Pretermination Codons of p53 Modulate Functional Activity of 3D Structure and Genetic Susceptibility in the Cases of Wilms' Tumour

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ABSTRACT

BACKGROUND
The tumor suppressor gene (p53) is also known as “Guardian of Cell” and play a central role in the variety of biological processes including cell-cycle arrest, DNA damage, chromatin modification and cell- death. Wilms' tumor (WT) falls under the category of embryonic tumor and their complexity arises due to interaction between the mother and the foetus. Etiopathology of WT is highly complex and multifactorial due to involvement of unconstitutional genetic factors followed by changes in transcriptional events resulting in alteration of DNA methylation process and appearance of truncated (mutated) proteins.

METHODS
Present study has been designed to evaluate the frequency of p53 gene mutation and their copy number variations (CNVs) to assess the de-novo mutations with help of RT PCR and Sanger’s DNA sequencing procedure in the cases of WTs. Secondly, superimposed 3D protein structure of p53 was predicted on the basis of nucleotide changes (gene mutations) with the help of bioinformatic tools to detect the functional activity.

RESULTS
Interesting finding reveals that there is loss of 279bp DNA fragments in 7.5% cases of WT and CNV p53 gene mutation. Statistically analysis was carried out between cases and controls shows significant (p <0.05) differences. After DNA sequencing large number of de novo mutations were observe. Remarkably, after decoding of these new gene variants of p53 where, the amino acids proline substituted into threonine. These findings were further confirmed the functional susceptibility by prediction of 3D superimposed structure of p53 gene between normal and abnormal (mutated).


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CONCLUSION

These de novo mutations spectra and super imposed prediction of 3D structure confirm that these new templates of DNA act as pretermination codons (PTC) which altered functional activity by changing DNA methylation or mismatch DNA repair mechanism of p53 gene during tumorigenesis. These variants may also designate as “hot spots” or genetic diagnostic or prognostic markers in WT cases.

KEYWORDS

p53 gene; Wilms’ tumour; RT-PCR; Sanger sequencing; Superimposed 3D structure; Pretermination codon

INTRODUCTION

Wilms’ tumor (WT) is aggressive renal tumor of children below three years of age that shows variety of both structural and numerical complex chromosomal rearrangements (CCR) [1-3]. The epidemiological study reveals the frequency of WT is 1 in 10,000 children below the age of six years and the tumor arise from the remnant of immature kidney. WT is also associated with specific congenital abnormalities, including genitourinary anomalies, sporadic aniridia, mental retardation, and hemihypertrophy. These congenital defects have been linked with specific genetic loci implicated during tumorigenesis [4,5].

The findings of the earlier studies shows that involvement of p53 gene mutation remain inconclusive during tumor progression in WT cases [6-10]. In vitro analysis shows p53 interact with WTI gene and modulates the functional activity of the protein to their respective target sites [11,12]. The association of these two factors also implicated the rare occurrence of WT in some Li-Fraumeni families who carry constitutional alterations of the p53 gene [13]. The p53 gene is located on the human short arm of chromosome-17, which encodes a 53 kD nuclear phosphoprotein and act as negative regulator cell signaling during proliferation and in tumorigenesis [14]. In addition, p53 also act as a transcription activator which suppresses abnormal cell proliferation at G1 phase of cell-cycle and protect the cell or DNA damage [15,16].

In human, the alterations of the p53 gene and its encoded protein are frequently encountered genetic events during every type of sporadic tumor progression [17-19].

In human genome, p53 gene regulates multiple biological process such as DNA damage response for such as, DNA damage-binding protein 2, cell-cycle arrest, glycolysis, apoptosis and also act as post-translation event during protein synthesis [13-16]. The present study has been designed with the aims to examine the frequency of p53 gene mutation and further identify new de novo nucleotide gene mutations like deletion or insertion or substitution of nucleotide. To test the functional susceptibility, the 3D superimposed mutated structure of p53 was develop and compare control with the help of bioinformatic tool. Only few research work has been studied regarding functional aspect in WT cases, where the role of p53 mutations has been associated with tumor progression. However, till date no clear association between 3D structural modification due to p53 gene mutation has been established [6-10]. Therefore, in the present study first identified the frequency of p53 gene mutation with the help of RT-PCR and Sangers DNA sequencing used to identify comprehensive de novo gene mutations. Secondly the 3D structure of p53 was constructed with the help of bioinformatic tool to confirm functional activity during tumorigenesis. However, such study helps to evaluate diagnostic and prognostic potential role of p53 gene.
mutations in WT cases deterring the role in clinical outcome.

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 (AIIMS-P), India was included in the study. Blood sample (1.0 ml) were collected (n = 40) from cases of WT and controls (n = 25), after written consent from the guardians of the cases and controls. The study was approved by Institute Ethical Committee of AIIMS-Patna. 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.

DNA extraction and PCR
Genomic DNA was isolated from the blood using Promega kit as per manufacturers protocol. p53 expression and mutational analysis was studied using forward and reverse primers in cases and controls. Specific forward and reverse primers selected for PCR reaction were as follows:

- Forward primer 5’-TGA AGT CTC ATG GAA GCC AGC-3’
- Reverse primer: 5’-GCT CTTT TTC ACC CAT CTA CAG-3’

Total reaction volume was 25 µl that included 50 mg - 100 ng of genomic DNA, 20 pmol of primer set, dNTPs (200 µM each)) along with Taq buffer (10 mM Tris HCl pH 8.3, 50 mM KCL), 3.0 mM MgCl2, and 3 units of Taq polymerase (Promega, USA) was prepared for Taq amplification. Amplification was carried out in a thermal cycler (Agilent Technologies). Cycling conditions includes- initial denaturation for 94°C, 3 min; 1 minute at 60°C for annealing, followed by 35 cycles, and final extension for 7 minutes at 72°C to ensure a complete extension of all PCR products.

DNA Sequencing for the Identification of p53 New Variants
Genomic DNA was isolated and purified before initiation for Sangers DNA Sequencing (ABL Pvt Ltd Pune, India). Alignment to the reference genomic database was obtained from National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/search/all/?term=p53) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Structural Modelling of Protein Structure
In homology modelling of 3D protein of a target sequence is generated by inducing experimental information from a closely related protein structure that serves as a template. SWISS-MODEL, is the best way to generate 3D structure of protein on the basis of target-template alignment search with help of Sequence Database (https://www.ncbi.nlm.nih.gov/protein/), Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) [20]. However, homology modelling done by using SWISS Model server (https://swissmodel.expasy.org/) where 6RZ3.pdb used as template to evaluate a homology model. SWISS-MODEL server uses GMQE (Global Model Quality Estimation) is a quality estimation which combines properties from the target-template alignment and the template structure and QMEAN is a composite estimator based on different geometrical properties and provides both global (i.e. for the entire structure) and local (i.e., per residue) absolute quality estimates on the basis of one single model. Visual molecular dynamic program has been used to design superimposed globular protein structure and to display molecular assemblies (biopolymers) of p53 protein. Visual molecular dynamic is simultaneously display variety of structures using different styles and colours methods [21,22].

Statistical Analysis
Chi square (χ²) two tailed test was applied to find out the level of significance differences (p = value) between the cases of Wilms tumor and their respective controls. The
odd ratio (O.R) and confidence interval (C.I) at 95% were also calculated to evaluate the minimum and maximum variations in the observed values of 'p' indicating the probability of significant difference was taken as <0.05 for comparison of the data.

RESULT
In the present study of WT cases (n = 40) along with respective controls (n = 25) were analyzed for p53 gene mutation/expression along with respective controls. DNA was isolated from blood samples and PCR was performed using p53 specific primers which was further analyzed on 1.5 % agarose gel for the expression of 279 bp bands as shown in Figure 1. The p53 gene was analyzed in terms of complete disappearance (null) of 279 bands, overexpression, or regression (under expression) in WT cases which were compared with controls. Our results indicated that 7.5% cases of WT showed null mutation in p53 (Figure 1). Further, CNV of p53 gene was assessed between cases and controls which showed a mean intensity in cases = 685.01 ± 43.3 and control = 259.02 ± 39.99; SE 38.0, with calculated value of odd ratio 0.12 and C.I. at 95% 0.014-1.047; showing significant (p <0.05) upregulation of the tumor suppressor gene.

![Figure 1: PCR based analysis of p53 gene mutations frequency in WT cases and controls.](image)

Further, sanger DNA sequencing was performed in p53 gene in WT cases and results are presented in table 1A - table 1C. Further, protein structure was constructed based on p53 DNA sequencing data after decoding to the corresponding amino acids. Most of the mutated sites corresponded to be the active part of introns regions leading to lack of functional activity. Interestingly, in the study population more than thirty-two (32) new gene mutations of p53 were observed based on Sanger’s sequencing analysis. The detailed data is documented in table 1A - table 1C that demonstrate various mutations such as substitution of nucleotide, seven insertions of nucleotides presented in table 1B, while twelve deletions of nucleotide are shown in table 1C. Present study was further extended to confirm the functional activity after construction of superimposed 3D structure based on the mutated spectra of p53 gene sequence and compared with normal p53 gene coded protein (globular) as showing in figure 2. Using bioinformatic tools to perform superimposition of p53 mutant protein structure with the normal shows major structural changes to overlap more than sixty percentage as shown in figure 2. The mutated protein structure is highlighted with the red colour and grey colour denotes the structure of normal p53 proteins, these results of superimposition further confirm our findings that normal structure of p53 protein is mutated and visualized in overlapped (superimpose) structure after gene (p53) mutation in the cases of Wilms’ tumour.

![Figure 2: Prediction of superimposed 3D structural analysis of p53 globular protein showing normal (grey) and mutated (red) form representing the coverage (overlap) of the structural homology (similarity) between two by using VMD technique.](image)
DISCUSSION

Wilms’ tumor is the most complex embryonic tumor of paediatric age group. Although, several genetic factors play a crucial role in the etiopathology of WT, still there is lack of identification of predisposition gene(s) or their complex interaction during embryological developmental events [23]. Genetic heterogeneity is commonly seen in the cases of Wilms’ tumor involving WT1 and WT2 genes assigned on chromosome-11 at two different 11p13 and 11p15 loci [23]. Tumour suppressor p53 gene and encoded protein have been reported to play a crucial role in a variety of human neoplasm, however, its role in Wilms’ tumor is inconclusive [17-19]. Earlier, different groups studied role of p53 mutation in Wilms’ tumor, including findings from Malkin et. al. that demonstrated p53 mutations in such cases [6]. The earlier study of Cheah et al. demonstrated the expression of p53 protein based on immunohistochemical in Asian population in WT cases and the findings were correlated with histological classification and accumulation of other than p53 protein in WT case for stabilization [7]. Further, Ooms et. al. found that stage III and IV in WT shows abnormally significantly higher expression with mortality rate is (61%) compared to the wild type (13%) of WT cases. These findings suggested p53 can act as a surrogate marker within the tumor and may determine the poor prognosis and management of the patient [9]. However, the role of p53 in WT cases still remains inconclusive due to lack of studies in different population with large sample size.

Recently, study from our group reported that the frequency (7.5%) of WTI gene mutation in WT cases [24] and present study is the extended part of the study to identify de-novo new gene variants based on nucleotide sequencing in the same cases. Our findings identified the frequency (7.5%) of null (complete absent of 279bp amplicon) mutation of p53 gene along with significant variations (p <0.5) of upregulation in copy number variations between WT cases and controls. Such findings suggests that these two genes

Table 1: A) p53 gene showing transcription and translational event after decode into respective amino acids during substitution, B) Insertion and C) loss of nucleotide (Deletion) into corresponding amino acid when compared with their mutated amino acid (red text).
(p53 and WT1) which are structurally different in nature but show functional similarity i.e., act as tumor suppressor and regulate various cellular processes such as cell - cycle and differentiation of cells, show similar frequency in mutation. Our study also supports the possibility of an association between p53 and WT1 gene as suggested earlier by another groups [11,12].

Further study was carried out to determine mutation in p53 using DNA Sanger sequencing. Although, PCR is a highly sensitive technique, there are limitations to detect mutations that are likely to exist outside of the protein coding region. Further, since there is lot of heterogeneity in tumor cell population, it further becomes relevant to identify the range of mutations associated with the p53 gene. Our study identified 32 different mutations associated with p53 gene in WT cases. Further, structural analysis predicted that these mutations effect the functional activity of p53 protein as compared to the normal structure. These results indicate that the target genes (p53) may either lose their functioning or may be over expressed thus resulting in disbalanced cell death (apoptosis), cell proliferation and differentiation. In our earlier studies, we identified variety of mutations in TGF-βR1 in Wilms’ tumor cases that modulates the DNA binding domain of WT1 (tumor suppressor gene) and as a result altered transcriptional events [25]. Such study indicates that there might be multiple genetic factors that play a crucial role in tumorigenesis of WT. Identification such novel factors is required to understand the etiopathology of Wilms’ tumor which is required for the management of such cases.

This is a novel study reporting first time the involvement of de novo p53 gene variants in WT cases in Eastern part of India. Present findings identify mutational spectra that may be involved in predisposition of “new variants” consider as hot spots that results in either loss or gain of function of p53 gene after DNA sequencing. Similarly, authors also hypothesized that these mutations arise during embryonic development, and it is quite possible that foetuses are exposed antenatally to the teratogen (s), that might lead to the onset congenital anomalies of urogenital system such as tumorigenesis (WT) with or without other anomalies such as ambiguous genitalia (hypospadias) or cryptorchidism [26].

**CONCLUSION**

In conclusion, present study indicates that these de-novo p53 gene variants could be used as potential prognostic “genetic biomarker” in WT cases. However further studies are required including larger sample size to make the data more conclusive. Although, first time the superimposition technique is used to confirm the gene -protein interaction after prediction of 3D structural analysis based on the variations in nucleotides of p53 gene consider as “hot spots” after obtains sequencing data for the validity of the findings in tumor biology.

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**CONFLICT OF INTEREST**

All the authors have agreed with the content of the manuscript. There is no conflict of interest between the authors.
REFERENCES


