International Journal of Research
Available at https://edupediapublications.org/journals
e-ISSN: 2348-6848
p-ISSN: 2348-795X
Volume 03 Issue10
June 2016

Detection of Notch2, Kcnq1 and Mtnr1b Variant Genes in Risk Assessment of Type 2 Diabetes Mellitus

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Abstract-*Diabetes mellitus (DM), often simply referred to as, is a group of metabolic diseases that is characterized by a chronically elevated serum glucose level. T2DM is a metabolic disorder, characterized by persistent hyperglycemia, recent polymorphism studies have shown that several genes are related to T2DM and GDM. Therefore, our study was aimed at the identification of genes, role of KCNQ1, NOTCH2 and MTNR1B genes polymorphisms associated with T2DM and the risk of T2DM in our population. We have carried out a case control study including 40patients with T2DM, 40 control subjects. The isolation of genomic DNA from EDTA-blood samples were carried out followed by PCR-direct sequencing analysis The results of the present study reveal that three single nucleotide polymorphisms (KCNQ1, NOTCH2 and MTNR1B genes, respectively) are associated with both T2DM. The results of our study suggest a role of KCNQ1 and notch2 gene variants in the increased risk of T2DM in south Indian population.*

Key words: PCR, SNP, polymorphisms, t2dm

I. INTRODUCTION

Diabetes has become a common global health problem that affects >170 million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million (www.who.int). The majority of diabetes (~90%) is type 2 diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver [1, 2]. According to Wild [3] et al the 'top' three countries in terms of the number of T2D individuals with diabetes are India (31.7 million in 2000; 79.4 million in 2030), China (20.8 million in 2000; 42.3 million in 2030); and the US (17.7 million in 2000; 30.3 million in 2030). Clearly, T2D has become an epidemic in the 21st century where India leads the world with largest number of diabetic subjects.

In this study total of 100 subjects (50 diabetic and 50 non-diabetics) aged between 30 and 65 years volunteered for the study. All the subjects were from the Hyderabad telangana State, India. The subjects were collected from the Institute Of Genetics And Hospital For Genetic Diseases Hyderabad, Telangana. Subjects were classified as patients and controls, the working professions of subjects were some of them working as a labor and others were office work job. The ratios of the subjects were in equal ratio i.e 1:1. The subject's family history is also to be taken for results analysis.

Why we choose this topic is, Diabetes has become a common global health problem that affects >170 million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million [4]. The majority of diabetes (~90%) is type 2 diabetes (T2D).According to Wild et al the 'top' three countries in terms of the number of T2D individuals with diabetes are India (31.7 million in 2000; 79.4 million in 2030). In India Hyderabad is number one place in type 2 diabetes mellitus. That's why we choose this topic 1. Demographic and clinical characteristics in patients with Diabetes.2 are studied. To determine association of the genetic polymorphisms of notch2, kcnq1 and mtnr1b genes with type 2 Diabetes.3.

Significance of the Study

T2DM is a metabolic disorder, characterized by persistent hyperglycemia, whereas PTDM is a condition of abnormal glucose tolerance, with variable onset after organ transplant. The KCNQ1 and KCNJ11 gene encode potassium channels, which mediate insulin secretion from pancreatic b-cells, and KCN gene mutations are correlated with the development of diabetes. However, no studies have been carried out to establish an association between KCNQ1 and KCNJ11 gene polymorphisms and T2DM and PTDM. Therefore, our study was aimed at the identification of the role of KCNQ1 and KCNJ11 gene polymorphisms associated with T2DM and the risk of developing PTDM in the Asian Indian population. We have carried out a case control study including 100 samples, Out of 100 cases, 20 samples had been sequenced with sanger sequencing method at Eurofins private limited (out sourcing) of which 7 each had been done for NOTCH2, KCNQ1 and 6 samples of MTNR1BThe results of the present study reveal that two single nucleotide polymorphisms (rs2283228 and rs5210, of the KCNQ1 and KCNJ11 genes, respectively) are associated with both T2DM. The results of our study suggest a role of KCNQ1 and KCNJ11 gene variants in the increased risk of T2DM and PTDM in the Asian Indian population.

Aim and Objectives

1. To study demographic and clinical characteristics in patients with Diabetes
2. To determine association of the genetic polymorphisms of notch2, kcnq1 and mtnr1b genes with type 2 Diabetes
3. To establish the prevalence of gene polymorphisms with Diabetes in our population

II. MATERIALS AND METHODS

Materials Required

- Autoclaved eppendorf tubes (2 ml tubes)
- Autoclaved micro tips
- Distilled water
- Vortex mixer
- Refrigerated Centrifuge
- Pipettes
- pcr
- Gel dock
- Balance
- Conical flask
- Microwave
- Gel tray with comb

Reagents required for DNA Isolation:

All the chemicals were of either biochemical or molecular biology grade (Qualigen, Mumbai, India).

1. Tris-HCl, KCl, $MgCl_2$, EDTA (TKM-I, pH 7.6)
2. Tris-HCl, KCl, MgC_{l2}, EDTA, NaCl (TKM-II, pH 7.6)
3. 10% Sodium Lauryl Sulphate (SLS)
4. Triton-X 100
5. 6M NaCl
6. Tris-EDTA buffer, pH 8.0 (TE buffer)

Protocol

1. Blood sample (300 µl) was taken in autoclaved eppendorf tube. To the blood 800 µl of TKM-1 and 100 µl of 1% Triton X-100 was added, vortexed well, and incubated for 15 minutes at room temperature (RT).
2. The mixture was centrifuged at 10,000 rpm (fixed angle microlitre rotor, 24 × 1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 5 minutes, and then the supernatant was discarded.
3. To the pellet 800 µl of TKM-1 and 100 µl of 1% of Triton-X was added. The first and second steps were repeated until a white pellet was obtained.
4. To the pale pellet, 260 µl of TKM-2 and 70 µl of 10% SLS was added and incubated for 30 minutes at RT.
5. To the mixture 90 µl of 6 M NaCl was added and mixed well by tapping for 2-3 minutes. Then the mixture was centrifuged at 10,000 rpm (fixed angle microlitre rotor, 24 × 1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 5 minutes.
6. The supernatant was carefully transferred to 300 µl of pre-chilled absolute ethyl alcohol. Then the mixture was centrifuged at 10,000 rpm (fixed angle microlitre rotor, 24 × 1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 5 minutes.
7. The supernatant was discarded and to the DNA pellet 300 µl of 70% ethyl alcohol was added. Then the tubes were again centrifuged at 10,000 rpm (fixed angle microlitre rotor, 24 × 1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 5 minutes and the supernatant was discarded band the pellet was air dried.
8. The DNA pellet was suspended in 80 µl of TE buffer and the quality and quantity were monitored.

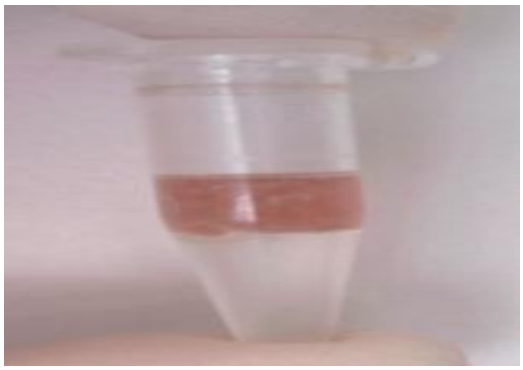

Figure 1: The formation of two phases after addition of phenol

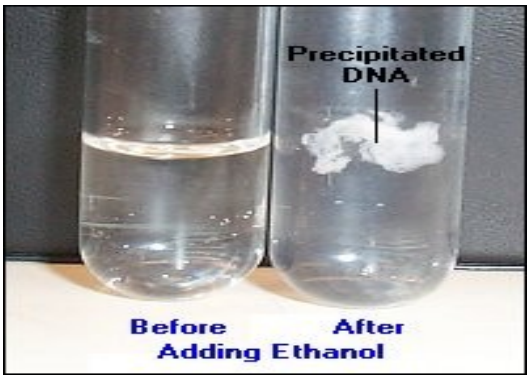

Figure 2: Precipitation of DNA on addition of Ethanol

Figure 3: Pellet on air dry condenses as a thick white mass

Quantization of DNA

a) By spectrophotometer

DNA was quantified by spectrophotometer. 5ml of isolated genomic DNA was diluted to 1ml with autoclaved distilled water and O.D was measured at 260 nm and at 280nm. The ratio of readings at 260nm and 280nm (O.D 260/280) provides an estimate of the purity of the DNA. The 260 / 280nm values were between 1.8 and 2 and indicated good quality of DNA.

b) By Agarose gel electrophoresis

5µl of isolated genomic DNA was electrophoresed on 0.8 % agarose gel along with the DNA of known amount. Ethidium bromide stained gel was visualized under UVi gel documentation system. Comparison between the band intensity of known amount of DNA to that of unknown provides the concentration of DNA present in the unknown sample.

Agarose Gel Electrophoresis

Aim

To separate the amplified DNA using agarose gel electrophoresis

Reagents:

1X TBE Buffer
Agarose
Ethidium bromide

Principle

Electrophoresis is used to separate molecules, The phosphate molecules that make up the backbone of DNA molecules have a high negative charge, thus in agarose gel electrophoresis, the DNA is forced to move through a sieve of molecules made of agarose. The end result is that larger pieces of DNA move slower than small pieces of DNA. The gel is stained with Ethidium bromide and visualized in presence of UV rays, as Ethidium bromide intercalates in between DNA strands thus getting excited in the presence of UV rays.

Procedure:

Step 1: Mixing Gel

On the scale, weighed 1 gram of agarose onto a piece of weighing paper. Add agarose to conical flask. Add 100mL of 1X TBE to conical flask. Swirl vigorously to thoroughly mix agarose. Slurry was by opaque.

Step 2: Melting Gel

Put agarose and 1x TBE slurry into microwave. Heat the mixture for 160°C for 3 minutes.
Swirl until mixture is clear. Add 2µl of EtBr and mix properly.

Step 3: Pouring the Gel

Place gel tray into casting chamber. Added casting comb(s) into the appropriate slot(s).Poured agarose into gel tray to about 5-7mm.Let sit for at least 30 min, until gel was cool to touch and was opaque in appearance. Carefully removed combs by pulling them upwards firmly and smoothly in a continuous motion. The remaining depressions were the wells into which your samples will be loaded. Once set, place gel and tray into gel rig, with wells on the left (cathode) side. Then, filled gel rig with 1X TBE sufficient to cover the entire gel.

Step 4: Loading the Gel

Cut a piece of Para film and placed it flat on the bench top.Using a pipette, placed small dots of 6X loading dye (About 1-2uL) onto Para film, in rows of 8, 1 dot for Each DNA or PCR sample that would be loaded on the gel. It was not necessary to be exact, and it was not necessary to change the tip. Using a pipette took 3 µl of product and pipetted it onto its corresponding dye dot, then mixed the sample and dye by pipetting up and down. Then, pipetted up the dye/sample solution and released it into the proper well. Put gel box cover into place (this step was essential for your gel to run and to minimize the risk of electric shock).Turned on power supply. Run the gel for 75 minutes at 80 volts.

Step 5: Visualizing the Gel

Turn off power supply. Using gloved hands, removed the cover from the gel box. Removed the gel and casting tray from rig, avoiding dripping buffer all over the benctop. Carefully place the gel inside the gel documentation system. Observe the gel under UV light.

Analysis:

After electrophoresis the gel was illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation). The illuminator apparatus also contained imaging apparatus that takes an image of the gel, after illumination with UV radiation. The ethidium bromide fluoresces reddish-orange in the presence of DNA, since it had intercalated with the DNA. The DNA band could also be cut out of the gel, and could then be dissolved to retrieve the purified DNA. The gel was then photographed usually with a digital or polaroid camera. Although the stained nucleic acid fluoresces reddish-orange, images were usually shown in black and white.

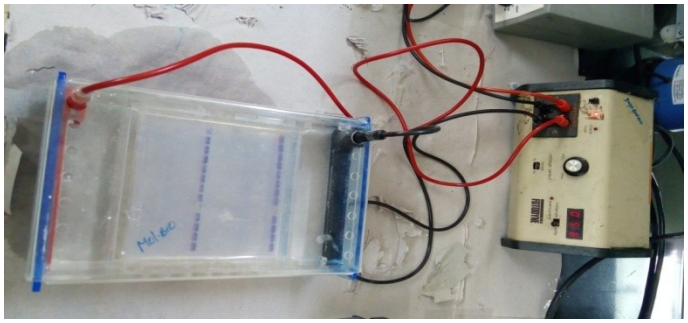

Figure 4: Gel electrophoresis

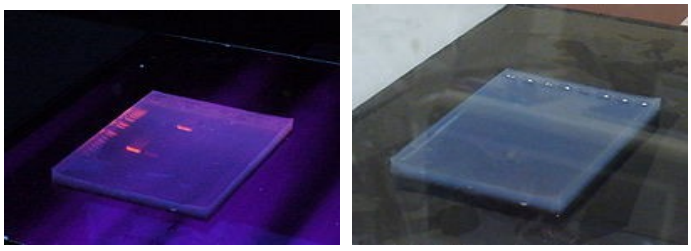

Figure 5: The gel without and with illumination (left to right)

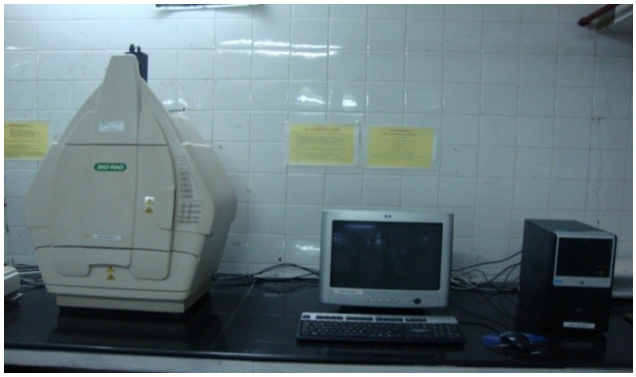

Figure 6: Gel Documentation unit

III. DETECTION OF APOC3 GENE VARIANTS

Detection of the –455 and –482 APOC3 promoter polymorphisms has been carried out using an amplification and restriction enzyme digestion technique by the modified method of Guettier et al., 2004.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Principle

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

A basic PCR set up requires several components and reagents. These components include:

a) DNA template that contains the DNA region (target) to be amplified.

b) Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.

c) Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C.

d) Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.

e) Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

f) Divalentcations, magnesium or manganese ions; generally Mg^{+2} is used, but Mn^{+2} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{+2} concentration increases the error rate during DNA synthesis (Pavlov AR)

g) Monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction

Procedure:

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three . The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

Initialization step:

This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermo stable polymerases are used), which is held for 1–6 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step:

This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step:

The reaction temperature is lowered to 57 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. The melting temperature of a specific oligonucleotide primer (Tm) can be calculated by the following simple equation:

International Journal of Research
Available at https://edupediapublications.org/journals
e-ISSN: 2348-6848
p-ISSN: 2348-795X
Volume 03 Issue10
June 2016

Tm= 2 (A+T) + 4 (G+C) - 5ºC

Extension/elongation step:

The temperature at this step depends on the DNA polymerase used.Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation

This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold:

This step at 4º–15 °C for an indefinite time may be employed for short-term storage of the reaction mixture.

Table 1: PCR Master mixture for kcnq1gene: (Assay 1 for 1 reaction µl)

water	11 µl
Forward primer	1 µl
Reverse primer	1 µl
Pcr master mix	10 µl
DNA sample	2 µl
Total	25 µl

47 µl master mix + 3 µl DNA in each reaction mixture

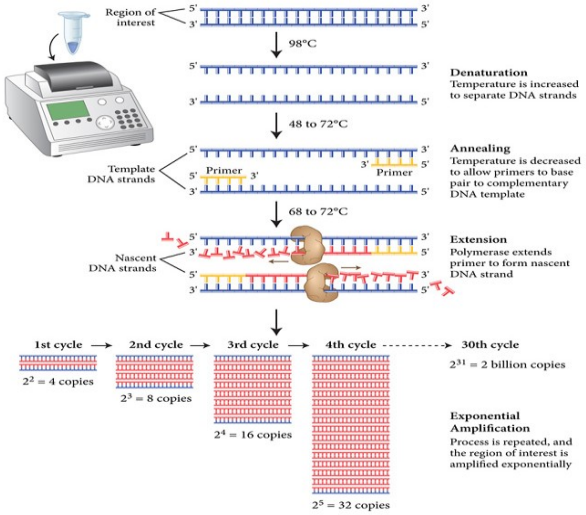

Figure 7: Schematic drawing of the PCR cycle.

Figure 8: Thermocycler

Table 2: Primer sequence for kcnq1 gene

Primer	Sequence
Forward primer	5'- CCAGGAGTGGGTGGTTCTAC -3'
Reverse primer	5'- GCCAGCACTAAAGATCTTGC -3'

Table 3: PCR Conditions for kcnq1

Step 1	Initial denaturation	94˚C for 6 min
Step 2	Denaturation	94˚ C for 1min
Step 3	Annealing	58˚C for 1min
Step 4	Extension	72˚C for 2 min
Repeat step 2-4 for 34 cycles		
Step 5	Final extension	72 ^{0}C for 8 min.
	Hold - 4^{0}C for 1 Hour	

Reaction is cycled 34 times. PCR reaction comprises, denaturation at 94ºC for 1 min, annealing at 580C for 1 min, and

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e-ISSN: 2348-6848
p-ISSN: 2348-795X
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June 2016

extension at 72ºC for 1 min. The size of the amplified PCR product is 196bp as shown in Fig.

Table 4: Primer sequence for notch2 gene

Primer	Sequence
Forward primer	5'- GCTGCAAGTATGCATCAGTGC -3'
Reverse primer	5'- GATTTGAGCATCACAGCCAATT -3'

Table 5: PCR Conditions for notch2

Step 1	Initial denaturation	94˚C for 6 min
Step 2	Denaturation	94˚ C for 1min
Step 3	Annealing	58˚C for 1min
Step 4	Extension	72˚C for 2 min
Repeat step 2-4 for 34 cycles		
Step 5	Final extension	72 ^{0}C for 8 min.
	Hold - 4^{0}C for 1 Hour	

Table 6: Primer sequence for MTNR1B gene

Primer	Sequence
Forward primer	5'-CTCAATACCCACCCTCAA-3'
Reverse primer	5'-CCAACAGAAGAATGGATAAG-3'

Table 7: PCR Conditions for mtnr1b

Step 1	Initial denaturation	95˚C for 6 min
Step 2	Denaturation	94˚ C for 30sec
Step 3	Annealing	57˚C for 30sec
Step 4	Extension	72˚C for 30sec
Repeat step 2-4 for 34 cycles		
Step 5	Final extension	72 ^{0}C for 7 min
	Hold - 4^{0}C for 1 Hour	

IV. RESULTS AND DISCUSSION:

Collection of blood samples: A total of 100 subjects (50 diabetic and 50 non-diabetic) aged between 30 and 65 years volunteered for the study. All the subjects were from the Hyderabad telangana State, India. The subjects were collected from the Institute Of Genetics And Hospital For Genetic Diseases Hyderabad, Telangana.

The results obtained are presented in two sections:

I. Genomic DNA extraction and purity
II. Detection of polymorphism
   a) PCR and Direct Sequencing

I. Genomic DNA extraction and purity:

Genomic DNA was extracted by the salting out method as described in the methods and materials section. The extracted DNA from all the t2dm samples and normal subjects appeared to be pure and intact as judged by the electrophoretic mobility of the DNA bands.

M= ladder 1, 2, 3, 4, 5, 6, 7, 8=samples

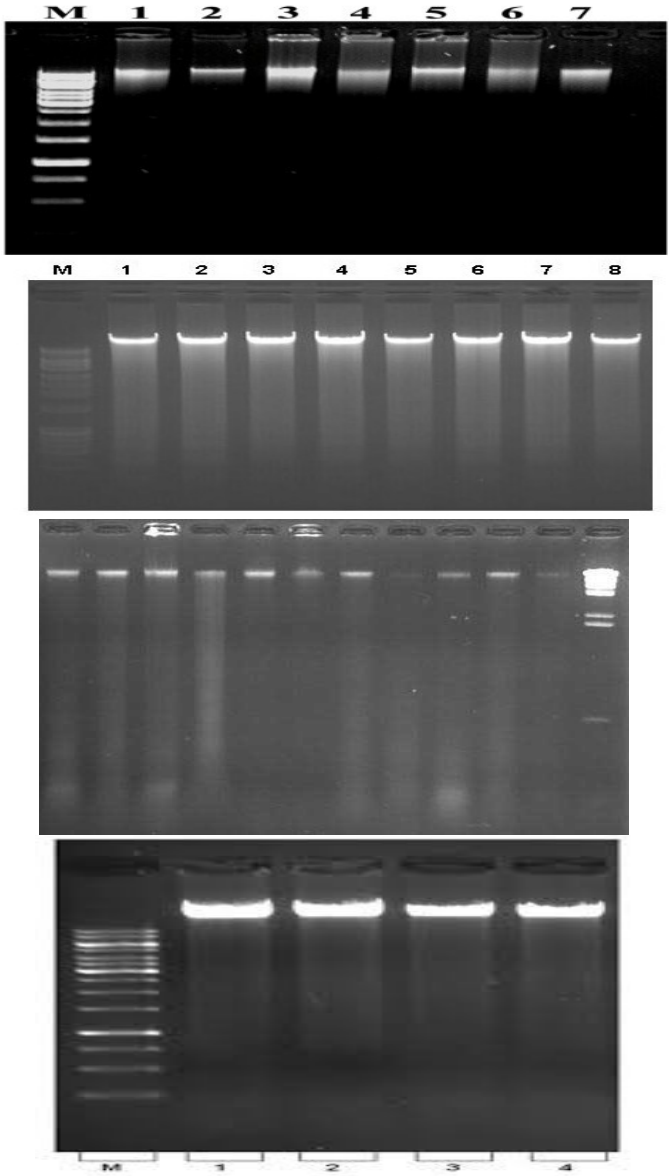

Figure 9: the gel pictures which we isolated.dna from the blood samples

Pcr producrt size: the size of Pcr producrt was determined by using gel dock

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e-ISSN: 2348-6848
p-ISSN: 2348-795X
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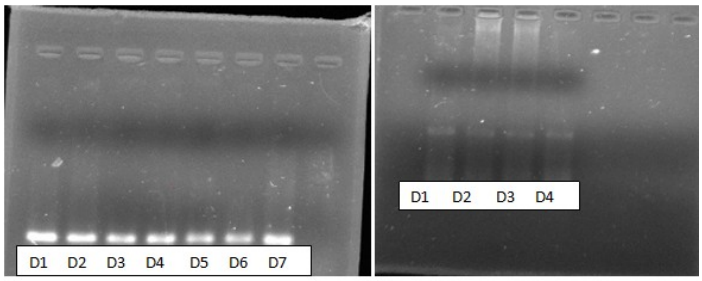


Figure 10: MTNR1B gel pic: 242bp; KCNQ1 gel pic
D1, D2, D3, D4, D5, D6, D7: diseased samples

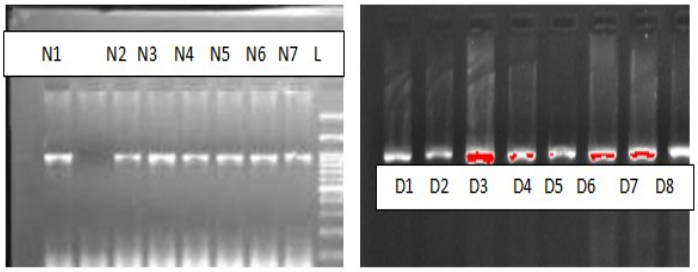


Figure 11: NOTCH2 Gene Control, NOTCH2 Gene Diseased

- N1 N2 N3 N4 N5 N6 N7 are control PCR products.
- D1 D2 D3 D4 D5 D6 D7 D8 diseased PCR products.
- L-LADDER
- Product size notch2 is **952bp**. Kcnq1 product size is **240**
- Given N1, N3,D1,D2,D5&D8 samples for sequencing

Overall Results from the Collected Samples

Patient Samples

PCR assay was conducted in over 100 samples and sequencing had been done for 20 samples to look for SNP's for KCNQ1, NOTCH2 and MTNR1B. For genotype analysis, genomic DNA was extracted from whole blood using the method tritonx method and stored at -20°C after confirming that the concentration of genomic DNA was 10-20 mg/L. PCR was performed to amplify the target genes. Primer sequences were as follows: kcnq1 upstream primer, 5'CCAGGAGTGGGTGGTTCTAC3'downstreamprimer5'GC CAGCACTAAAGATCTTGC -3'.Notch2 upstream primer 5'-GCTGCAAGTATGCATCAGTGC-3', downstream 5'-GATTTGAGCATCACAGCCAATT-3'. Upstream primer, 5'-CTCAATACCCACCCTCAA-3';downstream primer, 5'-CCAACAGAAGAATGGATAAG-3'. PCR was carried out using 0.2 mL genomic DNA in a total volume of 20 mL, with 35 cycles of initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 7 min. PCR products were evaluated by running 2 mL on 1.5% agarose-TBE gels, using 250 base pairs as the standard molecular weight. PCR products and upstream primers were sequenced by the Eurofins Genomics India Pvt Ltd., DNA sequence analysis was performed using the direct sequencing method.

Clinical History

A total of 100 subjects (50 diabetic and 50 non-diabetic) aged between 30 and 65 years volunteered for the study. All the subjects were from the Hyderabad telangana State, India. The subjects were collected from the Institute Of Genetics And Hospital For Genetic Diseases Hyderabad, Telangana. Subjects were classified as patients and controls, the working profession of subjects was some of them working as a labor and others were office work job. The ratio of the subjects was in equal ratio i.e 1:1. The subjects' family history is also to be taken for results analysis. Out of 100 cases, 20 samples had been sequenced with sanger sequencing method at Eurofins private limited (out sourcing) of which 10 controls and 10 patients each had been done for NOTCH2, KCNQ1 and MTNR1B as listed below in the table 8.

Table 8: Clinical history of the patients

S. no	Characteristics	Cases (n=10)	Healthy Controls (n=10)
1	age(years)	41 to 65	30 to 60
2	Males/females	20	20
3	BMI(kg/m^2)	23.47 ± 5.48	22.99 ± 4.97
4	Medication	5	0
5	Non medication	5	0
6	Profession	Office/Labor	Labor/Office

NOTCH2 Mutation

After sequencing the samples, the results were analysed by multiple sequence alignment (CLUSTAL W) by taking the actual reference sequence from DbSNP of NCBI of **NOTCH2** and comparing with the ab1 sequence (applied Biosystems) of Sanger sequencing results of the same SNP of control and diseased separately as given below.

For control sample: Notch2

Reference sequence for NOTCH2 (rs10923931)

CTTGTTGCTCCATCCTCTGGCTTCA[G/T]GCTGAACA AGTAAGATTATGGGCAC

Reference sequence for NOTCH2, forward sequence:

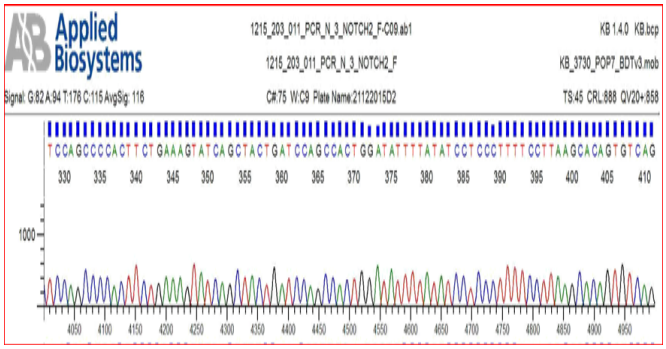
CTTGTTGCTCCATCCTCTGGCTTCAGGCTGAACAAG TAAGATTATGGGCAC

Sequence of NOTCH2_ab1

TGAGCACGACCCGTGTGTATGCTGTTATAATCCATT
TAAATTGGGTTTTTTCCATCAAACCCAATTAATTGA
ACCAACAAAAATAATTACTTCTGCCCTGAGATAAG
CAGATTAAGTTTGTTCATTCTCTGCTTTATTCTCTCC
ATGTGGCAACATTCTGTCAGCCTCTTTCATAGTGTG
CAAACATTTTATCATTCTAAATGGTGACTCTCTCCC
TTGGACCCATTTATTATTCACAGATGGGGAGAACCT
ATCTGCATGGACCTCTGTGGACCACAGCGTACCTG
CCCCCTTGTTGCTCCATCCTCTGGCTTCAGTGCTGA
ACAAGTAAGATTATGGGCACGCCACTGGATATTTT
ATATCCTCCCTTTTCCTTAAGCACAATGTCAGACCA

AATTGCTTGTTTCTTTTTCTTGGACTACTTTAATTTG
GATCCTTTGGGTTTGGAGAAAGGGAATGTGAAAGC
TGTCATTACAGACAACAGGTTTCAGTGATGAGGAG
GACAACACTGCCTTTCAAACTTTTTACCGATCTCTT
AGAACAGGTTTCAGTGATGAGGAGGACAACACTGC
CTTTCAAACTTTTTACCGATCTCTTAGATTTTAAGA
ACTCTTGAATTGTGTGGTATCTAATAAAAGGGAAG
GTAAGATGGATAATCACTTTCTCATTTGGGTTCTGA
ATTGGAGACTCAGTTTTTATGAGACACATCTTTTAT
GCCACTTTCTCATTTGGGTTCTGAATTGGAGACTCA
GTTTTTATGAGACACATCTTTTATGCCATGTTTAGT
TTTAAGCATCATTTTCTTTATGTGAGGTGGGGGAAG
GGAAAGGTATGAGGGAAGAGATTCTG

For Control Notch2



Multiple sequence alignment: >1215_203_012_PCR_N_CONTROL _NOTCH2_F-D09.ab1

REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TGAGCACGACCCGTGTGTATGCTGTTATAATCCATT
TAAATTGGGTTTTTTCCATCAAAC
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
CCAATTAATTGAACCAACAAAAATAATTACTTCTG
CCCTGAGATAAGCAGATTAAGTTTG
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TTCATTCTCTGCTTTATTCTCTCCATGTGGCAACATT
CTGTCAGCCTCTTTCATAGTGTG
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
CAAACATTTTATCATTCTAAATGGTGACTCTCTGCC
CTTGGACCCATTTATTATTCACAG

REFSEQ_NOTCH2_F ----------
----------CTTGTTGC

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
ATGGGGAGAACCTATCTGCATGGACCTCTGTGGAC
CACAGCGTACCTGCCCCCTTGTTGC
****

REFSEQ_NOTCH2_F
TCCATCCTCTGGCTTCAGTGCTGAACAAGTAAGATT
ATGGGCAC----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TCCATCCTCTGGCTTCAGTGCTGAACAAGTAAGATT
ATGGGCACGCCACTGGATATTTTA
****

REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TATCCTCCCTTTTCCTTAAGCACAATGTCAGACCAA
ATTGCTTGTTTCTTTTTCTTGGAC
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TACTTTAATTTGGATCCTTTGGGTTTGGAGAAAGGG
AATGTGAAAGCTGTCATTACAGAC
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
AACAGGTTTCAGTGATGAGGAGGACAACACTGCCT
TTCAAACTTTTTACCGATCTCTTAG
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
ATTTTAAGAACTCTTGAATTGTGTGGTATCTAATAA
AAGGGAAGGTAAGATGGATAATCA
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
CTTTCTCATTTGGGTTCTGAATTGGAGACTCAGTTT
TTATGAGACACATCTTTTATGCCA
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
CGTATAGATCCTCCCCTGCTATTTTTGGTTTATTTTT
ATTGTTATAAATGCTTTCTTTCT
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TTGACTCCTCTTCTGCCTGCCTTTGGGGATAGGTTT
TTTTGTTTGTTTATTTGCTTCCTC
REFSEQ_NOTCH2_F ----------

1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
TGTTTAGTTTTAAGCATCATTTTCTTTATGTGAGGTG
GGGGAAGGGAAAGGTATGAGGGA
REFSEQ_NOTCH2_F ----------
1215_203_012_PCR_N_4_NOTCH2_F-D09.ab1
AGAGATTCTG

Result: No snp identified in the diseased sequence of ab1 at rs no of NOTCH2 (rs10923931) gene. The **GG genotype** (meaning

both copies of the gene contain the "**G**" allele) is referred to as "homozygous normal.

Notch2 Diseased sample

NOTCH2(rs10923931)Reference sequence :

CTTGTTGCTCCATCCTCTGGCTTCAG/TGCTGAACAAGT
AAGATTATGGGCAC

NOTCH2 Reference sequence for Diseased:

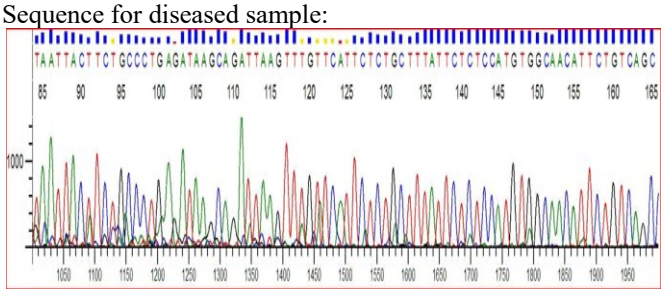
CTTGTTGCTCCATCCTCTGGCTTCATGCTGAACAAGT
AAGATTATGGGCAC

CLUSTAL O(1.2.1)

Sequence of _NOTCH2_F-C09.ab1

NNTTTGACTGTCTGTGGCCATATGGAACCCTGCGTGT
CTGTTGGCATAATCAATTTACAAATGGTTTTTTCCTTC
CTATACAAATTTATTGAACCAACAAAAATAATTACTT
CTGCCCTGAGATAAGCAGATTAAGTTTGTTCATTCTC
TGCTTTATTCTCTCCATGTGGCAACATTCTGTCAGCCT
CTTTCATAGTGTGCAAACATTTTATCATTCTAAATGG
TGACTCTCTGCCCTTGGACCCATTTATTATTCACAGA
TGGGGAGAACCTATCTGCATGGACCTCTGTGGACCA
CAGCGTACCTGCCCCTTTCTGCCTTGTTGCTCCATCCT
CTGGCTTCATGCTGAACAAGTAAGATTATGGGCACCT
GGTAAGATTATGGCCTCCCTTTTCCTTAAGCACAGTG
TCAGACCAAATTGCTTGTTCTTTTTCTTGGACTACTTT
AATTTGGATCCTTTGGGTTTGGAGAAAGGGAATGTG
AAAGCTGTCATTACAGACAACAGGTTTCAGTGATGA
GGAGGACAACACTGCCTTTCTGTGAAAGCTGTCATTA
CAGACAACAGGTTTCAGTGATGAGGAGGACAACACT
GCCTTTCAAACTTTTTACCGATCTCTTAGATTTTAAGA
ACTCTTGAATTGTGTGGTATCTAATAAAAGGGAAGGT
AAGATGGATAATCACTTTCTCATTTGGGTTCTGAATT
GGAGACTCAGTTTTTGGGAAGGTAAGATGGATAATC
ACTTTCTCATTTGGGTTCTGAATTGGAGACTCAGTTTT
TATGAGACACATCTTTTATGCCACGTATAGATCCTCC
CCTGCTATTTTTGGTTTATTTTTATTGTTATAAATGCT
TTCTTTCTTTGACTCCTCTTCTGCCTGCCTTTGGGGAT
AGGTTTTTGGCTGTGATGCCTCAGATCAGGGGGGGG
GGGGCA

Sequence for diseased sample:



Multiple sequence alignment of
1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1

REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
NNTTTGACTGTCTGTGGCCATATGGAACCCTGCGTG
TCTGTTGGCATAATCAATTTACAA

REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
ATGGTTTTTTCCTTCCTATACAAATTTATTGAACCA
ACAAAAATAATTACTTCTGCCCTG
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
AGATAAGCAGATTAAGTTTGTTCATTCTCTGCTTTA
TTCTCTCCATGTGGCAACATTCTG
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
TCAGCCTCTTTCATAGTGTGCAAACATTTTATCATT
CTAAATGGTGACTCTCTGCCCTTG
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
GACCCATTTATTATTCACAGATGGGGAGAACCTAT
CTGCATGGACCTCTGTGGACCACAG
REFSEQ_NOTCH2_FORWARD -------
CTTGTTGCTCCATCCTCTGGCTTCAGGCTGAACAAG
TAAGATTATGGGCAC
1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
CGTACCTGCCTTGTTGCTCCATCCTCTGGCTTCATG
CTGAACAAGTAAGATTATGGGCAC
****
REFSEQ_NOTCH2_FORWARD --ACAAG---
----TAAGATTATGG----------GCAC----------
1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
GAACAAGCCACTGGTAAGATTATGGCCTCCCTTTTC
CTTAAGCACAGTGTCAGACCAAAT
****
****
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
TGCTTGTTTCTTTTTCTTGGACTACTTTAATTTGGAT
CCTTTGGGTTTGGAGAAAGGGAA
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
TGTGAAAGCTGTCATTACAGACAACAGGTTTCAGT
GATGAGGAGGACAACACTGCCTTTC
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
AAACTTTTTACCGATCTCTTAGATTTTAAGAACTCT
TGAATTGTGTGGTATCTAATAAAA
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
GGGAAGGTAAGATGGATAATCACTTTCTCATTTGG
GTTCTGAATTGGAGACTCAGTTTTT

REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
ATGAGACACATCTTTTATGCCACGTATAGATCCTCC
CCTGCTATTTTTGGTTTATTTTTA
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
TTGTTATAAATGCTTTCTTTCTTTGACTCCTCTTCTG
CCTGCCTTTGGGGATAGGTTTTT
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
TTGTTTGTTTATTTGCTTCCTCTGTTTTGTTTTAAGC
ATCATTTTCTTATGTGAGGTGGG
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
GAAGGGAAAGGTATGAGGGAAAGAGAGTCTGAAA
ATTAAAATATTTTAATATAAGCAATT
REFSEQ_NOTCH2_FORWARD ----------

1215_203_011_PCR_N_3_NOTCH2_F-C09.ab1
GGCTGTGATGCCTCAGATCAGGGGGGGGGGGGCA

Result: SNP identified in the diseased sequence of ab1 at NOTCH2 (rs10923931)gene. The GT **genotype** is described as "heterozygous,T replaced by G.

MTNR1B Control Sample: After sequencing the samples, the results were analysed by multiple sequence alignment (CLUSTAL W) by taking the actual reference sequence from DbSNP of NCBI of mtnr1b and comparing with the ab1 sequence (applied Biosystems) of Sanger sequencing results of the same SNP of control and diseased separately as given below.

MTNR1B Reference no (rs4753426) forward sequence

CAACATATTTGTGATTAATCCATGC[C/T]GTTGTGTAGG
TCAATCATCTTTATT

MTNR1B Reference Sequence For Controle:

CAACATATTTGTGATTAATCCATGCCGTTGTGTAGGTC
AATCATCTTTATT

Sequence of mtnr1b_r-d02.ab1

CGACTATTTATTTTCCTCACAAATGTACTTACCTCTCTA
ATTGTTCAGTAAATAACATCTCAACATATTTGTGATTA
ATCCATGCCGTTGTGTAGGTCAATCATCTTTATTTTTCC
CCTTTTTCTTCCTCTTTCCCCCCTCTTTCCTTTCTTTCTCT
TCCCTCTCTCCTTCTTCTCCTTTCTCTCTCTTTATCTCCC
ACTCCCTTACGTCTCCCCCCCTTCTCTCTCTTCCCCCTTT
CTCCATCCCCTCCCCTCCCTCTCTCCTCCCTCTTTCCCCC
CCCATCCTCCTCCCTCTCACATCACTTCCAAACTCCACT
AATTCCTCTAATCTTTGTACTCTCCTTTCTCTCCTTGCTT
TCCCACCTCTCACCCCCCTTA

Multiple sequence alignment CLUSTAL O(1.2.1)

>1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
MTNR1B ----------
----------CAACAT--
1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
CGACTATTTATTTTCCTCACAAATGTACTTACCTCTCTA
ATTGTTCAGTAAATAACATCT
******
MTNR1B -----
ATTTGTGATTAATCCATGCCTGTTGTGTAGGTCAATCAT
CTTTATT--------
1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
GTGCTAATTTGTGATTAATCCATGCCTGTTGTGTAGGTC
AATCATCTTTATTTTCCCCTT
****
MTNR1B ----------

1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
TTTCTTCCTCTTTCCCCCCTCTTTCCTTTCTTTCTCTTCC
CTCTCTCCTTCTTCTCCTTT
MTNR1B ----------

1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
CTCTCTCTTTATCTCCCACTCCCTTACGTCTCCCCCCCTT
CTCTCTCTTCCCCCTTTCTC
MTNR1B ----------

1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
CATCCCCTCCCCTCCCTCTCTCCTCCCTCTTTCCCCCCC
CATCCTCCTCCCTCTCACATC
MTNR1B ----------

1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
ACTTCCAAACTCCACTAATTCCTCTAATCTTTGTACTCT
CCTTTCTCTCCTTGCTTTCCC
MTNR1B ----------
1115_245_004_PCR_D4_MTNR1B_R-D02.ab1
ACCTCTCACCCCCCTTA

RESULT: No SNP identified in the diseased sequence of ab1 at rs no of MTNR1B (rs4753426) gene. The **CC genotype** (meaning both copies of the gene contain the "**C**" allele) is referred to as "homozygous normal.

MTNR1B Diseased sample: MTNR1B (rs4753426) After sequencing the samples, the results were analysed by multiple sequence alignment (CLUSTAL W) by taking the actual reference sequence from DbSNP of NCBI of mtnr1b and comparing with the ab1 sequence (applied Biosystems) of Sanger sequencing results of the same SNP of diseased as given below.

Reference sequence

CAACATATTTGTGATTAATCCATGC[C/T]GTTGTGTAGG
TCAATCATCTTTATT

Reference sequence Diseased

CAACATATTTGTGATTAATCCATGCCGTTGTGTAGGTC
AATCATCTTTATT

sequence 1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
Diseased

GCGATATCTAGCCAGTCTGCGTATTTACTGAACAATTA
GAGAGCTAAGCACTTTTTAGATACTATATAATTTAATT
GCCGTATGAGTCACCCTTAGTTTTCAAACAAAGATGTG
TTAAAGTTTAACTGAAATTTCTGCGGCGTAAATATGGG
TAGGTCTAGTTGTGCTTCCTTGTGAGGGTAGATAGCCA
CGAATTTCCCCACCAGAAAGAATGTCATCCGGCGGCTT
TCAAGCGGAGCTTTACCCCAAACAACTTCCAGGAACG
GATTTCCTGGCTCTCGCATCGATAAATCAACGCAGCGA
AATGCAATAAGTACGTTAAATGTGCGATCATTGAGTCA
ATCACTCATTGAACGCGGCTCGCCCTCCCTGTTAACCC
AAGGAGCCAACATATTTGTGATTAATCCATGCTGTTGT
GTAGGTCAATCATCTTTATTCATCTTTATTGCTTGAATG
CCGGGGGTTTGCCTGCGGGGTGCAGGGGGAGCCCCGC
GCCCCTCAAATGGGTAACCGATCTCGAAAG

CLUSTAL O(1.2.1) Multiple sequence alignment
1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
GCGATATCTAGCCAGTCTGCGTATTTACTGAACAATTA
GAGAGCTAAGCACTTTTTAGAT
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
ACTATATAATTTAATTGCCGTATGAGTCACCCTTAGTTT
TCAAACAAAGATGTGTTAAAG
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
TTTAACTGAAATTTCTGCGGCGTAAATATGGGTAGGTC
TAGTTGTGCTTCCTTGTGAGGG
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
TAGATAGCCACGAATTTCCCCACCAGAAAGAATGTCAT
CCGGCGGCTTTCAAGCGGAGCT
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
TTACCCCAAACAACTTCCAGGAACGGATTTCCTGGCTC
TCGCATCGATAAATCAACGCAG
MTNR1B ----------

1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
CGAAATGCAATAAGTACGTTAAATGTGCGATCATTGAG
TCAATCACTCATTGAACGCGGC
MTNR1B ----------
CAACATATTTGTGATTAATCCATGCCTGT-----
1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
TCGCCCTCCCTGTTAACCCAAGGAGCCAACATATTTGT
GATTAATCCATGCCTGTCCTCA

****

MTNR1B -----
TGTGTAGGTCAATCATCTTTATT----------
1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
CCCCTTGTGTAGGTCAATCATCTTTATTGCTTGAATGCC
GGGGGTTTGCCTGCGGGGTGC
****
MTNR1B ----------
1115_245_003_PCR_D4_MTNR1B_F-C02.ab1
AGGGGGAGCCCCGCGCCCCTCAAATGGGTAACCGATC
TCGAAAG

Result: NO SNP identified in the diseased sequence of ab1 at rs no of MTNR1B (rs4753426) gene. The **CC genotype** (meaning both copies of the gene contain the "**C**" allele) is referred to as "homozygous normal.

KCNQ1 Control Sample: rs1057128

After sequencing the samples, the results were analysed by multiple sequence alignment (CLUSTAL W) by taking the actual reference sequence from DbSNP of NCBI of KCNQ**1** and comparing with the ab1 sequence (applied Biosystems) of Sanger sequencing results of the same SNP of control and diseased separately as given below.

Reference sequence:
TGCGGGACGTCATTGAGCAGTACTC[A/G]CAGGGCCAC
CTCAACCTCATGGTGC
Reference sequence For Control
TGCGGGACGTCATTGAGCAGTACTCACAGGGCCACCTC
AACCTCATGGTGC
Sequence_ of _KCNQ1_F-F09.ab1

NNNTATNTCNCNAGATACGTGCTGACCGCCTGGCAGA
GGACCCTGCCAAGCCCAGGTACC
GTGCCCGCCAGCGGAGGGACGTCATTG--
AGCAGTACTCAGCAGGGCCACCTCAACCTCA
TGGTGCTGTCCTGTGCCGGATGTCTGGGTTTAACCCTG
CAACGTCGCCCACAATCCGAGA AGTCTTGTTCTTTTC
Multiple sequence alignment KCNQ1
1215_203_014_PCR_Kc_7_control_KCNQ1_F-F09.ab1
CLUSTAL O(1.2.1) multiple sequence alignment
KCNQ1 ----------TGCG----------
1215_203_014_PCR_KJ_7_KCNQ1_F-F09.ab1
NNNTATNTCNCNAGATACGTGCTGACCGCCTGGCAGA
GGACCCTGCCAAGCCCAGGTACC
***
KCNQ1 ----------GGACGTCATTG-
-TGCGGGACGTCATTGAGCAGTACTCAAACCTCA
1215_203_014_PCR_KJ_7_KCNQ1_F-F09.ab1
GTGCCCGCCAGCGGAGGGACGTCATTG--
TGCGGGACGTCATTGAGCAGTACTCAAACCTCA
****
KCNQ1 TGGTGC----------
1215_203_014_PCR_KJ_7_KCNQ1_F-F09.ab1
TGGTGCTGTCCTGTGCCGGATGTCTGGGTTTAACCCTG
CAACGTCGCCCACAATCCGAGA
******

KCNQ1 ----------
1215_203_014_PCR_KJ_7_KCNQ1_F-F09.ab1
AGTCTTGTTCTTTTC

Result: No SNP identified in the diseased sequence of ab1 at rs number of KCNQ1 (rs1057128) gene. The **AA genotype** (meaning both copies of the gene contain the "**A**" allele) is referred to as "homozygous normal.

Diseased Sample: KCNQ1 Gene (rs1057128) After sequencing the samples, the results were analysed by multiple sequence alignment (CLUSTAL W) by taking the actual reference sequence from DbSNP of NCBI of KCNQ**1** and comparing with the ab1 sequence (applied Biosystems) of Sanger sequencing results of the same SNP of diseased as given below.

Reference sequence:
TGCGGGACGTCATTGAGCAGTACTC[A/G]CAGGGCCAC
CTCAACCTCATGGTGC

Reference sequence Diseased
TGCGGGACGTCATTGAGCAGTACTCGCAGGGCCACCTC
AACCTCATGGTGC

KCNQ1 ab1 _sequence:
TNNANNTCCCNGAGATACGTGCGGGACGTCATTGA
GCAGTACTCGCAGGGCCACCTCAACCTCATGGTGC
AGCGGAGGGCCCGCTTTGTGTCCAAGCAGGGCCAC
CTCCACCTCGCCCACACATGGTGCCCTGGGCCAGA
TGTCTGGGTTTACCCTGCAACGTCGCCCACAATCGN
AAGTTTTAGAACTTGT

Multiple sequence alignment KCNQ1
1215_203_013_PCR_KJ_6_KCNQ1_F-E09.ab1
CLUSTAL O(1.2.1) multiple sequence alignment

KCNQ1 ----------
TGCGGGACGTCATTGAGCAGTACTCAG----------
1215_203_013_PCR_KJ_6_KCNQ1_F-E09.ab1
TNNANNTCCCNGAGATACGTGCGGGACGTCATTGA
GCAGTACTCAGCCAAGCCCAGGTAC
****

KCNQ1 ----------
--CAGGGCCACCTCAACCT-------
1215_203_013_PCR_KJ_6_KCNQ1_F-E09.ab1
CGTGCCCGCCAGCGGAGGGCCCGCTTTGTGTCCAA
GCAGGGCCACCTCCACCTCGCCCAC
****

KCNQ1 -CATGGTGC----------

1215_203_013_PCR_KJ_6_KCNQ1_F-E09.ab1
ACATGGTGCCCTGGGCCAGATGTCTGGGTTTACCCT
GCAACGTCGCCCACAATCGNAAGT
****

KCNQ1 ----------
1215_203_013_PCR_KJ_6_KCNQ1_F-E09.ab1
TTTAGAACTTGT

Result: SNP identified in the diseased sequence of ab1 at rs number of **KCNQ1** (rs1057128) gene. The **AA genotype** (meaning both copies of the gene contain the "**A**" allele) is referred to as "homozygous normal.

DISCUSSION

The results of the present study reveal that there is an association between Kcnq1 mtnr1b and Notch2 and gene polymorphisms in T2DM. Previous studies found that all the genotyped KCNQ1, mtnr1b, notch2 variants were significantly associated with type 2 diabetes in Dutch and Japan population, and the association of knq1 was the strongest. The individuals carrying the same at-risk alleles, as reported in the Japanese studies , had a modestly increased risk of developing type 2 diabetes, with a population attributable risk from 0.6% to 4.3%.

In the case of notch2 Previous studies found that all the genotyped notch2 variants were significantly associated with type 2 diabetes, with a population attributable risk from 0.5% to 4%.In the case of MTNR1B Several studies showed the association of impaired fasting glucose with homozygosity for the risk alleles of certain single nucleotide polymorphisms falling outside as well as within the coding sequences of MTNR1B.mtnr1b variants were significantly associated with type 2 diabetes in asian population and rsikis from 0.3% to 2%. Moreover, these data confirm the observations from the previous studies in which the relationship between the type 2 diabetes risk alleles in variant genes and reduced levels of various measures of insulin secretion have been reported.

Several studies demonstrated that the polymorphism of notch2,KCNQ1 and Mtnr1b are a risk factor for developing T2D. In the stratified analysis by ethnicity, significant associations were observed among different populations in all genetic models among East Asian, and South Asian populations, which suggested a similar role of the polymorphism in different ethnicity with different genetic backgrounds and living environment By considering sample size, significantly increased T2D susceptibility in KCNQ1 risk allele carriers was also found both in large and small studies for all genetic models. However, our results suggest an overestimation of the true genetic association by small studies.

In our study, In the case of **NOTCH2** 10 contols and 10 diseased samples of notch2 (rs10923931) have been done for sequence. Mutational analysis had been done after sequencing using multiple sequence alignment (clustal w).we found that there is no snp (single nucleotide polymorphism) in control sample. We found that snp only mediation and non medication patients. Two single nucleotide polymorphisms were observed in those who were working in office and one snp found in labour. This concludes that notch2 gene most significant association with t2dm.

In the case of **KCNQ1**, same sample size 10contol and 10disased samples of Kcnq1 (rs1057128) have been done for sequence. Mutational analysis had been done after sequencing using multiple sequence alignment (clustal w).we found that there is no snp (single nucleotide polymorphism) in control sample. we found that two snp only mediation and non medication patients. This concludes that Kcnq1 gene significant association with t2dm

In the case of **MTNR1B** (rs4753426),contol and diseased samples of Mtnr1b have been done for sequence. Mutational analysis had been done after sequencing using multiple sequence alignment (clustal w).we found that there is no snp (single nucleotide polymorphism) in control sample. We did not found snp in mediation and non medication patients. This concludes that notch2 gene significant association with t2dm.this concludes that Mtnr1b is though significant variant gene not found in our population study, might be present if the population size had been large. We found that the association NOTCH2, KCNQ1 and MTNR1B genes

Clearly results of the present study reveal that three single nucleotide polymorphisms (KCNQ1, NOTCH2 and MTNR1B genes, respectively) are associated with T2DM. The results suggest a role of KCNQ1 and notch2 gene variants in the increased risk of T2DM in those who were working in office compared with labor. May be the lack physical activity and family history also considered. Low physical activity levels and high saturated fatty acid diets, both of which may be inhibit expression these genes leads to dysfunction beta cell, that effects on decrease the formation insulin.

V. CONCLUSION

Many studies have revealed that genetic variants are associated with a susceptibility to T2DM. These disease entities share common pathophysiological background, including β-cell dysfunction and insulin resistance. Based on this background, notch2,mtnr1b and KCNQ1 were selected in this study because they have been reported to exhibit SNPs in patients with T2DM. Impaired β-cell function (kcnq1), insulin resistance (notch2) and obesity are major pathophysiological features known to be associated with loci variants and susceptibility to T2DM.

The limitations of the current study include a relatively smaller number of enrolled subjects than previous studies. Another limitation is that we were only able to select a specific SNP from one gene each. To summarize, our results showed that not only clinical factors, but also polymorphisms in notch2, and kcnq1 were associated with a higher prevalence of t2dm.

In conclusion, we identified the genotype by using pcr amplification followed by direct sequencing method. Our results suggested that prevalence of notch2, kcnq1 gene is more in our population. We propose that genetic polymorphisms could also be of value in predicting the occurrence and diagnosis of t2dm. These genome wide association studies will help us to give proper medications to the t2dm patients and we can control the t2dm.

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[2] Wolfs MGM, Hofer MH, Wijmenga C, van Haeften TW. "Type 2 diabetes mellitus: new genetic insights will lead to new therapeutics." Current Genomics (2009): 10:110-118.

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