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ARAŞTIRMA / RESEARCH

Molecular investigation of mechanisms considered to cause preterm premature membrane rupture

Preterm erken membran rüptürüne yol açtığı düşünülen mekanizmaların moleküler düzeyde incelenmesi

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Abstract

Purpose: The aim of this study was to investigate the mRNA expression level of p16, CDK4, CDK6, Cyclin D, RB1, and E2F genes in preterm premature rupture of membrane (PPROM) cases and their roles in etiopathogenesis of PPROM.
Materials and Methods: Twenty-one pregnancies with PPROM before 34th gestational weeks (study group) were compared with twenty pregnancies with no complication, who gave birth after 37th gestational-week (control group). Both groups chorioamniotic membranes were compared for mRNA expression of p16, cyclin D, CDK4, CDK6, RB1 and E2F genes.
Results: The mRNA expression levels of p16, cyclin D, CDK4, CDK6, RB1and E2F genes decreased in the PPROM group compared to control group at a statistically significant level.
Conclusion: Our findings have shown that oxidative stress may not act on the p16 pathway in these cases. In order to understand the molecular mechanism of PPROM, biomarkers of oxidative stress and aging should be evaluated together with other pathways related to aging and oxidative stress in future studies.
Keywords:. PPROM, p16, cyclin D, CDK4, CDK6, RB1 and E2F

Öz

Amaç: Bu çalışmanın amacı, preterm erken membran rüptürü (PEMR) vakalarında, p16 ile CDK4, CDK6, siklin D, RB1, E2F genlerinin mRNA ekspresyon seviyelerini ve bu genlerin etiyopatogenezdeki rollerini saptamaktır.
Gereç ve Yöntem: Çalışmamızda 34. gebelik haftasından önce membran rüptürü olan 21 gebe (çalışma grubu) ile herhangi bir durumla komplike olmamış ve 37. gebelik haftasından sonra doğum yapmış 20 gebenin (kontrol) doğum sonu koryoamniyotik membranları incelendi ve bu iki grubun koryoamniyotik membran örnekleri p16, siklin D, CDK4, CDK6, RB1 ve E2F genlerinin mRNA ekspresyon seviyeleri açısından karşılaştırıldı.
Bulgular: Çalışmamızda, kontrol grubu ile kıyaslandığında p16, siklin D, CDK4, CDK6, RB1 ve E2F genlerinin ekspresyon seviyelerinin PEMR grubunda anlamlı derecede azaldığı saptandı.
Sonuç: Bulgularımız bu vakalarda oksidatif stresin p16 yolağı üzerinden etki etmeyebileceğini göstermiştir. PEMR'nin moleküler mekanizmasını anlamak için, gelecekteki çalışmalarda oksidatif stres ve yaşlanma biyobelirteçleri ile yaşlanma ve oksidatif streste görevli diğer yolaklar birlikte değerlendirilmelidir.
Anahtar kelimeler: PEMR, p16, siklin D, CDK4, CDK6, RB1 ve E2F

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INTRODUCTION

Preterm premature rupture of membranes (PPROM) is the rupture of fetal membranes before 37 weeks of gestation, complicates approximately 3-4% of all pregnancies, and causes 40-50% of all preterm births[1]. Neonatal mortality and morbidity is higher in PPROM, which also gradually increases inversely with the week of birth, mainly before the 34th weeks[2,3]. Although, history of preterm birth, smoking, multiple pregnancy and anatomical disorders of the genital system are known risk factors of PPROM[44], in many cases there has not been any identified specific risk factor[5]. Intraamniotic infection is thought to be the main cause of PPROM, with or without a known risk factor, however clinically proven intraamniotic infection has been shown in only 15-25% of these cases[6]. Even though infection plays an important role in the PPROM etiopathogenesis, it is insufficient to explain most cases therefore it is necessary to investigate other mechanisms, including endocrine signals that support fetal membrane apoptosis originating from the fetus[2].

Progressive accumulation of senescent cells in mitotic tissues is thought to be one of the aging factors. Senescent cells in tissues change their normal cellular functions and their behavior towards neighboring cells, degrade structural components such as the extracellular matrix, suppress stem and progenitor cells and cause a decrease in tissue regeneration, moreover all these processes lead the initiation and progression of various pathological processes in human tissues and organs[7]. In the light of our current knowledge, PPROM is a disease of the fetal membranes, which results in the deterioration of the chorioamniotic membranes structural integrity. PPROM also plays a role in the development of cellular pathways induced by the inflammation-oxidative stress axis. Cellular aging is an irreversible cessation of cell proliferation triggered by internal and external stimuli or stress factors[6]. In the placental (chorioamniotic) membranes of PPROM cases; there has been increased DNA damage and oxidative stress, decreased antioxidant capacity and telomere length , and induced p38MAPK (p38 mitogen activating protein) expression observed[8]. Stress factors that trigger senescence act through two main pathways, namely p53 tumor suppressor protein stabilization and cyclin-dependent kinase (CDK) inactivation. Suppression of CDKs is mediated by the p21, p16 (p16INK4a, cyclin dependent kinase inhibitor 2A), and pRB (RB transcriptional corepressor) proteins. Upon p53 activation p21, the transcriptional target of p53, becomes active. The pRB protein, which is activated by the transcriptional activation of p21 and p16, suppresses the transcription factor E2F1 (E2F transcription factor) and halts the cell cycle[7]. p16 is an important cyclin-dependent kinase inhibitor and tumor suppressor gene encoded in the 9p21 region of the human genome. The classical role of p16 is to control the cell cycle in the early G1 phase and to prevent the cell cycle transition from G1 to S phase[9]. Extracellular signals induce cyclin D1 expression in cells entering the cell cycle, where cyclin D1 binds to cyclin-dependent kinases CDK4/6 and activates them. The resulting complex causes RB phosphorylation and cleavage from the E2F transcription factor, which is required for expression of genes essential for cell cycle progression towards S phase. By inhibiting CDK4 and CDK6, p16 retains RB bound to E2F therefore the cell cycle cannot progress[10].

Previous studies have suggested that oxidative stress and related aging may play a role in premature rupture of membranes. The p16 pathway has not been previously studied in the case of PPROM despite a potential role in influencing senescence and oxidative stress. In this study, we aimed to determine the mRNA expression levels of p16, CDK4, CDK6, Cyclin D1, RB and E2F in patients with PPROM.

MATERIALS AND METHODS

Sample collection

The study was carried out at Department of Obstetrics and Gynecology Clinics of Cukurova University Faculty of Medicine and ethics committee approval was obtained on 02.11.2018 with a decision number of 54. It was a prospective controlled study. The study included patients whom were diagnosed with PPROM before 34 weeks of gestation by clinical (pooling of fluid in the vagina or leakage of fluid from the cervix) and/or laboratory methods (alkaline pH of the vaginal fluid as determined by Nitrazine paper). Diagnosis was made one of the 4th year resident and confirmed by one maternal fetal medicine fellow. As control group, healthy term pregnants were chosen. Pregnancies that complicated any other medical or obstetrics problems like diabetes, preeclampsia, gestational hypertension were excluded. All the patient information including time of diagnosis and delivery was prospectively recorded into hospital

database. All the patients underwent cesarean section and following delivery, the amnion and chorion membrane were separated, tissue biopsies were taken, placed in RNAlater solution and stored at -80 °C until RNA isolation.

RNA isolation and real time-PCR

Total RNA was isolated using Tri Reagent Kit (Sigma-Aldrich), according to the manufacturer's protocol. The concentration and purity of RNA samples were measured using a Nano photometer (Implen). cDNA was synthesized using Abm's OneScript® Plus cDNA Synthesis Kit with 1µg of total RNA, and quantitative PCR analyses were performed using Applied Biosystems 7500 instrument (Applied Biosystems). Ampliqon RealQ Plus Master Mix Green was used to determine the gene expressions levels. Sequences of primers used for Real-time PCR are shown in Table1. Primers were used as previously reported[11-15]. The housekeeping gene β-actin was used as an internal control for normalization.

Table 1. Sequences of primers used for quantitative (real-time) PCR

Gene	Primer
p16[11]	F:CCCACTACCGTAAATGTCCAT R: TCAAGAGAAGCCAGTAACCC
CDK4[12]	F: CTGTGCCACATCCCGAACTG R: GCCTCTTAGAAACTGGCGCA
CDK6[12]	F: CCGAAGTCTTGCTCCAGTCC R: GGGAGTCCAATCACGTCCAA
Cyclin D1[13]	F:TGATGCTGGGCACTTCATCTG R:TCCAATCATCCCGAATGAGAGTC
RB1[12]	F:GACCCAGAAGCCATTGAAATCT R:GGTGTGCTGGAAAAGGGTCC
E2F1[14]	F: CATCAGTACCTGGCCGAGAG R: TGGTGGTCAGATTCAGTGAGG
Beta-actin[15]	F: TGGCACCCAGCACAATGAA R: CTAAGTCATAGTCCGCCTAG

Statistical analysis

Statistical analyzes were performed in SPSS V22.0 (IBM, Armonk, NY) program. Power analysis was performed for the number of samples included in the study. (The sample size was determined with a standard deviation of 5% between groups, alpha 0.05, and power of 0.8). Statistical analysis of gene expression between two groups was performed using unpaired-t test. Statistical analysis of demographic characteristics was performed using Student's T-test. $p<0.05$ was accepted as significant.

RESULTS

Samples from a total of 21 PPROM and 20 control patients were acquired. Demographic characteristics of participants are given in Table 2. Both group participants were non smokers. p16, cyclinD, CDK4, CDK6, RB1 and E2F gene expression leves decreased significantly ($p=0.0001$, $p<0.05$) in the PPROM group compared to the control group (Figure1).

Table 2. Demographic characteristics of participants

	PPROM	Control	p
Mother ages (years)	29.9 ± 7.0	29.9 ± 3.9	0.7
Birth week (weeks)	32.2 ± 2.3	38.2 ± 2.2	< 0.001
Birth weight (grams)	1880 ± 548	3112 ± 352	< 0.001

PPROM: Preterm premature rupture of membranes

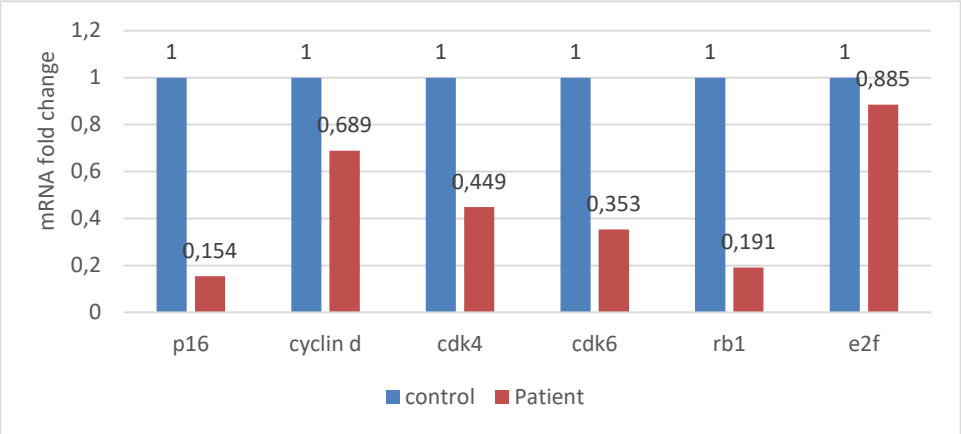

Figure 1. Expression profile of p16, cyclin D, CDK4, CDK6, RB1 ve E2F genes in PPROM and control groups (p=0.0001, p<0.05).

DISCUSSION

In this study, the expression levels of p16, Cyclin D, CDK4, CDK6, RB1 and E2F genes, in the chorioamniotic membranes were significantly lower in the PPROM group than the control group. Although it is known that as the gestational week progresses, apoptosis and senescence in the membranes increase, the lower expression levels of these genes in PPROM might suggest that oxidative stress, which is thought to play a role in the etiopathogenesis of the disease, reflects its effects through other pathways.

Unlike our results, some studies investigating other oxidative stress pathways, reported that oxidative stress triggers cellular senescence, which may contribute to PPROM pathology[16].

In a study based on this hypothesis, Feng et al. showed that hydrogen peroxide induced oxidative stress significantly induced cell senescence and p38 MAPK phosphorylation, and decreased siruin 3 (SIRT3) expression in fetal membranes and chorion cells. They also showed that hydrogen peroxide induced senescence, p38 MAPK phosphorylation, and SIRT3 downregulation were enhanced in Progesterone Receptor Membrane Component 1 (PGRMC1) knockdown cells and it has been determined that PGRMC1 had an important role in maintaining a healthy pregnancy and membrane integrity[17]. Likewise, another study showed that oxidative stress was higher and antioxidant enzymes were lower in women with PPROM compared to women, who had spontaneous premature delivery without PPROM. At the same time, the number of cells with DNA damage and the activation of p38 MAPK, which stimulates senescence, were found to be higher in PPROM[18].

In another study, p53, p21, phospho-p38 MAPK were investigated in women with PPROM and spontaneous preterm delivery and in term fetal membrane tissue in which cellular senescence was stimulated by oxidative stress (cigarette smoke extract) in vitro. In 80% of the cells with PPROM, more than 60% of the term fetal membrane cells, the cellular senescence markers; p53, p21 and phospho-p38 MAPK were found to be positive, moreover they were found in higher concentrations than those of the preterm delivery[19].

Contrary to these findings, in our study, the expression levels of the p16 gene and other genes involved in this pathway were found to be lower in pregnant women with PPROM than in term pregnant women.

Fetal membrane senescence induced by oxidative stress is mediated by telomere reduction, activation of p38MAPK, and the development of sterile inflammation called the senescence-associated secretory phenotype (SASP)[20]. p16 and p53 are targets of p38. As a result of inhibition of different cyclin-dependent kinases in both pathways, Rb cannot be phosphorylated and remains bound to the transcription factor E2F[21,22].

Therefore it is plausible to think that if oxidative stress takes place in the sample group we studied, decreased p16 expression in the patient group in our study may stem from the progression of oxidative stress-induced cell senescence via the p53 pathway.

Yet again, there are studies reporting that p21 and p27, which inhibit CDK2 and CDK4/6, cause not only dephosphorylation but also degradation of Rb[23]. The fact that CDK4 and CDK6 mRNA expressions were found to be lower in the PPROM group compared to the control, strengthens the hypothesis that senescence mechanism in PPROM proceeds via the p53-p21 pathway. On the other hand, in a study investigating the mechanism of early-onset preeclampsia, found that oxidative stress can affect the p16 pathway[24].

In another study, in which scientists opted to study telomere homeostasis and senescence expression in trophoblasts from Placenta Percreta (PP), they found that senescence markers p15 and p21 expression were higher in PP compared to the controls and in accordance with our findings p16 and p53 expressions were lower in PP group[25].

In a study conducted to investigate the possible implication of senescence in pregnancy complications, Gal et al. focused on intrauterine growth restriction (IUGR) and similar to our results they found that expressions of the senescence markers p16, p21 p53, and DCR2 were also reduced in the IUGR placentas compared with the normal placentas of the same gestational age and they thought that the disruption of the senescence mechanism might underlie IUGR pathogenesis[26].

In a study aiming to determine the relationship of placental aging and oxidative stress markers with different gestational ages in women with uncomplicated pregnancies (term and post-term pregnant) and diagnosed with preeclampsia, p16 mRNA expression was found to be lower in preeclamptic and post-term pregnancies compared to term pregnancies, but the reduction was not found statistically significant. However, it was determined that p16 protein expression was higher in post-term placenta than in term placenta and had similar expression in pre-eclamptic placenta. In the same study, it was determined that placental p21 mRNA expression was higher in preeclamptic placenta and post-term placenta compared to term samples[27].

In a study examining the effect of oxidative stress in mature, post-mature and pathological human placentas, it was found that the p21 level was significantly lower in term control placentas than both preterm controls or preeclamptic placentas, and there was no significant difference in p16 protein between the three groups[28].

Although the current findings suggest that oxidative stress plays a role in different obstetric problems, it strengthens the idea that different pathways are involved for each pathology. The most important limitation of our study was that the patient and control groups were at different gestational weeks. However, as a control group, it was theoretically almost impossible to find a healthy pregnancy that gave birth at this gestational week. In previous similar studies, healthy pregnant women, who gave birth at term were taken as the control group[24]. In our study, spontaneous preterm birth cases could be taken as a third group without PPROM, however in almost all previous studies, it has been shown that oxidative stress and senescence were lower in this group of patients compared to patients with PPROM or term pregnant, therefore inclusion of this group in our study would not make an additional contribution to the current literature. Along with those, one of the prominent strengths of our study was that the p16 pathway was investigated for the first time in this patient group. Moreover, the prospective design of our study, together with the fact that both groups had similar characteristics in terms of delivery type and conditions that may cause additional oxidative stress such as smoking, are other strengths.

In conclusion, although our study does not exclude the presence of oxidative stress and aging in the etiopathogenesis of PPROM, it can be inferred that these processes may not act through the p16 pathway. Future studies investigating oxidative stress and aging biomarkers such as β-galactosidase (SA-β-Gal) and mRNA and protein expressions of other genes in these pathways will contribute to elucidating the mechanisms of PPROM.

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