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RESEARCH ARTICLE

Role of *MMP-1*, *MMP-8* and *MMP-9* gene polymorphisms in preterm birth

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Abstract. Novel approaches to preterm births are underway building upon our prior discoveries and probing into unknown discovery pathways. The recent findings showed a high affinity of MMP-9 in serum and its polymorphisms for preterm birth. This study, which is a hospital-based case–control study, aims to investigate the association of *MMP-1*, *MMP-8* and *MMP-9* polymorphisms, and levels of MMP-9 in preterm birth. Increased level of MMP-9 was reported in cases as compared to control. The significant association of *MMP-9* (−1562) CT (P = 0.001; OR = 1.44 (CI = 0.97–2.14)) and TT genotype (P = 0.05; OR = 2.6 (CI = 1.46–4.69)) were reported in preterm birth. Our findings suggest that the MMP-9 plays an important role in contributing preterm labour and this can be used as a diagnostic tool during pregnancy.

Keywords. infection; inflammation; matrix metalloproteinases; polymorphism; preterm birth.

Introduction

Preterm birth (PTB) is the most prevailing and persistent problem causing enormous morbidity and mortality among infants (Pandey *et al.* 2017). India itself accounts for 21% of the total global burden (Indian Foundation of Premature Babies 2014, http://www.dayofbangalore.com/news/healthcare/indianfoundation-premature-babies-ifpb-marks-worldprematurity (accessed 17 August 2018). The involvement of genetic and environmental factors are quite evident in several studies (Knight and Smith 2016; Pandey *et al.* 2018). However, the role of particular gene and genetic pathway are still needs to be elucidated.

The causes of preterm birth and premature rupture of the membranes are associated with infection and inflammation driven pathway (Boyle *et al.* 2017). The maintenance of tensile strength of amniochorion is regulated by intracellular matrix metalloproteinases (MMPs) (Vadillo-Ortega and Estrada-Gutiérrez 2005). These MMPs are zinc-dependent enzymes that takes part in the collagen processing and mainly expressed in the placenta and foetal membranes (Majali-Martinez *et al.* 2016). A large portion of the extracellular membrane (ECM) and parts of the cellular layer is underlined by the network of metalloproteinases (MMPs) (Bowers *et al.* 2010). *MMP1*, *MMP-8*, *MMP-9* are the most studied candidate genes (Fanjul-Fernández *et al.* 2010). Their altered gene expression may be an attributing factor of causing preterm birth (Sheikh *et al.* 2016). The functional polymorphisms situated in *MMP-1*, *MMP-8* and *MMP-9* promoter region may be a contributing element (Fanjul-Fernández *et al.* 2010). As reported by a study, an insertion deletion of single nucleotide polymorphism, i.e. single guanine (1G or 2G) situated at nucleotide 1607 in the *MMP-1* promoter site, and the extra guanine prompts up to a four-fold increased secretion (Gonçalves *et al.* 2013). Likewise, the +17C/G and –799 C/T polymorphic sites are present on the promoter region of *MMP-8* (González-Arriaga *et al.* 2008). Also, a single nucleotide polymorphism at nucleotide 1562 in the *MMP-9* promoter region causes the substitution of cytosine (C) with thymine (T), which builds promoter action (Jafari *et al.* 2014).

However, there is limited data concerning changes in MMP level in PTB, therefore, here we aim to assess the association of MMP-9 level and *MMP-1*, *MMP-8* and *MMP-9* gene polymorphism in PTB.

Methods and materials

A case–control study was conducted between December 2015 to December 2017 on mothers aged between 18 to 40 years in King George's Medical, Lucknow. For this

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Table 1. Summary of *MMP-1*, *MMP-8*, *MMP-9* gene polymorphisms rsID, location, primer, restriction enzyme, PCR product and allele frequency.

Gene	rs ID	Location	PCR primer	Restriction enzyme	PCR product (bp)	Allele	Allele frequency		*P* value
							Case	Control	
MMP-1 (−1607 1G/2G)	rs1799750	Intronic	(F)TGACTTTTAAAACATAGTCTATGTTCA (R)TCTTGGATTGATTTGAGATAAGTCATAGC	*AluI*[14]	269	1G	0.51	0.51	1
						2G	0.49	0.49	
MMP-8 (+17C/G)	rs2155052	5′ UTR variant	(F)CTG TTG AAG GCC TAG AGC TGC TGC TCC(R) CAT CTT CTC TTC AAA CTC TAC CC	*DdeI*[20]	579	C	0.93	0.93	1
						G	0.07	0.07	
MMP-8 (−799C/T)	rs11225395	Intron variant	(F)CAGAGACTCAAGTGGGA (R)TTTCATTTGTGGAGGGGC	*SfcI*[28]	968	C	0.52	0.55	0.67
						T	0.48	0.45	
MMP-9 (− 562C/T)	rs3918242	Upstream gene variant	(F) GCCTGGCACATAGTAGGCCC (R) CTTCCTAGCCAGCCGGCATC	*SphI*[14]	436	C	0.79	0.68	0.07
						T	0.21	0.32	

study, ethical approval was obtained from the Institutional ethics committee.

Aggregately, 510 subjects were recruited, of which 255 cases and 255 controls were enrolled as per inclusion and exclusion criteria. Women who delivered a live singleton newborn before 37 weeks of gestation without any complications (such as, preeclampsia, hypertension, antepartum haemorrhage, diabetes, intra uterine growth retardation (IUGR)) were included as cases and those who delivered a live singleton neonate at ≥37 weeks of gestation were enrolled as controls. Those women who delivered neonate with congenital anomaly, twin delivery and not giving consent were excluded from this study.

Demographic and clinical data and sampling

All data were recorded in the predesigned questionnaire. Gestational age was calculated according to last menstrual period (LMP) and reconfirmed by modified Ballard score (Ballard *et al.* 1991) within 48 h of live birth, by a medical scientist.

Clinical data such as prenatal complications (cervical surgery, probable venous thromboembolism, uterine abnormality, previous pregnancy outcomes, past obstetric history etc) and family history were taken into consideration. However, nothing was reported significant (data not given). Anthropometric detail of mothers, like weight and maternal mid upper arm circumference (MMUAC) were recorded. Neonatal weight, height, foot length, head circumference, chest circumference were also measured. Mothers were asked for demographic details, medical and obstetric history and use of substances like tobacco, pan masala and alcohol, lifestyle, and nutritional status during pregnancy. Environmental data, including residential environment and cooking environment were also recorded to find its association with PTB (data not given).

Genotyping

For the molecular analysis, a total of 3 mL (1 mL EDTA and 2 mL plain vial) peripheral venous blood was collected from mother's DNA. using salting out method. Quality of DNA was checked on 0.8% agarose gel electrophoresis and quantity and purity estimation was performed by using the spectrophotometer.

Genotypic analysis of *MMP-1* (–16071G/2G), *MMP-8* (+17C/G, −99C/T), *MMP-9* (–562 C/T) was performed by using polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP). The PCR amplification was performed by reported primers (table 1). After running PCR, an aliquot of 5 μL of each PCR product was digested overnight with 1U of restriction enzyme. Scoring of a gel was performed.

Table 2. Demographic and clinical characteristics of mothers and neonates enrolled as cases and controls.

Characteristics	Case (n = 255)	Control (n = 255)	P value
Maternal			
Age (yrs in mean ± SD)	26.74 ± 4.2	25.55 ± 3.8	0.46
Post delivery weight (kg in mean ± SD)	51.17 ± 6.3	54.28 ± 7.2	< 0.0001
Maternal mid upper arm circumference (cm in mean ± SD)	23.62 ± 2.3	24.54 ± 1.9	**< 0.0001**
Average kcal intake perday	1630.38 ± 268.54	1881.62 ± 329.73	**< 0.0001**
Average protein intake perday	33.70 ± 7.5	38.28 ± 7.79	**< 0.0001**
Neonatal details			
Birth weight (gm)	1850 ± 379	2814.74 ± 423.4	**< 0.001**
Foot length (cm)	6.32 ± 0.6	7.61 ± 2.84	**< 0.001**
Length (cm)	43.36 ± 2.4	46.77 ± 3.7	**< 0.001**
Head circumference (cm)	30.3 ± 1.6	33.23 ± 1.18	**< 0.001**
Chest circumference (cm)	28.0 ± 1.2	31.12 ± 1.16	**< 0.001**
Gravida			
<3	178 (69.76%)	192 (75.13%)	0.052,0.87 (0.77–0.99)
≥3	77 (30.23%)	63 (24.86%)	
Parity			
<3	193 (75.84%)	202 (79.24%)	0.19,0.90 (0.79–1.03)
≥3			
Total number of antenatal visit			
<3	78 (30.59%)	58 (22.71%)	**0.0036**,1.21 (1.07–1.37)
≥3	177 (69.40%)	197 (77.28%)	
No antenatal care	16 (6.45%)	8 (3.04%)	**0.007**

Values in bold represent significance, i.e. $P<0.05$.

Immunoassay

Levels of MMP-9 were measured by Ray Bio Human ELISA kit (Norcoss, GA30092 USA) which quantitatively measures the MMP-9 pro and active forms in serum. Microplates (96-wells) were coated with MMP-9 and samples and standards were pipetted into the wells. These wells were blocked by immobilized Ab (anti-human MMP-9 Ab) for 1–2 h at 4°C. After washing the unbound Ab HRP, conjugated streptavidin was added. Further, for stopping this reaction, the stop solution was added which changed the colour from blue to yellow in proportion to the amount of MMP-9 bound. The intensity of colour was measured at 450 nm by using UV spectrophotometer.

Statistical analysis

Data was entered in MS Excel and analysed using software SPSS (v15, Chicago, Illinois, USA). Univariate analysis was performed to study the frequency distribution of the variables. Chi-square and student's *t*-test were used to test the association between categorical and continuous variables. The analysis was done blinded to the status of women delivering preterm or term. Allelic and genotypic distribution of selected polymorphisms *MMP-1* (–16071G/2G), *MMP-8* (+ 17C/G, −799C/T), *MMP-9* (–1562 C/T) was calculated by Pearson's chi-square test taking 95% of confidence interval (CI). Hardy–Weinberg equilibrium was checked for each genotype. Enzyme -linked immunosorbent assay levels were calculated by taking the mean of the levels and comparing them with controls. All the statistical results were considered significant with a P value ≤ 0.5. SNP analyser 2.0 was used to determine the haplotype contribution of *MMP-8* (+ 17C/G, −99C/T) gene polymorphisms in PTB. The ethical approval was taken from Institutional Ethical Committee of King George's Medical University.

Results

Demographic and clinical analysis

The subjects enrolled as cases (n = 255) involved < 1% of women belonging to extreme preterm group (≤ 32 weeks) and 21% were in the very preterm group (32 ≥ 35 weeks). Demographic and clinical features of mothers and anthropometric features of neonates are detailed in table 2. The mean age of case and control was 26.74±4.2 years and 25.55±3.8 years, respectively.

Genetic association

We selected four polymorphisms in the minor allele frequency of at least 10% in Indian population. Each polymorphism is predicted to cause a functional change in the gene expression as it is located in a coding region. All genotypes were in Hardy–Weinberg equilibrium. The genotypic distribution of *MMP-1* (–16071G/2G), *MMP-8* (+17C/G, −799C/T) and *MMP-9* (–1562 C/T) was compared between the cases and controls, depicted in table 3.

Table 3. Genotypic distribution of MMP-1, MMP-8, MMP-9 polymorphisms in subjects recruited as cases and controls.

Gene polymorphism	Case (n = 255)	Control (n = 255)	P value	OR (95%CI)
MMP-1 (–10671G/2G)				
1G/1G	56	63		1
1G/2G	150	138	0.89	1.03 (0.61–1.76)
2G/2G	49	54	0.52	0.86 (0.57–1.34)
Dominant model				
1G/1G	56 (22.1%)	63 (24.7%)		1
1G/2G + 2G/2G	198 (78%)	192 (75.3%)	0.48	0.86 (0.57–1.30)
Overdominant model				
1G/1G + 2G/2G	104 (40.9%)	115 (45.1%)		1
1G/2G	151 (59.1%)	140 (54.9%)	0.34	0.84 (0.59–1.20)
Recessive model				
1G/1G + 1G/2G	206 (80.7%)	203 (79.6%)		1
2G/2G	49 (19.2%)	52 (23.3%)	0.67	1.10 (0.71–1.70)
MMP-8 (+17C/G)				
CC	224	223		1
CG	30	30	0.57	0.4 (0.45–5.5)
GG	1	2	0.59	0.51 (0.44–6.0)
Dominant model				
C/C	223 (88%)	222 (87%)		1
C/G + G/G	32 (12.2%)	33 (12.4%)	0.8	1.07 (0.63–1.61)
Overdominant model				
C/C + G/G	224 (88.2%)	224 (87.8%)		1
C/G	30 (11.8%)	31 (12.25%)	0.9	1.03 (0.61–1.76)
Recessive model				
C/C + C/G	254 (99.6%)	253 (99.2%)		1
G//G	1 (0.4%)	2 (0.8%)	0.56	2.00 (0.18–3.17)
MMP-8 (–799C/T)				
CC	67	72		
CT	132	138	0.24	1.35 (.81–2.2)
TT	56	45	0.26	1.30 (0.82–2.05)
Dominant model				
C/C	66 (26%)	72 (28.2%)		1
C/T + T/T	188 (74%)	183 (71.8%)	0.57	0.89 (00.60–1.32)
Overdominant model				
C/C + T/T	122 (48%)	117 (45.9%)		1
C/T	132 (52%)	138 (54.1%)	0.63	1.09 (0.77–1.54)
Recessive model				
C/C + C/T	198 (78%)	210 (82.3%)		1
T/T	57 (22%)	45 (17.6%)	0.21	0.76 (0.49–1.17)
MMP-9 (–1562C/T)				
CC	167	134		1
CT	69	79	.001	1.44 (0.97–2.14)
TT	19	42	.05	2.6 (1.46–4.69)
Dominant model				
C/C	166 (65.3%)	134 (52.5%)		1
C/T + T/T	88 (34.6%)	121 (47.5%)	0.003	1.70 (1.19–2.43)
Overdominant model				
C/C + T/T	186 (73.2%)	176 (69%)		1
C/T	68 (26.8%)	79 (31%)	0.29	1.23 (0.84–1.84)
Recessive model				
C/C + C/T	234 (92%)	213 (83.5%)		1
T/T	21 (8%)	42 (16.5%)	0.002	2.31 (1.31–4.05)

Different genetic models (dominant/over dominant and recessive models) were tested for identifying any association of these studied polymorphisms with PTB (table 4). Increased risk of PTB was found in mothers carrying CT (P = 0.001; OR = 1.44 (95%CI = 0.97–2.14)) and TT (P = 0.05; OR = 2.6 (95% CI = 1.46–4.60)) genotype of *MMP-9* (–562C/T). The frequency of CC/CT and TT genotypes in preterm mothers were 65%, 27% and 8% as compared with mothers with term birth 53%, 31% and 16%, respectively. Comparison of genotype frequency of cases and controls with different genetic models revealed positive association of *MMP-9* (–1562C/T) recessive model (CC

Table 4. Haplotypic distribution of MMP-8 (+17C/G, −799C/T) gene polymorphisms among subjects.

	Haplotype	Case	Control	OR (95%CI)	*P* value
1	CC	0.41	0.43	1	–
2	CT	0.21	0.26	1.16 (0.88–1.53)	0.3
3	GC	0.20	0.20	0.32 (0.21–0.48)	0.57
4	GT	0.18	0.11	1.36 (1.14–1.62)	**< 0.0001**

Values in bold represent significance, i.e. $P<0.05$.

+ CT vs T/T, $P = 0.002$; OR = 2.31(CI = 1.31–4.05)) and dominant model (C/C vs C/T + T/T, $P = 0.003$; OR = 1.70 (CI = 1.19–2.43)) in PTB.

Haplotype association

We also carried out the haplotypic analysis to find the putative association of *MMP-8* in PTB. The expected haplotypes of genetic variants of *MMP-8* are (+17C/G and –799C/T):H1 (CC), H2 (CT), H3 (GC) and H4 (GT). The GT haplotypes of MMP-8 (+17C/G and –799C/T) were observed significantly associated with PTB (table 4). These two polymorphisms were in close proximity with each other and are in linkage disequilibrium ($P < 0.0001$).

Immunoassay analysis

The immunoassay of MMP-9 was performed by Raybiotech ELISA kit. The evaluation was done after taking the reading from Biorad iMark Microplate Reader. The specificities of the ELISA were verified by serial dilution. The levels were measured and compared between the cases and controls. The significant higher levels of MMP-9 was found in cases (135.38 ± 91.76 ng/mL) ($P = 0.05$) when compared with control (108.466 ± 52.26 ng/mL) (figure 1).

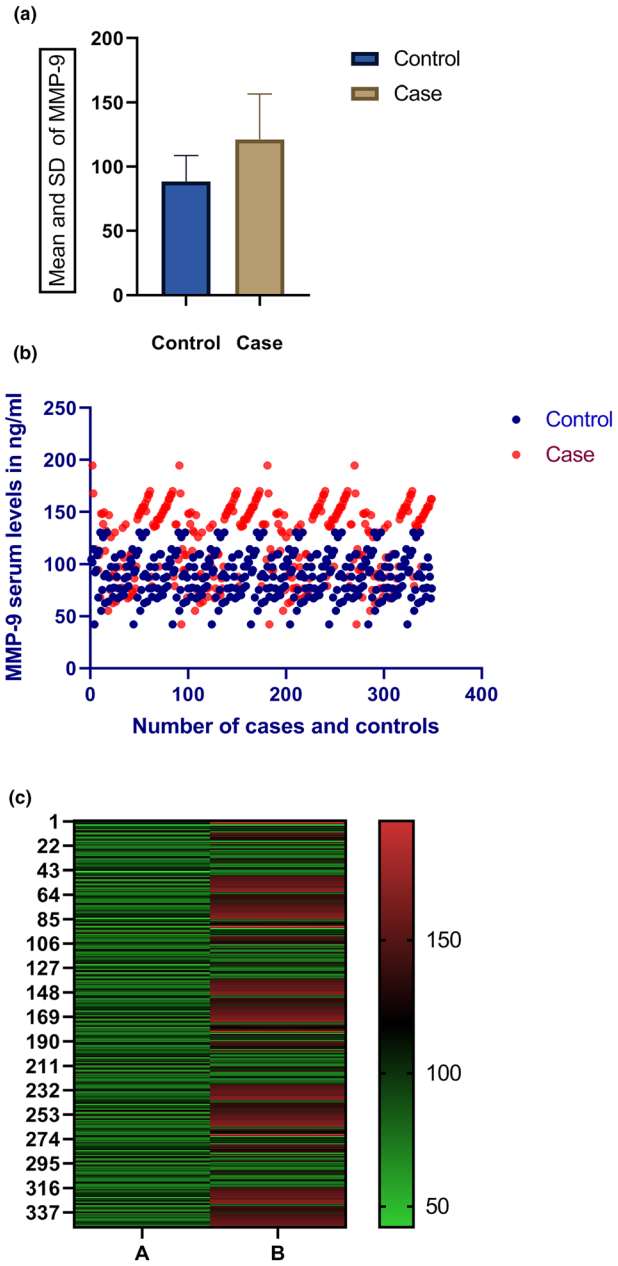

Figure 1. The serum level of MMP-9 analysed by ELISA kit. (a) Bar graph representing the mean and standard deviation, namely case = 135.38 ± 91.76 ng/mL; control = 108.46 ± 52.26 ng/mL; (b) scatter plot; (c) heat map of differentially expressed protein levels between the cases and controls, where A shows the distribution in controls and B in cases.

Discussion

On the basis of the present findings, it is postulated that an increased risk of PTB was associated with increased level of MMP-9 protein and increased risk of PTB was also seen in mothers bearing *MMP-9* 1562 TT genotype. However, no evident association was found between MMP-1 (1607 C/T), MMP-8 (+17C/G, –799C/T), polymorphisms in PTB.

The cause of PTB can be understood by abnormal degradation of the extracellular matrix in the amniochorion, which leads to rupturing of a membrane (Lee *et al.* 2011). Amniochorionic extracellular matrix is composed of several different types of collagen arranged in a complex framework, maximizing its mechanical resistance (Vadillo-Ortega and Estrada-Gutiérrez 2005; Kumar *et al.* 2016). Major components are type I, III, IV, V and VI collagens and abundant proteoglycans in this layer are contributing to mechanical strength (Gillies and Lieber 2011). During labour, these collagens losses their mechanical strength because of increased mymetrial contraction thus causing the delivery to occur. If this mechanism occurs aberrantly, i.e. abnormal activation of labour, it will lead to PTB (Vadillo-Ortega and Estrada-Gutiérrez 2005).

MMP plays a crucial role in normal physiological processes like embryonic development, reproduction and tissue

remodelling, and also involved in diseases like arthritis and cancer (Fanjul-Fernández *et al.* 2010). Thus, it has a key role in monitoring the gestation period as they help in collagen formation (Yoshida *et al.* 2014). Therefore, any imbalance in the MMP-levels can terminate into PTB (Tency *et al.* 2012). The human MMP family consists of 26 members (Jabłońska-Trypuć *et al.* 2016). Preliminary studies proclaimed that alterations of *MMP-1* and *MMP-9* gene expression in terms of increased levels in serum, amniotic fluid, foetal membranes, cervical fibroblasts, and cervical mucus plug in women with PTB compared to women with the term delivery (Pereza *et al.* 2014).

In our study, no association was revealed between *MMP-1* (1607C/T) and *MMP-8* (+17C/G, –799C/T) polymorphisms and PTB. However, the haplotypic association was observed in *MMP-8* (+17C/G, –799C/T) polymorphisms and PTB. This haplotypic association was prior investigated and confirmed by Wang *et al.* (2004). Various studies have reported heterogeneous results about the role of *MMP-1* gene (Fujimoto *et al* 2002; Pereza *et al.* 2014) and *MMP-8* gene polymorphisms (Maymon *et al.* 2000; Wang *et al.* 2004).

The previous studies reported the potential role of the *MMP-9*-1562 C/T gene polymorphism predisposition to PPROM in African American (Ferrand *et al.* 2002) and Chinese women (Fang *et al.* 2010), and one study included nonHispanic white women with PTB (Wu *et al.* 2012). A recent study by Pereza *et al.* (2014) also revealed no significant association of PTB with the polymorphisms in admixed European Caucasian population. As reported by earlier studies Sundrani *et al.* (2012), the levels of MMP-9 were higher in PTB, a similar depiction was done by our study. Our study was also corroborated with Sorokin *et al.* (2010) which was carried out in European population.

We have considered a large sample size. However, the limitations are also accompanied with the study. The foetal genotype contribution towards PTB and the previous history of recurrent PTB of the mother should also be considered for a better understanding of pathophysiology.

In this our study, we conclude that *MMP-9* plays a crucial role in identifying the term and preterm delivery.

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