Imbalance between matrix metalloproteinases and their tissue inhibitors in preeclampsia and gestational trophoblastic diseases

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Abstract

The invasion cascade exhibited by placental trophoblasts and cancerous cells bears many similarities, and it is attributed to extracellular matrix degradation mediated by matrix metalloproteinases (MMPs). Although proper and controlled invasion by trophoblasts into the maternal uterus is an essential requirement for maintenance of normal pregnancy, any abnormality in this phenomenon results in the development of invasion-related disorders such as gestational trophoblastic diseases (GTDs) and preeclampsia. We studied the epigenetic basis of differential expression of two placental MMPs (*MMP2* and *MMP9*) and tissue inhibitors of metalloproteinases (*TIMP2* and *TIMP1*) during normal gestation and invasion-related disorders, i.e., preeclampsia and GTDs. Our study suggests the association of H3K9/27me3 with differential expression of these MMPs and their inhibitors, which regulate the placental invasion during normal pregnancy, whereas no role of CpG methylation was observed in the differential expression of MMPs/TIMPs. Further, development of GTDs was associated with abnormally higher expression of these MMPs and lower levels of their inhibitors, whereas the reverse trends were observed for MMPs and their TIMPs in case of preeclampsia, in association with abnormal changes in H3K9/27me3. These results suggest the involvement of higher levels of MMPs in an aggressive invasive behavior depicted by GTDs, whereas lower levels of these MMPs in shallow and poor invasive phenotype associated with preeclampsia. Thus, our study shows the significance of a proper balance regulated by histone trimethylation between differential expression of MMPs and their TIMPs for maintaining normal pregnancy and its deregulation as a contributing factor for pathogenesis of invasive disorders during pregnancy.

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Introduction

To achieve proper attachment of the developing fetus with the maternal uterine wall and to establish an efficient exchange of nutrient and waste material between developing fetus and mother, placental extravillous trophoblasts rapidly proliferate, migrate and invade into myometrium and remodel maternal spiral arteries during implantation (Pijnenborg *et al.* 1981). This process of placental invasion bears many similarities with the growth of cancer cells, which includes the expression of endopeptidases that degrade extracellular matrix (ECM) and help in tissue remodeling (Soundararajan & Rao 2004) called matrix metalloproteinases (MMPs). The activities of MMPs are tightly regulated by their proper transcription, translation, activation of the precursor zymogens, and inhibition of their active forms by endogenous tissue inhibitors of MMPs (TIMPs) under

physiological conditions (Nagase & Woessner 1999). TIMPs are known to play regulatory role by controlling the activities of MMPs in tissues (Visse & Nagase 2003).

Gelatinases, especially *MMP2* and *MMP9*, are known for their important role during pregnancy, being involved in the degradation of collagen IV, which is the prime component of maternal basement membrane (Staun-Ram & Shalev 2005). In addition, these MMPs can also degrade other components of ECM-like gelatins, type V and XI collagens, and laminin (Nagase *et al.* 2006). Moreover, the expressions of the mentioned MMPs have been shown to mediate the invasiveness of cultured trophoblasts into the Matrigel (Fisher *et al.* 1989). Therefore, *MMP2* and *MMP9* are regarded as the key enzymes during implantation. Although *TIMP1* and *TIMP2* inhibits the active forms of all MMPs, these preferentially bind to both active and latent forms of *MMP9* and *MMP2* respectively (Nagase *et al.* 2006).

Under normal physiological conditions, MMPs are known to regulate cellular migration, invasion, and angiogenesis during embryonic implantation, whereas under pathological conditions, increased expression and activity of MMPs result in tumor growth, migration, and metastasis, leading to the development of cancer (Corthorn *et al.* 2007). The controlled expression and a proper balance between MMPs and their inhibitors (TIMPs), which determine the net MMP activity (Seval *et al.* 2004) and hence regulate the invasive potential of placental trophoblasts, are pivotal for maintaining normal placentation (Staun-Ram & Shalev 2005). In general, any imbalance in this equilibrium leads to the development of pathological conditions (Verma & Hansch 2007), and specifically during pregnancy, this imbalance disturbs the trophoblastic invasive cascade, leading to the development of placental disorders such as preeclampsia and gestational trophoblastic diseases (GTDs). These placental disorders are believed to be associated with abnormal invasive potential of placental trophoblasts: preeclampsia is associated with shallow and poor trophoblast invasion into maternal uterus, resulting in the formation of poor connection with maternal arteries (Fisher 2004), whereas uncontrolled and excessive invasion of trophoblasts, which might even be metastatic, leads to the development of GTDs such as hydatidiform mole and choriocarcinoma (Hui *et al.* 2005). In this context, a previous study reported reduced invasion potential of JAR cells (choriocarcinoma cell line) associated with their decreased secretion of *MMP2*, upon treatment with preeclamptic serum (Mahameed *et al.* 2005). Hence, this phenomenon draws a special attention toward studying the expression and regulation of MMPs and their inhibitors during pregnancy. Earlier studies have reported the expressions of *MMP2* and *MMP9* within the first-trimester placenta (Staun-Ram & Shalev 2005), whereas high expression levels of *MMP2* and *MMP9* have been observed in highly invasive extravillous trophoblasts (Seval *et al.* 2004). Our previous study (Rahat *et al.* 2014) and few recent studies (Dokras *et al.* 2006, Rahnama *et al.* 2006) have highlighted the importance of epigenetic mechanisms in the regulation of gene expression during placentation. Therefore, we aimed to find the significance of differential mRNA expressions and the epigenetic regulatory mechanisms of *MMP2* and

MMP9 and their inhibitors in the development of pathological pregnancies, with reference to normal physiological pregnancies.

Methods

Study design and sample collection

Pregnant women diagnosed with normal singleton pregnancy in the first trimester undergoing suction evacuation for medical termination of pregnancy (6–11 weeks, $n=30$), second trimester undergoing medical termination of pregnancy (16–20 weeks, $n=30$), and third trimester (37–40 weeks, $n=30$) or pregnancy-related disorders such as preeclampsia (clinical symptoms of 140/90 mmHg systolic/diastolic pressure; proteinuria >300 mg in 24 h, or 1+ protein on dipstick, $n=30$) and hydatidiform mole (diagnosed by ultrasonography during early gestation and further confirmed by histopathology, $n=15$) were included in this study. The demographic characteristics of pregnant women included in this study are summarized in Table 1, and the clinical characteristics of preeclamptic women included in this study are summarized in Table 2. There was no significant difference between gestational age of preeclampsia cohort and normal third-trimester cohort. Further, the blood pressure levels of preeclampsia group significantly increased compared with its control group. Written consent was obtained from each subject after clearly informing about the study, and Institute Ethics Committee approved this study. From each subject, 10 mL maternal peripheral blood was collected before any obstetric procedures and used for the isolation of plasma and maternal blood leukocytes (Chiu *et al.* 2001). Placental villous tissue samples were collected via caesarean sections in case of pregnant women with normal third-trimester pregnancy or preeclamptic pregnancy, whereas in the case of normal first-trimester, second-trimester, and molar pregnancies, placental villous tissue samples were collected after elective termination of their pregnancies via suction evacuation or dilation and curettage. All the collected placental samples were immediately processed for isolation of placental villi. The isolated villous tissue was rinsed in sterile ice-cold phosphate-buffered saline and then stored at -80°C for further analysis.

Cell line

In order to study the level of mRNA expression and the pattern of promoter region methylation for *MMP2* and *MMP9* and *TIMP2* and *TIMP1* in choriocarcinoma, we selected JEG-3 cell line, which is a placental choriocarcinoma-derived cell line (Source: American Type Culture Collection). JEG-3 cells

Table 1 Demographic characteristics of the pregnant women included in different groups in the study.

Study group	Number of pregnant women included in this study	Mean age in years $\pm$ s.d.	Gestational age in weeks $\pm$ s.d.	Gestational age range in weeks
Normal first trimester	30	27.9 $\pm$ 3.4	8.2 $\pm$ 1.4	6–11
Normal second trimester	30	27.6 $\pm$ 3.9	18 $\pm$ 1.6	16–20
Normal third trimester	30	28 $\pm$ 3.5	38 $\pm$ 0.8	37–40
Preeclampsia	30	26 $\pm$ 3.3	35.3 $\pm$ 2.5	34–39
Hydatidiform mole	15	25.1 $\pm$ 2.6	13.6 $\pm$ 2.9	9–18

Table 2 Clinical characteristics of the preeclamptic women included in this study.

S. no	Clinical characteristics of preeclamptic women	
	Proteinuria: as protein on dipstick	Blood pressure: systolic/diastolic pressure in mmHg
1	2+	160/100
2	2+	150/100
3	3+	160/90
4	3+	160/110
5	2+	140/100
6	4+	160/90
7	3+	150/120
8	2+	160/90
9	2+	140/90
10	3+	200/110
11	3+	160/130
12	3+	160/120
13	2+	150/110
14	2+	200/100
15	4+	170/120
16	3+	150/100
17	3+	160/120
18	3+	150/110
19	3+	140/100
20	2+	160/110
21	2+	250/110
22	2+	140/80
23	3+	160/110
24	2+	140/106
25	3+	140/100
26	3+	190/110
27	3+	160/110
28	4+	160/120
29	2+	160/110
30	3+	160/120

were maintained in Dulbecco's modified Eagle medium-HG, with 4500mg/L glucose supplemented with L-glutamine and sodium bicarbonate (3.7 and 2 g/L respectively), HEPES (25 mM), and fetal bovine serum (10%).

Isolation of cytotrophoblast from first-trimester villi

Cytotrophoblasts were isolated from first-trimester placentas (6–11 weeks) by enzymatic digestion of first-trimester villi as explained previously (Rahat *et al.* 2014). Briefly, 2 g dissected first-trimester villi was subjected to sequential trypsin digestion. After each step, cell debris was removed by passing the cell suspension through pre-equilibrated 60 μ M mesh and the filtrate with isolated cytotrophoblasts was centrifuged. The obtained cell pellet was resuspended in phosphate-buffered saline and subjected to enrichment on a preformed 10–70% Percoll gradient at 800 g for 20 min at 20°C. The top cellular layer of mononuclear cells containing cytotrophoblasts was collected and immunopurified for the pure population of cytotrophoblasts by negative selection method using EasySep Human CD45 Depletion Kit and EasySep magnet (STEMCELL Technologies, Vancouver, BC, Canada). The purity of obtained cytotrophoblasts was confirmed by flow cytometry using trophoblast marker cytokeratin-7 (Epitomics, Burlingame, CA, USA), showing >90% cells as cytokeratin-7 positive.

Quantitative mRNA estimation

Total RNA isolated via TRIzol (Ambion, Life Technologies) from placental villous samples, maternal blood leukocytes, and JEG-3 cells was used in mRNA quantification studies. Isolated RNA (1 μ g) was used in reverse transcription to obtain cDNA using RevertAid™ M-MuLV-RT kit (MBI Fermentas, Life Sciences, Waltham, MA, USA). For endpoint analysis, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out in Applied Biosystems real-time PCR system (Life Technologies) on reaction mixture prepared for each sample by mixing SYBR Green master mix (5 μ L) with 60 ng of the obtained first-strand cDNA template (1 μ L), 500 nM gene-specific primers, and 1.5 mM MgCl₂ in a total reaction volume of 10 μ L. PCR product amplification was carried out by initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, primer-specific annealing temperature for 1 min, followed by a melt curve of 95°C for 10 s, 60°C for 1 min, and 95°C for 15 s. This was followed by normalization of transcript levels to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as the cycle threshold (Ct) values were observed to be constant across different trimesters. Relative fold changes in gene expression between different groups were calculated using the comparative threshold cycle or Ct method ($\Delta\Delta$ Ct) method (Livak & Schmittgen 2001). The primers used for mRNA estimation assay are presented in Table 3.

DNA isolation and methylation-sensitive high-resolution melting

Total genomic DNA isolated by DNA isolation kit (Real Genomics, Real Biotech Corporation, Taipei, Taiwan), from placental villi samples, maternal blood leukocytes, JEG-3 cell line, and circulating DNA isolated by Miniprep DNA isolation kit (Bioserve, Beltsville, MD, USA), from maternal plasma, was used to estimate the promoter region methylation percentage by methylation-sensitive high-resolution melting (MS-HRM) as described previously (Rahat *et al.* 2014). Briefly, DNA methylation standards were prepared for their use in the estimation of DNA methylation of study samples. Fully methylated DNA (100% methylated) was prepared by methyltransferase treatment of genomic DNA (M.SssI enzyme; New England Biolabs, Beverly, MA, USA), whereas 0% methylation standard was obtained commercially from Qiagen (EpiTect Control DNA). This was followed by bisulfite conversion using EZ DNA Methylation-Gold Kit (Zymo Research, USA), of these methylation standards and 1 μ g isolated DNA from each subject. The converted DNA was then analyzed for promoter region methylation using gene-specific primers (Table 3). MS-HRM analysis was performed by real-time-based PCR amplification in Applied Biosystems StepOnePlus Real-Time PCR, followed by melt curve analysis. For each assay, a series of different methylation standards ranging from 0 to 100% CpG methylation were obtained by mixing 100 and 0% DNA methylation standards in proper proportion. Finally, the raw melt curves were analyzed using MS-HRM software version 3.0.1. from Applied Biosystems to get various methylation-predicting graphs. The data obtained from graphs were used to calculate the exact percentage methylation of unknown samples using Polyfit interpolating function within program MatLab (The MathWorks, Natick, MA, USA).

Table 3 Primer sequences and their parameters used in the study.

Assay/Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing temperature °C/ Amplicon size bp/No. of CpGs in amplicon*	Genbank accession number
qRT-PCR				
<i>MMP2</i>	AGATCTTCTTCTCAAGGACCGGT	GGCTGGTCAGTGGCTTGGGGTA	64/225	NM_001127891.2
<i>TIMP2</i>	CTCGCTGGACGTTGGAGGAAAGAA	CCATCTGGTACCTGTGGTTCA	64/152	NM_003255.4
<i>MMP9</i>	TTGGTCCACCTGGTTCAACT	ACGACGTCTTCCAGTACCGA	64/95	NM_004994.2
<i>TIMP1</i>	CATCCTGTTGTGCTGTGGCTGAT	GTCATCTTGATCTCATAACGCTGG	64/168	NG_012533.1
<i>GAPDH</i>	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG	60–65/228	NM_001256799.2
MS-HRM				
<i>MMP2</i>	TTGAGTCGGGTAAA TTTTAGGTTAT	CCGCCTAATTAACCTACTC	58/84/9	NG_008989.1
<i>TIMP2</i>	TAGTAAATATATTCGTAGAAGGTAG	CGCAAATTTCTCTCCTCTTTA	56/148/23	NC_000017.11
<i>MMP9</i>	CGTTGTTGTTTTTTAGAAGTAAT- GTTTTGT	CGCCCTCAAAAATTTAAATC- TACCCAA	60/129/9	NG_011468.1
<i>TIMP1</i>	TTTTAGCGTGATATTTATTTTATG	CCGAACCTACTTACCTCTAA	56/96/7	NG_012533.1
qChIP-PCR				
<i>MMP2</i>	CCGGCGTCCCTCCTAGTAGT	TGTCGCTCTAGGCCCTTGAC	62/64	NG_008989.1
<i>TIMP2</i>	GCCAGGCGCACTTAAATTC	GGGTGGCCTATTGAGAACTGA	60/71	NC_000017.11
<i>MMP9</i>	CCCTTTACTGCCCTGAAGA	AAGGAATGGGCTCTGCTAGG	62/90	NG_011468.1
<i>TIMP1</i>	GGTGGGTGGATGAGTAATGCA	AGCGGGTGC GGAAACC	60/59	NG_012533.1

*In case of only MS-HRM assays. qRT-PCR, quantitative Reverse transcriptase polymerase chain reaction; MS-HRM, methylation-sensitive high resolution melting; qChIP, quantitative Chromatin immunoprecipitation assay.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed only on placental villi samples to estimate the levels of H3K9me3 and H3K27me3 at promoter region of genes as described previously (Rahat *et al.* 2014). Briefly, 25 mg placental villi were fixed in 1.5% formaldehyde to cross-link histones with DNA, followed by its disaggregation into single-cell suspension. The cell pellet was resuspended in lysis buffer for sonication in order to shear chromatin, using Bioruptor XL (Diagenode, Liège, Belgium) at 20 pulses of 10 s each. Sheared chromatin was analyzed on agarose gel to confirm proper shearing of chromatin. The sheared chromatin lysate was then divided into aliquots to be used for input DNA, immunoprecipitation with anti-trimethyl H3-K9/K27Ab (Abcam), and normal rabbit immunoglobulin-G in proper proportion. The immunoprecipitated complexes were collected using protein A/G-polyacrylamide beads, followed by reverse cross-linking and proteinase K treatment. The immunoprecipitated DNA as well as input DNA was isolated by phenol chloroform method and then quantified using specific primers (Table 3) on real-time PCR. ChIP assays for each histone modification specific to a particular gene were done in triplicates. Data were normalized with input DNA and calculated relative to non-specific antibody as fold enrichment.

Statistical methods

Data were expressed as mean $\pm$ S.E.M. Student's *t*-test was used in order to compare the significance of variance between two groups, whereas ANOVA and then Fisher's *post hoc* test was used between more than two groups. Further, the correlation between different parameters was estimated by Pearson's correlation analysis. In addition, multiple regression analysis was done in order to study the effect of epigenetic regulatory mechanisms on the mRNA expression. Data were considered statistically significant at $P < 0.05$. IBM SPSS statistical

program (v.16) and GraphPad Prism (v.5.00.288) were used for statistical analysis.

Results

mRNA expression of MMPs and their inhibitors

Placental trophoblasts are known for their excessive invasive potential invading maternal uterus for proper attachment. This phenomenon requires balanced expression levels of MMPs and their TIMPs. Thus, we analyzed the mRNA expression of *MMP2* and *MMP9* and their respective inhibitors, i.e., *TIMP2* and *TIMP1*, in normal gestational first-, second-, and third-trimester groups and placental disorders (preeclampsia and molar pregnancy) and JEG-3 cells (choriocarcinoma cell line) (Fig. 1A, B and C). The mRNA expressions of *MMP2* and *MMP9* and their respective inhibitors *TIMP2* and *TIMP1* were observed to show a reverse trend with advancing normal gestation. *MMP2* expression decreased by 2.5-fold ($P < 0.01$) in the second-trimester placental villi and 4.3-fold ($P < 0.001$) in the third-trimester placental villi, whereas *TIMP2* expression increased by 9.5-fold ($P < 0.001$) in the third-trimester placental villi, with respect to the first-trimester placental villi. Similarly, expression of *MMP9* decreased by 8.4- and 15.3-fold ($P < 0.001$), respectively, in the second and third trimester relative to the first trimester, whereas that of *TIMP1* increased by 2.5-fold till midgestation ($P < 0.001$), then remained almost constant till term gestation (Fig. 1A). The expression levels of *MMP2* and *TIMP2* were also observed to show a reverse trend in maternal blood leukocytes showing 2.9- to 3.3-fold decrease after midgestation ($P < 0.05$) and 2.3-fold ($P < 0.01$) increase in the third trimester, respectively, with reference to

Figure 1 Relative fold change in the mRNA expression of MMPs and TIMPs normalized with *GAPDH*. Differential mRNA expression among (A) placental villous samples and JEG-3 cells, (B) maternal blood leukocytes (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs first trimester, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs second trimester, € $P < 0.05$, €€ $P < 0.01$, €€€ $P < 0.001$ vs third trimester and ¥¥¥ $P < 0.001$ vs molar), and (C) placental villi samples with reference to their corresponding maternal blood leukocytes (** $P < 0.01$, *** $P < 0.001$). The data are presented as mean of the observed fold change $\pm$ S.E.M.; $n = 30$ per group.

the first trimester. However, expression of *MMP9* was observed to show non-significant variation within maternal blood leukocytes, whereas expression of *TIMP1* decreased by 3.5- to 4.8-folds after midgestation ($P < 0.001$) (Fig. 1B).

Development of preeclampsia was observed to be associated with a gross decrease in the levels of *MMP2* and *MMP9* (2.7-fold, $P < 0.01$, and 5.6-fold, $P < 0.001$, respectively) and increased levels of *TIMP2* and *TIMP1* (2.4-fold, $P < 0.001$, and 1.7-fold, $P < 0.01$, respectively) in preeclamptic villi relative to normal term placental villi. Preeclamptic maternal blood leukocytes also showed significantly increased *TIMP2* and *TIMP1* (28- and 3.4-fold, $P < 0.001$, respectively) and *MMP2* levels (2.7-fold, $P < 0.05$) with respect to normal third-trimester maternal blood leukocytes.

Development of GTDs demonstrated increased expression in molar villi and JEG-3 cells for *MMP2* (1.8- and 3.5-fold, $P < 0.001$, respectively) and *MMP9* (2.5- and 8.2-fold, $P < 0.001$, respectively), whereas the mRNA levels were observed to be decreased in

molar villi as well as JEG-3 cells for *TIMP2* (2.9- and 3.8-fold, $P < 0.001$, respectively) and *TIMP1* (2.5- and 9.2-fold, $P < 0.001$, respectively), with respect to first-trimester placental villi. Further, molar maternal blood leukocytes also demonstrated reduced levels of *MMP2* (3-fold, $P < 0.05$), *TIMP1* (2.3-fold, $P < 0.01$), with respect to first-trimester maternal blood, and *TIMP2* (2-fold, $P < 0.01$), with respect to the normal second-trimester maternal group.

Comparing the mRNA expression of *MMP2* and *MMP9* and *TIMP2* and *TIMP1* in placental villi groups with their respective maternal blood leukocytes, it was observed that the expression of these genes were significantly higher in placental villi samples in all groups ($P < 0.01$ to < 0.001), except for *TIMP1* in the first-trimester villi and molar villi, where it was higher in maternal blood ($P < 0.001$) (Fig. 1C).

Pearson's correlation analysis was performed in order to find the correlation between the mRNA expression of these genes during normal gestation (first-, second-, and third-trimester placental villi) and placental

A *MMP2* **B *TIMP2*** **C *MMP9*** **D *TIMP1***

Figure 2 Promoter region DNA methylation as predicted by HRM software. HRM difference plots for (A) *MMP2* (B) *TIMP2* (C) *MMP9*, and (D) *TIMP1*. Each representing three difference plots: first one for methylation standards in different colors (M100 to M0%, which stands for DNA methylation standards with 100–0% methylation) normalized to the 0% methylated standard DNA, second and third difference plots for few selected samples from placental villous groups and cell lines and maternal leukocyte groups represented in different colors and methylated standard curve of M100 to M0% represented as black curves. Note: “Trim” stands for trimester.

disorders (preeclamptic and molar villi) in JEG-3 cells, which revealed significantly higher positive correlation between *MMP2* vs *MMP9* ($r=0.98$, $P<0.001$) and *TIMP1* vs *TIMP2* ($r=0.85$, $P<0.05$). Further, moderately higher negative correlation was observed between mRNA expressions of *MMP2* vs *TIMP2* ($r=-0.6$) and *MMP9* vs *TIMP1* ($r=-0.73$) among these groups.

Promoter region DNA methylation of MMPs and their inhibitors

To elucidate the role of CpG methylation in mediating the differential expression of *MMP2* and *MMP9* and their inhibitors *TIMP2* and *TIMP1* during normal advancing gestation and placental abnormalities, we estimated CpG site DNA methylation at their promoter

regions in placental villi samples, maternal blood cells, and placental cell lines. *MMP2* and *TIMP2* promoter regions (Fig. 2A and B) were detected to be either hypomethylated or completely unmethylated with the exception of *MMP2* promoter region in JEG-3 cells ($96.8\pm1.3\%$ methylated, $P<0.001$). However, some variations were detected in the promoter region methylation of *MMP9* and *TIMP1* (Fig. 2C and D). *MMP9* and *TIMP1* promoter regions were observed to be hypomethylated with less than 5% average methylation in normal gestational placental villi groups. Development of preeclampsia and molar pregnancy was observed to be accompanied with increased methylation of *MMP9* by 2-fold ($P<0.05$) and 3.5-fold ($P<0.001$), respectively, in comparison with their respective control groups. Further, similar

Figure 3 Promoter region DNA methylation for *MMP9* and *TIMP1*. (A) Box-and-whisker plot to show % CpG methylation among different placental villous samples and JEG-3 cells. (B) Box-and-whisker plot to show % CpG methylation among maternal blood leukocytes ($***P<0.001$ vs first trimester, $***P<0.001$ vs second trimester, $^{\epsilon}P<0.05$ vs third trimester and $***P<0.001$ vs molar), and (C) % CpG methylation in villous samples with reference to their corresponding maternal blood leukocytes ($*P<0.05$, $**P<0.01$, $***P<0.001$); $n=30$ per group.

to *MMP2*, the promoter regions of *MMP9* and *TIMP1* were also detected to be hypermethylated in JEG-3 cells ($81.6 \pm 6.2\%$ and $26.3 \pm 1.1\%$ methylation respectively) (Fig. 3A). Analysis of promoter region methylation of *MMP9* and *TIMP1* in maternal blood cells revealed non-significant variation between different groups (Fig. 3B), whereas promoter region methylation of *MMP9* and *TIMP1* was detected to be significantly higher in maternal blood samples relative to their corresponding placental villi groups (Fig. 3C).

Pearson's correlation analysis predicted a positive correlation between mRNA expression and promoter region methylation of *MMP2* ($r=0.86$, $P<0.05$) and *MMP9* ($r=0.97$, $P<0.01$) among placental villi groups and JEG-3 cells. In addition, a high positive correlation ($r=0.99$, $P<0.001$) was observed among DNA methylation at promoter regions of *MMP2* and *MMP9* among these groups.

Confirmation of DNA hypomethylation at promoter regions of MMPs and their inhibitors in isolated first-trimester cytotrophoblasts

In order to confirm our DNA methylation data, we further analyzed the DNA methylation status of *MMP2* and *MMP9* and *TIMP2* and *TIMP1* in cytotrophoblasts isolated from first-trimester placenta. Our data from first-trimester cytotrophoblasts also revealed hypomethylation at the promoter regions of *MMP2*, *TIMP2*, *MMP9*, and *TIMP1*, with average methylation of 1.1 ± 0.95 , 0.78 ± 0.65 , 2.16 ± 1.56 , and $4.9 \pm 2.12\%$, respectively, in cytotrophoblasts, thus confirming no role of DNA methylation in regulating the differential expression of these MMPs and TIMPs. The methylation levels of these genes in cytotrophoblasts and first-trimester villi samples are presented in Fig. 4.

Figure 4 Promoter region DNA methylation for MMPs and TIMPs. Bar diagram to show % CpG methylation at promoter regions of *MMP2* and *MMP9* and *TIMP2* and *TIMP1* in isolated first-trimester cytotrophoblasts; $n=30$ per group.

Histone trimethylation at the promoter regions of MMPs and their inhibitors

In order to estimate the transcription regulatory effect of histone modifications, especially histone trimethylation at lysine 9 and 27, on *MMP2* and *MMP9* and *TIMP2* and *TIMP1*, we quantified the levels of H3K9/K27me3 at their promoter regions in normal placental villi groups as well as preeclampsia and molar villi groups via chromatin immunoprecipitation (ChIP) assay (Fig. 5A and B). ChIP analysis detected some significant and reverse pattern of changes in the levels of H3K9/27me3 with advancing gestation at *MMP2* vs *TIMP2* and *MMP9* vs *TIMP1* promoter regions. H3K9me3 and H3K27me3 levels were observed to be raised by 2.2-fold ($P<0.01$) and 2-fold at *MMP2* promoter region and decreased by 1.2- and 2.6-folds ($P<0.05$) at *TIMP2* promoter region, respectively, in the third-trimester placental villi relative to normal first-trimester villi. Similarly, *MMP9* promoter region revealed 1.2- and 4-fold ($P<0.05$) increase in H3K9me3 and H3K27me3 levels, respectively, whereas *TIMP1* promoter region was observed to show 2.5- and 2.1-fold, $P<0.05$, decrease in H3K9me3 and H3K27me3 levels, respectively, by full-term normal pregnancy. Development of preeclampsia was observed to be associated with 1.8- and 1.7-fold ($P<0.05$) increase in H3K9me3 levels at *MMP2* and *MMP9* promoter regions in relative to its control group. Molar placental villi also showed relatively higher levels of H3K9me3 at *MMP2* (2.2-fold, $P<0.05$) and *TIMP2* (1.9-fold, $P<0.05$) and H3K27me3 at *TIMP1* (1.8-fold, $P<0.05$) promoter regions, whereas H3K9me3 levels were relatively lower in molar villi at *MMP9* (1.5-fold, $P<0.01$) promoter region, in comparison with normal first-trimester placental villi.

Multiple regression analysis was performed to analyze the effect of epigenetic regulation on differential mRNA expression of these genes in normal gestational placental villi groups and two placental disorders: preeclamptic and molar villi showed high regression coefficient between mRNA expression of *MMP2*, *MMP9*, and *TIMP1* and their respective H3K9me3, H3K27me3, and promoter region DNA methylation levels ($r^2=0.84$, 0.92 and 0.93, respectively) (Table 4).

Discussion

This study shows the importance of proper balance between *MMP2* and *MMP9* and their inhibitors *TIMP2* and *TIMP1* through the epigenetic regulation in maintaining normal pregnancy. The invasion and migration of trophoblasts into maternal uterus and degradation of the components of ECM resembles the growth of malignant tumors. These invasive properties of placental trophoblasts (Librach et al. 1991) and metastatic phenotype of cancer cells (Bernhard et al. 1994, Gokaslan et al. 1998) have been associated with

Figure 5 Quantification of histone modifications at promoter regions of MMPs and TIMPs among placental villous groups. Fold enrichment relative to non-specific IgG acting as negative control and normalized with input DNA in (A) H3K9me3 and (B) H3K27me3. The data are presented as mean of the observed fold change $\pm$ S.E.M.; $n=4$ per group. * $P<0.05$, ** $P<0.01$ vs first trimester, # $P<0.05$, ## $P<0.01$ vs second trimester, € $P<0.05$ vs third trimester.

the activity of MMPs, whereas the inhibition of metastasis correlates with the action of TIMPs (DeClerck *et al.* 1992). Thus, the precise balance between the levels of MMPs and TIMPs regulate these pathways (Gomez *et al.* 1997). In light of the current literature, we analyzed the differential expression and the epigenetic regulation of MMPs and TIMPs, mediated by promoter region DNA methylation and histone trimethylation.

Our data revealed decreased expressions of *MMP2* and *MMP9* with advancing gestation, associated with reduced invasive potential of trophoblasts, supporting an earlier study showing the role and *MMP9* in the invasiveness of cultured cytotrophoblasts in Matrigel (Fisher *et al.* 1989). Further, the observed reverse trend in the expressions of *TIMP2* and *TIMP1*, respective inhibitors of *MMP2* and *MMP9*, suggests effective high activity of these MMPs in early placental villi, supported by the reported highest activity of *MMP9* in the first trimester of gestation (Librach *et al.* 1991, Cross *et al.* 1994).

Estimation of DNA methylation revealed hypomethylation at the promoter regions of *MMP2* and *MMP9* in placental villi, suggesting a similar role of DNA hypomethylation in activating expression of these invasion-promoting genes in placental tissue, as observed in the cancerous tissue (Shukeir *et al.* 2006, Farias *et al.* 2012). Emerging evidences suggest a significant role of chromatin-remodeling complexes in regulating expression of MMPs (Yan *et al.* 2003, Chang

et al. 2006). In this context, we studied the involvement of histone trimethylation in regulating the expression of MMP expression in the placenta. Our data suggested the association of increased H3K9/K27me3 with decreased mRNA levels of *MMP2* and *MMP9* and decreased H3K9/K27me3 with increased mRNA levels of their inhibitors *TIMP2* and *TIMP1*, with advancing gestation, relative to the first trimester. Thus, the reverse trends of H3K9/27me3 levels at the promoters of MMPs and their inhibitors (TIMPs) might be regulating the observed reverse trends in their mRNA expressions during normal gestation. Our study is the first to show the role of histone modifications in regulating the expression of MMPs/TIMPs and thus supports the recently reported alteration in the expression levels of MMPs/TIMPs, upon use of chromatin-modifying agents (Vincent *et al.* 2015).

Deregulation in the invasive behavior of placental trophoblasts leads to the development of abnormal pregnancies such as GTDs (Hui *et al.* 2005) and preeclampsia (Brosens *et al.* 1972). Development of molar pregnancy and choriocarcinoma was found to be associated with abnormally high expression levels of *MMP2* and *MMP9* and low expression levels of *TIMP2* and *TIMP1*, suggesting the association of raised MMPs: TIMPs ratio with the abnormal trophoblastic invasiveness observed in these disorders. Such a phenomenon is also observed in metastatic cancers (Nuovo *et al.*

Table 4 Pearson correlation and multiple regression analysis for *MMP2* and *MMP9* and *TIMP2* and *TIMP1* in all five placental villous groups.

Gene	<i>r</i>		mRNA vs DNA methylation	Multiple regression analysis with mRNA expression as dependent variable: <i>r</i> ² (SEM) [#]
	H3K9me3	H3K27me3		
<i>MMP2</i>			0.45	0.84 (0.01)
mRNA	−0.4	−0.77		
DNA methylation	0.45	0.04		
<i>TIMP2</i>			ND	0.56 (0.03)
mRNA	−0.53	−0.73		
DNA methylation	UD	UD		
<i>MMP9</i>			0.76	0.92 (0.001)
mRNA	−0.72	−0.62		
DNA methylation	−0.2	−0.14		
<i>TIMP1</i>			0.3	0.93 (0.008)
mRNA	−0.9*	−0.85*		
DNA methylation	−0.2	0.03		

r, Pearson's coefficient, *r*², regression determination coefficient, S.E.M., standard error of measurement, ND, not determined due to unmethylated gene promoter. [#]Equation for Prediction for each gene was 'mRNA expression = β₁H3K9me3 + β₂H3K27me3 + β₃DNA methylation + α', where β₁, β₂, β₃ are regression coefficients and α is intercept of test. *P < 0.05.

1995). Our findings are in accordance with the earlier studies correlating high expression of MMPs to the high invasiveness in GTDs (Singh *et al.* 2011) and inverse correlation between invasiveness of human cells and the levels of TIMPs (Hicks *et al.* 1984). Decreased levels of H3K9me3 at *MMP9* and increased levels of H3K9me3 at *TIMP1* and both H3K9/27me3 at *TIMP2* were detected in molar villi, relative to early trimester normal villi. The association of such changes in H3 trimethylation levels and mRNA expression of these genes were also supported by observed higher Pearson's correlation in our study. Therefore, these observations emphasize the role of aberrant trimethylation at H3K9/K27 in the pathology of molar pregnancy. Aberrant histone modifications at the promoter regions of MMPs are also reported to be involved in the pathogenesis of other diseases such as skin disorders and diabetic retinopathy (Kim *et al.* 2009, Zhong & Kowluru 2013). Silencing of TIMPs via promoter methylation is a prominent feature of cancer cells and such DNA methylation-mediated repression of gene expression was observed for *TIMP1* in choriocarcinoma cell line (JEG-3 cells) (Yuan *et al.* 2004). Notably, JEG-3 cells revealed hypermethylation at the promoter regions of *MMP2* and *MMP9*. However, it was not found to down-regulate the expression levels of these genes, whereas a positive Pearson's correlation coefficient was observed between mRNA expression and the respective promoter region DNA methylation of *MMP2* and *MMP9*. Similar examples of the up-regulation of hypermethylated genes have been cited earlier as well (Jones 1999, Renner *et al.* 2013). The transcriptional regulation of such overexpressed and hypermethylated genes can be either independent of DNA methylation or promoter hypermethylation might be affecting gene transcription via some different epigenetic mechanisms (Jones & Baylin 2007). Furthermore, high methylation was in fact found to be correlated with enhanced invasiveness. Such a correlation has been reported previously for placental first-trimester explants, where

increased methylation of *MMP2* was found to be associated with high invasiveness of these explants (van Dijk *et al.* 2012).

Shallow interstitial invasion and limited endovascular invasion by trophoblasts have been associated with the pathogenesis of preeclampsia (Norwitz *et al.* 2001). In this study, the development of preeclampsia was found to be associated with deregulation in the levels of H3K9/27me3, showing increased levels of H3K9me3 and both H3K9/27me3 associated with low expression of *MMP2* and *MMP9* respectively. These results support the documented deficient expression of *MMP2* and *MMP9*, in preeclampsia (Lockwood *et al.* 2008, Zhang *et al.* 2011). MMPs also play important role in controlling the bioactivity of growth factors, angiogenic factors, cytokines (Staun-Ram & Shalev 2005), and release of VEGF (Monaco *et al.* 2006), thus their deficient expression in preeclampsia might also be hampering the VEGF release, as supported by lower levels of VEGF reported in preeclampsia (Levine *et al.* 2004). Hence, these factors might be contributing to poor migration and proliferation of the endothelial cells and poor development of new blood vessels. The observed high expression of *MMP2* in maternal blood in preeclampsia is consistent with the documented high level of *MMP2* in preeclamptic plasma (Myers *et al.* 2005) and might have a role in regulating vascular tone, as highlighted by a recent study that suggested the role of MMP1 expression in vasoconstriction (Mousa *et al.* 2012). Consistent with our results, high levels of *TIMP1* and *TIMP2* have been demonstrated in preeclamptic endothelial cells (Merchant *et al.* 2004). High expression levels of *TIMP2* and *TIMP1* in preeclampsia might be further decreasing the activities of already compromised levels of *MMP2* and *MMP1*, respectively, hence leading to decreased invasion of uterine vasculature. Thus, the reported decrease in the invasive potential of JAR cells after their treatment with preeclamptic serum (Mahameed *et al.* 2005) might be due to high levels of TIMPs present in preeclamptic

serum. Overexpression of *TIMP1* and *TIMP2* is known to reduce tumor growth (Bai *et al.* 2005), and keeping in view the homology between normal placentation and tumor growth, it can be commented that their increased expression might also be contributing to the specific phenotype of preeclampsia involving intrauterine growth retardation and lighter placentas.

Significantly higher expressions of MMPs and TIMPs except *TIMP1*, in placental villi with reference to their respective maternal blood, support the role and importance of these genes in placental invasiveness. Relative lower expression of *TIMP1* in first-trimester and molar placental villi with respect to their corresponding maternal blood might be associated with the minimum inhibition of *MMP9* in these placental tissues.

Our study analyzed the role of only H3K9/27me3, as we aimed to study the effect of transcription-repressing epigenetic mechanisms such as DNA methylation and histone trimethylation; however, future study can be carried out to analyze the role of transcription-enhancing histone modifications such as histone acetylation to provide more insight into the role of various histone modifications on the differential expression of MMPs/TIMPs during placentation. Moreover, this study has only analyzed the mRNA expression of MMPs irrespective of their activity, as we intended to access gene regulation at the DNA level.

Conclusion

In light of our results, we suggest the role of balanced expression of MMPs and their respective TIMPs, mediated by H3K9/27me3, as the strong regulatory factor for maintaining normal pregnancy and the deregulation of these modifications associated with abnormal expression of MMPs and their respective TIMPs, involved in the development of placental disorders such as preeclampsia and hydatidiform mole. The observed aberrations in histone modifications and the changes induced in mRNA expression of MMPs and their TIMPs can be further evaluated for their potential as targets to design therapeutic strategies for pregnancy-related disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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