Analysis and Homology Modeling of Proteins Derived from *NKX2.5* Non-synonymous Single Nucleotide Polymorphisms Involved in Congenital Heart Disease

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Abstract: Congenital heart disease (CHD) is the most common type of birth defect, affecting 1% of all live births and is the leading noninfectious cause of death in the first year of life. With the progress in molecular genetics and developmental biology, many genes associated with heart development have been identified. NKX2.5 is an important transcription factor for heart development and the single nucleotide polymorphisms (SNPs) in *NKX2.5* are associated with various CHD phenotypes. Bioinformatics analysis may help to discriminate between synonymous SNPs and non-synonymous SNPs (nsSNPs), located in coding regions and resulting in amino acid variation in the protein products of genes. We made an attempt to analyse the nsSNPs of *NKX2.5* which are involved in CHD by using bioinformatics tools to predict protein structural changes. 61 nsSNPs of *NKX2.5*, which are involved in CHD were analysed. We edited nsSNPs into the control sequence manually and made all 61 sequences with nsSNPs. Translated these sequences into amino acid polymorphisms and were subjected for protein secondary structure prediction. We analysed the 3-D structure of all the mutated proteins of the *NKX2.5* nsSNPs. This investigation revealed the side chain differences of the amino acids in the 3-D structure of the protein. [Nature and Science 2010;8(9):27-38]. (ISSN: 1545-0740).

Key words: Congenital heart disease; Single nucleotide polymorphisms; *NKX2.5*

1. Introduction

Congenital heart disease (CHD) is the most common type of birth defect, affecting 1% of all live births and is the leading noninfectious cause of death in the first year of life (Hoffman and Kaplan, 2002; Jing-bin et al., 2009). CHD is a multifactorial complex disease with environmental and genetic factors playing important roles (Kohl, 1985; Jing-bin et al., 2009). With the progress in molecular genetics and developmental biology, many genes associated with heart development have been identified (Jing-bin et al., 2009). A number of selected congenital heart defects and genetic syndromes have been found to be associated with SNPs in a variety of single genes (Jing-bin et al., 2009). Among them, NKX2.5 is an important transcription factor for heart development and the SNPs in *NKX2.5* are associated with various CHD phenotypes such as Atrial Septal Defect (ASD), Ventricular Septal Defect (VSD) and Tetrology of Fallot (TOF) (Smitha and Ramachandra, 2005; Jing-bin et al., 2009). Biochemical analysis showed that, several mutations of *NKX2.5* are responsible for reduced DNA binding activity of homeodomain and no transcriptional activity function (Scott et al., 1998; Kasahara et al., 2000; Kasahara and Benson, 2004; Chen et al., 2007). Molecular genetic testing of *NKX2.5* gene mutations is more widespread and clinically applicable. Such testing will form the basis for improved diagnosis and therapy of human congenital heart disease.

Analysis of DNA and protein sequences may help to discriminate between synonymous SNPs (sSNPs) which constitute the majority of genetic variation and non-synonymous SNPs (nsSNPs) with likely functional importance, located in coding regions and resulting in amino acid variation in the protein products of genes (Ramensky et al., 2002).

Earlier preliminary studies from our laboratory revealed a prevalence of CHDs ranging from 6.6 to 13.06 per 1000 live births in India (Smitha et al., 2006). Among the different CHDs, VSDs were the most common phenotype observed with high rate of consanguinity among these patients (Smitha and Ramachandra, 2006). Earlier studies from our laboratory also reported 18 CHD patients with numerical and 3 with structural chromosomal anomalies and also more than 114 CHD patients with normal chromosomes (Smitha et al., 2006; Smitha et al; 2007; Smitha et al., 2010). However, molecular studies

on CHDs in India are limited. Therefore, further molecular analysis of the candidate genes involved in CHDs may aid in the understanding of the genotype-phenotype associations. In the light of this, we have screened SNPs of NKX2.5 in 150 CHD patients and 70 controls in south Indian population and found three nsSNPs namely 608A>G (E203G), 646C>T (R216C), 852G>A (D226N). We also found sSNPs 239A>G (E21E), 896C>A (A240A) and 1212G>T in the 3' UTR region (data not published). Perusual of the literature revealed that 61 nsSNPs of *NKX2.5* have been reported (Scott et al., 1998; Benson et al., 1999; Goldmuntz et al., 2001; Ikeda et al., 2002; Gutierrez-Rolens et al., 2002; McElhinney et al., 2003; Reamon-Buettner et al., 2004; Gutierrez-Rolens et al., 2006; Riafai et al., 2007; Tian et al., 2007; Bjornstad and Leren, 2009; Hamanoue et al., 2009; Gioli-Pereira et al., 2010). Further, bioinformatics analysis of these SNPs is very limited in exploring the molecular mechanisms of CHD. In view of this, we made an attempt to analyse the nsSNPs of *NKX2.5* which are involved in CHD by using bioinformatics tools to predict protein structural changes.

2. Material and Methods:

Secondary structure analysis

We obtained 61 nsSNPs of *NKX2.5*, by perusual of literature, which are involved in CHD (Scott et al., 1998; Benson et al., 1999; Goldmuntz et al., 2001; Ikeda et al., 2002; Gutierrez-Rolens et al., 2002; McElhinney et al., 2003; Reamon-Buettner et al., 2004; Gutierrez-Rolens et al., 2006; Riafai et al., 2007; Tian et al., 2007; Bjornstad and Leren, 2009; Hamanoue et al., 2009; Gioli-Pereira et al., 2010). We edited nsSNP into the control sequence manually and created all 61 sequences with nsSNPs. These sequences were translated into amino acid sequence to identify polymorphisms. The differences in the nucleotide and protein sequences were compared through multialignment and also secondary structure for all the 61 nsSNPs has been predicted by Chou Fasman secondary structure algorithm by using Accelrys gene software.

Comparative modeling of 3-D structure for homeodomain protiens of NKX2.5 nsSNPs.

The effect of each amino acid changes on protein structure was determined using a homeodomain protein template model using http://swissmodel.expasy.org/workspace With the availability of reference homeodomain reference homeobox protein structure (VND=NKX2.5) between 138-197 amino acid residues (PDB Id: 1QRYA) (Guex et al., 1997; Schwede et al., 2003; Arnold et al., 2006), 14 nsSNPs of this region were analysed.

Evaluation and validation of 3-D structure

Evaluation and validation of the 3-D structure was done using Swiss-pdb viewer. The overall quality of the protein and the amino acid residues in the allowed, disallowed region were assessed by Ramachandran plot analysis. The modeling for control and mutated protein was carried out. The structural superimposition of both control and mutant proteins were visualized using Pymol.

3. Results and Discussion:

Human single nucleotide polymorphisms (SNPs) represent the most frequent type of human population DNA variation and one of the main goals of SNP research is to understand the genetics of the human phenotype variation and especially the genetic basis of human complex diseases (Ramanskey et al., 2002). The nsSNPs comprise a group of SNPs that, together with SNPs in regulatory regions are believed to have the highest impact on phenotype (Vage and Lingaas, 2008). The nsSNPs also known as single amino acid polymorphism (SAPs) that causes amino acid changes in proteins which have the potential to affect both protein structure and function (Hu and Yan, 2008). Some of the mutations in SAP sites are not associated with any changes in phenotype and are considered functionally neutral, but others bringing deleterious effects to protein function and are responsible for many human genetic diseases (Hu and Yan, 2008). By the analysis of the new incoming data on SNPs by mapping them onto three-dimensional structures of proteins, problems concerning population, medical and evolutionary genetics can be addressed (Sunyaev, 2001; Chen et al., 2009).

Multialignment of both nucleotide and protein sequences of *NKX2.5* in both control and mutant are presented in Figure 1a and 1b. Analysis of secondary structure in 61 mutated proteins derived from nsSNPs of *NKX2.5* revealed that, 28 mutated proteins were positive in their structural changes (Table 1). Secondary structural changes of 28 mutated proteins with nsSNPs of *NKX2.5,* compared with control protien are presented in Figure 2. These proteins showed changes in their helix, sheet and turn of secondary structure. In this study, we explore the feasibility of classifying the nsSNPs of *NKX2.5* on the basis of protein structural changes.

An attempt is also made to analyse the 3-D structure of all the mutated proteins of the *NKX2.5* nsSNPs. One limitation of this analysis is they are not applicable to the cases where reference protein structures are not available. Therefore, the available homeodomain reference protein structure was used to analyse the 14 nsSNPs involved in mutation of protein. For the modeling of proteins, evaluation and validation of 3-D structure was carried out by using

Ramachandran plot analysis for the template model structure (Figure 3). The mutated homeodomain proteins showed structural differences while visualising 3D structures (Figure 4). The structural variations such as side chain differences compared to control protein were observed (Figure 4). The homeodomain portion of the protein forms three alpha helices, and helix 3 is responsible for DNA-binding specificity (Gioli-Pereira et al., 2010). Homeodomain binds to the DNA regulating the gene expression, nsSNP Q149X is expected to encode a truncated protein of only 148 amino acids and this results in an incomplete homeodomain, prevents its binding to DNA, and makes the protein non-functional (Bjornstad and Leren, 2009). The structural changes in the analysed homeodomain mutated protein may responsible for non functional NKX2.5 proteins.

However, we found only side chain differences of the amino acids in the 3-D structure of the protein. Theoretical accuracy of prediction of the tertiary structure of a protein from a sequence is 90% (Saunders and Deane, 2010). Local conformation of a protein varies under the native conditions. These limitations are also imposed by secondary structure prediction's inability to account for tertiary structure. As only one amino acid difference occurs by particular nsSNP, modeling tool considers the same template for all nsSNPs and models almost the same structure except side chain difference for the mutated.

In this study, we have observed differences in the side chain atoms such as, Delta Carbon (CD), Epsilon Nitrogen (NE), Zeta Carbon (CZ), Gamma Carbon (CG),

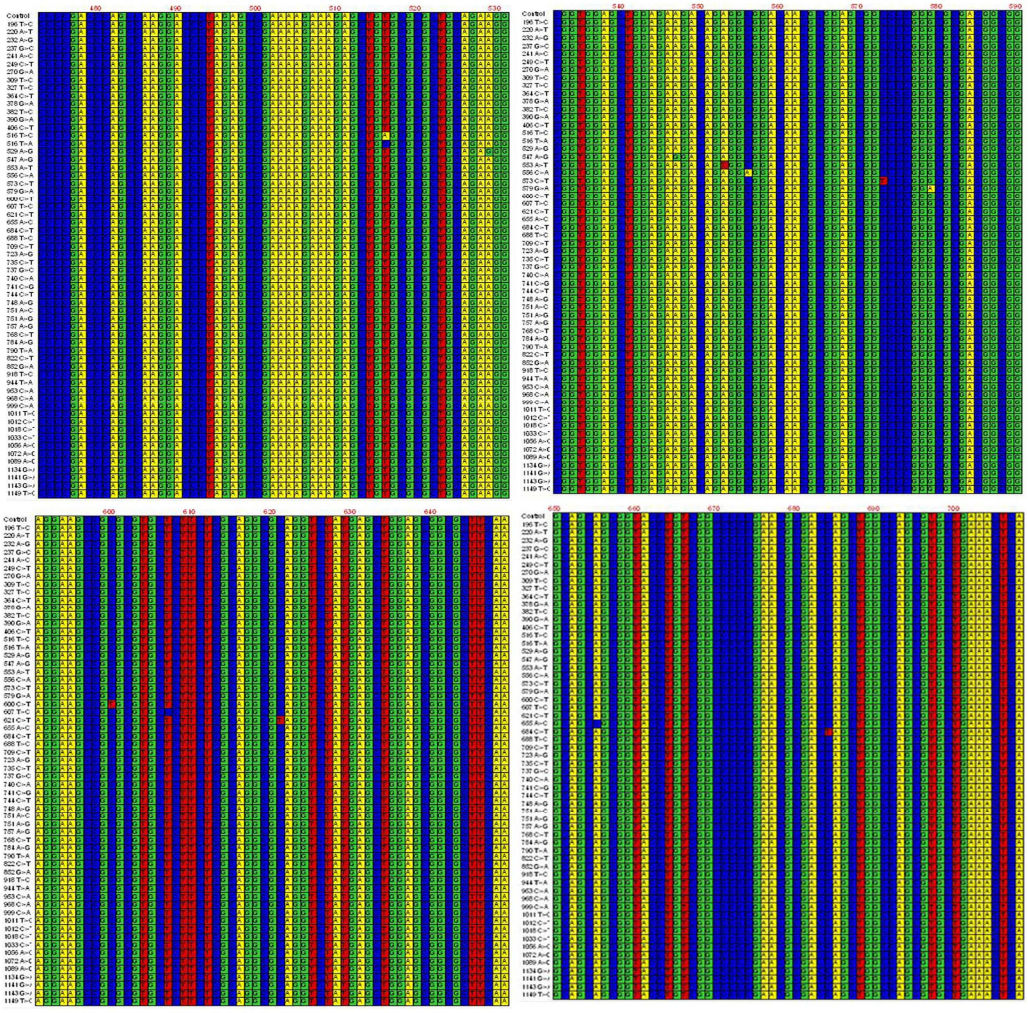


Figure 1a. Multialignment of *NKX2.5* nucleotide sequences of both control and mutant by using Accelrys gene software. Conserved sequences were not shown in this Figure. Alighnments of 61 nsSNPs edited sequences were made with control sequence. The four nucleotides ATGC were represented with different coloures (A=Yellow, T=Red, G=Green, C=Blue) in the alignment Figure 1a.

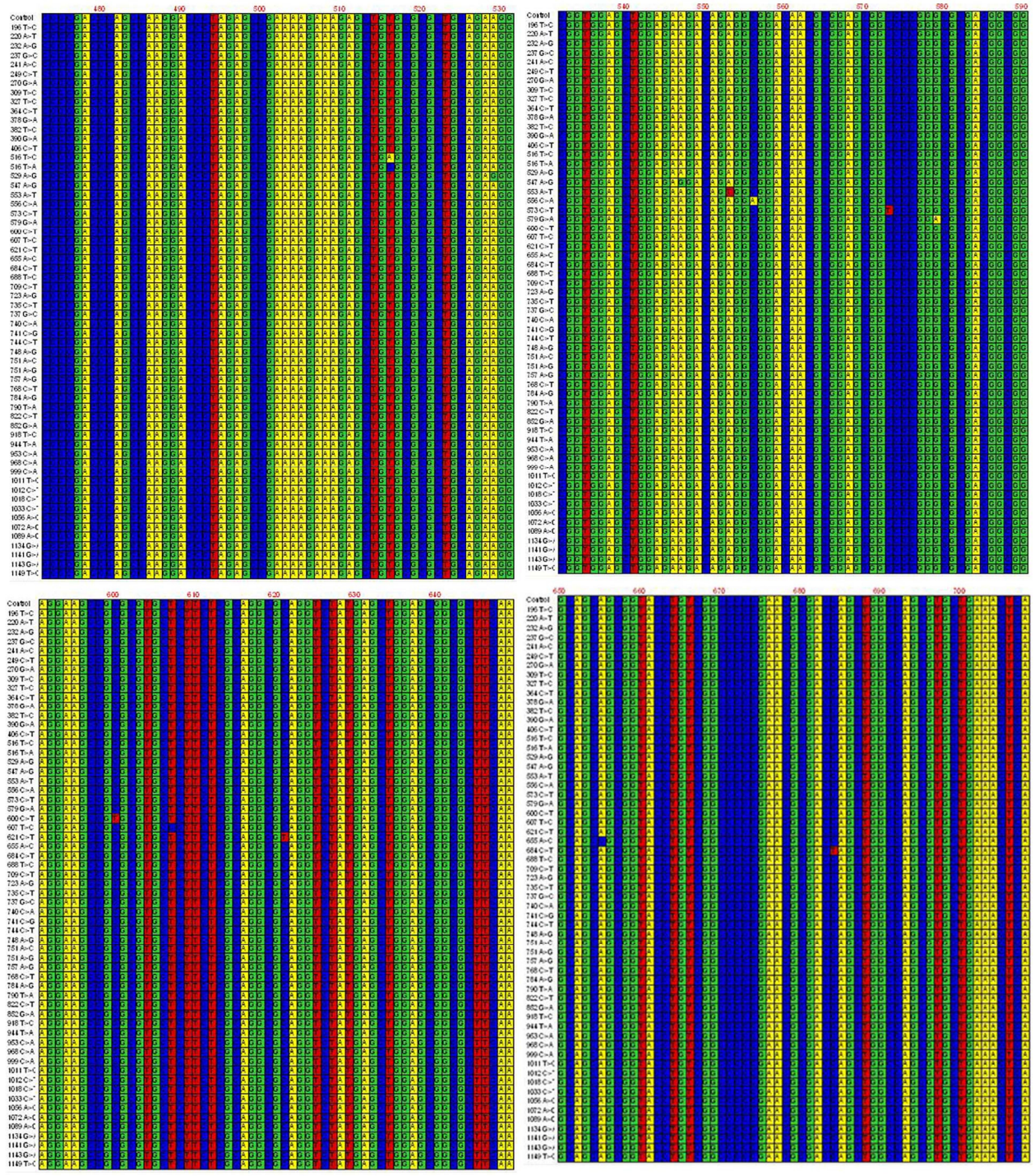


Figure 1a Continued (1)

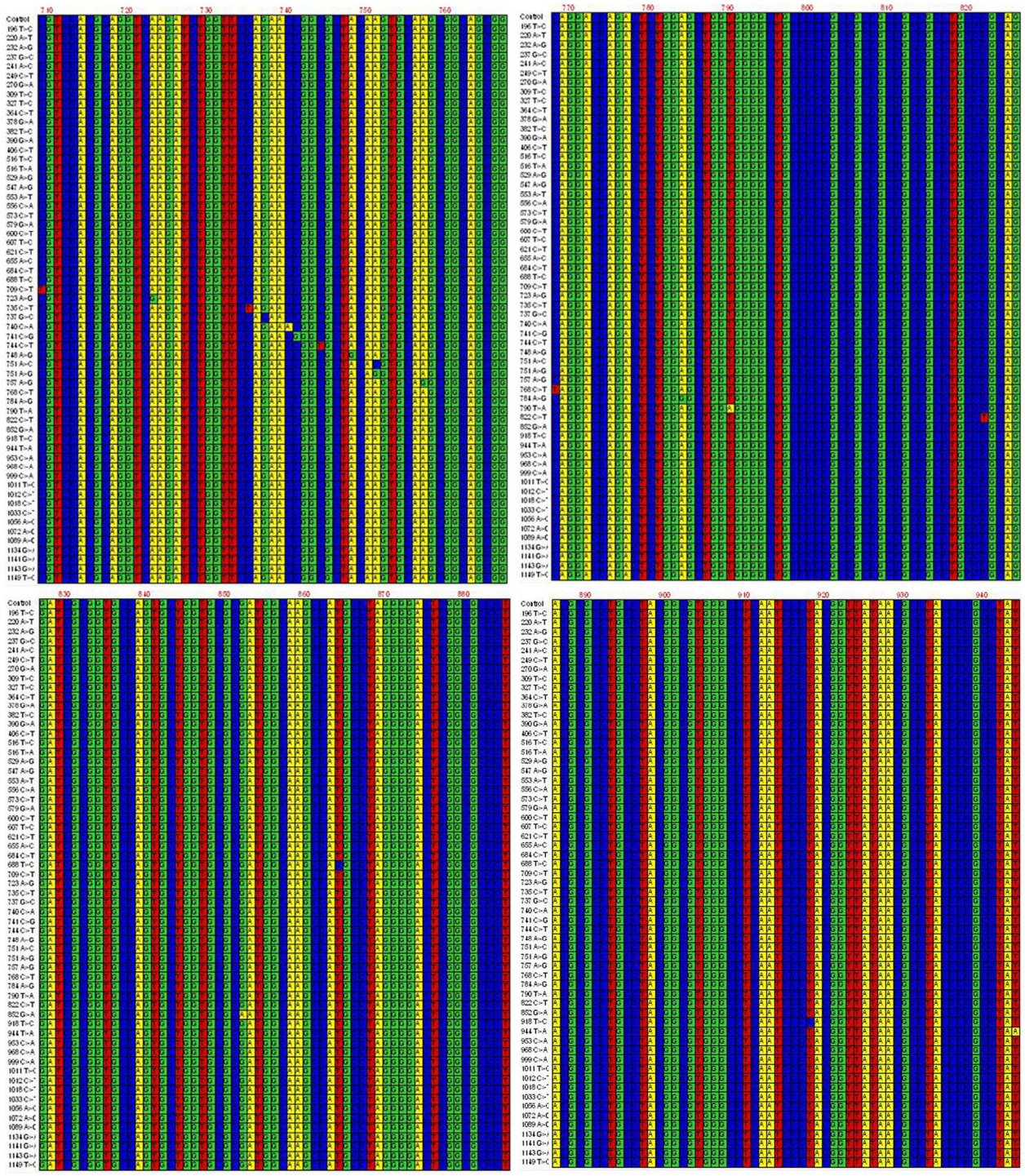
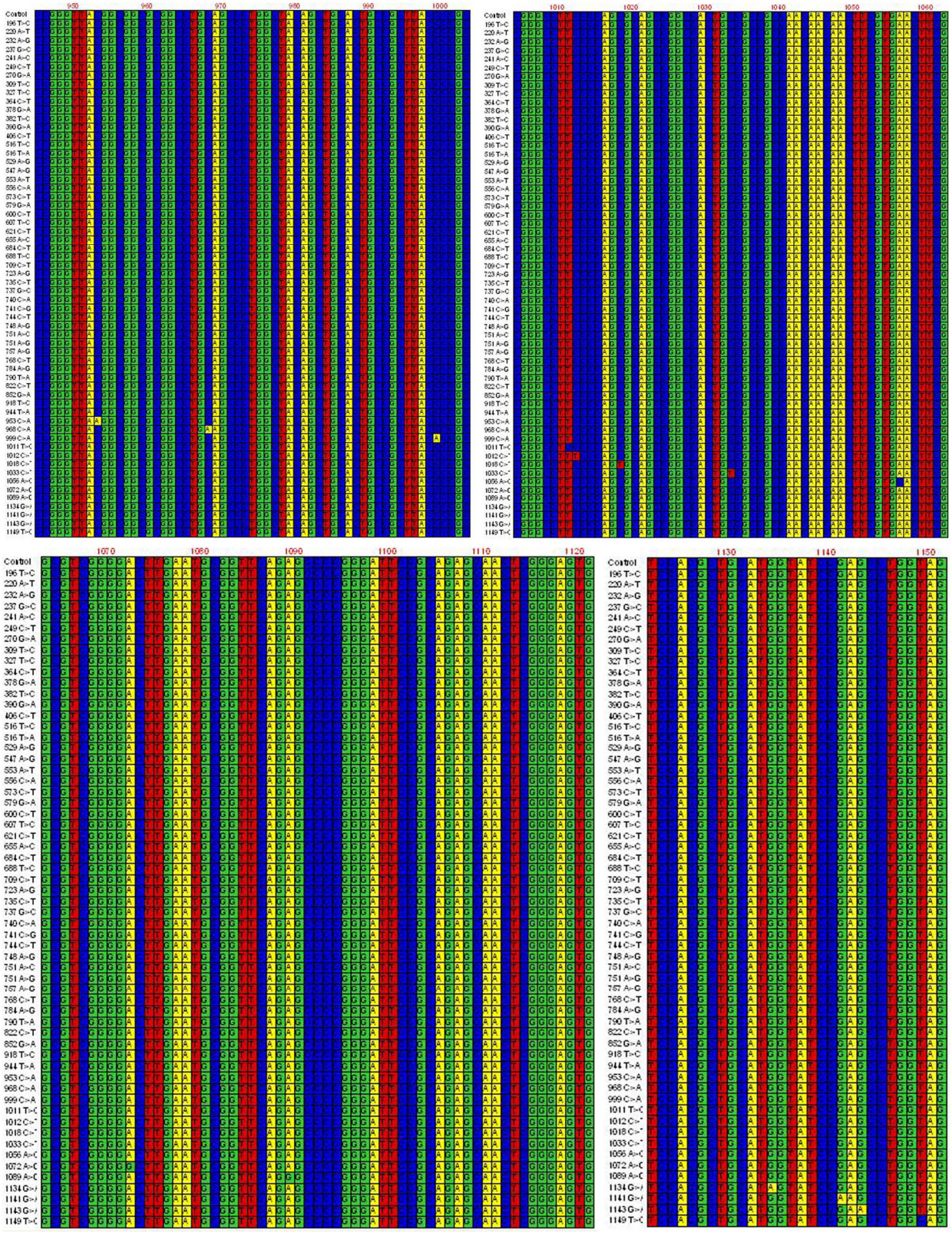


Figure 1a Continued (2)



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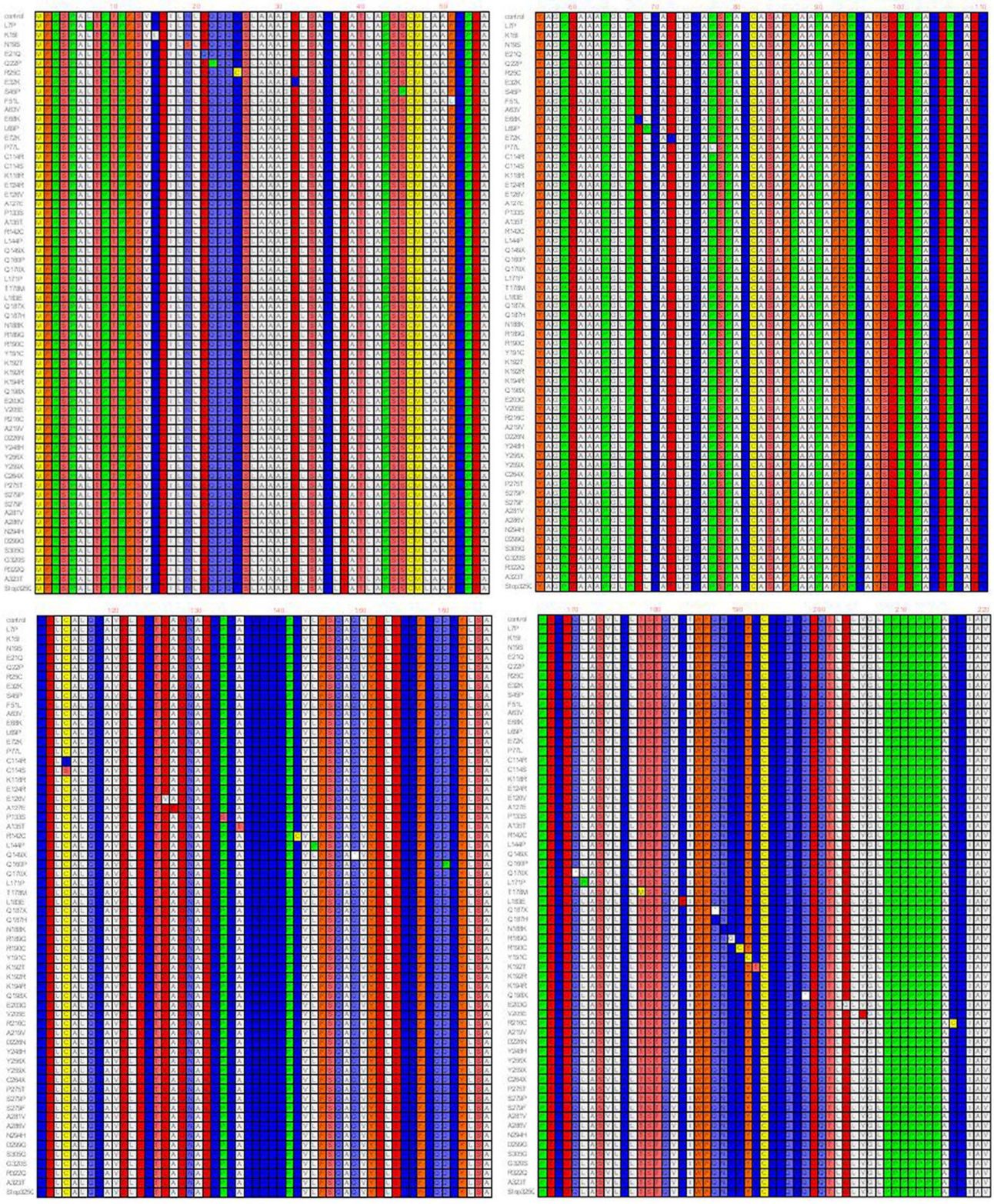

Figure 1b. Multialignment of NKX2.5 protein sequences for both control and mutant by using Accelrys gene software. Alignments were made control protein sequence with 61 amino acid polymorphisms edited protein sequences. Each colour represents particular amino acid.

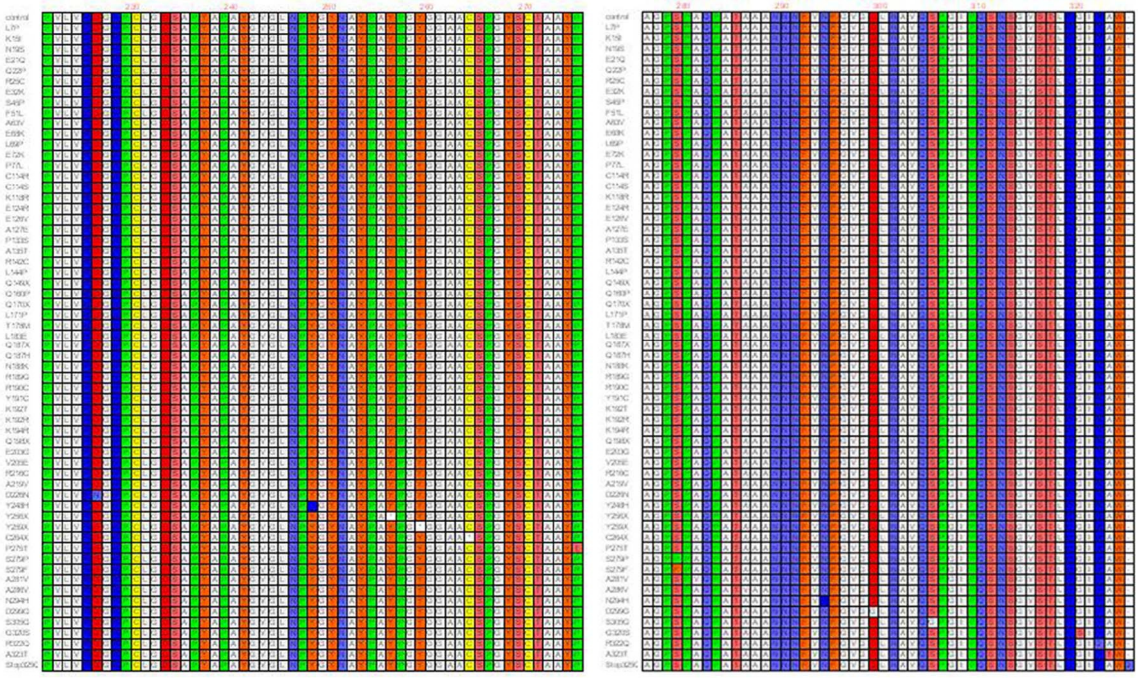

Figure1b.Continued

Table.1. Non-synonymous SNPs of NKX2.5 and their impact on secondary structure of protein ('+' indicates the secondary structure changes of the *NKX2.5* protein) Gamma Sulphur (SG), Gamma Oxygen (OG1), Delta Sulphur (SD) in the 3-D structures of control and mutant protein.

Sl. No	SNP	Amino acid change	Secondary structure of the protein		
			Helix	Sheet	Turn
1	196 T>C	L 7 P	-	-	+
2	220 A>T	K 15 I	+	+	-
3	232 A>G	N 19 S	-	-	-
4	237 G>C	E 21Q	+	+	-
5	241 A>C	Q 22 P	-	-	-
6	249 C>T	R 25 C	+	+	-
7	270 G>A	E 32 K	-	-	-
8	309 T>C	S 45 P	-	-	-
9	327 T>C	F 51 L	-	-	-
10	364 C> T	A 63 V	-	-	-
11	378 G>A	E 68 K	-	-	-
12	382 T>C	L 69 P	-	-	+
13	390 G>A	E 72 K	-	-	-
14	406 C>T	P 77 L	+	-	-
15	516 T>C	C 114 R	+	+	-
16	516 T>A	C 114 S	+	+	-
17	529 A>G	K 118 R	-	+	-
18	547 A>G	K 124 R	-	-	-
19	553 A>T	E 126 V	-	-	-
20	556 C>A	A 127 E	-	-	-
21	573 C>T	P 133 S	-	-	-
22	579 G>A	A 135 T	-	-	-
23	600 C>T	R142C	-	-	-
24	607 T>C	L 144 P	+	+	+
25	621 C>T	Q 149 X	+	+	-
26	655 A>C	Q 160 P	-	-	-
27	684 C>T	Q 170 X	+	-	-
28	688 T>C	L 171 P	+	-	-
29	709 C>T	T 178 M	+	+	-
30	723 A>G	L 183 E	-	-	-
31	735 C>T	Q 187 X	-	+	+
32	737 G>C	Q 187 H	-	-	-
33	740 C>A	N 188 K	-	-	-
34	741 C>G	R 189 G	-	-	+
35	744 C>T	R 190 C	-	-	+
36	748 A>G	Y 191 C	-	-	-
37	751 A>C	K 192 T	-	+	+
38	751 A>G	K 192 R	-	-	-
39	757 A>G	K 194 R	-	-	+
40	768 C>T	Q 198 X	+	-	-
41	784 A>G	E 203 G	+	-	-
42	790 T>A	V 205 E	+	-	-
43	822 C>T	R 216 C	+	-	-
44	832 C>T	A 219 V	+	-	-
45	852 G>A	D 226 N	-	-	-
46	918 T>C	Y 248 H	-	-	-
47	944 T>A	Y 256 X	-	-	-
48	953 C>A	Y 259 X	-	-	+
49	968 C>A	C 264 X	-	-	+
50	999 C>A	P 275 T	-	-	-
51	1011 T>C	S 279 P	-	-	-
52	1012 C>T	S 279 F	-	-	-
53	1018 C>T	A 281V	+	+	-
54	1033 C>T	A 286 V	+	+	-
55	1056 A>C	N 294 H	-	-	-
56	1072 A>G	D 299 G	-	-	-
57	1089 A>G	S 305 G	-	-	-
58	1134 G>A	G 320 S	-	-	-
59	1141 G>A	R 322 Q	-	-	-
60	1143 G>A	A 323 T	-	-	-
61	1149 T>C	Stop 325 Q	-	-	-

Side chain of an amino acid is specific to each amino acid of a protein. The carbon atom next to the carbonyl group is called the α–carbon and amino acids with a side chain bonded to this carbon are referred to as alpha amino acids (Creighton, 1993; Laufer et al., 2009). In the alpha amino acids, the α–carbon is a chiral carbon atom, with the exception of glycine (Creighton, 1993; Trbovic et al., 2009). In amino acids that have a carbon chain attached to the α–carbon are labeled in order as α, β, γ, δ, and so on (Jones, 2006). In some amino acids, the amine group is attached to the β or γ-carbon, and these are therefore referred to as beta or gamma amino acids (Creighton, 1993; Trbovic et al., 2009). The side chain can make an amino acid a weak acid or a weak base, and a hydrophile if the side chain is polar or a hydrophobe if it is nonpolar (Creighton, 1993; Laufer et al., 2009; Trbovic et al., 2009). The distribution of hydrophilic and hydrophobic amino acids determines the tertiary structure of the protein, and their physical location on the outside structure of the proteins influences their quaternary structure (Creighton, 1993; Laufer et al., 2009; Trbovic et al., 2009). These properties are important in protein structure and protein–protein interactions (Creighton, 1993; Laufer et al., 2009; Trbovic et al., 2009).

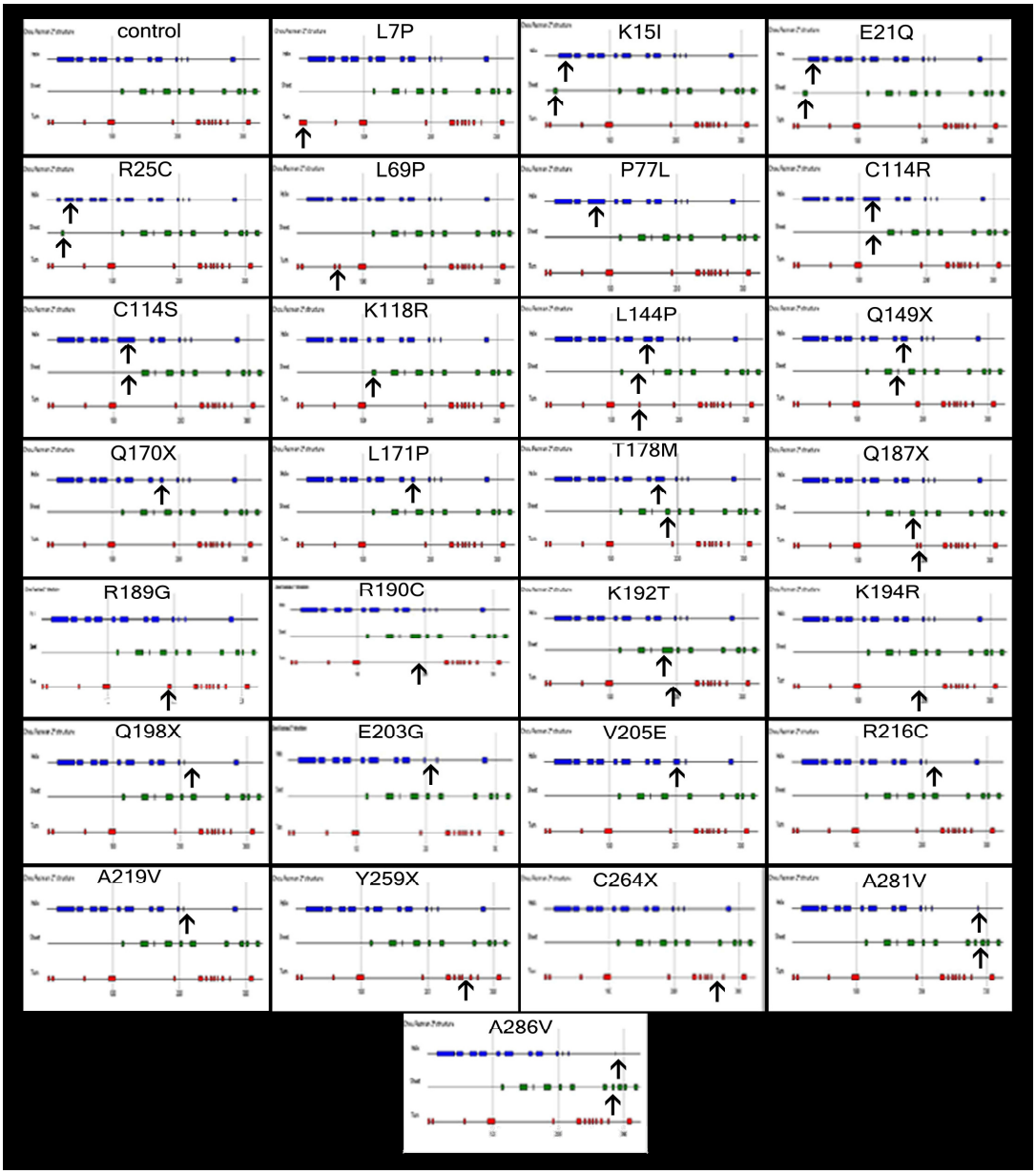

Figure 2. Secondary structure of NKX2.5 mutated proteins derived from 29 nsSNPs shows structural changes in their helix (blue), sheet (green) and turn (red) compare to control protein.

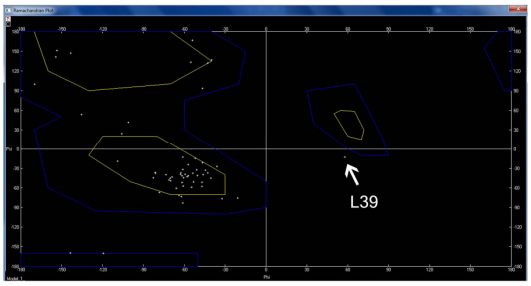

Figure 3. Ramachandran plot was analysed using Spdbv for the evaluation and validation of 3-D structure of the reference homeobox protein, ventral nervous system defective (VND=NKX2.5) (PDB Id: 1QRYA). Most of the amino acid residues fall in allowed region bounded by curves in yellow (no contact) and blue (frequently observed moderate contact) except L39 in the forbidden region.

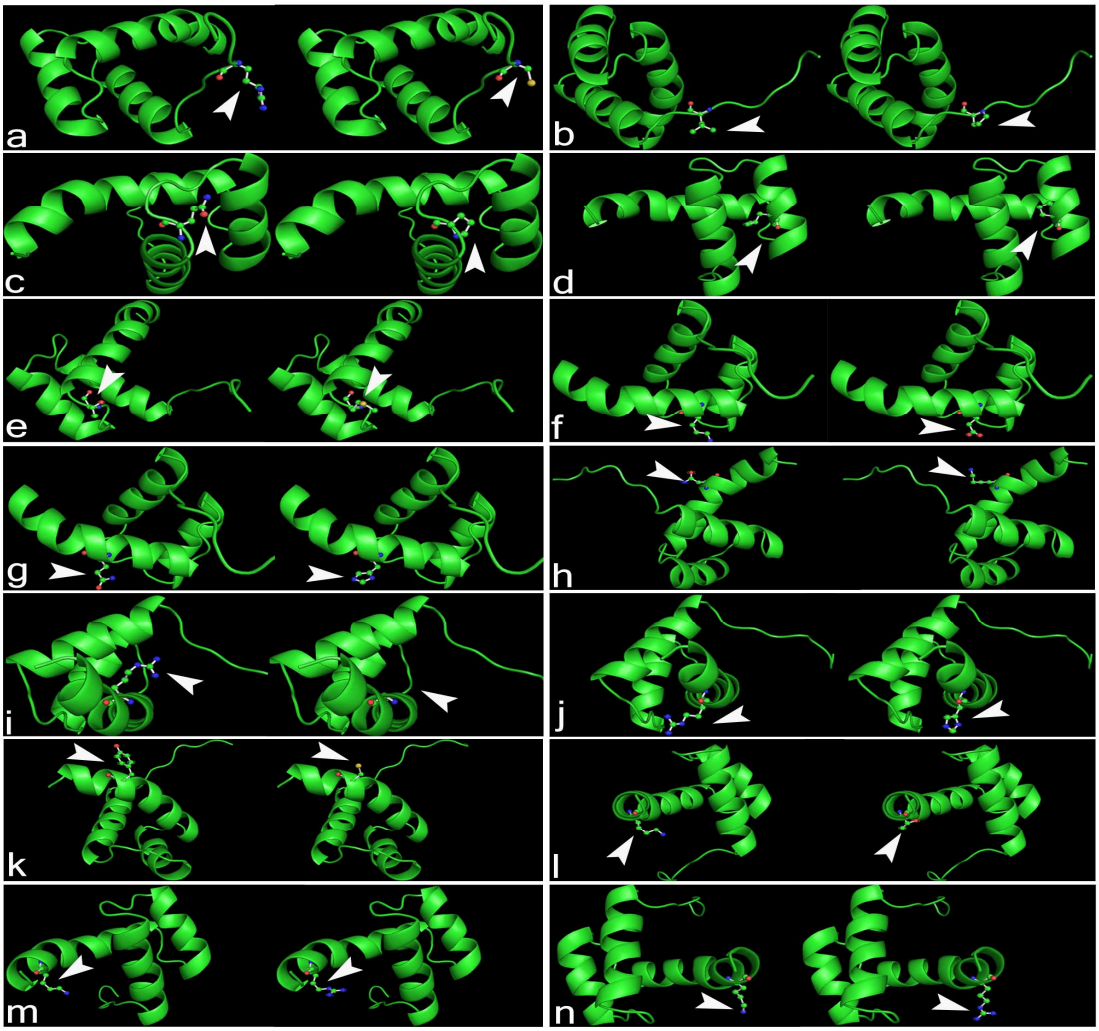

Figure 4. 3-D models of mutated proteins derived from 14 ns SNPs of homeodomain region of *NKX2.5*. Each model was superimposed by control (left side of the each image) and mutated protein (right side of the image). Models were done using homeobox protein of ventral nervous system defective (VND) protein (PDB_Id:1QRY) and also for mutant. Ball and stick representation in both the models showed the side chain structural differences between control and mutated proteins. (a)R142C: In control - Gamma Carbon (CG), Delta Carbon (CD), Epsilon Nitrogen (NE), Zeta Carbon (CZ) and in mutant - NH1, Gamma Sulphur (SG). (b) L144P: In control - Delta Carbon (CD) and in mutant -CD1, CD2. (c) G160P: In control - NE2 and in mutant Epsilon Oxygen (OE 1). (d) L171P: In control absence of CD2 and in mutant presence of CD2.(e) M178T: In control - CG2, Gamma Oxygen (OG1), CG, Delta Sulphur (SD) and in mutant - CE.(f) K183E: In control - CE, NZ and in mutant -OE1 and OE2. (g) Q187H: In control - CD, NE2, OE1 and in mutant - CD2, ND1, CE1, CE2. (h) N188K: In control - ND2, OD1 and in mutant - CD, CE, NZ. (i) R189G: In control - CB, CG, CD, NE, CZ, NH1 and in mutant - NH2. (j) R190H: In control - CD, NE, CZ, NH1, NH2 and in mutant- CD2, ND1, CE1, NE2.(k) Y191C: In control- CG, CD1, CD2, CE1, CE2, CZ, OH and in mutant- SG.(l) K192T: In control- CG, CD, CE, NZ and in mutant- CG2, OG1.(m) K192R: In control- CE, NZ and in mutant NE, CZ, NH1, NH2.(n)K194R:In control- CE, NZ and in mutant- NE, CZ, NH1, NH2

4. Conclusion

Secondary structure analysis of 61 ns SNPs showed structural changes in their helix, sheet and turn of the 28 nsSNPs. 3-D structure visualization of 14 nsSNPs of homeodomain showed structural differences in the side chain due to the mutation by nsSNPs involved in CHD. This study will lighten to further study on the side chain differences by mutations of nsSNPs involved in CHD. These structural changes of the mutated proteins further will help us to distinguish the CHD-causing nsSNPs from neutral SNPs. A subset of nsSNPs is known to functional that is they likely alter the protein product function. Thus, abnormal side chain changed *NKX2.5* protein is not efficiently involved in regulation of the down stream genes. Therefore, these SNPs and SAPs are important for normal function of *NKX2.5* in normal heart development. This information will facilitate further probing and be utilized for pharmacogenetics study and also for biomedical applications.

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