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Research Article

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Penetrance of WT1 and WT2 Gene Mutation and Loss of Heterozygosity in Wilms' tumors in Indian Population

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Annotation

Wilms tumor is a nephroblastoma of pediatric age group and heterogeneous in nature. WT1 and WT2 gene mutations are involved in onset of tumorigenesis in syndromic and non syndromic cases of Wilms tumor. Present study has been designed with aim to evaluate the frequency of WT1 and WT2 gene mutation, loss of heterozygosity (LOH) and DNA copy number variation (CNV) in clinically diagnosed cases of Wilms tumor using RT-PCR based analysis. Findings reveals that there was vast difference in the mutation frequency between WT1 (7.5%) and WT2 (17.5) gene, and statistical analysis shows significant difference ($p < 0.05$) in WT2 mutation and calculated value of C.I. varies between 0.886-1.990 at 95% with odd ratio (7.52). Interestingly, the frequency of LOH (loss of heterozygosity and DNA copy number variations (CNVs) shows significant difference ($p < 0.01$), suggesting increase of genetic susceptibility and penetrance of gene in proband of the family tumor resulting increase of risk factor after using three different microsatellite DNA markers (DS11S935, DS11S904 and DS11S1363). Genetic heterogenecity were also observed by calculating Tm values, between cases and controls (GAPDH), and C.I. varies between 0.292 - 2.270 at 95% with O.R = 0.54 in D11S1363 and maximum value of C.I. was 1.165-2.165 at 95% with O.R = 0.50 in D11S1905 and the $p = 0.194$ & $p = 0.522$ respectively, between cases and controls..

Keywords: Wilms Tumor, WT Gene Mutation, Loss of Heterozygosity, Copy Number Variation

Introduction

Wilms' tumor (WT), an embryonic renal tumour of children belong to different paediatric age group i.e. 1 to 9 years, and is accounting 1 in 10,000 live births [1,2]. Cytogenetically, WT1 and WT2 gene locus mapped on chromosome - 11p13 and 11p15 respectively, and 10% cases shows loss of heterozygosity (LOH) in Wilms tumor [3,4]. WT1 gene is also known as tumor suppressor gene and play an important role during urogenital development [5]. The short arm (p) of chromosome-11 invariably involved in loss of heterozygosity and reduction of copy number variation (CNVs).The concept of loss of heterozygosity (LOH) and WT1 gene mutation has been commonly involved in WAGR syndrome (*Wilms -Aniridia - Genitourinary anomalies - mental Retardation*) of Wilms tumor. The loss of heterozygosity were observed only in 20% non- syndromic cases of Wilms tumor [6,7]. The frequencies of familial cases are rare account only 1 - 2% and majority (98%) of cases are sporadic in nature [8,9]. It is still not clear that how constitutional WT1 gene mutation have been associated to either unilateral or bilateral disease with congenital anomalies in the family of Wilm's tumour [10]. The discrepancy in the frequency of WT1 gene mutation varies between 10 - 20% has been reported individually in sporadic cases of Wilms tumor [6,7]. Similarly, the second locus of WT2 gene is mapped on chromosome -11p15.5 - 11p15.4 and shows high frequency (20%) of genetic heterozygosity in syndromic cases of Beckwith –Wiedemann Syndrome of Wilm's tumor [11-13]. The complexity of predisposition of WT2 gene mutation including copy number variations (CNVs) failed to explain the

mode of inheritance during loss of maternal allele (~10Mb - 800kb) in heterozygous condition [14]. The loss of heterozygosity is also plays an important role in genomic imprinting containing two domains i.e. IGF21H19 and KIP21LITI during tumor progression [15]. However, these studies showing a lack of evidence of linkage between familial (heritable) and non-familial (sporadic) cases of Wilm's tumor, where the loci were lost during mutation (deletion) of 11p region of chromosome. In tumor biology, the DNA copy number variations (CNVs) are the important component of genetic susceptibility in spectrum of diseases and becomes an emerging field to explain either gain or loss of DNA (> 1kb) fragment in overlapping regions during constitutional gene mutations. The genetic diversity of CNVs is highly complex and together with single nucleotide polymorphism (SNP) increase "risk" in disease phenotypes and designated as "hot spot" regions distributed all over the human genome [16]. The informations regarding loss of heterozygosity (LOH) and copy number variation (CNVs) are scanty in nature in the family of Wilms tumors. WT1 gene encodes a zinc-finger protein and plays an important role in transcription and gene expression [17,18]. The functional domain of WT1 locus outside the zinc finger regions has failed to answer enough to investigate the relevance of this mutation. WT1 also act as transcription factor to regulate gene expression with stop codon in transforming growth factor receptor (TGFR) in Wilms tumor [19,20]. Wilms tumors are genetically heterogeneous and contradictory findings in the variation of frequency of WT1/WT2 gene mutation required further validation of gene variants in syndromic and compare to non- syndromic cases. The mechanism by which the loss of maternal allele occurs is still not clear in the cases of Wilms tumor. The several genome – wide association studies (GWAS) on genetic susceptibility and genomic imprinting has been documented in USA, UK and Chinese population but lacking in Indian Population in the cases of Wilms tumor [21]. Therefore, a comprehensive study has been designed with the aim - (1) to evaluate the (%) frequency of WT1/WT2 gene mutation, (2) secondly the loss of heterozygosity (LOH) using microsatellite DNA markers, and (3) to assess the DNA copy number variations (CNVs) to determine "risk factors" of the disease who carrying WT gene mutation. This study will help to explore the molecular mechanism of pathogenicity and penetrance of gene - flow in Wilms tumor in Indian population.

Materiels and Methods

Present study has been carried out in clinically diagnosed cases of (n=90) of Wilms tumor with age matched controls (mean age 3.7 year) were referred to Molecular Genetics Laboratory, Department of Pathology/Lab Medicine of All India Institute of Medical Sciences Patna, India for genetic analysis. Whole blood (W.B) samples (1.0 ml) were collected from the proband of Wilms tumor under sterile condition after written consent from the legal guardian or parent. Present study is approved by Institute Ethical Committee (IEC) of All India Institute of Medical Sciences Patna. Family history of the proband was recorded to know the mode of transmission the disease and exposure either with radiation or any drug or chemicals on prescribed performance.

Isolation of Genomic DNA and Mutational Analysis of WT1 and WT2 gene

Genomic DNA was isolated from the Promega kit (USA) and stored at -80°C till further study. The polymerase chain reaction (PCR) was performed by using kit (Promega Biotech India) and quantitative analysis of genomic DNA was carried out by Nanodrop spectrophotometer (Eppendrof, USA) before initiation of PCR and RT- PCR analysis. Mutational analysis of the WT 1 / WT 2 gene and loss of heterozygosity (LOH) were carried out using a set of specific forward and reverse primers obtained from Eurofines (USA), after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov./Blast.cgi). Polymerase chain reactions were carried out in ~50 ng DNA, 5X Green Go Taq buffer, 10 mM dNTP mix, 1µl each of 10 pM forward and reverse primer, and 0.2 µl of 5units/µL Go Taq DNA polymerase, with a final volume of 25 µl. The reaction mixture was amplified for 35 cycles, followed by each amplification cycle consist of denaturation at 95°C for 1 minute, and annealing temperatures of each candidate genes vary as documented in Table 1, and extension at 72°C for 1 minute, with an initial denaturation at 95°C for 4 minutes, and final extension at 72°C for 8 minutes. Syber green was used as fluorescence dye for RT PCR analysis. The post amplification, the amplicons were analysed on ultrapure 1.5% agarose gel and stained with ethidium bromide. The banding pattern were identified and visualization on Gel Doc XR+ (Bio red USA) system.These mutations of WT1 and WT2gene were obtained and compared from the catalogue of somatic cancer database (http://www.cancer.sanger.ac.uk/cosmic).

Table 1: List of the forward (F)/reverse (R) primers used for WT1 /WT2 gene mutation and loss of heterozygosity (LOH) during based RT PCR analysis

Sl.	Gene(s)	Forward/Reverse Primer (5'-3')	Program	bp
1	WT1A	F- GTGAGCCACACTGAGCCTTT R- GGCCGGTAAGTAGGAAGAGG	(94˚C, 56.1˚C, 72˚C) × 35 cycle	253bp
2	WT1B	F- GGCTTAAAGCCTCCCTTCCT R- TGAGAGCCTGGAAAAGGAGC	(94˚C, 58.2˚C, 72˚C) × 35 cycle	200bp
3	WT1C	F- CCAGGCTCAGGATCTCGTGT R- AAGGACCCAGACGCAGAGC	(94˚C, 58.2˚C, 72˚C) × 35 cycle	237bp
4	WT1D	F- TGCTTTTGAAGAAACAGTTGTG R- GGAAAGGCAATGGAATAGAGA	(94˚C, 55˚C, 72˚C) × 35 cycle	178bp

5	WT1E	F- CATTGTTAGGGCCGAGGCTA R-CTTTTCCAATCCCTCTCATCA	(94˚C, 57˚C, 72˚C) × 35 cycle	218bp
6	WTII	F – GGGCAGAGGCAGTGGAG R- GCATGTTTCGGGGGTG	(94˚C, 51.1˚C, 72˚C) × 35 cycle	226bp
Microsatellite DNA markers (primers) used for LOH analysis				
7	D11S935	F-TACTAACCAAAAGAGTTGGGG R- CTATCATTCAGAAAATGTTGGC	(94˚C, 47.2˚C, 72˚C) × 35 cycle	206bp
8	D11S904	F –ATGACAAGCAATCCTTGAGC R -CTGTGTTATATCCCTAAAGTGGT	(94˚C, 56˚C, 72˚C) × 35 cycle	199bp
9	D11S1363	F- GAAAATGGTATTTAGAAACCAA R- CCCAAGGGCTTACAAC	(94˚C, 47.2˚C, 72˚C) × 35 cycle	248bp

Results

PCR based Analysis of WT1 and WT2 Gene Mutation

Genomic DNA was isolated from clinically diagnosed samples from both syndromic and non- syndromic cases of Wilms tumor to determine the frequency of constitutional mutation of WT1 and WT2 gene. The standard procedure was apply to determine the amplicons analysis PCR and different sets of primers (forward and reverse) were used as details are documented in Table 1. Figure 1A showing the frequency (7.5%) of WT1 gene mutation in different set of primer f-TGCTTTTGAAGAAACAGTTGTG and r-GGAAAGGCAATGGAATAGAGA with loss of DNA fragment consist of 178bp, (arrow head) mapped on chromosome 11p13. The calculated value of C.I. at 95% varying from 0.0058 - 2.2827 with O.R value 0.11 showing lack of significance (p = 0.155) differences with respect to controls. Similarly, the frequency of WT2 gene mutations (17.5%) were observed after using specific f- GGGCAGAGGCAGTGGAG and r-GCATGTTTCGGGGGTG primers with loss of DNA fragment consist 226 bp as shown in Figure 1B (arrow). Statistical analysis showing significance difference ($p < 0.033$) with respect to controls and calculated value of C.I. at 95% vary from 0.886 - 1.9991 with odd ratio (7.52) as details data are documented in Table 2.

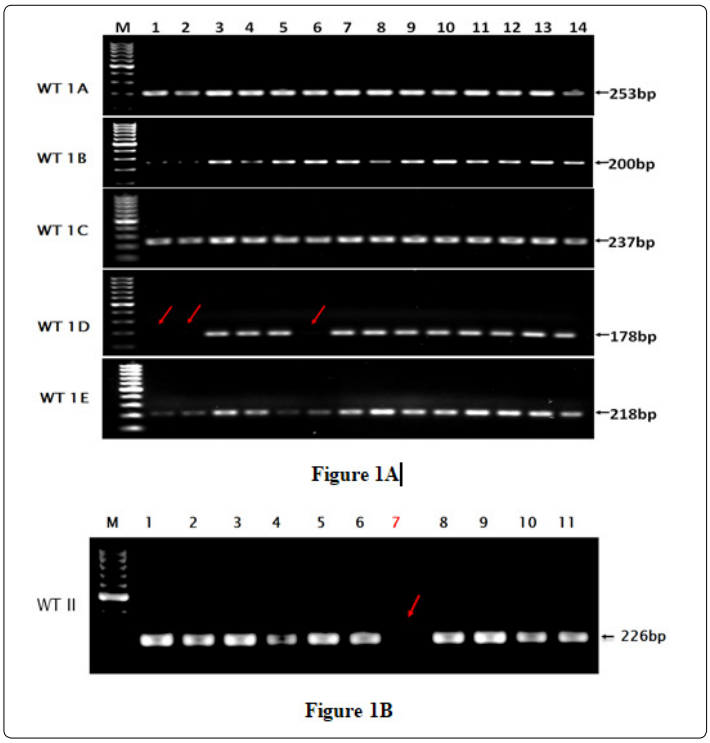

Figure 1: PCR based of analysis of WT1 gene mutations (→) using series of forward / reverse primers in cases of WT and control (Figure 1A), and WT2 gene mutation as shown in Figure 1B. Amplicons of different base pairs including 178bp for WT1 and 226bp for WT2 (arrow head) were analysed on 1.5 % agarose gel after staining with ethedium bromide and bands were visualized on Gel Doc system.

Table 2: Statistical analysis showing the frequency (%) of WT1 and WT2 gene mutation in the cases of Wilms tumors

Types/Genes	Total number and frequency (%) of mutation		Odd Ratio (O.R)	Confidence Interval (C.I.) at 95%		p- value
	Cases	Controls		Min	Max	
WT I	40 (7.5)	50(0)	0.1146	0.0058	2.2827	0.155
WT II	40 (17.5)	43 (2.32)	7.525	0.8861	1.9901	0.033*
*Significant ($p < 0.05$) difference were observed between cases and control						

Analysis of Loss of Heterozygosity (LOH) using RT-PCR.

The epicentre of LOH is mapped on chromosome between 11p13 & 11p15.5 and still unclear that how WT1/WT2 gene variants has been associated to the uncontrolled cell proliferation during syndromic cases of Wilms tumor. We have identify the specific and highly variable polymorphic loci of chromosome 11p15 , after using three set of microsatellite DNA markers (D11S935, D11S904 and D11S1363) for the study of loss of maternal allele (Table 1). The 35% cases of Wilms tumor shows loss of allele (D11S935) with significance difference ($P<0.05$) after calculation of C.I.

values varying at 95% from 1.433-7.824 and value of O.R was (27.32). Similarly, again showing the significance difference in the loss of allele frequency between 7.5% to 35.5%, after using D11S1363 (248bp) and D11S904 (199bp) microsatellite DNA markers with respect to controls as details are depicted in Table 3. However, the findings explore that D11S935 marker seems to be most "sensitive" for loss of heterozygosity during tumor progression in syndromic cases Wilms tumor. The data of genetic heterogenecity was further analysed to calculate Ct and *Tm* values, (Figure 2A and 2B). Showing lack of significant between cases and controls differences (Table 4).

Table 3: Statistical analysis showing the frequency (%) of loss of heterozygosity in the cases of Wilms'tumor using microsatellite DNA markers

Sl. No.	Markers	Total no. and frequency (%) of gene mutations		Odd Ratio (O.R.)	Confidence Interval (C.I.) at 95%		p-value
		Cases	Controls		Min	Max	
1	DS11S935	40 (35)	45 (0)	27.32	1.433	7.824	0.013*
2	DS11S904	40 (30)	45 (8.8)	1.55	1.003	8.175	0.015*
3	DS11S1363	40 (7.5)	50 (0)	3.45	0.0352	30.34	0.01*
*Significant ($p < 0.05$) difference were observed between Cases vs Controls							

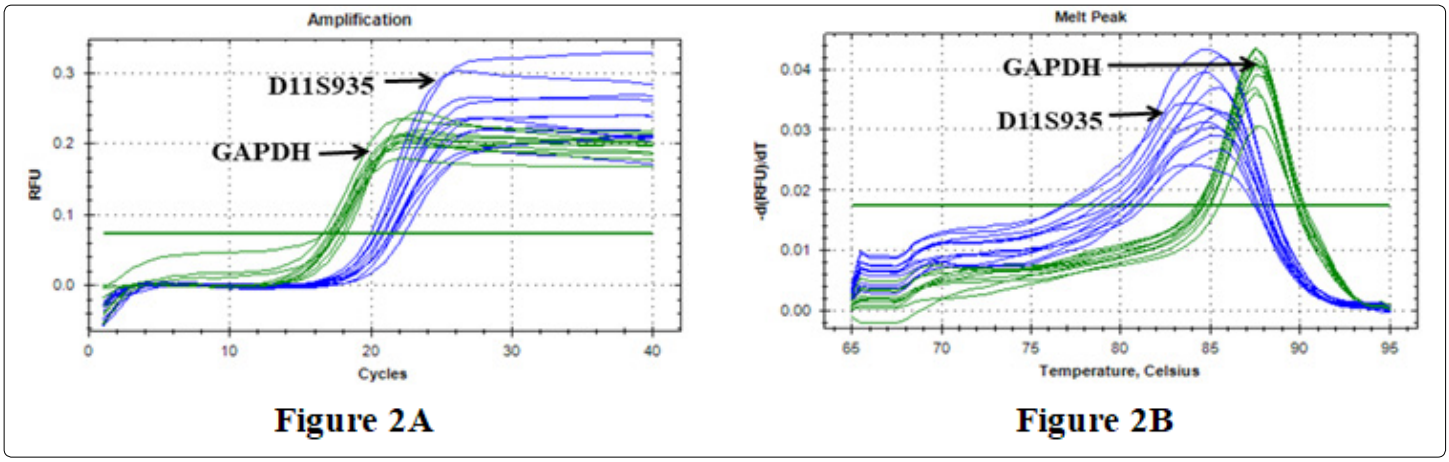

Figure 2A Figure 2B

Figure 2: Showing the Ct values after 25 cycles (Figure 2A) and melt peak (*Tm*) values using three different microsatellite DNA markers (D11S935, D11S904, D11S1363) in cases of Wilms tumor and compare with controls (GAPDH) (Figure 2B).

Table 4: Statistical analysis showing the mean value, C.I & Odd ratio, values of Ct and Tm during analysis of LOH in cases of Wilms tumors using microsatellite DNA markers.

Sl. No.	Marker	Mean ± SD		Odd Ratio (O.R)	Confidence interval (C.I) at 95 %		p values
		Case	Control		Min	Max	
1	D11S935	84.9 ± 0.62	84.66 ± 0.84	0.54	0.7241	1.2041	0.5947
2	D11S904	87.7 ± 1.24	88.0 ± 0	0.5	1.1655	2.1655	0.5224
3	D11S1363	83.0 ± 0.92	84.50 ± 0	0.54	0.2927	2.7073	0.194
*Significant ($p < 0.05$) difference were observed between Cases vs Controls							

Analysis of DNA Copy Number Variations

The short arm (p) of chromosome-11 invariably involved in tumor progression and mitotic recombination (non-disjunction) during cell - division resulting variations in DNA copy number (CNVs) in the family of Wilms tumor. Figure 3 showing a comprehensive analysis of data of CNVs between refractive fluorescence unit (rfu) and number of tumor cases, where, GAPDH was used as positive control for housekeeping gene. The significant trends of down regulation were appeared, after using three set of microsatellite DNA markers D11S935, D11S904 and D11S1363 belongs to different size of DNA. Interestingly, the statistical analysis were carried out which showing significant variation of CNVs in all the three sets of microsatellite DNA markers used. The highly significant ($p = 0.026$) variations were observed in D11S1363, after calculated value of C.I vary at 95% varying from 0.0464 - 0.01044 and value of odd ratio (0.256), suggesting highly sensitive maker for the study of genomic instability or fragility consider as hot spot in Wilms tumor (Table 5).

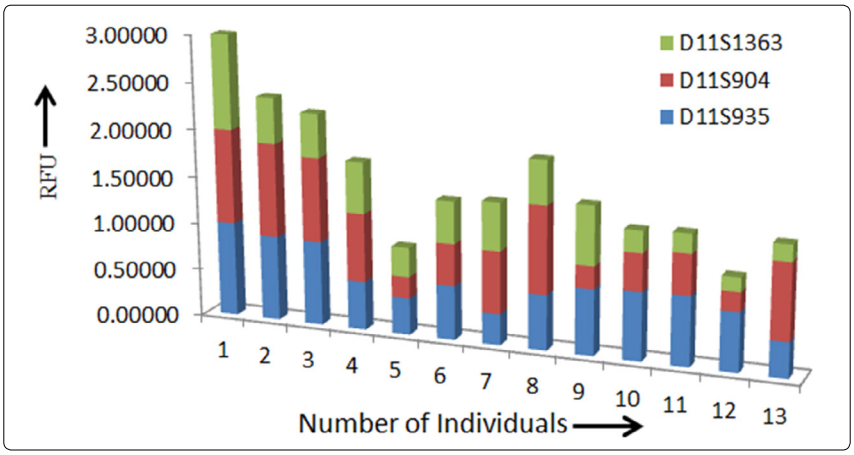

Figure 3: Bar diagram showing the significant differences after comparative analysis of the DNA copy number variations using three different microsatellite DNA markers (represented in different colors) between relative fluorescence unit (rfu) of Wilms tumor cases and GAPDH act as control.

Table 5: Statistical analysis showing DNA copy number variation between cases and controls

Sl. No.	DNA Marker	Mean ± SD		Odd Ratio (O.R)	Confidence interval (C.I) at 95 %		p values
		Case	Control		Min	Max	
1	D11S935	0.92 ± 0.05	0.55 ± 0.14	1.011	0.0821	5.0370	0.045*
2	D11S904	0.96 ± 0.04	0.50 ± 0.24	0.333	0.0231	2.8511	0.038*
3	D11S1363	0.64 ± 0.24	0.36 ± 0.16	0.256	0.0464	0.1044	0.026*
* Highly significance ($p < 0.05$) were observed between cases and controls after using students t-test significant							

Discussion

Chromosome variations are the characteristic feature of tumor biology. The variation in the frequency of gene mutations (WT1/ WT2) occurs either due to different pathological stages or unknown environmental factors [22]. Present study shows the unconstitutional variation in the frequency of WT1/ WT2 gene mutation and loss of heterozygosity makes the mechanism more complex during tumorigenesis of Wilms tumor. The involvement of new locus (chromosome -6 and 16) other than known regions of loss of alleles assigned on different chromosomes (1p, 2q, 7p, 9q, 14q, 11p15 & 22) 11p13 and 11p15 might have responsible to increase the frequency loss of heterozygosity and suggesting alterations during urogenital development in Wilm's tumors [7,23,24]. Earlier study of the chromosome rearrangement (specific break point) reveals loss of genomic DNA, which increase the genetic susceptibility known as "Hot Spots" help to understand the primary event of pathogenecity of cancer [25]. Gessler M et al [26] were observed 20% mutation of WT1 gene in the sporadic cases , whereas present study reveals the variation in the frequency of WT1 and WT2 gene varying between 7 - 17% either may be due to penetrance of gene in proband individually or variation in genetic susceptibility of proband in the family (heritable) of Wilms tumor. These constitutionally WT1 mutations encode essential (histidine ,Isoleucine, threonine and valine) and non essential (alanine, aspartic acid, serine) amino acids and might be alter either to DNA zinc finger domain or encode truncated proteins which have interfare during congenital developmental of congenital anomalies like ambiguous genitalia or cryptorchidism or hypospadias in syndromic cases of Wilms tumor. Present study reveals low frequency (7.5%) of WT1 gene mutation either due to lack of penetrance or genetic susceptibility towards environmental factors. More than 90% cases of Wilms tumor belongs to Denys-Drash syndrome - a rare developmental disorder related to gonadal dysgenesis due to missence mutation [10]. Although, the in present study reveals unexpected low frequency of WT1 gene mutation either due to loss of heterozygosity at 11p13 locus or inactivation of tumor suppressing gene variants. Earlier study of our group, showing two "new locus" assigned on chromosome - 6q21 and 16q23 might have lost the maternal alleles during non-disjunction and contribute significantly in participation to increase the frequency of WT2 gene mutation, LOH and copy number variations followed by increase of risk factors in Wilms tumor [27]. However, till to date the functional role of WT1 gene (tumor suppressor gene) variants outside zinc finger domain has not been documented with enough evidence to evaluate the significance in tumor biology, but present findings of mutational spectra must interfare to the mismatch DNA repair mechanism. Similarly, author also hypothesized that these mutations arises in the foetus where the mother exposed antenatally to the strong teratogen like cyclophosphamide during "critical period" at the time of sex differentiation leading to develop urogenital anomalies such as either ambiguous genitalia (hypospadias) or cryptorchidism [28]. The second locus 11p15 of WT2 gene mutation which is highly sensitive following significant variation in the frequency again support and validate the data of LOH and CNVs associated "risk factor" in syndromic cases other than sporadic of Wilms tumor during onset of tumorigenesis [29].

Conclusion

The present study has been concluded with non random variation in the mimicry of WT1 and WT2 gene mutation in Wilms tumor are as follows – (1) either due to heterogeneous cell population which significant increase frequency LOH of maternal allele by incorporation two additional loci 6q21 and 16q23, or (2) Significant variation of DNA Copy number variations determining "risk factor". These sequential changes occur either due to different environmental factors or penetrance of gene (s) in the proband of Wilms tumor. Still, we are unable to conclude that how many of mutations are still unknown or hidden in new locus of syndromic/ non- syndromic cases. Further, the study might have help to explain the genetic diversity to help the understanding of "gene flow" during management of syndromic cases of Wilms tumor. Lastly we suggest that genetic screening of all individuals in the family should further continue on the basis of DNA sequencing to find out new gene variants and to reduce the burden of "risk" of the disease in community.

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Authors Contribution

AKS, for manuscript preparation, VS, VK, PS help for Clinical Diagnosis and provide sample of Wilms tumor, while, MT, RK, CKS, associated to the technical assistance during research work.

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