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Non-synonymous *de novo* gene mutations in Wilms' Tumor: Identification and characterization of new variants of WT1 and WT2 loci in Indian Population

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ABSTRACT. Wilms tumor (WT) is a complex pediatric disease involving both genetic and epigenetic factors. There is strong evidence that mutations at WT1 and WT2 loci are associated with the etiopathology of WT cases. In humans, large numbers of predisposition genes are distributed throughout the genome, and their functional aptitude has not been defined clearly in tumor biology. We examined new variants of WT1 and WT2 in WT cases in Eastern India by performing gene analysis using Sanger sequencing. The study was further extended to translate nucleotide variants into the corresponding amino acids to explore the mechanisms of tumorigenesis. Using bioinformatics tools, the WT1 locus showed single nucleotide substitutions in the sequence, i.e. TGT → CGT or ACC→GCC, resulting in changes in amino acids; i.e. arginine is replaced by cysteine or alanine by threonine, suggesting that these changes might either alter zinc finger DNA binding domains or be involved in the synthesis of altered proteins during tumor cell differentiation. Similarly, the WT2 locus showed non-synonymous *de-novo* gene mutations, CAC→TAA, TAT→TAA, TGA→ GGA, and these new variations correspond to either histidine or tyrosine or glycine, respectively, resulting in failure to

regulate transcription, suggesting that these nucleotide sequences can be considered as “stop codons” or pre termination codons. In conclusion, we identified new variants of WT1 and WT2 loci, leading to truncated proteins that may play a role in tumorigenesis in WT cases.

Key words: Wilms’ Tumor; Gene Mutation; Stop Codon; DNA sequencing

INTRODUCTION

Wilms tumor (WT) is a common embryonic tumor of pediatric age group (1 to 9 years old); the frequency is about 1 in 10,000 live births (Apoznanski et al. 2015; Hohenstein et al. 2015). The WT1 and WT2 loci are mapped on chromosome 11p13 and 11p15, respectively. More than 10% of cases of WT show loss of heterozygosity and copy number variations (Feinberg, 2005). The concept of gene mutation at WT1 locus has been pragmatic in WAGR syndrome (*Wilms -Aniridia - Genitourinary anomalies - mental Retardation*). Etiopathology of WT cases are complex in nature due to infrequently involvement of several loci of chromosomes1p, 2q, 7p, 9q, 14q, 11p15, 16q, 22q (Pelletier et al., 1991; Huff, 1998; Yuan et al., 2005; Ruteshouser et al., 2008). Interestingly, the majority of the cases (98%) are sporadic and only 1-2% cases of WT are familial in nature (Dome & Coppes, 2002). Earlier study has been documented that loss of genes of WT1 locus has been associated with rare syndrome like Li-Fraumeni (*TP53*), Fanconi anemia (*BRCA2*), CLOVES (*PIK3CA*) and Perlman (*DIS3L2*). Similarly, the genetic variability has been observed between 10 -20% in sporadic cases with 20 % case alone shows genetic heterozygosity (20%) in syndromic cases of WT (Wadey et al., 1990; Maw et al., 1992; Maher et al., 2008).

Maternal chromosome plays a crucial role in genomic imprinting and loss of heterozygosity containing two domains i.e., IGF21H19 and KIP21LITI during tumor progression (Feinberg, 2000). Recent study shows 2-3% cancer predisposition genes variants TRIM28, FBXW7, NYNRIN and KDM3B have close linkage to the truncated protein by Sanger sequencing in sporadic cases of WT (Yost et al., 2017; Mahamdallie et al., 2019). WT1 gene encodes a zinc-finger protein and plays a crucial role during gene expression (Call et al., 1990). Hence, the present study based on gene mutations becomes imperative to explore the mechanism of tumorigenesis in WT cases in eastern population of India. The findings of mutational spectra further extended to compare between syndromic and non-syndromic cases of WT based on DNA sequencing (Sanger’s). These nucleotide variants either gain (insertion) or loss (deletion) or replacement (substitution) regulate transcriptional and translational machinery during protein assembly play an important role in tumor cell biology. There is very well evident that WT1 acts as transcription factor to regulate eukaryotic gene expression either through transforming growth factor (TGF) or their transforming growth factor receptor (TGFR) to regulate cell-signaling in tumour cell. Interestingly, zinc proteins encoded by WT1, is rich in proline and glutamine contain carboxy terminus regions involving premature termination codons (PTCs) or stop codon due to assembly of truncated (Little et al., 1992; Gessler et al., 1994; Saxena et al., 2019).

Several genome wide association studies (GWAS) have been carried out in USA, UK and Chinese populations, but lacking in the Indian population (Treger et al., 2019). The rationale behind this study is to discover novel gene variants (nucleotide sequence) that become relevant to identify “hot-spots” on human genome to explore the role of stop codon in regulating transcription followed by translational events during protein synthesis-the one of the most important components of cell-kinetics in tumor biology. WTs are embryonic tumor and protein synthesis is a highly sensitive during organogenesis and there is lack of knowledge on fetal -

maternal interaction which might have possible to expose the knowledge of antenatal exposure with strong teratogen resulting abnormal development during organogenesis resulting origin of tumor in the proband of WT family.

However, these new variants could be use as "genetic biomarker" for clinical diagnosis during tumor progression in WT cases. Therefore, the present study has been designed with the aim to evaluate *de novo* gene mutations based on Sanger's sequencing. The study further extended to translates these nucleotide sequences or decodes into respective amino acid residues to correlate the functional activity of differentiating tumor cell. The study will also help to explore the knowledge of truncated protein and their association with zinc finger DNA binding domain during loss of heterozygosity in tumorigenic process. The present study explores new mechanistic approach on the basis of spectrum of new gene variants in WT cases, reporting first time in Indian population.

MATERIAL AND METHODS

Present study was carried out on clinically diagnosed cases (n=90) of Wilm's tumors with mean age (3.7 year) group. Family history of the proband was observed to construct pedigree analysis to evaluate the mode of inheritance and the nature of the disease (familial or sporadic). Blood samples (1.0 ml) were collected in sterile vial under sterile conditions for genome analysis after written consent from the legal guardian or parent and after approval from the Ethical Committee (IEC) of the Institute. The study was carried out in the molecular genetics laboratory of Department of Pathology/Lab Medicine, All India Institute of Medical Sciences, Patna.

Isolation of Genomic DNA

Genomic DNA was isolated by Promega kit (USA), quantitative analysis was carried out by nanodrop spectrophotometer and DNA samples were stored at -80^{0}C till further study. The RT PCR analysis was performed by using GoTaq DNA Polymerase, followed by agarose gel electrophoresis with the help of forward and reverse primers procured from Eurofines (USA). After confirmation of gene mutations of WT1 and WT2 locus, the PCR product (n=6) were used for DNA sequencing by Sanger's method to analyzed *de novo* non synonymous mutations. These sequencing data was further analyzed to evaluate different type of gene mutations i.e. either in the form of substitution, deletion and insertion and compare the same with controls. These sequencing data was based on mutation and further searched by ensemble genome database (http://www.ensembl.org/index.html), while gene coded protein by Biological database (https://www.ncbi.nlm.nih.gov/protein). These gene mutations of WT1 and WT2 locus were further confirmed by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared from the catalogue of somatic cancer database (http://www.cancer.sanger.ac.uk/cosmic) databases as described previously (Saxena et al 2019).

DNA sequencing analysis of the gene at WT1 and WT2 loci

Mutational analysis of the WT1 and WT2 locus were carried out using a set of forward & reverse primers after confirmation from NCBI (BLAST/http://blast.ncbi.nlm.nih. gov./Blast.cgi). Polymerase chain reaction reactions were carried out in ~50 ng DNA in 5X Green Go Taq buffer, 10 mM dNTP mix, 1µl each of 10 pM with specific used of primers f-

TGCTTTTGAAGAAACAGTTGTG and r- GGAAAGGCAATGGAATAGAGA for WT1 of 178bp and f- GGGCAGAGGCAGTGGAG and for WT2 r- GCATGTTTCGGGGGTG primers with 226 bp, and 0.2 µl of 5unit/µl of Taq DNA polymerase in final volume of 25 µl. The amplification was carried out in 35 cycles and each cycle consist of denaturation at 95^0C for 1 minute, with different annealing temperatures (55.0^0C,56.1^0C,57.0^0C,$58.^0$C) for WT1 genes locus and 51.1^0C for WT2 locus with extension at 72^oC for 1 minute, with an initial denaturation at 95^0C for 4 minutes, and final extension at 72^0C for 8 minutes as detailed by Saxena et al (2019).

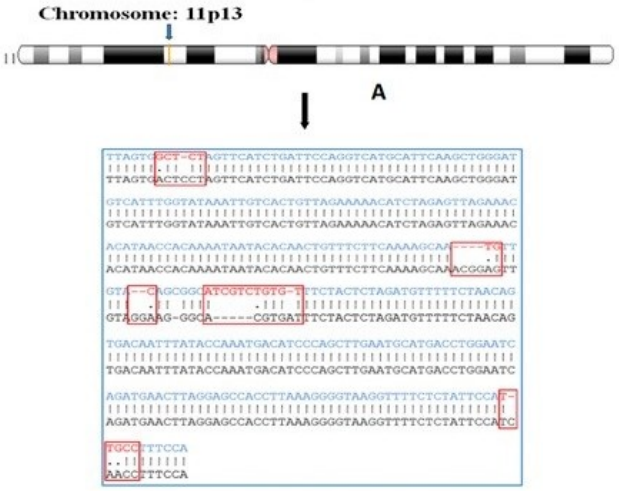
RESULTS

Analysis of DNA Sequencing by Sanger's method.

The genomic DNA was isolated from clinically diagnosed cases of WT using RT - PCR based analysis for the characterization of gene mutations of two different WT1 and WT2 locus. The PCR products from randomly selected cases of the WT (n=6) were further processed for Sanger's sequencing to characterize *de novo* mutations. These genomic data show spectrum of gene mutations based on involving either single nucleotide base or multiple nucleotide base pairs and classify three type of mutations - substitution (replacement), insertion (addition) and deletion (loss). Present data of the sequencing analysis, further confirmed by pair wise alignment through NCBI-BLAST and verify the translational event after decoding into corresponding amino acids with the help of bioinformatics tools.

Findings of DNA Sequencing Analysis of WT1 and WT2 Genes.

Figure-1A. shows cytogenetic locus of WT1 gene mapped on chromosome 11p13 and spectrum of gene mutations observed by DNA sequencing (Sanger's method) and further confirmed by NCBI-BLAST, which shows more than 91% homology during sequencing alignment between cases and their respective controls. There are more than 6.2% total gaps were observed during alignments of nucleotide as documented in figure - 1B. These sequencing data of the nucleotide was further characterized which showing the variety of constitutional mutations in the form of substitution, insertion and deletion as shown in figure-1C. The genes of WT1 locus show substitution of single nucleotide sequence (code) TGT→ CGT, ACC→GCC and GCT →ACT, followed by changes in the corresponding amino acids i.e., arginine is replaced by cysteine and alanine by threonine. During deletion of single nucleotide or more than one base pair of nucleotides change in WT cases also change series of amino acid such as glycine, aspartic acid, serine, proline, threonine, and glutamine when compared with controls (figure-1C). Further, the sequencing data also shows insertion of either single single or multiple nucleotides to penetrate in WT1 locus affecting interference of translational followed by translational activity. Such changes might lead to initiation of abnormal protein synthesis resulting in the origin of truncated protein due to changes in essential amino acid like isoleucine, valine and serine during tumour progression in WT cases.



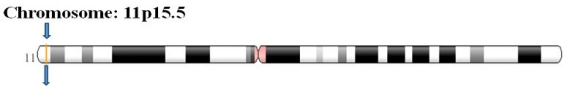
Substitution (Replaced)	
Genetic Code (Normal? Case)	Amino Acid (Normal? Case)
TGT ? CGT	Arg ? Cys
ACC ? GCC	Ala ? Thr
GCT ? ACT	Ala ? Thr
Insertion (Addition)	
- - - ? ATC	- - - ? Ile
- - - ? GTC	- - - ? Val
AG - ? AGC	- - - ? Ser
Deletion (Loss)	
GGA ? - - C	Gly ? - - -
GAT ? G - T	Asp ? - - -
TCA ? T T	Ser ? - - -
CCT ? -	Pro ? - - -
ACG ? - - -	Thr ? - - -
GAG ? - TG	Glu ? - - -

C

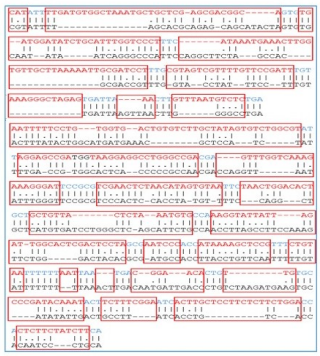
Figure 1. A, B and C. The location of WT I gene locus on chromosome 11p13 **(**1A**),** and DNA sequencing data with mutation (red box) as shown in 1B. Further analysis of coding data after sequencing of substitution, insertion, and deletion, as shown in black (text) represented as normal (control) and mutated sequences of WT1 cases are shown in red text (1C**).**

Figure -2 A, showing second locus of WT2 gene assigned on chromosome 11p15 with sequencing data of nucleotides (figure-2B) and translational event in to corresponding amino acids as documented in figure 2C. These spectra of genomic data show more than 95% homology during alignment and after confirmation by NCBI-BLAST, which reveals total frequency (29.6%) of gene mutations (nucleotide gaps) were observed as shown in figure -2B (box) between cases and controls. These genomic data of WT2 gene further characterized and showing substitution (replacement), insertion (addition) and deletion (loss). Bioinformatic tools help for decoding the translational event into corresponding amino acids as shown in figure - 2C. Interestingly, after comparative genomic analysis between WTI and WT2 locus genes, WT2

shows more new variants such as seven time more substitution of single nucleotide changes in corresponding base pairs and that consider as “stop codons”. These substitutions failed to translate in to corresponding amino acids and these three codons are CAC→TAA (Histidine → Stop), TAT→TAA (Tyrosine → Stop) and TGA→GGA (Stop → Glycine) in the cases of WT. Further analysis of the sequencing data after decoding were carried out which revels that serine is replaced by arginine, glycine, tyrosine, and phenylalanine due to single nucleotide substitution between cases of WT and their corresponding control. This genomic data shows that maximum number of mutations were observed in the form of “insertion” either alone (single) or in pair or in triplet form and again appear as “stop codon” (T-T→TAA), (---→TAA), (-GC→TGA) and (---→TAA). Similarly, this genomic data again repeats the loss of (deletion) of nucleotide either single or in pair leading to form sequences, which failed to translate in to corresponding amino acids leading to alter gene expression during protein synthesis and designated as “stop codon” (TAA→-AA) (stop→---) of these nucleotides as documented in figure-2C.



A



Substitution (Replaced)	
Genetic Code (Normal? Case)	Amino Acid (Normal? Case)
TGT ? CGT	Arg ? Cys
ACC ? GCC	Ala ? Thr
GCT ? ACT	Ala ? Thr
Insertion (Addition)	
- - - ? ATC	- - - ? Ile
- - - ? GTC	- - - ? Val
AG - ? AGC	- - - ? Ser
Deletion (Loss)	
GGA ? - - C	Gly ? - - -
GAT ? G - T	Asp ? - - -
TCA ? T - T	Ser ? - - -
CCT ? - CT	Pro ? - - -
ACG ? - - -	Thr ? - - -
GAG ? - TG	Glu ? - - -

B

Substitution (Replaced)	
Genetic Code (Normal? Case)	Amino Acid (Normal? Case)
CAC ? **TAA**	His ? **Stop**
TAT ? **TAA**	Tyr ? **Stop**
TGA ? **GGA**	**Stop** ? Gly
Insertion (Addition)	
T-T ? **TAA**	--- ? **Stop**
--- ? **TAA**	--- ? **Stop**
-GC ? **TGA**	--- ? **Stop**
--- ? **TAA**	--- ? **Stop**
Deletion (Loss)	
TAA ? -AA	**Stop** ? ---

C

Figure 2. A, B and C. Localization of WT2 gene locus on chromosome 11p15.5 (fig.2A), and data of DNA sequencing showing spectrum of gene mutations as in boxes represented with red (substitution, insertion, deletion), while blue and black text represent as cases of Wilms tumor and normal, respectively (.2B). Further analysis of coding data after sequencing of substitution, insertion, and deletion, where shown in black (text) represented as normal (control) and mutated sequences of WT1 cases are shown in red text (2C**).**

DISCUSSION

The basic concept of tumor biology is highly complex due to invariable rearrangements of chromosome aberrations (structural and numerical) and their percentage frequency during uncontrolled cell differentiation. These constitutional genetic variations occur either due to different pathological stages or loss of genetic heterozygosity (Mitelman et al., 2005). Interestingly, present study reveals the spectrum of *de novo* new gene variants at WT1 and WT2 locus in the cases of WT first time in Indian population and has not been reported earlier. These new variants of WT locus genes explore the mechanistic approach of penetrance of mutant nucleotide sequence makes the pathology more complex in syndromic cases of WT. This is either due to involvement of variants (substitution) lead to alters transcriptional event during RNA assembly or due to change in DNA binding to transacted zinc finger domain of WT1 locus gene. Since WT is embryonic tumor may changes in conserve amino acid sequences during protein synthesis at the time of gonadal - differentiation, and might be possible that these truncated proteins are responsible for tumour progression during the time of organogenesis (Huff, 1998). In the earlier study of Francke et al. (1979) and Huff (1998) shows 10-20% frequency of gene mutations at WT1 locus were observed in sporadic cases of WT, whereas, our findings based on DNA sequencing shows only 6.2% of nucleotide variations, almost three time less, suggesting either due to different pathological states or other geographical or environmental factors (Gessler et al., 1994).The present study of WT1 locus gene mutations become relevant because of association to germ line mutation and might be increase “risk factors” for penetrance of gene in proband in syndromic cases inducing genitourinary anomalies. However, more than 90% cases of WT belong to Denys-Drash syndrome - a rare developmental disorder related to gonadal dysgenesis due to missense mutation. Earlier study of the same author found two “new loci” assigned on chromosome - 6q21 and 16q23, which might have played a significant correlation either due to loss of heterozygosity or copy number variations in syndromic cases of WT (Saxena et al., 2020). The variation in the frequency of WT1 and WT2 gene may be due to tissue specific genetic heterogeneity (Oji et al., 2002; Yang et al., 2007).

Further, the sequencing data analysis helps to identify the spectrum of new constitutional nucleotide gene variant at WT1 and WT2 locus and designated as deletion, substitution and insertion. Interestingly, the sequencing data after analysis shows two important findings that firstly, the frequency of gene mutation of WT2 locus shows three-time higher frequency than WT1 locus gene. Secondly, the appearance or origin of pretermination codons (PTC) or stop codon at WT2 locus gene showing new findings that have not been reported earlier. Authors further extend the study with help of bioinformatics after translation of these nucleotide variants i.e. decode essential amino acids histidine, isoleucine, threonine and valine while non-essential are alanine, aspartic acid, serine perhaps significantly involved to alter binding capacity to DNA zinc finger domain result origin of truncated proteins. These are relevant findings might be associated with abnormal sexual development leading to ambiguous genitalia or cryptorchidism or hypospadias, suggesting increase “risk factor” for syndromic cases of WT. The sequencing data after decoding shows that essential amino acids such as arginine, glutamine and proline might have interfered either in metabolism or cell - singling pathway during stress in differentiating tumor cells. Hence, the present study becomes relevant and reporting first time on embryonic tumors after exhaustive exercise on genomic data based on sequencing analysis.

Therefore, the role of non - coding regions (stop codon) is not much known in the literature, but the author(s) try to make interesting in genome wise association study (GWAS), suggesting might have play a significant role during modification of epigenetic factor during

chromatin folding at locus WT1 and WT2 genes. Most of the WT1 locus gene mutations play a significant role in tumor by changing zinc finger DNA binding domain after replacement of the key amino acid histidine by tyrosine or proline by serine and destroy the relevant DNA binding domain (Gessler et al., 1994). Present study incorporates "new gene variants" of WT1 gene after substitution of nucleotide (code) TGT → CGT, ACC → GCC and GCT→ACT, and encodes three amino acid arginine is replace by cysteine, alanine by threonine (repeat). The authors suggests that this missense mutation might have interfere either to DNA binding protein or disturb translational machinery to synthesize alanine rich truncated WT1 protein during tumorigenesis. Similarly, unlike change of amino acids- arginine and alanine play a key role to modulate gene expression and unfolding of chromatin structure during synthesis of truncated protein in WT cases (Lieu et al., 2020). However, to date the functional role of WT1 gene variants outside zinc finger domain has not been documented clearly and present data based on DNA sequencing incorporate additional knowledge of either gain or loss of nucleotide (gene) and interference of the transcription followed by translational events after decoding of the essential amino acid influence mismatch DNA repair mechanism too. Moreover, WT1 is a transcription factor of 45-49 KDa protein which has four Cys-His zinc finger domain and associated mutations (deletion) are known to increase the risk of tumorigenesis (Haber et al., 1991; Drummond et al., 1994)

The sequencing analysis of WT2 gene shows (29.6%) close association to the earlier findings (Sheffield et al., 1995) and in the present study of variants of gene mutations translates in essential nonsynonymous amino acids i.e. leucine, glutamine, histidine during substitution and valine, leucine, glycine during insertion which might have modulates the cell signaling pathway at the time of tumor progression. Johnson et al 2014 suggests that locus of WT2 gene encodes histidine and play a major role in the onset of disease other than cancer. Previous study also suggests that such mutations, where single nucleotide substitution (A→T) translates amino acid residue serine into glycine leading to develop atypical protein structure and interfere during development of urogenital organ (Mahamdallie et al., 2019). These modifications might be associated with clinically diagnosed abnormal phenotypes including ambiguous genitalia or hypospadias or cryptorchidism, confirming the role of "stop codon" during translational event leads to play an important role (truncated protein) in syndromic cases of Wilms tumor. Interestingly, authors have reporting highly unformatting illustration first time in Indian population. Earlier, study of the Wilms tumor has shown that "stop codon" TGA → TCA and TGA → CCC failed to translate the functional activity of the amino acids serine and proline in transforming growth factor β receptor (TGFβR) followed by loss of binding capacity to drug (Saxena et al., 2019). However, the role of "stop codon" become more evident that play a significant role in modulation by copy number variations during assembly of encoded protein after point mutation or substitution of single nucleotide in homozygous conditions. Recently, our research group identified novel methylene tetra hydro folate reductase (MTHFR) gene variants in cases of WT in Indian population (Saxena et al., 2020). However, the exact role of these amino acids is still not clear in cancer metabolism and seems to modulates cell metabolism, cell-signaling, cellular-proliferation and protective role in therapeutic regime by altering mismatch DNA repair mechanism during tumorigenesis in the WT.

CONCLUSIONS

The frequencies of WTI and WT2 gene mutations are highly variable in nature and WT2 gene mutates three times more than WT1, perhaps due to tissue specific genetic susceptibility. Appearance of non-coding sequences designated as stop codons in WT2 might be

responsible for interference in a zinc finger DNA binding domain, resulting in a truncated protein due to substitution of serine by glycine or arginine or tyrosine or phenylalanine. These gene variants originate either in the maternal or paternal genome of the proband followed by an increased risk of developing Wilms tumor. Therefore, we cannot conclude how many mutations are still hidden in the human genome in Wilms tumor. Genetic screening is essential to understand the pathogenicity of Wilms tumor in different populations.

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AUTHOR CONTRIBUTIONS

Author (AKS) was involved in the designing and implementation of this study, and VK made the clinical diagnoses, MT was responsible for data analysis, while SK and UK helped with finalizing the results and writing the manuscript. All authors revised and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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