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SNPs AND TRANSCRIPTIONAL ACTIVITY OF GENES OF INNATE AND ADAPTIVE IMMUNITY AT THE MATERNAL-FETAL INTERFACE IN WOMAN WITH PRETERM LABOUR, ASSOCIATED WITH PRETERM PREMATURE RUPTURE OF MEMBRANES

DOI: 10.36740/WLek202001104

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ABSTACT

The aim is to identify mRNA expression of innate (TLR2 and TLR4) and adaptive (IL1 β, IL17A, FoxP3, Tbet, Roryt) immunity in maternal-fetal interface and evaluate the contribution of SNP genes of IL1β (rs1143627), TNFα (rs1800629), IL4 (rs2243250), IL10 (rs1800896, rs1800872) and RLN2 (rs4742076, rs3758239) to PTB, associated with PPROM in 26-34 weeks of gestation.

Materials and methods: We had done open cohort randomized research during period 2016-2018 years. The case group consisted of 50 women with PPROM in preterm pregnancy, 26-34 weeks of gestation. For the control group we collected samples from 50 women without previous history of PTB. To determine the level of mRNA target genes we used thermocycler CFX96™Real-Time PCR Detection Systems ("Bio-Rad Laboratories, Inc.", USA) and set of reagents Maxima SYBR Green / ROX qPCR MasterMix (2x) (Thermo Scientific, USA).

Results: In the population of the Zaporizhzhia region, there is no reliable clinical association between the IL1β and TNFα genes and a high risk of PTB. We obtained high reliable data on SNP genes RLN2 rs4742076 and rs3758239 in Zaporizhzhia women. The distribution of the rs2243250 gene polymorphism alleles of the IL4 gene of the main study group – TT homozygotes were determined in 2 (4%) cases, CT heterozygotes were found in 11 (22%), CC homozygotes in 37 (74%) cases. In the study of polymorphism rs1800872 of the IL10 gene, the main group of homozygous TT studies was identified in 7 (14%) cases, TG heterozygotes were found in 18 (36%), GG homozygotes in 25 (50%) cases. The range of all obtained values of the relative normalized expression of TLR2 gene in the placenta of 0.79-163.44 (median – 31.06), in the fetal membranes – 1.1-126.06 (median – 10.22). The placement of all obtained values compared to mRNA expression of the TLR4 gene was lower than the TLR2 in the placenta, which was 0.39-43.85 (median – 7.74) and higher in the fetal membranes – 0.18-216.01 (median – 40.04). We observed an 8.33-fold decreased expression in FoxP3 in decidua, especially in 31-32 weeks of PPROM manifestation (27.03-fold). In amniotic membranes a similar trend of reduction of FoxP3 expression was found, overall level decreased in 2.33 times, especially in 31-32 weeks of PPROM manifestation (10.64-fold).

Conclusions: Among Zaporizhzhia population, combination of IL4 (rs2243250), IL10 (rs1800896 and rs1800872), RLN2 (rs4742076 and rs3758239) supports the role for functional polymorphisms in immunoregulatory genes in the susceptibility to PTL, associated with PPROM. Marked increased transcriptional activity of components of innate (TLR2, TLR4), adaptive (Th1, Th17) immune system and conversely decreased expression of Treg (FoxP3) in the maternal-fetal interface are involved in immune pathways of PTB and contribute in the fetal inflammatory response syndrome.

KEY WORDS: single nucleotide polymorphism, transcriptional activity, preterm birth, preterm premature rupture of membranes

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INRODUCTION

Today, in Ukraine, the number of normal births is an average of 32.6% of their total. The frequency of registration of the prematurity in different regions of the country ranges from 3 to 12%. Spontaneous preterm birth (PTB) and preterm premature rupture of membranes (PPROM) are major contributors to neonatal mortality and serious neonatal morbidity worldwide [1, 2, 3].

It has been well-established that infection of the amniotic cavity can cause PTB via the activation of inflammatory processes resulting the onset of labour, rupture of membranes and dilatation oh the cervix [4]. This pathological inflammatory process can be caused by microorganisms invading the amniotic cavity (i.e. intra-amniotic infection) or danger signals/alarmins released during cellular stress or death (i.e. sterile intra-amniotic inflammation) [5]. In both scenarios, cytokines, such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) among others, play a central role in the pathophysiology of PTB [6]. Of all of these cytokines, IL-1β is a central mediator in the pathological process of PTB since it can stimulate the expression and release of other labor mediators, such as prostaglandins [7].

The epidemiological and clinical data regarding the proportions and relative incidence of intrauterine inflammation and infection in preterm deliveries are quite discordant

across different studies [8, 9]. Different patient populations exhibit different rates of vaginal dysbiosis, intrauterine infection and degrees of inflammatory responses [10, 11, 12].

Since inflammation is often invoked as an etiologic factor in spontaneous preterm birth, the question1 of whether spontaneous preterm birth has a genetic predisposition in the case of pathologic inflammation has been of long-standing interest to investigators. In recent years a great attention also has been paid to associative search of polymorphic markers with different diseases and as a result creation the genetic platform for personalized medicine. Most of the single nucleotide polymorphism (SNP) genes of the cytokines are found in the regulatory regions of the gene and directly affect their transcriptional activity and the concentration of the cytokine in the blood [13]. However, many findings are controversial what demonstrates the importance of standardized methods and reproductive techniques as well as strictly performed evaluation adjusted for potential confounding factors. Additionally, great part of inconsistencies found in the literature can be due to differences in genetic background and environmental exposures, parameters that vary greatly among distinct populations.

It is very important to identify changes in mRNA expression of immune cells at local level, to investigate candidate gene association with susceptibility to PTB induced PPROM and to integrate these results with our and other previous findings [14, 15, 16]. Through this mRNA analysis and integration with candidate gene sequencing data from the same individuals, we obtain a better understanding of the transcriptional regulation that occurs in the context of PTB.

THE AIM

To identify mRNA expression of innate (TLR2 and TLR4) and adaptive (IL1 β, IL17A, FoxP3, Tbet, Roryt) immunity in maternal-fetal interface and evaluate the contribution of SNP genes of IL1β (rs1143627), TNFα (rs1800629), IL4 (rs2243250), IL10 (rs1800896, rs1800872) and RLN2 (rs4742076, rs3758239) to PTB, associated with PPROM in 26-34 weeks of gestation.

MATERIALS AND METHODS

We had done open cohort randomized research during period 2016-2018 years. The case group consisted of 50 women with PPROM in preterm pregnancy, 26-34 weeks of gestation. For the control group we collected samples from 50 women without previous history of PTB. Control patients delivered normal infants at term without labor via elective cesarean section. Patients with diabetes mellitus, severe cardio-vascular morbidity, multiple gestations, fetal demise in utero or fetal anomalies were excluded.

Placental decidua and amniotic membranes tissues were obtained from patients (n=30) with PTB, associated with PPROM and control group (n=30). We are limited in our ability to perform extensive characterization, stratification/clustering or any predictive analyses of these mRNAs due to inadequate statistical power resulting from a small sample size. The study was approved by the Molecular-Genetic Research Division of the Medical and Laboratory Center of Zaporizhzhia State Medical University, and all patients who participated provided written informed consent. The tissues were collected at Regional perinatal center Zaporizhzhia city. The objects for molecular genetic studies with using of the real-time reverse transcription polymerase chain reaction (RT-PCR) techniques. Total RNA was procured from samples by use of "NucleoZOL" (Macherey-Nagel, Germany). For reverse transcription and obtaining cDNA, we used RevertAid First Strand cDNA Synthesis Kit ("ThermoScientific", USA). To determine the level of mRNA target genes we used thermocycler CFX96™Real-Time PCR Detection Systems ("Bio-Rad Laboratories, Inc.", USA) and set of reagents Maxima SYBR Green / ROX qPCR MasterMix (2x) (Thermo Scientific, USA). Specific primer pairs (5'-3') for analysis of target and reference genes were selected by the software PrimerBlast (www.ncbi.nlm.nih.gov/tools/primer-blast) and produced by ThermoScientific (USA). Normalized relative quantity of cDNA target genes was determined by the method $_{\Delta\Delta}$Ct. Statistical data analysis of PCR were conducted using available software CFX Manager ™ (Bio-Rad, USA).

We performed SNP sequencing in whole blood from women undergoing PTB induced PPROM with active contractions (26-34 weeks of gestation, n=50) matched for gestational age to healthy pregnant non-labouring controls (>37 weeks' gestation, n = 50) who later delivered at term.

The genotyping using TaqMan tests was done on amplifier CFX96™Real-Time PCR Detection Systems («Bio-Rad Laboratories, Inc.», USA). Polymerase chain reaction (PCR) for TaqMan genotyping was performed according to the instructions Applied Biosystems, USA. Studied SNPs genes were selected based on existing evidence in the literature for a role in the pathogenesis of the studied conditions for comparison with Ukrainian population.

Statistical data processing was carried out using the software package «Statistica 6.0» (StatSoftInc, No. AXXR712D-833214FAN5). The comparison of qualitative indices was carried out using the χ^2 criterion with Yates correction and Fischer's exact criterion (F). In order to evaluate the contribution of gene polymorphism to the probability of development of PPROM in preterm pregnancy, odds ratios (OR) were calculated with 95% confidence interval (CI). The differences were considered to be significant at $p < 0,05$.

RESULTS AND DISCUSSION

The data that we received in our work were compared with the results of other scientists. In addition, the results of studies of the same gene polymorphisms are determined by the design of the study are controversial in populations, and the expression of these mutations depends on a combination of culturological, socioeconomic and semantic factors, which are determined by the style of life in general and the style of habitation, in particular.

Table 1. Association between polymorphic locks of cytokine genes and high risk of PPROM and PTL in Zaporizhzhia region

SNPs	Allel / Genotype	Public location	OR	95 % CI	χ^2	p-value
IL1β *rs1143627*	Allele G Genotype GA Allele A	112836810	1,13 1,54 0,89	0,65-1,98 0,68-3,49 0,51-1,55	0,18	0,67
TNFα *rs1800629*	Allele A Genotype AG Allele G	31575254	0,8 0,67 1,25	0,42-1,54 0,28-1,62 0,65-2,39	0,44	0,51
IL4 *rs2243250*	Allele C Genotype CT Allele T	132673462	3,94 4,42 0,25	2,0-7,76 1,15-16,97 0,13-0,5	16,77	4×10^{-5} *
IL10 *rs1800896*	Allele C Genotype CT Allele TT	206773552	3,24 0,26 0,8	1,07-11 0,05-0,93 0,32-0,84	6,1	0,05 *
IL10 *rs1800872*	Allele T Genotype TG Allele G	206773062	0,33 0,2 24,0	0,18-0,58 0,08-0,47 5,25-109,65	14,7	0,0001 *
RLN2 *rs4742076*	Allele C Allele T	5309831	0,03 33,0	0,00-0,23 4,37-249,1	25,46	5×10^{-7} *
RLN2 *rs3758239*	Allele A Allele G	5306824	12,57 0,08	3,68-42,98 0,02-0,27	23,86	1×10^{-6} *

Note. *– reliability of the difference, $p<0.05$.

In connection with the above, there was a need for the study of frequent mutations of genes IL4 (rs2243250), IL10 (rs1800896 and rs1800872), IL1β (rs1143627), TNFα (rs1800629) and RLN2 (rs4742076 and rs3758239) in Zaporizhzhia population to determine their role in the pathogenesis of PPROM and PTL in order to identify patterns and mechanisms for the formation of PPROM. In Ukraine, the study of such a combination of genes in the pathology of software at the start of this study was not conducted.

Based on the genotyping results of rs1800896 gene polymorphism IL-10 gene TT homozygotes were detected in 37 (74%) cases of the main study group, CT heterozygotes in 2 (4%) and CC homozygotes in 11 (22%) cases, consequently. In the study of polymorphism rs1800872 of the IL10 gene, the main group of homozygous TT studies was identified in 7 (14%) cases, TG heterozygotes were found in 18 (36%), GG homozygotes in 25 (50%) cases. The distribution of the rs2243250 gene polymorphism alleles of the IL4 gene of the main study group – TT homozygotes were determined in 2 (4%) cases, CT heterozygotes were found in 11 (22%), CC homozygotes in 37 (74%) cases.

IL10 is an anti-inflammatory cytokine and attenuates the inflammatory response through effects on pro-inflammatory cytokines and reduces the function of host immune cells, such as neutrophils and macrophages. We proved association of IL4 rs2243250 with high risk or PPROM in Zaporizhzhia region, as in the study performed by Heinzmann A. in German population [17, 18].

In the study of polymorphism rs1143627 of the IL1β gene, the main group of homozygous AA studies was identified in 17 (34%) cases, GA heterozygotes were found in 21 (42%), GG homozygotes in 12 (24%) cases. The distribution of the rs1800629 gene polymorphism alleles of the TNFα gene of the main study group – AA homozygotes were determined in 5 (10%) cases, GA heterozygotes were found in 12 (24%), CC homozygotes in 33 (66%) cases. In the population of the Zaporizhzhia region, there is no reliable clinical association between the IL1β and TNFα genes and a high risk of PTB.

We have got statistically significant differences of rs4742076 polymorphisms (TT, CT, and CC) of the RLN2 gene in the study groups. We also detected statistically significant differences in all alleles of the rs3758239 polymorphism (AA, GG and AG) of the gene RLN2, respectively $p< 0.05$, indicating the reliability of the received prognostic markers (Table 1). The received data of frequency of alleles/genotypes distribution in case and control group is set on the figure 1 and 2.

A recent study by Vogel I. with homogeneous Danish population improved that women who are homozygous for specific SNP in the promotor region of RLN2 have a genetic susceptibility for PTL [19, 20]. In a study performed by Frederico G. Rocha et al. the contribution of SNP rs4742076 and rs 3758239 in the RLN2 promoter in Filipino population was shown [21, 22]. We obtained high reliable data on SNP genes RLN2 rs4742076 and rs3758239 in Zaporizhzhia women.

Summarizing the data of repertoire of SNP genes involved in our study, we have got significant difference in all markers, except IL1β (rs1143627), TNFα (rs1800629). The presence of these alleles may disrupt the balance between pro- and anti-inflammatory cytokines, modify the inflammatory response, increasing the risk for PPROM and PTB. Being a global problem preterm birth warrants global solutions. Recent genomic approaches are beginning to reveal the information for understanding the causes of PTL. To fully appreciate and

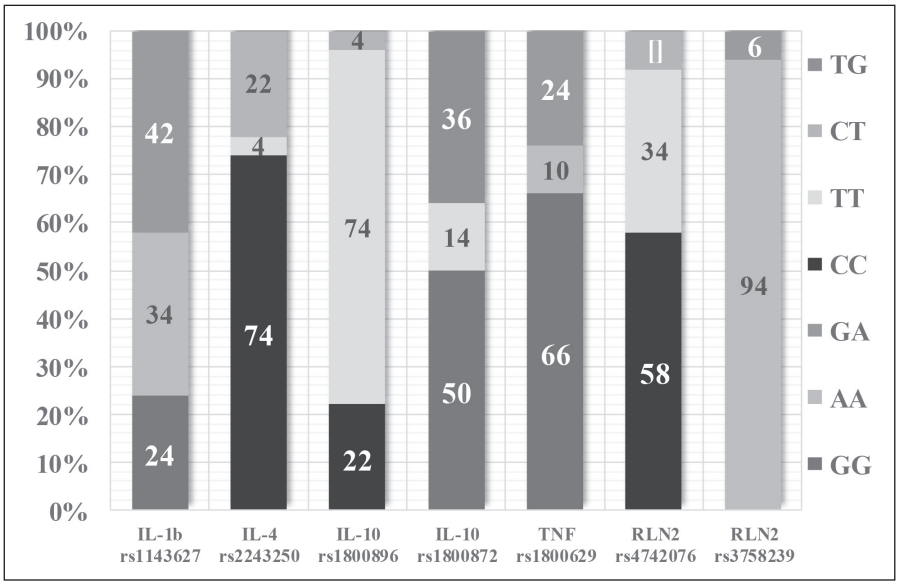

Fig 1. Frequency of genotypes distribution in women with PTB, associated with PPROM (%)

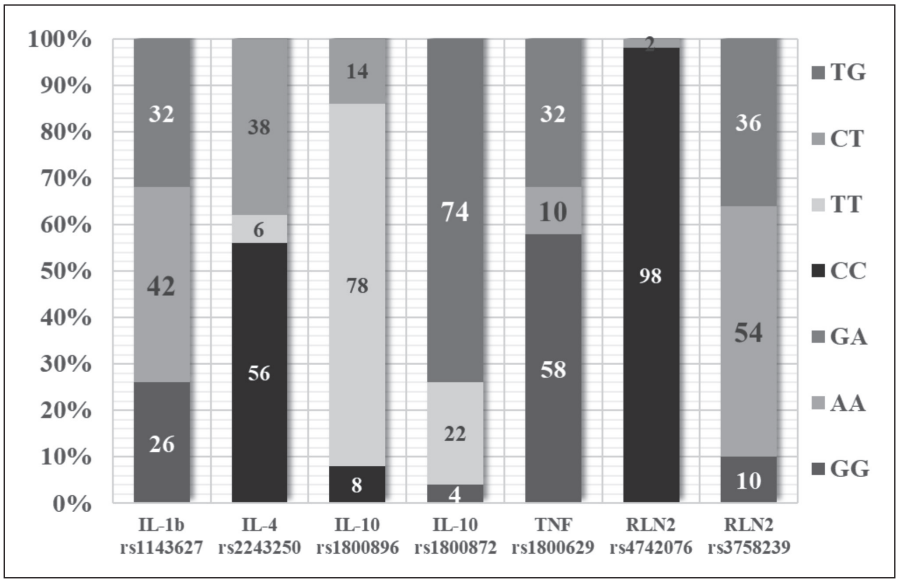

Fig 2. Frequency of genotypes distribution in control group (%)

understand the complexity of PPROM and PTL, further approaches using high-throughput genome sequencing methods such as genome wide association (GWAS), whole exome sequencing (WES) studies are essential.

Toll-like receptors (TLR)-2 and TLR-4 are innate immune receptors that recognize the microorganisms most frequently involved in amniotic cavity infections, which are associated with activating the inflammatory response at the maternal-fetal interface during PTB [12, 22, 23]. Transcriptional profile of innate and adaptive immunity genes in key tissues depending on the term of manifestation PPROM is shown in Table 2. The range of all obtained values of the relative normalized expression of TLR2 gene in the placenta of 0.79-163.44 (median – 31.06), in the fetal membranes – 1.1-126.06 (median – 10.22). The placement of all obtained values compared to mRNA expression of the TLR4 gene was lower than the TLR2 in the placenta, which was 0.39-43.85 (median – 7.74) and higher in the fetal membranes – 0.18-216.01 (median – 40.04).

The range of all obtained values of the relative normalized expression of pro-inflammatory IL1β gene mRNA in the placenta was 0.61-227.93 (mean – 25.08), in fetal membranes – 1.23-139.24 (mean – 23.83). The range of relative normalized expression of mRNA of the IL-17A gene in the placenta was 0.04-62.77 (mean – 5.69), in fetal membranes – 0.36-130.67 (mean – 19.31).

We identified significant decreased expression of FoxP3 (Treg) in women with PTB, induced PPROM compared with controls. We observed an 8.33-fold decreased expression in FoxP3 in decidua, especially in 31-32 weeks of PPROM manifestation (27.03-fold). In amniotic membranes a similar trend of reduction of FoxP3 expression was found, overall level decreased in 2.33 times, especially in 31-32 weeks of PPROM manifestation (10.64-fold).

The mRNA expression of TLR4 in the fetal membranes is significantly higher (40.04-fold) in patients with PTB, associated PPROM than in control group. In 19.31 times IL17A and 24.43 times RoRγt (Th17) higher transcriptional activity was present in membranes compared with

Table 2. Transcriptional profile of innate and adaptive immunity genes in key tissues depending on the term of manifestation PPROM (Mean (L-H))

Gene / local level	26-34 weeks n=30	26-30 weeks n=6	31-32 weeks n=10	33-34 weeks n=14
TLR2 *placenta*	31,06 (0,79-163,44)	46,31 (0,79-163,44)	37,74 (6,91-149,60)	21,22 (1,59-52,83)
TLR2 *membranes*	10,22 (1,1-126,06)*	3,27 (2,32-5,49)*	5,99 (1,1-28,88)*	15,61 (1,39-126,06)*
TLR4 *placenta*	7,74 (0,39-43,85)	11,09 (1,82-26,43)	3,08 (0,40-6,30)	10,00 (0,39-43,85)
TLR4 *membranes*	40,04 (0,18-216,01)*	34,38 (0,69-133,4)*	24,77 (0,53-152,97)*	53,53 (0,18-216,01)*
IL1β *placenta*	25,08 (0,61-227,93)	47,13 (1,43-227,93)	16,49 (0,61-132,74)	23,21 (1,63-91,02)
IL1β *membranes*	23,83 (1,23-139,24)	39,81 (4,47-77,11)	28,66 (2,79-139,23)*	12,85 (1,23-38,32)*
IL17A *placenta*	5,69 (0,04-62,77)	3,59 (1,15-5,62)	3,05 (0,04-5,73)	4,16 (0,92-62,77)
IL17A *membranes*	19,31 (0,36-130,67)*	15,46 (3,3-27,54)*	17,36 (0,36-49,74)*	13,73 (1,64-130,67)*
FoxP3 (Treg) *placenta*	0,12 (0,008-2,01)	0,093 (0,018-0,229)	0,037 (0,013-0,088)	0,191 (0,08-2,01)
FoxP3 (Treg) *membranes*	0,38 (0,013-0,986)	0,512 (0,143-0,927)	0,094 (0,013-0,167)	0,49 (0,07-0,98)
T-bet (Th1) *placenta*	20,29 (0,63-376,73)	79,27 (2,36-376,73)	5,48 (1,65-14,55)	9,8 (0,63-98,19)
T-bet (Th1) *membranes*	16,91 (0,51-101,71)	27,32* (0,51-71,93)	10,04* (1,43-44,45)	17,35* (1,27-101,71)
RoRyt (Th17) *placenta*	4,77 (0,692-44,41)	3,61 (1,25-6,14)	3,24 (1,09-6,26)	6,4 (0,69-44,41)
RoRyt (Th17) *membranes*	24,43* (0,75-141,89)	63,44* (6,26-141,89)	19,75* (0,75-87,68)	13,83* (0,86-100,93)

Note: * – statistically significant differences p <0,05 (Mann-Whitney U test) compared to the expression level of the studied gene in the placenta

term deliveries This may be reflective of the inflammatory and immune response that occurs as a breakdown in maternal-fetal tolerance or inflammation related to PTB. Our results shed light on potential mechanisms by which mRNAs may play a role in mediating systemic inflammatory response in pregnant women that deliver prematurely.

The modern molecular genetic method of research has allowed to reveal key immunoregulatory components of development of PROM at the local level during the 26-34 weeks' gestation period in the form of an expression violation of the receptors of innate immunity and consequently cell pool disbalance of T-helpers Th1/ Th17/ Treg.

CONCLUSIONS

1. There is evidence suggests that the nature and intensity of an inflammatory response in women with PTB induced PPROM is under genetic control. Among Zaporizhzhia population, combination of IL4 (rs2243250), IL10 (rs1800896 and rs1800872), RLN2 (rs4742076 and rs3758239) supports the role for functional polymorphisms in immunoregulatory genes in the susceptibility to PTL, associated with PPROM.
2. Marked increased transcriptional activity of components of innate (TLR2, TLR4), adaptive (Th1, Th17) immune system and conversely decreased expression of Treg (FoxP3) in the maternal-fetal interface are involved in immune pathways of PTB and contribute in the fetal inflammatory response syndrome.

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Conflicts of interest:

Authors declare no conflict of interest.

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