

Genetic Study for G-Protein Coupled Receptor from *Saccharomyces Cerevisiae* and From Sera of Patients with Heart Thrombosis

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Abstract

Aim of the present study is Identification of specific gene for GPCR using specific primers .and identification of difference in PCR analysis in patients with heart thrombosis and compared with healthy, Sequencing of PCR product regarding GPCR compared for all three subject, Identification the similarity of human GPCR with local strain of yeast fifty healthy control and fifty patients with thrombosis which diagnosed medically with cardiac specific troponin t, troponin I levels and electro myocardiogram ECG. The aged for all subjects ranged (39-75) years patients were lying in cardiac care unit at Ibn- al- Nafees teaching hospital and Sheikh Zayed teaching hospital. Genomic DNA of whole blood was extracted from buffy coat and cell cultured handbook protocol using Bioneer- kit and Genomic DNA fungus/yeast kit was used in isolation and purification of DNA. patients divided into three groups according to their age: group A (60-75) years , group B (50-59) years , group C (39-49) years the results of genomic DNA isolation from blood cells extracted in pure form which ensured by the absorbance ratio (260/280) was (1.6 – 1.9) with a concentration of 50µg/ml and one DNA band with high resolution in gel electrophoresis. The result of genomic DNA extracted from the local strain of *S. cerevisiae* showed that DNA extracted with high purity because the absorbance ratio (260 /280)was (1.7 to 2.0) with a concentration of 60 µg/ml and presence one DNA band with high resolution in gel electrophoresis. primers were designed depending on the sequence of the gene responsible for the production of GPCR on the chromosome 11 , GPCR contain three exons which covered with six primers to detect a defect in gene sequence among. Results of gel electrophoresis are showed that primer GPRX1 gave one band for (Control , A,B) groups but absent amplified band in the patient eight and nine from group C. with molecular weight of this band is 1000 bp. The GPRX2 primer used to amplify second exon in the GPCR gene ,the molecular weight of amplified bands are 400 bp were present in all control samples and three groups of thrombosis patients and yeast. GPRX2A primer that designed to amplify part two from second exon of GPCR gene by PCR gave one band for all samples which include control and patient, the molecular weight of this band is 500 bp. PCR analysis showed one amplify band for all control and patients group with molecular weight 500 bp for GPRX3 primer and 400 bp for GPRX3A ,300 bp for GPRX3B. The specific primers which designed to covering GPCR gene used to amplification genomic DNA of the local strain *S.cerevisiae* by PCR technique. Results showed all six primers which gave one band with difference molecular weight for each primer. All samples demonstrate identity planned sizes to control and local strain of *S. cerevisiae* samples except patient number 8 and 9 in the group(C) that showed non specialist bands in specific primer with first exon (GPRX1) .So the genetic sequence analysis of these two case based on the sequence of the remainder exons to detect the genetic defect in these case. The sequence of the first part for the second exon (X2) was identity standard sequence found on the NCBI web site for case(8). The case (9) showed identity with the sequence present in the human gene bank but some difference in the first of sequence which neglected because it is in the place link of primer. The results for case 8 showed some mutation for Exon X2(part2). but case (9) demonstrate one deletion and one substitution. The results, also, illustrated that the ether48 thrombosis patients didn't appeared any mutation despite the positive results for (Troponin) that gives strong indication of thrombosis. The conclusion Primer GPRX1 gave one band for (Control , A,B) groups but absent amplified band in the patient eight and nine from group C. The molecular weight of this band is 1000 bp. The amplified band with molecular weight 400 bp were present in all control samples and three groups of thrombosis patients with primer GPRX2 and 500 bp with primer GPRX2A.PCR analysis showed one amplify band for all control and patients group with molecular weight 500 bp for GPRX3 primer.,400 bp with GPRX3A and 300 bp for primer GPRX3B.Similarity between results given by healthy group and local strain of yeast. Genetic study showed that there are only two case of patients eight and nine demonstrated mutation in nucleic location on exon two and three from GPCR gene

Keyword: GPCR, Genetic Study, *Saccharomyces cerevisiae*, Patient with Thrombosis.

1. Introduction

Thrombosis is a disease occurred when there are deficiencies of natural anticoagulants in plasma such as protein c, protein s, antithrombin and mutations in genes (Bender et al 2015). The recruitment of additional platelets is mediated by a variety of locally accumulating mediators that are produced or released once platelet adhesion has been initiated and some level of platelet activation through platelet adhesion receptors has accrued. These mediators include ADP/ATP and thromboxane A₂ (TXA₂) which are secreted or released from platelets and thrombin which is produced on the surface of activated platelets, these diffusible mediators have in common that they act via G protein-coupled receptors (GPCRs) through the activation of G-protein-mediated signaling pathways, they can further increase their own formation and release, therefore acting as positive-feedback mediators that amplify the initial signals to ensure the rapid activation and recruitment of platelets into a growing thrombus (Nisar et al 2015).

Regulator of G-protein signaling proteins attenuate G_x activity by accelerating their intrinsic GTPase activated via GTPase activating protein. Platelets contain both G_{sx} and G_{ix}, who play the same role in the regulation of cAMP formation as they do in other cells. Agents that increase cAMP levels in platelets, such as pituitary adenylyl cyclase activating peptide do as via G_{sx}. Despite most cells cAMP has a positive effect on cell function, in platelets cAMP is an inhibitor that dampens platelet responsiveness. The phosphoinositide Pathway results in the formation of inositol 1,4,5-triphosphate (IP₃) by phospholipase c (PLC) after activation of G_{9x}. IP₃ release {ca}⁺² from the platelet dense tubular system and contributes to the rise in cytosolic free {ca}⁺² concentration that usually accompanies platelet activation (Kimple 2009).

Amongst several signaling pathways regulated specific guanine nucleotide exchange factors (GEFs). Thrombin is the main effector protease of the coagulation system and is among the most effective activators of platelets. Previous study revealed that in platelets GPCRs mediate diverse effects including shape change, granule secretion and platelet aggregation. During the platelet activation process, binding of VWF and collagen generate the release of several agonists of platelet GPCRs, resulting in platelet degranulation and binding successful platelet therapeutics (Dawal and Flaumenhaft 2010).

GPCR malfunctions due to mutations have been associated with many diseases, including immunological, metabolic and reproductive disorders, cancer and neurodegenerative diseases, but only a fraction of disease-associated GPCR mutations have been characterized functionally (Heng et al 2013).

Mutations altering GPCR function are usually classified according to the net change in signaling ability as follows: mutations affecting GPCR basal activity (Lebon et al 2012, Tao and Segaloff 2005), mutations affecting ligand binding (Stoy and Gurevich 2015, Venkatakrishnan et al 2013), mutations affecting GPCR-G protein interaction (Rasmussen et al 2011, Rasmussen et al 2011, Kang et al 2014). Mutations affecting cell surface expression (Tao 2006, Ward et al 2012, Ulloa-Aguirre et al 2013). The aim of the present study is identification of specific gene for GPCR using specific primers and identification of difference in PCR analysis in patients with heart thrombosis and compared with healthy, Sequencing of PCR product regarding GPCR compared for all three subjects, Identification the similarity of human GPCR with local strain of yeast.

2. Material and method

Five ml of blood samples were collected from fifty healthy control and fifty patients with thrombosis which diagnosed medically with cardiac specific troponin t, troponin I levels and electrocardiogram ECG. The aged for all subjects ranged (39-75) years patients were lying in cardiac care unit at Ibn- al- Nafees teaching hospital and Sheikh Zayed teaching hospital in the period of 3/3/2014- 1/9/2014. patients divided into three groups according to their age: group A (60-75) years, group B (50-59) years, group C (39-49) years. The blood placed containing anti-coagulant reagent (K₂EDTA) and kept at -20 °C until further use for genomic DNA extraction.

Loop fully of yeast colonies which grown on (YEPA) medium and inoculate in (YEPDB) medium were taken, yeast cells were grown overnight on (YEPDB) medium at 220 rpm and 30 °C.

Genomic DNA of whole blood was extracted from buffy coat and cell cultured handbook protocol using Bioneer- kit. The eluted genomic DNA is stable and can be used immediately, or stored at 4 °C for long-term DNA storage. Genomic DNA fungus/yeast kit was used in isolation and purification of DNA. The procedure was done according to the manufactured company (GeneAid, Taiwan). The elute DNA stored for later analysis at -20 °C. Concentration and purity of DNA solution were estimated by using nanodrop spectrophotometer, by

adding 2µl of DNA solution into the photocell of the apparatus. The concentration and purity (ng/ ml) of DNA were calculated by the following equation .

$$\text{DNA concentration (ng/ ml)} = \text{abs}(260\text{nm}) \times (50 \text{ mg/ml})$$

$$\text{DNA purity} = \text{Abs at } 260 \text{ nm} / \text{Abs at } 280$$

GPCR gene were amplified as shown in table(1) by using PCR machine amplification procedure:

Steps	Temp C°	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	60	30 sec	35
Extension	72	1 min	35
Final extension	72	5 min	1

Stock primer (100 pmol) used to prepared dilution primer (10 pmol) by addition Nuclease-Free Water(1:10) and stored at -20C°

Primers were used in this study ,as shown in table(2) , primers were designed depending on the sequence of the gene responsible for the production of GPCR on the chromosome 11

Table (2): Primers were used in the GPCR gene amplification .

Oligonucleotide primer	Sequence (5 --- 3)	Size (bp)
GPRX1 F	CCAACCATCACAACCTTGGAC	21
GPRX1 R	CCTTCATCCACCTCAGACGC	20
GPRX2 F	TGCTGAGCATCTTGGAAAGGG	20
GPRX2 R	CTCTGGAGAGCCAGCTTGAG	20
GPRX2a F	AGGAGCGCCTTCTCTGTCTA	20
GPRX2a R	CAGGTACAGCCTGGTCAGTG	20
GPRX3 F	AAGCAGACCCTGAGCTTCAC	20
GPRX3 R	CCAGCCACGCGATTGTAATG	20
GPRX3a F	GTGGTTCTCTGTGGGTCCAG	20
GPRX3a R	GGGCAGAGGTTCTTCCTCAC	20
GPRX3b F	GGCAGCGTCAAAATAGGCAG	20
GPRX3b R	CGAAGACCTTGAGGGACCAC	20

Horizontal electrophoresis unit was used in analyzed PCR product , Agarose gel (1%) was prepared in 1X TBE buffer , Gel was left to solidify in the tray , which transferred to the electrophoresis tank of the device and immersed with TBE buffer ,The DNA samples was loded into the wells. electrophoresis was performed at 5 V/cm for 60 min ,The gel was stained with ethidium bromide stock solution for 15 min .the DNA bands were visualized on a UV transilluminator and photographed by using Gel Imaging System. DNA ladder (100bp) (BIONEER) was used to determine the molecular weight of DNA band.

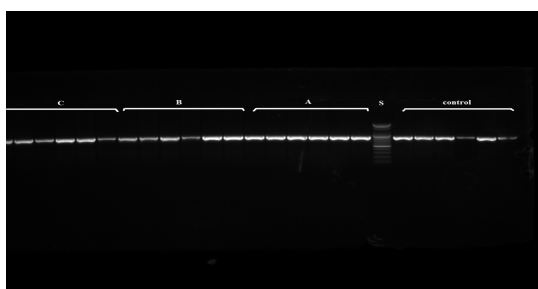
3. Result and Discussion

The results demonstrated that all patients show positive result with troponin test. The results of genomic DNA extracted from the local strain of *S. cerevisiae* showed that DNA extracted with high purity because the absorbance ratio (260 /280)was (1.7- 2.0) with a concentration of 60 µg/ml and presence one DNA band

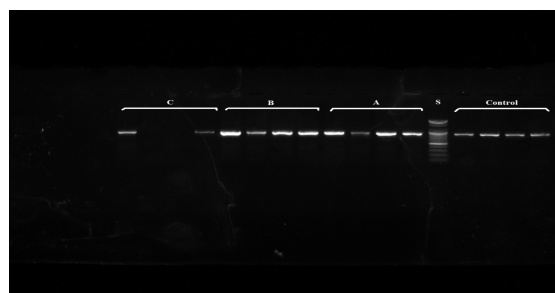
with high resolution in gel electrophoresis. Aliquots of this DNA were taken and used in amplification of GPCR gene. The results of genomic DNA isolation from blood cells extracted in pure form which ensured by the absorbance ratio (260/280) was (1.6- 1.9) with a concentration of 50µg/ml and one DNA band with high resolution in gel electrophoresis. GPRX1 primer was designed to covered first exon in the GPCR gene .

Genomic DNA of thrombosis patients and control was used to amplify first exon of GPCR gene, then PCR products were analyzed on agarose gel electrophoresis to identify the products of amplified fragments in the presence of 100 bp DNA ladder .

Results of gel electrophoresis are documented in figure (1) and figure (2) showed that primer GPRX1 gave one band for (Control , A,B) groups but absent amplified band in the patient eight and nine from group C. The molecular weight of this band is 1000 bp.



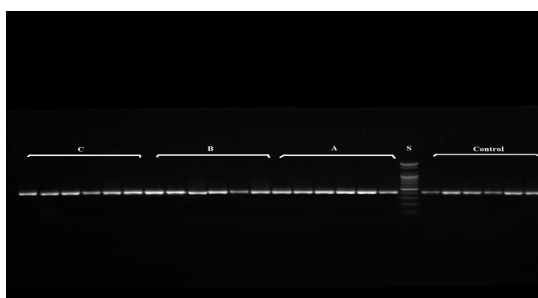
Figure(1):PCR analysis for GPRX1primer



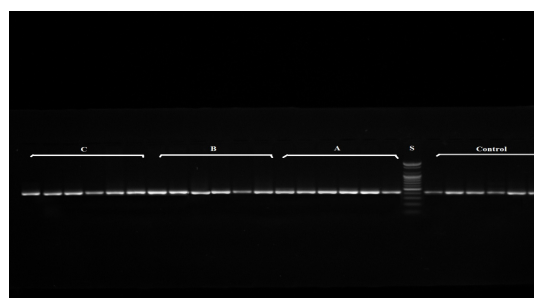
Figure(2):PCR analysis for GPRX1

Lane S : Maker DNA Ladder , Lane A : Represent amplified band in the group A Patients , Lane B : Represent amplified band in the group B Patients , Lane C : Represent amplified band in the 7 and 10 from group C but absent in the Patient eight and nine .

The GPRX2 primer which has sequence mentioned in table (1) used to amplify second exon in the GPCR gene . The bands amplified across the lanes as shown in the figure (3) and (4) . The molecular weight of these bands are 400 bp. The amplified band with molecular weight 400 bp were present in all control samples and three groups of thrombosis patients.

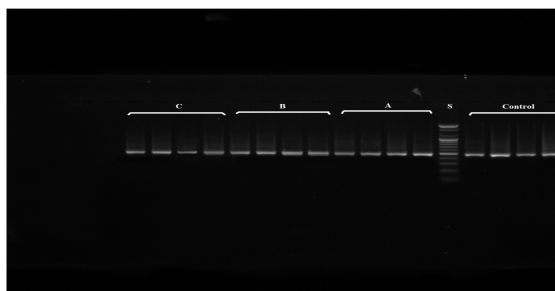
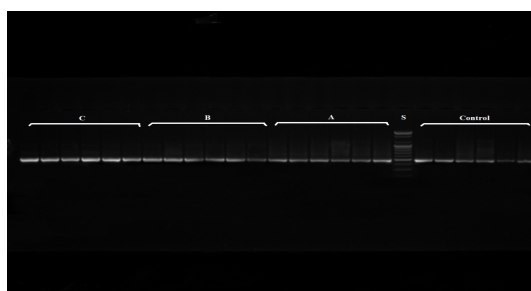


Figure(3) PCR Analysis for Amplified Band



Figure(4) PCR Analysis for Amplified Band

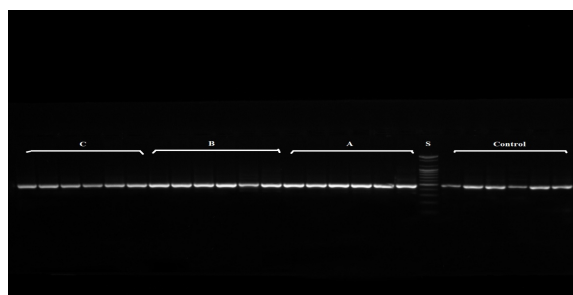
The GPRX2A primer that designed to amplify part two from second exon of GPCR gene by PCR gave one band for all samples which include control and patient, the molecular weight of this band is 500 bp as shown in the figure (5)and(6).



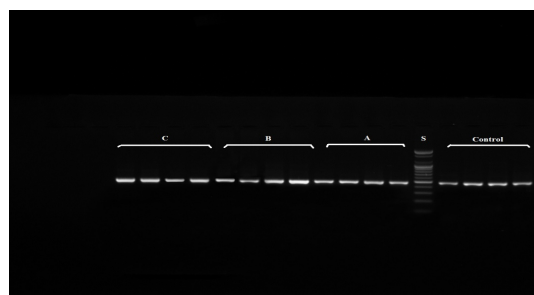
Figure(5) : PCR analysis for all samples Figure(6) : PCR analysis for all samples

Lane S : DNA Ladder . Lane (A, B, C) : represent amplify band in the (A, B, C) patients group. Lane Control : represent amplify band in the normal samples .

The GPRX3,GPRX3A,GPRX3B primers designed to cover exon number three in the GPCR gene . PCR analysis showed one amplify band for all control and patients group with molecular weight 500 bp for these primers. Electrophoresis performed to analyzed PCR products as shown in the figure (9)and(10).



Figure(7): PCR Analysis for GPRX3 Primer.



Figure(8): PCR Analysis for GPRX3 Primer

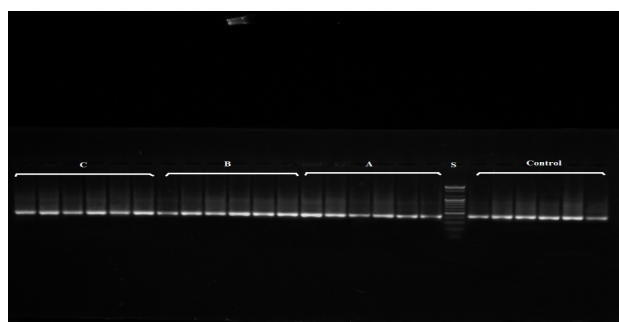


Figure (9): PCR for GPRX3A Primer

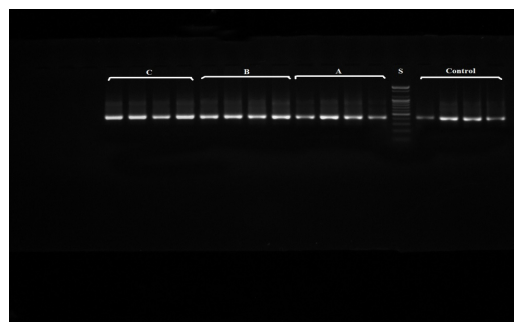


Figure (10): PCR for GPRX3A primer

Results of agarose gel electrophoresis for PCR analysis that display in the figures (11),(12) showed amplify band for all samples with molecular weight 300 bp for GPRX3B primer

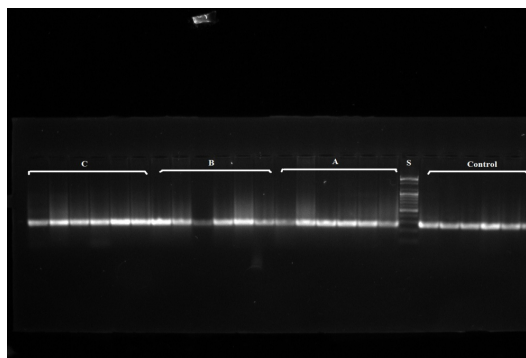
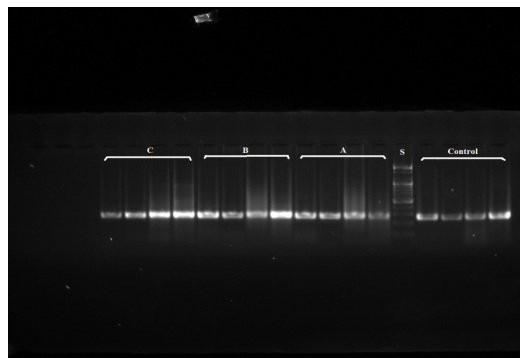


Figure (11):PCR analysis for samples.



Figure(12):PCR analysis for samples.

The specific primers which designed to covering GPCR gene used to amplification genomic DNA of the local strain *S.cerevisiae* by PCR technique .

Results are documented in figure (13) showed all six primers which gave one band with difference molecular weight for each primer .

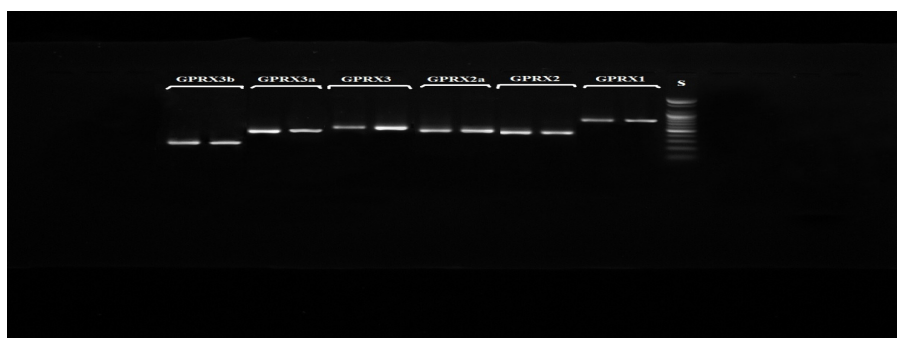


Figure (13):PCR analysis for *S.cerevisiae* with all primers

All samples demonstrate matching sizes to control and local strain of *S. cerevisiae* samples except patient number 8 and 9 in the group(C) that showed non specialist bands in specific primer with first exon (GPRX1) .So the genetic sequence analysis of these two case based on the sequence of the remainder exons to detect the genetic defect in these case. The results of the genetic sequences for all cases explained that there are only two case of patients eight and nine demonstrated mutation in nucleic location on exon two and three from GPCR gene. The results summaries in table(2).

Table (2): Mutation detection on chromosome 11 regarding GPCR location.

LOCATION (nuc)	MUTATION	TYPE
19056167	Transversion (substitution)	T -- A
19056177	Transion (substitution)	T -- G
19056178	Transversion (substitution)	C -- A
19056184	Deletion	T-- -
19056185	Transversion (substitution)	G -- C
19056017	Transion (substitution)	T -- C
19056029	Transion (substitution)	A -- G
19056037	Transversion (substitution)	G -- T
19056044	Transversion (substitution)	G -- T
19056066	Transion (substitution)	G -- A
19056004	Transversion (substitution)	G -- C
19055903	Transversion (substitution)	G -- C
19055943	Transversion (substitution)	C -- G
19055944	Transversion (substitution)	A -- C
19056177	Transion (substitution)	A -- G
19056187	Deletion	T -- -
18137414	Transversion (substitution)	C -- A
18137622	Transion (substitution)	A -- G
18137708	Transversion (substitution)	A -- T
18137367	Transversion (substitution)	C -- A
18137380	Transversion (substitution)	G -- T
18137414	Transversion (substitution)	C -- A
18137622	Transion (substitution)	A -- G
18137707	Transion (substitution)	A -- G
18933773	Transversion (substitution)	G -- C
18933774	Transversion (substitution)	T -- A
18933775	Transion (substitution)	G -- A
18933796	Transion (substitution)	G -- A
18933865	Transion (substitution)	T -- C

18933866	Transion (substitution)	G -- A
18933915	Deletion	A -- -
18933904	Deletion	G -- -
18138164	Transion (substitution)	A -- G
18138178	Transion (substitution)	A -- G
18138212	Transion (substitution)	T -- C
18138213	Transion (substitution)	G -- A
18138225	Transversion (substitution)	C -- G
18138256	Transversion (substitution)	T -- A
18138259	Transion (substitution)	T -- C
18138268	Transversion (substitution)	A -- C
18138275	Transversion (substitution)	C -- G
18137824	Deletion	A -- -
18137868	Insertion	- - - C
18137871	Deletion	C - - -
18137898	Transversion (substitution)	T -- G
18137901	Transion (substitution)	C -- T
18137910	Transversion (substitution)	G -- T
18137911	Transversion (substitution)	G -- T
18137912	Transversion (substitution)	C -- T
18137921	Transion (substitution)	C -- T
18137922	Transversion (substitution)	C -- A
18137923	Transion (substitution)	A -- G
18137932	Transversion (substitution)	C -- G
18137949	Transversion (substitution)	A -- T
18137961	Transversion (substitution)	G -- T
18137967	Transion (substitution)	A -- G
18137973	Transversion (substitution)	C -- A
18138106	Transion (substitution)	G -- A
18138111	Transion (substitution)	A -- G
18138114	Transversion (substitution)	T -- G

18138126	Transion (substitution)	G -- C
18138128	Transversion (substitution)	T -- C
18138129	Transversion (substitution)	C -- G
18138130	Transversion (substitution)	T -- G
18138140	Transversion (substitution)	C -- A
18138146	Transversion (substitution)	T -- G
18138149	Transversion (substitution)	C -- A
18137986	Transversion (substitution)	G -- A
18137991	Transversion (substitution)	T -- A
18138010	Transion (substitution)	A -- G
18138011	Transion (substitution)	T -- C
18138026	Transversion (substitution)	T -- G
18138043	Transversion (substitution)	C -- G
18138055	Transversion (substitution)	C -- G
18138059	Transversion (substitution)	A -- C
18138062	Transversion (substitution)	T -- G
18138072	Transion (substitution)	T -- C
18138077	Transion (substitution)	A -- G
18138078	Transion (substitution)	G -- A
18138084	Transion (substitution)	T -- C

The results, also, illustrated that the ether48 thrombosis patients didn't appeared any mutation despite the positive results for (Troponin) that gives strong indication of thrombosis. The study demonstrated that Human Platelet disorders occurred when there are defect in the signaling of (G-Protein) that accompanied with defect in GPCRs, which leading to an isolated thrombopathy. Mutations in specific genes of platelet result thrombosis such as genes coding for G-proteins and their effectors platelet defect (Van Geet et al 2009).

The recent study illustrated the coding and splice section of eighteen GPCR gene about 8000 exons from about 1000 genomes in thirty one cases with inherited platelet function disorders , that proved rare , SVVS in GPCR genes for this type from patients (Jones et al 2015). Another study demonstrated the essential role of genetic factors in thrombosis risks in Spanish population which a GPCR genetic sequence defect that case increased risk of thrombosis (Souto et al 2000). The recent study revealed mutations in (6) candidate genes which entire to the etiology of venous thrombosis in Indian subjects (Kumari et al 2014).

changes in phenotype or in disclosure gene that relation of protein production these changes doesn't relation with sequence of nucleic acid in DNA but occur as a result of added molecule such as methyl group to gene consequently affect production of protein . Some studies indicate that epigenetics which changes in gene function that happen by gene methylation ,histone modification or RNA mechanisms led to injury with thrombosis(MacLeod, et al 2015). Another study suggested that epigenetics may be occur as a result of heritable ,lifestyle and environmental factors(McCance et al 2015).

4. Conclusion

1-Primer GPRX1 gave one band for (Control , A,B) groups but absent amplified band in the patient eight and nine from group C. The molecular weight of this band is 1000 bp.

2-The amplified band with molecular weight 400 bp were present in all control samples and three groups of thrombosis patients with primer GPRX2 and 500 bp with primer GPRX2A.

3-PCR analysis showed one amplify band for all control and patients group with molecular weight 500 bp for GPRX3 primer.,400 bp with GPRX3A and 300 bp for primer GPRX3B.

4-Similarity between results given by healthy group and local strain of yeast.

5-Genetic study showed that there are only two case of patients eight and nine demonstrated mutation in nucleic location on exon two and three from GPCR gene

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