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"A TALE OF GENETIC VARIATION IN THE HUMAN SLC22A1 GENE ENCODING OCT1 AMONG TYPE 2 DIABETES MELLITUS POPULATION GROUPS OF WEST BENGAL, INDIA"

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ABSTRACT

The organic cation transporter 1, OCT 1 (also called SLC22A1-Solute Carrier Family 22 member 1), appears to play a role in the efficacy and disposition of variety of organic cation including drugs. Genetic polymorphisms in the drug transporter have been increasingly recognized as a possible source of variation in drug disposition and response. Genetic variants in OCT1 have been identified largely in European, Asian (Japanese, Chinese and Korean) populations. Interestingly, eight genetic variations were found in the human *SLC22A1* gene, which encodes OCT 1, from 50 type 2 diabetes mellitus individuals (T2DM), in West Bengal population. The purpose of this study was to investigate genetic variants of OCT1 in West Bengal populations. We detected the three previously reported non-synonymous variations, 480 G>C (L160F); 1022 C>T (P341L); 1222 A>G (M408V) and one synonymous variations 156 T>C (S52S) at a minor allele frequencies (MAF) of 0.63, 0.20, 0.43 and 0.27 respectively. We also found four previously reported intronic variations: IVS1-43(T>G), IVS2 -99(C>T), IVS5 -61(G>A), IVS9 +43(C>T) with minor allele frequencies of 0.20, 0.17, 0.18, and 0.37 respectively.

KEYWORDS: SLC22A1, OCT1, Non-Synonymous Single Nucleotide Polymorphisms, Type 2 Diabetes Mellitus, West Bengal Population

Abbreviations: OCT, Organic Cation Transporter; SLC22A1, Solute Carrier Family 22 Member 1; MAF, Minor Allele Frequency; NS SNP, Non-Synonymous Single Nucleotide Polymorphisms; T2DM, Type 2 Diabetes Mellitus, BLAST, Basic Local Alignment and Search Tool; SIFT, Sorting Intolerant from Tolerance; PSIC, Position-Specific Independent Counts

INTRODUCTION

Human organic cation transporter 1 (OCT1), belongs to the largest superfamily of transporters, the solute carrier family and is encoded by the *SLC22A1* gene [5]. This transporter is predicted to have 12 transmembrane domains (Figure 1) and involve in translocating large variety of organic cations including drugs [6].

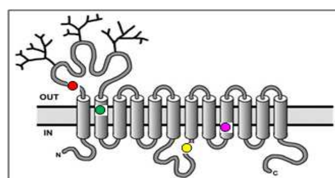


Figure 1: Predicted Secondary Structure of OCT1. The Protein is thought to Contain 12 Transmembrane Domains with both N and C Terminus Located Intra Cellularly. The First Large Extracellular Loop Contains Three Putative *N*-Linked Glycosylation Sites (Indicated by Branches) Cytoplasmic (IN) and Extracellular (OUT) Orientation are Indicated● Ser52Ser; ● Leu160Phe; ● Pro341Leu; ● Met408Val

In humans, OCT1 is expressed in the basolateral membrane of hepatocytes and is the primary mediator of hepatic substrates (e.g. drugs) uptake [1, 13]. Indeed, in *SLC22A1* (-/-) mice, significantly less anti-diabetic drug (e.g. Metformin) is distributed to the liver compared to control mice [13]. *SLC22A1,* located at chromosome 6q23.3 (Figure 2), is adjacent to *SLC22A2* (encoding OCT2) and *SLC22A3* (encoding OCT3). It is consists of 11 exons spanning approximately 37 kb [14]. In many previous studies, others showed that human *SLC22A1* is highly polymorphic in ethnically diverse populations[8, 10].

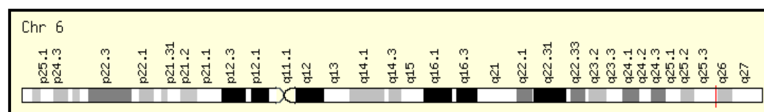


Figure 2: Human *SLC22A1* Gene (Cytogenetic Band: 6q25.3)

Many non-synonymous polymorphisms of *SLC22A1*, mostly found in Caucasian populations, exhibit reduced activity in cellular assay. From Caucasians, Kerb*et al.* showed 25 polymorphisms, including eight were non-synonymous, three of which showed reduced activities [7]. However, genetic variants of *SLC22A1* that are related to decreased metformin uptake (i.e., S14F, R61C, S189L, G220V, G401S, 420del, G465R) have been identified primarily in populations with European ancestries and have not been identified in Asian American, Chinese, Korean, and Japanese populations [12].

In one study with 33 Japanese patients with type 2 diabetes mellitus, two *SLC22A1* polymorphisms (intron1 –43T>G, M408V), which do not exhibit altered function, were shown to have no significant effects on the clinical efficacy of metformin [9]. Kerb*et al.* reported that significantly reduced (P341L) transport activities using tetraethylammonium as a substrate [7]. The goal of the study is to identify SNPs (single nucleotide polymorphisms) by sequencing all exons and the surrounding introns of *SLC22A1* from 50 T2DM individuals, in the population of West Bengal. In this study, eight variants including three non-synonymous, one synonymous and four intronic SNPs were identified.

MATERIALS AND METHODS

Collection of Study Samples: Study subjects consisted of 50 Indian patients with T2DM who were on cationic anti hyperglycaemic drug such as Metformin. Patients were recruited from Diabetes outdoor center of IPGME&R, SSKM Hospital, Kolkata. The clinical features of the subjects were 24 men and 26 women, age at the study was 46 ± 12.72 (mean ± SD), body mass index (BMI) was 26.57 ± 5.00 kg/m^2, fasting blood sugar was 154.13 ± 24.89 mg/dL, postprandial blood sugar was 227.66 ± 41.17 mg/dL and HbA1c was (Glycated hemoglobin) 08.78 ± 2.18 %. The ethical review board of the IPGME&R, SSKM Hospital, Kolkata approved this study. Written informed consent was obtained from all participating subjects.

Collection of Blood Samples and Genomic DNA Preparation: Approximately 10 ml peripheral blood samples were collected from the subjects with the help of the collaborating doctors. EDTA was used as an anticoagulant. Genomic DNA extracted from fresh whole blood by QIA amp Blood Kit (Qiagen, Hilden, Germany). Genomic DNA used as a template in the polymerase chain reaction (PCR).

Polymerase Chain Reaction: PCR was carried out in a total reaction volume of 30 µl containing 50-100 ng genomic DNA, standard buffer, $MgCl_2$ (as appropriate), 0.2 mM of each dNTP, 0.5 µM of each primer, and 0.8 units of *Taq*DNA polymerase (Invitrogen, Carlsbad, CA) in a Veriti™ 96-well thermocycler (model# - 9700, Applied Biosystems, Foster City, CA). The exons were amplified using the primer sequences and PCR conditions described in Table 1.

Table 1: Primer Sequences and PCR Conditions Used for the Analysis of Human *SLC22A1*

Exon	Primer Sequence	Amplicon (Bp)	PCR Condition
EXON 1	FR. 5'- ACTTGGTTGCCTTCCAGATGTT-3' RV. 5'- AACTCCCATGTTACAGAGGCTT-3'	584	94° C 30 Sec, 60° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 2	FR. 5'- AAACAGCCCAGGGATACCGAGTTT-3' RV. 5'- TCCAACTGGTCATGTTCTCCACCA-3'	332	94° C 30 Sec, 62° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 3	FR.5'- GAGCGCTCAGACTCCTCTTCAGAC-3' RV.5'- GAGGAGGCCATTCTAGCCCATGTC-3'	530	94° C 30 Sec, 60° C 45 Sec, 72° C 1min for 40 cycle using 1.0mM $Mgcl_2$
EXON 4	FR. 5'- GCATAACGTCCACACCTCCTGTTT-3' RV. 5'- ATGTGGACACCATGGCCTTTGGAA-3'	332	94° C 30 Sec, 60° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 5 & 6	FR.5'- CCGAGGAAAATGCCAGATAG-3' RV. 5'- CCCCCCCTCTTTTTCCACCTG-3'	577	94° C 30 Sec, 60° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 7	FR. 5'-TTGAAACCTCCTCTTGGCTCAGGT-3' RV. 5'- GCCTGGGAAATGATGAAAGCAGAC-3'	276	94° C 30 Sec, 62° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 8	FW.5'-TTTACAGCCCAGGAAACCAAGCTG-3' RV.5'-ATGGGTTGACCCTCTCTTGATGCT-3'	558	94° C 30 Sec, 60° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 9	FR. 5'-ACGGATGGCTCATACCCACTTTCA-3' RV.5'-GAGCATGCATCATCCTTGCCTTCT-3'	497	94° C 30 Sec, 58° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 10	FR.5'-TCTCCCATCTGTGTTGTCTCTTCC-3' RV.5'-TTTGGAGGGCGTGTCAGACTCAT-3'	263	94° C 30 Sec, 62° C 45 Sec, 72° C 1min for 40 cycle using 1.5mM $Mgcl_2$
EXON 11	FR.5'- CAGGCTGTAGTTTGCTATGCCCTT-3' RV.5'- TACAGAGAAGTGAAGGCGTCTAGG-3'	324	94° C 30 Sec, 58° C 45 Sec, 72° C 1min for 40 cycle using 1.0mM $Mgcl_2$

The PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized under UV light. Only those PCR products that had a single amplification product with no evidence of nonspecific amplification were used for DNA sequencing as described below (Figure 3).

Figure 3: 1.5 % Agarose Gel to Check the PCR Products

DNA Sequencing: The PCR products free of contaminating bands due to nonspecific amplification were column-purified using a Qiagen PCR-purification kit (Qiagen, Hilden, Germany), and bidirectional sequencing was performed in an ABI Prism 3130 DNA sequencer (Applied Biosystems, Foster City, CA) using dye-termination chemistry.

Nucleotide changes were promptly detected by identifying ‘double peaks’ in the chromatogram due to heterozygosity of the DNA sample analyzed and confirmed by sequencing from the opposite direction. Additionally, the sequences were analyzed using pairwise BLAST to examine if there were any changes from the normal sequence available in the database.

PREDICTION OF THE FUNCTIONAL EFFECT OF AMINO ACID SUBSTITUTIONS

Multiple Sequence Alignment: Sequences of representative transporters were downloaded from NCBI (National Center for Biotechnology Information). Multiple sequence alignments were obtained using ClustalW and visualized using Jalview. The align residues were colored based on their type (using “Clustalxcolour scheme in Jalview) and their level of conservation (more conserved the residue in a given position, the stronger the colour).

Grantham Value: Low values indicate chemical similarity and high values indicate radical difference.

BLOSUM62: Where negative values indicate less acceptable and non-negative values indicate more acceptable substitution.

SIFT: (Sorting Intolerant from Tolerance; *http://blocks.fhcrc.org/sift/SIFT.html*).

SIFT is a sequence homology-based tool that presumes that important amino acids are conserved in the protein family. Hence, changes occurring at well-conserved positions are generally predicted to be deleterious. Substitutions at each position showing normalized probabilities less than a chosen cutoff value are predicted to be deleterious, and those greater than or equal to the cutoff value are predicted to be tolerated (Tolerant if score >0.05; intolerant if score ≤0.05).

PolyPhen: The *PolyPhen* programme, which structurally analyses the amino-acid polymorphism and is used to predict whether an amino acid change is likely to be deleterious to protein function. The prediction is based on the position-specific independent counts (PSIC) score derived from multiple sequence alignments of observations. *PolyPhen*score: if score > 0.50 - Intolerant (probably damaging), if score ≤0.50- tolerant (Benign).

STATISTICAL ANALYSIS

In general, data are expressed as mean ± standard deviation. Allele frequencies were estimated by the gene counting method, and x^2 test was used to identify significant departures from Hardy-Weinberg equilibrium. A $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

We sequenced all the *SLC22A1* exons (exons 1-11) and their flanking sides from 50 type 2 diabetes mellitus patients of West Bengal, and found eight genetic variations (Table 2).

Table 2: Summary of Variations of the *SLC22A1* Gene in 50 T2DM Patients of West Bengal

Genomic Location	Nucleotide Change	Amino Acid Change ★	Variant Allele Frequency (*n*=100)	*P* Value	Location in Protein	Kinase/ Phosphatase Motif	Binding Motif	Mutation / SNP
Exon1	c.156 AGT>AGC	52 Ser>Ser	0.27	0.091	TP	Yes	Yes	S
Intron 1	IVS1-43 T>G	NA	0.20	0.008	NA	NA	NA	NA
Exon 2	c.480 TTG>TTC	160 Leu>Phe	0.63	0.0002	TM	No	No	NS

Table 2: Contd.,

Intron 2	IVS2-99 C>T	NA	0.17	0.147	NA	NA	NA	NA
Intron 5	IVS5-61 G>A	NA	0.18	0.022	NA	NA	NA	NA
Ex 6	c.1022 CCG>CTG	341 Pro>Leu	0.20	0.376	TP	Yes	Yes	NS
Ex 7	c.1222 ATG>GTG	408 Met>Val	0.43	0.663	TM	Yes	No	NS
Intron 9	IVS9+43 C>T	NA	0.37	0.084	NA	NA	NA	NA

★Positions are relative to the ATG start site and are based on the cDNA sequence from Gene Bank accession no. NM_003057. NA: Not applicable. TP: Topological Domain; TM: Transmembrane Domain, S: Synonymous; NS: Non-synonymous. *n*: Number of chromosomes

We detected the three previously reported non-synonymous variations, 480 G>C (L160F); 1022 C>T (P341L); 1222 A>G (M408V) and one synonymous variations 156 T>C (S52S) at minor allele frequencies of 0.63, 0.20, 0.43 and 0.27 respectively. We also found four previously reported intronic variations: IVS1-43(T>G), IVS2 -99(C>T), IVS5 -61(G>A), IVS9 +43(C>T) with frequencies of 0.20, 0.17, 0.18, and 0.37 respectively (Table 2). Previously, 25 polymorphisms were identified from 57 Caucasian subjects [(7)]. Among them, 156T>C, IVS1 -43T>G, 480C>G (L160F), 1222 A>G (M408V), IVS9 +43C>T were also detected in our study. Most of their frequencies were different between the Caucasians and our study. Over two - fold difference were observed in IVS1 -43T>G (0.085 in Caucasians and 0.20- in our study), 480C>G (L160F) (0.216 in Caucasians and 0.63- in our study). One SNP 1022 C>T (P341L) were also detected our study at a frequency of 0.20 (0.168 in Japanese) (Figure 4).

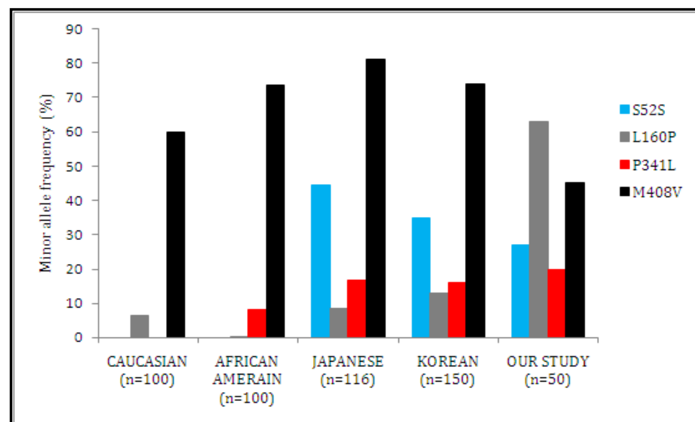


Figure 4: Comparison of Minor Allele Frequencies of Four Coding Single Nucleotide Polymorphisms in *SLC22A1* between Our and Other Population Groups *N*: Number of Subjects

These SNPs, especially IVS1 -43T>G may be ethnic specific [(4)]. Shu*et al.* detected 15 non-synonymous polymorphisms from 5 different ethnic groups, Afriran Americans (100 subjects), European Americans (100 subjects), Asian Americans (30 subjects), Mexican Americans (10 subjects) and Pacific Islander (7 subjects) [(11)]. Regarding Asian Americans, they identified 480 G>C (L160F); 1022 C>T (P341L); 1222 A>G (M408V), which are also detected in our study.

The discrepancy of the allele frequencies of these SNPs in this study may be caused by different sample size. We screened the DNA sequence and the electropherograms for these SNPs are shown in Figure 5.

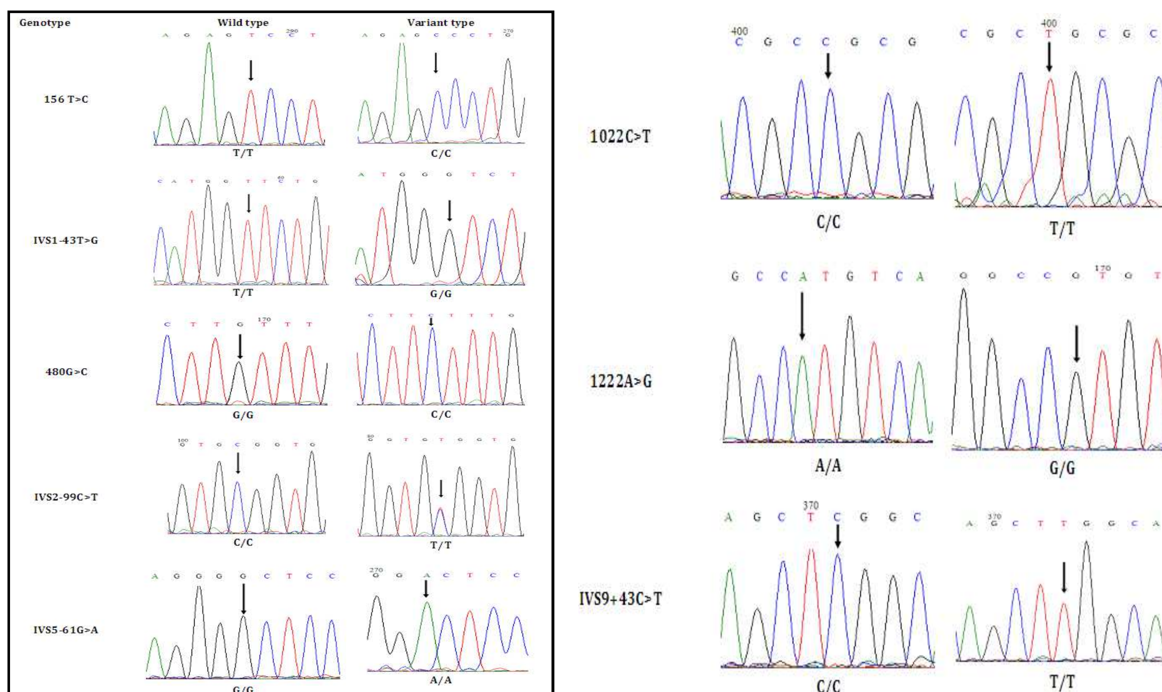


Figure 5: Electropherograms of Eight SNPs of *SLC22A1* Gene. Arrow Indicates the Position of Nucleotide Change

The SNP P341L located in exon 6, coding a topolocial domain in cytoplasmic side (Table 2). The replacement of a rigid proline with a leucine, which has a relatively flexible side chain, could possibly change the local structure of OCT1. The effects of some amino acid substitutions on OCT1 structure have been evaluated (Table 3)

Table 3: Characteristics of Founded nsSNPs in *SLC22A1*

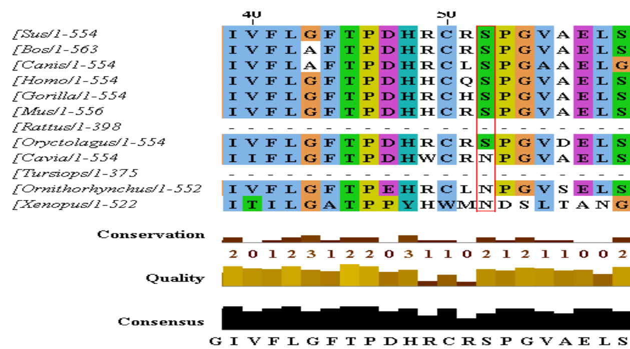
Amino ★ Acid Change	Function	Scoring System for Non-Synonymous Variants						
		Grantham	EC/ EU	BLOSU M62	Tolerance Index	SIFT Prediction	PSIC SD	*PolyPhen* Prediction
L160F	+	22	EC	0	0.00	Intolerant	0.187	Benign
P341L	+/-	98	EC	-3	0.00	Intolerant	2.083	Probably damaging
M408V	+	21	EU	1	0.08	Tolerant	0.609	Benign

★ Positions are relative to the ATG start site and are based on the cDNA sequence from Gene Bank accession no. NM_003057; +, function (as measured by MPP^+ uptake) similar to that of reference OCT1; +/-, reduced function; EC/EU indicates evolutionary conserved/ evolutionary un conserved; SIFT, Sorting Intolerant from Tolerance; PSIC SD, position-specific independent counts score difference by different criteria (evolutionary conservation, amino acid substitution and chemical changes)

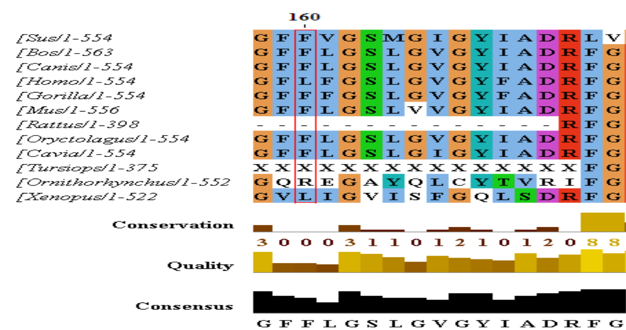
We use amino acid scoring systems, especially Grantham and BLOSUM62 values [2, 3]. It was shown in Table 3 the variants (P341L) with reduced or diminished activity gave much higher Grantham value (98), indicative larger chemical changes and more negative BLOSUM62 value (-3) showing evolutionary unfavorable changes than those (22 and 0 respectively) for the variant Leu160Phe with unchanged function[8].

Evolutionary conservation is a strong predictor of allele frequency, indicating that substitutions at evolutionary conserved (EC) positions are more deleterious than those at evolutionary unconserved (EU) positions [11]. In our study, we also investigate these variants among different species for OCT1 by Multiple sequence alignment, and it was shown that 3 of them were highly conserved (Figure 6).

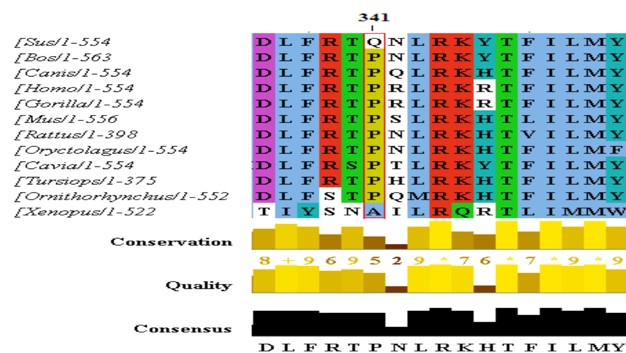
- **EXON 1 (S52S)**



- **EXON 2 (L160F)**



- **EXON 6(P341L)**



- **EXON 7(M408V)**

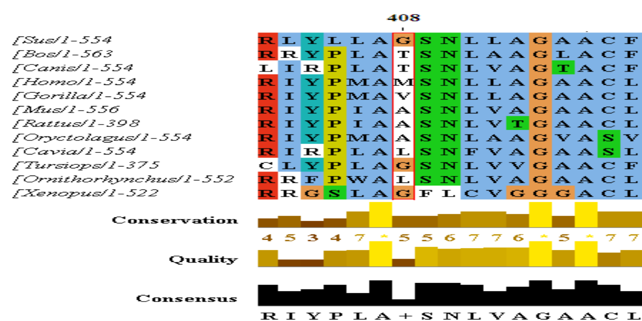


Figure 6: Multiple Sequence Alignments were Obtained Using ClustalW and Visualized Using Jalview. The Align Residues Were Colored Based on Their Type (Using "Clustalxcolour Scheme in Jalview) and Their Level of Conservation (More Conserved the Residue in a Given Position, the Stronger the Colour)

To predict the potential effects of missense mutation on protein function, we obtained estimates of the impact of three missense mutations through the use of two sequence homology-based programmes, SIFT and *PolyPhen*. Moreover, several studies indicate that analysing non-synonymous mutations at the structural level is very important in understanding the functional activity of the protein of interest. The SIFT programme was applied to prioritize three missense mutations in the exon 2, 6 and 7 coding region of *SLC22A1*. The tolerance index score for L160F and P341L mutation were 0.00, predicting a deleterious effect for each, but for M408V was 0.08 (Table 3). The structural levels of alteration were then determined by applying the *PolyPhen*programme in which a PSIC score difference. One missense mutations, P341L predicted to be possibly damaging to protein structure with PSIC scores of 2.083. L160F and M408V were predicted to be a mutation with a benign effect, indicated by a PSIC score of 0.187 and 0.609 respectively (Table 3). Thus, one missense mutations, P341L was identified as potentially important in the identification of protein function.

CONCLUSIONS

In conclusions, we identified eight genetic variants including four exonic ones in *SLC22A1* in 50 type 2 diabetes mellitus patients of West Bengal. This is the first report of *SLC22A1* variations among Indian, especially West Bengal's type 2 diabetes mellitus patients. The present results would be useful for haplotype analysis and pharmacogenetic studies on OCT1.

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