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Correlation Between Long Chain Non-Coding RNAs, ADAMTS15 mRNA and Collagen in Premature Rupture of Membranes

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Abstract— The incidence of premature rupture of membranes is approximately 12% of all pregnancies, although this varies between 2-18% of pregnancies. The pathogenesis and epigenetic aspects of premature rupture of membranes are still unclear. lncRNA, ADAMTS15 mRNA and collagen are associated with premature rupture of membranes (PROM). Thus, this study would like to analyze the correlation of lncRNA BF328678, ADAMTS15 mRNA expression and collagen levels in premature rupture of membranes. The samples taken from two groups of subject, that consist of 20 people with premature rupture of membranes and 20 people without premature rupture of membrans. The mean of lncRNA expression in spontaneous labor with premature rupture of membranes is: 139.1 ± 28.94, compared to spontaneous labor without premature rupture of membranes, is: 267.2 ± 55.84, with $p \leq 0.05$. The mean of ADAMTS15 mRNA expression in spontaneous labor with premature rupture of membranes is: 39.4 ± 10.01, compared with the results of expression on spontaneous labor without premature rupture of membranes, is: 6.95 ± 2.32, with $p \leq 0.05$. The mean of collagen levels in spontaneous labor samples with premature rupture of membranes is: 5.92 ± 1.95, compared to spontaneous labor without PROM, is: 6.95 ± 2.32, with $p \leq 0.05$. It can be concluded that premature rupture of membranes increased expression of ADAMTS 15 mRNA.

Keywords— LncRNA, mRNA ADAMTS15, collagen, premature rupture of membranes

1. Background

Premature rupture of membranes (PROM) is a rupture of the amniotic membrane that occurs before delivery at 37 to 41 weeks of gestation. The effect of PROM is the risk of developing intrauterine infection and progressing to labor. It is even more problematic if there is PROM in a very preterm gestational age, so that the baby is born with not mature organ function. As many as one third of preterm deliveries are preterm premature rupture of membranes (PPROM). Preterm labor is the leading cause of neonatal death and an estimated 1 million neonates die each year. Even in developed countries, preterm delivery remains the leading cause of neonatal death [1].

According to the World Health Organization (WHO), the incidence of PROM in the world is approximately 12% of all pregnancies, although this varies between 2-18% of pregnancies [2]. The incidence of PROM in Indonesia ranges from 4.5% - 6% of all pregnancies. Approximately 70% of PROM in cases of term pregnancy. Budijaya and Surya Negara (2016) reported cases PROM at the Sanglah Central General Hospital in Denpasar as many as 212 cases out of 1450 deliveries (14.62%) [3]. The incidence of delivery with PROM at term gestation (≥37 weeks) was 179 cases (84.43%), while at preterm were 33 cases (15.57%) [3].

The pathogenesis of PROM is unclear. Inflammation is indicated as the cause. What is still unclear is the epigenetic aspect. The epigenetic aspect plays an important role in the pathogenesis of various diseases, this is due to its ability as a regulator of gene expression. Epigenetics is currently growing rapidly and has the potential as a biomarker. Epigenetics includes methylation, acetylation, and noncoding RNAs (ncRNAs) where ncRNAs were able to attract the attention of researchers because ncRNAs were the largest portion of the total ribonucleic acid (RNA) produced by the body. As it is known, the total RNA includes protein-coding RNA (mRNAs) and protein-coding RNA (ncRNAs). Interestingly, it turns out that mRNAs are only 1-2% while ncRNA reaches 98-99%. The ability of ncRNAs is to regulate the expression of a gene and ncRNAs are divided according to the length of the strand, namely short strands (<200 nucleotides) and long strands (> 200 nucleotides). Short chains include microRNAs (miRNAs) and long chains include long chains of noncoding RNAs (lncRNA). Recent studies have demonstrated the role of lncRNA in regulating the ubiquitin-proteasome and inflammation-infection pathways [4].

In previous studies, there was a correlation between PROM with lncRNA BF328678 expression, mRNA expression a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS15) and collagen in placental tissue [4]. So in this study, we want to know correlation between PROM with lncRNA BF328678 expression, ADAMTS15 mRNA expression and collagen in the amniotic membrane.

2. Methods

This study was a cross-sectional analytic observational study. Conducted on all women who come to the Maternity Room Emergency Unit (ER) and Obstetrics and Gynecology Clinic Sanglah Hospital Denpasar with gestational age ≥ 37 with spontaneous labor, PROM and all women with gestational age ≥ 37 with spontaneous delivery without PROM, collected consecutively from targeted populations and have met the inclusion and exclusion criteria. Informed consent was given verbally and in writing. Inclusion criteria included women ≥ 37 weeks pregnant, single live fetus and head presentation. The exclusion criteria were maternal diseases such as diabetes mellitus, hypertension, and heart disease, overdistention (polyhydramnios), cervical incompetence and trauma.

This research was conducted after obtaining ethical clearance from the Health Research Ethics Committee of Sanglah Hospital, Denpasar, and implemented for 6 months from January 2018 to June 2018. The sample size was determined based on the prevalence of premature rupture of membranes in the previous study, which was 12%. The sample size was calculated using the Araoye [5] formula: The sample size calculation in this study used $\alpha = 0.05$, $d = 0.10$, so that a sample of 40.6 was obtained. To anticipate the sample dropout, 10% is added to make it 44.6. So that the total sample is 45 people.

Amniotic membrane sampling is taking the place where the amniotic rupture occurs. Samples were taken approximately 10 cm in a circle at the site of the rupture of the membranes. Samples were taken after the baby was born. RNA extraction method: weigh and take a sample (maximum 15-20 mg), crush and homogenize the sample by grinding until smooth (using a mortar and pestel), put the tissue into an eppendorf tube, add 600 µl RLT buffer, homogenize with a 20 G needle minimum 5x suction-out, Centrifuge lysate for 3 minutes at full speed, take the supernatant carefully and put the supernatant into a

new eppendorf tube, add 50% ethanol 1: 1 and mix through pipetting, take the mixture (maximum 700 µl, including the precipitated there may be) into the RNA spin column, then centrifuge 15 seconds 8000 x g. Discard the flow-through (reused collection tube), repeat the above steps until all the mixture is put into the spin column, add 700 µl RW1 buffer into the spin column, centrifuge for 15 seconds at 8000 xg. Discard flow-through (reused collection tube), add 500 µl RPE buffer into the spin column, centrifuge 15 seconds at 8000 xg, discard flow-through (collection tube is reused). Add 500 µl of RPE buffer to the spin column, centrifuge 2 minutes at 8000 xg. Discard flow-through. Transfer the spin column to a new 2 ml collection tube, centrifuge for 1 minute at maximum speed. Transfer the spin column to the 1.5 ml tube, add 30-50 µl of free-water RNAase directly to the membrane spin column, centrifuge for 1 minute at 8000 xg. Repeat the steps or use to elute and re-centrifuge. Store at -20^{0}C before proceeding to the next step.
Relative quantification of 1-step qRT-PCR was carried out using primers (Table 1):

Table 1. Primers

Target	Sequence 5'-3'
Forward lncRNA BF328678	CACATGCACGCAAATCTGTC
Reverse lncRNA BF328678	TCCCACCCACTTTATGATGG
Forward Gen ADAMTS15	TGAAGATCTCCCGAATGGAC
Reverse Gen ADAMTS15	GCTTGCCATCCATTCTCATC
Forward Gen ACTB	AGCGGGTATGAGGAGTGCAT
Reverse Gen ACTB	GGGAGCATCCTTCGTGTCTG

Amplification will be carried out in a total volume of 20 µl, consisting of 50 ng RNA from the sample, Kappa SYBR Fast qRT distilled water to 20 µl. Amplification will be carried out on a thermal cycler machine for 40 cycles. The amplification product was analyzed by electrophoresis of 1% agarose gel with 3X Gel Green dye. Gel documentation will be carried out on the Dark Reader DR46B Clare Chemical to ensure the size of the PCR product and the comparison of color intensity using Image J software. The data obtained were analyzed by doing descriptive analysis, then continued with the Kolmogorov-Smirnov normality test and the Levene test homogeneity and the T-Test to find out the mean difference between the two groups. The data analysis process was carried out using SPSS 13 for windows software.

During the study period, 45 samples were collected consisting of 24 samples of spontaneous labor with premature rupture of membranes and 21 samples of spontaneous labor without premature rupture of membranes. From the 5 sample obtained the concentration of RNA did not meet the standard. So that the total examination committed of 20 samples of spontaneous labor with PROM and 20 samples of spontaneous labor without PROM.

3. Result

Normality test with Kolmogorov-Smirnov and homogeneity test with Levene test, Obtained homogeneous results and followed by T-Test and the results of the mean lncRNA expression are as follows (Table 2):

Table 2. Mean difference test lncRNA expression

	Group	N	Average + SD	Difference Mean	p
lncRNA	Spontaneous labor PROM (+)	20	139.1 + 28.94	130.40	≤ 0.05
	Spontaneous labor PROM (-)	20	267.2 + 55.84		

In the mean difference test of lncRNA expression above, the mean expression of lncRNA in spontaneous labor samples with PROM was: 139.1 + 28.94, compared with the results of lncRNA expression in spontaneous labor without PROM, namely: 267.2 + 55.84, with $p \leq 0.05$. After the Kolmogorov-Smirnov normality test and the Levene test for homogeneity were carried out, homogeneous results were obtained and continued with the T-Test and the mean results of the ADAMTS 15 mRNA expression were as follows (Table 3):

Table 3. Mean difference test ADAMTS 15 mRNA expression

	Group	n	Average + SD	Difference Mean	p
mRNA ADAMTS 15	Spontaneous labor PROM (+)	20	39.4 + 10.01	17.09	≤ 0.05
	Spontaneous labor PROM (-)	20	10.23 + 4.68		

In the mean difference test of ADAMTS15 mRNA expression above, the mean of ADAMTS15 mRNA expression in spontaneous labor samples with PROM was: 39.4 + 10.01, compared with the results of ADAMTS15 mRNA expression in spontaneous labor without PROM namely: 10.23 + 4.68, with $p \leq 0.05$.

After the normality test with the Kolmogorov-Smirnov test and the homogeneity test with the Levene test, homogeneous results were obtained and continued with the T-Test and the results of the average collagen percentage were obtained as follows (Table 4):

Table 4. Mean difference test percentage of collagen

	Group	n	Average + SD	Difference Mean	p
%Collagen	Spontaneous labor PROM (+)	20	5.92 + 1.95	2.27	≤ 0.05
	Spontaneous labor PROM (-)	20	6.95 + 2.32		

In the mean difference test for collagen above, the mean percentage of collagen in samples of spontaneous labor with PROM was: 5.92 + 1.95, compared with the percentage of collagen results in spontaneous labor without PROM was: 6.95 + 2.32 with $p \leq 0.05$.

4. Discussion

4.1 Mean difference test lncRNA expression

Analysis of the correlation between lncRNA expression in PROM used mean as a relative measure of decreased lncRNA expression in PROM. The significance of the correlation between decreased lncRNA expression in PROM was tested by T-test with a significance level of $p \leq 0.05$.

Based on the results of the significance analysis with the T-test, the mean expression of lncRNA in samples of spontaneous labor with PROM was: 139.1 ± 28.94, compared with the results of lncRNA expression in spontaneous labor without PROM, namely: 267.2 ± 55.84, with $p \leq 0.05$. This means that there is a significant decrease in lncRNA expression in PROM.

In recent years there has been an increasing focus on non-coding RNA (ncRNA). About 98% of the total human genomic DNA has been recovered and transcribed into ncRNA. Although the roles of small ncRNAs (such as siRNA and microRNA) are well defined whereas those of lncRNA are more well characterized [6]. LncRNA is a transcribed RNA molecule of more than 200 nucleotides in length, involved in various cellular processes such as cell differentiation, imprint control, immune response, human disease and tumorigenesis. Knockdown and overexpression studies have shown that an increasing number of lncRNA play an important role in regulating a wide spectrum of processes, including splicing, transcription, localization and arrangement of subcellular compartments. Underlining the important role of lncRNA regulation is the emergence of LncRNA as a key player in the etiology of several disease conditions [7]. A number of lncRNA have been tested to alter cancer gene expression in humans and are regulated by specific oncogenic and tumor suppressor pathways, such as p53. It can be concluded that lncRNA regulates tumor suppressor genes which are important regulators of the cell cycle [8].

Previous studies of PROM have suggested that PROM is associated with genital infection and inflammatory reactions of the cytokine and chemokine pathways and involves the extracellular matrix. However, the molecular pathogenic mechanisms underlying pathogenic pathogens in PROM appear to be more complicated, wherein lncRNA regulates gene expression, can regulate different metabolic pathways and participate widely in biological processes [9].

Infection will induce the activation of matrix metalloproteinases (MMPs) so that MMPs increase, recently it has been shown that this is related to the weakness of the collagen membrane that causes the membranes to rupture. Infection of microorganisms and recognition of pathogens further suggests that genital infection and inflammatory reactions are important pathological factors in PROM. In addition, in the group with PROM it was found that p53 is an important pathway that leads to apoptosis and has been shown to play a role in the development of PROM [10]. The regulated p53 signaling pathway may explain that microorganism infection can initiate apoptosis of cell membranes via the p53 signaling pathway. It is hoped that lncRNA plays a suppressor function for gene expression resulting in increased mRNA transcription. This may be the reason it is possible for the lncRNA to be placed in the same or the antisense strands of the associated mRNA locus. However, in the PROM group low lncRNA was found. A possible mechanism of mRNA regulating lncRNA is that it can bind to miRNA, protecting mRNA from targeting and repression of miRNA [4].

All RNA is transcribed in the nucleus, but it can be processed and exported differently. MicroRNAs (miRNAs) and long noncoding RNAs (lncRNA) can also be imported back into the nucleus where they can influence gene regulation through chromatin structure and epigenetic modification. The full function of lncRNA and circular RNAs (circRNAs) still needs further explanation [11].

In another study found specific differences in lncRNA expression observed between placenta and amniotic membrane, studies in mice found lower lncRNA in virus-infected than in those not virus-infected. Among the lncRNA found in virus-infected placentas, the differential expression of lncRNA associated with the proteasome (prosome, macropain) of the 26S subunit and the non-ATPase subunit was determined to be up or down regulation. Viral infection is rare, but viral particles are almost undetectable in human amniotic fluid in pregnancy. Mostly in humans, viral infection creates intolerance or an immune imbalance and creates an environment predisposing to secondary bacterial infection. Recent studies in animal models have shown that viral infection at the implantation site influences the immunological response to bacteria. products by promoting an inflammatory response that leads not only to preterm labor but also the possibility of a wider range of outcomes. Currently, there are no biomarkers to document viral infection. It would be interesting to know whether this differently expressed lncRNA can also be found in the human placenta and fetal membranes from 'idiopathic' PTB cases and can serve as a biomarker for viral infection during pregnancy [12].

Identification of thousands of lncRNA expressed differently from human placenta with cases of PPROM, full term birth (FTB), preterm birth (PTB), and PROM providing evidence that lncRNA can participate in physiological processes and pathogenic human pregnancy relevant to reproductive conditions and disorders. Further characterization of the metabolic pathways supports previous findings that infection and inflammatory response, extracellular matrix (ECM) receptor interactions, apoptosis and smooth muscle contraction are the main pathogenic mechanisms involved in the development of PPROM, along with PROM and PTB. Although the detailed function and pathogenesis of how individual lncRNA play their role in PPROM and PTB is still unknown [13].

Although lncRNA has been reported to be involved in many diseases that occur in humans, such as neurodegenerative, psychiatric, cardio, autoimmune and carcinogenic, there are still very few new publications on pregnancy and lncRNA [4].

4.2 Mean difference test of ADAMTS15 mRNA expression

Analysis of the correlation between ADAMTS15 mRNA expression in PROM used mean as a relative measure of the correlation between increased ADAMTS15 mRNA expression in PROM. The significance of the relationship between increased ADAMTS15 mRNA expression in PROM was tested by T-test with a significance level of $p \leq 0.05$.

Based on the results of the analysis of the significance with the T-test, the mean ADAMTS15 mRNA expression in spontaneous labor samples with PROM was: 5.92 ± 1.95, compared with the results of ADAMTS15 mRNA expression in spontaneous labor without PROM, namely: 6.95 ± 2.32, with $p \leq 0.05$. This means that there is a significant increase in ADAMTS15 mRNA expression in PROM.

In studies where lncRNA and associated mRNA were in different strands (lncRNA in the antisense chain, and mRNA in the main chain) and had opposite expression patterns, containing the genes ADAMTS15, STAM, and EML [4]. ADAMTS15 is coded as a member of the ADAMTS protein family. Proteins in the ADAMTS family share several different protein modules, including propeptide regions, metalloproteinase domains, disinterring-like domains, and type 1 thrombospondin motifs [14].

Researchers have found that the degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) in the fetal membrane plays an important role in membrane rupture in PPROM. Moreover, several other non-MMPs with proteinase domains, such as serine proteases, cysteine proteases, and members of the ADAMTS family, can also degrade extracellular matrix (ECM) amniochorion [15]. The expression of ADAMTS15-regulated mRNA in the PPROM group may indicate that ADAMTS15 may function as an

MMP that participates in the regulation of collagenolytic and ECM degradation in PPROM [16].

The host inflammatory response induced by different proinflammatory cytokines (including IL-1β, IL-6, and TNF-α) is closely related to PPROM. Apoptosis has been shown to play an integral role in PPROM, and IL-6 has also been shown to promote MMP activation or fetal membrane apoptosis in PPROM. Since the lncRNA and mRNA from ADAMTS15 are located in opposite strands, the lncRNA in this category can be complementary to the associated mRNA. Although there is no evidence to date to suggest that lncRNA can directly downgrade mRNA via RNA-like pathways such as miRNA, the expression patterns of lncRNA and mRNA in this category may explain with other recent findings that lncRNA could be the host gene for small RNA. These lncRNA can suppress their mRNA via RNA-like pathways, which may be the mechanism by which lncRNA regulates mRNA in this category [4].

4.3 Mean difference test of percentage of collagen level

Analysis of the collagen correlation in PROM used mean as a relative measure of the correlation between collagen reduction in PROM. The significance of the relationship between collagen reduction in PROM was tested by T-test with a significance level of $p \leq 0.05$. Based on the results of the significance analysis with the T-test, the mean collagen in samples of spontaneous labor with PROM was: 5.92 ± 1.95, compared with the mean results of collagen in spontaneous labor without PROM, namely: 6.95 ± 2.32, with $p \leq 0.05$. Which means that there is a significant decrease in collagen levels in PROM. Recent studies have focused on the pathophysiological features of matrix degradation, mainly due to infection and inflammation. Increases in matrix metalloproteinases (MMPs) with associated decreases in MMP inhibitors have been demonstrated in PROM [17].

The fetal membrane consists of two distinct parts: the amnion and the chorion. Chorion contributes to membrane strength and elasticity, but to a lesser extent than amnion. Regardless of the initiation of the pathway, MMP, proinflammatory cytokines, apoptosis, and oxidative stress are the main factors causing the loss of extracellular matrix and collagen, reduced membrane tensile strength, and PPROM. Extracellular collagen is determined by various MMPs, and MMP can degrade many types of extracellular collagen, cell surface receptors, release apoptosis-inducing ligands (such as FAS ligands), and activate cytokines and chemokines. Fetal membrane apoptosis, mediated by the TNFα pathway or the p53 pathway, has also been associated with PPROM. Studies consistently show higher rates of apoptosis in membranes of women with PPROM when compared with women with preterm labor and intact membranes [18].

There is a difference from the collagen content in the amniotic membrane in cases of PROM, so that collagen content is reported in cases of PROM. lower than the normal amniotic membrane [19]. In the current study, it was found that there was a reduction of collagen type III in cases of PROM. As is well known, type II collagen is the interstitial collagen that is the pillar of the extracellular matrix, which supports the tissue almost similar to type I collagen. The tissue contains a lot of type II collagen which will be very elastic. The decrease of type III collagen will cause a decrease in the elasticity of the amniotic membrane [20].

Epigenetic regulation of lncRNA in the ubiquitin proteasome system (UPS), and collagen remodeling in PROM. The differences in expression of lncRNA identified from human placentae and fetal membranes indicated that the decrease in collagen was not only due to collagen degradation but also influenced by suppression of the expression of collagen lncRNA [21].

5. Conclusion

Low lncRNA expression was found in the amniotic membrane at the PROM group with ADAMTS 15 mRNA expression which was high in the amniotic membrane at the PROM group and low collagen levels at amniotic membrane at the PROM group, caused the strength of the membranes to be weakened and easily ruptured.

6. References

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