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Low Methylation of Matrix Metalloproteinase 1 *(MMP1)* is Associated with Preterm Labour in Malaysian Mothers

(Metilasi Rendah Matriks Metalloproteinase 1 (*MMP1*) dikaitkan dengan Kelahiran Bayi Pramatang dalam kalangan Ibu di Malaysia)

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ABSTRACT

Preterm births comprise 10.6% of livebirths worldwide and account for 35% of deaths among newborn babies. Understanding DNA methylation may offer basic knowledge in the understanding of pathogenesis of preterm labour. The study was undertaken to determine DNA methylation of matrix metalloproteinase 1 (*MMP1*) promoter in term and preterm labour using methylation-specific polymerase chain reaction (MSP). Thirty maternal venous blood samples (n=15 each) of term and preterm labour was subjected to bisulfite treatment prior to MSP. This result was then validated using DNA sequencing. Evaluation of the sequencing results by CpG islands analysis was performed using the ClustalW and SPSS software. Primers for *MMP1* were located between -1226 and -1378 upstream from the transcription start site (TSS) that consisted five CpG islands. Preterm labour group had significantly lower methylated CpG islands with 39 out of total 75 (52%) compared to the term labour that has 49 out of 75 methylated CpG islands (65.33%) ($t=0.694$, $p<0.05$). Methylation occurred in 4 out of 5 methylated CpG islands in the *MMP1* promoter where it only involved 2 preterm samples (13.33%) and 7 term samples (46.47%). This data suggested there were significant lower percentage of methylated *MMP1* in preterm labour. Higher percentage of methylated *MMP1* as observed in the term labour, will probably reduce the expression of *MMP1*, thus maintaining fibrillar collagen strength on the amniotic membrane and subsequently maintain the pregnancy till term. In conclusion, preterm labour has higher percentage of methylated CpG compared with term labour in *MMP1* gene.

Keywords: CpG islands; DNA methylation; matrix metalloproteinase 1; methylation-specific PCR; preterm labour

ABSTRAK

Kelahiran bayi pramatang merangkumi 10.6% kelahiran hidup di seluruh dunia dan menyumbang 35% kematian dalam kalangan bayi yang baru lahir. Pemahaman mengenai metilasi DNA boleh menyumbang kepada asas pengetahuan dalam memahami patogenesis kelahiran bayi pramatang. Kajian ini dilakukan untuk menentukan metilasi DNA matriks metalloproteinase 1 (*MMP1*) promoter dalam kelahiran bayi matang dan bayi pramatang menggunakan analisis bisulfit, metilasi khusus tindak balas rantaian polimerase (MSP). Tiga puluh sampel darah vena ibu (n=15 setiap kumpulan) daripada kelahiran bayi matang dan bayi pramatang menjalani rawatan bisulfit dan seterusnya MSP. Keputusan MSP disahkan menggunakan penjujukan DNA. Penilaian keputusan penjujukan bagi analisis gugusan CpG dibuat menggunakan perisian ClustalW dan SPSS. Primer bagi *MMP1* terletak di antara -1226 dan -1378 daripada lokasi permulaan transkripsi (TSS) gen *MMP1* yang mengandungi lima gugusan CpG. Kumpulan kelahiran bayi pramatang mempunyai gugusan CpG metilasi yang signikannya lebih rendah iaitu 39 daripada keseluruhan 75 (52%) berbanding kelahiran bayi matang yang mempunyai 49 daripada jumlah keseluruhan 75 CpG (65.33%) ($t=0.694$, $p<0.05$). Metilasi dikesan pada 4 daripada 5 gugusan CpG dalam promoter *MMP1* dan ia hanya melibatkan 2 sampel pramatang (13.33%) dan 7 sampel kelahiran matang (46.47%). Data ini menunjukkan terdapatnya peratusan signifikan metilasi *MMP1* yang

lebih rendah dalam kelahiran bayi pramatang. Peratusan metilasi *MMP1* yang lebih tinggi dalam kelahiran bayi matang berpotensi menyebabkan ekspresi *MMP1* berkurangan dan mengekalkan kekuatan kolagen fibrilar membran amnion, seterusnya mengekalkan kehamilan sehingga tempoh matang. Kesimpulannya, kelahiran bayi pramatang mempunyai peratusan gugusan CpG metilasi yang lebih rendah berbanding kelahiran bayi matang bagi gen *MMP1*.

Kata kunci: Gugusan CpG; kelahiran bayi pramatang; matriks metalloproteinase 1; metilasi DNA; metilasi khusus tindak balas rantaian polimerase (MSP)

INTRODUCTION

Spontaneous preterm labour is defined as labour resulting in birth before 37 completed weeks (259 days) of gestational age (WHO 1970). Preterm births comprise 10.6% of livebirths worldwide in 2014 and account for 35% of deaths among newborn babies in 2017 (Chawanpaiboon et al. 2019; Hug et al. 2017). The overall statistics at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) recorded 5822 deliveries in the year 2008. Of the total, 139 (2.39%) were preterm deliveries, which was 1 in every 50 term babies. This percentage may not fully reflect the experience elsewhere in Malaysia and may be an underestimate of the real numbers of preterm deliveries as a nationwide registry data is unavailable.

The etiology of preterm labour is unknown in more than 50% of cases (Phillips et al. 2017). About 30 to 40% of preterm labour, however, is associated with preterm premature rupture of membrane (PPROM) (Phillips et al. 2017; Tchirikov et al. 2018). The underlying pathology related to PPROM is related to the under synthesis of fetal membrane collagen or the degradation of fibrillar collagens (Menon & Richardson 2017; Modi et al. 2017; Shah 2017). This negative pregnancy outcome has been linked with epigenetic alterations (de Andrade Ramos & da Silva 2018; Linner & Almgren 2020; Zakar & Paul 2020).

We chose DNA methylation, a well-documented component of epigenetics, to address its role in obstetrics area especially in preterm labour (Hong et al. 2018; Konwar et al. 2018; Santos et al. 2019). Its inheritance nature, capability in regulating gene expression and has the storage mechanism for lifelong molecular information (Kim & Kaang 2017; Skinner et al. 2018). Moreover, this process is reversible and preventable (Dugué et al. 2020; King et al. 2016). Methods such as microarray and methylation specific polymerase chain reaction (MSP) are used to determine the DNA methylation status in a disease (Liu & Maekawa 2003; Soozangar et al. 2018). The MSP was chosen for this research in view of its simplicity and cost effectiveness (Herman et al. 1996; Sestakova et al. 2019).

We focused our interest on the matrix metalloproteinase 1 *(MMP1)* gene. This gene encodes a secreted enzyme that degrades extracellular matrix through breaking down the type II and III interstitial collagens (Amar et al. 2017; Eo et al 2016; Nagase & Woessner 1999). The gene is localized at chromosome 11q22.3 (Krane 1995). A previous large cross-sectional study was conducted to determine whether spontaneous rupture of membranes was associated with a change in the amniotic fluid concentration of *MMP1* (Maymon et al. 2000). High concentration of *MMP1* was observed in the advanced stage of pregnancy and during parturition in normal pregnancies (Geng et al. 2016). Furthermore, several studies have suggested that the imbalance between MMPs and tissue inhibitor metalloproteinase 1 (*TIMP-1*) in amniotic fluid will promote the PPROM (Litwiniuk et al. 2017; Lombardi et al. 2018; Vadillo-Ortega et al. 1996). PPROM, either with the presence or absence of infection was associated with an increase in the amniotic fluid *MMP1* concentration (Maymon et al. 2000; Myntti et al. 2017).

MMP1 also plays a role in term and preterm labour by an inflammatory mechanism that involved in softening and dilatation of the cervix (Frey et al. 2016; Geng et al. 2016). An Indian study reported that placental mRNA levels of *MMP-1* was higher, and *TIMP-1* were lower in the placentae of women delivering preterm as compared to term. Higher mRNA level of *MMP-1* was seen in the placentae of those delivering preterm as compared to term in both groups of women with spontaneous vaginal deliveries and preterm labor (Sundrani et al. 2017). A much earlier interesting study documented that inhibition of DNA methyltransferase with 5-aza-2'-deoxycytidine in amnion fibroblasts has resulted in a significant increase in the *MMP1* gene transcription (Wang et al. 2008). These results correlated with the reduction of DNA methylation at a particular site, which was at -1538bp from the transcription start site (TSS) in the *MMP1* promoter. A single nucleotide polymorphism [AF007878.1 (MMP1):g.3447 T>C] was also observed in the *MMP1* promoter. This study concluded that in addition to genetic mutation, DNA methylation may play

a role in controlling *MMP1* expression thereby influenced the risk for preterm labour (Wang et al. 2008).

There is paucity of data on the relationship between DNA methylation and preterm labour specifically in Malaysia and in Asian countries. The outcome of this research could help on further understanding of epigenetics changes involving *MMP1* in preterm labour. We hypothesized that DNA methylation pattern in the *MMP1* promoter was associated with its abnormal expression in preterm labour. In addition, this study has the aim to assess the effectiveness of MSP as a qualitative method to detect the methylation marker, *MMP1* in term and preterm labour.

Materials and Methods

CLINICAL SAMPLES

Patients were selected among women who underwent vaginal delivery or Caesarean section with spontaneous preterm labour at gestational ages between 24 and 36 weeks and 6 days, singleton pregnancy, normal pregnancy and no known chronic medical history. The babies were considered healthy with normal external physical appearance. Exclusion criteria were patients who were having obstetrical history of preterm birth or PPROM, medical complications of pregnancy requiring induction of labour, multiple gestations and placental abnormalities such as placental praevia, chorioamnionitis and babies with major fetal anomalies. Control samples were obtained from mothers of singleton pregnancies delivered at term with no prior history of PPROM or preterm labour. Blood samples were taken within one hour following the labour. The blood was aliquot to six tubes containing 0.5 mL blood each to preserve the purity of the sample during the sample processing and kept in -80 °C freezer until further analyzed.

GENOMIC DNA EXTRACTION AND BISULFITE TREATMENT

DNA extraction and bisulfite treatment from blood was carried out using the EZ DNA Methylation-Direct™ Kit (Zymo Research Corporation, Orange, California, USA) according to the manufacturer's protocol (Ørntoft et al. 2017). All cytosines will be converted to uracil, except those that were methylated (5-methylcytosine), which were resistant to modification and remained as cytosine (Pajares et al. 2020; Singer 2019). The end product of this treatment was the recovered bisulfite-converted DNA, single stranded with methylated and unmethylated DNA. The purity and concentration of the DNA were determined by the spectrophotometer (NanoDrop ND-1000) (Thermo Scientific, United Kingdom).

METHYLATION-SPECIFIC PCR (MSP)

Bisulfite-treated genomic DNA was amplified using either methylation-specific primers or unmethylated-specific primers (Herman et al. 1996). Sequences for methylation-specific forward primer were 5'-AGGGAAGTTATGGTGTTATCG-3' and reverse primer were 5'- ACGCACCTAATAACTATTCGAC-3'. Sequences for unmethylated-specific forward primer were 5'-AGGGAAGTTATGGTGTGTTATT-3' and reverse primer were 5'- ACTACACACCTAATAACTATTCAAC-3'. These primers covered a region in between -1226 and -1378 upstream from the TSS of *MMP1* promoter region, which consisted of five CpG islands with the total number of 75 for both term and preterm groups. CpG islands are defined as regions of DNA 500-1500 bp long with a CG: GC ratio of more than 0.6, and they are normally found at promoters and contain the 5' end of the transcript (Cross & Bird 1995).

MSP was performed using the EpiTech MSP PCR kit (Qiagen; Hilden, Jerman) as stated by the manufacturer's protocol. The MSP was carried out using cycle parameters of initial activation step at 95 ºC and denaturing at 94 ºC; and amplification reaction was performed by annealing process at 56.1 ºC for 30 s for *MMP1* methylated and 53.7 ºC for *MMP1* unmethylated. Final step of the MSP was an extension at 72 ºC. All these steps were conducted using the thermal cycler MyCycler (BioRad, United Kingdom). PCR products were separated by 1.2% agarose gel and bands were scanned with an ultraviolet transilluminator (Alpha Innotech Corporation, San Leandro, CA). Each PCR product was purified using the QIAquick PCR purification kit (Qiagen; Hilden, Jerman) prior to DNA sequencing.

DNA SEQUENCING

DNA sequencing was carried out using 3130xl Applied Biosystems Genetic Analyzer sequencing machine (ABI, California, USA). The reaction contained 0.2 µL Big Dye Terminator v3.1, 0.3 µL Big Dye v3.1 buffer (ABI, California, USA), 20 ng/µL of purified PCR product as the template and forward and reverse oligonucleotide primers, which were similar as in the MSP method, to the final volume of 20 µL. The whole reaction underwent purification process using 2 µL 3M sodium acetate Ambion (Life Technologies, California, USA), 2 µL 0.5 M acid ethilendiaminetetraacetic (EDTA) (Amresco, Ohio, USA), 150 µL 100% ethanol (Fisher Scientific,

United Kingdom) and 10μL HiDi Formamide (ABI, California, USA). This reaction underwent cycle sequencing process in the thermal cycler Takara PCR Gradient Model TP600 (Takara, Japan). Data of the total methylated CpGs and CpGs sequences distribution in the DNA sequencing was recorded in the ClustalW software.

DATA ANALYSIS

We used an online tool, which is ClustalW at http://www.ebi.ac.uk/Tools/msa/clustalw2/ to align multiple sequences of *MMP1* that were highly conserved to evaluate the sequencing results by CpG islands analysis (Chang et al. 2020; Hung & Weng 2016). The score table shows the pairwise scores calculated for every pair of sequences that is to be aligned. Pairwise scores are simply the number of identities between the two sequences, divided by the length of the alignment (Hung & Weng 2016; Thompson et al. 1994). The study is one to one comparison or case-by-case comparison. Using the probability (power) of 0.951, we have calculated a total of 30 samples for the whole experiment with 15 samples for each sample and control groups. Statistical data analysis was done using the SPSS version 19 software, which include paired t-test and correlation test.

RESULTS

DEMOGRAPHICAL DATA

The summary of demographical data of the women is presented in Table 1. The mean age for the control group was 27.267 ± 4.964 years old, while for the preterm labour was 27.867 ± 6.632 years old. The preterm labour women have significantly shorter gestation age, 244.667 ± 15.656 days as compared to the term labour women, which is 275.467 ± 7.308 days ($p<0.01$). Preterm babies had significant lower birth weight, which was 2.440 ± 0.446 kg compared to term babies (3.281 ± 0.284 kg) ($p<0.01$). Other variables which include age, gravida and parity were not significantly different between both groups. Correlations between variables in the study are shown in Table 2. The correlation for preterm labour and gestational days was strong positive correlation with r equals to 0.794 and $p<0.01$. There was also strong positive correlation for birth weight and gestational days with r equals to 0.877 and $p<0.01$. Preterm labour and birth weight have strong positive r value (0.738, $p<0.01$).

TABLE 1. Demographical data of term and preterm groups with t-test result. Only gestation days and birth weight showed significant t-test

Variable	Preterm	Mean ± SD	T-test	p
Age (years)	Yes No	27.867 ± 6.632 27.267 ± 4.964	t=0.185	p=0.781 (>0.05)
Gravida	Yes No	2.000 ± 1.558 1.933 ± 1.280	t=0.839	p=0.899 (>0.05)
Parity	Yes No	0.800 ± 1.082 0.733 ± 1.280	t=0.634	p=0.879 (>0.05)
Gestation (days)	Yes No	244.667 ± 15.656 275.467 ± 7.308	t=6.904	p=0.000000167** (<0.01)
Birth weight (kg)	Yes No	2.440 ± 0.446 3.281 ± 0.284	t=6.156	p=0.000001200** (<0.01)

SD = standard deviation, **p <0.01

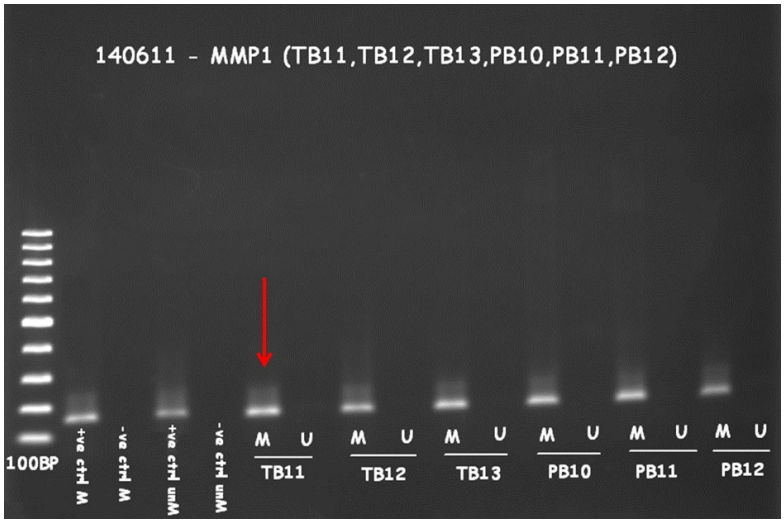
TABLE 2. Correlations between variables of preterm labour, gestation days and birth weight

Variable	r	p
Preterm labour Gestation (days)	0.794	0.0000001670**
Preterm labour Birth weight (kg)	0.738	0.0000012000**
Birth weight (kg) Gestation (days)	0.877	0.0000000002**

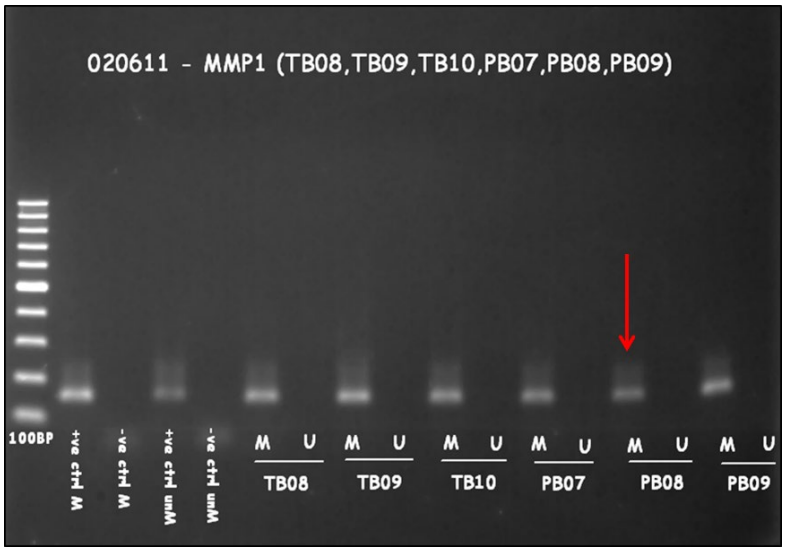
All variables show positive strong correlations, r>0.7, **p<0.01

METHYLATION SPECIFIC PCE, DNA SEQUENCING AND DATA ANALYSIS

Post bisulfite treatment, there are 31 sites of cytosine in the DNA product (*MMP1*) will be converted to thymine. The bisulfite treatment would convert all unmethylated cytosine which are not located within the CpG islands to thymine, however the methylated cytosine within the five CpG islands in *MMP1* would remain as cytosine. This region of methylated *MMP1* primers contained five CpG sites. The first CpG was located at -1246, the second CpG -1299, the third CpG -1310, the fourth CpG -1356 and the fifth CpG -1373 of TSS of *MMP1* promoter. These locations were based on the sequence of promoter region of *MMP1* (NCBI accession no: AJ002550.1) with a size of 1899 bp. Thus, each sample for both term (n=15) and preterm (n=15) consisted of five conserved CpG sites. All the term (TB) and preterm (PB) samples showed positive bands after amplification with methylated primers (Figure 1). Using the methylated *MMP1* primers, the PCR reaction gave a product of 150 bp in length. The gel electrophoresis of the MSP products showed positive bands with different density in all methylated samples of TB and PB. Despite the presence of positive bands in all samples, the degree of methylation was most likely different between term and preterm sample.



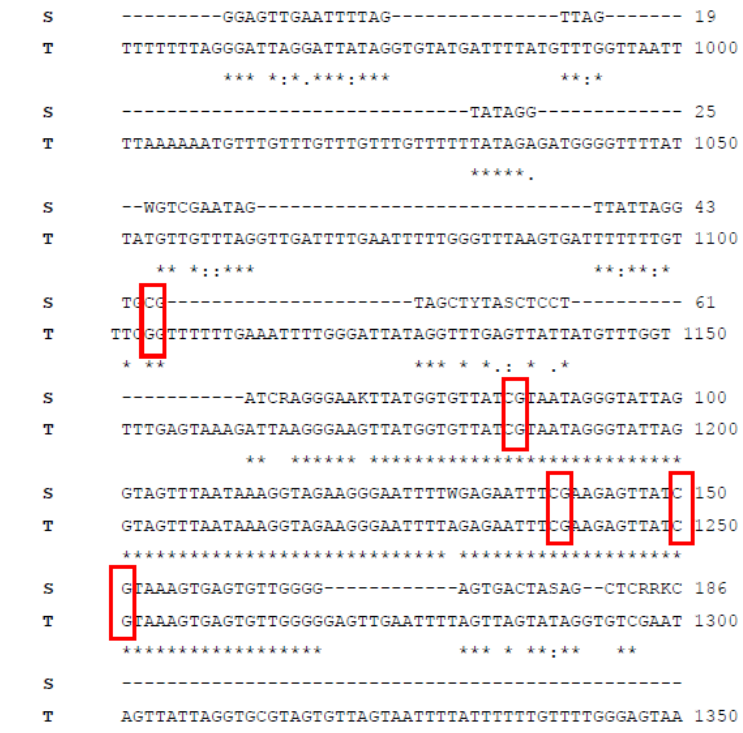
(a)



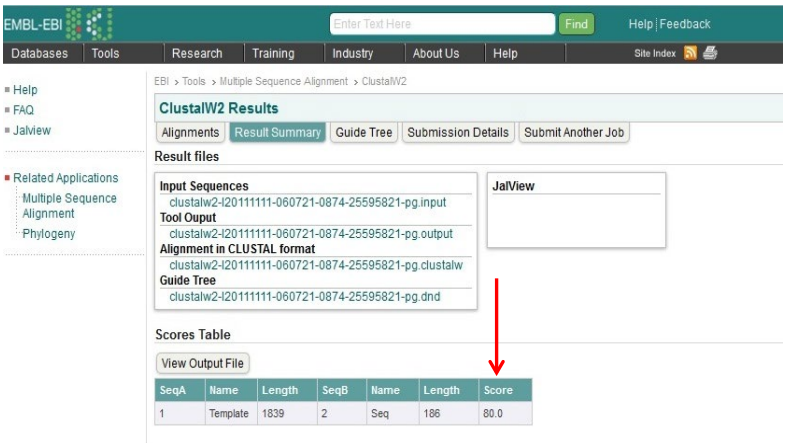
(b)

FIGURE 1. MSP agarose gel electrophoresis. For methylated *MMP1*(M)*:* '+ve ctrl M' is positive control and '-ve ctrl M' is negative control. For unmethylated *MMP1*(U)*:* '+ve ctrl unM' is positive control and '-ve ctrl unM' is negative control. TB: term and PB: preterm. The MSP agarose gel electrophoresis showed positive bands with different density in all methylated samples of TB and PB. (a) MSP agarose gel electrophoresis on samples TB11, TB12, TB13, PB10, PB11 and PB12. Red arrow shows sample TB11. (b) MSP agarose gel electrophoresis on samples TB08, TB09, TB10, PB07, PB08 and PB09. Red arrow shows sample PB08

These samples with positive bands detected from the gel electrophoresis were subjected to DNA sequencing to confirm the presence of methylated *MMP1*. The chromatograph results were analyzed using the ClustalW software to confirm that the sequence of *MMP1* was true and within the acceptable score range. All samples (n=30 for both groups) scored between 78 and 92% matching, which was interpreted as good similarity, after the alignment of the sequencing product templates (150 bp) compared with the bisulfite conversion of methylated *MMP1* sequence (1839 bp). Figures 2 and 3 show *MMP1* sequence in TB11 and PB08 was confirmed by software ClustalW score table. The percentage scoring for TB11 was 80% and PB08 was 78%.



(a)



(b)

FIGURE 2. *MMP1* sequence in a sample of term birth (TB11). (a) *MMP1* sequence confirmed by software ClustalW alignment. 4 CpGs islands marked by red box. S: sequence of TB11 and T: template of bisulfite conversion of methylated *MMP1* sequence. (b) *MMP1* sequence in TB11 was confirmed by software ClustalW score table. The percentage scoring for TB11 shown by red arrow (80%)

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S      ------------GTTGAATTTTAGTT----AGTATAGGAG---------- 24
T      TATGTTGTTTAGGTTGATTTTGAATTTTTGGGTTTAAGTGATTTTTTTGT 1100
                  *****:*** *.**    .**:**.*:*

S      -TCG--------------------AATAG-------TTATTAGG------ 40
T      TTCGGTTTTTTGAAATTTTGGGATTATAGGTTTGAGTTATTATGTTTGGT 1150
        ***                    :****       ****** *

S      --TGCRTACK-----AGGGAAGTTATGGTGTTATCGTAATAGGGTATTAG 83
T      TTTGAGTAAAGATTAAGGGAAGTTATGGTGTTATCGTAATAGGGTATTAG 1200
         **. **.      ************************************

S      GTAGTTTAATAAAGGTAGAAGGGAATTTTARAGAATTTCGAAGAGTTATC 133
T      GTAGTTTAATAAAGGTAGAAGGGAATTTTAGAGAATTTCGAAGAGTTATC 1250
       ****************************** ******************

S      GTAAASTGAGTGTGGGG-------------------------------- 150
T      GTAAAGTGAGTGTTGGGGGAGTTGAATTTTAGTTAGTATAGGTGTCGAAT 1300
       *****.******* ***

S      AGARCTTAGMCGC--------AGKSKST---------------------- 170
T      AGTTATTAGGTGCGTAGTGTTAGTAATTTTATTTTTTGTTTTGGGAGTAA 1350
       **: .****  **        **.: :*
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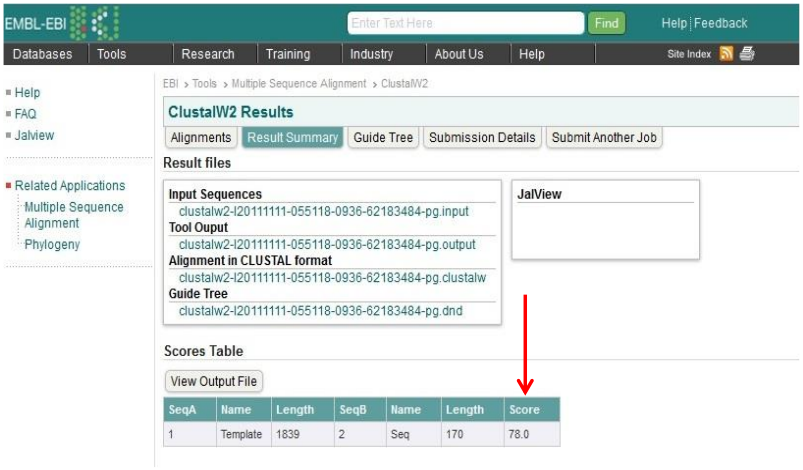
(a)



(b)

FIGURE 3. *MMP1* sequence in a sample of term birth (PB08). (a) *MMP1* sequence confirmed by software ClustalW alignment. 4 CpGs islands marked by red box. S: sequence in PB08 and T: template of bisulfite conversion of methylated *MMP1* sequence. (b) *MMP1* sequence in PB08 was confirmed by software ClustalW score table. The percentage scoring for PB08 shown by red arrow (78%)

Table 3 shows the total methylated CpG islands of *MMP1* promoter in term and preterm groups. In this analysis, there were 75 total CpG islands in each group of 15 term samples and 15 preterm samples. Term labour had a higher number of methylated CpG islands of 49/75 (65.33%), as compared to preterm labour group, which was only 39/75 (52%). There was also significant difference of mean methylated CpG islands of *MMP1*

promoter between term (3.267 ± 0.799) and preterm groups (2.600 ± 0.910) with t=0.694 and p<0.05 (Table 4). Interestingly, there was also significant correlation with p<0.05 between preterm labour (r=0.374) and birth weight (r=0.444) with total number of methylated CpG islands of *MMP1* promoter (Table 5).

TABLE 3. Total methylated CpG islands of *MMP1* promoter in term and preterm groups (15 samples × 5 CpG islands, n=75 for each group)

CpG islands	Total methylated CpG islands in term	Total methylated CpG islands in preterm
Total number of methylated CpG islands of *MMP1* promoter – number (%)	49/75 (65.33)	39/75 (52.00)

TABLE 4. T-test for mean methylated CpG islands of *MMP1* promoter in term and preterm groups

Group	N	Mean (no) ± SD	T-test	p
Term	15	3.267 ± 0.799	t=0.694	p=0.042*
Preterm	15	2.600 ± 0.910		

*p <0.05

TABLE 5. Correlation between variables of preterm labour and birth weight with total number of methylated CpG islands of *MMP1* promoter

Variable	r	p
Preterm labour Total number of methylated CpG islands	0.374	0.042*
Birth weight (kg) Total number of methylated CpG islands	0.444	0.014*

*p< 0.05

Data in Table 6 shows total methylated CpG islands of *MMP1* promoter in each term and preterm groups in relation to number and sequences location of methylated CpG islands. Seven out of 15 term samples (46.47%) had the highest number of methylated CpG sites of *MMP1* promoter involved, which were four methylated CpGs out of five sites. On the other hand, only two out of 15 preterm labour cases (13.33%) had four methylated CpG sites involved. Just below 50%, 46.67% of preterm labour cases had three out of five methylated CpG sites involved. Further analysis on the number of methylated CpG islands with regards to the five CpG islands sequences location showed that the highest methylation was at the -1356 site (methylated 4th CpG) of *MMP1* promoter for term (15/15, 100%) and preterm samples (14/15, 93.33%) (Table 6). The lowest number of methylated CpG islands in the preterm and term groups was seen at the -1373 site (methylated 5th CpG) with 6.7% and 33.33% respectively. Figure 4 shows number of methylated CpG islands in *MMP1* promoter in term group were statistically significant at region CpG2 (t=0.867, p<0.05), CpG3 (t=0.018, p<0.01) and CpG5 (t=0.001, p<0.01) as compared to the preterm group. However, there were no statistically significant differences for CpG1 or CpG4.

TABLE 6. Total methylated CpG islands of *MMP1* promoter in each term and preterm groups in relation to number and sequences of methylated CpG islands

Methylated CpG islands	Term labor (n=15)	Preterm labor (n=15)
Number of methylated CpG islands in each group based on total 5 CpGs – number (%)		
One methylated CpG	0/15 (0)	2/15 (13.33)
Two methylated CpGs	3/15 (20.00)	4/15 (26.67)
Three methylated CpGs	5/15 (33.33)	7/15 (46.67)
Four methylated CpGs	7/15 (46.67)	2/15 (13.33)
Five methylated CpGs	0/15 (0)	0/15 (0)
Number of methylated CpG islands in each group with regards to the five CpG islands sequences location – number (%)		
Methylated 1st CpG (CpG1)	10/15 (66.67)	12/15 (80.00)
Methylated 2nd CpG (CpG2)	7/15 (46.67)	3/15 (20.00)
Methylated 3rd CpG (CpG3)	12/15 (80.00)	9/15 (60.00)
Methylated 4th CpG (CpG4)	15/15 (100.00)	14/15 (93.33)
Methylated 5th CpG (CpG5)	5/15 (33.33)	1/15 (6.67)

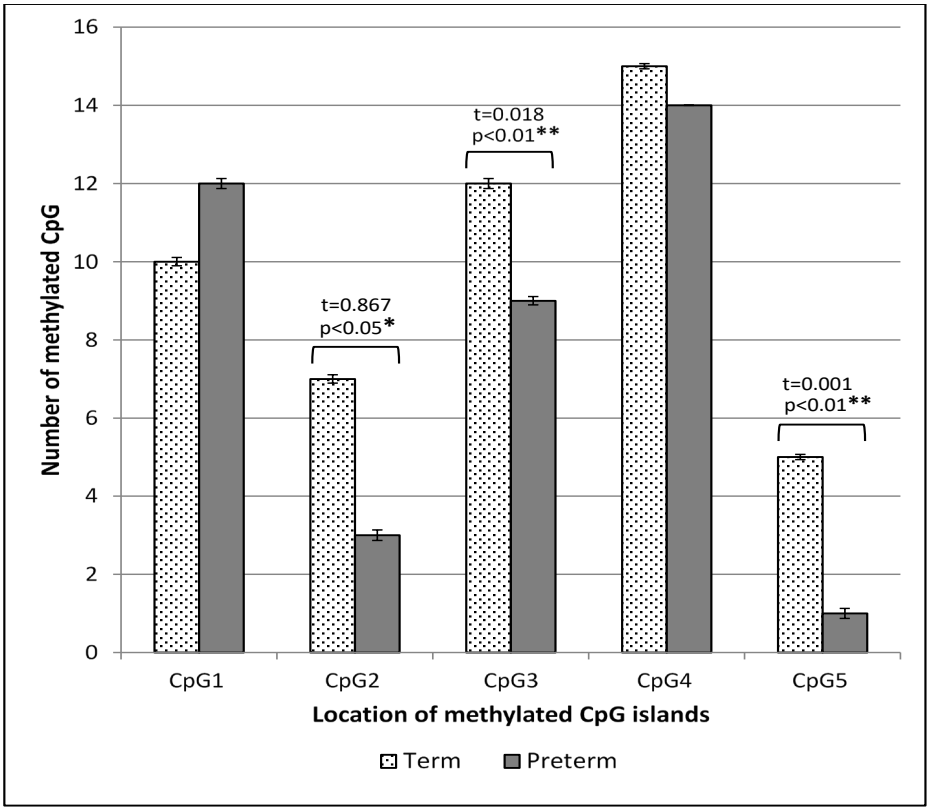


FIGURE 4. Number of methylated CpG islands in *MMP1* promoter with regards to the five CpG islands sequence's location in the term and preterm groups. Results of significant t-test for methylated CpG2, CpG3 and CpG 5 islands were also depicted. CpG1: methylated 1st CpG, CpG2: methylated 2nd CpG, CpG3: methylated 3rd CpG, CpG4: methylated 4th CpG and CpG5: methylated 5th CpG

DISCUSSION

As predicted, two variables in our demographic data were statistically significant, which were gestational days and birth weight. Our study reported that mean birth weight for term and preterm labour was 3.281 ± 0.284 and 2.440 ± 0.446 kg, respectively (Table 1). Previous studies concluded that there was strong association between comorbidity and clinical type of preterm labour with gestational age (Hafström et al. 2018; Houben et al. 2019; Phillips et al. 2017). Baer et al. (2016) reported that small or large for gestational age (SGA/LGA) status by gestational age affect the risk on neonatal mortality and morbidity. Results showed that compared with appropriately grown infants of similar gestational age, SGA infants were at increased risk for infant mortality. Risk of preterm morbidity was increased for SGA infants born between 28 and 38 weeks but decreased for LGA infants born before 37 weeks. This study demonstrated the importance of considering birth weight for gestational age when evaluating morbidity and mortality risks.

To the best of our knowledge, our current study is among the pioneer in determining the DNA methylation status in term and preterm labour in local Malaysian population and in Asian countries. Till date, there is only one study that reported *MMP1* methylation in non-Asian mothers, which was in African American women by Wang et al. (2008). This similar study was conducted to investigate the DNA methylation status and single nucleotide polymorphism on the activity of *MMP1* promoter in preterm labour. They concluded that the hypomethylation at -1538 site increased *MMP1* expression that enhanced the rupture of fetal membrane (Wang et al. 2008).

There are a few differences between Wang et al. (2008) and our current study which are: participant racial distribution, sample size, type of clinical samples and research method. The participants for our current research were Malaysian women who underwent vaginal delivery or caesarian section with spontaneous preterm labour in UKMMC. Thus, the subjects from different racial distribution may affect in term of genetic predisposition, which may play a role in DNA methylation (Chan et al. 2017; Kader & Ghai 2017). Sample size for our current research was 15 subjects for both term and preterm labour. The sample size by Wang et al. (2008) was larger than us, with 47 subjects for both term and preterm labour. Although smaller sample size was used, the power of our current research is good (0.951 or 95.1%), which is considered strong and the results are reliable based on the sample size population. Findings that were due to chance and recall bias were avoided by random variation and counter check participant information from reliable source as medical records.

Furthermore, our study used maternal blood as the clinical sample in order to determine whether similar findings can be observed in the circulating DNA of maternal blood as previously reported (Wang et al. 2008). Wang et al. (2008) used amnion fibroblast from the placental tissue as the clinical samples. The usage of maternal blood in our study was preferred because this clinical sample is easily accessible as compared to placental tissue (Gil et al. 2019; Munchel et al. 2020). It also has a potential as a screening method in the future for impending preterm labour diagnosis by identifying the level of the methylated *MMP1*. Thus, mothers with high risk of having preterm labour could be closely monitored and early preventive measure could be taken to reduce perinatal and maternal morbidity.

Our current study used MSP method that has advantages as compared to complicated method of direct bisulfite sequencing as used in the Wang et al. (2008) and other previous studies (Hattori & Ushijima 2017; Ramalho-Carvalho et al. 2018). Advantages of MSP include: (1) it avoids the use of restriction enzymes and resultant problems associated with incomplete enzymatic digestion; (2) it is very sensitive, permitting the analysis of small, heterogeneous samples; (3) it is specific for relevant CpG sites, not just those in restriction sites; (4) it is not prone to false-positive results; (5) simple, rapid and cost effective; (6) does not require radioactive agents and (7) ability to analyze large number of samples (Hattori & Ushijima 2017; Herman et al. 1996; Ramalho-Carvalho et al. 2018). The reliability of MSP was further enhanced by the usage of the EZ DNA Methylation-Direct™ kit that combined DNA denaturation and bisulfite conversion processes into a single step. The kit has an advantage of completing DNA bisulfite conversion directly from blood, tissue, and cells without the prerequisite for DNA purification. In addition, the kit has >99.5% of conversion efficiency and >80% DNA recovery (Ørntoft et al. 2017). Both features will prevent DNA loss and preserve the purity of the samples in our study.

We also demonstrated the different degree of methylation observed between term and preterm samples. This can be described by the presence of different densities of MSP product bands in the gel electrophoresis in both study groups. Some bands appeared as clearer and whiter appearance (*strong band*) as in term labour sample number 11 (TB11), while others appeared as vague white bands (*weak band*) as in preterm sample number 8 (PB08)

(red arrows in Figure 1). Similar ways of describing MSP analysis was reported in several studies which include study on methylation of tumor-suppressor gene, *p53* gene, in 8-week-old mice (Okazaki et al. 2011). A previous study in preeclampsia, has shown hypomethylation of a CpG near the transcription start site in *MMP9* gene correlating with its over-expression (Wang et al. 2010). A more recent study observed hypomethylation status at non-CpG and CpG loci in HIF-1α promoter and H3K9ac modification contribute to maintain higher HIF-1αactivity in invasive breast cancer cells (Li et al. 2019).

MSP is a qualitative and not quantitative approach to measure methylation, therefore the level of methylation of gene *MMP1* could not be measured directly from the study. The methylation level can be interpreted as hypermethylated, hypomethylated, partial methylation or slight methylated as in quantitative methods such as reverse transcription-PCR and microarray (Chu et al. 2018; Weeding et al. 2018; Zhang et al. 2019). Another study reported that *MMP9* gene promoter was hypomethylated in preterm placenta as compared to term placenta, while the mRNA levels were comparable between the two groups (Sundrani et al. 2017).

A practical and reliable method to identify the level or different degree of methylation in the CpG islands of the specific gene following the analysis of MSP products is by analyzing CpG islands cluster in the end product post DNA sequencing. This is because the DNA sequencing used the DNA template from MSP product that had positive bands detected in the gel electrophoresis. Post DNA sequencing, the chromatograph results were analyzed using the ClustalW software (Chang et al. 2020; Hung & Weng 2016). In this study, we managed to obtain scores between 78 and 92% matching for the positive bands, which was interpreted as good similarity after the alignment of the sequencing product templates (150 bp) with the bisulfite conversion of methylated *MMP1* sequence (1839 bp).

Analysis of the methylated CpG islands of *MMP1* promoter has demonstrated a higher percentage of methylated CpG (65.33%) in term labour as compared to preterm labour cases (52%) (Table 3). Significant correlations ($p<0.05$) between variables of preterm labour with total number of methylated CpG islands of *MMP1* promoter indicate that number of methylated CpG islands influence the outcome of term or preterm labour for gestational age (Table 5). This data was further confirmed with the analysis of individual CpG sites in the *MMP1* promoter. Our study explored specific five CpG sites that were located between -1226 and -1378 upstream from the TSS of *MMP1* promoter. CpG4 is the most sequence location site (out of the total 5 CpG) with methylated CpG and the least was CpG5 in both term and preterm groups (Table 6). The probability of preterm labour was significantly influenced by total number of methylated CpG at sequence location CpG2 ($p<0.05$), CpG3 ($p<0.01$) and CpG5 ($p<0.01$) (Figure 4). This may provide a suggestion for future research on the reasons these 3 CpG islands site were likely to be methylated as compared to the remaining two sites. Mean for methylated CpG of *MMP1* promoter in term samples showed significantly higher number methylated CpG sites for *MMP1* promoter ($t=0.694$, $p<0.05$) as compared with preterm labour (Table 4). Alternatively, preterm labour cases have less methylated CpG sites.

This study initial objective was to investigate whether *MMP1* is methylated in term labour and unmethylated in preterm labour. The hypothesis mechanism was that in term sample, methylated *MMP1* play a role in gene suppression causing the level to decrease, maintaining the strength of the fibrillar collagen in the amnion whereby the gestation will persist till full term delivery. The unmethylated *MMP1* in preterm samples would cause gene expression causing the level to increase, thus weakening the strength of the fibrillar collagen in the amnion, which predispose to preterm delivery.

However, our study data indicated slightly different findings. It suggested that all term and preterm sample had methylated *MMP1* gene, but the level and degree of methylation varies, as evidenced by the different densities of MSP product bands in the gel electrophoresis. The methylation status of CpG islands may be one of the mechanisms that play a role to distinguish suppressed or activated genes (Saif et al. 2018; Sarda et al. 2017; Xu et al. 2016). Genes that are transcribed actively would have unmethylated CpG sites in the CpG islands of the *MMP1* promoter regions. Lower percentage of methylated *MMP1* will presumably increase *MMP1* expression in blood. This will reduce fibrillar collagen strength at the amniotic membrane, therefore decrease the possibility of term delivery.

An increase in the methylation at the CpG sites of gene's promoter is associated with a decrease in gene expression, also known as transcriptional silencing (Jang et al. 2017; Spainhour et al. 2019; Zhang et al. 2017). The same can be applied in our study whereby high level of methylation occurred in the *MMP1* promoter leading to likely low expression of the gene. Without *MMP1* expression, the strength of the fibrillar collagen in the amnion is maintained. As a result, the gestation is sustained till full term before delivery can take place.

Nonetheless, in preterm labour cases, there was less DNA methylated in *MMP1* promoter led to probably overexpression of the gene. Overexpression of *MMP1* would lead to weakening of the fibrillar collagen in the amnion, thus encouraging the process of labour occurs earlier.

Conclusion

The methylation status of CpG islands provides one of the important mechanisms for distinguishing genes that are most likely involved. This significant new finding in this study requires further evaluation using more advance and comprehensive different quantitative methylation method such as methylation specific multiplex ligation-dependent probe amplication, DNA methylation microarray and pyrosequencing (Pajares et al. 2020; Sestakova et al. 2019; Singer 2019). A simple screening method using the mothers' blood to identify the high-risk pregnancy is desirable for close monitoring so that early preventive measures could be taken. In conclusion, understanding the epigenetic different in term and preterm labour will hopefully provide more fundamental knowledge on the pathogenesis of this problem.

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