The expression and clinical significance of three lncRNAs in patients with a missed abortion

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Abstract. Missed abortions are common complications that occur in early pregnancy, and impaired trophoblast functions have been indicated to be associated with their pathogenesis. The long noncoding RNAs (lncRNAs) Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1)*, HOX Transcript Antisense RNA (*HOTAIR)* and Maternally expressed gene 3 (*MEG3)* have been demonstrated to serve a crucial regulatory role in the mobility of trophoblast cells and embryo implantation. However, the expression profile and role of each of these three lncRNAs in patients with a missed abortion remain unclear. The expression of *MALAT1*, *HOTAIR*, and *MEG3* in decidual and villous tissues from 26 patient exhibiting a missed abortion and 26 healthy controls was detected using reverse-transcription quantitative PCR. Serum TNF-α, IL-1β, IL-6 and IL-10 levels were measured using ELISA, and serum estradiol and progesterone levels were measured with electrochemiluminescence immunoassays. Additionally, the correlations between lncRNA expression and the levels of cytokines and hormones were further analyzed. *MALAT1*, *HOTAIR* and *MEG3* expression was significantly higher in villous tissues of patients exhibiting a missed abortion compared with healthy controls. *MALAT1* expression was higher in decidual tissues of patients exhibiting a missed abortion compared with healthy controls. Serum IL-10 levels were significantly lower in patients exhibiting a missed abortion compared with healthy controls. Serum estradiol and progesterone levels were significantly lower in the group of patients exhibiting a missed abortion compared with the control group. Furthermore, *MALAT1* expression in villous tissue was inversely related to serum progesterone levels. The results of the current study suggest that *MALAT1* may be associated with the pathogenesis of missed abortions.

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Introduction

Missed abortions are a common complication in early pregnancy, affecting ~15% of clinically recognized pregnancies (1). The incidence of missed abortions in China is 13.4% and has been rapidly increasing annually (2). The majority of missed abortion cases are asymptomatic, which are discovered during routine ultrasound examination and are currently treated by dilation and curettage, which causes physiological and psychological harm to women and their families (3). Previous studies have indicated that the risk factors for a missed abortion include chromosomal abnormalities, infections, maternal systemic diseases, endocrine disorders and anatomical defects (4-6). However, the cause of ~30% of missed abortions is still unclear.

Long noncoding RNAs (lncRNAs) are a subtype of noncoding RNAs with transcripts >200 nucleotides that do not encode proteins. Recent studies have indicated that three lncRNAs, Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1)*, HOX transcript antisense RNA *(HOTAIR*) and Maternally expressed gene 3 (*MEG3)*, serve a crucial role in regulating the migratory and invasive ability of tumor cells (7-10). Trophoblasts share a number of common features with tumor cells, such as high proliferative capacity, invasive nature, secretion of growth factors and hormones and immunosuppressive activities (11). Trophoblast dysfunction has also been associated with adverse pregnancy outcomes, including missed abortion (12). Insufficient trophoblast invasion may result in inadequate vascular remodeling and placental perfusion, which can lead to a variety of pregnancy complications, such as miscarriage, preeclampsia and intrauterine growth restriction (13). Over-invasion of trophoblasts in the first trimester may induce immune response at the maternal-fetal interface, leading to failure in the induction of immune tolerance and miscarriage (14). Therefore, the precise regulation of trophoblast invasion is essential for maintaining a successful pregnancy (15). The MALAT1, HOTAIR and MEG3 lncRNAs are thought to be involved in the mobility of trophoblast cells and embryo implantation (12). However, the expression profile and role of the three lncRNAs in patients with missed abortion remain unclear.

In the present study, the expression levels of the MALAT1, HOTAIR and MEG3 lncRNAs were examined in decidual and chorionic tissue from women who had a missed abortion and in women with normal pregnancies. Additionally, serum TNF-α, IL-1β, IL-6 and IL-10 levels in the two groups were detected. The correlation between lncRNA expression and the levels of cytokines, estradiol and progesterone in patients with a missed abortion were evaluated.

Patients and methods

Clinical samples. A total of 52 patients who underwent dilation and curettage and a median age of 31 years (age range, 20-45 years) were enrolled into the present study. Decidual and villous tissue as well as serum (1.5 ml per patient) were obtained during or before the dilatation and curettage procedure in the Department of Obstetrics and Gynecology, Beijing Luhe Hospital, Capital Medical University (Beijing, China), between October 2017 and May 2018. The patients exhibiting a missed abortion (n=26) and control individuals (n=26) were diagnosed via ultrasound examination 6-12 weeks of gestation. The inclusion criteria for missed abortion include: i) Individuals harboring a fetus <5 mm with either no growth or heartbeat; ii) a fetus of ≥5 mm without heartbeat; or iii) an empty gestational sac of ≥20 mm. Women with obvious risk factors for missed abortion, including chromosomal abnormalities, autoimmune disorders, infections, endocrine diseases, twin pregnancy, anatomical abnormalities, those currently on medication within 1 month, those with insufficient amount of villous samples and poor quality RNA were excluded. Written informed consent was obtained from all participants, and the present study was approved by the ethics committee of the Medical Faculty, Beijing Luhe Hospital, Capital Medical University.

RNA Extraction and reverse-transcription quantitative (RT-q) PCR. Total RNA was isolated from decidual and villous tissues using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed with 1 μg of total RNA, using the temperature protocol of incubation at 55˚C for 15 min and 85˚C for 5 sec using RevertAid First Strand complementary (c)DNA synthesis kits (Thermo Fisher Scientific, Inc.). cDNA was quantified using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the following conditions: Pre-denaturation at 95˚C for 10 min followed by 40 cycles of amplification at 95˚C for 15 sec and 60˚C for 1 min. Each sample was examined in triplicate. The primer sequences (GENEWIZ) are listed in Table I. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (16). All samples were normalized to GAPDH, which served as an endogenous control.

Measurement of serum tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 levels. Blood samples were obtained before vacuum aspiration. The samples were placed in 4˚C for blood clotting, and serum was acquired after centrifugation at 1,620 x g for 15 min at 4˚C. Serum was then collected and frozen at -80˚C for cytokine analysis. The levels of serum TNF-α, IL-1β, IL-6 and IL-10 were measured with respective human TNF-α (cat. no. ml064303), IL-1β (cat. no. ml058059), IL-6 (cat. no. ml028583) and IL-10 (cat. no. ml064299) ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.) according to the manufacturer's protocols. Experiments were repeated in triplicate.

Table I. The primer sequences used for reverse-transcription quantitative-PCR.

Gene	Primer sequences (5'-3')
MALAT1	
F	AAAGCAAGGTCTCCCCACAAG
R	GGTCTGTGCTAGATCAAAAGGCA
HOTAIR	
F	GGTAGAAAAAGCAACCACGAAGC
R	ACATAAACCTCTGTCTGTGAGTGCC
MEG3	
F	GCATTAAGCCCTGACCTTTG
R	TCCAGTTTGCTAGCAGGTGA
GAPDH	
F	CCTGGTATGACAACGAATTTG
R	CAGTGAGGGTCTCTCTCTTCC

F, forward; R, reverse; *MALAT1*, Metastasis Associated Lung Adenocarcinoma Transcript 1; *HOTAIR*, HOX Transcript Antisense RNA; *MEG3*, maternally expressed gene 3.

Measurement of serum estradiol and progesterone levels. Serum was obtained via centrifugation as aforementioned and serum estradiol and progesterone levels were measured via ECL immunoassays using the Roche Cobas E601 Analyzer. The dilution for estradiol and progesterone is 1:10 (cat. nos. 06656021190 and 07092539190; Roche Diagnostic GmbH) according to the manufacturer's protocol.

Statistical analysis. Data are presented as the mean ± SD. Expression differences between groups were analyzed by the Mann-Whitney U test. Correlations were measured using Spearman's correlation test. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 5.0 software (GraphPad Software, Inc.) was used to perform data analysis.

Results

Patients' general and clinical information. Table II summarizes the characteristics of missed abortion patients and healthy controls. No significant differences were indicated in age, BMI, gestational age, gravidity, parity, number of previous pregnancies, number of live births or number of previous miscarriages between the two groups.

MALAT1, HOTAIR and MEG3 expression is upregulated in villous tissue from patients with missed abortion. To explore the role of MALAT1, HOTAIR and MEG3 lncRNAs in the pathogenesis of missed abortion, the expression of these three lncRNAs were evaluated using RT-qPCR in first trimester

Table II. Characteristics of patients with missed abortion and healthy controls.

Characteristic	Healthy controls (n =26)	Patients with missed abortion (n =26)	P-value
Maternal age (years)	29.81±1.30	32.73±1.22	0.14
BMI (kg/m^2)	21.97±0.67	24.39±1.73	0.44
Gestational age (days)	51.65±1.80	51.23±1.44	0.89
Gravidity	2.46±0.27	2.81±0.31	0.53
Parity	0.65±0.11	0.69±0.12	0.87
Number of previous pregnancies (%)			0.98
0	1 (3.8)	0 (0.0)	
1	7 (26.9)	6 (23.1)	
2	6 (23.1)	6 (23.1)	
3	5 (19.2)	7 (26.9)	
4	5 (19.2)	4 (15.4)	
≥5	2 (7.7)	3 (11.5)	
Number of previous miscarriages (%)			0.05
0	26 (100.0)	21 (80.8)	
≥1	0 (0.0)	5 (19.2)	
Number of live births (%)			P>0.99
0	10 (38.5)	10 (38.5)	
1	15 (57.7)	14 (55.8)	
2	1 (3.8)	2 (7.7)	

decidua and villous tissue. MALAT1 expression was significantly upregulated in decidua tissue of patients with missed abortion (P=0.025; Fig. 1A), while HOTAIR and MEG3 expression indicated no significant differences between the missed abortion and healthy control groups (Fig. 1C and E). As presented in Fig. 1B, D and F, the results of villous samples showed that MALAT1 (P<0.001), HOTAIR (P=0.001) and MEG3 (P=0.002) expression were significantly upregulated in patients with missed abortion compared with healthy controls.

Expression of IL-10 in serum of patients with missed abortion is downregulated. The serum levels of the proinflammatory factors TNF-α, IL-1β and IL-6 and the anti-inflammatory cytokine IL-10 was detected in two groups using ELISA. The results indicated that the levels of TNF-α, IL-1β and IL-6 slightly increased in patients with a missed abortion, but there were no significant differences (Fig. 2A-C). Serum IL-10 levels were significantly lower in the missed abortion group compared with the control group (P<0.05, Fig. 2D). The correlation of serum IL-10 levels with the expression of three lncRNAs was further analyzed and the results indicated that there was no significant correlation of serum IL-10 with MALAT1 expression in decidua and villous tissue or with HOTAIR and MEG3 expression in villous tissue (Fig. 3). The expression of HOTAIR and MEG3 in decidual tissues showed no significant differences between the two groups, therefore the correlation of serum IL-10 levels with the HOTAIR and MEG3 expression in decidual tissues was not analyzed.

MALAT1 expression in villous tissues inversely correlates with serum progesterone levels. The levels of the serum hormones progesterone and estradiol were subsequently detected and the results indicated that serum progesterone (P<0.001) and estradiol (P<0.001) levels in patients exhibiting a missed abortion were significantly lower compared with the healthy controls (Fig. 4). Additionally, the current study determined whether the expression of the three lncRNAs was association with the levels of serum progesterone and estradiol. No statistically significant correlation was indicated between HOTAIR or MEG3 expression in villous and serum progesterone and estradiol levels (Fig. 5D-H). However, MALAT1 expression in villous tissues was significantly inversely associated with serum progesterone levels (Fig. 5C), but there was no significant correlation between serum progesterone and estradiol levels and MALAT1 expression in decidua (Fig. 5A and B).

Discussion

lncRNAs serve an important role in normal cell and tissue development and differentiation, including in embryo implantation (17). Increasing evidence has demonstrated that multiple lncRNAs are dysregulated in pregnancy complications such as miscarriage (18-20), preeclampsia (21,22) and intrauterine growth restriction (23). A growing body of evidence has indicated that *MALAT1, HOTAIR* and *MEG3* serve a key role in regulating the mobility of trophoblast cells. Knockdown of *MALAT1* and *MEG3* in HTR-8/SVneo cells was revealed to result in suppressed cell invasion, decreased cell viability and increased cell apoptosis, while overexpression of *MALAT1* and *MEG3* had the opposite effect on trophoblast cells (24,25). Zhang *et al* (26) reported that overexpression of *HOTAIR* in HTR-8/SVneo trophoblast cells, which was measured using

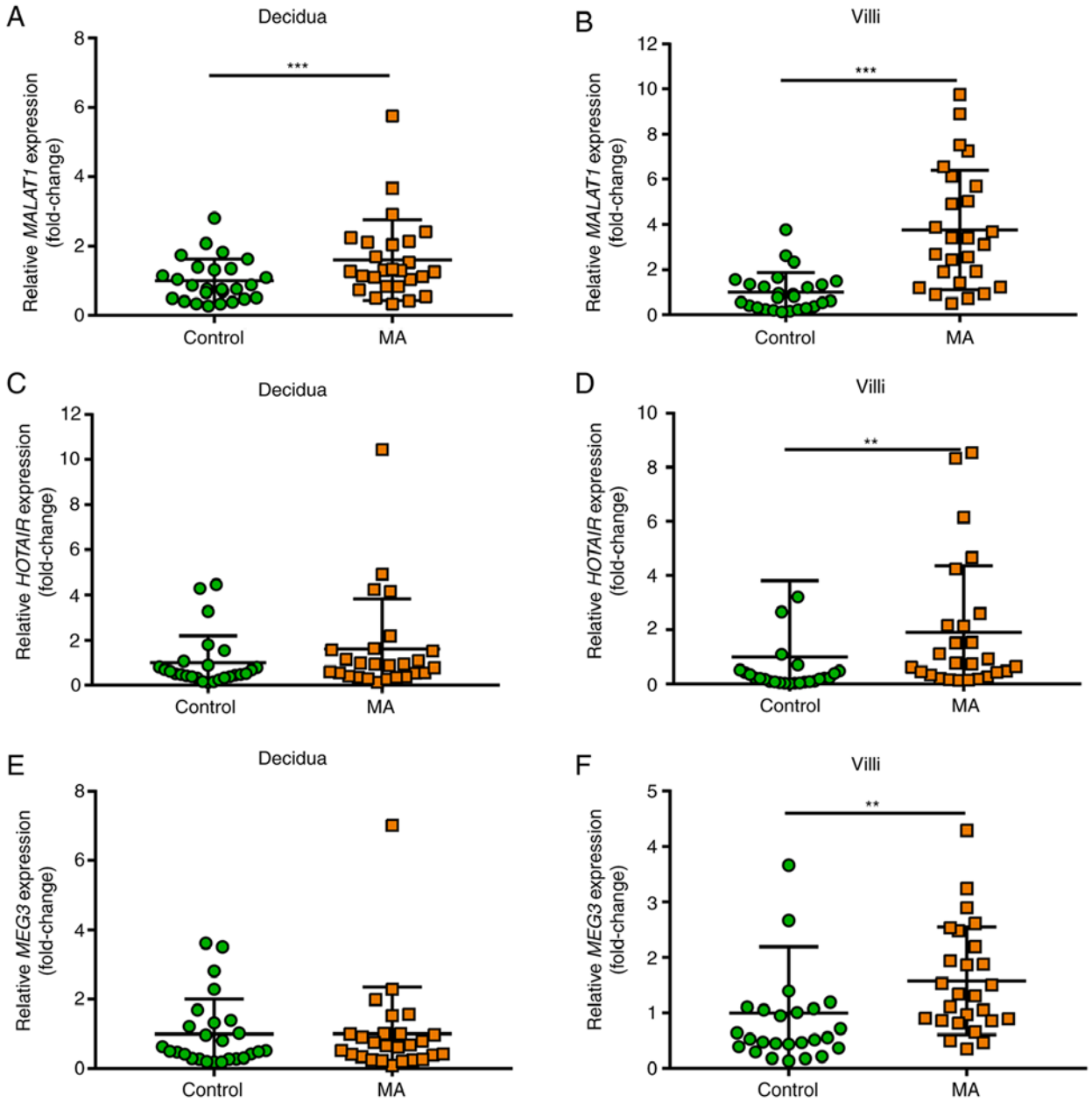

Figure 1. Expression of *MALAT1*, *HOTAIR* and *MEG3* in decidual and villous tissue from patients with missed abortion and healthy pregnancies detected using reverse-transcription quantitative PCR. Expression of *MALAT1* in (A) decidual and (B) villous tissues of patients with missed abortions and healthy controls. Expression of *HOTAIR* in (C) decidual and (D) villous tissues of patients with missed abortions and healthy controls. Expression of *MEG3* in (E) decidual and (F) villous tissues of patients with missed abortions and healthy controls. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001. MA, missed abortion; *MALAT1*, Metastasis Associated Lung Adenocarcinoma Transcript 1; *HOTAIR*, HOX Transcript Antisense RNA; *MEG3*, Maternally expressed gene 3.

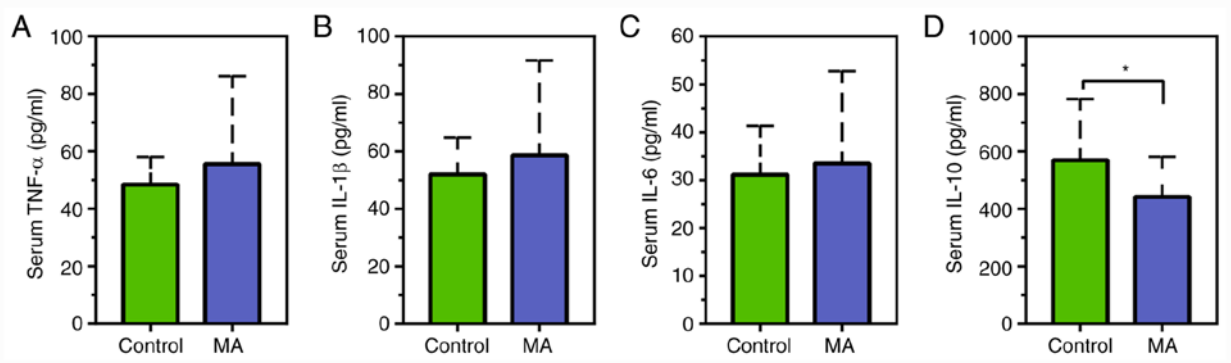

Figure 2. Level of serum inflammatory cytokines detected by ELISA. The levels of serum (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-10 in missed abortion patients and healthy controls. Data are presented as the mean ± SD. *P<0.05. MA, missed abortion.

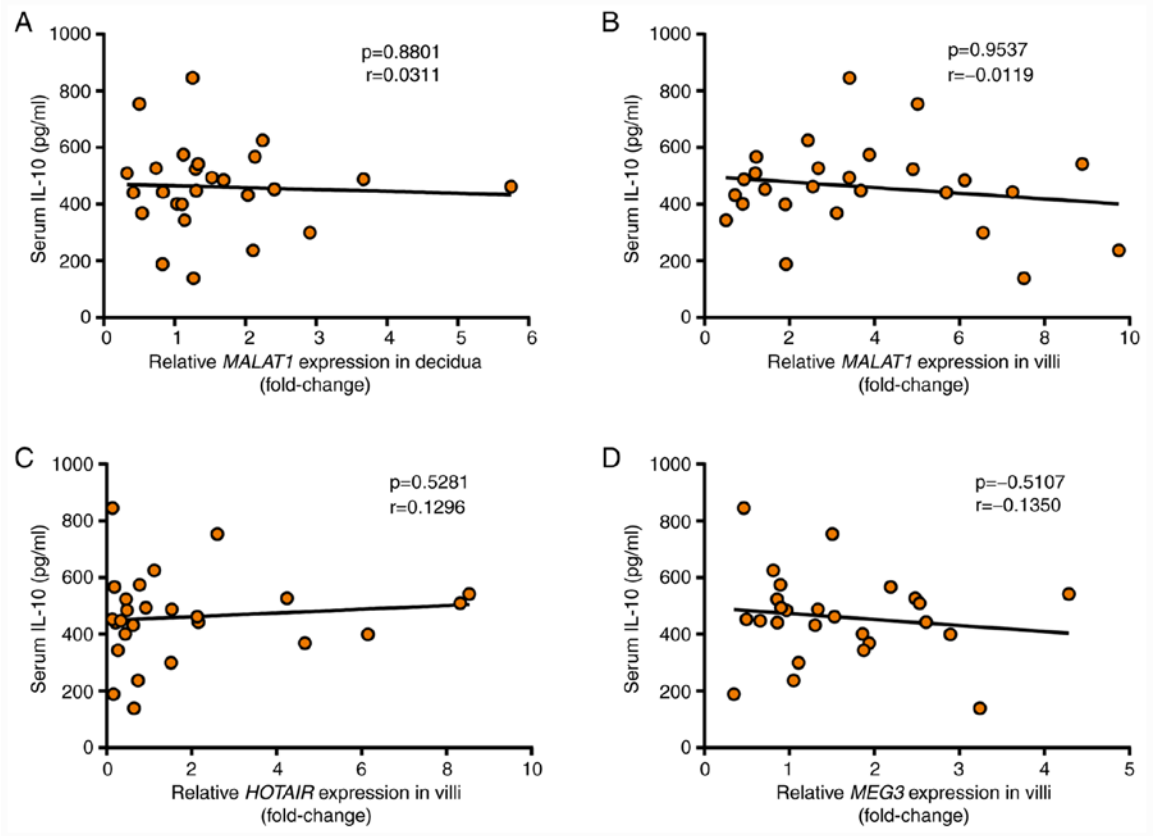

Figure 3. Correlation analysis of *MALAT1*, *HOTAIR* and *MEG3* expression with serum IL-10 levels in patients exhibiting missed abortion. Correlational analysis between *MALAT1* in (A) decidual and (B) villous tissue and serum IL-10 levels, and correlational analysis between (C) *HOTAIR* and (D) *MEG3* in villous tissue and serum IL-10 levels. *MALAT1*, Metastasis Associated Lung Adenocarcinoma Transcript 1; *HOTAIR*, HOX Transcript Antisense RNA; *MEG3*, maternally expressed gene 3.

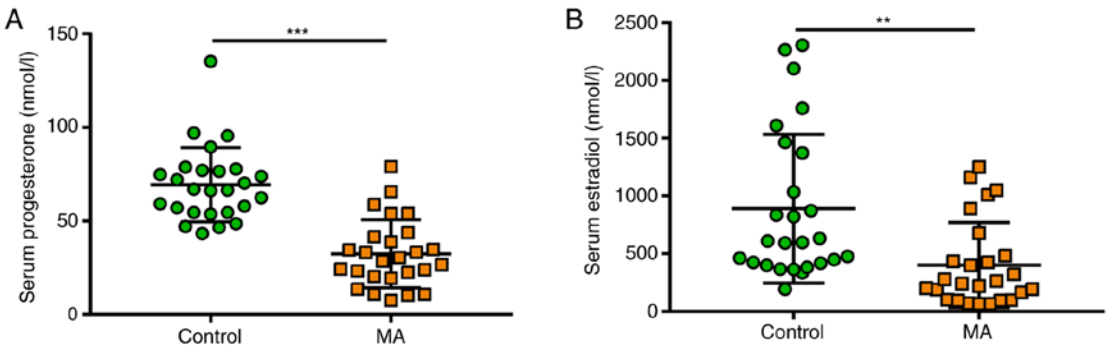

Figure 4. Serum progesterone and estradiol levels in patients with missed abortion and healthy controls. (A) Serum progesterone and (B) serum estradiol levels in patients with missed abortion and healthy controls. Data are presented as the mean ± SD. **P<0.01, ***P<0.001. MA, missed abortion.

Matrigel cell invasion assays and *ex vivo* explant culture models, leads to increased invasive properties (26). Abnormal expression of these three lncRNAs have been associated with the pathogenesis of adverse pregnancy outcomes. In preeclamptic placenta, *MALAT1* and *MEG3* were demonstrated to be downregulated, which suppressed the migratory and invasive capabilities of trophoblast cells and resulted in preeclampsia (27). Conversely, *MALAT1* is overexpressed in placenta previa increta/percreta and has been strongly associated with the high invasion ability of trophoblast cells (28). Overexpression of MALAT1 may lead to over-invasion of trophoblast cells, which contributes to failure of immune tolerance, leading to pathological pregnancy outcomes (29). *HOTAIR* was reported to exhibit significantly higher expression in placenta from patients with preeclampsia (30). Additionally, the expression of *HOTAIR* in the human placenta was reported to be negatively correlated with the expression of vascular endothelial growth factor A (VEGFA), and *HOTAIR* was reports to suppress the angiogenesis of the human placenta by inhibiting VEGFA expression (31). Defective angiogenesis in the first trimester contributes to a majority of early missed abortions.

In the present study, *MALAT1*, *MEG3* and *HOTAIR* lncRNAs were demonstrated to be significantly overexpressed

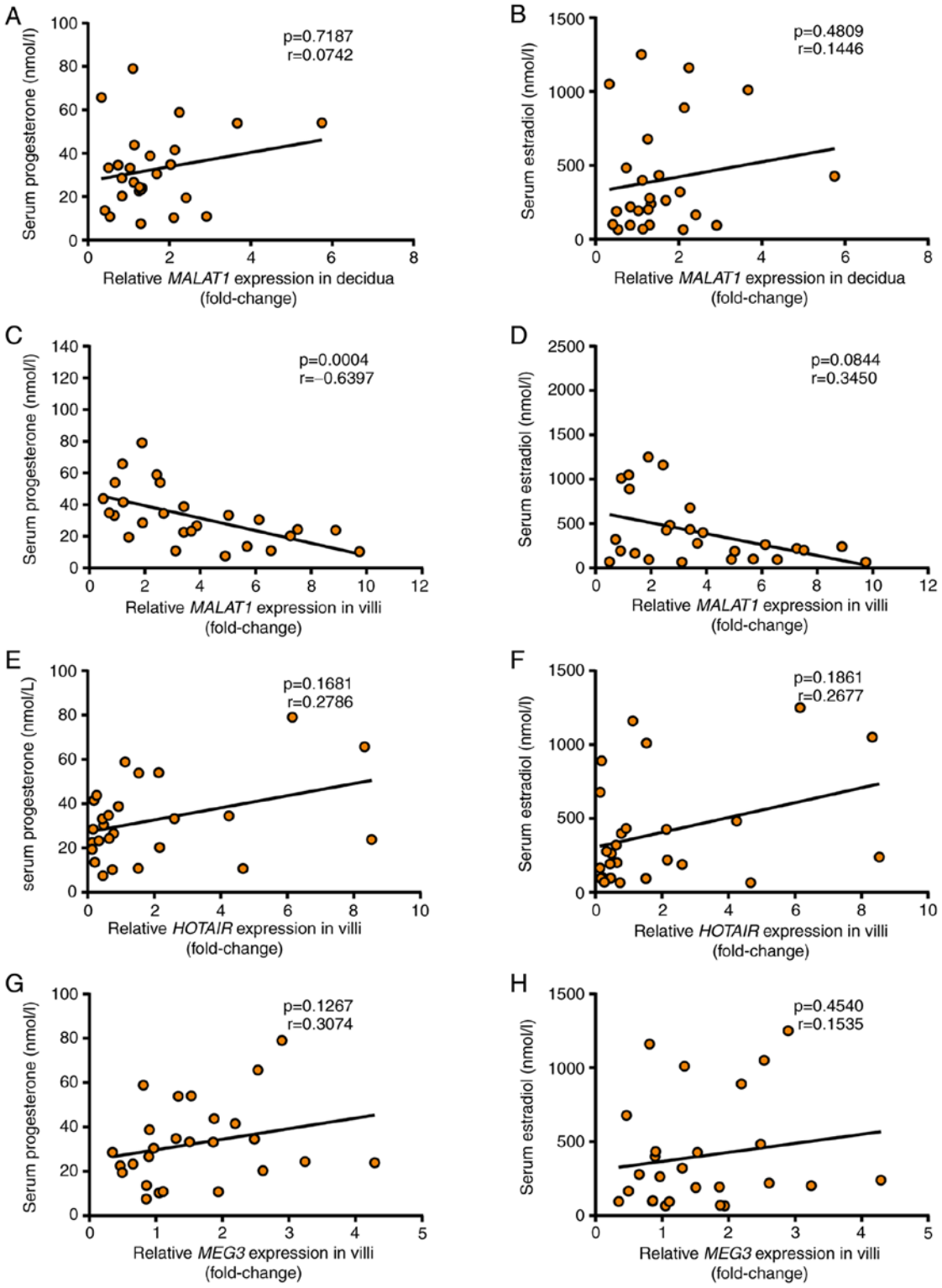

Figure 5. Correlation analysis of *MALAT1*, *HOTAIR* and *MEG3* expression with serum progesterone and estradiol levels in patients with missed abortion. Correlation analysis between decidual *MALAT1* and (A) progesterone and (B) estradiol levels and villous *MALAT1* and (C) progesterone and (D) estradiol levels in patients with missed abortion. Correlation analysis between villous *HOTAIR* and (E) progesterone and (F) estradiol in patients with missed abortion. Correlation analysis between villous *MEG3* and (G) progesterone and (H) estradiol in patients with missed abortion. *MALAT1*, Metastasis Associated Lung Adenocarcinoma Transcript 1; *HOTAIR*, HOX Transcript Antisense RNA; *MEG3*, Maternally expressed gene 3.

in villous tissues from patients exhibiting a missed abortion, which implies that aberrant expression of these three lncRNAs may be associated with the pathology of missed abortions by regulating the function of trophoblast cells in the first trimester. Furthermore, the results of the current study demonstrated that *MALAT1* was expressed in decidual tissue from healthy pregnancies and was significantly upregulated in patients exhibiting missed abortion. Although the biological significance of *MALAT1* expression in decidual tissues remains unclear, it should be noted that *MALAT1* may be associated

with decidualization. The current study also detected serum progesterone and estradiol levels in the two groups and revealed that serum progesterone and estradiol levels were significantly lower in the missed abortion group compared with the healthy control group. The correlation between *MALAT1* expression and serum progesterone was also examined, and the results demonstrated that increased *MALAT1* expression was inversely correlated with serum progesterone levels. Progesterone is an important hormone in regulating endometrial cell decidualization, which is an essential step in the establishment and maintenance of pregnancy (32). The results of the current study indicated that *MALAT1* exerts biological roles in missed abortion by regulating the behavior of trophoblast cells and also by mediating decidualization.

An appropriate balance between cytokines produced by Th1 and Th2 cells is essential for a successful pregnancy (33). It is widely believed that the first trimester of pregnancy is dominated by proinflammatory Th1 cells to ensure successful implantation (34). A number of studies have demonstrated that the skewed balance of Th1/Th2 cells may result in adverse pregnancy outcomes (35,36), such as early pregnancy loss and preeclampsia (37). As an important cytokine in the Th2-dependent immune responses, IL-10 exerts a regulatory effect on Th1/Th2 balance. IL-10 may induce the expression of HLA-G in trophoblasts and monocytes at the maternal-fetal interface and reduce the expression of MHC-I and MHC-II antigens on the surface of monocytes (38). Additionally, IL-10 can also inhibit Th1 cytokines secreted by Th1 cells (39). Healthy pregnant women have significantly higher production of IL-10 and other Th2 cytokines (40). Previous studies have indicated that the reduced production of IL-10 may contribute to recurrent miscarriages (41). Patients with recurrent pregnancy loss exhibit a lower proportion of IL-10+ CD19+ B cells, and reduced levels of IL-10 in serum and supernatant of the stimulated B cell culture medium (42). The results of the current study are consistent with this, indicating that serum IL-10 was significantly lower in the missed abortion group. Furthermore, a number of studies have demonstrated that lncRNAs are associated with the regulation of Th1/Th2 balance and inflammation at the maternal-fetal interface (43,44). Ou *et al* (45) recently reported that *MALAT1* promotes the production of proinflammatory cytokines via activation of the NF-kB pathway, and exacerbates hypertension symptoms in the RUPP-induced rat model. In trophoblasts, downregulation of *MALAT1* inhibits inflammation by reducing the secretion of inflammatory factors TNF-α, IL-6 and TGF-β (18). However, correlations were not revealed between the expression of MALAT1 and serum IL-10, which may be partially explained by the small sample size. Future studies should investigate these issues.

In conclusion, the current study demonstrated that *MALAT1* is upregulated in decidual and villous tissue in patients exhibiting a missed abortion, and higher expression of *MALAT1* is inversely associated with serum progesterone levels. The results also revealed that *HOTAIR* and *MEG3* expression are increased in villous samples from patients with missed abortions. These data indicated that *MALAT1* may be involved in the pathogenesis of missed abortion. Further studies should aim to elucidate the molecular mechanisms underlying adverse pregnancy outcomes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

ML designed and executed the study, analyzed the data and prepared the manuscript. HX, LW and JZ collected the clinical data. JG and WM conceived the study and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the Medical Faculty, Beijing Luhe Hospital, Capital Medical University. Written informed consent was obtained from all individual participants included in the study prior to collection of tissue and blood samples.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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