

MUTATIONAL ANALYSIS OF *GJB2* GENE IN THE DIABETIC PATIENTS

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ABSTRACT

The main objective of this study was to find out mutations in the coding exon of *GJB2* gene in the diabetic patients. Samples were collected from Lahore. A total of 50 type-II diabetic patients were enrolled for this study. Blood samples were collected in EDTA vials. DNA was isolated from whole blood. PCR was done to amplify the exon of *GJB2* gene. The PCR products were purified. Sequencing was done, in order to identify mutations in the coding region of *GJB2* gene.

The sequence analysis of PCR products i.e., the coding exon of *GJB2* gene showed no mutations in the diabetic patients, to associate the gene with diabetes. Statistical analysis of these results also revealed no association of the *GJB2* gene with diabetes.

Key-words: Connexin 26, *GJB2* Gene, Gap-Junctional Communication, Diabetes.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyper-glycaemia that can often lead to several defects both at the molecular and cellular levels in a varied range of tissues (Kerner and Brückel, 2014). Several connexin changes are linked with the renovation of arterial wall, which takes place in response to hypertension i.e., the primary basis of diabetes. The prevalence of hypertension among the diabetics is 54%, greater than the normal subjects. In different representations of hypertension, the variable expression patterns of these connexin changes are described, and these changes in connexin molecules are also very complex (Haefliger *et al.*, 2004). 21 connexin genes are being stated in humans, as yet (Eiberger *et al.*, 2001). *GJB2* gene is a member of a gap junction protein family. It is present on chromosome 13q12.11. It structurally contains two exons, out of which exon 2 is the coding region and exon 1 is the non-coding one (Beyer and Berthoud, 2009). The coding region of this gene translates into connexin 26, the protein which is one of the six connexin subunits that oligomerize to form a connexon hemi-channel. When the extra-cellular part of this connexon harbors with a connexon from the opposite cell, a gap junction channel formulates. The gap junctions, thus formed, are tremendously specialized trans-membrane structures, formed of the channels spanning the two connecting cell membranes, leaving an 2-4 nm extra-cellular “gap”, hence their name (Laird, 1996). Connexin channels link the secretory cells of all the multi-cellular glands, including pancreatic islets, for the secretion of small biological molecules like insulin. The gap junctions, hence, may be convoluted in the pathogenesis of diabetes (Wright *et al.*, 2012). Also, connexins are involved in causing the hearing loss impairment, which may signal some other health issues, including diabetes as well, so it can evidently be the reason for the appearance of several diabetic symptoms in those people (Dalton *et al.*, 1998). Globally, the mortality percentage of diabetes is a serious issue. This persistent metabolic disorder is becoming a vastly increasing problem day by day in the whole world, with its enormous societal, viable and healthiness distresses. It is assessed that 415 million persons are alive with diabetes in the world i.e., 1 in 11 of the world's mature population (King *et al.*, 1998). The number of individuals with diabetes has increased from 108 million in 1980 to 422 million in 2014 i.e., the global prevalence of diabetes among adults having age of above 18 years, has risen from 4.7% in 1980 to 8.5% in 2014 (Guariguata *et al.*, 2014). It is greater in the developed states than in the developing ones. *GJB2* and *GJB6* genes undergo coordinated transcription, and their major expressing organs are cochlea, placenta, hepatocytes, skin, pancreas, kidney and intestine (*GJB2* gene), and astrocytes, cochlea (*GJB6* gene). Type-II diabetes accounts for 90–95% of the overall diabetes in a population (Dabelea *et al.*, 2014). The worldwide prevalence of this disease is anticipated to rise from 7.7% to 439 million by 2030 (Zhang *et al.*, 2010). The main aim and objective of this work was to find out mutations in the coding region of *GJB2* gene in a diabetic population. For, the mutations in the coding region of this gene may be a leading cause of diabetes. The work will certainly add certain information to the literature of science and also, will aid to treat diabetes by gene therapy, controlling beta cell survival and islet function by modulating the coupling of gap junctions, or solely by designing the different drugs.

MATERIALS AND METHODS

A total of 50 blood samples were collected from the diabetic patients of Lahore, Pakistan and 50 healthy controls with age and sex matched. A detailed family history, symptoms and the complications of these diabetic patients was collected. Blood samples were taken by using sterilized syringes and vacutainers, EDTA vials. The genomic DNA was extracted by one day protocol of DNA isolation. For the identification of isolated DNA, agarose gel electrophoresis was performed. Polymerase chain reaction was done to amplify the required sequence of *GJB2* gene from the whole of DNA. PCR was performed in a total volume of 50 μ l, under the optimized conditions. The product length i.e., 806 bps, was analyzed on 1.3% gel. DNA ladder of 1kb was used to compare the results. PCR products were mostly cleaned with ethanol precipitation. The sequencing results of these PCR products were analyzed by Sanger dideoxy method. The mutations, if any, were noted and analyzed statistically.

RESULTS

Among the selected 50 patients for study, the percentage of male diabetic patients was greater than the female ones. 39 patients (79%) were males and 11 (21%) were females. A huge number of patients were having the age, greater than 35 years. The distribution of age among the diabetic individuals was determined with the help of a bar graph. Most patients were within the age group between 40-60 years (Table 1). Majority of patients had no cousin marriages. Only 19 individuals (39%) had consanguinity. Rest of the 31 patients (61%) were with no cousin marriages (Table 2). Diabetic complications were observed in some of the patients. Only 14 patients (29%) were diabetic with the disease complications. 36 patients (71%) had diabetes with none of its complications (Fig. 1). Among them, individuals 36 (72%) had high blood pressure i.e., hypertension. It was the most obvious symptom of diabetes. Half of the patients were with severely high blood sugar level (Fig. 2). In some of the patients, long-term complications of diabetes were also observed. Among these, diabetic retinopathy was the most frequent one as it was observed in 7 (13%) patients (Fig. 3). Using one day protocol for DNA isolation, a clear DNA band of interest was obtained for each of the patients (Fig. 4).

Table 1. Age groups made among diabetic patients (n=50).

Age (Years)	Frequency (%)
20-30	7
31-50	74
50-65	19

Table 2. The proportion of consanguinity among diabetic patients (n=50)

Consanguinity	Frequency (%)
Cousin	39
No Cousin	61

The primer, used for PCR, amplified 806 bases of exon 2 of *GJB2* including few bases of intronic region. The exon 2 consists of 2140 bases. The sequence of the intron, exon and coding sequence is as follows. The CDS consists of 681 bases which encode gap junction beta 2 protein.

(Intron)GCTTACCCAGACTCAGAGAAGTCTCCCTGTTCTGTCCTAGCTAGTGATTCCCTGTGTTGTGTGCA
TTCGTCTTTTCCAG**(Exon)**AGCAAACCGCCCAGAGTAGAAG**(CDS)**ATGGATTGGGGCACGCTGCAGACG
ATCCTGGGGGGTGTGAACAAACACTCCACCAGCATTGGAAAGATCTGGCTCACCGTCTCTTCATTTTT
CGCATTATGATCCTCGTTGTGGCTGCAAAGGAGGTGTGGGGAGATGAGCAGGCCGACTTTGTCTGCAA
CACCCTGCAGCCAGGCTGCAAGAACGTGTGCTACGATCACTACTTCCCCATCTCCACATCCGGCTATG
GGCCCTGCAGCTGATCTTCGTGTCCACGCCAGCGCTCCTAGTGGCCATGCACGTGGCCTACCGGAGAC
ATGAGAAGAAGAGGAAGTTCATCAAGGGGGAGATAAAGAGTGAATTTAAGGACATCGAGGAGATCA
AAACCCAGAAGGTCCGCATCGAAGGCTCCCTGTGGTGGACCTACACAAGCAGCATCTTCTCCGGGTC

ATCTTCGAAGCCGCCTTCATGTACGTCTCTATGTCATGTACGACGGCTTCTCCATGCAGCGGCTGGTGA
 AGTGCAACGCCTGGCCTTGTCCCAACACTGTGGACTGCTTTGTGTCCCGGCCACGGAGAAGACTGTC
 TTCACAGTGTTTCATGATTGCAGTGTCTGGAATTTGCATCCTGCTGAATGTCACTGAATTGTGTTATTGTC
 TAATTAGATATTGTTCTGGGAAGTCAAAAAAGCCAGTTTAA(CDS)CGCATTGCCAGTTGTTAGATTAA
 ...Exon continues.

When PCR was performed on this extracted genomic DNA of the patients, the PCR product had the position of 806 bps with respect to the 1 kb DNA ladder. Henceforth, the required sequence of the *GJB2* gene was amplified with the help of PCR process, and clear bands of interest were obtained (Figure-5). Gradient PCR was done for optimization of the primers. PCR of samples was done at a temperature of 57 °C, 57.57 °C, 58.71 °C and 60 °C. The PCR condition was set as initial denaturation at 95 ° for 5 minutes, denaturation at 95 °C for 1 minute, annealing at 60 °C for 1 minute, initial extension at 72 °C for 1 minute and a final extension at 72 °C for 5 minutes. This PCR was set for 30 cycles.

Sequencing of the PCR product was done after purification by Sanger Sequencing. The samples were sent to the *Centre for Applied Molecular Biology (CAMB)* at University of the Punjab Lahore. Two samples were sequenced from Macrogen Company in Korea. The sequencing was done by genetic analyzer 3730. The sequencing results were analyzed by using Chromas version 2.6.4. Low-quality sequences on both sides were trimmed. All nucleotides were also checked manually to ensure any wrong base call. The similarity of the sequence was checked by the NCBI nucleotide blast. Diabetic patients had no association with the *GJB2* gene ("Fisher Exact Test" statistical value was 1). The result was non-significant at $p < .05$, when this test was applied on the sequencing results with mutated and non-mutated nucleotides of controls and subjects, dealing with the independent variables. Hence, no mutations were found to be associated with diabetes in a studied group of patients.

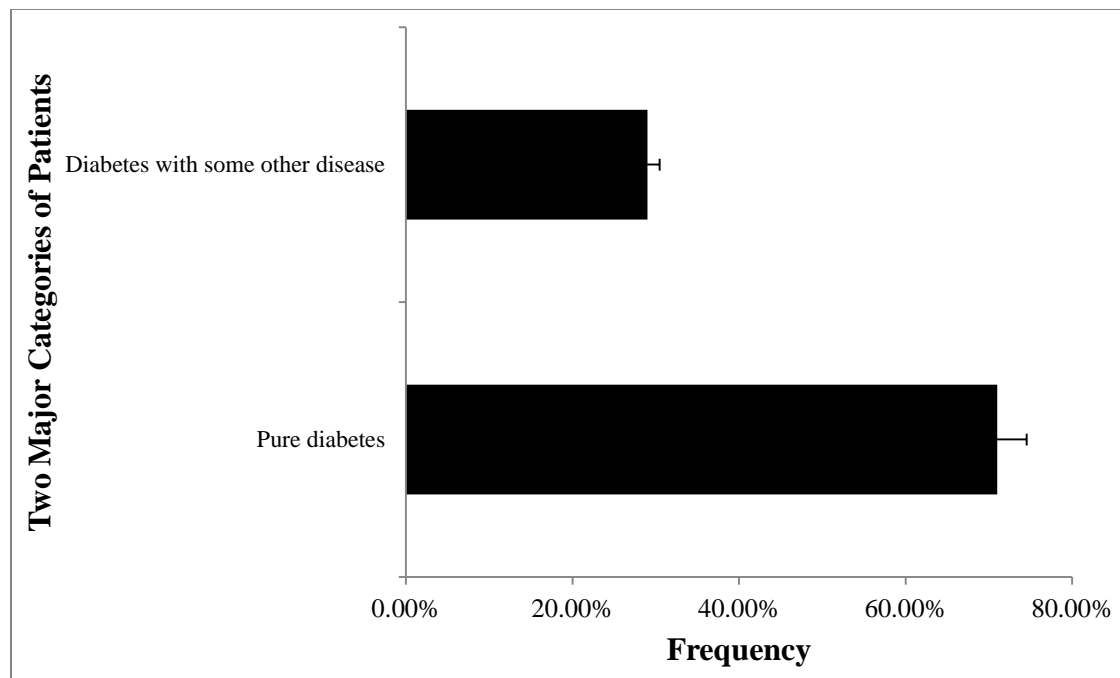


Fig. 1. Proportions of patients with diabetes and along with some other complications as well (n=50).

DISCUSSION

Gap junctions and junction-mediated cell-to-cell communications, obviously are the essential features for gland cells e.g., pancreatic islets, irrespective of the fact that their secretory product is what. Also, the endocrine and exocrine parts of the pancreas display contrasting connexin changes and coupling alterations in relative to the activation and inhibition of their secretory roles. This alternate expression of connexin isoforms in the pancreas is established in quite a few of other endocrine and exocrine glands as well. These interpretations direct that connexin-made channels have a fundamental part in the regulation of secretory actions of pancreas (Bosco *et al.*, 2011). To

date, experimentations have also revealed that at least, in vitro, the connexin channels contribute to the synchronized insulin biosynthesis as well as its release (Vozzi *et al.*, 1995). On the other hand, however, no work has demonstrated for the time being, whether connexin-dependent coupling is linked to the functioning of native β cells in vivo and if so, how it is assimilated with the other signaling mechanisms that subsidize to regulate the β -cell function i.e., insulin secretion (Meda, 2012). Also, the cells of pancreatic islets express a number of different connexins, whose defined scattering, performing a specific function there, persist to be discovered (Farnsworth and Benninger, 2014). Moreover, the *GJB2* gene has a very low expression in the pancreas. If the gene is expressed, secretion of connexin 26 protein is then also very limited (Meda *et al.*, 1993). In the light of all these perspectives, when this gene was checked for mutations in the pure diabetics to determine that whether they can be accounted as a cause of diabetes or not, no mutations were identified in them. It suggests that diabetes in the studied subjects was not due to the mutations in coding exon 2 of the *GJB2* gene. Low expression and secretion of connexin 26 in the pancreatic islets explain this kind of un-mutated.

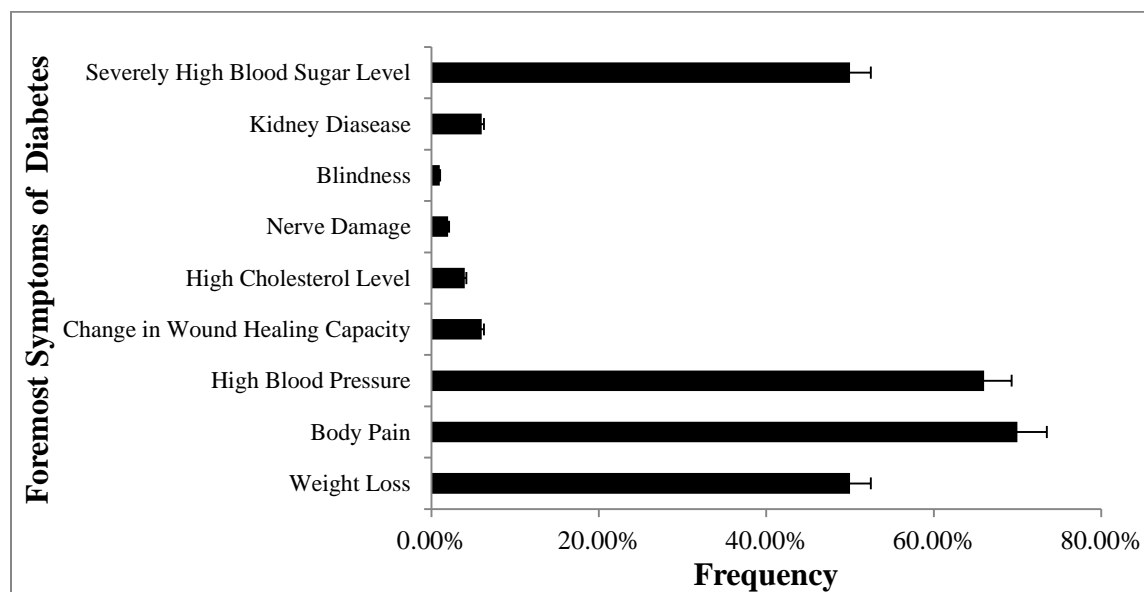


Fig. 2. Symptoms of Diabetes, Affecting Different Organs in Diabetic Patients (n=50).

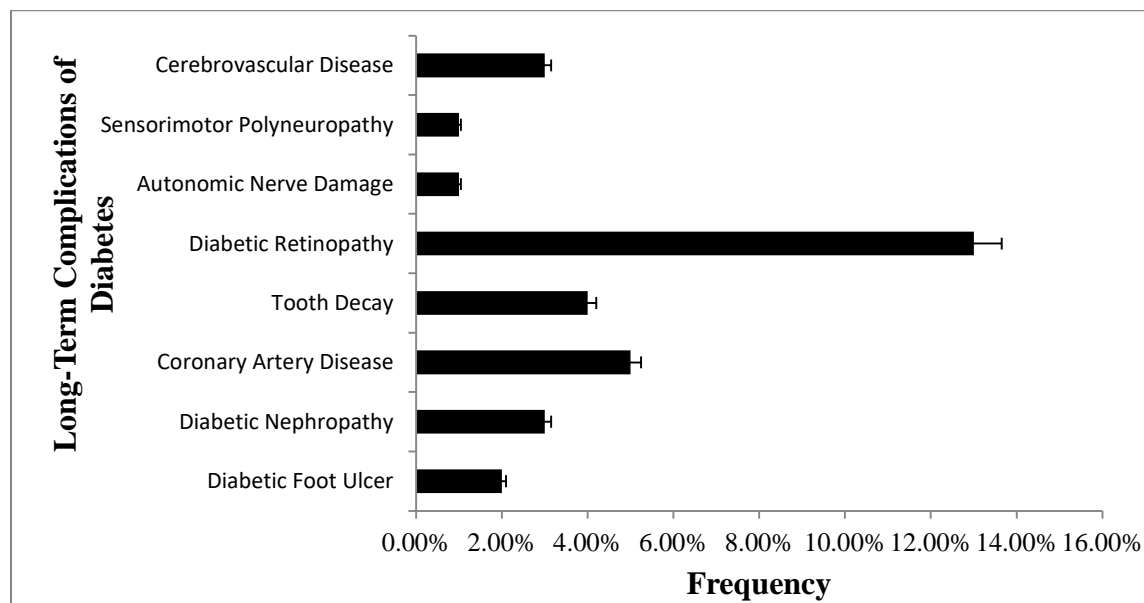


Fig. 3. Lifelong Complications of Diabetes in Diabetic Patients (n=50).



Fig. 4. Extracted DNA Bands on 1% agarose gel.

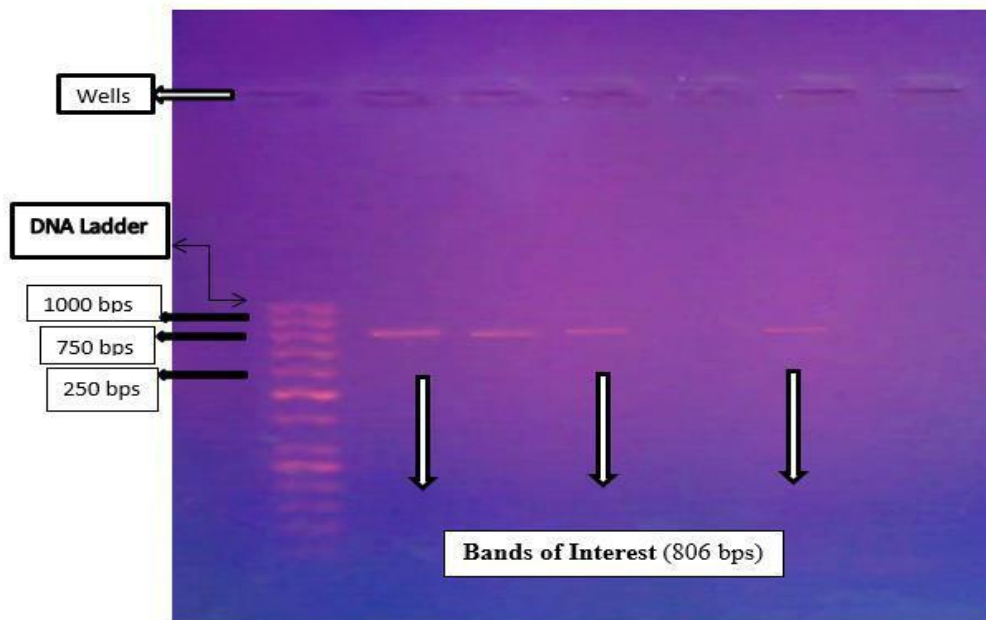


Fig. 5. Amplified PCR Products on 2% agarose gel.

PCR product of the selected diabetic patients. *GJB2* gene is basically a deafness gene and a mutation in this gene causes hearing loss in the corresponding individual. A lot of work has done, that reveals its association with hearing loss (Kelley *et al.*, 1998). In contrast, no cases have reported to date, on the association of this gene with diabetes, i.e., no experimental work has thus far been reported for it. Hence, they may not have any basis in the process of lowering the insulin secretion in pancreas i.e., diabetes, but they definitely are accounted for some other diseases, the most obvious example of which is hearing loss impairment (Wilcox *et al.*, 2000). The secretion process of these connexins, hence, is predominantly not connected with the high levels of glucose in blood, by means of

corresponding change in either the insulin secretion or in its action if it is secreted. Consequently, in the genetic causes of diabetes, *GJB2* gene mutations are not encountered as mentioned below.

Score: 1258 bits (681), E value: 0.0 Query cover: 100%, Length: 681, Identity: 681/681 (100.0%), Similarity: 681/681 (100.0%), Gaps: 0/681 (0.0%)

Query	1 ATGGATTGGGGCACGCTGCAGACGATCCTGGGGGGTGTGAACAAACACTC	50
Subject	1 ATGGATTGGGGCACGCTGCAGACGATCCTGGGGGGTGTGAACAAACACTC	50
Query	51 CACCAGCATTGGAAAGATCTGGCTCACCGTCCTCTTCATTTTTCGCATTA	100
Subject	51 CACCAGCATTGGAAAGATCTGGCTCACCGTCCTCTTCATTTTTCGCATTA	100
Query	101 TGATCCTCGTTGTGGCTGCAAAGGAGGTGTGGGGAGATGAGCAGGCCGAC	150
Subject	101 TGATCCTCGTTGTGGCTGCAAAGGAGGTGTGGGGAGATGAGCAGGCCGAC	150
Query	151 TTTGTCTGCAACACCCTGCAGCCAGGCTGCAAGAACGTGTGCTACGATCA	200
Subject	151 TTTGTCTGCAACACCCTGCAGCCAGGCTGCAAGAACGTGTGCTACGATCA	200
Query	201 CTA CT TCCCCATCTCCACATCCGGCTATGGGCCCTGCAGCTGATCTTCG	250
Subject	201 CTA CT TCCCCATCTCCACATCCGGCTATGGGCCCTGCAGCTGATCTTCG	250
Query	251 TGTCCACGCCAGCGCTCCTAGTGGCCATGCACGTGGCCTACCGGAGACAT	300
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Query	351 CATCGAGGAGATCAAAACCCAGAAGGTCCGCATCGAAGGCTCCCTGTGGT	400
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Query	401 GGACCTACACAAGCAGCATCTTCTTCCGGGTCATCTTCGAAGCCGCCTTC	450
Subject	401 GGACCTACACAAGCAGCATCTTCTTCCGGGTCATCTTCGAAGCCGCCTTC	450
Query	451 ATGTACGTCTTCTATGTCATGTACGACGGCTTCTCCATGCAGCGGCTGGT	500
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Query	501 GAAGTGCAACGCCTGGCCTTGTCCTTGTCCCAACTGTGGACTGCTTTGTGTCCC	550
Subject	501 GAAGTGCAACGCCTGGCCTTGTCCTTGTCCCAACTGTGGACTGCTTTGTGTCCC	550
Query	551 GGCCACGGAGAAGACTGTCTTCACAGTGTTTCATGATTGCAGTGTCTGGA	600
Subject	551 GGCCACGGAGAAGACTGTCTTCACAGTGTTTCATGATTGCAGTGTCTGGA	600
Query	601 ATTTGCATCCTGCTGAATGTCACCTGAATTGTGTTATTTGCTAATTAGATA	650

Subject	601 ATTTGCATCCTGCTGAATGTCAGTGAATTGTGTTATTTGCTAATTAGATA	650
Query	651 TTGTTCTGGGAAGTCAAAAAAGCCAGTTTAA	681
Subject	651 TTGTTCTGGGAAGTCAAAAAAGCCAGTTTAA	681

Conclusion: It is concluded that the *GJB2* gene is not associated with diabetes.

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