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Original Research

Polymorphisms in proinflammatory cytokine genes, effect on gene expression and association with preterm delivery in Saudi females

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Abstract: Genetic polymorphism in proinflammatory cytokine genes may be associated with the etiology of preterm birth (PTB). The current study was designed with the aim to explore the association of genetic polymorphisms and mRNA expression of IL-1α, IL-1β, and TNF-α gene with preterm birth in the Saudi population. Genotyping of genomic DNA of 50 PTB patients and an equal number of controls were carried out using TaqMan Genotyping assay kits. Gene expression of each gene was carried out using quantitative RT-PCR. The cytokine levels in the serum of PTB patients and controls were measured by ELISA. A statistically significant association was observed between the rs361525 alleles (G and A) of TNF-α and PTB, where the mutant A allele was significantly protective against PTB development (OR= 0.362; $\chi 2$=4.31; p=0.038). The gene expression of all studied genes (*IL1α, IL-1β, IL-6*, and *TNF-α*) was higher in the PTB patients, but the results reached significance only for *IL-1β* (p=0.035). Elevated gene expression was also evident from the level of these proteins in plasma, where the level of IL1α, IL-β were significantly higher in the serum of PTB patients compared to the controls, however, IL6 and TNF-*α* were significantly lower despite higher gene expression. For IL6, the lower level could be due to tocolytic treatment that was given to all women suffering from PTB. Receiver operating curves (ROC) were drawn for the studied cytokines. In conclusion, this study revealed that rs361525 polymorphism plays a role as a protective marker for PTB and the levels of IL-1α, IL-6, TNF- α can be used as predicative biomarkers for PTB I Saudi women.

Key words: Preterm birth; Cytokine; Genes; IL6; IL1. TNF- α.

Introduction

Multiple environmental and lifestyle factors are associated with preterm birth risk (1). However, the risks associated with preterm births remain unexplained as etiology is not yet fully understood (2). Infectious diseases and unavailability and accessibility of health care resources, conception among aged women, and usage of fertility drugs are considered as the main cause of preterm birth risk all over the world (3). However, genetic variations including polymorphism in immune response genes, particularly cytokine polymorphisms might contribute to the etiology of PTB (4). Cytokines, such as interleukin (IL)-1 (5) and IL-6 (6) are responsible for the onset of premature labor, and mutations in these genes may contribute to certain pathological states. Interleukin -1 family is a diverse group of proteins which stimulate prostaglandin E2 synthesis by the amnion, while IL6 typically released by macrophages and T-cells is a proinflammatory cytokine which stimulates inflammatory response during infection. When body gets infected with microbes or microbial metabolites, mature B cell or T cell secretes Tumour necrosis factor (TNF)-α to mediate the immunologic response of the host. TNFα shows major variability in populations due to the genetic polymorphisms. A large number of epidemiological studies are available showing the relationship between increased risk of premature labor and single SNPs in interleukin (IL)-1, (IL)-6 and TNFα genes (7-10). However, the various genetic contributors have not been studied well in Saudi Arabia. We conducted this study to explore the possible relationships between polymorphisms of proinflammatory cytokine genes and PTB in Saudi Arabia. We further investigated the mRNA expression of IL-1α, IL-1β, and TNF-α genes and the level of these proteins in serum.

Materials and Methods

Subject Recruitment

The study was carried out as a hospital-based case-control study in Riyadh during 2015-16. A total of 50 patients with PTB and 50 controls with full-term birth were recruited for the study. All controls and patients were healthy, not suffering from any disease or infection during the period of pregnancy and labor. The study was reviewed and approved by the Institutional Review Board of the Ethics Committee at King Khalid Univer-

Table 1. Details of primers and change in nucleotide position of the studied genes.

Gene	SNP ID	Gene location	Polymorphism	primers sequence
IL-1β	rs16944	promoter region (5 UTR)	A/G,Transition	-511 A/G
IL-6	rs1800796	promoter region	G/C, Transversion	-572 G/C
TNF-α	rs1800629	promoter region	G/A, Transition	-308 G/A
TNF-α	rs361525	promoter region	G/A, Transition	-238 G/A

sity Hospital (KKUH) in Riyadh, KSA. Each patient and control was required to sign a written informed consent prior to inclusion in the study. All patients and controls were attending the clinics of Dr. Babay, and the samples were collected in the delivery room at the time of delivery.

Data Collection

Each patient and control was interviewed, and detailed information was recorded on specially designed forms. The information collected included maternal age, maternal age at first pregnancy, maternal consanguinity, body mass index (BMI), number of children, gestational age at delivery, the new-born birth weight, and type of delivery (vaginal or cesarean). Information on gestational diabetic mellitus, hypertension hypotension, and hypothyroidism was also included.

Genetic and Biochemical Analysis

Blood collection and preparation

Five milliliters of venous blood was collected from each subject in sterilized plastic vials containing EDTA (0.5 M; pH 8.0) and stored at -80° C before DNA extraction. Genomic DNA was extracted from blood samples by using QI Amp DNA Blood Mini Kit (QIAGEN, Germany) according to manufacturer's protocol. The extracted DNA was quantified and stored at 4° C until used for further analysis. Another five ml of peripheral blood was collected in sterilized plastic vials without anti-coagulate to obtain the serum for measuring the level of cytokines. The clotted blood was centrifuged, and the serum was carefully separated and stored at -80^{0}C till required for analysis.

Tissue collection and preparation

Fresh human placentas were obtained after delivery from 10 PTB patients and 10 controls in the delivery room at King Khalid University Hospital. RNA was extracted from maternal (decidua) portions of each placenta. The tissue was placed in RNA later solution prior to RNA extraction process (Ambion, Life Technologies, USA) to maintain the quality and quantity of RNA then stored at -80°C until use.

Genotyping

Genotyping of four SNPs was carried out using a Taq-Man assay. PCR reaction for genotyping was carried out using 5.84 µl Tag man ® Genotyping Master Mix (Applied Biosystems, USA), 0.28 µl Tagman®Genotyping assays (Applied Biosystems, USA), 3.02 µl water, making the volume 9.14 µL, 2µL DNA sample was added in each well which gives 11.14 total volume. Sample analyses were carried out according to manufacturer's instruction. Steps included an initial denaturation for 15 minute at 95°C, then amplification for 55 cycles at (95°C denaturation for 15 seconds, 60°C for 1 minute annealing and detection of the signal, then 72 for 1 second), last cooling at 40°C for 30 seconds. The details of the SNPs investigated, their location in the gene and the base change are presented in Table 1

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from placenta tissue which has been placed in *RNAlater* solution using RNeasy Mini Kit (QIAGEN, Germany) and was subsequently treated with DNase I (DNA-free; Ambion Inc, Austin, TX) to remove genomic DNA. cDNA synthesis was performed using Reverse Transcription kit (Applied Bio systems, USA). Gene expression studies were performed using SYBR green method, and GAPDH was used as the internal normalizer.

Gene expression was studied using qRT-PCR by quantifying the transcribed mRNA (cDNA). Briefly, PCR reactions were prepared in 96 well Micro Amp®Fast Optical Reaction plate from Applied Biosystems® (Life Technologies, USA). Gene expression primers were designed by using primer 3 NCBI from Invitrogen (Thermo Fisher Scientific). Gene expression primer sequences used are listed in Table 2. GAPDH from CROGEN was used as the reference gene. The optimization of the primer concentration is essential and was carried out. Each set of primers work best at 10pmol/µl concentration conditions. The annealing temperature was optimized and restricted to 60°C, which

Table 2. Sequence of the gene expression primers used during this study.

Gene		Sequence for the gene expression primers
IL-1α	Frw(A8849A08)	GTGCTGCTGAAGGTCAGTTG
	Rev (A8849A09)	AGAGGTGTCTGCCTATTGGG
IL-1β	Frw (A8849A10)	TGTACCTGTCCTGCGTGTTG
	Rev(A8849A11)	CTTTTCCTAGGGATGGGGGC
IL – 6	Frw(A8849B06)	GCACCTCAGATTGTTGTTGTT
	Rev(A8849B07)	AGTGTCCTAACGCTCATACTTT
TNF- α	Frw(A8849B08,)	GCACACAGAAGACACTCAGGG
	Rev(A8849B09).	TGTTGGGGAGAAGGAGGATGG

corresponds to the optimal working conditions for the AmpliTaq Gold® DNA polymerase enzyme (Perkin Elmer). The Syber Green Master Mix in each well contains 6.25mL Power SYBR® Green PCR Master Mix 2X from Applied biosystems® (Life Technologies, USA), 0.25µL forward primer, 0.25µL reverse primer for each gene, and 3.75µL nuclease-free water making the volume 10.25µL, 2µL cDNA was added which gave 12.5 total volume in each well. Each sample of cDNA was analyzed in triplicates for both the cytokine genes and GAPDH (the reference gene). The plate was covered with ceiling and placed in ViiA™ 7 Real-Time PCR SystemApplied biosystems® (life technologies, USA). The qPCR- thermocycler, optimal conditions for each target gene were established and carried out as 10min at 95°C activation, followed by PCR stages: 45 cycles of 15 sec at 95°C denaturation, 1min at 60°C annealing, followed by melting curve stage: 15 sec at 95°C.

The specificity of cytokine primers assay used for the reaction was verified by the presence of single melting temperature peak. Results were then analyzed using $2^{-\Delta\Delta Ct}$, a variation of the Livak method, where ΔCt was calculated, and then the fold change values were measured using normalization method.

Measurement of cytokines level in maternal serum

Maternal Serum level of IL-1α, IL-1β, and TNF-α were measured by ELISA Kit based on standard sandwich enzyme-linked immune-sorbent assay technology (MyBiosource, USA), while for IL-6 was measured by double-sandwich ELISA technique (MyBiosource, USA).

Statistical Analysis

All the Statistical analysis was carried out using IBM SPSS version 22 statistical software (IBM SPSS, Chicago, IL, USA) and Microsoft Excel®. The genotypes and allele frequencies of the three SNPs were calculated manually. Independent sample T-test was used to achieve values of Odds Ratio, 95% Confidence Intervals, Chi square to determine the significance between the patients and controls. A significant difference was considered at p value <0.05.

Results

In this study, 50 PTB and an equal number of controls were recruited. The demographic characteristics and clinical data of cases and controls are presented in Table 3 and Table 4, respectively. There was no difference in maternal age, BMI, parity, number of children and age at first pregnancy between control and preterm subjects. While gestational age at delivery and the newborn birth weight were significantly lower among PTB.

Table 3. Demographic characteristics of study patients.

Characteristics	Controls (n=50)	PTB patients (n=50)	P value
Age (Yrs)	28.045 ±0.532	28.920 ±0.897	0.381
BMI (Kg/m2)	29.938 ±0.593	28.444± 0.862	0.159
Parity	2.642 ±0.200	3.200 ±0.352	0.144
No. of children	2.220 ±0.226	2.340 ±0.257	0.731
Age at first pregnancy	22.574±0.420	23.208 ±0.658	0.421
Gestational age (weeks)	38.800 ±0.279	29.816 ±0.761	0.0001
Weight (Kg) new born birth	3.224 ±0.054	1.496 ±0.111	0.0001

Table 4. Clinical data of study patients.

Characteristics	Controls (%)	PTB (%)	Fisher Exact Probability test
Previous PTB	0	86	-
Previous PROM	0	42	-
Infections	0	0	-
PCOS	4	12	0.0001
Gestational DM	2	14	0.002
Hypertension	5.4	10	0.200
Hypotension	8.6	2	0.09
Hypothyroidism	0	6	-
Mode of delivery (CS)	10	19	0.0001
Survival of the new born	100	90	0.0001

Table 5. Pearson correlation between measured parameters.

Parameters	R (Person Correlation)		Sig.	
Maternal Age(Years) ~ Maternal BMI(Kg/m2)	PTB Control	0.433 ** 0.460 **	0.002 0.0001	P[a]
Maternal Age(Years) ~ Gestational age (weeks)	PTB Control	-0.103 -0.199	0.538 0.478	N[b]
Maternal Age(Years) ~ New born weight (Kg)	PTB Control	0.036 0.220	0.809 0.125	P[a]
Maternal BMI(Kg/m2) ~ Gestational age (weeks)	PTB Control	-0.336 ** -0.007	0.0039 0.980	N[b]
Maternal BMI(Kg/m2) ~ New born weight (Kg)	PTB Control	0.023 0.273	0.878 0.055	P[a]
Gestational age (weeks) ~ New born weight (Kg)	PTB Control	0.663 ** 0.411	0.0001 0.128	P[a]

** Correlation is significant at the 0.01 level. [a]Positive Correlation. [b]Negative Correlation.

Table 6. Genotype and Allele frequencies of SNP under study of (IL)-1, (IL)-6 and TNFα gene in cases (PTB) and control group.

Polymorphism	Control, n (%)	Preterm, n (%)	OR (95% CI)	X^2	p-value
IL-1β rs16944 (A/G)					
AA	9 (18%)	6(12%)	Reference		
AG	21 (42%)	22(44%)	1.571 (0.476-5.184)	0.55	0.456
GG	20 (40%)	22(44%)	1.650 (0.498-5.464)	0.68	0.4131
AG+GG	41 (82%)	44(88%)	1.610(0.527-4.920)	0.71	0.400
Alleles					
A	39 (39%)	34(34%)	Reference		
G	61 (61%)	66(66%)	1.241 (0.697-2.209)	0.54	0.462
IL-6 rs1800796 (G/C)					
GG	42(84%)	41(82%)	Reference		
GC	7(14%)	9(18%)	1.317(0.448-3.868)	0.25	0.615
CC	1(2%)	0	0.341(0.014-8.622)	0.96	0.325
GC+CC	8(16%)	9(18%)	1.152(0.405-3.277)	0.07	0.790
Alleles					
G	91(91%)	91(91%)	Reference		
C	9(9%)	9(9%)	1.000(0.380-2.634)	0.00	1.000
TNF-α rs1800629 (G/A)					
GG	33(66%)	36(72%)	Reference		
GA	15(30%)	10(20%)	0.611(0.241-1.548)	1.09	0.296
AA	2(4%)	4(8%)	1.833 0.315-10.676)	0.47	0.494
GA+AA	17(34%)	14(28%)	0.755 (0.322-1.767)	0.42	0.516
Alleles					
G	81(81%)	82(82%)	Reference		
A	19(19%)	18(18%)	0.936(0.458-1.911)	0.03	0.855
TNF-α rs361525(G/A)					
GG	42(84%)	47(94%)	Reference		
GA	1(2%)	0	0.298(0.012-7.519)	1.11	0.293
AA	7(14%)	3(6%)	0.383(00.093-1.577)	1.87	0.171
GA+AA	8(16%)	3(6%)	0.335(0.083-1.346)	2.55	0.110
Alleles					
G	85(85%)	94(94%)	Reference		
A	15(15%)	6(6%)	0.362(0.134-0.975)	4.31	p=0.038

PCOS, gestational diabetic mellitus and cesarean delivery were significantly high in PTB, while survival of the new born was significantly low in PTB. A significantly positive correlation was found between maternal age and maternal BMI in PTB and controls, while a significantly negative correlation existed between maternal BMI and gestational age in PTB. Also, a significantly positive correlation was found between gestational age and new born weight in PTB (Table 5).

Allelic Frequencies and Genotype Analysis

Hardy-Weinberg equilibrium was estimated separately for each polymorphism in PTB and controls. The distribution of rs16944 in IL-1β -511 A/G, rs1800796 in IL-6 -572 G/C, rs1800629 and rs361525 in TNF-α -308G/A,-238 G/A, genotypes and alleles in control and PTB groups is presented in Table 6**.** There was a significant difference in TNF-α -238 (genotype GA, and AA) polymorphism between term and preterm groups. We found no statistically significant influence of polymorphism of the IL-1β -511 A/G, IL-6 -572 G/C, TNF-α -308G/A.

Expression profile of IL-1α, IL-1β, IL-6 and TNF-α genes

The expression of IL-1β was significantly higher in PTB placenta when compared to controls. Remarkably high expression of IL-1α, IL-6, and TNF-α was found in PTB placenta compared to the control group (Table 7). A significant positive correlation was found between IL-1α ~IL-1β, IL-1α ~TNF-α and IL- β ~ TNF- α. (Table 7).

Cytokine levels in serum of cases (PTB) and control

Table 9 presents the Mean ± S.D and the percentage change of the measured cytokines in both study groups. IL-1α and IL-1β were found to be higher in PTB, while IL-6 and TNF-α were found to be lower in PTB when compared to the control group. Receiver Operating Characteristic (ROC) Curves were constructed to determine the sensitivity and specificity of each cytokine for

Table 7. Expression of IL-1 α IL-1β, IL-6 and TNF-α genes in human placentas from PTB and controls mothers**.**

Genes	Group	Mean ± S.D.	P value
IL-1 α	Control PTB	1 ± .96 2.63 ± 2.59	0.11
IL-1β	Control PTB	1 ± 0.55 3.93 ± 3.52	0.035
IL-6	Control PTB	1 ± .81 4.18 ± 4.36	0.061
TNF-α	Control PTB	1± 1.32 4.08± 4.34	0.07

Table 8. Pearson correlation between all the cytokines.

Parameters	r (Person Correlation coefficient)	Sig.
IL1- α ~ IL- β	0.929	0.000
IL1- α ~ TNF- α	0.997	0.000
IL1- α ~ IL6	0.500	0.170
IL- β ~ TNF- α	0.917	0.001
IL- β ~ IL6	0.334	0.379
TNF- α ~ IL6	0.479	0.192

PTB, and the cut-off, the area under the curve (AUC), sensitivity, and specificity in the PTB group are presented in Table 10 and Figure 1. The results show that the combination of IL-1 α, TNF-α, and IL-6 are perfect marker and predictors of PTB.

Discussion

Cytokine-mediated inflammation and infection play a major role in preterm birth at early gestations (<30 weeks). Almost 50 cytokines/chemokines controls the process of preterm labour, but the mechanism involved in the process and exactly how these proteins interact and lead to preterm birth is not fully understood. In the present study, four proinflammatory cytokines were measured at the gene level, gene expression level and protein level and the serum levels of the serum cytokines were correlated with the prediction of PTB in Saudi females.

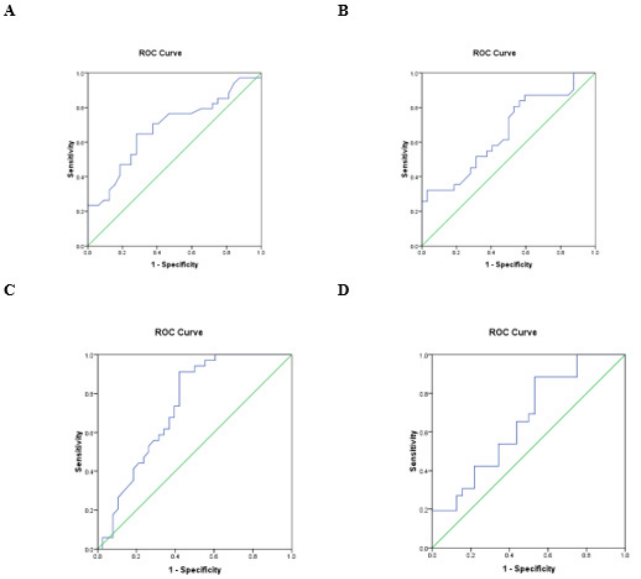

Figure 1. ROC Curve of **A**- IL-1a, **B**-IL-1β, **C**- IL-6, **D**- TNF-α in PTB group.

Our results show high expression of cytokines in PTB when compared with control group. The gene expression was studied in the placenta, since each tissue has a specific pattern of gene expression. Some genes are expressed in one tissue and not in another. Furthermore, the extent of gene expression differs in different tissues and under different conditions. In this regard it was considered essential to use the expression of cytokines in the placenta, which is most closely related to PTB. Cytokines are important factors in the evolution of the mature placenta and are also associated with the induction of placental apoptosis. There is a strong possibility that when labor is triggered early, gestational and labor-related differences may result in the tissue displaying a pattern of gene and protein expression specific to preterm labor (12). Overexpression of cytokines in patients with PTB may result in the excessive induction of the inflammatory response.

IL-1β together with TNF-α stimulates the amnion, decidua, and myometrium to produce prostaglandin which then provokes uterine contractions. Furthermore, IL-1 and TNF-α induce the release of IL-6 from decidual and chorionic cells (13) which in turn induce the degradation of the extracellular matrix of the lower uterine segment. Thus, the texture of the tissue is reduced and the cervix ripens (matures) under increasing uterine pressure or labor, and dilates (14). Thus, the results of the present study confirm that labor induces gene expression changes in chorio-amniotic membranes consistent with a localized acute inflammatory response, despite the absence of histological evidence of inflammation. This was further confirmed by the positive and significant correlation between IL-1-α, IL-1β, and TNF-α. The positive correlation between the gene expression of the four studied cytokines was an interesting

Table 9. Mean ± S.D. of all the measured cytokines in serum of cases (PTB) compared to the control group.

Parameters	Groups	N	Min	Max	Mean± S.D.	%Change	P value
IL-1α (pg/ml)	Control	32	4.74	12.03	8.01 ± 2.08	100.00	0.012
	PTB	34	3.67	15.59	9.78 ± 3.05	122.07	
IL-1β (pg/ml)	Control	32	0.13	1.04	0.54 ± 0.23	100.00	0.030
	PTB	31	0.32	2.93	0.98 ± 0.81	182.45	
IL-6 (pg/ml)	Control	38	1.08	42.45	16.68±13.44	100.00	0.001
	PTB	34	1.59	15.87	6.80 ± 3.36	40.74	
TNF-α (pg/ml)	Control	32	54.64	512.31	223.41±165.96	100.00	0.036
	PTB	26	30.9	353.98	131.17 ± 82.84	58.71	

Table 10. ROC-Curve of all measured cytokines in PTB group.

Group	AUC	Cutoff value	Sensitivity %	Specificity %
IL-1α	0.681	8.514	64.7 %	71.9 %
TNF-α	0.661	195.628	88.5 %	46.9 %
IL-6	0.733	11.128	91.2 %	57.9 %
Combining	0.941	-------	100.0 %	75.0 %

finding and indicated that not only individual cytokine gene expression is elevated, but also some mechanisms exist whereby each cytokine gene expression can influence the gene expression of other cytokines.

In our results, except TNF-α 238 G>A polymorphism, no other significant association between the gene polymorphisms of study proinflammatory cytokine gene and preterm birth in Saudi Arabia population was observed. TNF-α 238 G>A polymorphisms has been reported to be associated with high transcriptional activity (15). In addition, its location within a known regulatory region of the TNF-α gene may have a functional role or, alternatively, may be a genetic marker for another polymorphism (16). The high level of TNF-α may alter the delicate balance of anti-inflammatory/proinflammatory cytokines and induce PTB (17). Our results reveal that AA genotype when compared with the GG genotype (wild-type), G allele was more common among the subjects with symptomatic PTB than controls. AA genotype is significantly ameliorating against PTB development, and hence A is a protective allele with a OR: 0.362, CI: 0.134-0.975, and p:0.038. and has a dominant effect. Carriage of at least one A allele at this position decreases the risk of PTB in Saudi females. However, GG genotype significantly increased maternal serum level of IL-6.

Our study revealed that pro-inflammatory cytokine, TNF-α -238 G>A polymorphisms, may increase the risk of PTB in Saudi women and maternal serum levels of IL-1α, IL-6, TNF- α can be used as a predicator of PTB.

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