Asian Journal of Biomedical and Pharmaceutical Sciences
ISSN : 2249 - 622X

RESEARCH ARTICLE

Received on: 04-09-2014
Accepted on: 30-10-2014
Published on: 23-11-2014

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QR Code for Mobile users

Conflict of Interest: None Declared !

DOI: 10.15272/ajbps.v4i37.584

Analysis of polymorphism in Kir 6.2 gene in Type 2 diabetic patients

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Abstract

Targeted genomic studies are required to understand complex polygenic disorders such as Diabetes. In this context our approach was to detect the polymorphism in ATP-sensitive potassium (KATP) channel protein subunit Kir6.2. Blood samples of normal and type2 diabetic patients in and around the Chittoor district were obtained. Amplification of Kir6.2 gene was done by custom designed primers from genomic DNA of normal and type2 diabetic patients and amplification products were sent for sequencing. Three mutations i.e., R177G, C344R, V138L corresponding to the region of Kir6.2 has been identified in comparative analysis. The crystal structure of Kir6.2 protein model is not available so far in any database and hence we modeled the structure using Modeller software tool and validated the stereo chemical quality of modeled structure by using PROCHECK and ProsaWeb model. Mutated conformations were generated after the introduction of the R177G, C344R and V138L mutations and subsequent energy minimization and molecular dynamics were done. RMSDs were observed to be 5.406 Å, 5.123 Å and 5.449 Å for R177G, V138L and C344R mutated structures respectively and these conformational and RMSD variations were observed to affect the functionality of the K ATP sensitive channels there by leads to increased glucose levels.

Key words: KCNJ11, mutations, variants, K ATP sensitive channels

Cite this article as:
V. Praveen Chakravarthi, Y. Nanda Kumar, K. Mahamanjunath, A. Rukmini, D. Bhavani, J. A. Pradeep Kiran, T.Chiranjeevi, M. Ismail, J. Durga Prasad, M. Bhaskar. D Analysis of polymorphism in Kir 6.2 gene in Type 2 diabetic. Asian Journal of Biomedical and Pharmaceutical Sciences; 04 (37); 2014 ,15-20.

INTRODUCTION

Type2 diabetes is a late onset polygenic disease which is characterized by insulin resistance and relative insulin deficiency [1]. More than36 genes have been identified which are found to contribute the risk of type 2 diabetes [2]. Most of these genes are associated with different functions of β cells [3]. The genes associated with developing type2 diabetes include KIR6.2, SUR-1, HMGA1, Irs1, INS, HNF alpha1&4, MODY genes and glucokinase gene, TCF7L2, PPAG, FT0, NOTCH2, IGF2BP2, SLC30A8, JAZF1, HHEX, fatty acid binding protein [FABP2], Lipoprotein lipase [LPL GENE], ABCC8 and KCNJ11 etc [4-5]. Common polymorphism in these genes was associated with type2 diabetes. Identifying specific genes related to type 2diabetes is a difficult task as the disease is characterized by complex inheritance and the interaction with environment [6]. So far no single gene mutation has been identified as sole contributor to cause type2 diabetes [7].

ATP-sensitive potassium (KATP) present in the β cells of pancreas plays a crucial role in the release of insulin. KATP channel is an octameric protein with four Kir6.2 subunits and four SUR1 subunits. ABCC8 and KCNJ11 are the genes which codes for SUR1 and Kir6.2 subunits. When the glucose is plenty, it enters the β cells and metabolized which finally leads to increase in the concentration of ATP. Increased ATP causes the KATP channel closure that in turn causes the calcium channels to open that leads to calcium influx and insulin release. But some of the mutations in Kir6.2 especially E23K changes the conformation that makes the channel insensitive to increased concentration of ATP and the channel remains open even at the high levels of glucose in the environment. Open KATP channel causes the membrane hyperpolarization. This hyperpolarization keeps the voltage gated calcium channels to close preventing the influx of calcium and insulin release even at the high levels of glucose in the environment giving rise to hyperglycemic condition [8-9]. Polymorphism in KCNJ11 was found to be associated with both neonatal and type2 diabetes. Association of KCNJ11 polymorphism and neonatal diabetes were reported in several studies [7-8, 10]. Risk of type 2 diabetes and its association with polymorphism in KCNJ11 were reported earlier [11-13].

In this aspect the present study has been designed to analyze the polymorphism in Kir6.2 gene in type 2 diabetic patients of chittoor district. This study was also focused on building a crystal structure model for normal and mutated conformation and to study its impact on functionality of ATP sensitive potassium channels.

MATERIALS AND METHODS

The ethical committee of CKS Theja institution had reviewed the present study and given clearance to carry out the work under the reference number CKS/Ethical/JAN/2013 dated 21.01.2013. Chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Hi Media pvt ltd and plastic ware from Oxygen Co. Pvt.

Collection of Blood samples from normal and Type 2 diabetic patients:

Whole blood samples of 10 normal and 30 type 2 diabetic patients were obtained from Gayatri Diabetic Hospital, Tirupati. The samples were stored at 4^0C in refrigerator temporarily. These samples were used for Genomic DNA isolation.

Retrieval of Gene Sequence and primer designing

The gene sequence for Kir6.2 was retrieved from the gen bank (Gene ID: 3767 and Accession number: NC_000011). In this sequence the entire coding region was taken as target and primers were designed spanning the coding region that is from 1,162 to 2,414 bp by using primer 3 software (Table.1). Primers were verified for secondary structure and primer dimmer formation by using Sigma DNA Calculator an online tool (www.sigma-genosy s.com/calc/DNACalc.asp,Sigma Chemical Co). These custom designed primers were ordered and obtained from Sigma Chemical Co Pvt ltd.

Isolation and analysis of Genomic DNA from whole blood

Genomic DNA isolation from whole blood sample was carried out by salting out method as per the protocol of Lahiri et al, [14]. Concentration and purity of DNA was estimated by spectrophotometric analysis. Samples having purity in and around 1.8 were taken for study. Integrity of DNA was analyzed by running agarose gel electrophoresis.

Polymerase chain reaction:

A PCR reaction was set to amplify the Kir6.2 gene coding region of 1253bp by using the custom designed primers (Table.1). PCR reaction mixture includes 2x ready mix Taq PCR master mix (Sigma p4600), 500 ng of template DNA, and 0.5μM of forward and reverse primers. Thermal profile parameters include Initial denaturation at 95 0C for 10 minutes, Denaturation at 95 0C for 1minute, Annealing temperature at 59.4 0C for 40s, extension at 72 0C for 1 minute and final extension at 72 0C for 5 minute.

Sequence analysis

The obtained PCR products were sent for sequencing to Bio Corporals Pvt ltd, Chennai. After obtaining the sequences the sequencing analysis was done by using Clustal W online tool.

Modeling and validation of Kir6.2 protein:

The crystal structure of Kir6.2 protein model is not available so far in any database and hence we modeled the structure using Modeller software tool. The sequence has been searched through BLAST-P to find the proper template. A sequence alignment file has been generated in PIR format between the target and template sequences and a python script has been written to construct the homology models. The crystal structure of the G-Protein-Gated Inward Rectifier Potassium channel (3SYA) from mouse was taken as template which is having the maximum identity of 50% and query coverage of 83%. The stereo chemical quality of modeled structure was validated by using PROCHECK and ProsaWeb model validated servers [15].

Molecular dynamics studies:

All the in silico studies were carried out in the Molecular Operating Environment software tool (MOE 2011.10). The modeled wild type KIR structure was loaded into MOE working environment and subjected to protonation after the addition of polar hydrogen. Protonated structure was energy minimized in MMFF94x (Merck Molecular Force Filed) [16] and filed to Root Mean Square gradient of 0.05. The identified mutations (V138L, R177G and C344R) were introduced individually at the respective positions into the energy minimized wild type Kir6.2 structure and the energy minimization step was repeated with previously explained conditions.

All the energy minimized conformations of wild type and mutated Kir6.2 structures were subjected to molecular dynamic simulations individually in the same force field. The NPT (Number of particles, Pressure and Temperature) statistical ensemble was specified where both temperature and pressure were held fixed. The algorithm Nose-Poincare-Anderson (NPA) was specified to solve the equations of motion during simulations. The initial temperature was set to 30K and increased to a run time. The heat time was set at 0 picoseconds (ps), the total run time of simulations was carried out for 10 nano seconds (ns) with a temperature of 300K and pressure of 101 kPa. The total energy values of each conformation were plotted as graph to observe and correlate the energy variations among wild type and mutated KIR conformations.

Conformational Analysis:

The detailed structural analysis and the respective pictorial representations of all the 3D conformations were generated using the PDBSum web interphase [17]. The stabilized conformations of intact and mutated KIR structures obtained at the end of MD simulations were submitted to PDBSum and the conformational variations were identified which are due to respective mutations in the structure. The conformational variations were measured and correlated in terms of sheets, β-α-β units, hairpins, β–bulges, strands, helices, helix-helix interactions, β–turns and γ–turns. All the mutated KIR structures were superimposed with the wild type KIR and the variable RMSD values were defined.

RESULTS

Agarose gel electrophoretic analysis of isolated genomic DNA showed sharp bands below the well which indicates the well integrity of isolated genomic DNA (Fig.1).

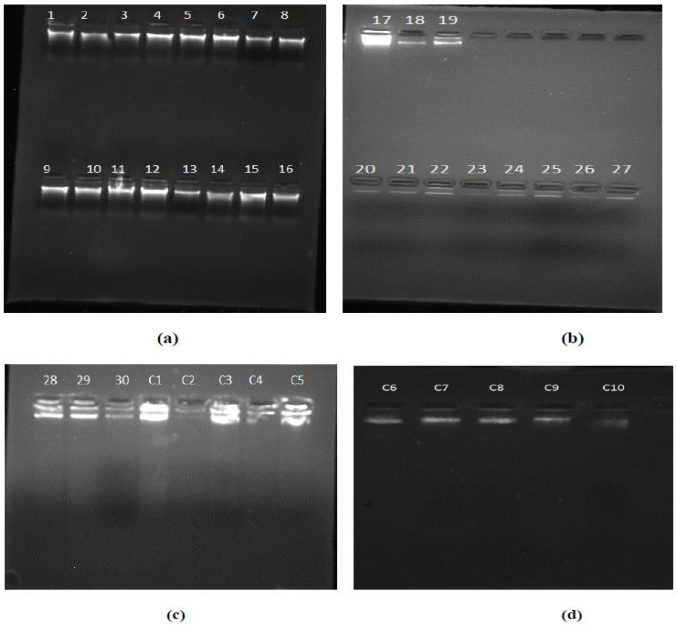


Fig.1: a) Lanes 1-16 genomic DNA from type 2 diabetic patients named as T2D1 to T2D16. b) Lanes 17 to 27 genomic DNA from type 2 diabetic patients named as T2D17 to T2D27. c) Lanes28-30 genomic DNA from type 2 diabetic patients named as T2D28 to T2D30, lanes C1 to C5 control DNA samples isolated from normal persons. d) Lanes C6 to C10 control DNA samples isolated from normal persons.

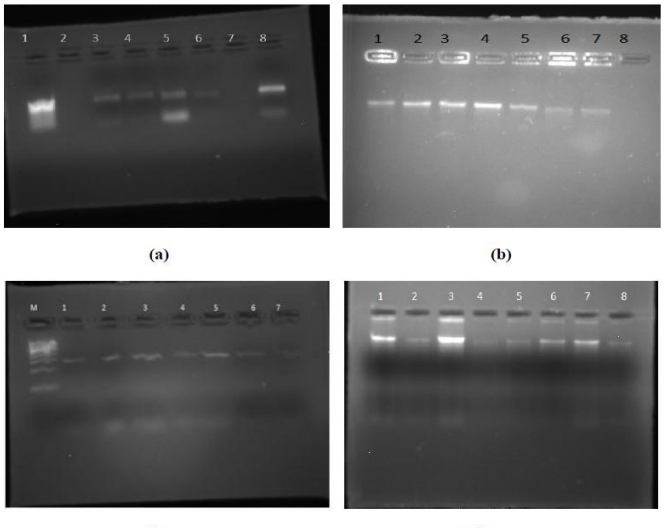


Figure.2: a, b, c, d : Amplified products of 1273 bp size obtained from custom designed primers in type 2 diabetes patients blood samples.

Amplified products when run on 0.8% agarose gel showed ~1273 bp products which were evident when compared with molecular marker (Fig.2). The genetic analysis of Kir6.2 gene in 30 type 2 diabetic patients (KIRT2D1, KIRT2D7 and KIRT2D13) revealed the presence of three mutations i.e. R177G, C344R and V138L respectively. Mutated sequences were deposited in genbank and the genbank IDs were KF646304, KF646305, KF646306. No mutations were observed in the remaining patients and normal controls.

Figure 3: The homology model of KIR protein.

The Kir6.2 protein model was constructed using Modeller tool (Fig.3) and the stereo-chemical quality of the model was validated. The Ramachandran plot generated from ProCheck validation server revealed that 91.8% residues are in most favored region, 7.2% are additional allowed regions, 1% in generously allowed regions and no residues were found in disallowed regions. The ProsaWeb analysis revealed that the structures fallen in the range of X-Ray crystallographic structures with a Z-score of -5.97 that indicates the overall model quality (Fig.4).

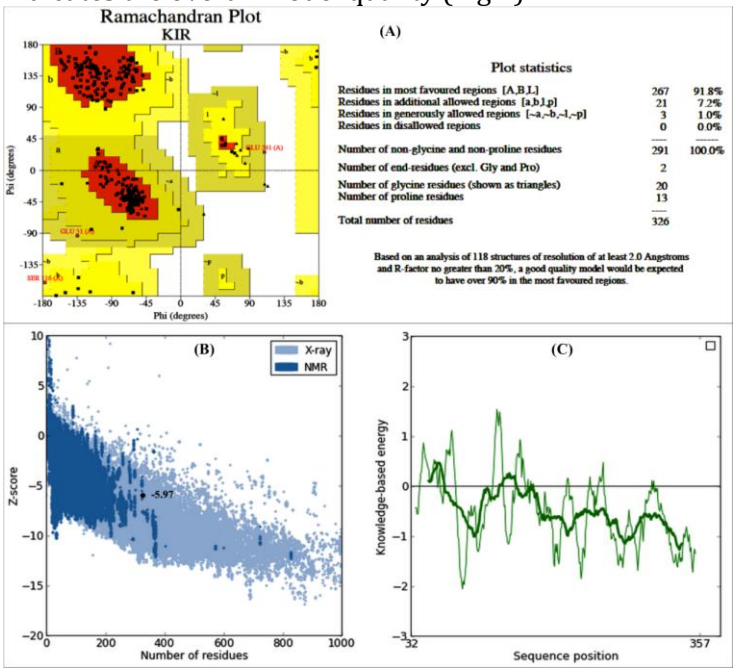


Figure 4: (A) Ramachandran plot explaining the stereo-chemical quality of KIR homology model. (A) Z-Score plot and (B) Residue score plot of KIR homology models.

The validated structure was subjected to energy minimization followed by molecular dynamics simulations to get the stabilized conformation. The mutated conformations were generated after the introduction of the R177G, C344R and V138L mutations and subsequent energy minimization and molecular dynamics. These conformations were correlated in terms of PDB Sum analysis and the results showed that the mutated conformations showed variable number of Sheets, Beta-hairpins, Beta-bulges, Strands, Helices, Helix-Helix interactions, Beta-turns and Gamma-turns where as no change was observed with the number of disulphide linkages (Table.2). RMSDs were observed to be 5.406 Å, 5.123 Å and 5.449 Å for R177G, V138L and C344R mutated structures respectively (Fig.5).

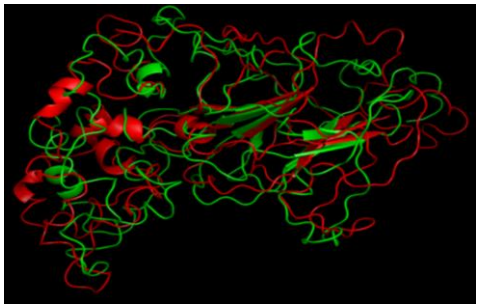
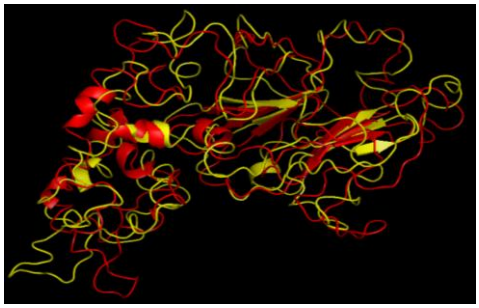
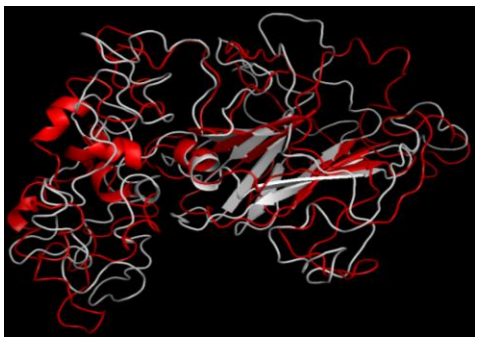
Mutation	Superimposed conformations of mutated KIR with wild type KIR	RMSD (Å)
R177G		5.406
V138L		5.123
C344R		5.449

Figure.5: The superimposed conformations of wild type and mutated KIR proteins indicating the impact of the mutation in terms of RMSD.

Gene	Primer Sequence	Annealing temperature	Product size
Kir 6.2	F: 5'GACTCTGCAGTGAGGCCCTA 3'	59.4	1253bp
	R: 5'CTCAGGACAGGGAATCTGGA 3'		

Table.1 Showing the primer sequences annealing temperature and product size

KIR	Sheets	Beta hairpins	Beta bulges	Strands	Helices	Helix-Helix interactions	Beta turns	Gamma turns	Disulphide bonds
Wild Type	4	5	4	12	9	4	70	27	1
R177G	3	4	2	10	5	-	74	45	1
C344R	3	4	-	10	2	-	87	51	1
V138L	3	3	2	8	5	1	81	40	1

Table 2: PDBSum analysis of KIR proteins showing the conformational variations due to mutations.

DISCUSSION

In the present study the genetic analysis of Kir 6.2 gene in type 2 diabetic people revealed the presence of three mutations corresponding to three patients out of 30 which were not reported earlier. Thus the three novel mutations identified were R177G, C344R and V138L. Common polymorphism observed in Kir6.2 gene was E23K and reported in many studies [11, 17-24]. But this mutation was not observed in our study. Other mutations of Kir6.2 observed especially in type 2 diabetic patients were L270V, and I337V [25], E10k, l270V, I337V [11], F35V, Y330C, F33I [7], Q52R, I296L and L164P [26], G53R, G53S, I182V, E227K, and E229K [27]. It was reported in many studies that mutations in KIR6.2 was the major cause in increasing the risk of type 2 diabetes [11-12, 28].

The model structure built by modeler tool and Ramachandran plot for validation which showed no errors in the homology model of protein. The ProsaWeb analysis with a Z-score of -5.97 indicates that these validations explain that the model was constructed with good stereo chemical quality and can be used for further investigations. The mutated conformations indicates that these mutations might affect the wild type conformation of Kir6.2 protein (Fig.5). This is more strengthened from the structure alignment of wild type conformation with mutated conformations. These conformational and RMSD variations (Fig.5) might affect the functionality of the molecule there by causing the increased glucose levels.

In conclusion the present study has identified 3 novel mutations in type 2 diabetic patients for which mutated conformational structures have been built and proved to some extent that these mutation plays a major role in causing increased blood glucose levels there by the risk of type 2 diabetes.

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