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Expression of Liver Receptor Homolog-1 (LRH-1) in Villi and Decidua of Patients with Unexplained Recurrent Spontaneous Abortion

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: In view of the important function of nuclear receptor liver receptor homolog 1 (LRH 1) in various biological processes and the physiological changes accompanying unexplained recurrent spontaneous abortion (USRA), our study was carried out to investigate the potential roles of LRH-1 in USRA.

Material/Methods: Thirty patients with URSA at early the early state of pregnancy were selected, and 30 patients with normal early pregnancy were also selected from Aug 2015 to Sep 2016 as a control group. The expression of LRH-1 protein in decidua and villi were detected by immunohistochemistry and Western blot analysis, and the expression of LRH-1 mRNA was detected by RT-PCR. The expression levels of CYP19 and P450scc were detected by RT-PCR and Western blot analysis at mRNA and protein levels, respectively.

Results: The levels of LRH-1, CYP19, and P450scc mRNA and protein in villi of the patients in the URSA group were significantly lower than in the control group. There were no significant differences between the URSA group and control group in the levels of LRH-1, CYP19, and P450scc mRNA and protein in villi in decidua.

Conclusions: URSA was related to the reduced expression level of LRH-1 in villous tissues but not in decidua, and expression of LRH-1 may be related to the expression of CYP19 and P450scc. We believe that the expression level of LRH-1 can be used as a marker in the early diagnosis of URSA, and the regulation of LRH-1 expression many lead to new URSA treatments.

MeSH Keywords: Abortion, Induced • Chorionic Villi • Decidua

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Background

RSA is a disease that can cause 3 or more consecutive spontaneous abortions within the first 24 weeks of pregnancy [1]. RSA affects 2–5% of couples who want to reproduce [2]. RSA can be induced by various factors, including genetic factors, anatomic abnormalities, autoimmunologic disorders, and infections [3–6]. However, in some cases RSA cannot be explained by any of these factors, and RSA without clear causes were called URSA. To date, no safe and effective method has been developed for the early diagnosis and treatment of USRA. Previous studies have shown that mixed lymphocyte reaction blocking factor (MLR-Bf) is a potential biomarker for the diagnosis of URSA [7]. A study has shown that the presence of MLR-Bf is a good indicator for the outcome of pregnancy in patients with 3 or more URSAs [8]; however, this method can only be used for patients who had never had demonstration of embryonic cardiac activity. This method does not work for patients with demonstration of embryonic cardiac activity or patients with less than 3 URSAs [8]. In addition, the high false-negative and false-positive rates of this method also limit the application of MLR-Bf as a biomarker in diagnosis of URSA. Therefore, it would be of great clinical value to identify new biomarkers with higher accuracy and efficiency in the diagnosis of URSA.

The nuclear receptor liver receptor homolog 1, which is also called LRH-1, is a transcription factor that plays pivotal roles in various physiological processes, including lipid metabolism [9], cancer development [10], and anti-inflammatory activities [11]. Previous studies have shown that the expression level of LRH-1 in ovaries was significant higher than that in other tissues [12,13], indicating the important functions of LRH-1 in this organ. It was also found that LRH-1 could regulate the ovarian intake of cholesterol to facilitate steroidogenesis [14]. Experiments in a mouse model using LRH-1 knockout found that LRH-1 can regulate ovulation [15]. Progesterone synthesis during pregnancy is important for the maintenance of pregnancy, and cholesterol side-chain cleavage enzyme (P450scc) catalyzes the synthesis of progesterone [16,17]. CYP19, which is also called aromatase cytochrome P450, is the key rate-limiting enzyme involved in the synthesis of estrogen. CYP19 can also regulate the expression of LRH-1 to play pivotal roles in placental development [18,19]. All those previous studies suggest that LRH-1, P450scc, and CYP19 may have important functions in URSA.

In our study, the potential role of LRH-1, P450scc, and CYP19 in URSA were explored by measuring their expression levels in patients with URSA and patients with normal pregnancy. We found that the low expression level of LRH-1 in villous tissues but not in decidua was correlated with URSA. Therefore, the detection of LRH-1 in villous tissues can be used in the diagnosis of URSA, and the regulation of LRH-1 expression may be useful in new treatments of URSA.

Material and Methods

Objects

Thirty patients with URSA in the early state of pregnancy, who were treated in the gynecology clinic of our hospital were selected from August 2015 to September 2016 to serve as the URSA group. At the same time, 30 patients with early pregnancy were also selected in the gynecology clinic of our hospital to serve as the normal pregnancy group (control group).

In the URSA group, vaginal bleeding after menopause and positive urinary HCG were detected in all the patients, and no fetal heart beat in the intrauterine fetal sac was detected by B-scan ultrasonography. The average age of the URSA patients was 27.61±2.93 years old and the average gestational age was 9.69±0.21 weeks. All the URSA patients had experienced at least 3 unexplained recurrent spontaneous abortions. Based on gynecological examinations and pelvic ultrasonography, we excluded URSA patients who had any genital abnormalities; chronic hypertension; diabetes; kidney, cardiovascular, and thyroid diseases; autoimmune diseases; or infectious diseases within 1 month before selection. In addition, the semen samples from URSA patients' partners were all normal.

In the control group, pregnancy was confirmed by pelvic ultrasonography and urine HCG test. The mean age of the patients in the control group was 27.23±1.54 years old and the average gestational age was 9.81±0.34 weeks. All the patients in the control group were pregnant for the first time. No pregnancy risk factors and infectious diseases within pregnancy were found in patients in the control group. They were not treated with any drugs and all patients in the control group were willing to terminate pregnancy. All patients in both the URSA group and the control group signed informed consent. This study was approved by the Ethics Committee of our hospital.

Specimen collection and treatment

Specimen collection was done during uterine surgery or artificial abortion. The fresh villi and decidual tissue were collected under aseptic conditions. A part of the specimen was fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) and subjected to immunohistochemistry. The rest of specimen was stored in liquid nitrogen for use in other experiments.

Immunohistochemistry to detect the expression of LRH-1 in decidua and villi

After fixation, decidua and villi tissue were routinely dehydrated, embedded with paraffin (Sigma-Aldrich, USA) and sliced to 5-μm continuous sections. Immunohistochemical S-P method was used to detect the expression of LRH-1 in decidua and

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villi according to the instructions of the kit (ZYMED, USA). After staining, the expression of LRH-1 was observed under an Olympus BX51 microscope (Olympus, Japan). Five 10×40 high-power fields were randomly selected, and the degree and percentage of cell staining were scored. The results were semi-quantitatively analyzed by software. The expression of LRH- 1 protein was expressed by the percentage of positive expression.

The visible brown dots in the right positions with clear background were the positive signals. The degree and percentage of cell staining were scored according to the following criteria: no staining, 0 point; light yellow color, 1 point; brown color, 2 points; dark brown color, 3 points. The percentage of stained cells over all the cells ≤5% is 0 points; 6–25% is 1 point; 26–50% is 2 points; ≥51% is 3 points. The staining degree of each slide was multiplied by the percentage of stained cells to get the final score. The final scores between 0 and 1 indicate negative expression (–); between 2 and 3 indicate weak positive expression (+); between 4 and 6 indicate medium positive expression (++); and 6 points indicates strong positive expression (+++).

RT-PCR to detect the expression of LRH-1, CYP19 and P450scc

The villi and decidual tissue were collected and immediately putted into Trizol reagent (Invitrogen, USA) for RNA extraction. A Nanodrop 2000 trace ultraviolet spectrophotometer (Thermo Fisher Scientific, USA) was used to detect the RNA concentration and purity at 260 nm and 280 nm. The reverse transcription procedure was performed according to the instructions of the kit (Takara, Japan). QRT-PCR amplification was performed using SYBR Green (Roche Molecular Biochemicals) with β-actin as endogenous control. Two replicates were used for each experiment. The primers were designed using Primer5.0 and the sequences of the primers used in this study were: F: 5'-CTGATACTGGAACTTTTGAA-3' (sense) and R: 5'-CTTCATTTGGTCATCAACCTT-3' (anti-sense) for LRH-1; F: 5'-TTGGAAATGGTCAACCCGAT-3' (sense) and R: 5'-CAGGAATCTGCC GTGGGAGA-3' (anti-sense) for CYP19; 5'-TCACTAACGTCATTTCTGGGGAGCGCCAGGG-3' (sense) and 5'-CCCTGGCGCTCCCCAGAAATGACGTTAGTGA-3' for P450scc; 5'-CGAGCCACATCGCTCAGACA-3' (sense) and 5'-CGATGCCGTGCTCGATGGGG-3' (anti-sense) for β-actin. The primers were synthesized by Sangon Biotech (Shanghai, China). Reaction conditions were: 95°C for 30 s, followed by 35 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The CFX Connect™ qRT-PCR system was used to detect the fluorescence threshold cycle (Ct) values for each sample. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of the target gene mRNA.

Western blot analysis to detect the expression of LRH-1, CYP19, and P450scc protein

The total protein was extracted from villi and decidual tissue by conventional method. The bicinchoninic acid assay (BCATM Protein Assay Kit, Pierce, USA) was used to quantify the protein. Then, the protein was subjected to 10% SDS-PAGE electrophoresis (Mini-Protean-3, Bio-Rad, USA), and the membrane was blocked with 5% skim milk. Mouse anti-human LRH-1 primary antibody (1: 1000, Abcam, USA) was added and incubated at 4°C overnight. After that, the membrane was washed 3 times with TBST for 10 min for each time. HRP-labeled goat anti-mouse secondary antibody (Abcam, USA) diluted with TBST (1: 500) was added and incubated at room temperature for 1 h. After that, ECL was added for exposure. β-actin was used as the endogenous reference. ImageJ2.1 (National Institutes of Health, USA) software was used to analyze the results. The relative expression levels of LRH-1, CYP19, and P450scc proteins were expressed by the ratios of the values of LRH-1, CYP19, and P450scc protein to that of β-actin.

Statistical analysis

SPSS19.0 statistical software was used to analyze the variance of monitoring data. All data are expressed as mean ± standard deviation ($\bar{\chi}\pm$SD). The comparisons between 2 groups were performed by t test, and $p<0.05$ was considered to be statistically significant.

Results

Clinical indicators of the objects

The average age, average gestational age, and the average body mass index (BMI) of the patients in the URSA group were 27.61±2.93 years, 9.69±0.21 weeks, and 24.41±1.14 kg/m^2, respectively, and the average age, average gestational age, and the average BMI of the patients in the control group were 27.23±1.54 years, 9.81±0.34 weeks, and 26.63±2.02 kg/m^2, respectively. There were no significant differences in age, gestational age, or BMI between the 2 groups ($p>0.05$). Therefore, the patients we selected were suitable for the comparison studies.

Expression of LRH-1 in villi and decidua of patients in the 2 groups

Figure 1 shows that LRH-1 protein in the URSA group and normal pregnancy group were detected in the villi, but the expression of LRH-1 in villi of patients in the URSA group was significantly lower than that in villi of patients in the normal pregnancy group ($p<0.05$). No significant difference was found in expression of LRH-1 in decidual tissue between the

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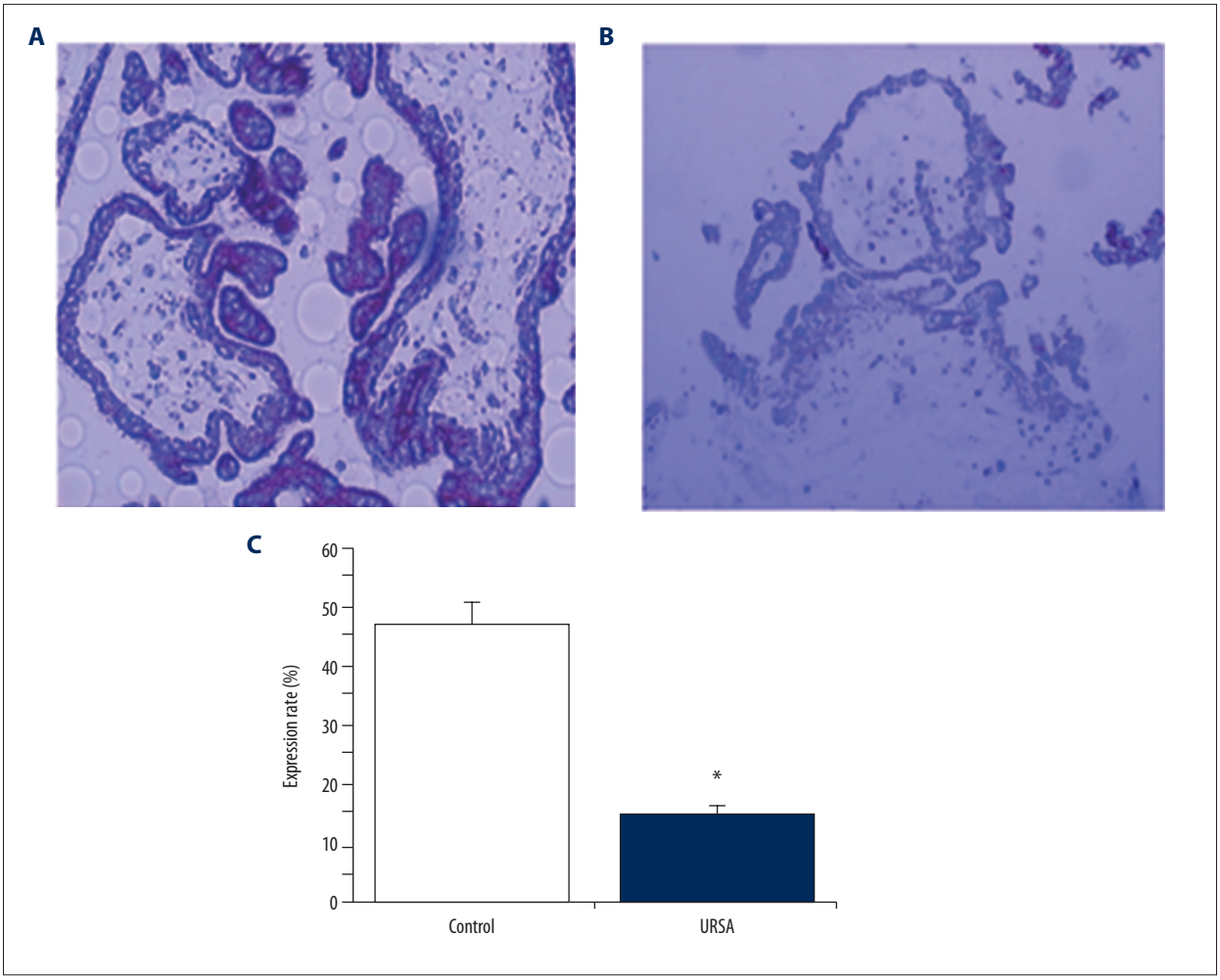

Figure 1. LRH-1 expression in villi of the patients in both groups (×100). (**A**) Representative result of villi staining of patients in control group. (**B**) Representative result of villi staining of patients in URSA group. (**C**) Positive expression rate of LRH-1 in villi of patients of the 2 groups. * Compared with the control group, $p<0.05$.

2 groups (Figure 2). Our data suggest that URSA can induce decreased expression of LRH-1.

The expression of LRH-1, CYP19, and P450scc mRNA in villi and decidua

The expression levels of LRH-1, CYP19, and P450scc mRNA were detected by RT-PCR. Figure 3 shows that the level of LRH-1 mRNA in villi of the patients in the URSA group was significantly lower than in villi of patients in the control group ($p<0.05$). There was no significant difference in the level of LRH-1 mRNA in decidua between the URSA group and control group ($p>0.05$). CYP19 and P450scc showed similar expression patterns to that of LRH-1; the levels of CYP19 and P450scc mRNAs in villi of patients in the URSA group were significantly lower than in villi of patients in the control group ($p<0.05$), but no significant difference was found in levels of CYP19 and P450scc mRNAs in decidua between the URSA group and control group ($p>0.05$). Our data suggest than USRA can reduce the expression of RH-1, CYP19, and P450scc mRNA in villi but not in decidua.

The expression of LRH-1, CYP19, and P450scc protein in villi and decidua

Western blot analysis was used to detect the expression levels of LRH-1, CYP19, and P450scc protein. The relative expression levels of LRH-1, CYP19, and P450scc protein were expressed by the ratio of the value of LRH-1, CYP19, and P450scc protein to that of β-actin. Figure 4 shows the level of LRH-1 protein in villi of the patients in URSA group was significantly lower than in villi of patients in the control group ($p<0.05$). There was no significant difference in the level of LRH-1 protein in decidua between the URSA group and control group ($p>0.05$). CYP19 and P450scc protein showed similar expression patterns to that of LRH-1; the levels of CYP19 and P450scc protein in villi of the

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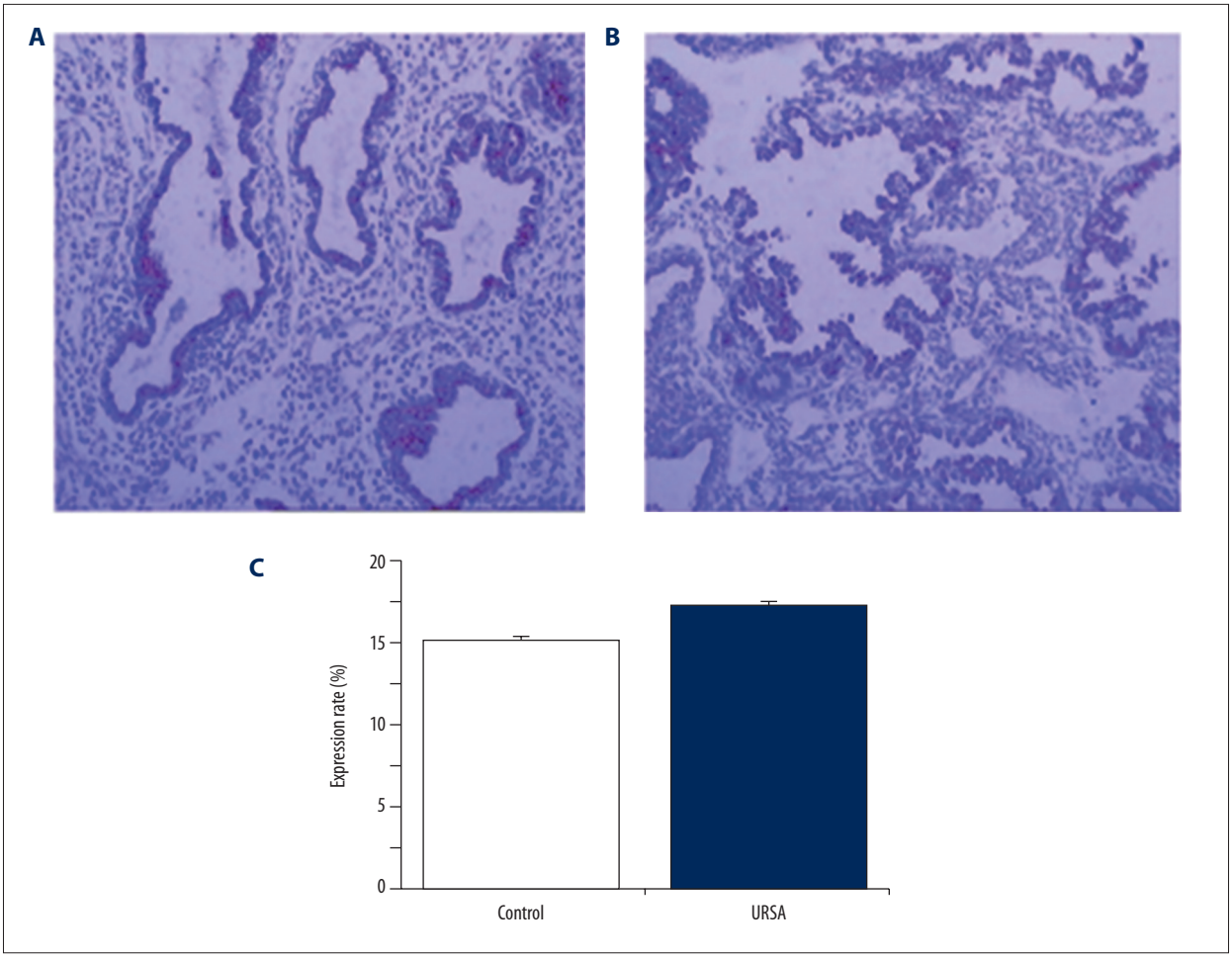

Figure 2. LRH-1 expression in decidual tissue of the patients in both groups. (**A**) Representative result of decidual tissue staining of patients in control group (×200). (**B**) Representative result of decidual tissue staining of patients in URSA group. (**C**) Positive expression rate of LRH-1 in decidual tissue of patients in the 2 groups (×100).

patients in URSA group were significantly lower than those in villi of patients in the control group ($p<0.05$), but no significant difference was found in levels of CYP19 and P450scc protein in decidua between the URSA group and control group ($p>0.05$). Our data suggest than we can reduce the expression of RH-1, CYP19, and P450scc at protein level in villi, but not in decidua.

Discussion

Genetic factors [20], nutritional and psychological status of pregnant women [21], diseases [22], and other internal or external factors were found to affect pregnancy outcomes. The patients with 3 or more consecutive spontaneous abortions within the first 24 weeks after pregnancy were usually diagnosed with RSA [1]. All the factors involved in pregnancy maintenance may directly or indirectly affect the occurrence of RSA. However, in some cases of RSA, the cause cannot be identified by routine investigation. Therefore, all those RSA cases are referred to as unexplained recurrent spontaneous abortion (URSA), which is not a definitive diagnosis. To date, no safe and effective method has been developed for the diagnosis and treatment of URSA. Previous studies have reported various biomarkers that can be used in the diagnosis of URSA, such as Dickkopf-1 (DKK1) [23] and MLR-Bf [7]. The level of serum DKK1 was much higher in patients with URSA than in normal pregnant women [23]. Therefore, the detection of DKK1 in serum can be used as a marker for diagnosis, which avoids the problem of decidual tissue collection. The level of MLR-Bf was also increased in patients with USRA compared to normal pregnant women [7]. However, the high false-positive and false-negative rates of those biomarkers limited the applications in the diagnosis of USRA. As a transcription factor, LRH-1 plays pivotal roles in different aspects of pregnancy. Previous studies have shown that LRH-1 regulates the ovarian intake of cholesterol to facilitate steroidogenesis [14]. LRH-1 can also participate in ovulation regulation [15]. These studies suggest that LRH-1 may have important positive roles in pregnancy by inhibiting USRA.

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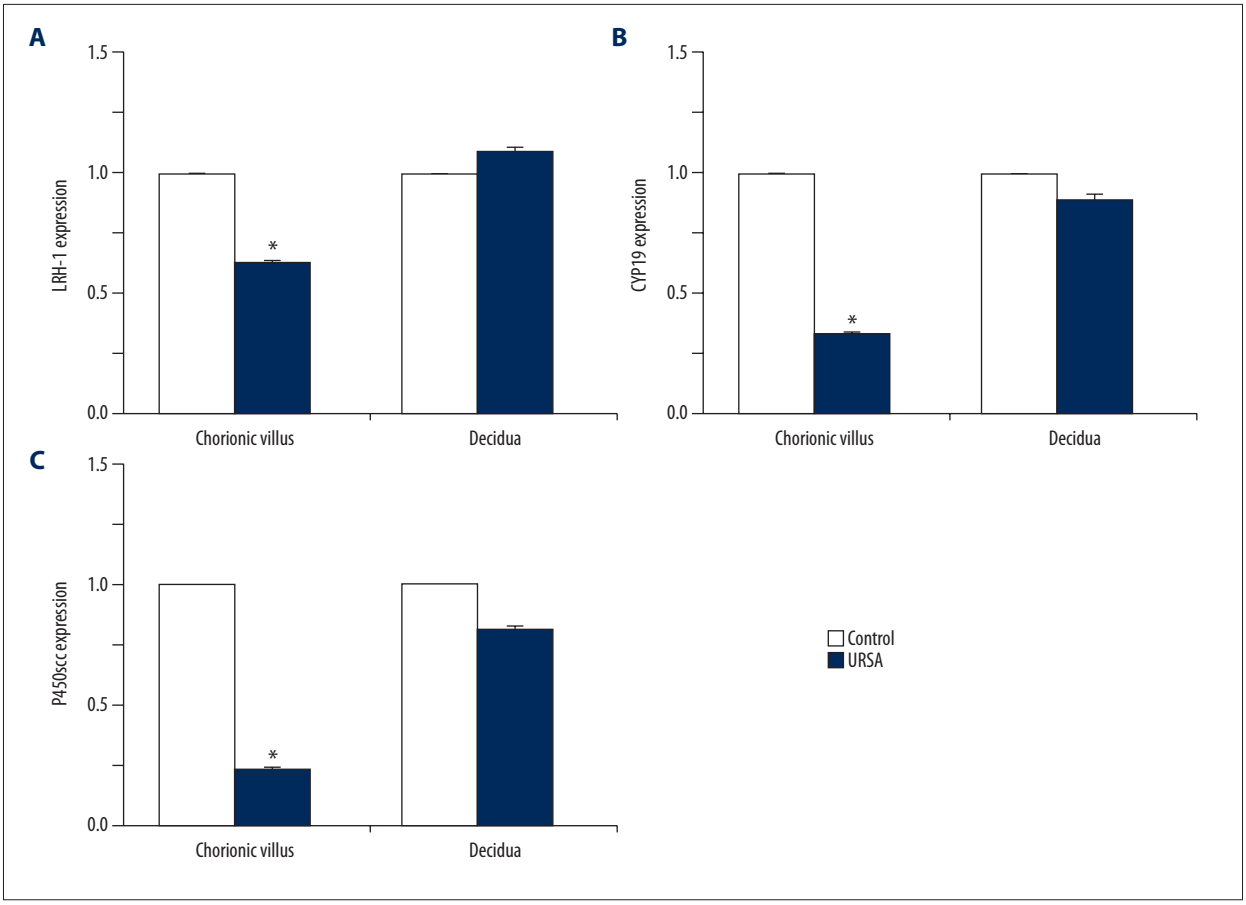

Figure 3. Expression levels of LRH-1, CYP19, and P450scc in villi and decidua. (**A**) Expression level of LRH-1. (**B**) expression level of CYP19. (**C**) Expression level of P450scc. * Compared with the control group, $p<0.05$.

Table 1. Clinical indicators of the objects.

	URSA	Control
Sample size	30	30
Age (years)	27.61±2.93	27.23±1.54
Gestational age	9.69±0.21	9.81±0.34
BMI (kg/m^2)	24.41±1.14	26.63±2.02
Previous number of pregnancies	3.63±0.41	0
Previous gestational age before miscarriage	9.72±1.57	0

To investigate the potential roles of LRH-1 in USRA, we selected 30 patients with URSA in the early stage of pregnancy, and selected 30 patients in early pregnancy to serve as normal pregnant controls. After comparing the basic information of those 2 groups, no significant differences were found in any tested indicators between the patients with URSA and normal pregnant women (Table 1), indicating that the patients we selected were suitable for our comparison studies to identify the roles of LRH-1 in various aspects of USRA. Previous studies using mouse models found the expression level of LRH-1 in the ovaries was significantly higher than in other tissues (e.g., 4 times higher than in the liver) [12,13], indicating that LRH-1 may have a similar expression pattern in humans and plays an important role in pregnancy. In this study, we found that the expression level of LRH-1 in villi of patients in the URSA group was significantly lower than in villi of patients in the control group at mRNA and protein levels (Figures 1–4). However, no significant difference in the expression level of LRH-1 in decidua was found between

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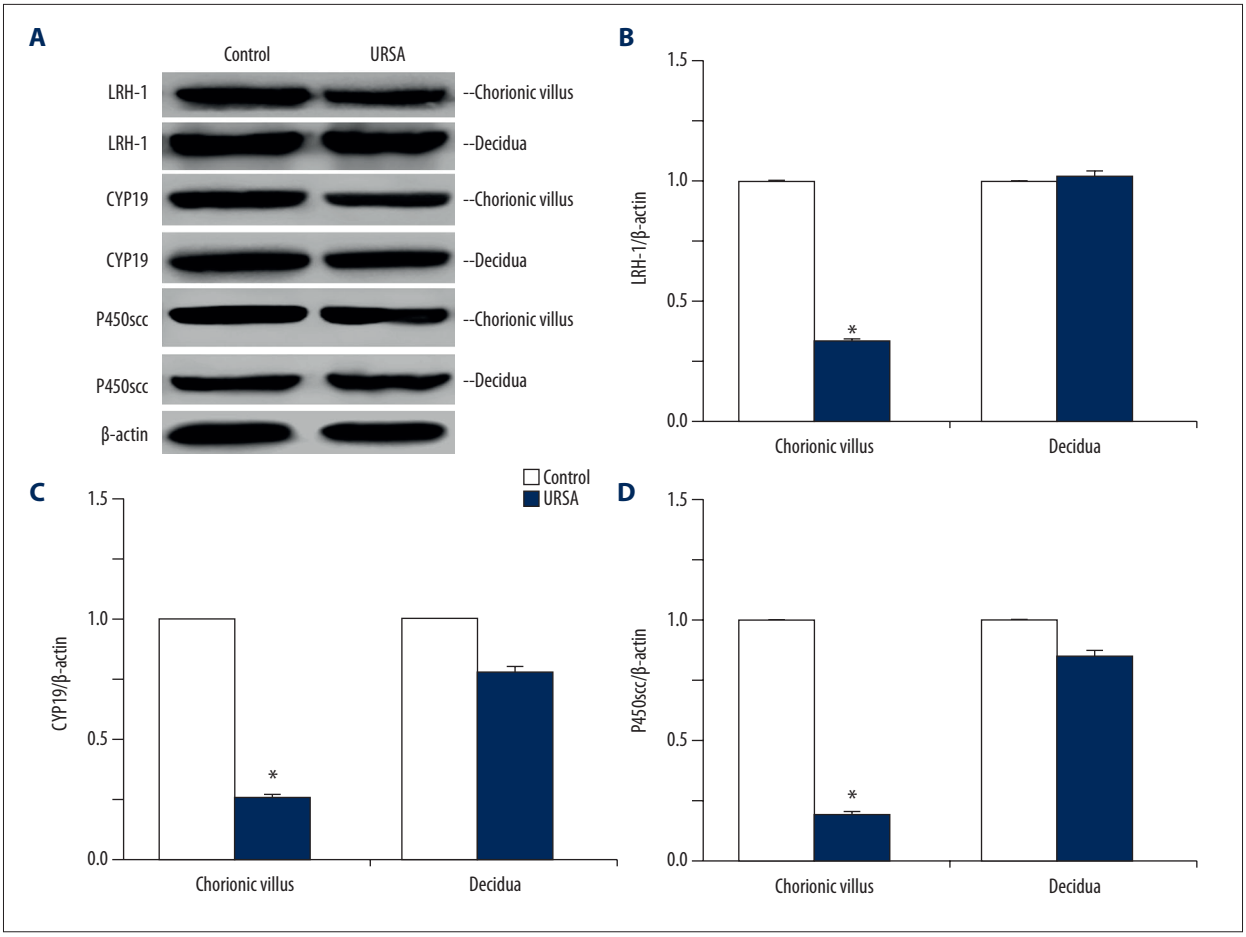

Figure 4. The expression levels of LRH-1, CYP19, and P450scc protein in villi and decidua detected by Western blot. (**A**) Results of Western blot analysis. β-actin was used as an endogenous control. (**B**) The expression levels of LRH-1 protein in URSA and control groups. (**C**) The expression levels of CYP19 protein in URSA and control groups. (**D**) The expression levels of P450scc protein in URSA and control groups. * Compared with the control group, $p<0.05$.

the URSA group and control group. Those data lead us to conclude that the expression of LRH-1 can be specifically reduced by URSA in villi but not in decidua. Therefore, the expression of LRH-1 in villi may be a new biomarker for the diagnosis of USRA.

Pregnancy is a very complex procedure and any stimulation that affects this procedure can directly or indirectly lead to the ending of pregnancy-spontaneous abortion. During pregnancy, a larger amount of progesterone is produced to control the physiological status of the body so as to maintain pregnancy. P450scc has been shown to play a pivotal role in the synthesis of progesterone, and loss of function of P450scc can lead to reduced levels of progesterone [16,17]. Estrogen also has an essential role in the maintenance and development of pregnancy [16]. CYP19, which functions upstream of LRH-1, regulates the expression of LRH-1 and has pivotal roles in the synthesis of estrogen. Therefore, P450scc and CYP19 may also have important functions in URSA. In our study, we found that the expression levels of CYP19 and P450scc in villi of patients in the URSA group were significantly lower than in villi of patients in the control group at mRNA and protein levels (Figures 3, 4). However, no significant difference in the expression levels of CYP19 and P450scc in decidua between URSA group and control group were found l (Figures 3, 4). In view of the expression patterns of P450scc and CYP19 and the interaction among P450scc, and CYP19, and LRH-1, we may safely conclude that P450scc and CYP19 exert their functions in USRA by interacting with LRH-1.

Conclusions

URSA was related to low expression level of LRH-1 in villous tissues, and expression of LRH-1 was closely related to the expression of CYP19 and P450scc. Therefore, the expression level of LRH-1 can be used as a maker for the early diagnosis of URSA, and the regulation of LRH-1 expression may be a new way to treat URSA.

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