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Stop Codons of *TGF β RI* Gene Modulate the Functional Activity of 3D Structure and their Genetic Susceptibility in the Case of Wilms' Tumour

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Abstract

Genetic variants of transforming growth factor beta receptors type-1 (*TGF-βR1*) are involved in cellular signalling pathway and their mutations encoded amino acids involved in protein structure has not been defined. Present study evaluate the frequency of *TGF-βR1* gene mutation, copy number variation (CNV) and DNA sequencing for nucleotide changes followed by prediction of 3D protein model for ligand binding sites. Clinically diagnosed cases of Wilm's tumour were used for genetic studies using RT-PCR for determine the frequency of gene mutations, CNVs and changes in nucleotide were observed by DNA sequencing (Sanger's method). Frequency of *TGF-βR1* gene mutation was 18.18% observed in WT cases with respect to controls. Similarly, the Tm value (mean) was 90.70 shifted to 91.0 showing significant differences (p=0.24) and C.I. at 95% varying between 2.09-7.09 with copy number variations showing S.D=0.37 and C.I. at 95% 0.337- 0.906. Sequencing data reveals the appearance of two nucleotide sequences TGA→TCA and TGA→CCC, which translates amino acid serine and proline, respectively and consider as "stop codon". Further mutations were indentified in the form of Insertion/Deletions and 3-D helical structure was predicted for the ligand binding capacity to develop new molecules for cancer therapeutics based on pharmacogenomics.

Keywords: *TGF-βR1*; Wilms' tumour; DNA sequencing; Molecular docking

Introduction

Wilms' tumor (WT), one of the rare childhood tumor and their incidence is 1: 10,000 between 1 to 5 years age group [1,2]. WT occurs both in hereditary and sporadic forms and WTI gene associated with tumors and is mapped on chromosome 11p13. The protein encoded by the *WT1* gene contains an amino terminus rich in proline and glutamine residues and a carboxy terminus containing four zinc fingers protein act as transcription factor to regulates gene expression [3-5].

The transforming growth factor beta (*TGF-β*) is polypeptides of highly conserved and abundant dimeric proteins of 25 kd, ubiquitously expressed in eukaryotes that modulate the function of glomerular cell which is responsible to increases the production of collagen and fibronectin in mesenchymal epithelial cells [6-8]. *TGF-β* signalling pathway regulates the cellular proliferation, differentiation, migration and apoptosis in tumours [9,10]. There are three *TGF-β* isoforms (*TGF-β1, TGF-β2* and *TGF-β3*) are expressed in epithelium and each gene is encoded in tissue-specific manner. *TGF-βR1* based protein kinase activity results in interaction with other transcription factors to promote angiogenesis and immunosuppressive activity [11,12].

TGF-βRI can also participate in variety of cellular functions including invasion, extracellular matrix (ECM) formation and migration of cancer cells [13,14]. *TGF-βRI* is rich in serine/threonine kinase receptor that is a member of the *TGF-β* signalling pathway and exhibits metastatic properties by invading surrounding cells [15]. It is still not clear whether *TGF-β* bind to ligands either type-I or type-II receptor during signalling at the time of angiogenesis. The present study explores for the first time in India, a comprehensive role of *TGF-βR1* gene mutation and their frequency after DNA sequencing includes the substitution, deletion and insertion in WT cases. The bioinformatics tools (docking) were used to decode amino acids and to predict the 3-D structural and functional role of *TGF-βR1* and their interaction with methotrexate (MTX) after the molecular docking to ligand. Therefore, the present study become relevant to understand the mechanism of oncogenesis through gene-protein interaction causing dysregulation of signal transduction and in future enhance effective management of WT cases.

Materials and Methods

In the present study clinically diagnosed patient of WT and age matched controls referred to genetics laboratory of department of Pathology/Lab Medicine at All India Institute of Medical Sciences-Patna, India. Blood sample (1.0 ml) were collected (n=48) from cases of WT and controls, after written consent from the parent. The study was approved by Institute Ethical Committee. The median age group consist of 3.5 years and none of the proband have family history of cancer or exposure of radiation or drug previously.

Genomic DNA was isolated, quantified by spectrophotometer subjected to RT PCR analysis using syber green as florescence dye. Initially the protocol consisted of an denaturation step (94°C for 3 min) followed by amplification and quantification steps repeated for 40 cycles (94°C for 20 s, 56°C for 10 s, '72°C for 20 s with a single fluorescence measurement at the end of the elongation step at 72° curve analysed the data and reaction was terminated by cooling to 40°C. GAPDH gene was used as positive control of homeobox region.

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Melting curves were constructed by lowering the temperature to 65°C and later increasing the temperature by 0.2°C/s to 98°C to measuring the change fluorescence consistently. Tm values were assigned to develop plot generated by the RT-PCR of the negative derivation of fluorescence versus temperature (dF/dT) of the melting curve for amplification products measured at 530 nm *TGF ßR1* gene amplify with initial denaturation at 94°C for 4min, annealing at 56 °C followed by 35 cycles and final extension at 72°C for 10 min, using specific forward and reverse primers, 5'-TTTCGCCTTAGCGCCCACTG -3'5'- GAAGTTGGCATGGTAGCCCTT-3' respectively of 414 bp to evaluate the frequency of mutation, copy number variations (CNV) and genetic heterogeneity of *TGF-ßR1*.

DNA sequencing study was performed using Sangers method to find out nucleotide changes (new mutation) like substitution, deletion and insertion and compare the same with controls. Gene coded protein sequences searched in Biological database (https://www.ncbi.nlm.nih.gov/protein) and mutational aspect were obtained by searching Ensemble genome databases (http://www.ensembl.org/index.html). The prospective *TGF-ßR1* sequences were confirmed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification of functional significance of *TGF-ßR1* gene somatic mutations were extracted from the Ensemble genome databases as described previously [15,16]. Mutations of *TGF-ßR1* gene were obtained from the catalogue of somatic cancer database (http://www.cancer.sanger.ac.uk/cosmic) and protein structure 5E8S.pdb was obtained from structural database (https://www.rcsb.org/).

Identification of the binding site

Structure - based design begins with the identification of the target molecule to pocket with a variety of potential hydrogen bond donors and acceptors, hydrophobic characteristics, and molecular surface sizes. These are the active site for enzyme, act as assembly site for protein during binding to ligand which may vary for a disease state [17,18]. The ligand binding site predictions of a protein are based on relevant template library, selected for alignment of sequence and evaluation by Raptor X (http://raptorx.uchicago.edu/BindingSite/) [19]. MTX is an inhibitor of tetrahydrofolate dehydrogenase and prevents the formation of tetrahydrofolate, necessary for synthesis of thymidylate, an essential component of DNA synthesis MTX is entered into the S-phase of the cell-cycle affecting rapidly dividing cells of the growing foetus, germ cells, liver and bone marrow leads to inhibit DNA replication and finally cell-death (https://www.drugbank.ca/drugs/DB00563). Molecular docking is commonly used for predicting binding modes and energies of ligands to proteins. It help to determine accurate complex geometry and binding energy estimation during calculations of partial charges. AutoDock software was widely used docking programs help for the calculation of van der Waals and the electrostatic forces between protein and ligand [20,21].

Results

Figure 1A, showing mutation of *TGF-ßR1* frequency (18.18%), while, RT PCR revealed Ct mean value 23.63, S.D. 0.94 and C.I. at 95% 0.717- 1.280 and P=0.53 value showing lack of significant in WT cases with controls. Calculated mean Tm value was observed 90.70 which shifted to 91.0, showing significant differences (P=0.24) with S.D.4.28 and C.I. at 95% varying between 2.09-7.09 using student't'- test, GAPDH were used as positive control. CNVs also showing S.D.=0.37 and C.I. at 95% 0.337- 0.906 having significant difference (P=0.351) (Figures 1B-1E). Cytogenetic locus of *TGF-ßR1* gene assigned on chromosome-9q22.33 and DNA sequencing data showing changes in nucleotides as substitution, insertion and deletion (represented in red) as documented in Figures 2A and 2B. The detailed spectrum of *TGF-ßR1* nucleotide changes and their encoded corresponding amino acids after analysis of bioinformatics tools are depicted in Tables 1A and 1B.

The 3D-structure of *TGF-ßR1,* after changes of amino acid residues as compared with normal structure showing substitution (red) and insertion (green) as shown in Figure 2B. Non-polar hydrogen atoms were merged with rotatable bonds and the interaction of protein with ligand binding sites with polar and hydrophobic bonding is documented in Figures 3A and 3B. Free energy binding sites with their respective minimum interacting energy is shown in Table 2 obtained using docking calculations to maintain accuracy for ligand and protein that can be visualized with amino acid residues (ASP 351, LEU340, LEU 240, VAL 219 and ILE 211). The interaction between MTX and protein after calculation of root mean square deviation (RMSD) using docking calculations are shown in Figures 3C and 3D. HB plot presenting protein structure as shown in Figure 4 with new approach for description of the three-dimensional folding and flexibility of a protein. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets method [21]. Thus, it can be concluded that good geometry prediction help

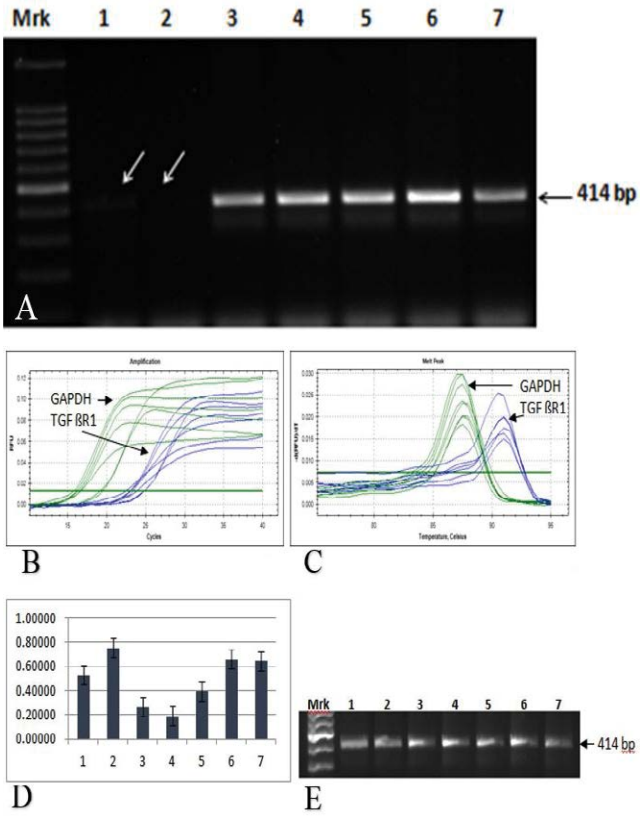

Figure 1: (A) PCR based analysis of TGF βR1 showing disappereance of 414 bp band in lane 1,2, using specific primers (forward/reverse) on 1.5% agrose gel after staining withethedium bromide and bands were visualized on Gel Doc system, **(B)** Ct value of the cases (blue) 25 cycle showing lack of significant differences with respect to control 23 cycle, **(C)** Melt peak analysis showing significant differences in cases of WT and changes in *Tm* value with respect to controls, **(D)** Copy number variations also showing significance difference, **(E)** intensity of bands further analysed on agarose gel showing different intensity correlated to CNVs.

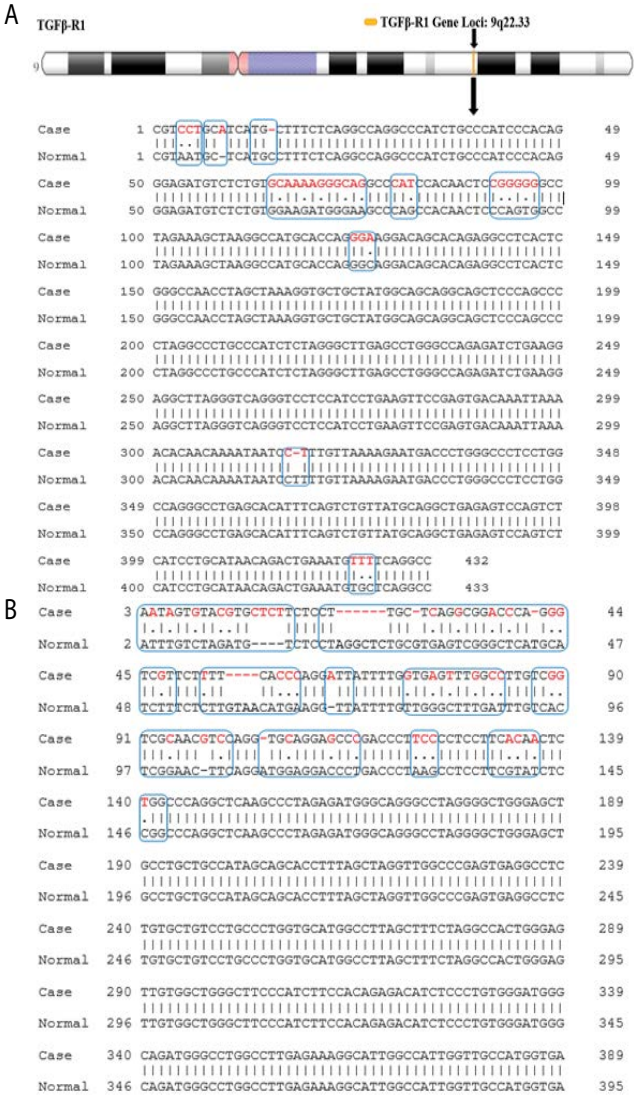

Figure 2: (A) Represent cytogenetic loci of TGF-□ R1 on Chromosome 9q22.33, **(B)** TGF-βR1 sequencing data represents in red colour substitution, insertion and deletion.

		(A)Substitution
S. No.	**Genetic Code (Normal→Case)**	**Amino Acid (Normal→Case)**
1.	AAT → CCT	Asn → Pro
2.	GGA → GCA	Gly → Ala
3.	AGA → AAA	Arg → Lys
4.	TGG → GGG	Trp → Gly
5.	GAA → CAG	Glu → Gln
6.	CAT → CAG	His → Gln
7.	CGG → CCA	Arg → Pro
8.	GGG → GTG	Gly → Val
9.	GGA → GGC	Gly → Gly
10.	TTT → TGC	Phe → Cys
11.	ATT → AAT	Ile → Asn
12.	TGT → AGT	Cys → Ser
13.	CTA → GTA	Leu → Val
14.	ATT → AAT	Ile → Asn
15.	CAT → CGT	His → Arg
16.	TGA → TCA	Terminator X → Ser
17.	GTC → GGC	Val → Gly
18.	GGG → GGA	Gly → Gly
19.	CTC → CCC	Leu → Pro
20.	CAT → GGT	His → Gly
21.	CTT → CGT	Leu → Arg
22.	CTT → TTT	Leu → Phe
23.	TGA → CCC	Terminator X → Pro
24.	TTG → GTG	Leu → Val
25.	GGC → AGT	Gly → Ser
26.	TTT → TTG	Phe → Leu
27.	GAT → GCC	Asp → Ala
28.	TCA → TCG	Ser → Ser
29.	CTC → GTC	Leu → Val
30.	GGA → GCA	Gly → Ala
31.	TTC → TCC	Phe → Ser
32.	GAG → CAG	Glu → Gln
33.	GAC → GAG	Asp → Glu
34.	CCT → CCC	Pro → Pro
35.	AAG → TCC	Lys → Ser
36.	TCG → TCA	Ser → Ser
37.	TAT → CAA	Tyr → Gln
38.	CGG → TGG	Arg → Trp

	(B) Deletion	
S. No.	**Genetic Code (Normal→Case)**	**Amino Acid (Normal→Case)**
1.	TGC → TG-	Cys → - - -
2.	CTT → C - T	Leu → - - -
3.	CTA → CT-	Leu → - - -
4.	GGC → - - -	Gly → - - -
5.	TCT → - -T	Ser → - - -
6.	GCG → GC-	Ala → - - -
7.	ATG → A-G	Met → - - -
8.	GTA → - - -	Val → - - -
9.	ACA → -CA	Thr → - - -
10.	ATG → -TG	Met → - - -
	Insertion	
1.	GCT → GCA	Ala → Ala
2.	G - - → GCT	- - - → Ala
3.	- - T → CTT	- - - → Leu
4.	- TT → ATT	- - - → Ile
5.	C - T → ACG	- - - → Thr

Table 1: (A) Detailed mutational spectra of TGFβR1 after DNA sequencing showing Substitution, **(B)** Deletion and Insertion with corresponding amino acids.

to contribute accurate binding energy estimation. Further, our results were compared to the experimentally designed complex structures of different ligand binding sites suggested that our predicted 3-D model of *TGF-βR1* derived the best energy based ligand binding sites [22].

Discussion

In this study, we have demonstrated that the *TGF-β* has been associated with development of WT. It is well known that *WT1* expression is essential for podocytes function, and *TGF-β* is able to repress its expression [23]. *TGF-β* superfamily consists of more than 60 secreted proteins that play critical roles in regulating diverse biological processes during embryonic development and in adults [24]. WTI protein has been designated as a transcription factor because it contains two discrete functional domains, one comprising the glutamine and proline rich amino terminal domain responsible for transcriptional

Rank	Est. Free Energy of Binding	Est. Inhibition constant, KI	vdW + Hbond + desolv Energy	Electrostatic Energy	Total Intermolec. Energy	Frequency	Interact Surface
1	-4.33 kcal/mol	674.44 uM	-6.48 kcal/mol	-0.1 kcal/mol	-6.59 kcal/mol	30%	1023.086
2	-3.91 kcal/mol	1.36 mM	-5.35 kcal/mol	-2.00 kcal/mol	-7.35 kcal/mol	30%	900.539
3	-0.68 kcal/mol	317.94 mM	-2.24 kcal/mol	-0.34 kcal/mol	-2.58 kcal/mol	10%	951.902
4	3.49 kcal/mol		1.28 kcal/mol	-0.93 kcal/mol	0.35 kcal/mol	10%	958.318
5	11.26 kcal/mol		4.73 kcal/mol	+0.41 kcal/mol	5.13 kcal/mol	10%	873.882
6	22.86 kcal/mol		21.34 kcal/mol	-0.94 kcal/mol	20.4 kcal/mol	10%	926.31

Table 2: Showing rank 1 most stable docking position on the basis of total estimated energies.

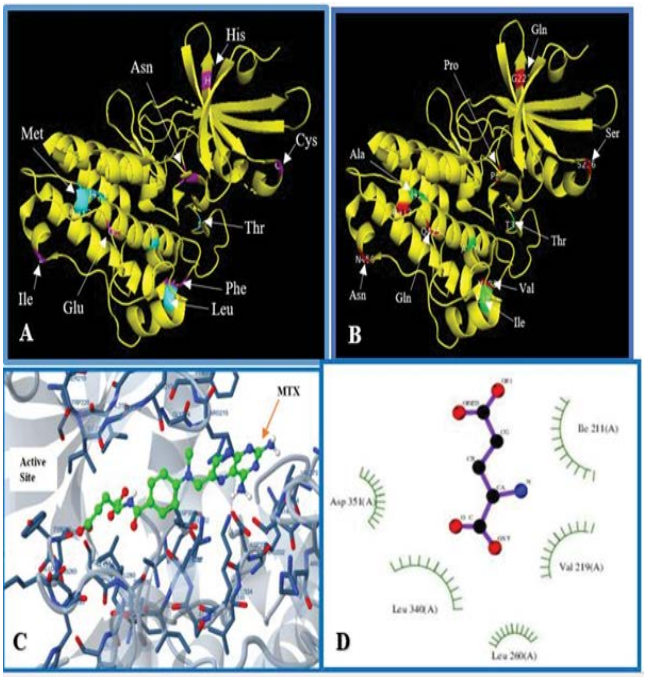

Figure 3: (A) 3D Helical normal structure showing active binding sites of TGF-βR1, **(B)** Mutated structure showing change in the amino acid residues sites represents in three different colours red, green and pink, **(C)** Interaction of amino acid with ligand in ball & stick model and **(D)** Interaction of amino acid residues with ligand binds with polar, hydrophobic, electrostatic force and VDW forces.

repression and four-zinc finger DNA binding domain. Mutations in *TGF-βR1* have been identified in cell lines and decreased *TGF-βR1* expression has been shown to decrease tumorigenicity [25,26]. In accordance, previous studies also demonstrated a significantly higher frequency of mutation of 6A allele in cancer patients as compared to controls, suggesting its role in genetic susceptibility [27]. This allele is located on the human *TGF-βR1* promoter, which is required for WTl-mediated repression of transcription. Mutation of several nucleotides in the WTl/Egr-1 response element that prevent or partially prevent WTl mediated transcriptional repression and mediating down regulation of *TGF-βR1* promoter activity by Egr-1 and play role in angiogenesis of tumor [28].

Previous findings suggested that the DNA binding domain of WTI is inactivated in tumours along with its transcriptional activities as a result, the target genes may be over expressed and the cell may lose control of normal cellular proliferation and differentiation [29,30]. WTl has been shown to be modified by alternative splicing and one alternative spliced product generates a protein with a 17-amino acid insertion N-terminal to the zinc finger domain; this protein retains its ability to repress the *TGF-βR1* promoter [31]. A second form of human WTl protein contains an insertion of three amino acids (Lys-Thr-Ser) between zinc fingers protein does not regulate the *TGF-βR1* promoter and probably binds to a sequence distinct from Egr-I /WTl [32].

Accumulating evidence has demonstrated that the *TGF-βR1* gene mutation plays vital role in progression of WT cases. Furthermore, such mutations might depress *TGF-βR1* activity, increased collagen production [33]. Hence, our findings are with the agreement of previous studies that WTl might play a significant role in regulating *TGF-βR1* expression during matrix production in WT cases. The bioinformatics tools were used for prediction of *TGF-βR1* 3D structures based on identified mutations help in providing knowledge of ligand binding sites in the gene coded amino acid residues (protein) with MTX as model anticancer drug [34]. Present study reveals the best interaction between protein and ligand with lowest binding energy. Interestingly, the mutation of *TGF-βR1* genes becomes relevant that how to reduce the mutagenicity either by changes in structural remodelling or decoding or supplementation of amino acids. Hence, the present study has widened the scope of developing new derivatives based on gene protein drug interaction for pharmacogenomics and personalized medicine for the management of the disease like Wilms' tumor.

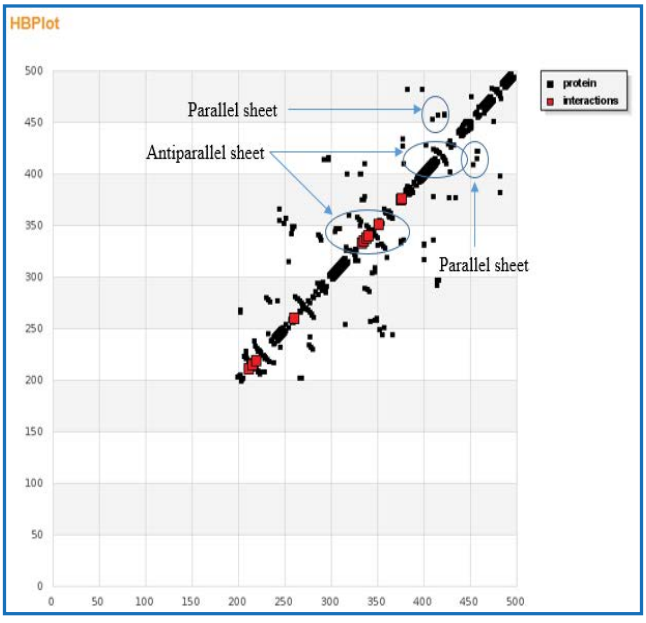

Figure 4: HB plot representation of a protein containing parallel and antiparallel beta sheets.

Conclusion

The present findings demonstrate a significant role of *TGF-βR1* gene mutations as major determinants in the WT cases, suggesting the significance of genetic alterations based on DNA sequencing increase high risk in cancer patients. Further, the realistic approach to provide better understanding of structural and functional genomics interactions (protein or drug) make the study more relevant. Hence this study has explored the scientific arena to develop derivatives of promising drug for disease like tumour, otherwise the study will be remaining incomplete.

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Conflict of Interest

All the authors have equally participated during preparation of the manuscript. There is no conflict of interest between the authors.

Authors Contributions

AKS, Aprajita is responsible for genetics analysis, VS, PS responsible for clinical diagnosis and management Mt, AK, CKS and RK is responsible structural analysis during preparation of the manuscript.

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