanks and appreciations to Medicare laboratories team for their practical assistance. Last but not least, I'm grateful to all who participated in this study, without them this thesis would not have been possible.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل عنوان:

Factor VIII intron 22 Inversion in Severe Hemophilia A Patients in Palestine

أقر ما اشتملت عليه هذه الرسالة انما هي نتاج جهدي الخاص، باستثناء ما تمت الاشارة اليه حيثما ورد، وان هذه الرسالة ككل لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحث لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: **إسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

°C	Celsius
µmol	Micromolar
λ	Lambda
3'UTR	3'-untranslated region
5'UTR	5'-untranslated region
7-deaza-dGTP	7´-deaza-deoxyguanosine triphosphate
∞	Infinity
A1, A2, A3, B, C1, C2	domains of FVIII
A230	Absorbance at 230 nm
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
AAV8	Adeno Associated Virus serotype 8
ADH	Antidiuretic Hormone
AHF	Antihaemophilic factor
APC	Activated Protein C
APTT	Activated Partial Thromboplastin Time
Arg	Arginine
BLAST	The Basic Local Alignment Search Tool
bp	basepair
BU	Bethesda Unit
CaCl2	Calcium Chloride
CD8+	Cluster of Differentiation 8
CP	Cryoprecipitate
CpG	cytosine-phosphate-guanine
D.W	Distilled Water
DDAVP	1-deamino-8-D-arginine vasopressin
DEPC	Diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DIC	Disseminated Intravascular Coagulation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside triphosphate
EDTA K3	Tripotassium ethylenediaminetetraacetic acid
FDPs	Fibrin Degradation Products
FFP	Fresh Frozen Plasma
FI	Clotting factor I
FIa	Activated clotting factor I
FII	Clotting factor II
FIIa	Activated clotting factor II

FIII	Clotting factor III
FIIIa	Activated clotting factor III
FIV	Clotting factor IV
FIVa	Activated clotting factor IV
FIX	Clotting factor IX
FIXa	Activated clotting factor IX
FV	Clotting factor V
FVa	Activated clotting factor V
FVII	Clotting factor VII
FVIIa	Activated clotting factor VII
FVIII	Clotting factor VIII
FVIIIa	Activated clotting factor VIII
FX	Clotting factor X
FXa	Activated clotting factor X
FXI	Clotting factor XI
FXIa	Activated clotting factor XI
FXII	Clotting factor XII
FXIIa	Activated clotting factor XII
FXIII	Clotting factor XIII
FXIIIa	Activated clotting factor XIII
GP	Glycoprotein
GPIb	Glycoprotein 1b
GPIIb/IIIa	Glycoprotein II b/IIIa
HAMSTeRS	Haemophilia A Mutation, Structure, Test and Resource Site
HBsAg	Hepatitis B Surface Antigen
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HMWK	High Molecular Weight Kininogen
IgG	Immunoglobulin G
IgG1	Immunoglobulin G subtype 1
IgG4	Immunoglobulin G subtype 4
Int1-h1	Intron 1 homolog 1
Int1-h2	Intron 1 homolog 2
Int22-h1	Intron 22 homolog 1
Int22-h2	Intron 22 homolog 2
Int22-h3	Intron 22 homolog 3
Inv1	Intron 1 inversion
Inv22	Intron 22 inversion
IRB	Institutional review board
ISTH	International Society on Thrombosis and Hemostasis
IU	International unit

IV	Intravenous
Kb	kilo base
MgCl2	Magnesium Chloride
ml	milliliter
mmole	millimolar
mm:ss	Minute: Second
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
ng	nanogram
nm	nanometer
nmol	nanomolar
OMIM	Online Mendelian Inheritance in Man
ORF	Open reading Frame
PCR	Polymerase chain reaction
PF3	Platelet factor 3
pH	potential Hydrogen
PT	Prothrombin Time
RNA	ribonucleic acid
scAAV	self-complementary AAV
S-PCR	Subcycling Polymerase Chain Reaction
TBE	Tris/Borate/EDTA
TF	Tissue factor
TFPI	Tissue Factor Pathway Inhibitor
TLR9	Toll-like receptor 9
t-PA	Tissue Plasminogen Activator
vWF	von Willebrand factor
WFH	The World Federation of Hemophilia
WHO	World Health Organization
WT	Wild type

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Abstract

Hemophilia A is X-linked recessive bleeding disorder affecting 1 in 5000 male births, and caused by mutations in FVIII gene which lead to deficiency or dysfunction of coagulation factor VIII protein. In severe cases, intron 22 inversion (Inv22) of the FVIII gene is a major cause of disease worldwide accounting up to 50% of all FVIII mutations. While intron 1 inversion (Inv1) and small scale mutations of FVIII mutations are responsible for 5% and 45%, respectively. The aim of the present study was to investigate intron 22 inversion mutations among severe hemophilia A patients as well as carrier status among hemophilia A patients in Palestine. A cohort of 77 hemophilia A individuals (72 males and 5 females) from 52 unrelated families in West Bank area of Palestine was studied. FVIII activity was measured for all male individuals by one stage coagulation assay. Subcycling PCR (S-PCR) was performed for detection of Inv22 mutation for all severe male cases as well as mothers. FVIII activity results showed that 41.7% of our patients with severe hemophilia

A, 22.2% with moderate hemophilia A and 36.1% with mild hemophilia A. Analysis of Inv22 by S-PCR revealed that 37% of the screened patients with severe hemophilia A have this inversion. This finding is similar to that reported among various populations worldwide. To the best of our knowledge, no previous studies are available in Palestine to investigate intron 22 inversion mutations among severe hemophilia A. The findings of the present study can be used as a step for genetic counseling. Further investigation on Inv1 mutation and full gene sequencing to look for novel mutations for those patients who approved to be negative for Inv22 mutation would be valuable.

Chapter One

Introduction

1.1 Overview

1.1.1 Hemostasis

Coagulation is a process that leads to formation of fibrin clot through interactions between several coagulation factors and can occur via thrombosis or hemostasis. Thrombosis is a coagulation process that occurs in pathological state and might lead to intravascular clotting and vessel occlusion depending on severity of formed occlusion. Hemostasis is a coagulation process that occurs in physiological state to seal breaks in vasculature and protect its integrity in a balanced manner to prevent neither excessive bleeding nor excessive clot. The process involves three main stages which are platelets aggregation, clot formation and fibrinolysis, which are also termed primary, secondary and tertiary hemostasis, respectively (Stassen et al., 2004; Verhamme & Hoylaerts, 2006).

1.1.1.1 Primary hemostasis

Primary hemostasis involves platelets' adherence, activation and aggregation. After an injury to vascular wall, sub-endothelial collagen fibers are exposed allowing the platelets to adhere to vessel wall via adhesion molecules such as von Willebrand factor (vWF), a multimeric protein synthesized by endothelial cells. Platelets activation and aggregation can be accomplished by platelet glycoprotein (GP) receptors which include GPIb-V-IX and GPIIb/IIIa. Interactions of GPIb-V-IX with vWF will lead to platelets activation, whereas interactions of GPIIb/IIIa with vWF cause platelets aggregation thus, enabling platelets plug

formation (Verhamme & Hoylaerts, 2006; Broos et al., 2011). Activated platelets provide phospholipid surfaces for the assembly of blood clotting factors (Ivanciu et al., 2014).

1.1.1.2 Secondary hemostasis

1.1.1.2.1 Coagulation factors

Coagulation factors are substances mostly synthesized by the hepatocytes and act in specific sequence to stop bleeding by formation of insoluble fibrin clot. These factors are classified into three groups which are fibrinogen group, prothrombin group and contact group. Fibrinogen group is usually consumed during the coagulation process and includes factors I, V, VIII and XIII (FI, FV, FVIII and FXIII, respectively). Prothrombin group depends on vitamin K during their synthesis and includes factors II, VII, IX and X (FII, FVII, FIX and FX, respectively). The contact group is moderately stable and is not consumed during coagulation process and consists of factors XI, XII, (FXI, FXII), prekallikrein (Fletcher factor) and high molecular weight kininogen (HMWK). Other cofactors are also required such as factors III and IV which are mainly involved in the initiation of coagulation process (Table 1.1). Normally, during injury these factors act in specific coagulation pathways (intrinsic and extrinsic) to control bleeding. These pathways are also known as contact and tissue factor (TF) pathways, respectively. These pathways are converging into final common pathway to give the final outcome represented by formation of stable fibrin clot as described in Figure 1.1 (Turgeon, 2012; Palta et al.,

2014). In addition, these pathways are highly regulated by anticoagulant proteins to prevent excessive clot formation such as protein C, thrombomodulin, protein S, tissue factor pathway inhibitor (TFPI) and antithrombin (Kato, 2002; Dahlbäck &Villoutreix, 2003).

Table 1.1: Coagulation factors (Turgeon, 2012; Palta et al., 2014).

Factor	Descriptive Name	Alternate Terms	Pathway
I	Fibrinogen		Intrinsic / Extrinsic
II	Prothrombin		Intrinsic / Extrinsic
III	Tissue factor	Tissue thromboplastin	Extrinsic
IV	Ionized calcium		Intrinsic / Extrinsic
V	Proaccelerin	Labile factor	Intrinsic / Extrinsic
VII	Proconvertin	Stable factor	Extrinsic
VIII	Antihaemophilic factor (AHF)	Antihemophilic factor A	Intrinsic
IX	Christmas factor	Antihemophilic factor B	Intrinsic
X	Stuart–Prower factor	Stuart factor	Intrinsic / Extrinsic
XI	Plasma thromboplastin antecedent	Antihemophilic factor C	Intrinsic
XII	Hageman factor	Contact factor	Intrinsic
XIII	Fibrin-stabilizing factor	FSF	Intrinsic / Extrinsic
Others	Prekallikrein HMWK	Fletcher factor Fitzgerald factor	Intrinsic Intrinsic

1.1.1.2.2 Coagulation pathways

1.1.1.2.2.1 Extrinsic pathway

It is named extrinsic because the main component is normally extrinsic to the circulation, which is TF that is derived from phospholipids of damaged tissues. This pathway is initiated by the entry of TF into the circulating blood after a vascular injury and become exposed to FVII, then FVII binds to TF and becomes activated FVII (FVIIa), which is a potent enzyme that able to activate FX to active FX (FXa) in the presence of ionized calcium. TF within the circulation also provide a surface for the conversion of factors II and V to their active form (FIIa and FVa, respectively). The final

step is the conversion of fibrinogen to fibrin clot by thrombin (Morrissey, 2001, Palta et al., 2014; Turgeon, 2012). The fibrin clot is additionally stabilized by activated FXIII (FXIIIa) which catalyze the links between adjacent fibrin molecules (Ichinose, 2001).

1.1.1.2.2.2 Intrinsic pathway

It is named intrinsic because all of the components are already present in the circulation. This pathway is initiated by activation of FXII to activated FXII (FXIIa) by the HMWK-kallikrein complex, then FXIIa converts FXI to activated FXIa (FXIa), FXIa converts FIX (FIX) to active FIX (FIXa). FIXa reacts with active FVIII (FVIIIa), platelets factor 3 (PF3) and calcium to form tenase complex which is in turn converted FX to FXa. After the formation of FXa, this activated factor binds to FV in the presence of Ca2+ and PF3 to form prothrombinase complex which converts prothrombin to thrombin that converts fibrinogen to fibrin monomers. FXIII is activated to FXIIIa by thrombin and catalyzes the cross-linking of soluble fibrin monomers to an insoluble fibrin meshwork. FV, FVIII and FXIII circulate as inactive pro-cofactors, and are activated to functional cofactors by the action of thrombin, which is another role of thrombin in coagulation cascade (Brown, 2002; Turgeon, 2012; Palta et al., 2014).

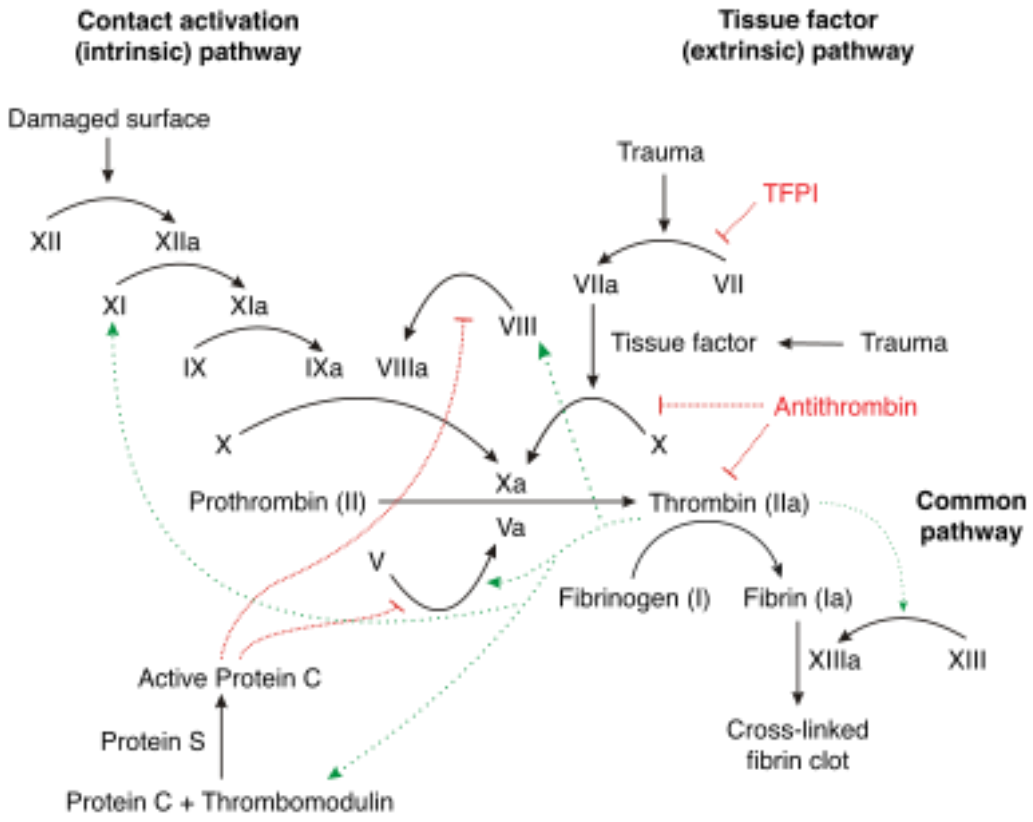

Figure 1.1: Blood coagulation pathways (adopted from Ilesanmi, 2010).

1.1.1.3 Tertiary hemostasis

Tertiary hemostasis is also called fibrinolytic system which is composed of plasminogen, plasmin, tissue plasminogen activator (t-PA) and plasminogen inhibitors. These components have important roles in coagulation system by ensuring that excess fibrin clot does not occur. Fibrinolytic process begins when t-PA activate plasminogen and convert it into plasmin, the latter interacts with fibrin and dissolves it into fibrinogen and fibrinogen degradation products (FDPs). Plasmin activity is controlled by α-2 antiplasmin thus, preventing the widespread of fibrinolysis (Cesarman-Maus & Hajjar, 2005; Palta et al., 2014).

1.2 Bleeding disorders

These disorders mainly are due to pathological inhibition, excessive destruction or defective production of coagulation factors which lead to inability of blood to clot properly. Bleeding disorders can be classified into acquired and congenital coagulopathies. Acquired coagulopathies such as disseminated intravascular coagulation (DIC) and vitamin K deficiency related coagulopathies, whereas congenital coagulopathies occur due to gene defects such as hereditary hemophilia (Turgeon, 2012; Palta et al., 2014).

1.3 Hemophilia

1.3.1 History of hemophilia

Abnormal bleeding was first recorded hundreds of years ago. Jewish rabbinical writings on laws and traditions, from the 2nd century AD, stated in Talmud that baby males should not have to be circumcised if two of their brothers had previously died from the circumcision (Rosner, 1969). Abu Al-Qasim Al-Zahrawi (Albucasis), an Arab physician considered as the greatest medieval surgeon (936-1013 AD), described families whose male relatives died from uncontrolled bleeding after only minor traumas (Kaadan & Angrini, 2010). In 1803, Dr. John Otto (1774-1844 AD), an American physician published the first article that recognizes a hemorrhagic bleeding disorder primarily affected men and passed down in certain families by healthy females (Nilsson, 1994). The term "hemophilia" is derived from the term "hemorrhaphilia" postulated in 1828 by Dr. Schonlein and his student Friedrich Hopff at the University of Zurich (Schramm, 2014). In

1905, Paul Morawitz was the first to assemble coagulation factors into the scheme of coagulation and describe four important components for blood coagulation which are calcium, thrombokinase, prothrombin and fibrinogen (Key et al., 2007). In 1937, anti-hemophilic globulin was discovered (Patek &Taylor, 1937). In 1947, hemophilia A and hemophilia B were found to be separate diseases (Pavlovsky, 1947). In 1952, hemophilia A and B were recognized and described (Aggeler et al., 1952; Biggs et al., 1952).

Hemophilia blood disorder became known as the royal disease, because Queen Victoria of England (1837‑1901 AD) was a carrier, and spread the disease to the royal families of England, Germany, Russia and Spain in the 19^{th} and 20^{th} centuries. The queen passed the mutation responsible for hemophilia to her son Leopold, through some of her daughters who in turn transmitted it on to several of their children (Graw et al., 2005; Faridi et al., 2014). Hemophilia pedigree in Queen Victoria family is shown in Figure 1.2 (Lannoy, 2015).

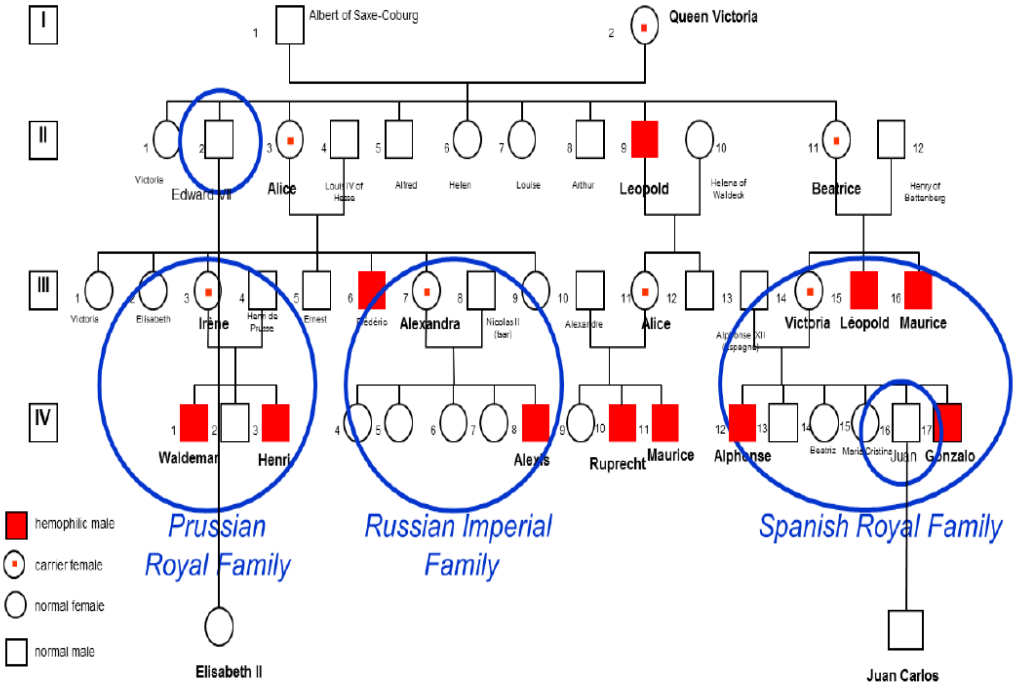

Figure 1.2: Hemophilia pedigrees in Queen Victoria family (adopted from Lanoy, 2015).

1.3.2 Definition, types and epidemiology of hemophilia

Hemophilia is a coagulation disorder by which the blood cannot clot normally at the site of injury leading to prolonged bleeding. Both inherited and acquired disorder forms exist. The inherited form of hemophilia is distinguished by being associated with bleeding problems at early stages of life and has three types known as hemophilia A (OMIM 306700), hemophilia B (OMIM 306900) and hemophilia C that caused by deficiency of FVIII, FIX and FXI, respectively. Acquired hemophilia occurs due to production of autoantibodies in adult life that inactivate FVIII (Franchini et al., 2005; Palta et al., 2014).

Hemophilia A is six times more prevalent than hemophilia B, the incidence of hemophilia A and B is approximately 1 in 5000 and 1 in 30000 male live

births, respectively (Tahiraj, 2012). The World Federation of Hemophilia (WFH) estimates that about 400,000 individuals worldwide with hemophilia of which around 315,000 with hemophilia A type (Skinner et al., 2004; Owaidah et al., 2009).

1.3.3 Hemophilia A

Hemophilia A is bleeding disorder that is inherited in an X-linked recessive pattern and therefore affects males almost exclusively. It is characterized by deficiency in FVIII due to defect in the gene encoding FVIII that lead to prolonged bleeding after injuries (Hwang et al, 2009; Key et al., 2009; Knobe & Berntorp, 2011).

1.3.4 Severity and symptoms of hemophilia A

Hemophilia A is classified to severe, moderate and mild disease based on FVIII activity in plasma and these classes represent 40%, 10% and 50% of patients, respectively. In severe hemophilia A patients, the most common symptoms are deep muscle and spontaneous joint bleeding (hemarthrosis) with average two to five bleeding episodes each month, and those patients are diagnosed within the first year of life when FVIII activity is <1% (<0.01 IU/ml). In moderate hemophilia A individuals, no spontaneous bleeding, however patients can develop joint and muscle hematoma after mild trauma or dental extraction. The bleeding episodes vary from once a month to once a year and excessive bleeding may occur after minor surgery such as dental extraction, those patients can be diagnosed before the age of five years when FVIII activity is 1-5 % (0.01-0.05 IU/ml). In mild

hemophilia A, patients do not bleed spontaneously and bleeding may occur only with major injury or surgery, those patients usually diagnosed at later stage of life where FVIII activity is >5-40% (>0.05-0.40 IU/ml). The classification of severity of hemophilia is set out in the Table 1.2 (White et al., 2001; Key et al., 2009; Knobe & Berntorp, 2011; Konkle et al., 2014).

Table 1.2: Classification of severity of hemophilia A (White et al., 2001).

Severity	FVIII activity % (Level in IU/ml)
Severe	< 1% of normal* (< 0.01)
Moderate	1%-5% of normal (0.01-0.05)
Mild	> 5%-40% of normal (> 0.05-0.40)

* Normal is 1 IU/ml of FVIII concentration (100%).

FVIII activity in carrier female is usually about 50% and do not experience problems due to lyonization phenomenon (X chromosome inactivation), but in certain cases such as skewed lyonization, biased X chromosome inactivation, homozygosity for the hemophilia gene (father with hemophilia and mother is a carrier) or Turner syndrome (XO) may suffer from the disease similarly to hemizygous males (Seeler et al., 1999; Favier et al., 2000; Rossetti et al., 2011).

1.3.5 Treatment of hemophilia A

The treatment of hemophilia may involve prophylaxis, management of bleeding episodes, treatment of FVIII inhibitors and rehabilitation of musculoskeletal complications. Use of factor VIII replacement products and other medications is typically required.

1.3.5.1 Fresh frozen plasma (FFP) and cryoprecipitate (CP)

In the late 1950s, FFP was used to treat hemophilia A and B types. To stop bleeding episodes, large volume of FFP with low amount of FVIII should be administrated intravenously. In mid-1960s, CP was prepared from FFP, this process allowed intravenous administration of more FVIII in a smaller volume. Concerns about the safety and quality of FFP and CP, are not recommended by World Federation of Hemophilia (WFH), and can only be justified in situations where clotting factor concentrates are not available. This due to FFP and CP are not subjected to viral inactivation agents, which increased risk of viral infections such as hepatitis B surface antigen (HBsAg), hepatitis C (HCV) and human immunodeficiency virus (HIV) (Bosch, 1999; Chuansumrit et al., 1999).

Many hemophilic patients who received FFP or CP from 1979 to 1985 infected with HIV or viral hepatitis, other patients infected with both. This occurs due to absence of advanced technologies for screening and detection of various pathogens (Wilde, 2004; Graw et al., 2005; Ludlam et al., 2005; Arnold et al, 2006).

1.3.5.2 Factor VIII concentrate

By the late 1960s, scientists developed methods to separate FVIII pooled plasma, resulting in neatly packaged bottles of lyophilized viral inactivated FVIII concentrate. Each bottle had a label indicating the amount of FVIII

it contained, allowing more accurate dosing which enable the patients to calculate their suitable dose by multiplying the patient's weight in kilograms by the FVIII level desired multiplied by 0.5. Due to quality, safety and early control of hemorrhages and musculoskeletal damage, WFH strongly recommends the use of FVIII concentrate (plasma derived or recombinant) in preference to CP or FFP for the treatment of hemophilia A (Bosch, 1999; Srivastava et al., 2013).

Dose calculated to achieve a FVIII activity of 30-40% (0.30-0.40 IU/ml) for most mild and moderate bleeds, and at least 50% (0.50 IU/ml) for severe bleeding states or prophylaxis of major surgery, and 80-100% (0.80-1 IU/ml)in life-threatening cases such as intracranial bleeding (Srivastava et al., 2013).

Prophylaxis is intravenous (IV) injection of FVIII concentrate to prevent anticipated bleeding episodes; this could be achieved through episodic (on demand treatment) or continuous prophylaxis. Episodic treatment involves IV administration of FVIII at the time of bleeding. Continuous prophylaxis could be primary, secondary or tertiary prophylaxis. Primary prophylaxis is regular continuous treatment which is started before the age of years and after no more than one joint bleed, whereas secondary prophylaxis is regular continuous treatment started after two or more joints bleed. Tertiary prophylaxis is regular continuous treatment which is started after the onset of joint disease is diagnosed (Berntorp et al., 2003; Coppola et al., 2012; Srivastava et al., 2013). Several observational studies approved that

prophylaxis started in early years of life, can protect from hemarthrosis and joint damage (Richards et al., 2010; Collins et al., 2013).

1.3.5.3 Desmopressin

Desmopressin or 1-deamino-8-D-arginine vasopressin (DDAVP) is a synthetic analogue of antidiuretic hormone (ADH) that temporarily boosts the plasma levels of FVIII and VWF after administration three to six folds above baseline. The compound is used for treatment of mild and moderate types of hemophilia A. The main advantages of the use of this compound over FFP and CP are lower cost and absence of risk of viral infection transmission (Mannucci, 1997; Franchini et al., 2010). DDAVP is contraindicated in patients with vascular disease because it increased the risk of thrombosis (Mannucci, 2000; Castaman, 2008).

1.3.5.4 Gene therapy

Hemophilia A and B is considered suitable for gene therapy mainly because it is caused by a single gene abnormality. The main goal of gene therapy for this disease is to replace the defective gene with a corrected sequence to eliminate the disease permanently rather than complete gene replacement. Currently, two possible approaches exist to gene therapy for this gene, these approaches depend on the delivery mechanism to provide a correct copy of the defective gene in somatic cells without removal of the error-containing genomic sequence, which are the *ex vivo* and the *in vivo* gene therapies.

Ex vivo gene therapy involve cells isolation from the patient followed by genetic modification that allows gene expression and secretion of FVIII by the isolated cells. After that isolated cells re-implanted into the patient leading to sustained production of FVIII in circulation. Different cell types have been considered as potential targets for *ex vivo* hemophilia gene therapy. Roth et al., 2001 used dermal fibroblasts from hemophilia A patients by skin biopsy and they grow these cells in culture media and transfected them with a plasmid containing sequences of the gene that encodes FVIII. Cells expressing FVIII were selected and administered to the patients. Increased of FVIII activity were reported in these patients compared with the levels observed before the procedure and the increase in FVIII activity coincided with a decrease in bleeding and a reduction in the use of exogenous FVIII, or both. Although rise in FVIII level lasted for 10 months, it seems to show a promise for the use of such techniques.

The *in vivo* gene therapy, involve the direct injection of patients with gene vectors encoding FVIII leading to *in situ* genetic modification of the target cells. Gene vectors could be non-viral or viral vectors such as retrovirus and adeno-associated virus (Kaufman, 1999; Chuah et al., 2001; Roth *et al.*, 2001; Matsui, 2012). Adenoviruses were used as vectors for hemophilia gene therapy and they considered as the best for this mission, due to several attractive features, such as the genomic size of such viruses which has a large packaging capacity. Also these viruses can infect and express genes at high levels in many different cell types without need the host cell replication, this due to absence of at least one of the essential viral

regulatory genes, so non dividing cells such as liver cells are readily infected. Another advantage using these viruses is that no malignant cells reported after their use (Thorrez et al., 2004; Aburubaiha, 2012).

At the recent, *in vivo* gene therapy for hemophilia is in clinical trials. Certain studies approved the effectiveness of gene therapy for hemophilic patients involving adenovirus vectors. International Society on Thrombosis and hemostasis (ISTH) reported data for a patient who has achieved FIX levels of 20-25% after hepatic gene transfer with adeno-associated virus serotype 8 (AAV8) vectors, this accomplished by incorporating a missense mutation into a codon-optimized FIX sequence, which improves FIX enzymatic activity by 5 to 10 folds and sustained correction from severe to mild hemophilia. However, others treated patients lost therapeutic expression because of $CD8^+$ T-cells responses, which activated by Toll-like receptor 9 (TLR9) signaling against AAV8 capsids. Other clinical trial reported the success of treatment of seven hemophilic B patients by new molecular method that devoid TLR9 activation which decrease immune responses against AAV8, this involve incorporation of FIX expression cassette into a self-complementary AAV (scAAV), to eliminate the need for second-strand synthesis, which limits traditional AAV vectors (Herzog, 2015).

The *in vivo* gene therapy is more cost-effective treatment than *ex vivo* gene therapy. However, the main disadvantage of *in vivo* gene therapy based on viral vectors is that a host immune response towards the viral vector would prevent vector re-administration, if more than one injection would be

required to achieve therapeutic FVIII levels (Aburubaiha, 2012). Several clinical trials based on AAV vectors had to be discontinued because of acute inflammatory responses and severe morbidity and mortality in some patients (Savers, 2002; Herzog, 2015).

1.4 Treatment of complications in hemophilia A

1.4.1 Factor VIII inhibitors

Factor VIII inhibitors are immunoglobulin G (IgG) composed of IgG1 and IgG4 antibodies subtypes that neutralize FVIII activity in plasma (Reding et al., 2002). Inhibitors development is the first challenging complication of FVIII replacement therapy among severe hemophilia A patients. About 30% of severe patients face this complication within the first 50 days of treatment (Kreuz et al., 2003; Oldenburg et al., 2004). Inhibitors formation is very rare in patients with mild and moderate hemophilia A which occurs in about 3-13% of patients (Graw et al., 2005; Witmer &Young, 2012). Many risk factors are involved for inhibitors development such as age at first exposure to FVIII, number of FVIII exposure days, ethnicity, family history and mutation type (Oldenburg et al., 2004; Franchini et al., 2013).

Inhibitors are usually classified according to their levels in plasma as a high-titer inhibitors, those with the highest activity >5 Bethesda Units (BU)/ml or a low-titer inhibitor type. In hemophilia A approximately 60-70% of inhibitors are high titer inhibitors, and the remainders are low titer (White et al, 2001; Owaidah, 2012).

Bleeding episodes in patients with inhibitors can be controlled with high dose of FVIII or using other agents to bypass FVIII, such as recombinant factor VIIa (NovoSeven) which restores hemostasis through its ability to directly activate the coagulation cascade downstream of FVIII (Lollar, 2004; Coppola et al., 2009).

1.4.2 Musculoskeletal complications

Musculoskeletal complications usually occur among severe hemophilic patients before 2 years of age. The most common sites of bleeding are the joints and muscles of the extremities. Frequent bleeding if not properly managed might lead to deterioration of muscles and joints followed by acute hemarthrosis which cause muscles atrophy, joint deformity and synovitis. Failure to manage these signs with inadequate treatment will develop chronic hemophilic arthropathy from the second decade of life, resulting in irreversible musculoskeletal damage (Llinas, 2010; Rodriguez-Merchan, 2010).

1.5 Structure of the human gene encoding factor VIII

Factor VIII gene is located in the distal band of long arm of X-chromosome at position 28 (Xq28) and spans 186 kilo bases (kb). The gene has 26 exons, of which 24 exons were reported to vary in length from 69 to 262 base pair (bp) and 2 exons remarkably large ones which are exon 14 and 26 with 3106 bp and 1958 bp, respectively. Introns located within this gene vary in length; the largest are introns 22 and 1 with approximately 32 kb and 23 kb, respectively (Brown, 2002; Tizzano et al., 2003; Graw et al.,

2005; Keeney et al., 2005). Introns and exons length of FVIII gene are illustrated in Table 1.3 (Brown, 2002; Viel, 2007).

Table1.3: Exon and intron sizes for FVIII gene (Brown, 2002; Viel, 2007).

Exon	Length (bp)	Exon	Length (bp)	Intron	Length (kb)	Intron	Length (kb)
1	313	14	3106	1	22.9	14	22.7
2	122	15	154	2	2.6	15	1.3
3	123	16	213	3	3.9	16	0.3
4	213	17	229	4	5.4	17	0.2
5	69	18	183	5	2.4	18	1.8
6	117	19	117	6	14.2	19	0.6
7	222	20	72	7	2.6	20	1.6
8	262	21	86	8	0.3	21	3.4
9	172	22	156	9	4.8	22	32.4
10	94	23	145	10	3.8	23	1.4
11	215	24	149	11	2.8	24	1
12	151	25	177	12	6.3	25	22.4
13	210	26	1958	13	16		

1.6 Synthesis and structure of coagulation Factor VIII

Factor VIII is a complex heterodimeric glycoprotein that is primarily synthesized by liver cells and secreted to the circulation where it assembled with vWF for stability and protection against the proteolytic action of activated protein C (APC). Factor VIII gene is transcribed into mRNA segment with approximately 9 kb in length which comprises a short 5-untranslated region (5'-UTR), an open reading frame (ORF) plus stop codon and a long 3'-UTR with 150, 7056 and 1806 bp, respectively. It is translated into a precursor protein of 2351 amino acids. Following translation, it undergoes extensive glycosylation in endoplasmic reticulum and sulfation in Golgi apparatus. The signal peptide is comprised of 19

amino acids that is proteolytically removed from N-terminal sequence to give a mature factor VIII of 2332 amino acids. The mature FVIII protein consists of three homologous A domains, two homologous C domains and unique B domain, which are arranged in the order A1-A2-B-A3-C1-C2 from the amino terminus to the carboxyl-terminal end. Further processing events by thrombin occur to obtain a final product of activated FVIII (Figure 1.3). First, cleavage at arginine (Arg)1689 at the junction between domains B and A3 which generates a heavy chain, consisting of domains A1 and A2 and heterogeneous fragments of the partially proteolysed B domain, also A3 domain is released from the C-terminal product to form a light chain that consists of domains A3, C1 and C2. Further cleavages by thrombin at Arg740 which removes most of the B domain and cleavage at Arg372 betweenA1 and A2 domains which generates separated A1 and A2 domains, and then the structure is held in a complex with calcium to give activated FVIII protein (Brown, 2002; Graw et al., 2005; Hwang et al, 2009; Tantawy, 2010).

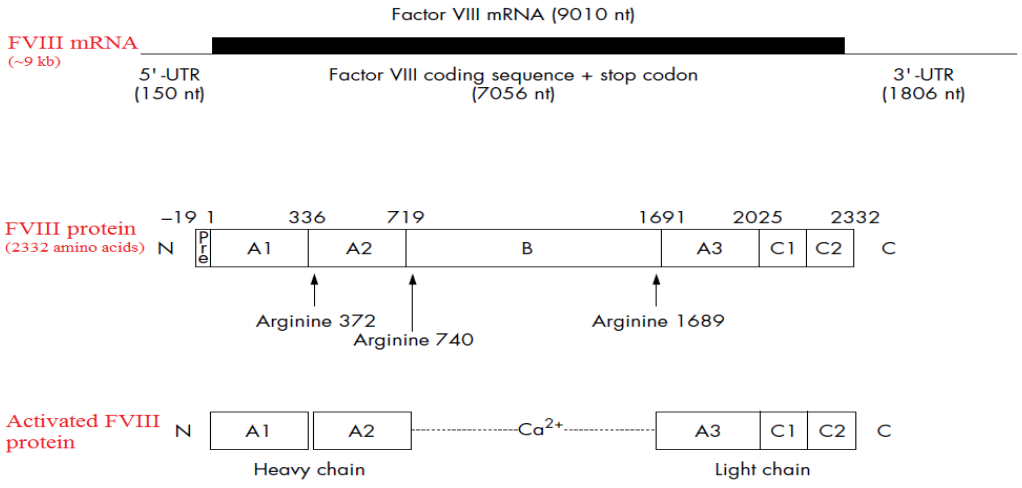

Figure 1.3: Features of FVIII mRNA and FVIII protein (adopted from Brown, 2002).

1.7 Genetic variation in severe hemophilia A

Both small scale mutations and large scale mutations were reported in hemophilia A gene that lead to more than a thousand recognized mutations varieties throughout this gene. Data on hemophilia A mutations structure can be obtained from the resource site (HAMSTeRS) (Lodish et al., 2000; Tantawy, 2010; Albanez et al, 2011).

The small scale mutations are responsible for hemophilia in approximately 45% of severe hemophilia A. These can affect FVIII gene in one or small number of nucleotides, and can be categorized as small insertions or deletions of genetic sequence, gross gene rearrangements and single base substitutions. The letter can lead to missense, nonsense, or splicing defects (Lodish et al., 2000; Key et al., 2009).

The large scale mutations alter the chromosomal structure, causing large insertions, large translocations, large duplications, large deletions and inversions (Lodish et al., 2000).

1.7.1 Intron 22 inversion

Intron 22 inversion is the most frequent inversion that affects FVIII gene, and is responsible for approximately 50% of individuals with severe hemophilia A. Besides being the only large intron, it contains a CpG island that act as a bidirectional promoter for two further small genes nested within FVIII gene, which are FVIIIA (OMIM 305423) and FVIIIB (OMIM 305424). CpG island and FVIIIA are contained within a stretch of DNA of

approximately 9.5kb which together termed as intron 22 homolog 1 (Int22-h1) that is located centromeric and intragenic to FVIII gene, and repeated at two positions with 99% homology located approximately 400 kb telomeric and extragenic to FVIII gene and termed as Int22-h2 and Int22-h3, also known as proximal and distal homologs, respectively. In wild type (WT), FVIIIA is transcribed in the opposite direction to FVIII gene, whereas FVIIIB is transcribed in the same direction to FVIII gene using a private exon within intron 22 that is spliced to exon 23 through exon 26, thus FVIII reading frame is maintained and creating a final overlapping transcript of 2.5 kb. In mutant type, due to the sequence homology of the three regions, these will work as hot spots of intra-chromosomal recombination between the intragenic int22h-1 and one of the two extragenic homologs (int22h-2 or int22h-3), resulting in a large inversion in which exons 1-22 are translocated away from exons 23-26, thereby disrupting FVIII gene, as described in Figure 1.4 (Brown, 2002; Graw et al., 2005; Ilić et al., 2013). This recombination occurs during the meiosis of spermatogenesis, and male germ cells show a >10 fold higher rate of this type of mutation than do females, in which this recombination event is inhibited during meiosis by homologous pairing of the X chromosomes (Rossiter et al., 1994).

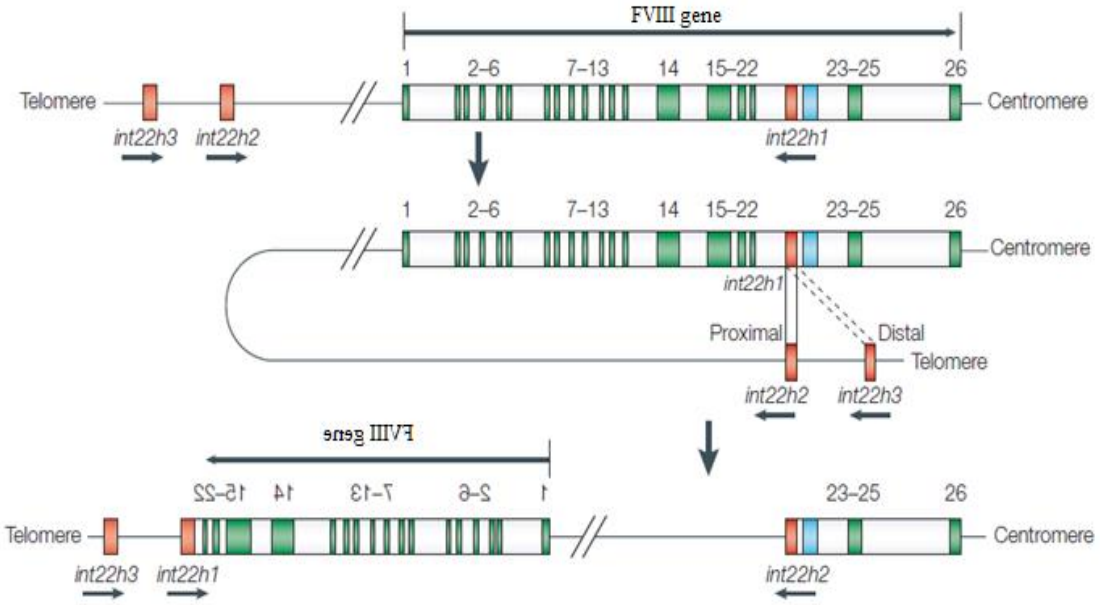

Figure 1.4: Intron 22 inversion of FVIII gene (adopted from Graw et al., 2005).

1.7.2 Intron 1 inversion

Intron 1 inversion is similar to Inv22 but less complicated, and occurs in about 2-5% of individuals with severe hemophilia A. Intron 1 homolog 1 (Int1-h1) is contained in intron 1 of 1041 bp which located centromeric and intragenic to FVIII gene, and repeated at one position located approximately 140 kb telomeric and extragenic to FVIII gene (Int1-h2). These repeats differ only at a single nucleotide and give homology of 99.9%. Intrachromosomal recombination occurs between intragenic Int1-h1 and extragenic Int1-h2 and causes an inversion that displaces exon 1 of FVIII gene by approximately 140 kb toward the telomere, thereby disrupting FVIII gene, as described in Figure 1.5 (Bagnall et al., 2002; Graw et al., 2005; Mantilla-Capacho et al., 2007).

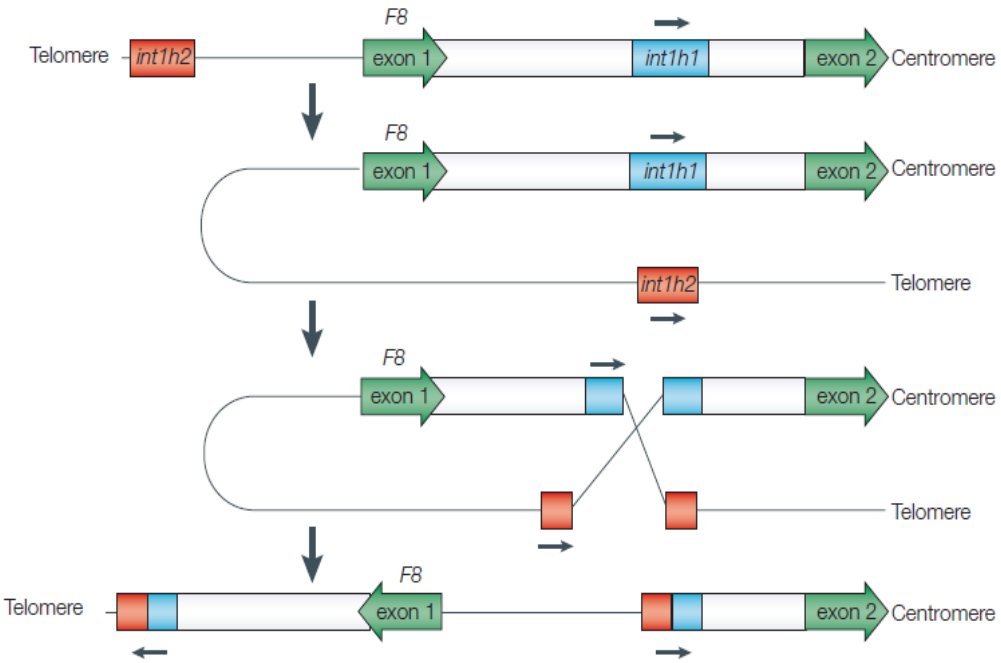

Figure 1.5: Intron 1 inversion of FVIII gene (adopted from Graw et al., 2005).

1.8 Testing and diagnostic strategy of hemophilia A

Evaluation of an individual with a suspected bleeding disorder can be done by many screening tests such as platelets count, bleeding time, activated partial thromboplastin time (APTT) and prothrombin time (PT). In individuals with hemophilia A, the above mentioned tests are normal except prolonged APTT, and in mild hemophilia A the APTT may be normal (Rodeghiero et al., 2008; Franchini et al., 2010; Peerlinck & Jacquemin, 2010). For this reason, there are certain strategies to diagnose individuals with hemophilia A, and can be employed after determining the degree of severity by measuring FVIII activity by coagulation assays. For Severe hemophiliacs A, they should be screened for the Inv22 followed by the Inv1 mutation; this approach could identify the underlying mutation in about 55% of severe hemophilia A patients. Remaining severe hemophilia

A patients should then be analyzed further either by linkage analysis or full gene sequencing. For mild and moderate hemophiliacs A, they require either linkage analysis or full gene sequencing and not require screening for Inv22 or Inv1 which are both uncommon (Keeney et al., 2005).

1.9 Aims

In this study, we aim to identify FVIII gene rearrangements involving intron 22 inversions, being a major causative of disease in severe hemophilia A patient in Palestine. The study represents a necessary step for genetic counseling and disease management for patients in the region.

Chapter Two

Materials and Methods

2.1 Study population

A group consisted of 77 hemophilia A patients (72 males and 5 females) from 52 unrelated families aged 1 month to 67 years (mean: 22.5 ±15.4 years, median: 19 years) from the West Bank area of Palestine were enrolled in the study. Table 2.1 and 2.2 shows the descriptive statistics for the study population.

All participants were contacted and interviewed; data were recorded using specially designed questionnaire which included Socio-demographic data, age, clinical and medical history, date of diagnosis, site of bleeding, frequency of bleeding and type of used treatment (Appendix A). Confidentiality was assured by the attribution of a coded identifier. A group of subjects was excluded which is consists of Subjects with bacterial or viral infection or those who had transfusion of FVIII concentrate in the preceding 3-5 days of blood sampling were excluded.

Table 2.1: Age and districts distribution of enrolled subjects

Variable		No.	Percentage (%)
Gender	Male	72	93.5
	Female	5	6.5
Age groups (year)	0.1-10	22	28.5
	11-20	17	22.1
	21-30	17	22.1
	31-40	10	13
	41-50	8	10.4
	51-60	2	2.6
	61-70	1	1.3
Districts	Al-Bireh	3	3.9
	Bethlehem	10	13
	Hebron	14	18.2
	Jenin	8	10.4
	Jerusalem	2	2.6
	Nablus	18	23.3
	Qalqilya	2	2.6
	Ramallah	10	13
	Salfeet	1	1.3
	Tubas	1	1.3
	Tulkarem	8	10.4

Table 2.2: Age and districts distribution of each subject.

ID	Sex	Age	District	ID	Sex	Age	District	ID	Sex	Age	District
S 1	M	44	Tubas	S 25	M	30	Hebron	S 60	M	67	Nablus
S 2	M	31	Jenin	S 26	M	21	Hebron	S 67	M	19	Nablus
S 3	M	6	Jenin	S 27	M	48	Hebron	S 72	M	25	Nablus
S 4	M	33	Jenin	S 44	M	10	Hebron	S 73	M	26	Nablus
S 5	M	7	Jenin	S 45	M	9	Hebron	S 35	M	17	Qalqilya
S 6	M	18	Jenin	S 46	M	5	Hebron	S 36	M	14	Qalqilya
S 66	M	37	Jenin	S 69	M	15	Hebron	S 53	F	29	Bethlehem
S 68	M	19	Jenin	S 74	M	42	Hebron	S 54	M	5	Bethlehem
S 71	M	29	Jenin	S 75	M	4	Hebron	S 55	M	3	Bethlehem
S 7	M	12	Al-Beira	S 76	M	2	Hebron	S 56	M	11	Bethlehem
S 8	M	9	Al-Beira	S 22	M	15	Jerusalem	S 57	F	30	Bethlehem
S 10	M	8	Al-Beira	S 23	M	15	Jerusalem	S 48	M	6	Bethlehem
S 9	M	36	Ramallah	S 28	M	55	Nablus	S 49	M	0.1	Bethlehem
S 15	M	23	Ramallah	S 29	M	41	Nablus	S 50	M	6	Bethlehem
S 16	M	15	Ramallah	S 30	M	16	Nablus	S 51	F	37	Bethlehem
S 17	F	35	Ramallah	S 31	M	38	Nablus	S 52	M	5	Bethlehem
S 18	M	9	Ramallah	S 32	M	49	Nablus	S 58	M	12	Tulkarem
S 19	M	22	Ramallah	S 33	M	5	Nablus	S 59	M	24	Tulkarem
S 20	M	30	Ramallah	S 34	M	50	Nablus	S 61	M	10	Tulkarem
S 21	M	29	Ramallah	S 38	M	47	Nablus	S 62	M	14	Tulkarem
S 24	M	19	Ramallah	S 39	M	60	Nablus	S 63	M	3	Tulkarem
S 77	M	6	Ramallah	S 40	M	22	Nablus	S 64	M	4	Tulkarem
S 11	M	33	Hebron	S 41	M	14	Nablus	S 65	F	25	Tulkarem
S 12	M	42	Hebron	S 42	M	23	Nablus	S 70	M	31	Tulkarem
S 13	M	30	Hebron	S 43	M	6	Nablus	S 37	M	40	Salfeet
S 14	M	25	Hebron	S 47	M	18	Nablus				

2.2 Ethical consideration

The research protocol was approved by the institutional review board (IRB) at An-Najah National University (Appendix B). Purpose and objectives of the study were explained to the participants or their guardians through consent form (Appendix C).

2.3 Samples collection

After obtaining informed consent from all involved subjects, 10 ml of peripheral blood samples were collected in two types of tubes; tripotassium ethylene-diamine-tetraacetate (EDTA K3) tubes and 3.2% trisodium citrate tubes (Greiner Bio-One, Austria). EDTA K3 tubes were stored at 4°C and were used later on for genomic DNA extraction, whereas the 3.2% trisodium citrate tubes processed immediately; centrifuged at 5000 rpm for 10 minutes (EBA20, Hettich ZENTRIFUGEN , Germany), then the plasma was separated and stored at -20°C and used later on for FVIII activity measurements. All blood samples were taken at least 1 week following FVIII infusion.

2.4 FVIII activity measurements

Factor VIII activity was measured by one-stage clotting assay using FVIII immune-depleted plasma. This method is based on the measurement of APTT, which measures the ability of patient plasma or standard through serial dilutions to shorten APTT of FVIII deficient plasma; where all factors are present and in excess except FVIII that is depleted from the

tested sample. The intrinsic coagulation pathway is activated by the addition of cephaline to mixture containing FVIII deficient plasma and sample to be tested, the clot formation is produced by adding calcium to the mixture and the time needed to generate the clot reflect the APTT. Time of measured samples is converted into percentages using a standard curve that was constructed from standard with known FVIII activity concentrations through serial dilutions. Each dilution is related to corresponding FVIII activity, by which the place where the test line passes through certain activity is read from the standard line that gives the concentration of the test in percentage of standard, as described in Figure 2.1 (Kitchen et al., 2000; Mackie et al., 2013; Moser, 2014).

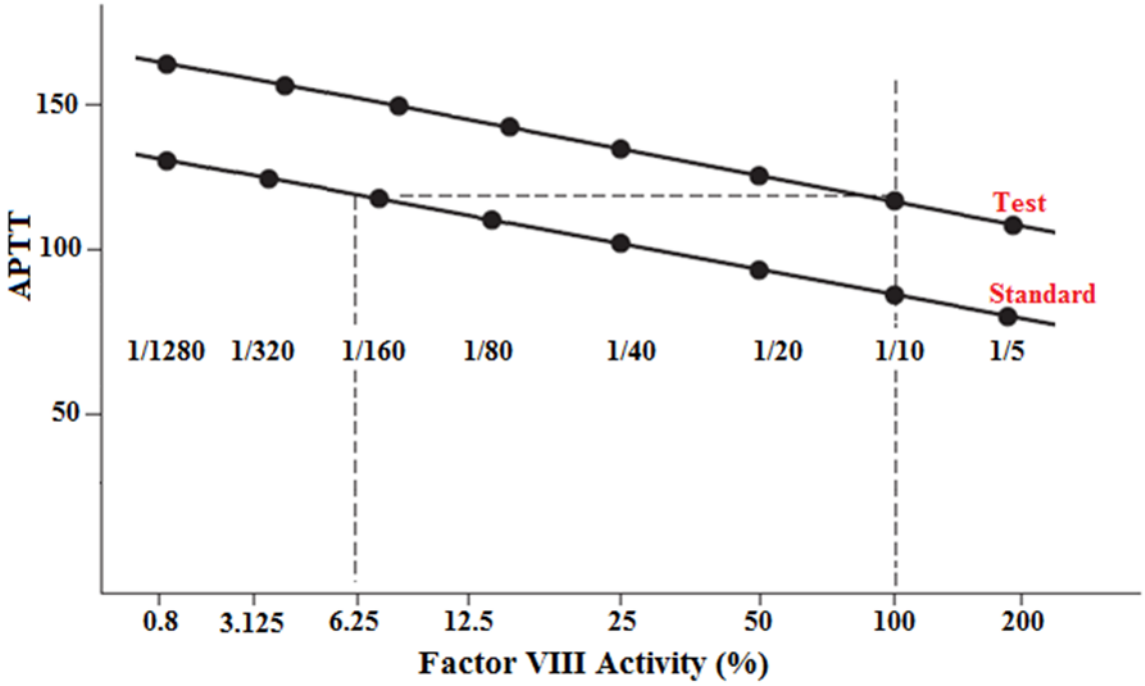

Figure 2.1: Graph of FVIII activity assay.

Except where otherwise stated, all materials used for FVIII activity measurement are from Diagnostic Stago Company (Diagnostica Stago, Asnieres, France). The FVIII deficient plasma vials of STA Deficient VIII kit were reconstituted according to manufacturer's instructions (Appendix D), and then eight serial dilutions of both citrated test plasma and standard; containing 100% FVIII activity; were prepared in glass tubes using buffered saline as a diluent. These dilutions are 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:1280 which are equivalent to 200%, 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 0.8% activity, respectively. For 0.1 ml of each dilution, 0.1 ml of FVIII deficient plasma were added and transferred to 37°C water bath (NÜVE, Turkey), then 0.1 ml of APTT reagent which contains cephalin were added to the mixture and incubated for 5 minutes, then the mixture was transferred to semi-automated coagulometer with build in stop watch (RAL Clot 2B, S.A, Spain) followed by addition of 0.1 ml of 0.025 M $CaCl_2$, stop watch started automatically and stopped when the clot formed. The clotting times which reflect the APTT were recorded and plotted on FVIII assay graph, as described in Figure 2.1. Blanked test was used as negative (low) control and performed in same procedures of tested plasma or standard with replacing the tested sample with undiluted buffered saline. Also, sample expected to be normal was used as a positive control.

2.5 DNA extraction

Genomic DNA was extracted from EDTA K3 anticoagulated whole blood using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA purity, quality and quantity were assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) according to manufacturer's instructions. Briefly, 1µl sample was pipetted onto the lower measurement pedestal, and then a spectral measurement was initiated using NanoDrop 2000 software (Appendix E). Isolated genomic DNA was stored at -20°C until tested.

2.6 PCR primers

Four primers; P, Q, A and B were used in the PCR reaction, as illustrated in Table 2.3. All PCR primers sequences are indicated from 5' to 3' end and most of them contained several additional nucleotides to serve as a GC-clamp, these sequences were obtained from the literature and checked for specificity through two tools; UCSC In-Silico PCR (www.genome.ucsc.edu) and NCBI Primer-BLAST.

Table 2.3: PCR Primers sequences used to detect factor VIII Inv22 (Liu & Sommer, 1998; Keeney et al., 2005; Mühle, 2008).

Primer	Sequence (5'→3')	Length	GenBank Accession No.	Specificity	PCR product sizes
P	GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC	38	AF062514	Forward for int22h1	AB=10 kb (WT, carrier, inversion)
Q	GGC CCT ACA ACC ATT CTG CCT TTC ACT TTC AGT GCA ATA	39	X86012	Reverse for int22h1	PQ=12 kb (WT, carrier)
A	CAC AAG GGG GAA GAG TGT GAG GGT GTG GGA TAA GAA	36	AF062515	Forward for int22h2&3	PB=11 kb (carrier, inversion)
B	CCC CAA ACT ATA ACC AGC ACC TTG AAC TTC CCC TCT CAT A	40	AF062516	Reverse for int22h2&3	AQ=11 kb (carrier, inversion)

These primers were used to differentiate between normal, inversion patients and carrier females. As described in Figure 2.2, the upper box indicates int22h1, whereas the lower box indicates int22h2 and int22h3, and the dashed lines indicate the flanking sequences. Primers P&Q are specific to flanking sequences of int22h1 that located at -1212 bp before and at +1334 bp after the homolog, and produce 12 kb segment in DNA of wild type and carrier females. Primers A&B are specific to the flanking sequences of int22h2 and int22h3 that located at -167 bp before and at +118 bp after the homologs and always produce 10 kb segment, because at least one copy of Int22h2 and Int22h3 remains intact and could serve as a positive control. Inv22 can occur by homologous recombination between Int22h1 and either Int22h2 or Int22h3 which was represented by dotted line, thus each P&B and A&Q produce 11 kb segments in DNA of male

patients and carrier females(Liu & Sommer, 1998; Poláková et al., 2003; Keeney et al., 2005; Mühle, 2008).

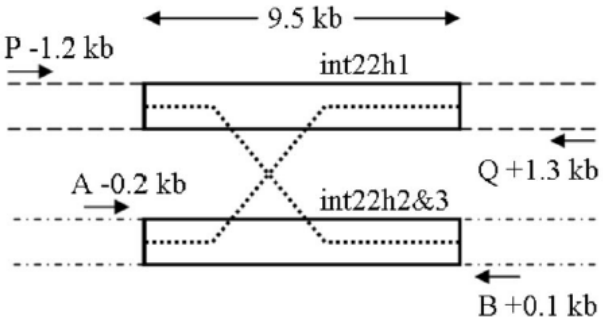

Figure 2.2: Scheme of primer location (Liu et al., 1998; Poláková et al., 2003).

2.7 Inv22 detection

Subcycling PCR was performed using Expand Long Template PCR kit (Roche, Mannheim, Germany) as previously described by modification of the original protocol (Liu &Sommer1998; Liu et al., 1998). The primers were reconstituted according to manufacturer's instructions (Appendix D). Four combinations of two primers A&B, P&Q, A&Q and P&B per sample were done for detection of Inv22 in the FVIII gene, which is performed in four single reactions for severe hemophilic A patients as well as for expected carriers samples (Appendix F). A total of 25 µl reaction volume in 0.2 ml thin wall tubes contained 2.5 µl buffer 3, 0.75 mM Mgcl2, 1.75 U enzyme mix, 200 µmolol 7-deaza-dGTP (Roche, Mannheim, Germany), 300 µmolol dGTP and 500 µmolol of each of the other dNTPs (Sigma, Deisenhofen, Germany), 0.2 µmolol of each forward and reverse primer (Hylab, Ltd., Israel), 7.5% dimethyl sulphoxide (DMSO) and 50-100 ng of human genomic DNA template. Bio-Rad Thermal Cycler (T100™, Foster

city, California, USA) was used for DNA amplification, and the S-PCR conditions were: initial denaturation at 95°C for 2 minutes, the first 10 cycles were 95^0C for 30 seconds followed by four subcycles of annealing/elongation step alternating temperature between 63°C for 2 minutes and 68°C for 2 minutes for each subcycle. The remaining 20 cycles were modified by the addition of an extra 3 seconds per cycle for each step of the annealing/elongation step, followed by a final elongation step of 68°C for 7 minutes. The tubes were left at a hold temperature of 4°C, as described in Table 2.4.

Table 2.4: Thermal Cycler Program for S-PCR.

Step	Temperature	Time (mm:ss)[a]		Cycles
Denaturation	95°C	02:00		1
Denaturation	95°C	00:30		10
Annealing/Elongation	63°C	02:00	4 subcycles	
	68°C	02:00		
Denaturation	95°C	00:30		20
Annealing/Elongation	63°C	02:00 + 00:03/cycle	4 subcycles	
	68°C	02:00 + 00:03/cycle		
Final Elongation	68°C	07:00		1
Cooling	4°C	∞[b]		

[a] Minute: Second

[b] Infinity

2.8 Agarose gel electrophoresis

For 1% agarose gel preparation, 1 gram of dried agarose (Sigma, Deisenhofen, Germany) were dissolved in a flask containing 100 ml of 1x TBA buffer (Promega, Madison, USA) previously prepared according to manufacturer's instructions (Appendix D) , then the solution was placed in

microwave and boiled for 2 minutes. After cooling the solution to 60^0C, 10 µl of ethidium bromide (Promega, Madison, USA) were added. The solution was poured into a gel tray carrying a comb for wells formation, and then the comb was removed when the gel solidified. The gel placed in a horizontal gel chamber within electrophoresis power supply apparatus (Jencons, UK) and filled with 1x TBE buffer about 0.5cm above the gel surface. PCR products were mixed with DNA loading dye (Thermo Scientific, Waltham, MA) before loading in the gel in ratio 1:6, by which 10 µl of was mixed with 2 µl of 6x loading dye (Fermentas) and loaded into the well, and then the gel was run at 70 volts for 2 hours in parallel to a lambda DNA Hind III marker (Thermo Scientific, 0.1 kb-23 kb). Bands were visualized and documented using ultraviolet trans-illuminator documentation system (Uvitec, Cambridge, UK).

Chapter Three

Results and Discussion

3.1 FVIII activity levels

Factor VIII activity was measured in duplicates for all hemophiliacs A patients as previously described in materials and methods (section 2.4). The quality of the assay was controlled through low (blank) and a normal control, where FVIII activity levels of the blank reflects the quality of FVIII deficient plasma and should be to less than 1%. According to FVIII activity patients with levels less than 1%were classified as severe, 1-5% of FVIII activity in the blood were classified as moderate and >5-40% were classified as mild (White et al., 2001; Knobe & Berntorp, 2011).

The distribution of patients according to FVIII activity levels and disease severity are shown in and Table 3.1. According to our findings, 30 (41.7%) cases were grouped as severe, 16 (22.2%) cases as moderate and 26 (36.1%) of the studied cases as mild hemophilia A.

Table 3.1: FVIII activity levels among study population.

ID	FVIII activity (%)	Clinical severity	ID	FVIII activity (%)	Clinical severity	ID	FVIII activity (%)	Clinical severity
S 1	<1	Severe	S 62	<1	Severe	S 14	16.7	Mild
S 3	<1	Severe	S 63	<1	Severe	S 15	39.5	Mild
S 6	<1	Severe	S 75	<1	Severe	S 21	7	Mild
S 7	<1	Severe	S 76	<1	Severe	S 23	10.5	Mild
S 8	<1	Severe	S 77	<1	Severe	S 24	10.8	Mild
S 10	<1	Severe	S 2	2.4	Moderate	S 25	28	Mild
S 16	<1	Severe	S 4	3.1	Moderate	S 26	38	Mild
S 18	<1	Severe	S 5	1.3	Moderate	S 28	39	Mild
S 19	<1	Severe	S 29	3.1	Moderate	S 31	6	Mild
S 20	<1	Severe	S 32	4.5	Moderate	S 38	6.2	Mild
S 22	<1	Severe	S 34	4.9	Moderate	S 39	5.2	Mild
S 27	<1	Severe	S 40	3.4	Moderate	S 50	16.5	Mild
S 30	<1	Severe	S 41	3.8	Moderate	S 54	39	Mild
S 33	<1	Severe	S 42	3.1	Moderate	S 66	10.1	Mild
S 35	<1	Severe	S 44	1.2	Moderate	S 67	15.6	Mild
S 36	<1	Severe	S 45	1.5	Moderate	S 68	5.4	Mild
S 37	<1	Severe	S 46	3.1	Moderate	S 69	16.7	Mild
S 43	<1	Severe	S 47	4.2	Moderate	S 70	16.2	Mild
S 48	<1	Severe	S 58	3.5	Moderate	S 71	32.4	Mild
S 49	<1	Severe	S 60	2.5	Moderate	S 72	16.9	Mild
S 52	<1	Severe	S 64	2.7	Moderate	S 73	15.8	Mild
S 55	<1	Severe	S 9	17	Mild	S 74	13.1	Mild
S 56	<1	Severe	S 11	6.3	Mild	N*	98	-
S 59	<1	Severe	S 12	11	Mild	L**	<1	-
S 61	<1	Severe	S 13	6.3	Mild			

* N: Normal control
** L: Low control

3.2 Inv22 status among study population

According to previously reported literature, most studies showed that Inv22on X-Chromosome seems to be associated with disease severity (Graw et al., 2005; Ilić et al., 2013). To elucidate the role of this inversion in our population, all severe cases (30 patients) were tested for the presence of this inversion. In addition, five mothers of these severely classified patients were randomly selected and included for evaluation of the carrier

status of this Inv22. Wild type healthy male controls (15) were also included for the analysis for this inversion using PCR.

Among tested the 30 severe hemophilia A patients, 11 were found to be hemizygous for Inv22 on the basis of absence of the PQ amplified product as shown in Figures 3.1 and 3.2.

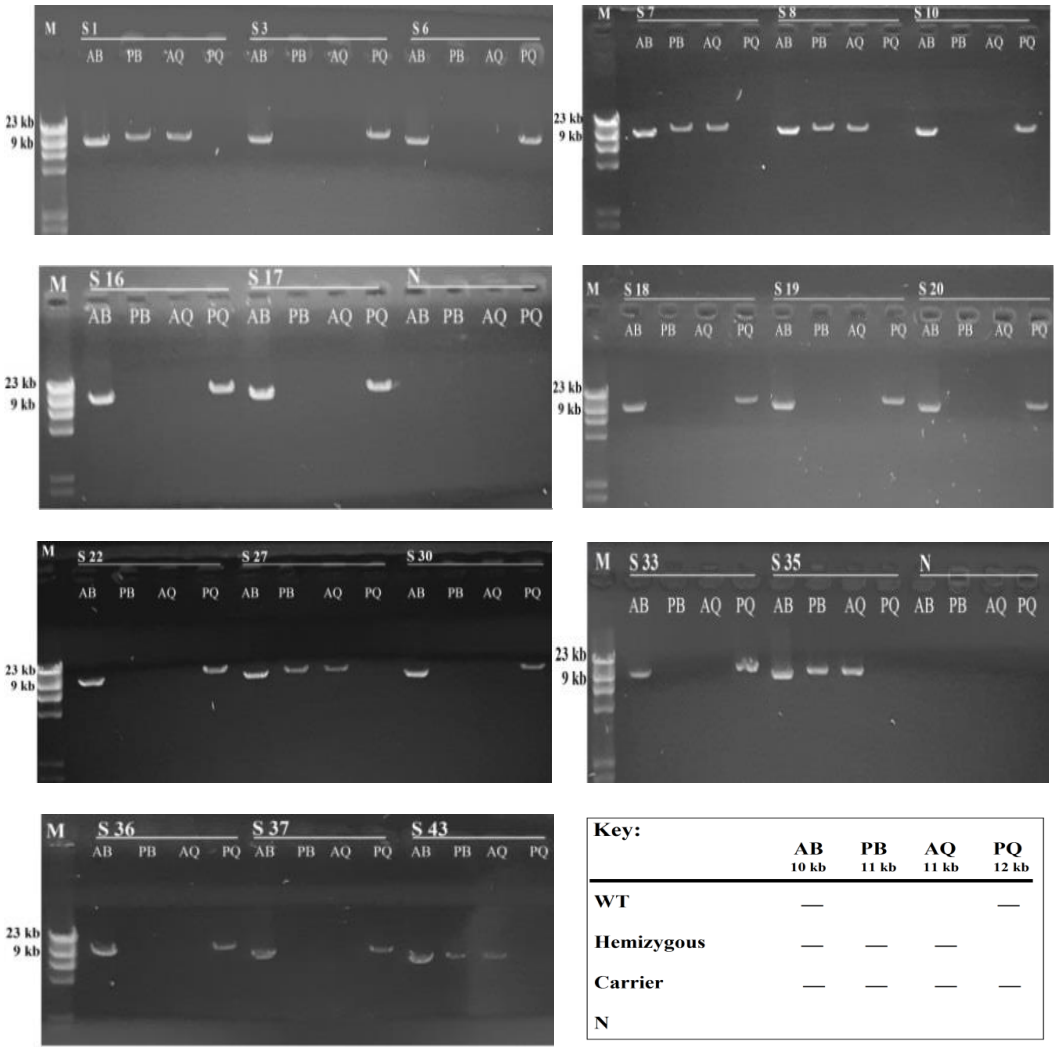

Figure 3.1: S-PCR analyses for the FVIII intron 22 related inversion using 1% agarose gels. AB, PB, AQ and PQ are primers related bands for samples 1, 3, 6, 7, 8, 10, 16, 17, 18, 19, 20, 22, 27, 30, 33, 35, 36, 37 and 43. M indicates □□DNA HindIII ladder marker (0.1 kb-23 kb). N indicates negative control (no template). S indicates sample number**.**

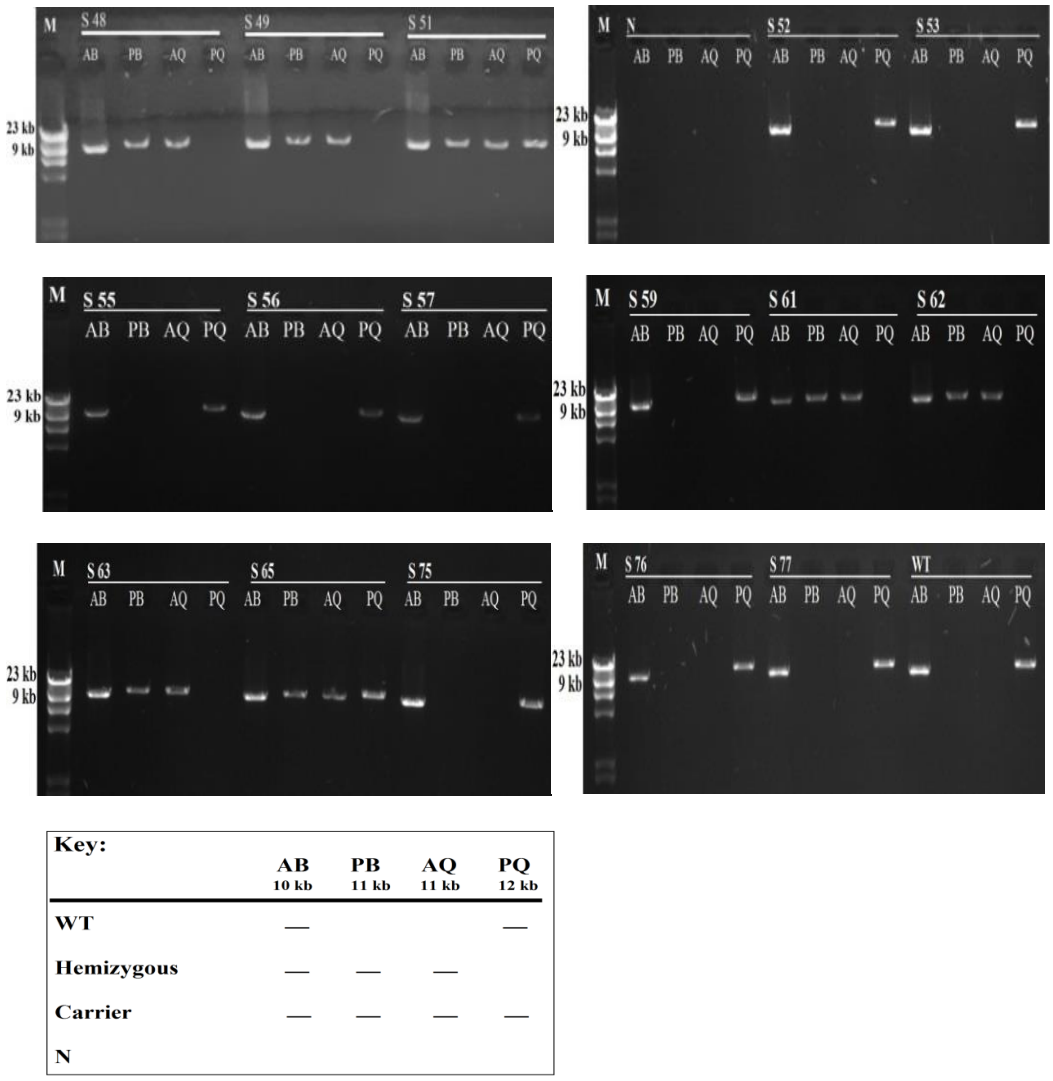

Figure 3.2: S-PCR analyses for the FVIII intron 22 related inversion using 1% agarose gels. AB, PB, AQ and PQ are primers related bands for samples 48, 49, 51, 52, 53, 55, 56, 57, 59, 61, 62, 63, 65, 75, 76 and 77. M indicates λ DNA HindIII marker (0.1 kb-23 kb). N indicates negative control (no template). WT indicates wild type control. S indicates sample number.

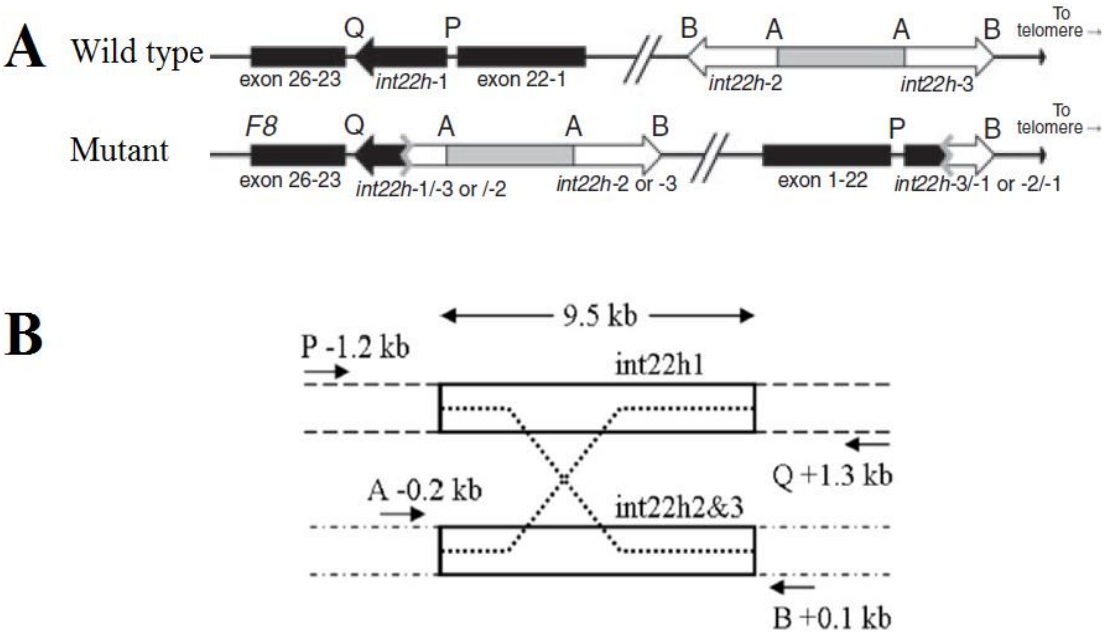

Figure 3.3: Schematic presentation of FVIII intron 22 inversion. (A) A scheme of expected structures of wild and mutant type (Adopted from Fujita et al., 2012). (B) A scheme of primer location and sizes of PCR products (Adopted from Poláková et al., 2003).

The diagram in Figure 3.3 illustrate the nature of the band resulted in both normal wild type and mutant states. For each tested DNA sample, the first lane is a 10 kb amplified fragment represent the AB product which is amplified in wild type, carrier and inversion cases. The second lane is an11 kb amplified fragment represent the PB product which is amplified in carrier and inversion cases, and was from int22h-1/-3 or int22h -1/-2. The third lane is an 11 kb amplified fragment represents the AQ product which is amplified in carrier and inversion cases, and was from int22h-3/-1 or int22h -2/-1as clarified in Figure 3.3. The fourth lane is a 12 kb amplified fragment represent the PQ product and amplified in both carrier and wild type states.

Among the tested 5 mothers (S 17, 51, 53, 57 and 65), Inv22 was detected in only 2 mothers, as shown in Figure 3.4. Of these five mothers, two mothers S 51 and S 65 (mothers of patients S 48, S 49 and S 63) were carriers as indicated by the presence of PQ products. As illustrated in Figure 3.4, one should expect that female carrier are expected to show positive amplifications of all bands (AB, PB, AQ and PQ), while affected male showed a positive amplification of three bands (AB, PB and AQ).

Since the hemophilia is an X linked disorder it was reasonable to carry out pedigree analysis involving the carrier female cases reported in this study. Data presented in Figure 3.4 clearly showed this mode of inheritance as both mothers transmitted this mutation to their sons. The mother has one altered copy and one normal copy of FVIII gene, and she can pass either of these gene copies to her children. So, there is 50% chance that each son will have hemophilia and 50% chance that each daughter will be a carrier for the hemophilia gene (Zimmerman & Valentino, 2013).

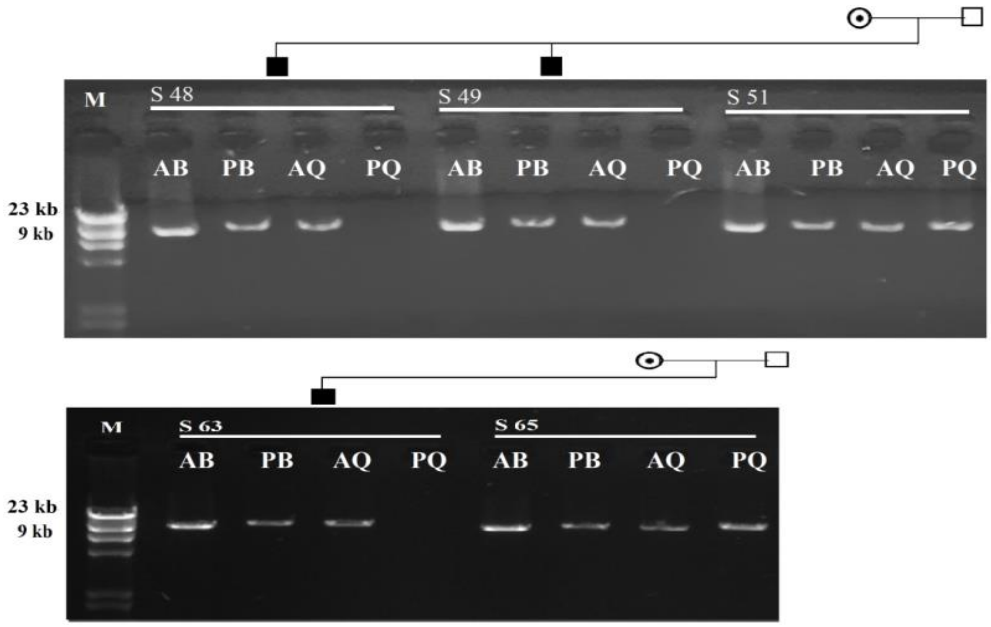

Figure 3.4: Family pedigree analysis of the carrier females included in the current study. AB, PB, AQ and PQ are primers related bands. Θ= carrier, ■= affected male, □= unaffected male. M indicates λ DNA HindIII marker (0.1 kb-23 kb). S indicates sample number.

A total of 11 patients were hemizygous for this inversion, thus the observed Inv22 frequency in this study was 37%. This frequency is similar to that reported in Iraq (39%), Japan (33.3%), Brazil (39.4%) and China (37%) and lower than frequency reported in Jordan (52%), Saudi Arabia (50%) and Egypt (46.1%), but higher than reported for Lebanon (29%), Tunis (22.7%) and Albania (10.5%), as illustrated in Table 3.2.

The low prevalence of Inv22 in Tunisian patients with severe hemophilia A was attributed to the limited size of sample involved in the study, and these data must be confirmed with a larger number of studied patients (Elmahmoudi et al., 2012). No explanation was reported for the low prevalence of this inversion among Albanian patients and it was suggested that this issue should be addressed by further studies (Castaman et al., 2007). The reported Inv22 high frequency of 55% among Arab patients

with severe hemophilia A (Abu-Amero et al., 2008), which is higher than the results reported in various worldwide populations, was attributed by the authors to ethnic variations and the small cohort studies.

Table 3.2: Inversion 22 frequency worldwide among hemophilia A patients.

Country	Inv22 %	Reference
Palestine	37 %	Current study
Jordan	52 %	Awidi et al., 2010
Lebanon	29 %	Djambas Khayat et al., 2008
Iraq	39 %	Hassan &Jabber, 2016
Saudi Arabia	50 %	Owaidah et al., 2009
Egypt	46.1%	Abou-Elew et al., 2011
Tunis	22.7 %	Elmahmoudi et al., 2012
Arab patients	55%	Abu-Amero et al., (2008)
Turkey	42 %	EL-Maarri et al., 1999
Iran	47 %	Roozafzay et al., 2013
Spain	41.2 %	Tizzano et al., 1994
Italy	42 %	Acquila et al., 2003
United Kingdom (UK)	45 %	Bagnall et al., 2002
Serbia	42 %	Ilić et al., 2013
Albania	10.5 %	Castaman et al., 2007
Hungary	52 %	Andrikovics et al., 2003
Poland	50.4 %	Sawecka et al., 2005
Japan	33.3 %	Inaba et al., 1995
China	37 %	Lin et al., 1995
Taiwan	42.7 %	Lin et al., 2008
Thailand	29.8 %	Yenchitsomanus et al., 2003
Venezuela	41 %	Albanez et al, 2011
India	46 %	Jayandharan et al., 2004
Pakistan	44 %	Khanum, 2014
Brazil	39.4 %	Soares et al., 2001
Mexico	45.2 %	Mantilla-Capacho et al., 2007
Argentina	41 %	De Brasi et al., 2000

3.3 Clinical manifestations

All patients tested for Inv22 were vary with respect to family history, treatment regimen, first bleeding site and age at first bleeding, as shown in Figure 3.5 and Table 3.3 and nothing seems to be commonly shared among

this group. All 5 mothers of severe patients were with a positive family history of bleeding and 2 of them were found to be carrier for Inv22 based on PCR analysis. Of the 30 severe hemophilia A patients included in the current study, family history of bleeding was presented in 29 (96.7%), and 11 patients of this group were found to have Inv22 mutation. In absence of this mutation, other genetic testing should be considered, which are Inv1 mutation on the same chromosome and full gene sequencing.

Age at first bleeding varied from less than 1 month to 2 years, a high proportion of first bled occurred at circumcision which represents 83.3% of severe patients, other types of first bleeding occurred post traumatic (6.7%) or due to muscle hematoma (10%).

According to treatment regimen, all individuals were treated with FVIII concentrate (plasma derived) and 76.7% of them are on-demand treatment, while 23.3% are receiving prophylactic treatment.

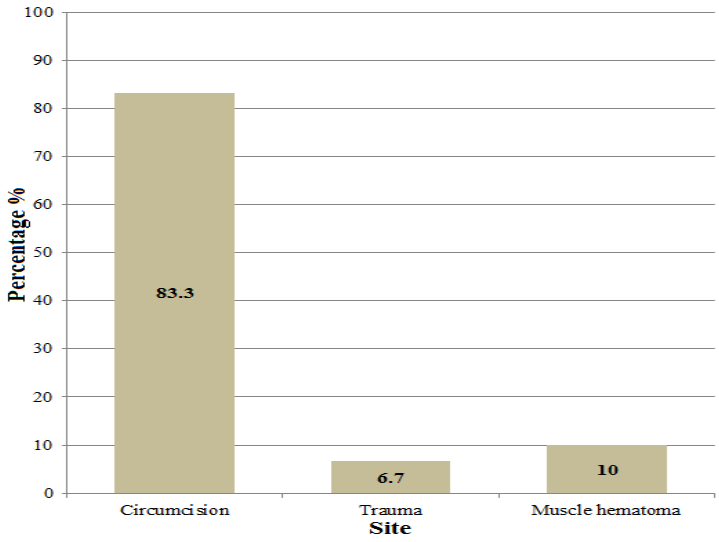

Figure 3.5: First bleeding sites among severe hemophilia A individuals**.**

Table 3.3: General characteristics of subjects screened for Inv22.

ID	Sex	Age at first bleeding	Clinical severity	Treatment regimen	Family History	Inv22
S 1	M	2 years	Severe	Prophylaxis	+ve	+ve
S 3	M	< 1 month	Severe	on-demand	+ve	-ve
S 6	M	2 years	Severe	on-demand	+ve	-ve
S 7	M	< 1 month	Severe	on-demand	+ve	+ve
S 8	M	< 1 month	Severe	on-demand	+ve	+ve
S 10	M	3 months	Severe	on-demand	+ve	-ve
S 16	M	< 1 month	Severe	Prophylaxis	+ve	-ve
S 17	F	-	-	-	+ve	-ve
S 18	M	< 1 month	Severe	on-demand	+ve	-ve
S 19	M	< 1 month	Severe	on-demand	+ve	-ve
S 20	M	< 1 month	Severe	on-demand	+ve	-ve
S 22	M	< 1 month	Severe	on-demand	+ve	-ve
S 27	M	< 1 month	Severe	Prophylaxis	+ve	+ve
S 30	M	< 1 month	Severe	on-demand	+ve	-ve
S 33	M	6 months	Severe	Prophylaxis	+ve	-ve
S 35	M	< 1 month	Severe	on-demand	+ve	+ve
S 36	M	< 1 month	Severe	on-demand	+ve	-ve
S 37	M	< 1 month	Severe	on-demand	+ve	-ve
S 43	M	< 1 month	Severe	Prophylaxis	+ve	+ve
S 48	M	6 months	Severe	on-demand	+ve	+ve
S 49	M	< 1 month	Severe	on-demand	+ve	+ve
S 51	F	-	-	-	+ve	Carrier
S 52	M	< 1 month	Severe	on-demand	+ve	-ve
S 53	F	-	-	-	+ve	-ve
S 55	M	< 1 month	Severe	Prophylaxis	+ve	-ve
S 56	M	< 1 month	Severe	Prophylaxis	+ve	-ve
S 57	F	-	-	-	+ve	-ve
S 59	M	< 1 month	Severe	on-demand	+ve	-ve
S 61	M	< 1 month	Severe	on-demand	+ve	+ve
S 62	M	< 1 month	Severe	on-demand	+ve	+ve
S 63	M	< 1 month	Severe	on-demand	+ve	+ve
S 65	F	-	-	-	+ve	Carrier
S75	M	< 1 month	Severe	on-demand	+ve	-ve
S 76	M	< 1 month	Severe	on-demand	-ve	-ve
S 77	M	< 1 month	Severe	on-demand	+ve	-ve

+ve: Positive
-ve: Negative

Chapter Four

Recommendations and Concluding Remarks

This is the first study to investigate Inv22 mutation in x-chromosome among severe hemophilia A patients as well as suspected carriers in Palestine. This mutation is responsible for approximately 50% of individuals with severe hemophilia A worldwide, and should be the first line for testing severe hemophilia A. In absence of this mutation, other genetic testing should be considered, which are Inv1 mutation on the same chromosome and full gene sequencing. Unfortunately, no sufficient data available for hemophilia A complications such as FVIII inhibitor development, musculoskeletal complications, anemia and infectious diseases, which unable to correlate these complications with severity of the disease. Therefore, this study should be addressed by further studies. On contrary, some benefits were achieved from the study; the results obtained can be helpful for genetic counseling, prenatal diagnosis and carrier detection of this disease. In addition, certain strategies could be applied to improve the quality of life for hemophilia A patients including:

1- Establishment of promotional and educational guidelines for hemophilia A individuals about the nature of disease.

2- FVIII inhibitor development; which is the most common complication among hemophiliac A; should be monitored through programmed testing strategy.

3- Most of hemophiliac A individuals in Palestine had minimal access to FVIII concentrate and treated on demand instead of prophylactically, and certain strategies should be taken to ensure adequate supply of safe FVIII

concentrate, which decreases the risk of infectious diseases and musculoskeletal complications.

4- Use of FVIII concentrates instead of FFP or CP to minimize the risk of infectious diseases such as HBsAg, HCV and HIV.

5- Establishment of annual testing strategy for monitoring of infectious diseases among hemophiliac patients.

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Appendix A
Hemophilia Questionnaire

Demography:

Name:_____**Sex:** Male□/ Female□

Date of Birth: _____/_____/_____ Age **group:**_____

Address: _____Type of locality: _____ District:

_____

Phone_____Occupation: _____

Clinical History:

Height:_____ **Weight:** _____**Smoker:** □Y /□N

HBs-Ag_____ **Anti-HCV**_____ **Anti-HIV**_____**HBs-Ag Vaccine** _____

1. Date of diagnosis?_____
2. Type of hemophilia?

HemophiliaA _____ HemophiliaB _____ Von Willebrand's Disease_____

3. Classification of hemophilia?

____Mild hemophilia: Clotting factor VIII or clotting factor IX level is 5% of normal or greater. Mild hemophilia might not be recognized unless there is excessive bleeding after a major injury or surgery.

____Moderate hemophilia: Clotting factor VIII or clotting factor IX level is 1% to 5% of normal. Bleeding usually follows a fall, sprain orstrain.

____Severe hemophilia: Clotting factor VIII or clotting factor IX level is less than 1% or normal. Bleeding often happens one or more times a week for no apparentreason.

4. Treatment?_____
5. Number of visits per month or 6 month? _____ Date of last visit _____
6. Prophylaxis? _____
7. On medication(s)? □Yes □No
   If yes, name, dosage_____
8. Any other major health problems? (Diabetes, cancer, cardiovascular, other)?_____

I represent that all statements and answers to the questions are complete and true to the best on my knowledge and belief.

Patient signature _____ **Date of interview**_____

Appendix B

An-Najah university Institutional review board (IRB)

AN-NAJAH UNIVERSITY

PROTOCOL FOR HUMAN SUBJECTS RESEARCH

CONTINUING PROJECTS ONLY

Investigator's Assurance

By submitting this protocol, I attest that I am aware of the applicable principles, policies, regulations, and laws governing the protection of human subjects in research and that I will be guided by them in the conduct of this research.

To apply for human subjects continuing review:

1. *Download the continuing projects protocol and save it on a floppy disk or on your hard drive. You may then open it, type in all requested information, save the file (Please use your last name and Continuing Protocol as the title: e.g., Musmar Continuing Protocol), and send the file as an e-mail attachment, along with your informed consent letter(s), to the Institutional Review Board at "**irb@najah.edu".***

It is essential that you answer all questions on this form since this is the primary source of information used by Board members to make their decisions. Also, only include information necessary to answer the questions. Please keep your responses as free of jargon as possible. Please

send, by campus mail or regular mail, all supporting that cannot be e-mailed (e.g., measures, permission letters from off-campus sites) to the **IRB Office, An-Najah University, Nablus**, **Palestine.** If you are proposing changes to your approved project, please send any necessary supporting materials. **If you are continuing to enroll human subjects, be sure to send your Informed Consent, printed on An-Najah letterhead, for stamping with the new IRB approval inclusion dates.**

PLEASE DO NOT INCLUDE THIS PAGE IN YOUR SUBMISSION

PLEASE BE SURE TO COMPLETE ALL SECTIONS:

Principal Investigator:

Department/School_____

Room # where mail can be sent _____

Phone _____ E-mail _____

Date of Current Submission:

Issue date of most recent Report of Action (ROA):

Expiration date of most recent ROA:

Title of Research:

PURPOSE OF RESEARCH

_____

Briefly describe the objective(s) of the research (please keep description jargon free and use 100 words or less). You may paste the paragraph from your original IRB New Project Protocol.

Dates Human Subjects Portion of Research Scheduled: from: _____ to _____.

STATUS OF RESEARCH

Number of subjects anticipated <u>for the entire project</u>: _____

Number of subjects from or about whom data has been gathered <u>to date</u>: _____

Number of subjects for whom you have signed consent forms: _____

Number of subjects who refused to participate: _____

Please describe reasons, if known:

Number of subjects who withdrew from participation: _____

Please describe reasons, if known:

Please describe any complications or deviations from the approved research:

Please describe any adverse events or unanticipated problems involving risks to subjects or others:

Appendix C
Consent form

استمارة موافقة للأشتراك في بحث علمي

فريق البحث: د.نائل أبو الحسن، د.فكري سمارة، قيصر أبو عرّه.

البحث: انعكاس انترون 22 لجين العامل الثامن في الهيموفيليا (أ) الحادة في فلسطين

مكان اجراء البحث: جامعة النجاح الوطنية.

المشترك الكريم:

انت مدعو للموافقة على اجراء فحوصات جينية لمرض الهيموفيليا، حيث تاتي هذه الفحوصات في اطار دراسة علمية يجريها فريقنا بهدف تحسين الخدمات المقدمة لمرضى الهيموفيليا في بلادنا، فنأمل من حضرتك الموافقة حيث نتعهد من طرفنا بضمان خصوصية المعلومات الطبية بشكل تام. سنقوم نحن كفريق من الباحثين باخذ عينة الدم لأجراء الفحص المطلوب، بالأضافة الى اجراء فحوصات أخرى عليها بصورة موسعه على ان يتم ادراجها في الدراسة مع المحافظة التامة على الخصوصية وابقاء نتائج الفحوصات واسماء المشاركين طي الكتمان. بامكانكم طلب اي ايضاحات او معلومات اضافية من الباحث ، تجدر الاشارة ان المطلوب منك اضافة الى الموافقة على سحب عينة الدم الاجابة على اسئلة الاستبيان، وانه لا يترتب على مشاركتك في هذه الدراسة اي اثار جانبية او تكاليف للفحوصات التي سيتم اجراؤها.

ختاما، ان مشاركتك في البحث تسهم في فهم افضل لتحديد إحدى أهم الطفرات الجينية المسببة لمرض الهيموفيليا والتي قد تفيد الطبيب المعالج في اتخاذ الاجراء المناسب بخصوص كل حالة، إذا كان لديك أسئلة حول البحث بشكل عام أو عن دورك في الدراسة ، فلا تتردد في الاتصال – قيصر محمود أبو عرّه (0598316147) مرشح ماجستير في علم الأحياء في جامعة النجاح الوطنية او من خلال البريد الالكتروني (caesar@medipal.ps) .

موافقة المشترك:

لقد قرأت استمارة القبول هذه وفهمت مضمونها، وبعد الاجابة على كل الاستفسارات التي احتاج اليها. وعليه فإنني حرا مختارا اوافق على الاشتراك في البحث واستخدام العينات والنتائج لأغراض البحث العلمي.

اسم المشترك	**توقيع المشترك / ولي الأمر**	**توقيع الباحث**	**التاريخ**
.....	.....	.....	.....

Appendix D

Molecular and chemicals preparation

Primer A

To prepare the 100 µmol primer stock solution, 386.1 µl sterile DEPC water were added to 38.61 nmol of the lyophilized primer. To generate 10 µmol working dilution of the primer, 10 µl of the stock solution were added to 90 µl sterile DEPC water (1:10).

Primer B

To prepare the 100 µmol primer stock solution, 446.3 µl sterile DEPC water were added to 44.63 nmol of the lyophilized primer. To generate 10 µmol working dilution of the primer, 10 µl of the stock solution were added to 90 µl sterile DEPC water (1:10).

Primer P

To prepare the 100 µmol primer stock solution, 289.2 µl sterile DEPC water were added to 28.92 nmol of the lyophilized primer. To generate 10 µmol working dilution of the primer, 10 µl of the stock solution were added to 90 µl sterile DEPC water (1:10).

Primer Q

To prepare the 100 µmol primer stock solution, 503.5 µl sterile DEPC water were added to 50.35 nmol of the lyophilized primer. To generate 10 µmol working dilution of the primer, 10 µl of the stock solution were added to 90 µl sterile DEPC water (1:10).

TBE buffer:

To prepare 1 liter of 2x TBE buffer (0,9 M Tris, 0.9 M boric acid, 0.5 M EDTA pH 8), 1.488 g EDTA (pH 8), 21.6 g Tris-base and 11 g boric acid were dissolved in 1 liter of sterile D.W, then the solution was autoclaved for 20 minutes and stored at room temperature . To prepare 1x TBE buffer, equal volumes of 2x TBE buffer and sterile D.W were mixed.

Deficient VIII reagent preparation

Each vial was reconstituted with 1 ml of D.W, and then the reconstituted reagent allowed standing at room temperature for 30 minutes. Upon use, the vial was mixed gently to obtain homogenous solution.

Appendix E

Measurements of extracted DNA concentrations (quality and quantity) using NanoDrop 2000 spectrophotometer.

ID	DNA conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}	ID	DNA conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
Blank	0	0	0	0	S 37	67	1.342	0.716	1.87
S 1	57	1.39	0.654	1.74	S 43	54	1.08	0.61	1.84
S 3	36	0.710	0.382	1.86	S 48	25	0.492	0.260	1.89
S 6	33	0.661	0.369	1.79	S 49	25	0.423	0.234	1.81
S 7	75	1.491	0.827	1.8	S 51	28	0.557	0.296	1.88
S 8	28	0.588	0.3	1.86	S 52	36	0.719	0.409	1.76
S 10	30	0.596	0.326	1.83	S 53	25	0.48	0.265	1.81
S 16	50	1.003	0.563	1.78	S 55	35	0.688	0.368	1.87
S 17	48	0.963	0.532	1.81	S 56	33	0.654	0.351	1.86
S 18	48	0.958	0.556	1.72	S 57	44	0.869	0.455	1.91
S 19	31	0.626	0.356	1.76	S 59	45	0.903	0.480	1.88
S 20	54	1.078	0.593	1.82	S 61	47	0.932	0.521	1.79
S 22	39	0.785	0.429	1.83	S 62	63	1.259	0.690	1.83
S 27	66	1.322	0.741	1.79	S 63	61	1.212	0.648	1.87
S 30	34	0.681	0.401	1.7	S 65	40	0.741	0.421	1.88
S 33	63	1.26	0.71	1.82	S75	37	0.735	0.417	1.76
S 35	49	0.982	0.562	1.75	S 76	54	1.076	0.6	1.79
S 36	26	0.522	0.287	1.82	S 77	39	0.785	0.424	1.85

A_{260}: displays absorbance at 260 nm normalized to a 10 mm path length.
A_{280}: displays absorbance at 280 nm normalized to a 10 mm path length.
$A_{260/280}$: ratio of absorbance at 260 nm and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA; a ratio of ~2.0 is generally accepted as pure for RNA.

Appendix F

Master mixes preparation

Master mix1 PQ	V_1			n		V_f	
dATP, dCTP, dTTP 2,5mM	5	µl	x	0	=	0.00	µl
dGTP 2,5mM	3.1	µl	x	0	=	0.00	µl
7-deaza dGTP 10 mM	0.5	µl	x	0	=	0.00	µl
Primer P & Q 10 mM	1	µl	x	0	=	0.00	µl
DMSO 100%	1.87	µl	x	0	=	0.00	µl
DEPC H2O	5.33	µl	x	0	=	0.00	µl
Master mix1 AB	V_1			n		V_f	
dATP, dCTP, dTTP 2,5mM	5	µl	x	0	=	0.00	µl
dGTP 2,5mM	3.1	µl	x	0	=	0.00	µl
7-deaza dGTP 10 mM	0.5	µl	x	0	=	0.00	µl
Primer A & B 10 mM	1	µl	x	0	=	0.00	µl
DMSO 100%	1.87	µl	x	0	=	0.00	µl
DEPC H2O	5.33	µl	x	0	=	0.00	µl
Master mix1 PB	V_1			n		V_f	
dATP, dCTP, dTTP 2,5mM	5	µl	x	0	=	0.00	µl
dGTP 2,5mM	3.1	µl	x	0	=	0.00	µl
7-deaza dGTP 10 mM	0.5	µl	x	0	=	0.00	µl
Primer P & B 10 mM	1	µl	x	0	=	0.00	µl
DMSO 100%	1.87	µl	x	0	=	0.00	µl
DEPC H2O	5.33	µl	x	0	=	0.00	µl
Master mix1 AQ	V_1			n		V_f	
dATP, dCTP, dTTP 2,5mM	5	µl	x	0	=	0.00	µl
dGTP 2,5mM	3.1	µl	x	0	=	0.00	µl
7-deaza dGTP 10 mM	0.5	µl	x	0	=	0.00	µl
Primer A & Q 10 mM	1	µl	x	0	=	0.00	µl
DMSO 100%	1.87	µl	x	0	=	0.00	µl
DEPC H2O	5.33	µl	x	0	=	0.00	µl
Master mix 2	V_1			4n		V_f	
Buffer 3 10X	10	µl	x	0	=	0.00	µl
Expand Taq 5U/mL	1.4	µl	x	0	=	0.00	µl
MgCl2 25 mM	3	µl	x	0	=	0.00	µl
DEPC H2O	14.4	µl	x	0	=	0.00	µl

جامعة النجاح الوطنية

كلية الدراسات العليا

انعكاس انترون 22 لجين العامل الثامن في الهيموفيليا (أ) الحادة في فلسطين

إعداد

قيصر محمود أبو عرّه

إشراف

د.نائل ابو الحسن

د. فكري سمارة

قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، في نابلس- فلسطين.

2017

انعكاس انترون 22 لجين العامل الثامن في الهيموفيليا (أ) الحادة في فلسطين

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إشراف

د.نائل ابو الحسن

د. فكري سمارة

الملخص

الهيموفيليا (أ) هو مرض نزف الدم الوراثي ذو صفة متنحية مرتبطة بالكروموسوم الجنسي (X) ويصيب 1 من 5000 من المواليد الذكور، و ينتج من طفرة في جين العامل الثامن التي تؤدي نقص أو عدم كفاءة بروتين العامل الثامن ، أحد البروتينات المهمة في عملية تخثر الدم. في حالات الهيموفيليا (أ) الحادة، يُعتبر إنعكاس انترون 22 لجين العامل الثامن المسبب الرئيسي للمرض على مستوى العالم حيث تشكل هذه الطفرة ما نسبته 50% من مجموع الطفرات، بينما انعكاس انترون 1 والطفرات ذات النطاق الصغير تصل إلى ما نسبته 5% و 45% على التوالي. الهدف من هذه الدراسة هو فحص طفرة إنعكاس انترون 22 لحالات الهيموفيليا (أ) الحادة و مدى حمل الصفة لمرضى الهيموفيليا (أ) في فلسطين. تم دراسة مجموعة مكونة من 77 مريض هيموفيليا (أ) من 52 عائلة مختلفة لا تجمعها صلة قرابة، حيث تضمنت 72 ذكر و 5 إناث من مناطق مختلفة في الضفة الغربية. تم قياس مستوى الدم من العامل الثامن لجميع المرضى الذكور بطريقة التخثر ذو المرحلة الواحدة. تم استخدام تفاعل البلمرة المتسلسل لكشف طفرة الانعكاس في الأنترون 22 لجميع مرضى الهيموفيليا (أ) الحادة بالإضافة الى جميع الإناث. أظهرت نتائج مستوى العامل الثامن في الدم أن ما نسبته 41.7% من المرضى مصابون بالنوع الحاد من الهيموفيليا (أ) و 22.2% مصابون بالنوع المتوسط من الهيموفيليا (أ) و 36.1% مصابون بالنوع الخفيف من الهيموفيليا (أ). أشار تحليل إنعكاس الأنترون 22 بتفاعل البلمرة المتسلسل إلى أن ما نسبته 37% من مرضى الهيموفيليا (أ) مصابون بهذه الطفرة. أظهرت النتائج أن هناك تشابه بين الدراسة الحالية بالمقارنة مع دراسات الدول الأخرى على مستوى العالم.

في الختام، هذه الدراسة تعتبر الدراسة الأولى في فلسطين التى تتعامل مع هذه المسألة المتعلقة بمرضى الهيموفيليا (أ) و التي يمكن إستخدامها كوسيلة للاستشارة الجينية. كما تؤكد الدراسة أن هناك حاجة لعمل دراسات أخرى خاصة بطفرة الإنعكاس في الأنترون 1 و تسلسل القواعد لكامل الجين للمرضى الغير مصابون بطفرة إنعكاس الأنترون 22.