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RESEARCH ARTICLE

ADENINE-TO-CYTOSINE 637 SINGLE NUCLEOTIDE POLYMORPHISM OF NPHS2 EXON 8 IN NEPHROTIC SYNDROME.

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Abstract

Introduction: The aim of this study was to determine the frequency of the A>C polymorphism at site 637 of exon 8 of NPHS2 and to assess the association of this SNP with demographic, clinical and laboratory data.

Patients and methods: This cross-sectional study was conducted in Al-Imamein Al-Kadhimein Medical City and Al-Nahrain College of Medicine from the 1st of August, 2016, to the 30th of November, 2016.
Demographic data were collected from each patient, and some laboratory results were recorded.
From each patient, 3 ml of venous blood was collected for molecular analysis.

Results: A total of 50 children with NS were divided into 24 patients with SSNS and 26 patients with SRNS.
Genetic analysis detected the mutated allele in 50 (100%) of the cases.
The wild-type allele was detected in 3 (6%) cases: 2 (8.3%) cases of SSNS and 1 (3.8%) case of SRNS.
The homozygous mutated genotype was observed in 47 cases, distributed into 22 (91.7%) SSNS and 25 (96.2%) SRNS cases. The heterozygous mutated genotype was observed in 3 cases, distributed into 2 (8.3%) SSNS cases and 1 (3.8%) SRNS case.
Our results showed no association of this polymorphism with any of the demographic, clinical or laboratory data for either the homozygous or heterozygous patients.

Conclusion: SNP 637 A>C in NPHS2 exon 8 was present in all cases and both groups.

Introduction:-

Nephrotic syndrome (NS) is one of the most common syndromes and is characterized by heavy proteinuria. The majority of NS occurs in sporadic form. Seven genes have been identified to have mutations that are responsible for severe forms of NS: NPHS1, NPHS2, ACTN4, CD2AP, WT1, TRPC6, and LAMB2. The proteins encoded by these

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genes (nephrin, podocin, alpha-actinin-4, an adapter protein anchoring CD2 and others) influence the function of the podocytes**.(1)**

Mutations in the NPHS2 gene lead to autosomal recessive steroid-resistant nephrotic syndrome (SRNS) (histologically, Focal segmental glomerulosclerosis, FSGS). It was concluded that patients with SRNS with homozygous or compound heterozygous mutations in NPHS2 have a reduced risk for the recurrence of FSGS in renal transplant cases (only 8%, in comparison with 35% in patients without NPHS2 mutation**.(1, 2)** Positional cloning demonstrated that NPHS2, which encodes podocin, was mapped to 1q25–q31. It was determined to be a causative gene in autosomal recessive steroid-resistant nephrotic syndrome, including FSGS**.**[(3)]

Approximately 50 NPHS2 gene mutations and variants and/or nonsilent polymorphisms have been reported and recognized as potentially involved in the development of proteinuria.[(4)]

Exon 8 of the NPHS2 gene carries many polymorphisms. Some of them were reported in the literature to be associated with amino acid changes, while others were not.**(5)**

Aims Of The Study
To determine the frequency of the A>C polymorphism at site 637 of exon 8 of NPHS2 in children with NS and to study the association of this SNP with the clinical presentation of SRNS.

Patients and Methods:-

This cross-sectional study was conducted in Al-Imamein Al-Kadhimein Medical City and College of Medicine, Al-Nahrain University, from the 1st of August, 2016, to the 30th of November, 2016.

The study sample included 50 children with NS, who were recruited from the Pediatric Nephrology Clinic at Al-Imamein Al-Kadhimein Medical City, where they were diagnosed, treated, and followed.

Data on patient gender, age, age of onset of NS, steroid responsiveness, family history of NS, consanguinity, hypertension, hematuria and renal biopsy (if completed) were collected. The following laboratory investigations were performed for all children: urinalysis, plasma albumin, blood urea, and serum creatinine.

Nephrotic syndrome was defined as the presence of proteinuria >40 mg/h/m^2 or >50 mg/kg/day or a protein/creatinine ratio >0.2 g/mmol (>2 g/g) and hypoalbuminemia <25 g/l with or without edema.**(6,7)**

Patients were classified into steroid-sensitive or steroid-resistant NS groups. Steroid-responsive NS was regarded as complete remission achieved with steroid therapy. Steroid-resistant NS was regarded as a failure to achieve remission following a 4-week course of prednisone (60 mg/m^2) followed by three methylprednisolone pulses.**(6, 7)**
Exclusion criteria: Congenital nephrotic syndrome (onset before the age of one year) and NS due to well-identified secondary causative factors.

Sample collection:
Venous blood samples (3 ml) were collected from each patient in an EDTA-containing blood collection tube. Samples were transferred to the Molecular Pathology Laboratory of the Department of Pathology and Forensic Medicine, College of Medicine, Al-Nahrain University for molecular study.

Materials:-

Easy PrepTM Genomic DNA Extraction kit, Real-time PCR-TaqMan Master Mix, and primers and probes for the detection of the exon 8 polymorphic site were all purchased from Bioland Scientific. Their sequences were adopted from the NCBI and are listed in Table (1.1).

Table 1.1:-Primer and probe sequences.

Primer/probe	**Sequence (5'→3' direction)**
Forward	GGTGAAGCCTTCAGGGAATG
Reverse	TTCTATGGCAGGCCCCTTTA
VIC-probe exon 8	5'VIC(GACATGTTTATAATGGAGATGCC) 3'BHQ

FAM-probe exon 8	5'FAM(CATGTTTCTAATGGAGATAGATGC) 3'BHQ

Normal values for serum albumin (3.9–4.5 g/dl), serum creatinine in children (27-62 µmol/L), serum creatinine in adolescents (44–88 µmol/L), blood urea for 1- to 2-year-old children (1.8–5.4 mmol/l), and blood urea for > 2-year-old patients (2.9–7.1 mmol/l) were set.[8]

Molecular studies:

TaqMan Real-Time PCR Genotyping Assay. The TaqMan probes used for SNP allelic discrimination included differentially labeled fluorescent probes

Constituents of TaqMan assay mix:

1. Step 1: Preparing the qPCR master mix
2. Step 2: Setting up individual reactions
3. Step 3: Running the qPCR: The thermal profile of real-time PCR used in the reaction
4. Step 4: Analysis of the results was conducted according to the machine's software. This analysis included recording the positive and negative fluorescent detection of each of FAM and/or VIC probe. The CT value for each amplification was recorded with the amplification curves.

Statistical Analysis:-

Data are presented as frequency and percentage. The comparison between two study groups was done using Fisher's exact test and the Chi square test. A P value less than 0.05 was considered significant; GraphPad Prism 6 software was used.

Ethical approval:

1. Informed written consent was obtained from parents or the child's guardian.
2. The study was approved by The Iraqi Medical Board for Medical Specialization.

Results:-

A total of 50 children with NS were enrolled in this study. They included 34 males and 16 females, with a mean age of (8.49 ± 3.92 years) and an age range from 1 to 18 years.

The study group was divided into 24 patients (SSNS) and 26 patients (SRNS).

The steroid-sensitive group consisted of 15 males and 9 females, with ages ranging from 2 to 11 years and a mean age of 7.54 ± 3.05 years.

The steroid-resistant group consisted of 19 males and 7 females, with ages ranging from 2 to 17 years and a mean age of 9.36 ± 4.47 years.

Genetic study

1. The allelic distribution of wild and mutated type alleles of NPHS2 exon 8 SNP 637 A>C are shown in Table 1.
2. The mutated type (FAM-labeled) allele was detected in 50 (100%) of the cases. These were distributed into 24 (100%) cases of SSNS and 26 (100%) SRNS.
3. The wild-type (VIC-labeled) allele was detected in 3 (6%) of the cases: 2 (8.3%) cases of SSNS and 1 (3.8%) case of SRNS, with no significant difference (p value = 0.6104).
4. The distribution of genotypes of NPHS2 according to types of nephrotic syndrome is shown in Table 2. The homozygous mutated genotype (C/C) was detected in 47 cases: 22 (91.7%) SSNS cases and 25 (96.2%) SRNS cases.
5. The heterozygous mutated genotype (A/C) was detected in 3 cases: 2 (8.3%) SSNS cases and 1 (3.8%) SRNS case.
6. The homozygous wild-type genotype (A/A) was not found in any case.
7. The difference was statistically not significant (p value =0.6020).
8. The distributions of genotypes of NPHS2 according to demographic data are shown in Table 3. The mean age at the diagnosis was 5.12± 5.12 for homozygous patients and 4.33 ± 4.04 for heterozygous patients. No significant difference was found (p value = 0.770). Males were more numerous than females in both homozygous and

heterozygous groups, accounting for 32/15 and 2/1 patients, respectively, with no significant difference (p value= 0.763).

9. Consanguinity was found in 34 (72.3%) homozygous and 2 (66.6%) heterozygous patients (p value = 0.814).
10. A positive family history was detected in 7 (14.8%) of the homozygous group members and 0 (%) of the heterozygous group members, with no significant difference (p value =0.630).
11. The distributions of genotypes of NPHS2 according to clinical and laboratory data are shown in Table 3.
12. Hypertension was detected in 16 (34%) patients in the homozygous group and 1 (33.3%) patient in the heterozygous group, with no significant difference (P value =0.736).
13. Hematuria was found in 8 (17%) homozygous and 2 (66.6%) heterozygous patients, with no significant difference (P value =0.098).
14. Serum albumin was low in 39 (82.9%) of homozygous patients and 1 (33.3%) heterozygous patient, with no significant difference (P value =0.098).
15. The blood urea was high in 14 (29.7%) of the homozygous patients and normal in all heterozygous patients, with no significant difference (P value = 0.364).
16. The serum creatinine was high in 7 (14.8%) of the homozygous patients and was not elevated in any patient the heterozygous group, but this difference was not significant (P value = 0.630).

Discussion:-

1. In this study, males were predominant in both the SSNS and SRNS groups. This result was similar to that reported by Kumar *et al* in India, [9] Madani et al in Iran, [10] and Rachmadi in Indonesia.[11] Nephrotic syndrome is more common in males than in females, with a ratio of 2:1. [6]
2. The mean age for SSNS was 7.54 ± 3.05 years, while the mean age for SRNS was 9.36 ± 4.47 years. These ages were comparable to those reported by Gbadegesin et al.[12]
3. The NPHS2 exon 8 polymorphic site A637C was not previously reported to be associated with SRNS. To the best of our knowledge, this is the first study to have identified this SNP.
4. The reported A>C change in the nucleotide sequence results in an amino acid substitution of isoleucine to leucine in the polypeptide chain. This was shown to be present as a homozygous mutation in the majority of children with nephrotic syndrome included in the present study, whether with SRNS or SSNS. It was detected as only a heterozygous mutation in 3 cases. These findings suggest that damage to exon 8 of this gene in a single allele is pathological and is associated with the development of NS. On the other hand, this mutation does not appear to be directly related to the steroid-resistant type of the disease. The latter phenotype might be due to other polymorphisms or mutations in the same or another exon.[13]
5. Mao et al. reported a single nucleotide polymorphism of 954T>C in exon 8 in 5 patients and 4 controls. Another polymorphism in exon 8, 1038A>G, was observed in 7 patients and 4 controls. Neither polymorphism causes an amino acid substitution (A318A & L346L). There was no significant difference in the genotypic or allelic frequencies of the 954T>C or 1038A>G polymorphisms in the NPHS2 gene between the patients and controls.[14]
6. Weber et al. performed a linkage analysis study on 62 families suggestive of autosomal recessive SRNS, using markers flanking the NPHS2 gene locus on chromosome 1q25-31. They identified pathogenic NPHS2 mutations in 25 of them.[15]
7. Guaragna et al. reported the identification of NPHS2 mutations in only 14.8% of both sporadic and familial SRNS cases in the Brazilian patients analyzed after screening for mutations in the NPHS2, NPHS1, and WT1 genes.[16]
8. Tory et al. focused on the R229Q polymorphism in exon 5. They observed that p.Arg229Gln podocin presented subcellular mislocalization when coexpressed with podocins carrying amino acid substitutions encoded in exons 7–8, but not with substitutions encoded in exons 1–6.[17]
9. Di Duca et al. described a phenomenon in an autosomal recessive disorder in which the pathogenicity of one allele depended on that of the other allele; they subsequently proposed a non-Mendelian pattern of mutation-dependent recessive inheritance in NPHS2-associated SRNS.[5]
10. Rachmadi et al. reported the identification of 6 NPHS2 polymorphisms (52G>T, c.101A>G, g.-117C>T, c.288C>T, c.954C>T, and c.1038A>G) in patients with a clinical diagnosis of SRNS. Homozygous NPHS2 c.954C>T in exon 8 was found in 9 subjects, leading to p.Ala318Ala. The other NPHS2 polymorphisms were heterozygous. In these cases, the homozygous and heterozygous NPHS2 polymorphisms had no implications for the clinical manifestation of SRNS; no changes (e.g., a decrease in GFR or increased hypertension) were reported.[11]

11. As in the present work, none of the described research papers reported a significant statistical association between the studied polymorphic sites and the clinical findings.

12. Renal lesions due to NPHS2 mutations should be inherited following a recessive pattern that produces an evident phenotype only in either homozygous or compound heterozygous cases. Therefore, the present study also concludes that there is a possibility of having a second mutation in the regulatory or noncoding regions of another allele of the NPHS2 gene. The possibility of a second mutation involving another podocyte gene that interacts with podocin via a 'digenic disease' mechanism therefore cannot be excluded.[13]
13. In this study, the presence of a C allele is indicative of the occurrence of nephrotic syndrome as a whole, regardless of the steroid sensitivity pattern. The presence of a C allele is suggestive of defective mutated protein.

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Table 1:-Allelic distribution of wild and mutated type alleles of NPHS2 exon -8 SNP 637 A>C between the SSNS and SRNS groups

Allele	**SSNS No. (%)**	**SRNS No. (%)**	Total **No. (%)**	**P value**
FAM (mutated)	24 (100)	26 (100)	50 (100)	0.6104
VIC (wild)	2 (8.3)	1 (3.8)	3 (6)	

Table 2:-Distribution of genotypes between the SSNS and SRNS groups

Genotype	**SSNS No. (%)**	**SRNS No. (%)**	**P value**
Homozygous mutated C/C	22 (91.7)	25 (96.2)	0.6020
Heterozygous mutated A/C	2 (8.3)	1 (3.8)	
Homozygous genotype A/A	0 (0)	0 (0)	
Total	24 (100)	26 (100)	

Table 3:-Comparison between the homozygous and heterozygous patients according to their demographic, clinical and laboratory data

Parameter		**Homozygous C/C=47**	**Heterozygous A/C=3**	**P value**
Age at diagnosis	Mean	5.12	4.33	0.770
	SD	5.12	4.04	
Sex	Male	32	2	0.763
	Female	15	1	
Consanguinity	Positive	34 (72.3%)	2(66.6%)	0.814
	Negative	13	1	
Family history	Positive	7 (14.8%)	0 (0%)	0.630
	Negative	40	3	
Hypertension	positive	16 (34%)	1(33.3%)	0.736
	negative	31	2	
Hematuria	positive	8 (17%)	2 (66.6%)	0.098
	negative	39	1	
S. albumin	normal	8	2	0.098
	low	39 (82.9%)	1 (33.3%)	
Bl. Urea	normal	33	3	0.364
	high	14 (29.7%)	0 (0%)	
S. creatinine	normal	40	3	0.630

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