Wilms’ tumour 1 gene mutations in south Indian children with steroid-resistant nephrotic syndrome

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Background & objectives: Clinically, nephrotic syndrome (NS) is a diverse group of symptoms; about 20 per cent of NS cases are resistant to steroid treatment, and within ten years they progress to end-stage renal disease. The present study was undertaken to identify the mutations of Wilms’ tumour 1 (WT1) gene in steroid-resistant NS (SRNS) children.

Methods: A total of 173 children with SRNS and 100 children in the control group were enrolled in the study. DNA extraction was done, screened for WT1 (exons 8 and 9) gene amplified by polymerase chain reaction and direct sequencing. Karyotype analyses were done for WT1 mutation cases.

Results: WT1 mutations were found in three of 173 SRNS cases (2 girls, 1 boy). All of them had intron 9 (IVS 9 + 4 C>T, 2; IVS + 5 G>A, 1) mutation. Of these three cases, one had familial and another two had sporadic history. Renal histology analysis showed two cases with focal segmental glomerulosclerosis (FSGS) and they had external female genitalia but 46,XY karyotype. Both of them had streak gonads. Of the three cases, one expired.

Interpretation & conclusions: The findings of the present study indicate that all females with SRNS-FSGS should be screened for WT1 gene mutation to diagnose whether they have FS for possible gonadectomy.

Key words Denys-Drash syndrome - focal segmental glomerulosclerosis - Frasier syndrome - Indian children - steroid-resistant nephrotic syndrome - Wilms’ tumour 1

Nephrotic syndrome (NS) is the most frequent renal-related syndrome in childhood. According to the clinical definitions, it requires the presence of oedema, massive proteinuria, hypoalbuminaemia and high cholesterol1. As per the treatment response, NS was categorized as steroid sensitive/remission, steroid responders/dependent (further this group divided into frequent and infrequent relapse) and finally, steroid non-responder/steroid resistant2. According to renal histological findings, NS is classified by minimal change nephrotic (MCN), focal segmental glomerulosclerosis (FSGS) and diffuse mesangial

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sclerosis (DMS). Many genes (NPHS1, NPHS2, WT1, LAMB2, CD2AP, α-ACT4 and PLCE1) have been identified and well documented in familial and sporadic NS cases. In India, the two available studies have focussed on NPHS2 gene only.

Wilms’ tumour (WT1) gene is originally a tumour suppressor gene; with a span of about 50 kb and consists of 10 exons which encode mRNA transcript of about 3.2 kb. In the hot spot sites (exons 5, 7, 8 and 9), small amount of mutant effect is sufficient to disrupt urogenital function; its immensity expression is linked to podocyte differentiation during nephrogenesis and gonadal development. Usually, 95 per cent of mutations within the WT1 gene causing steroid – resistant (SRNS) occur within the two exons 8 and 9. Frasier syndrome (FS) and Denys-Drash syndrome (DDS) are caused by the WT1 mutation. These disorders are characterised by NS, sex reversal, gonadoblastoma and WT. The WT, FS and DDS are successfully treated in majority (80%) of the cases. There has been no study on WT1 mutation from south India, thus the molecular mechanisms of WT1 gene and its link to SRNS are poorly understood. We therefore, undertook this study in a tertiary care hospital in south India to analyse the WT1 (exon 8 and 9) gene mutations in children with SRNS.

Material & Methods

This study was conducted in the department of Paediatric Nephrology, Institute of Child Health and Hospital for Children (ICH & HC), Madras Medical College between 2008 and 2012. Failure to respond to oral prednisolone of 2 mg/kg/day for four weeks followed by three doses of pulse methyl prednisolone was considered as steroid resistance. The children with SRNS were followed up for a minimum of six months before including in the study. All initial steroid-sensitive NS (SSNS) patients who subsequently turned to be SRNS (frequent relapse/late non-responders), chronic kidney disease (CKD) cases with a history of SRNS (aged 1-12 yr) were included in the study. Children with SSNS and history of positive HIV and HbsAg were excluded from the study. Children with minor illness (such as fever and diarrhoea) were taken as control, all of them aged between 3-12 years. Both cases and controls were selected randomly from the outpatient department. Ethical approval was obtained from the institutional ethical committee and written informed consents were obtained from the patient’s parents. Blood sample (5 ml) was collected; genomic DNA was isolated from blood leucocytes according to a standard salting out method.

The primer sets cover the WT1 exons (gene expressed region) and introns based on a previously published report, genomic DNA was amplified by polymerase chain reaction (PCR) using flanking specific sequences for confirmation of the genotype primer sets - forward 5'-CTT TTA ATG AGA TCC CCT TTT CC-3' and reverse 5'-GGG GAA ATG TGG GTG GTT TCC-3', forward 5'-CCTCAC TGT GCC CAC ATT GT-3' and reverse 5'-GCA CTA TTC CTT CTC TCA ACT GAG-3'. Amplification with the primer pairs resulted in 391 and 349 bp products. PCR reaction was performed in 25 µl reactions (0.5 µg genomic DNA, 200 pmol of each primer, 0.5 mM dNTPs, 3 mM MgCl₂, 1 unit of Taq DNA polymerase (Genet Bio, South Korea) and 10 PCR buffer (Genet Bio) with initial denaturation at 94°C for 10 min followed by 30 cycles of one min at 94°C, one min at 58°C (annealing) and two mins at 72°C (extension) in a thermal cycler (Eppendorf AG, Germany). After amplification, the PCR products were electrophoresed in 1.5 per cent agarose gels containing 0.5 µg/ml ethidium bromide. The gels were run in ×0.5 TBE (Tris-borate-EDTA) buffer for 40 min at 100 volt and visualized under ultraviolet transilluminator and documented in a gel documentation unit (Vilber Lourmat, France). The PCR product was purified in PureLink™ kit (Thermo Fisher Scientific, USA) and products were loaded in the sequencer (Genetic analyzer, 3500 from Applied Biosystems, USA).

Results & Discussion

A total of 173 children with SRNS were studied, including 107 boys (mean age 7.4 ± 3.76 yr) and 66 girls (6.7 ± 3.38 yr). Renal biopsy was done for 71 SRNS cases [MCN, 46 (31 boys, 15 girls); FSGS, 18 (11 boys, 7 girls); DMS, 7 (2 boys, 5 girls)]. One hundred (63 boys, 37 girls, age 6.19 ± 1.85 and 5.8 ± 1.77 yr, respectively) controls were also screened. Molecular genetic analysis confirmed that three patients (2 girls and 1 boy) had intron 9 mutation (Table, Figure) and no mutation was identified in control group. In mutation cases, the first girl had a history of familial NS with two previous (sisters) deaths; the other two had sporadic. Karyotype analysis was carried out for all the three cases. Two of them were phenotypically females, but genetically they were males (sex reversal). Their renal histology showed FSGS. Abdominal scans of these two patients showed normal uterus with streak gonads and considerable risk of gonadoblastoma. Gonadectomy was done for both cases, and tissue biopsy was reported as dysgerminoma stage I. The first patient underwent sex reversal, gonadoblastoma and WT.
a live donor (mother) renal transplant; and recovered with a stable graft function. The second was diagnosed as end-stage renal disease (ESRD) and underwent haemodialysis and finally died. The third case was a seven year old nephrotic boy; histopathology report was MCNs. He had normal genotype, and was on steroid treatment (infrequent relapses) with regular follow up.

Frasier et al\textsuperscript{16} initially reported a pair of 46,XY monozygotic twins with pure gonadal dysgenesis, gonadoblastoma and streak gonads. Most of the FS cases have proteinuria in childhood and progressively with age they develop ESRD. FS is caused by mutations in the intronic region of 8 and 9; it leads to loss of lysine, threonine and serine (KTS) isoforms. Drash et al\textsuperscript{17} described a child with male pseudohermaphroditism, WT and nephropathy in association with sex chromosomal abnormality. Most of the DDS cases have germ line, missense and heterozygous form mutations, and these occur in exons 7, 8 and 9\textsuperscript{18}. The classical presentation of DDS is a child with ambiguous genitalia, in exceptional cases they may be normal male or female\textsuperscript{19}. DDS usually has an early onset (first 3 years of life), but FS progresses slowly\textsuperscript{20} (puberty period).

Usually five nucleotides are altered in 9 intronic region these are +2T>C, +4 C>T, +5 G>A, +5 G>T, +6 T>A, most frequent being +4 C>T position\textsuperscript{7,10}. In our study two patients had +4 C>T nucleotide mutation. The onset was at five and six years, respectively. On the contrary, one Chinese study reported 4 C>T

| Table. Summary of Wilms’ tumour 1 mutation in steroid-resistant nephrotic syndrome cases |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Patient No.     | Sex             | Age at onset (yr) | Age at diagnosis (yr) | Karyotype     |
| 1.               | Female          | 5                | 9                | 46,XY FSGS    |
| 2.               | Female          | 6                | 8                | 46,XY FSGS    |
| 3.               | Male            | 2                | 7                | 46,XY MCN     |

<table>
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<th>Outcome</th>
<th>Biopsy (renal)</th>
<th>Biopsy (gonadal)</th>
<th>Syndrome</th>
<th>Tumour</th>
<th>Biopsy (gonadal)</th>
<th>Biopsy (renal)</th>
<th>Mutation</th>
</tr>
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<tbody>
<tr>
<td>Renal transplant, hormonal replacement therapy</td>
<td>Dysgerminoma stage I</td>
<td>Streak</td>
<td>Syndrome</td>
<td>Tumour</td>
<td>Biopsy (gonadal)</td>
<td>Biopsy (renal)</td>
<td>Mutation</td>
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<tr>
<td>ESRD, died</td>
<td>Dysgerminoma stage I</td>
<td>Streak</td>
<td>Syndrome</td>
<td>Tumour</td>
<td>Biopsy (gonadal)</td>
<td>Biopsy (renal)</td>
<td>Mutation</td>
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$\text{WT1}$, Wilms’ tumour 1; FSGS, focal segmental glomerulosclerosis; MCN, minimal change nephrotic; ESRD, end-stage renal disease

Figure. The chromatogram indicates heterozygous mutation in 9 exon/intron junction (arrow indicates Cytosine (C) to Thymine (T) conversion at position +4 intron region) was identified in the steroid-resistant nephrotic syndrome case.
mutation in a female child with streak gonad, who had ESRD occurring at 8 months\textsuperscript{21}, Fujita et al\textsuperscript{22} reported a three year old girl with SRNS and FS. Interestingly, one unusual FS case was reported with an IVS 9 + 4 C>T mutation, genotypically the case was a male and presented with diaphragmatic hernia, hypospadias and unilateral cryptorchidism\textsuperscript{33}. According to the reports, WT1 mutation mostly occurred in SRNS girls\textsuperscript{7,10-12,24}. Early (pre-pubertal period) diagnosis may help in the management of FS and to offer a reliable prognosis\textsuperscript{11,12,19,22}. Sinha et al\textsuperscript{13} reported SRNS-FSGS girls with 9.5 per cent mutation, it is higher than our study. In our study, only one female child had familial history, her first sibling had physical development delay, unable to sit, stand and walk without support; she died at the age of three years. The second sibling had NS with irregular follow up, she died at the age of 12. In this study, it was confirmed that the third girl had intronic mutation. Denamur et al\textsuperscript{14} reported a mother of a girl with NS having proteinuria since the age six, she had normal phenotype and normal genital development with pregnancy. Her child had DDS with 46,XY genotype, WT1 intronic mutation identified for both. In another report, siblings of two female patients had glomerular disease and developed CKD; both of them had intronic mutation\textsuperscript{26}. WT1 mutations occur rarely in boys, in our study, one boy had WT1 mutation; the same type also reported in an earlier study\textsuperscript{20}.

Genotype and phenotype correlations are not always possible in WT1 mutation cases\textsuperscript{27,28}. Previous reports\textsuperscript{29,30} and the present study indicate that WT1 gene alteration, and phenotype correlation between boys and girls are difficult to interpret. Early diagnosis of FS in girls helps trace the streak gonads from early gonadectomy. In conclusion, in our study, two of 66 girls and one of 107 boys with SRNS had WT1 mutation; the boy had no other morphological abnormality. The pathogenesis of WT1 mutations in the early-onset age in female NS children becomes imperative to be examined. In future, studies need to be performed on a large sample to confirm the findings.

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Conflicts of Interest: None.

References


