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Research Article

Amplification of ABCG2 Transporter Gene Exon 8 and 14 of Diabetic Patients

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Abstract

Diabetes is a serious illness affecting over 425 million people worldwide. Diabetes develops as a result of the failure of the pancreatic β-cells to produce the hormone insulin in the amount required to meet the body's needs. As a consequence of insulin deficiency, blood sugar levels rise and lead into macro vascular and micro vascular diseases of the kidneys, heart, eyes and nerves with passage of time. Elevated serum uric acid level is related with a variety of adverse health outcomes which includes gout, hypertension, diabetes mellitus, metabolic syndrome and cardiovascular diseases. Several genome-wide association studies on uric acid levels have implicated the ATP-binding cassette, subfamily G, member 2 (ABCG2) gene as being possibly causal. The aim of present study was to amplify exon 8 and exon 14 of ABCG2 gene of diabetic patients. Blood samples were collected from diabetic type II patients (n=25). Genomic DNA was extracted from blood using Phenol-Chloroform method, followed by amplification of Exon 8 and exon 14 was using Polymerase Chain Reaction (PCR). The size of amplified genes of DNA was analyzed by Gel Electrophoresis and then observed under gel documentation through UV rays.

Keywords: ABCG2; Transporter; SNP; BCRP; Diabetes.

Introduction

ABC (ATP Binding Cassette) Transporters are specified proteins for transmembrane pumps they utilize ATP to work as a carrier across the membranes. They have varieties of different

function for nutrition uptake, protection from toxic industrial compounds and also in homeostasis. They are highly involved in molecules from the inside cell, they may be out of the cell or into organelles within the cell. They contain endogenous compounds like metabolic products and lipids, as well as xenobiotics which are of great clinical interest [1]. Human's body contains approximately 48 different ABC transporters which are divided into groups ABCA to ABCG [2]. The ability of these transporters to efflux multiple, structure wise different drugs implicate them in multidrug resistance (MDR) and the efflux of compounds from cells, lowering intracellular concentration and show resistance to a large variety of drugs [3]. The ABC subfamily G isoform 2 (ABCG2) protein is a MDR pump with a lot of distribution in the human body, including the blood-brain barrier, small intestine, blood-placenta barrier, hepatocytes canalicular membranes, proximal convoluted tubule cells of the kidney and the mammary gland. ABCG2 was identified in 1998 in placenta and also found in multidrug resistant breast cancer cell lines. It is the one of the most expressed gene in all over the body [4]. It is clear from Single nucleotide polymorphism that it relates its function with its three-dimensional structure [5]. Both environmental and genetic factors are the causes of elevated serum uric acid levels. Prevalence increases with age, male gender, the duration of type 2 diabetes and hypertension [6]. Structure wise it is known that ABCG2 is a half transporter its two domains are named as one NBD (nucleotide binding domain) and one MSD (membrane spanning domain) in the polypeptide chain. For ABCG2 gene its necessary to dimerize or oligomerize after that it will become function [1]. Elevation in serum uric acid levels is concerned with a lot of variety of adverse health result that includes hypertension, gout, metabolic syndrome, diabetes mellitus and cardiovascular diseases. Different genome association shows broad studies on uric acid levels that implicated the ATP-binding cassette, subfamily G and its second member (ABCG2) gene as being possibly temporary. ABCG2 that is G2 family of ABC transporters is the carrier of many different types of substrates that also contain Uric Acids. It is also discussed earlier that ABCG2 abnormalities in expression has adverse effect upon the Diabetes and have role in increasing the prevalence of Diabetes. ABCG2 is up regulated in the Alzheimer's patients and 421CC genotyping represents that It significantly increased the susceptibility to Alzheimer's in comparison with AA and CA [7]. The two main reasons of hyperuricemia are the rate of synthesis of uric acid in the liver and the kidney rate of uric acid excretion [8]. An abnormal uric acid excretion is mainly due to the abnormal expression of urate transporter in the kidneys. As updated recently, the genetic factors account for nearly 40-70% cause for hyperuricemia that alters the renal uric acid transport system [9]. ABCG2 was exactly identified in 1998 in placenta and also in multidrug resistant breast cancer cell lines, hence it was given the names breast cancer resistance protein and placenta-specific ABC transporter [10]. ABCG2 belongs to the ATP binding cassette G2 family of transporters that secrete and transport a wide variety of substrates, including Uric acid [11]. ABCG2 is not only restricted to the placenta and clinical relations between ABCG2 and breast cancer staging and metastasis outcome are equivocal

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[12]. Traditional anticancer drugs and photodynamic therapy agents such as protoporphyrin A and pheophorbide A was verified as substrates, that influencing the effects of photodynamical therapy in some individuals [13]. The broad existence and wide specificity of ABCG2 make it possible an important determinant for the pharmacokinetics of various substrate of drugs [14]. ABCG2 is coded on the chromosome position at 4q22 [15]. Inside the kidney of humans ABCG2 expressed on the apical position of proximal convoluted tubular cells while the expression level decreases in the small intestine and hepatocyte cells for uric acid exportation. Indeed, ABCG2 activity as a urate carrier is known to be not present in gout [16]. In normal human's tissues, ABCG2 is expression level is extremely on the apical membrane of the placenta syncytia trophoblasts. ABCG2 expel out the drugs or toxic compounds from the fetal compartment and bring it back to the maternal blood circulation, limiting fetal exposure of the toxic substances and it plays a preliminary role in securing fetus against maternal derived toxins [17, 18]. Uric acid is an excretory product that is found in the blood. It is formed due to the breakdown of substances called "purines" that are part of a non-vegetarian diet. High amounts of uric acid in circulation may results in the formation of crystals inside the joints, leading to gout arthritis. However, hyperuricemia, may be the result of a variety of metabolic and physiological disturbances [17] Hyperuricemia is one of those syndromes that is highly inherited similar to obesity and body weight [19]. The SNP locus rs2231142 of the ABCG2 gene was found to be associated with hyperuricemia and found significant in obese alcoholic men who are suffering from type 2 diabetes and hypertension. Studies have proved that genetic polymorphism on the Q141K and V12M variants of the ABCG2 gene is responsible for the abnormal uric acid levels. Hence, people carrying these alleles are more likely to develop hyperuricemia. However, the extent of association of ABCG2 and hyperuricemia was found to vary with ethnicity. So, we have selected the same SNP Rs 2231142 of the variants Q141K, in order to establish the theory of hyperuricemia variation with ethnicity [20].

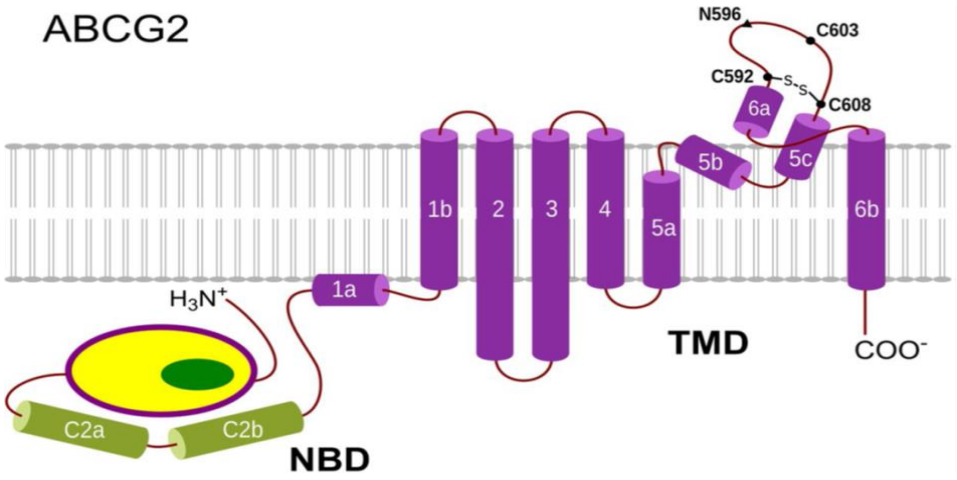

Figure 1: ABCG2 Membrane Topology Model.

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Aim of the Study

Aim of the study is to identify the difference of the expression of ABCG2 exon 8 and 14 through the Gel Documentation and Electrophoresis in the normal versus Diabetic patients.

ABC (ATP-Binding Cassette) Transporters

Represents the broad family of transmembrane protein that can bind ATP and utilizes the energy to make able the exchange of different molecules across cell membranes. Genes related to these families are subdivided further into seven sub-family (ABCA to ABCG).

ATP-binding Cassette Transporter G2 (ABCG2)

This ABCG subfamily is further composed of half transporters which is known to form homo and hetro dimers for these active transporters. The ABCG2gene is composed of 16 exons while 15 introns and it is located on chromosome locus 4q22 [21]. The gene encodes about 655 amino-acid ABC half transporter (ABCG2, also named as BCRP, ABCP and MXR) that is composed of one transmembrane region and one nucleotide binding fold, often referred to as an TM-NBF [22].

Physiology of ABCG2

In its primary function it is known that ABCG2 unidirectional that means transport or substrates outside the cell. Similar in the case of ABCB1 which is a P-glycoprotein, ABCG2 is expressed in apices of membranes of multiple and healthy organs, that includes the kidney, intestine, liver and blood brain barriers [23] and itis thought that its play an important role in removal of toxic substances from cells by preventing excessive accumulation in certain tissues and in reducing absorption. During lactation the expression of ABCG2 is strongly induced in different mammals [24]. Where it is likely involved in the secretion of certain important nutrients in the milk, such as riboflavin also known as vitamin B2 [25]. The normal physiologic function of ABCG2 might be related to the transport of a different natural substances across the cell membrane in order to prevent intracellular accumulation of toxic compounds.

ABCG2 is also an important mediator of resistance to a variety of anticancer drugs including mitoxantrone, topotecan, irinotecan, flavopiridol and methotrexate [26]. A decrease in the concentration of ABCG2 can leads to trigger the oxidative stress and also change the brain inflammatory response this can leads into aging and increase in free radicals inside the body called oxidants.

Future Perspective in Pharmacokinetics

It is a major clinical interest for scientists now to detect ABCG2 genetic changes and to know about their role in drugs resistance and pharmacokinetics, while currently there is no diagnostic test available. But the knowledge that was gained in the field of tumor and cancer biology encourages the adoption of strategies for the treatment based on the compatibility between the molecular profile of the disease and the drug to be administered. So, the future directions in this field will be mainly related with the development of genomic and proteomic

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techniques and with validation and subsequent clinical implementation of (Nano) technologies of genotyping and phenotyping of ABCG2 [27].

Substrates of ABCG2

The spectrum for substrate of ABCG2 was increased exponentially over that include physiological compound, malignant and benign therapeutics, drugs targeted molecularly and that of common dietary xenobiotics. The physiological efflux of ABCG2 involves multiple substrate that include organic ions which is conjugated heme or porphyrin [28].

It was discovered originally in drug resistant cells, the first compounds that was reported as (exogenous) substrates of ABCG2 were chemotherapeutics that include mitoxantrone [29] anthracycline, methotrexate, camptothecin derivatives and flavopiridol as well as tyrosine kinase inhibitors (TKIs) like imatinib and gefitinib [30].

Inhibitors of ABCG2

The mechanism of inhibition divides ABCG2 inhibitors into two Categories: "general" inhibitors that perform the inhibition of ATPase activity of the transporter such as Ko143 and FTC (fumitremorgin C).

(BCRP inhibitor) and inhibitors that is known as "substrate depending" that is actually ABCG2 substrates and thus act as competitive inhibitors [31].

In in-vivo process fumitremorgin C (FTC) was discontinued due to its neurotoxic effect unlike other inhibitor that was its analogues. FTC other analogue called Ko143 is also extremely potent against ABCG2 activities [32]. This reference prefers Ko143 inhibitors over FTC. Due neurotoxic effects of FTC its other analogue was preferred and both act as BCRP (Breast Cancer resistance protein) inhibiters.

One of the first and earliest inhibitors of ABCG2 was Fumitremorgin C (FTC). It was well known about FTC that it might inhibit mitoxantrone resistance in drugs selected cell line but before it ABCG2 was cloned but failed to have any effect upon [10].

Historical Background of Diabetes Type II

It is well known from the previous history of diabetes that is one of the oldest diseases first reported in Egypt before 3000 years ago [33]. It was confirmed in 1936 about type 1 and type 2 DM that these are different from one another. Type 2 diabetes east considered as a component of metabolic syndrome near 2000 [34]. (Hyperglycemia is the main condition of type 2 DM caused due to insulin resistance [35]. Different factors like genetic, behavioral and environmental are the three risk factors [36]. From historical background it is clear that the most common is Diabetes Mellitus also known as type II Diabetes. Peoples of developing countries are more vulnerable to this disease a lot of them are still remained undiagnosed.

Epidemiology

Epidemiology i.t is estimated that 422 million people had DM nowadays according to official website of World Health Organization while in 2030 this might have further rise to about 550million.This number of people

having type 2 DM is increasing, it is also clear that 80% of people linked with DM are from developing countries. About 4.8 million peoples were killed by this disease in 20 centuries. (Diabetic atlas fifth edition 2011 by Brussels. The incidence of type 2 DM varies substantially from one geographical region to the other as a result of environmental and lifestyle risk factors [37].

World Wide Prevalence of Diabetes

It was observed in 2017 that 453 million peoples having ages ranges from 18-99 faced with diabetes around the world. It was also expected these figures will increase to about 700 million in 2045. It was also analyzed 49% people living with diabetes are undiagnosed. People related to impaired glucose tolerance is about 380 million, abbreviated as IGT and some babies were considered to be related with hyperglycemia which ranges up to 22 million live births to women during their pregnancy. According to a survey of 2017, 5 million peoples were dead due to hyperglycemia and their ages were ranges from 20-99. Globally it was estimated that the expenses on DM patients are 850 billion USD in 2017.

Diabetes Type II Prevalence in Pakistan

This report shows that males are more suspicious to diabetes mellitus than females. The current prevalence of type 2 diabetes mellitus in Pakistan is 12%. In males the prevalence is 11% and in females 9%. The mean prevalence in Sindh province is 16% in males and 11% in females in Punjab province it is 12.14% in males and 9.83% in females. In Baluchistan province 13% in males, 9% among females; while in Khyber Pakhtunkhwa (KPK) it is 9.5% in males and 11.90% in females. The prevalence of type 2 diabetes mellitus in urban regions is 14.68% and 10.64% in rural areas of Pakistan [38].

Materials and Methods

Sample Collection

All of the observations were collected in order to start with a proper planning and to get the right results through clinical records such as essential Demographic factors that includes gender, clinical signs and symptoms and familial background of diabetes with significant pathological features were gathered from different patients. Blood Samples from patients (n=25) having either type I or type II diabetes were collected under sterile conditions through 5 cc syringes into an antiseptic vacutaining tubes having EDTA inside these tubes, which was used to avoid blood clotting. These tubes (samples) were taken from different hospitals and private laboratories with the help of authentic lab technicians and hospitals. The samples were transferred immediately after collection into molecular laboratory of Biochemistry department, AWKUM. Samples were stored at -20° to -19.

Genomic DNA Extraction from Human Blood

For amplification of ABCG2 gene (exon8 and exon14), genomic DNA from human blood was extracted and purified through DNA extraction protocol through organic method also known as phenol chloroform method. This DNA were then stored for further analysis to perform Polymerase Chain reaction. A volume of 0.76 ml blood and 0.74

ml of solution A were placed in a micro-centrifuge. Mixed via inversion and placed the Eppendorf at room temperature for up to 10 minutes at least. The Eppendorf's were centrifuged for up to 60 seconds at 13,000 rotation speed per minute. Then, after centrifugation, the Supernatant was thrown away and the pellet was re-suspended once more in 0.4ml of solution A, and centrifuged further for time of 1 minute at 13,000 rotations per minute. At the end of centrifugation, Supernatant was once more discarded and the Pellet existing in Eppendorf tubes were then re-suspended in 401 µl of Solution B.

Then 25µl 11% Sodium dodecyl sulphate (14 µl of 20% SDS) and 8.5 µl of Proteinase K (20mg/ml) was added and kept 37˚C for overnight and sometimes at 65˚C for three hours inside incubator. Following incubating condition, 0.6ml of a newly synthesized mixture, equal quantity of Solution C (401 µl of phenol and 10.1mM Tris) and then Solution D contains chloroform 24 ratio Isoamyl alcohol 1 ratio was added to sample, then homogenized by mixing and centrifuged for about 12 mints at 13,000 rotation per mints.

The aqueous layer (upper part) from that tube was isolated in fresh Eppendorf & an equivalent amount Solution D were also added, after that centrifuged for 12 minutes on 13000 RPM. In end of centrifugation the upper phase was removed once again in 1.6 ml Eppendorf. 55 microliters of 3 molar Sodium Acetate 6 pH value and approximately 501 µl Iso-propanol stored at -21˚C were added to the gathered upper Aqueous phase as replacement of Iso-propanol a similar quantity as Ethanol can be also be added as well.

It was ensured that the Eppendorf tube was inverted gently for DNA precipitation. Then the tube was centrifuged at 13000 RPM for fifteen mints. Following centrifugation, DNA pallet was retained while the aqueous phase was cautiously removed. For the washing purpose of the DNA pellet, 70% ethanol was taken as 350 µl of volume and stored at -21˚C, was used and then Centrifugation upon 13000 RPM for fifteen minutes. Removal of Ethanol was done and then DNA Pallet was dried. Pallet was kept that was dried at 65℃ for ten mints. DNA was dissolved in a proper volume of DNA dissolving buffer or PCR water.

Agarose Gel Electrophoresis

To check that DNA was extracted by qualitative and quantitative analysis. Detection was done by applying visualizing agent Gel red or ethidium bromide. Gel documentation system were used for the visualization of the extracted DNA under ultraviolet illuminator. To prepare 2% Agarose gel, 0.6 g of agarose was added and dissolved in 30 ml of 1X TBE buffer (or 1.3 g of agarose in 60 ml of 1X Tris Borate EDTA Buffer).

Beaker of solution were then heated upon the heater till it got clear appearance and boiled. The solution was then kept at room temperature to cool down and reach to 60 degree. After that 4µl ethidium bromide (11 mg/ml) was added to this solution for staining DNA to be visualized. After assembling the gel apparatus, agarose solution was poured into the gel cassette and a comb was kept carefully to form wells and left for 30 minutes to solidify.

Then removing the comb, the gel was shifted to gel tank containing 1X TBE buffer which touches the normal line on gel tank. PCR products was loaded to the wells here no need for loading dye because it was already present in master mix. Then the gel was run for about 30 minutes at 125V (50 mA) in 1 X TBE running buffer. The power supply was removed followed by gel documentation. The estimation of PCR products size was done in accordance to 100bp DNA ladder.

A 2% of agarose gel was prepared for the visualization of amplified exons of ABCG2 (the gel % was kept according to the gene size) and it was detected by the visualizing agent ethidium Bromide. 8 micro liters of PCR samples were loaded in the wells. Electrophoresis was done at 125 volts for at least 25 minutes in 1 X TBE buffer. The amplified PCR samples were visualized under UV radiations having a Wavelength of 365nm through Gel doc machine.

Polymerase Chain Reaction

PCR (Polymerase chain reaction) was conducted in a 0.2 ml of tubes. The ingredients that were used is shown in the Table 1 Below.

Ingredients used in PCR Compound	Amount with concentration
PCR Master mix	20µl
Forward Primer	1µl (10mM)
Reverse Primer	1µl (10 mM)
Genomic DNA	2 µl
PCR H2O	26 µl
Total Volume	50 µl

Table 1: Composition of PCR.

Thermo Cycling conditions used for PCR with 45X cycles

The reaction mixture was mixed by using vortex, then centrifugation of samples was done for few seconds to mix the contents thoroughly. The mixture of reaction used then kept in thermo cycling conditions comprising 5 mints at 95 degree for Template DNA. The denaturation followed by 45 cycles 3 steps composed of one minute at 95 degree for DNA denaturation into single which is shown in Table 2.

PCR Thermal Conditions		
Steps Followed	Temperature	Time duration
Lid Temperature	104 °C	
Initial Denaturation of DNA	96°C	1 minute
Annealing of primer	57-65°C	1 minute
Extension of complementary DNA	72 °C	1minute
Final extension	72 °C	1 minute

Table 2: Thermo Cycling conditions used for PCR with 45X cycles.

Primers used for PCR analysis

Strands 1 minute at 60 degree for primers hybridization or "annealing" with their sequence complementarity on either side of the target sequence and one minute at 72 degree for extension of DNA which has complementary strands from each primer, finally extended for 1 minutes at 72°C for Thermus aquaticus (Taq) DNA polymerase that synthesize each no extended strands left. The reaction mixture was set according to the following composition per tube. 2% of gel was used for the confirmation of amplified exon 8 and 14 and then visualized through Gel Documentation under the UV radiations.

ABCG2gene, exon 8 and 14 was amplified by PCR from the human genomic DNA of diabetic patients and also with normal individual (as a control). The two different primers for each set were used to amplifyABCG2exon 8 and 14 is given below in Table 3 and 4.

Primer Name	ABCG2 Gene Exon 8 Primers (seq 5`-3`)	Product Size
ABCG2_Exon8_fwd	5`AAAGTGAGTTCTCTTTGTTTTCCA3`	350 bp
ABCG2_Exon8_rev	5`GTTGACTGGTATCAGAAGACTGC3`	350 bp

Table 3: Primers used for PCR analysis.

Primer Name	ABCG2 Gene exon Primer (5 – 3)	Product Size
ABCG2_Exon14_fwd	AATAAGCAATCCCAAACATACGG	283 bp
ABCG2_Exon14_rev	TTATCAGAGCAAACACAGTTCAG	283 bp

Table 4: Primers used for PCR analysis.

Results

General Characteristics of Studied Diabetic Population

A total of 25 blood samples from clinically diagnosed and fasting plasma glucose confirmed diabetic (including both type I and type II diabetes) Blood were collected from different patients and different laboratories of district Mardan.

Demographic Characteristics of Diabetic Patients

As per inclusion criteria, the important demographic and bio-statistical details of diabetic patients were recorded. All the patients belong to a very low socio-economic status with unhealthy and sedative life styles. Table 5 show the demographic characteristics of diabetic patients and the clinical feature of diabetic patients is shown in Table 6.

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Demographic Characteristics	
Number of Diabetic Patients	N=25
No. of males	60% (N=15)
No. of females	40% (N=10)
Socio-economic status	Mostly low income
Life style	inactive
Age Lower limit	20 Years
Age Upper limit	70Years

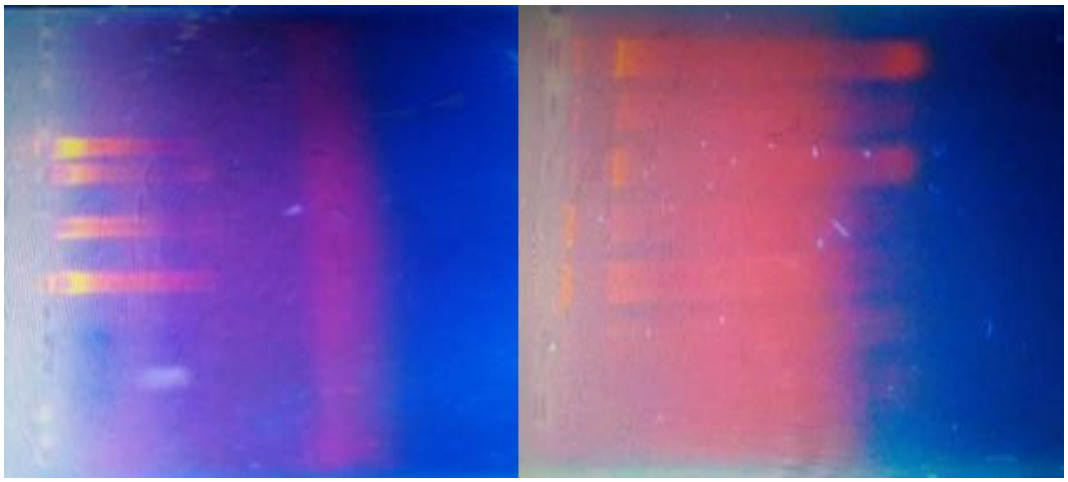
Table 5: Demographic Characteristics of Diabetic Patients.

Clinical Characteristics Number Percentage (%)		
Diabetes Insipidus	N = 2	8%
Diabetes Mellitus	N = 23	92%
Glucose Level		
Lower Limit	160	
Upper limit	450	

Table 6: Clinical Features of Diabetic Patients.

Results for DNA Extraction

Phenol / Chloroform method was used for all of the 25 samples. After the extraction of DNA, 1% of the gel electrophoresis was conducted in order to verify the extraction of DNA. The gel was then examined in gel doc and the pictures were captured in order to verify DNA presence which is shown in Figure 2.



Figures 2: Bands of extracted DNA sample 1-12.

Polymerase Chain Reaction (PCR)

After extraction of DNA, Polymerase Chain Reaction was done to amplify exon 8 and 14 of the ABCG2 gene.

Amplification of ABCG2 Gene (Exon8 and 14)

Set of both forward and reverse primers were used to amplify exon 8 and 14 of ABCG2 gene. Amplification of exon 8 by PCR is shown in Figure 3 and amplification of exon 14 is shown in the Figure 4.

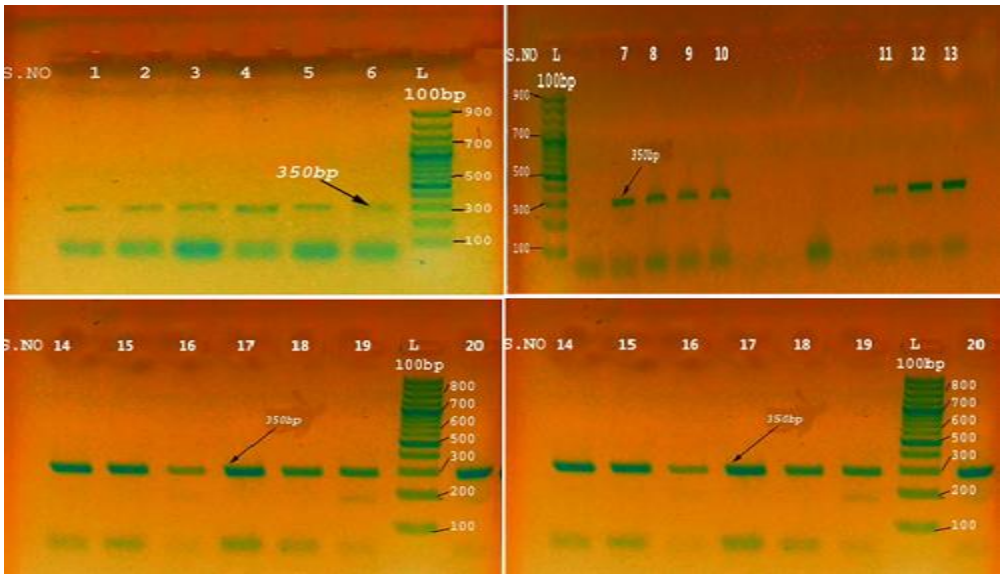


Figure 3: Exon 8 Amplification by PCR.25 amplified Samples of Exon 8. The optimized annealing temperature for exon 8 was 57˚C.

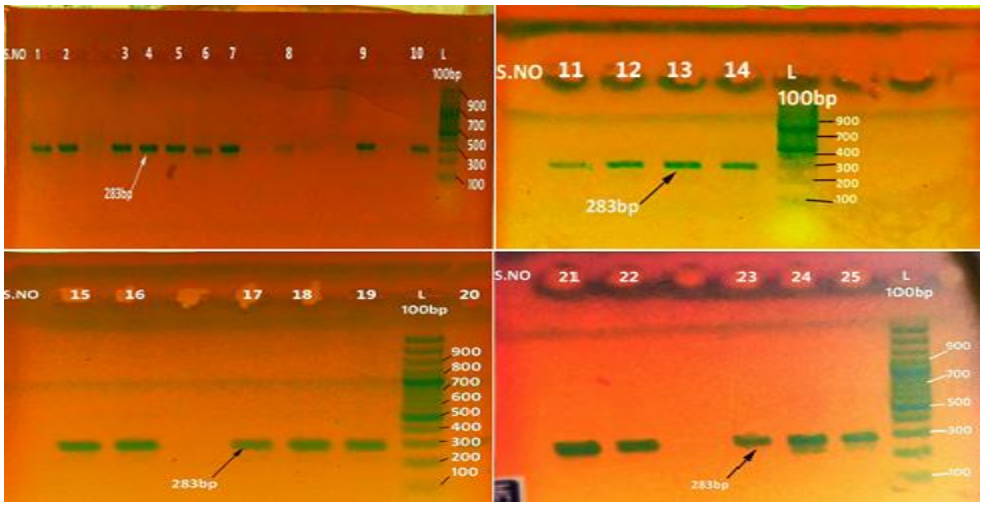


Figure 4: Exon 14 Amplification by PCR. 25 amplified Samples of Exon 14. The optimized annealing temperature for exon 14 was 60˚C.

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Discussion

The present work was aimed to evaluate the extracted DNA in order to amplify the most expressed gene of the body and to relate its expression rate with the Diabetes.ABCG2 gene expression occurs in the apical membranes of different healthy organs, that includes liver cells, kidney, small intestine and brain, It is thought that this plays important role in removal of toxic compounds from the cell and keep it safe from accumulation of uric acids and thus inhibit absorption.ABCG2 that is G2 family of ABC transporters is the carrier of many different types of substrates that also contain Uric Acids. It was also observed that a higher SUA (Serum Uric Acid) level means greater chance of Diabetes Type 2 according to the different Surveys. It is also discussed earlier that ABCG2 abnormalities in expression has adverse effect upon the Diabetes and have role in increasing the prevalence of Diabetes. This report shows consistency with the literature review as given earlier. The ABCG subfamily is composed of different kind of half transporters that are generally known to form hetero or homo-dimers to create the active transporter. TheABCG2 gene is consist of 16 exons and 15 introns and is located on chromosome 4q22 [39-41]. It was confirmed on Ensemble Genome website that exon and introns numbers are the same as in case of Spare boom A reference. The fungal toxin FTC (fumitremorgin C) is a potent and specific inhibitor of BCRP, but its neurotoxic effects made it to be discontinued use in vivo. K0143 is well known to be the most powerful BCRP inhibitor known for a long time [42].

The two inhibitors FTC and K0143 both was compared in light of some references in literature session that gives an idea that FTC is a potent BCRP (Breast Cancer Resistant Proteins) inhibitor but not much effective as K0143 because K0143 has no neurotoxic effect [43,44]. The SNP locus rs2231142 of the ABCG2 gene was found to be associated with hyperuricemia and found significant in obese alcoholic men who are suffering from type 2 diabetes and hypertension.

Conclusion

From our laboratory work we learned that it needs high precautions and to take the perfect number of chemical compounds in order to take the exact result. We achieved the amplification of ABCG2 gene but also, we were faced too many problems that were removed with passage of time. It was also concluded that the expression level of two exons of ABCG2 that is exon 8 and exon 14 varies in the normal individuals (control group) as compared to Diabetic Patients which was confirmed from its genotyping.

Author Contribution

Dr Shahid Ullah, Said Ali Shah and Farhan Ullah design and supervised the project, performed sample collection, data analysis, Experiments. All authors contribute to data analysis, wrote the manuscript and reviewed the manuscript.

Ethical Approval

The study of this report was endorsed by the ethical committee in AWKUM (Abdul Wali Khan University Mardan). Volunteer patients were informed, to obtain the fresh blood samples from them and it was ensured that

the patients are suffered just with diabetes in order to study the relevant disease.

Conflict of interest

The authors have no conflict of interest.

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