ejpmr, 2018,5(4), 146-152

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EFFECT OF IL6 C-174G POLYMORPHISM ON RESPONSE TO STEROID THERAPY IN EGYPTIAN CHILDREN WITH NEPHROTIC SYNDROME

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Article Received on 12/02/2018 **Article Revised on 04/03/2018** **Article Accepted on 24/03/2018**

ABSTRACT

Nephrotic syndrome (NS) is a common glomerular disorder in childhood. Interleukin-6 (IL-6) is a multifunctional cytokine its polymorphism is considered as an aggravating factor in the development and progression of renal disease. The aim of the current work was to investigate the serum level of IL6 and the association between IL 6 G-174C polymorphism and the response to steroid therapy in nephrotic syndrome. This study was conducted on 250 subjects classified into the following groups: Group I: included 100 healthy children, Group II: included 50 steroid sensitive nephrotic syndrome, Group III: included 100 steroid resistant nephrotic syndrome patients. Serum IL6 was estimated by ELISA technique. IL6 C-174G gene polymorphism was evaluated by PCR/RFLP method.

KEYWORDS: *Nephrotic Syndrome, IL6, gene polymorphism, Egyptian.*

1. INTRODUCTION

Nephrotic syndrome (NS) is a common glomerular disorder in childhood (Eddy and Symons, 2003). Idiopathic nephrotic syndrome (INS) is primary immune disease associated with immunoregulatory imbalance between T helper subtype 1 (Th1) and T helper subtype 2 (Th2) cytokines (Mathieson, 2002).

INS is characterized by the presence of proteinuria, which is accompanied with hypoalbuminemia, hyperlipidemia and odema (Safaei and Maleknejad, 2010). Patients with INS who not respond to glucocorticoids called steroid resistant (SR). Most of the children with INS get recovery following steroid treatment i.e., steroid sensitive (SS). Some children with steroid sensitive nephrotic syndrome (SSNS) will undergo relapse. 50% of the relapsers are either frequent relapsers or advanced to steroid dependence (Reidy K and Kaskel, 2007).

Most cases of NS in children are of unknown cause and the little is secondary to systemic diseases. Most of INS have minimal change NS (MCNS) (Gbadegesin and Smoyer, 2008).

In 50%-60% of children with steroid-resistant nephrotic syndrome (SRNS) the cause is unknown, whereas the remaining patients have a single genetic defect that affects glomerular podocyte. Most patients with SRNS not response to immunosuppressive treatment (Kim et al., 2005).

Interleukin-6 (IL-6) is a multifunctional cytokine, it has many functions including activation and differentiation of macrophages, B and T cells. IL-6 is produced by different cell types and it has pro-inflammatory, anti-inflammatory and immune suppressive properties (Müller-Steinhardt et al., 2002).

IL6 gene is located at chromosome 7p21 (Sehgal et al., 1986) and consists of 5 exons and 4 introns (Zilberstein et al., 1986).

IL-6 polymorphism is considered as an aggravating factor in the development and progression of renal disease (Aker et al., 2009). A biallelic polymorphism in the promoter of IL-6 gene involving a substitution of guanine by cytosine at position –174 (G-174C polymorphism) has been associated with altered in production of IL-6 (Fishman et al., 1998). Functional SNPs within the promoter area of these cytokine genes affect the activities of gene promoter and levels of gene product (Tindall et al., 2010).

The aim of the current work was to investigate the serum level of IL6 and the association between IL6 G-174C polymorphism and the response to steroid therapy in nephrotic syndrome.

2. PATIENTS AND METHODS

2.1. Ethics statements

All patients provided written informed consent and approval of the local ethics committee of Mansoura University was also received with reference code R/17.03.30.

2.2. Patients

This study was conducted on 250 subjects classified into the following groups: Group I: included 100 healthy children, 45 males and 55 females (mean age 8.69 ±3.952 years). Group II: included 50 steroid sensitive nephrotic syndrome, 21 males and 29 females (mean age 7.86±3.922 years). Group III: included 100 steroid resistant nephrotic syndrome patients, 45 males and 55 females (mean age 8.91±4.085 years).

All subjects included in the study will be recruited from Mansoura Univeristy Childern Hospital, from the period of March 2015 to October 2016.

2.3. Methods

Urine sample was collected to determine total protein (Orsonneau et al., 1989) [Spinreact S.A. 7E-17176 Spain.] and creatinine concentration and protein creatinine ratio was calculated.

Five ml venous blood was collected after an overnight fast. Two ml were transported into sterile vacutainer containing EDTA solution (50 µl) for DNA extraction. Three ml were transported into plain vacutainer tubes and serum was separated for creatinine, cholesterol, albumin and serum IL6 assessment. Serum creatinine, cholesterol, albumin and urine creatinine were assessed using [Cobas Integra - 400 plus, Roche Diagnostics. Switzerland].

2.3.1. Measurement of serum IL6

Serum IL6 was estimated by ELISA technique using human Interleukin-6 ELISA kit, Sun Red, catalogue No.: 201-12-009. The kit uses a double antibody sandwich enzyme linked immunosorbent assay to assay the level of human IL-6. This kits has sensitivity 2.11 ng/L.

2.3.2. DNA extraction

DNA was extracted from whole blood by (Gene JET Whole Blood DNA Purification Mini Kits) from Thermo Scientific.

2.3.3. Polymerase chain reaction (PCR)

A pair of primers were used for amplification of the region containing G174C gene polymorphisms of IL6 forward primer: 5'-GGAGTCACACACTCCACCT-3' and reverse primer 5'-GTGGGGCTGATTGGAAACC-3 (Tripathi et al., 2008).

PCR amplification was done using Tiangen 2×Taq PCR Master Mix (Cat. no. KT201, TIANGEN BIOTECH (BEIJING) CO., LTD).

The reaction mixture of total volume (25 µl) contained 12.5 µl Taq PCR Master Mix, 0.1 µl (100 pmol) forward primer, 0.1 µl (100 pmol) reverse primer, 1.0 µl (100 ng) template DNA and 11.3 µl water (nuclease-free). Initial denaturation at 94°C for 3 min followed by 35 cycles each consisting of 30 s denaturation at 95 ℃, 1 min annealing at 64℃, 1 min of extension at 72˚C and a final 10 min extension at 72˚C.

2.3.4. Restriction fragment length polymorphism (RFLP)

The PCR product was then digested with (New England Biolabs SfaN1 restriction enzyme). The reaction mixture of total volume (25 µl) contained 17 µl nuclease-free water, 2 µl 10X Buffer, 5 µl PCR product and 1 µl Digest enzyme. The components were mixed and incubated at 37˚C for 60 min. The restriction products were separated by electrophoresis in 3.5% agarose gels with ethidium bromide and were visualized in UV transilluminator.

Two band at (474 and 58 bp) identified homozygous for the wild type GG, one band at (532 bp) identified CC homozygous and three bands at (532, 474 and 58 bp) indicate a heterozygote GC.

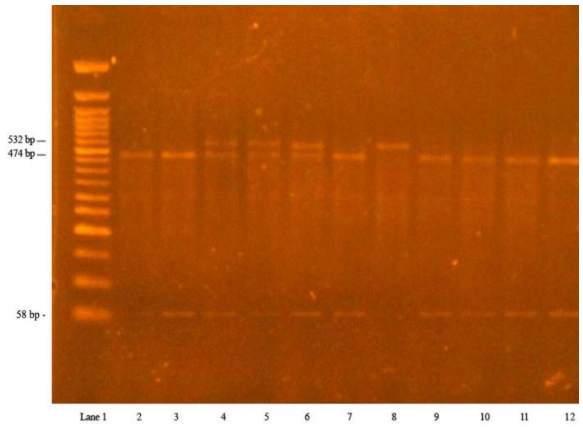

Figure 1: Shows IL6 G-174C polymorphism using Fast Digest restriction enzyme.
Lane 1= gene ruler.
Lanes 2,3,7,9 10, 11 and 12 = GG genotype (474 and 58 bp).
Lanes 8= CC genotype (532 bp).
Lanes 4, 5 and 6 = GC genotype (532, 474 and 58 bp).

2.4. Statistical analysis

Data were analyzed using SPSS version 20 (SPSS, Inc., Chicago, IL). Chi square and Fisher exact tests were used to compare groups. Variables were described as mean (standard deviation) or median (minimum, maximum). For comparison between two groups; student t-test and Mann-whitney test were used. For comparison between more than 2 groups; ANOVA and Kruskal Wallis tests

were used. The distribution of alleles and genotypes in the studied groups was examined for appropriate to the Hardy-Weinberg equilibrium. The associations between nephrotic syndrome and C-174G polymorphism were estimated by (ORs and their 95% CIs). Logistic regression was used for prediction of nephrotic syndrome with healthy control. Correlation coefficient was used for correlating between parameters. P <0.05 was considered significant.

3. RESULT

Mean age of NS patients was 8.6 years. They were 66 males (44%) and 84 females (56%). No significant differences in age between SSNS and SRNS groups and between them and healthy control group. Creatinine, cholesterol, protein creatinine ratio and serum IL6 showed significantly higher, while albumin showed significantly lower concentrations in NS cases in comparison to control group (*P*< 0.001 for all). Cholesterol and IL6 showed significantly higher (*P*< 0.001), while albumin showed significantly lower concentrations (*P*=0.045) in SRNS when compared to SSNS subgroups Table 1. No significant differences were found in serum IL6 according to IL6 genotypes in SSNS and SRNS group (data not shown).

IL6 GC, CC, GC+CC genotypes and C allele showed significant association with risk of NS development within healthy control subjects even after adjustment with age and sex (*P*< 0.001, =0.008, <0.001 &< 0.001 respectively) Table 2.

IL6 CC, GC+CC genotypes, C allele showed significant association with risk of SRNS development within NS even after adjustment with age and sex (*P*= 0.022, =0.045 &=0.022 respectively) Table 3.

ROC curves of IL6 concentration were conducted for discrimination between NS and healthy control subjects and discrimination between SSNS and SRNS patients. The area under curve (AUC) was (0.87 & 0.76 respectively). By using a cut off value (88.6 ng/L) between NS and healthy control subjects the sensitivity was 78% and specificity was 100% (Figure 2). As regard to SSNS and SRNS patients cut off value was (86.4 ng/L) and the sensitivity and specificity were (79% and 60%) (Figure 3).

Logistic regression analysis was conducted for prediction of NS within healthy subjects; applying serum IL6 concentrations and IL6 genotypes as covariates. IL6 concentration and IL6 GC+CC were associated with risk of NS development in uni and multivariate analysis (*P*< 0.001) Table 4. Regression analysis for prediction of SRNS within NS patients show that IL6 (GC+CC vs GG) was only associated with SRNS development within NS patients (*P*= 0.045) data not shown.

IL6 concentration showed significant positive correlations with cholesterol, protein creatinine ratio and urinary protein in SSNS subgroup (*P*=0.006, 0.001 & 0.0.01 respectively); creatinine, protein creatinine ratio and urinary protein in SRNS group (*P*< 0.001 for all); significant negative correlation with albumin in SSNS (*P*= 0.002) and SRNS subgroups (*P*< 0.001) Table 5.

Table 1: Comparison age and laboratory data between studied groups.

`		***Healthy control N=100***	***NS N=150***			***P*[1]**	***P*[2]**
			Total NS N=150	***SSNS N=50***	***SRNS N=100***		
Age (years)	Mean	8.7	8.6	7.9	8.9	0.337	0.130
	SD	2.9	2.4	2.9	2.1		
Creatinine (mg/dL)	Median	0.6	0.8	0.8	0.8	<0.001	0.954
	range	0.2-0.9	0.3-1.7	0.3-1.5	0.3-1.7		
Cholesterol (mg/dL)	Median	134.5	279.5	239	324.5	<0.001	<0.001
	range	100-183	132-708	132-400	170-708		
Albumin (g/dL)	Median	3.8	1.9	2.1	1.9	<0.001	0.045
	range	3.2-4.8	1.2-4.1	1.2-4.1	1.2-3.7		
Protein creatinine ratio	Median	0.13	2.6	2.4	2.8	<0.001	0.211
	range	0-0.27	0.07-10.8	0.12-8.47	0.07-10.8		
Serum IL6 concentration (ng/L)	Median	56.1	176.3	84.3	209.1	<0.001	<0.001
	range	20.6-88.6	18.4-634.5	18.3-467.4	78.45-634.5		

P1, comparison between NS versus healthy control; p2, comparison between SSNS versus SRNS.

Table 2: Comparison of IL6 genotypes and alleles between NS and healthy control subjects.

IL6	*Healthy control N=100*		*NS N=150*		*Crude*			*Adjusted**		
	N	%	N	%	*p*	*OR*	*95% CI*	*p*	*OR*	*95% CI*
GG	80	80	72	48.0		R			R	
GC	18	18	64	42.7	<0.001	3.951	2.142-7.287	0.013	3.076	1.268-7.458
CC	2	2	14	9.3	0.008	2.778	1.709-5.399	0.043	2.507	1.518-12.134
GC+CC	20	20	78	52.0	<0.001	4.333	2.413-7.781	0.009	2.958	1.305-6.704
G	178	89	208	69.3	<0.001	3.579	2.157-5.938	0.018	2.293	1.156-4.550
C	22	11	92	30.7						

*Adjusted for age and sex; HW p, Hardy Weinberg p value; R, reference genotype; OR, odds ratio; CI, confidence interval.

Table 3: Comparison of IL6 (genotypes and alleles) between SSNS and SRNS patients.

IL6	*SSNS N=50*		*SRNS N=100*		*Crude*			*Adjusted**		
	N	%	N	%	*p*	*OR*	*95% CI*	*p*	*OR*	*95% CI*
GG	29	58	43	43		R			R	
GC	20	40	44	44	0.275	1.484	0.731-3.012	0.308	1.455	0.707-2.995
CC	1	2	13	13	0.022	2.767	1.087-7.726	0.045	2.616	1.052-7.600
GC+CC	21	42	57	57	0.045	2.831	1.921-3.639	0.049	2.797	1.893-3.618
G	78	78	130	65	0.022	1.909	1.096-3.326	0.028	1.884	1.071-3.311
C	22	22	70	35						

*Adjusted for age and sex; HW p, Hardy Weinberg p value; R, reference genotype; OR, odds ratio; CI, confidence interval.

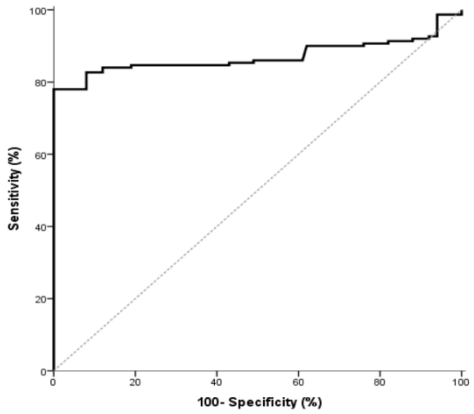

Figure 2: ROC curve of IL6 concentration for discrimination between NS and healthy control Subjects.

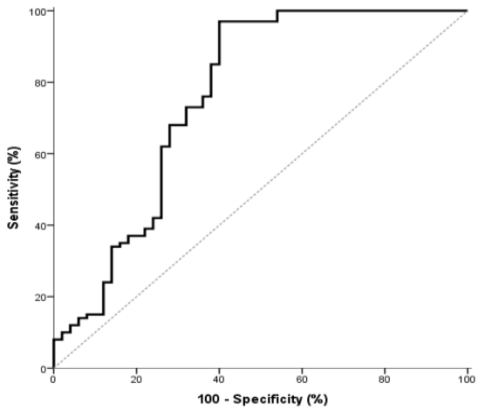

Figure 3: ROC curve of IL6 concentration for discrimination between SSNS and SRNS.

Table 4: Regression analysis for prediction of NS within healthy control subjects.

	Univariate				***Multivariate***			
	p	OR	95% CI		p	OR	95% CI	
IL6 concentration (ng/L)	<0.001	1.049	1.034	1.064	<0.001	1.039	1.021	1.057
IL6 (GC+CC vs GG)	<0.001	4.333	2.413	7.781	0.016	4.818	1.344	17.269

Table 5: Correlation between serum IL6 and studied parameters in different studied group.

Serum IL 6	***Control***		***SSNS***		***SRNS***	
	r	***p***	***r***	***p***	***r***	***p***
Age (years)	-0.046	0.651	-0.042	0.771	-0.044	0.664
Creatinine (mg/dl)	-0.132	0.189	0.231	0.107	0.445	<0.001
Cholesterol (mg/dl)	-0.086	0.395	0.382	0.006	-0.006	0.953
Albumin (g/dl)	0.100	0.321	-0.431	0.002	-0.547	<0.001
Protein/creatinine ratio	-0.078	0.438	0.464	0.001	0.413	<0.001
Urinary protein	-0.090	0.372	0.360	0.010	0.359	<0.001

r, Spearman correlation coefficient.

4. DISCUSSION

Cytokines play an essential role in NS. It act as mediators of inflammation and as progressive factors (Tenbrock et al., 2002). IL-6 and its gene polymorphism have been studied in different diseases which have susceptibility to recurrent infections and immunological changes (DeMichele et al., 2003), (Castellucci et al., 2006), (Tischendorf et al., 2007).

The current study shows significant increase in creatinine, cholesterol and protein creatinine ratio and significant decrease in albumin concentrations in NS cases in comparison to control group (P<0.001 for all). As regard to SRNS group cholesterol and showed significantly higher concentrations (*P*<0.001), while albumin showed significantly lower concentrations (P=0.045) when compared to SSNS subgroups Table 1. Similar results were obtained by Rizk et al., (2005) and Madani et al., (2014) who found an increase cholesterol and decrease albumin in NS patients when compared to controls. Also, Madani et al., (2014) found an increase protein creatinine ratio in SRNS when compared to SSNS. Increased IL-1β in NS stimulates hepatic protein synthesis due to hypoalbuminaemia and inhibits lipoprotein lipase (Ganong, 1999), which will lead to hypercholesterolemia. Although IL-1β increased hepatic protein synthesis, its vascular leak effects exceed it, with a net hypoproteinaemia (Rizk et al., 2005).

As regard to serum IL-6 this study reveals a significant increase in serum IL6 in NS patients when compared to the control group and in SRNS when compared to SSNS (*P*< 0.001) Table 1. IL6 concentration was associated with risk of NS development in uni and multivariate analysis (*P*< 0.001) Table 4. ROC curves of IL6 concentration were conducted for discrimination between NS and healthy control subjects and discrimination between SSNS and SRNS patients. The area under curve (AUC) was (0.87 & 0.76 respectively) (Figure 2&3). Also, our study found that IL6 concentration showed significant positive correlations with creatinine, protein creatinine ratio and urinary protein; significant negative correlation with albumin in SRNS subgroups Table 5. Similar results were obtained by Daniel et al., (1997), Ece et al., (1999) and Rizk et al., (2005). An important effect of increased IL-1β combined with IL-6 and TNF in NS is the increased leukocyte count from bone marrow and leucocytes attached to endothelial cells (Janeway et al., 1999). The infiltrating monocytes-macrophages are the main source of inflammatory cytokines, especially IL-1β, IL-6 and TNF (Ganong, 1999).

Some studies have showed that -174 polymorphism may change the rate of IL6 gene expression, where the G to C change at position -174 creates a potential binding site for the transcription factor *nuclear factor* I (NF-1), which act as repressor of IL6 gene expression, so that individuals of GG and GC genotypes have higher plasma IL6 levels and higher IL6 response than carriers of CC genotype (Fishman et al., 1998).

In our results, there was no significant differences were found in serum IL6 among different IL6 genotypes in SSNS and SRNS group (data not shown). Our results are in agreement with Lieb et al., (2003) and Elsaid et al., (2014) who reported the same result but in coronary artery disease.

In the present study IL6 GC, CC, GC+CC genotypes and C allele showed significant association with risk of NS development within healthy control subjects even after adjustment with age and sex (*P*< 0.001, =0.008, <0.001 &< 0.001) Table 2.

This is in concord with Madani et al., (2014) who found that IL6-G174C CC genotype and C allele are significantly higher in patients group than the control group (*p*= 0.003 and 0.009). Also, these results were in accordance with study on Indian population by Jafar et al., (2011), who revealed that IL6-G174C CC genotype and C allele were significantly higher in NS patient group than the control group (*p* < 0.001).

The current study revealed that IL6 CC, GC+CC genotypes, C allele showed significant association with risk of SRNS development within NS even after

adjustment with age and sex (*P*= 0.022, =0.045 &=0.022 respectively) Table 3. Regression analysis for prediction of SRNS within NS patients show that IL6 (GC+CC vs GG) was only associated with SRNS development within NS patients (*P*= 0.045) data not shown.

This is in agreement with Madani et al., (2014) and Jafar et al., (2011) who found that IL6-G174C CC genotype and C allele are increased significantly in SRNS group than SSNS group and this may be considered the cause of progression and steroid resistance.

On the other hand, Tripathi et al., (2008) reported that GG genotype of IL-6 -G174C is more common in the SRNS and responsible for poor prognosis.

The discrepancy between our results and some studies due to a number of limitations eg. sample size, ethnic difference and different techniques used for genotyping.

5. CONCLUSIONS

Serum IL6 level is significantly increased in NS group than the control group and in SRNS patients than SSNS patients. IL6-G174C gene polymorphisms affect the response to steroid therapy in Egyptian children have nephrotic syndrome and influence the clinical course.

6. ACKNOWLEDGMENTS

The authors would like to thank the Clinical Pathology Department and Children Hospital Mansoura Faculty of Medicine for their kind help and support to complete routine laboratory work.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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