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Original article

Genotyping of *PPAR-γ* gene polymorphism in Egyptian neonates affected with sepsis disease and its severity

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ABSTRACT

Background: Peroxisome Proliferator-Activated Receptor gamma (*PPARγ*) is a ligand-dependent transcription factor involved in inflammatory process. *PPAR-γ* gene was mentioned as having a modulating role in the pathological status of sepsis.
The present study aimed to make a correlation between The *Pro12Ala* polymorphism in *PPAR-γ* gene and occurrence of neonatal sepsis and its severity among a sample of Egyptian neonates suffering sepsis.
Subjects and methods: This case-control study included 30 neonates (11 females and19 males) newly admitted with neonatal sepsis at the intensive care unit (NICU) (mean age 10.3 days ± 6.23). The control group included 50 age and sex matched neonates (23 females and 27 males) (mean age 10.20 days ± 5.36 days). All the neonates (preterm and full term) included were with clinical signs and laboratory data consistent with neonatal sepsis. Genotyping for *PPARγ* gene region harboring the *Pro12Ala* variant locus were carried out using Tetra ARMS technique.
Results: About 56.7% of the patients group was homozygote (GG) for polymorphic locus (coding for Alanine/Alanine) while 30% was heterozygote for polymorphic locus (CG) (coding for Proline/Alanine) and up to 13.3% was homozygote for the polymorphic locus (CC) (coding for Proline/Proline). Compared to the control group where homozygotes for CC were the most prevalent (90%) and the CG were 10% with absence of GG genotypes. There was a strong statistical significant difference between patients and the normal control group as regards prevalence of *PPAR-γ* gene polymorphism in occurrence of neonatal sepsis and its severity. Also, there were strong relation between genotype GG and low birth weight, neonatal fever, prematurity and depressed neonatal reflexes.
Conclusion: PPAR-γ gene has been suggested to be a candidate gene for neonatal sepsis. Therefore, *Pro12Ala* polymorphism might be useful in predicting the risk factor of neonatal sepsis and its severity.

1. Introduction

Sepsis clinically may involve a massive systemic inflammatory response resulting in septic shock, multiple organ failure, and death [1]. Premature and ill infants are more susceptible to sepsis disease; thus, considerable care is required in those infants so that sepsis can be effectively identified and treated [2].

Peroxisome proliferator-activated receptor (PPAR) are ligand activated transcription factors that belong to the nuclear hormone receptor superfamily [3], which include 3 subfamilies (*PPARα*, *PPARβ/δ*, *PPAR-γ*). These transcription factors play a major role in sepsis and development of inflammatory disease. Peroxisome proliferator-activated receptor gamma (*PPAR-γ*) was described as playing a role in modulating the pathological status of sepsis by regulating energy metabolism, inflammation, and immune cell function [4]. PPAR-γ regulate inflammatory status by controlling the differentiation of macrophages and monocytes and by inhibiting the expression of inflammatory cytokines, such as interleukin-1 beta (*IL-1β*), tumor necrosis factor alpha (*TNF-α*), inducible cyclooxygenase-2 (*COX-2*). Therefore, *PPAR-γ* seems to be a candidate gene for sepsis [5].

SNP in the *PPAR-γ* gene rs1801282 is considered as a major functional polymorphism in codon 12 of exon B. This SNP leads a mutation in which Proline is replaced by Alanine [6].

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The present study tried to detect if there is relation between The Pro12Ala Polymorphism in PPAR-γ Gene and occurrence of neonatal sepsis and its severity among a sample of Egyptian neonates suffering sepsis.

2. Patients and methods

2.1. Patients

This case-control study included 30 neonates (11 females and19 males) newly admitted with neonatal sepsis at the intensive care unit (NICU) in the age range from 2 to 25 days (mean 10.3 days ± 6.23). The control group included 30 age and sex matched neonates (23 females and 27 males) (mean age 10.20 days ± 5.36 with range from 2 to 20 days). All the neonates (preterm and full term) included in the study were with clinical signs and laboratory data consistent with neonatal sepsis. Exclusion criteria included neonates with lethal or major congenital anomalies and neonates with suspected clinical features of chromosomal disorders.

Consents from all patients' and controls' parents/guardians were taken to approve sharing in the study after full description of the steps and aim of the study. The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki) for experiments involving humans and the Ethical Committee of Ain Shams University" for the same purpose.

- Detailed maternal and obstetric history were obtained regarding gravidity and parity, obstetric history including mode and place of delivery, premature rupture of membrane > 18 h, duration of labor, maternal pyrexia >38 °C or maternal illness. Neonatal history included gestational age confirmed by "Ballard score" [7], birth weight, onset of neonatal sepsis and duration of hospital stay.
- All of the neonates were subjected to complete neonatal examination: Newborns with evidence of at least one organ dysfunction and those with at least 2 of the following 4 criteria defining systemic inflammatory response syndrome (SIRS) were included in the study.

• Core temperature >38.5 or <36.0 °C.
• Tachycardia, defined as a mean heart rate >2 SD above normal for age, and bradycardia, defined as a mean heart rate <10th percentile for age.
• Mean respiratory rate >2 SD above normal for age [8].

2.2. Methods

Sepsis screen by qualitative CRP by CRP card, ESR, CBC (Leukocyte count elevated or depressed for age or >10% immature neutrophils of the total neutrophils count).

Genotyping: DNA was extracted from 250 μl peripheral blood by Chemagic DNA Blood250 Kit and Chemagic magnetic stand 2 × 12 (Art. No. CMG-300) (Chemagen Technology®, PerkinElmer©). The eluted DNA was stored at −20 °C till application. Peroxisome proliferator activated receptor-γ gene region harboring the Pro12Ala variant locus was amplified using the following primers [9]:

Tetra primers: Forward tetra primer: 5′-GAA ACT CTG GGA GAT TCT CCT ATT GTC C-3′, Reverse tetra primer: 5′-GTA TCA GTG AAG GAA TCG CTT TCA GC-3′. (C allele 221 bp while G allele 288 bp).

Outer primers 455 bp: Forward outer primer: 5′-AAC TTT TTG TCA CAG CTG GCT CCT AAT A-3′, Reverse outer primer: 5′-CAA CGA GCT AAG CAT TAA AAT ACT GGA-3′.

Tetra-primer ARMS-PCR method was used for amplification of approximately 200 ng of template genomic DNA in a 25 μL. PCR reaction volume containing 20 pmol of each of the 2 inner primers, 2 pmol of each of the 2 outer primers. 1.5 mM $MgCl_2$, 400 μM of each dNTP, 2 U Hot start Taq polymerase, 10 mM Tris-HCl and 1× PCR buffer. The PCR program (S24 thermal cycler, Quanta Biotech-England, UK) was: Predenaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 60 s, 63 °C for 60 s and 72 °C for 60 s, and followed by final extension at 72 °C for 7minutes. This yielded an amplified PCR fragment of 221 bp in length for the fragment harboring C allele and 288 bp for G allele in addition to 455 bp from the two outer primers. The genotypes of the PCR products were subjected to electrophoresis on 2% high resolution agarose gels stained with ethidium bromide in 1x Tris-EDTA (ethylenediamine tetraacetic acid)-Borate buffer (TBE) against 50 bp ladder molecular weight GeneRuler™ 50 bp DNA ladder (Fermentas™, #SM0373, Thermo Scientific/Fermentas, Vilnius, Lithuania). The PCR products were all documented by Gel Documentation System and Software for DNA analysis (InGenius Syngene™ – UK) (Fig. 1).

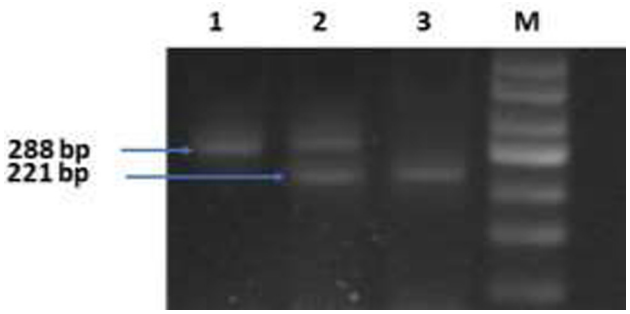

Fig. 1. PCR Products of a fragment from the PPARγ gene for the detection of the Pro12Ala mutation. Lane 1: show the mutant homozygous genotype (GG). Lane 2: show the heterozygous genotype (GC). Lane 3: show the normal homozygous genotype (CC). M: Marker 50 bp ladder.

2.3. Statistics

The distribution of genotypes and allele frequencies were all statistically compared in all patients versus healthy controls (Package for Social Science-IBM SPSS-version 20). Qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviations and ranges. *Kolmogrov-Smirnov test* of normality was used to assess the distribution of the studied data and found with normal distribution in all parameters. The comparison between two groups with qualitative data were done by using *Chi-square test* and/or *Fisher exact test* was used instead of Chi-square test when the expected count in any cell was found less than 5. The comparison between two independent groups regarding quantitative data with parametric distribution was done by using *Independent t-test.* The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as ($P < 0.05$: Significant, $P < 0.01$: Highly significant) and $P > 0.05$: Non significant.

3. Results

The present study included 30 neonates with sepsis and 30 healthy controls of matching age and sex. There were no significant differences between patients and control regarding age and sex.

About 56.7% of the patients group was homozygote (GG) for polymorphic locus (coding for Alanine/Alanine) while 30% was heterozygote for polymorphic locus (CG) (coding for Proline/Alanine) and up to 13.3% was homozygote for the polymorphic locus (CC) (coding for Proline/Proline). There was a highly significant statistical relationship between the pro 12 Ala polymorphism in

PPAR-γ gene and occurrence of neonatal sepsis. The incidence of wild type allele (C) in homozygosity (CC) and in heterozygosity (CG) was 28.33% while the incidence of mutant allele (G) in homozygosity (GG) and in heterozygosity (GC) was 71.67%. The tendency of allele G to segregate in homozygosity was more than allele C (56.66% vs. 13.33%). Among the patients group; the prevalence of the homozygote genotype for GG was 56.7% followed by the heterozygote for CG genotype 30% then the homozygotes for CC genotype which appeared in a percentage of 13.3% compared to the control group where homozygotes for CC were the most prevalent (90%) and the CG were 10% with absence of GG genotypes. There was a strong statistical significant difference between patients with neonatal sepsis and the normal control group as regards prevalence of PPAR-γ gene polymorphism in occurrence of neonatal sepsis and its severity. The percentage of C allele among patients group was nearly equally distributed between homo- and heterozygosity while the G alleles was more in tendency to be in homozygosity. On the other hand, the percentage of G allele among control group was 5% and appeared in heterozygosity only, while the percentage of C allele among control group was 95% with more prevalence to be in homozygosity (90%), thus there was statistical significant difference between patients group and the control group which may suggest high risk for occurrence of neonatal sepsis and PPAR-γ G allele (Table 1).

Table 1
Prevalence of genotypes and alleles among patients group compared to control group.

Genotype	Patients group		Control group		Chi-square test	
	No.	%	No.	%	X^2	p-value
Homozygote (CC) Proline/Proline	4	13.3%	45	90.0%	50.612	0.000**
Heterozygote (CG) Proline/Alanine	9	30.0%	5	10.0%		
Homozygote (GG) Alanine/Alanine	17	56.7%	0	0.0%		
Alleles:						
C	17	28.33%	95	95.0%	79.365	0.000**
Homozygosity	(8)	(13.33%)	(90)	(90%)		
Heterozygosity	(9)	(15%)	(5)	(5%)		
G	43	71.66%	5	5.0%		
Homozygosity	(34)	(56.66%)	(0)	(0)		
Heterozygosity	(9)	(15%)	(5)	(5%)		
Total	60	100%	100	100%		

** Highly significant.

Table 2
Correlation between wild type allele (C) and mutant (G) allele regarding age and gender among patients group.

Item		Wild (C)	Mutant (G)	Independent *t*-test	
		No. = 17	No. = 43	*t*	P-value
Age (days)	Mean ± SD Range	13.46 ± 5.83 3–23	7.88 ± 5.53 2–25	2.675	0.012**
Sex	Females Males	5 (38.50%) 8 (61.50%)	6 (35.30%) 11 (64.70%)	0.032	0.858*

* Insignificant.
** Highly significant.

Table 3
Correlation between genotypes, and mean values for laboratory findings among patients group.

Mean value		Homozygote (CC) Proline/Proline (221 bp)	Hetero-zygote (GC) Proline/Alanine (221 bp/288 bp)	Homozygote (GG) Alanine/Alanine (288 bp)	One Way ANOVA		Post hoc analysis		
		No. = 4	No. = 9	No = 17	F	P-value	P1	P2	P3
CRP (IU/UL)	Mean ± SD Range	27.50 ± 19.00 18–56	26.00 ± 15.87 12–48	32.71 ± 22.45 6–96	0.355	0.704*	0.884	0.674	0.435
RBCS ('106)/UL)	Mean ± SD Range	4.06 ± 1.16 2.86–5.32	4.78 ± 1.18 3.3–6.42	4.29 ± 1.06 2.28–5.78	0.804	0.458*	0.326	0.703	0.291
Hb (g/dL)	Mean ± SD Range	12.33 ± 3.65 9.3–17.5	14.28 ± 2.58 11–17.4	13.52 ± 3.37 7.2–19.3	0.527	0.596*	0.288	0.535	0.564
WBCS ('103)/UL)	Mean ± SD Range	11.98 ± 9.12 4.4–25.2	9.52 ± 3.38 4.8–13.9	13.61 ± 6.88 5.5–29.4	1.212	0.313*	0.479	0.690	0.108
Neutrophils (%)	Mean ± SD Range	47.63 ± 7.36 37.7–54.3	47.82 ± 11.47 21.4–60	50.44 ± 19.92 18.4–85.7	0.094	0.910*	0.976	0.788	0.721
Lymphocytes (%)	Mean ± SD Range	38.43 ± 8.97 28.4–48.2	41.37 ± 10.34 30.2–57.7	35.78 ± 17.38 10.3–72.2	0.422	0.660*	0.634	0.774	0.388
Monocytes (%)	Mean ± SD Range	13.95 ± 4.17 10.1–19.7	12.57 ± 4.10 6.9–20.9	13.50 ± 12.31 4–57	0.037	0.964*	0.587	0.944	0.828

* Insignificant.

Table 4
Correlation between the segregated alleles and laboratory findings among patients group.

Laboratory findings		Wild (C)	Mutant (G)	Independent *t*-test	
		No. = 17	No. = 43	t	P-value
CRP (IU/UL)	Mean ± SD Range	26.46 ± 16.09 12–56	32.71 ± 22.45 6–96	−0.849	0.403*
RBCS (‘106)/UL)	Mean ± SD Range	4.56 ± 1.17 2.86–6.42	4.29 ± 1.06 2.28–5.78	0.655	0.518*
Hb (g/dL)	Mean ± SD Range	13.68 ± 2.94 9.3–17.5	13.52 ± 3.37 7.2–19.3	0.130	0.897*
WBCS (‘103)/UL)	Mean ± SD Range	10.28 ± 5.46 4.4–25.2	13.61 ± 6.88 5.5–29.4	−1.434	0.163*
Neutrophils (%)	Mean ± SD Range	47.76 ± 10.06 21.4–60	50.44 ± 19.92 18.4–85.7	−0.442	0.662*
Lymphocytes (%)	Mean ± SD Range	40.46 ± 9.67 28.4–57.7	35.78 ± 17.38 10.3–72.2	0.871	0.391*
Monocytes (%)	Mean ± SD Range	12.99 ± 4.00 6.9–20.9	13.50 ± 12.31 4–57	−0.143	0.888*

* Insignificant.

Table 5
Correlation between clinical data and genotyping.

Clinical data		Wild (CC + CG)		Mutant (GG)		Chi-square test	
		No.	%	No.	%	X^2	P-value
Birth weight (kg)	<1 >2 1–2	1 4 1	16.7% 66.7% 16.7%	6 0 18	25.0% 0.0% 75.0%	18.722	0.000**
Gestation	Preterm Term	1 5	16.7% 83.3%	20 4	83.3% 16.7%	10.159	0.001**
Neonatal fever	Negative Positive	4 2	66.7% 33.3%	0 24	0.0% 100.0%	18.462	0.000**
Depressed neonatal reflexes	Depressed Normal	1 5	16.7% 83.3%	17 7	70.8% 29.2%	5.868	0.015**

** Highly significant.

There was significant statistical difference between alleles regarding age and neonatal sepsis (14 patients were early onset neonatal sepsis and 16 patients were late onset neonatal sepsis) while there was insignificant statistical difference between males and females and neonatal sepsis. Early and late neonatal sepsis occurred more with G allele (14 early-onset neonatal sepsis of whom 10 were homozygote GG and 2 were heterozygote CG and 16 late onset neonatal sepsis of whom 7 were homozygote GG and 7 were heterozygote CG (Table 2).

There was insignificant statistical difference for mean value of CRP, RBCS, HB, WBCs, neutrophils, lymphocytes and monocytes among patients group correlation with genotype. There was insignificant statistical difference between alleles G and C in relation to CRP, WBCs, neutrophils, lymphocyte and monocytes among patients group (Tables 3 and 4).

There was strong statistical relation between genotyping and risk factors for neonatal sepsis: There was strong relation between genotype GG and low birth weight, neonatal fever, prematurity and depressed neonatal reflexes. Furthermore, the sepsis was more severe with the risk factors which occurred mainly with the mutant genotype (GG) within PPAR Gamma gene where all cases with genotype GG died during the study (Table 5).

4. Discussion

The present study aimed to investigate the relationship between the *Pro12Ala* polymorphism of *PPAR* gene and sepsis disease severity in a sample of Egyptian neonates (preterm and term). Recently, peroxisome proliferator activated receptor gamma (PPAR-γ) was described as playing a role in modulating the pathological status of sepsis by regulating energy metabolism, inflammation, and immune cell function and that PPAR-γ has been suggested to be beneficial in sepsis [4].

In the current study, patients with neonatal sepsis showed strong significant prevalence of Ala allele (*PPAR-γ* gene polymorphism) in comparison with controls regarding occurrence of neonatal sepsis and its severity. It was demonstrated that the beneficial effects of *PPAR-γ* agonists appear to be mediated through the down regulation of inflammatory responses, reduction of oxidative stress, inhibition of apoptosis, and promotion of neurogenesis [10].

In our study, there was a strong relationship between (GC) (the *pro 12 Ala*) genotype polymorphism in *PPAR-γ* gene and occurrence of neonatal sepsis ($p < 0.01$). The incidence of mutant allele in both homozygosity (GG) and in heterozygosity (GC) together was 71.67%. On other hand, Ma et al. [11] found insignificant differences in the genotype distribution of the *PPAR-γ Pro12Ala* variant between the sepsis patients and healthy controls in the Chinese Han population. *Furthermore*, *they* found that the frequency of the mutant allele (G) in homozygosity alone (GG) (*Pro12Pro*) was much higher (96.59%) than current study. In a study by Wang et al., [12] on the effect of *PPAR-β/δ* on sepsis-induced acute lung injury, it was found that the *PPAR-β/δ* agonist significantly increased the survival of rats affected by sepsis, reduced histological damage of the lungs, and decreased inflammatory cytokines in serum and lung tissues of such affected rats with sepsis, however, the counts of peritoneal bacteria did not increase. Over-expression

of *PPARβ/δ* attenuated gene expression of *TNF-α*, *IL-1β* and *IL-6* in alveolar macrophages. Yang et al. [13] reported that a *PPARβ/δ* agonist has anti-inflammatory effects in experimental models of atherosclerosis, myocardial ischemia and proteinuric kidney disease.

In this study, the most prevalent genotype among our patients was homozygote (GG) for polymorphic locus (coding for Alanine/Alanine). This is in contrast to Ma et al., [11] who reported that the frequency of (CC) genotype was the most prevalent (93.18%) followed by GC (6.82%) in their patients group.

In our study, the percentage of G alleles among patient group was detected in 71.66% with more tendency to be in homozygosity (56.66%) compared to 5% only among control group where it appeared in heterozygosity only ($P < 0.05$). This may suggest high risk for occurrence of neonatal sepsis with *PPAR-γ* G allele. Stratification by subtypes (sepsis, septic shock, and severe sepsis) revealed a statistically significant difference in the frequency of the Ala allele and Ala-carrier genotype between the patients with the sepsis subtype and the healthy controls [11].

The frequency of the Ala allele C in the sepsis subtype was significantly higher than in the healthy controls and there were no significant differences in genotype distribution or allele frequency between the healthy controls and the septic shock or severe sepsis subtype. So that the *Pro12Ala* polymorphism allele might not serve as a marker for susceptibility to sepsis but could influence a patient's clinical outcome and risk of dying from sepsis. This is in accordance with Ma et al., [10] who found that significant differences were found in the frequency of the Ala allele and genotype between the sepsis survivors and non-survivors. In the survivors, the *PPAR-γ Pro12Ala* genotype was significantly associated with decreased disease severity and recovery time. Gong et al. [14] suggested that the Ala12 variant of *PPAR-γ* has potential gene-environment interactions. *PPAR-γ* is expressed in the inflammatory cells involved in the progression of sepsis; monocytes, macrophages and T-cells [15]. Some studies observed a protective effect of the *Ala* allele in the carrier state [16].

Sun et al. [17] reported that the *PPAR-γ* agonist Rosiglitazone may ameliorate coagulation disorders in septic rats. *PPAR-γ/NF-ΚB* transduction pathway plays an important role in septic coagulopathy. Brenneis et al. [18] reported that the level of peroxisome proliferator-activated receptor γ (*PPARγ*) expression in T cells from septic patients correlates with clinical outcome. Rosiglitazone increased bacterial clearance through *PPAR γ* activation and NET formation, combining immunomodulatory and host-dependent anti-bacterial effects and, therefore, warrants further study as a potential therapeutic agent in sepsis [19,20]. The potential role of *PPARs* in regulation of inflammation and angiogenesis is intriguing and warrants further studies. Standage et al. [21] reported that altered peroxisome proliferator-activated receptor-α-mediated cellular metabolism may play an important role in sepsis-related end-organ injury and dysfunction, especially in the heart.

In our study, there was an insignificant statistical difference between genotypes in correlation gender. This agrees with other studies. However, The *Ala12* allele frequency of the *Pro12Ala* SNP varies different widely among ethnic groups [22].

The difference between genotypes for patients group was statistically insignificant for mean value of CRP, RBCS, HB, WBCs, neutrophils, lymphocytes and monocytes. For homozygote GG there was a difference but not significant statistically for *CRP and WBCS*. It was stated that lipoxin A4 up-regulated expression of *PPAR-γ*, as well as neutrophil gelatinase associated lipocalin (NGAL) in adult neutrophils. In contrast, no effects were observed in neutrophils from neonates. Moreover, constitutive expression of *PPAR-γ* was significantly reduced in neonatal neutrophils, when compared with adult cells. These data suggest a potential mechanism underlying persistent activation of neonatal neutrophils at inflammatory sites [23]. *PPAR-β/δ* agonists and *PPARβ/δ* adenovirus down-regulate *TNF-α*, *IL-1β and IL-6* gene expression [12].

5. Conclusion

There was a strong association between the *Pro12Ala* polymorphism in *PPAR-γ* gene and the occurrence of neonatal sepsis. Moreover, (G) allele is considered as a risk factor for neonatal sepsis and its severity, as well as *PPAR-γ* gene has been suggested to be a candidate gene for neonate sepsis. Therefore, *Pro12Ala PPAR-γ* polymorphism might be useful in predicting the risk of neonatal sepsis and its severity.

Conflict of interest

The authors declare no conflict of interest. There are no financial or personal relationships with other people or organizations that could inappropriately influence the work.

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