



CDX1 Gene Expression in Recurrent Spontaneous Abortion under Extracellular Regulatory Protein Kinase1/2 Signaling Pathway

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ABSTRACT

The aim was to explore the mechanism of the gene expression of caudal-related homeobox transcription factor 1 (*CDX1*) in recurrent spontaneous abortion (RSA) based on the extracellular regulatory protein kinase 1/2(ERK1/2) signaling pathway. In this study, 25 non-pregnant female patients were grouped into the experimental group (group B), who were admitted to the Binzhou Medical University Hospital from August 15, 2022 to June 10, 2023. Besides, 25 non-pregnant healthy female employees were selected as the control group (group A). The DNA was extracted from venous blood, which was amplified by polymerase chain reaction (PCR). Besides, the uterine tissue samples were processed and subjected to immunohistochemistry to monitor the phosphorylation level of ERK1/2, so as to analyze the expression of decidua and villous tissue cells in cytoplasm from group A and B. The results showed that the decidua and villous tissue cells of patients with RSA were almost not expressed in the cytoplasm. Through the χ^2 test, the frequency of CC genotype in patients from group B was sharply lower than that of group A ($\chi^2=13.9$ and $P<0.05$). However, the frequency of TT genotype in patients from group B was markedly higher than that of group A ($\chi^2=9.1$ and $P<0.05$), and the frequency of TT genotype in patients with 3 or more than 3 abortions was higher than that of patients with less than 3 abortions. Therefore, it proved that there was a damage in the ERK1/2 signaling pathway in the cytoplasm of decidua and villous tissue cells of patients with RSA. RSA was also closely related to homocysteine, folic acid and vitamin B12 levels in serum. Moreover, the level of homocysteine in serum is associated with mutations of *CDX1*. In addition, patients with more than 3 abortions indicated a greater probability of *CDX1* abnormalities.

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Authors' Contribution

SC and AY collected the samples. WZ and FL analysed the data. XL conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript, and approved the final manuscript.

Key words

Extracellular regulatory protein kinase, 1/2 signaling pathway, Caudal-related homeobox transcription factor 1, Gene expression, Recurrent spontaneous abortion

INTRODUCTION

Recurrent spontaneous abortion (RSA) refers to three or more spontaneous abortions in a row, with the incidence of about 1% among pregnant women at present (Pereza *et al.*, 2017). The causes of RSA are complex and diverse, mainly including chromosomal abnormalities or gene defects, structural abnormalities of female reproductive system, cervical orifice relaxation, endocrine

disorders, and immune dysfunction (Li *et al.*, 2018). The risk factors for clinical onset mainly include age increase, increased number of abortions, drug abuse and obesity (Zhao *et al.*, 2019). Hyper-homocysteine sepsis is one of the independent risk factors for blood hyper-coagulable diseases. Methylene tetrahydrofolate reductase plays a crucial role in the metabolism process of homocysteine, which is mainly related to the level of homocysteine and folic acid in serum.

CDX1 gene is a transcriptional regulator (Yang *et al.*, 2017). Studies have pointed out that E7.5 starts to be expressed in the primitive streak area during the development of rat embryos and regulates the formation of multiple organs during the early embryonic development (Gaunt, 2018). *CDX1* gene mutation is a common type mutation in humans, which can decrease the activity of methyl-tetrahydrofolate reductase in the cell, and is the main reason for different folic acid levels in the population

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(Jin *et al.*, 2020). The main function of ERK is to transmit signals from surface receptors to the nucleus (Najafi *et al.*, 2019). When other signal pathways are greatly activated, the ERK signaling pathway can be activated, and other signal pathways can also be activated when the ERK signal pathway is obviously activated (Wu *et al.*, 2019). Studies have found that ERK signaling pathway is associated with the invasion of trophoblast cells in early pregnancy, including the proliferation, differentiation, apoptosis, and other biological functions of trophoblast stem cells (Tusa *et al.*, 2018). Some scholars have pointed out that the embryo ectoderm and blastoderm cone of rats with ERK mutation were poorly formed, and the embryos will die early after implantation (Dawid *et al.*, 2019). However, the ERK gene shifting into normality can diagnose and treat the abnormal development of the placenta of the rats with ERK mutation, suggesting that ERK has the function of maintaining normal development of pregnancy and supporting trophoblast cells. In this study, considering that the ERK1/2 signaling pathway might be involved in the emerging of RSA, and then, the mechanism of action of *CDX1* gene expression was further explored.

In this study, the expression of uterine decidua and villous tissue cells in cytoplasm was analyzed by detecting the phosphorylation level of ERK1/2, and the mechanism of *CDX1* expression in RSA was explored, so as to provide reliable reference for the etiology of RSA in medicine.

MATERIALS AND METHODS

Subjects

Twenty-five non-pregnant female patients aged 25-38 years were selected as group B, who visited Binzhou Medical University Hospital from August 15, 2020 to June 10, 2023. Besides that, 25 healthy non-pregnant female workers were selected as group A with an age of 26-37 years and had a normal birth history.

The criteria for inclusion were defined to include patients who suffered from spontaneous abortion more than twice, had normal chromosome karyotype in peripheral blood of both husband and wife, had no deformity of the genital detected by gynecology and B-ultrasound, and had normal endocrine function, normal menstrual cycle, normal thyroid function, and no other major diseases.

The criteria for exclusion were defined to include patients who suffered from disease caused by genetic mutation, had other mental diseases, had a history of fetal intrauterine development restriction, and suffered from stillbirth due to unnatural causes.

PCR amplification of *CDX1* gene

In this study, DNA samples were amplified by DNA

amplifiers made by Perkin-Elmer, USA. The 2mL of venous blood and 2mL of ethylene diamine tetraacetic acid (EDTA) anticoagulant blood from the research objects were collected under fasting state in the morning. The DNA extraction was in strict accordance with the instructions of the kit, and then, the DNA concentration was adjusted to 100µg/µL. The *CDX1* gene fragment was amplified by PCR, and the primer sequence of *CDX1* was as follows. The 5' end primer sequence was 5'-GAGCGACGACGTTTGACAAG-3' and the 3' end primer was 5'-CCGATTGCACGGAATGCGCCGC-3'. The PCR system contained 4µL of diethyl-nitrophenyl thiophosphate (dNTP), 2µL of upstream primers, 2µL of downstream primers, 2.5U of TaqDNA polymerase, and 2µL of DNA template, with a total reaction volume of 35µL. It was pre-degeneration at 95°C for 5 min, for renaturation at 62°C for 30 sec, and for extension at 72°C for 60 sec, with 35 cycles in total. Then, it was extended at 72°C for 5min again. The PCR amplification results were detected by 2% agarose gel electrophoresis. 6% polyacrylamide was subjected to vertical electrophoresis and stained with silver nitrate, so that the results were observed. Homocysteine in serum was determined by high performance liquid chromatography (HPLC). Folic acid and vitamin B12 were tested by ⁵⁷Co and ¹²⁵I double-standard radioimmunoassay, respectively.

Histopathological studies

Firstly, the uterine tissue samples of the research objects were selected and fixed with 4% paraformaldehyde. Then, the routine histopathological sections were embedded with paraffin. Immunohistochemistry was applied to monitor the phosphorylation level of ERK1/2, so as to analyze the expression of uterine decidua and villous tissue cells in cytoplasm. The section tissues were for HE staining and immune-histochemical detection. There were dewaxing, hydration, and tissue sections first. According to the special requirements of the primary antibody applied in this study, the tissue sections were pretreated, and then, incubated with 3% hydrogen peroxide for 5 min, followed by washing with phosphate buffer saline (PBS) for 2 min each. Next, universal immunoglobulin G (IgG) antibody-horseradish peroxidase (HRP) polymers were added by drop, the mixture was incubated at room temperature for 10-20 min and rinsed with PBS for 3 times (each rinsing for 2 min), followed by color development with diaminobenzidine (DAB) solution. Finally, it was rinsed with distilled water, re-dyed, dehydrated, and sealed.

Statistical analysis

SPSS19.0 version statistical software was employed to analyze this research data processing. The measurement data conforming to normal distribution were expressed as

the mean $\pm$ standard deviation ($\bar{x} \pm s$), and measurement data without conformity to normal distribution were represented by the count data percentage (%). What's more, the χ^2 test was for correlation comparison of genotype and allele frequency between groups with repetitive abortion, and risk rate was expressed as a relative risk (RR). The homogeneity of variance test was used for mean comparison among subjects of group A and B, and the t-test was adopted for the homogeneity of variance and the t'-test was employed for the heterogeneity of variance. The mean values among different genotypes were compared, so that they were analyzed by one-way variance (ANOVA). $P < 0.05$ revealed there was a statistically obvious difference.

RESULTS

There were 3 genotypes in the CDX1 locus among humans. First one was CC genotype with only 132bp fragment after enzyme digestion. Second one was CT genotype that had not only 132bp fragment, but also 72bp and 56bp fragment after enzymatic digestion, which became heterozygous mutation. Third one was TT genotype with only 72bp and 56bp fragments after enzymatic digestion, which became homozygous mutation. There was a comparison of CDX1 genotypes in subjects from the two groups. The frequency of CC genotype in patients from group B through the χ^2 test was steeply lower than that of group A ($\chi^2 = 13.9$ and $P < 0.05$). But TT genotype frequency of patients in group B was dramatically higher than that of group A ($\chi^2 = 9.1$ and $P < 0.05$). There was no huge difference in CT genotype among subjects of the two groups ($\chi^2 = 0.07$ and $P > 0.05$) (Fig. 1).

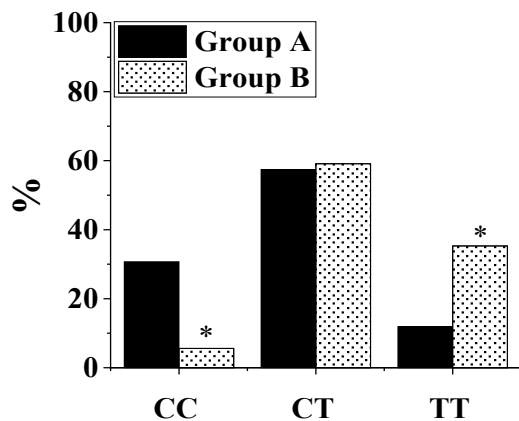


Fig. 1. Frequency of *CDX1* genotypes.

*shows there is a statistically substantial difference compared with group A ($P < 0.05$). Group A: Non-pregnant healthy female employees (control); Group B: Non-pregnant female patients.

The phosphorylation level of ERK1/2 in patients from group B was sharply reduced compared with group A, and the difference was statistically obvious ($P < 0.05$). Pathological analysis of ERK1/2 phosphorylation in the cytoplasm of uterine decidua and villous tissue cells from subjects in the two groups revealed that the expression in the cytoplasm of subjects in group A showed diffuse high density and the expression things were brown-yellow beaded. In addition, there was almost no expression in the cytoplasm of uterine decidua and villous tissue cells from patients in group B (Fig. 2). It suggested that the ERK1/2 signaling pathway in the cytoplasm of uterine decidua and villous tissue cells might be damaged in patients with RSA.

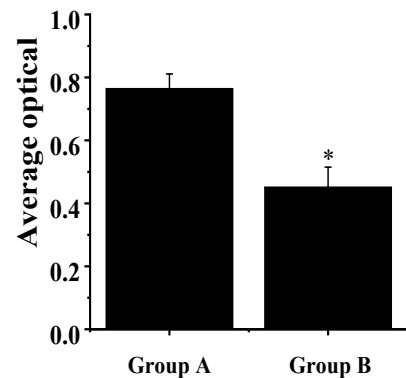


Fig. 2. Phosphorylation levels of ERK1/2 in subject from both groups.

* shows there is a statistically huge difference compared with group A ($P < 0.05$).

See Figure 1 for details of groups.

The frequency of alleles C and T in subjects from group A and B was consistent with the Hardy Weinberg equilibrium. The results indicated that the frequency of allele T in patients from group B was higher than the frequency of the control group, with a statistically great difference ($P < 0.05$) (Fig. 3).

Figure 4 showed that the homocysteine and folic acid levels in serums of patients with CC, TT, and CT genotypes were increased remarkably by comparing with group A ($P < 0.05$). The folic acid level of patients with TT genotype in group B was hugely lower than the level of patients with CC and CT genotypes ($P < 0.05$) (Fig. 5A). There was no obvious difference of vitamin B12 levels in serums among patients with the three genotypes, and the difference was not statistically remarkable ($P > 0.05$). In addition, ANOVA was applied to detect the homocysteine, folic acid, and vitamin B12 levels in serums among subjects with the three *CDX1* genotypes from group A, showing that the differences were not statistically substantial ($P > 0.05$).

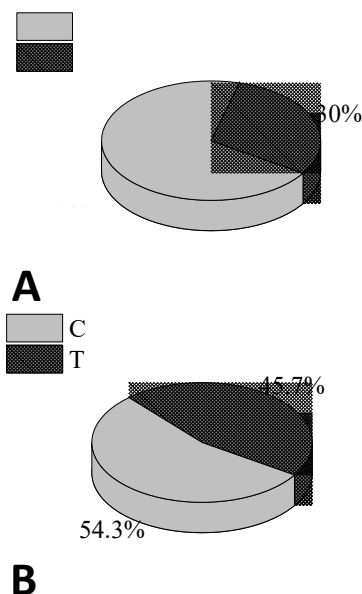


Fig. 3. *CDX1* allele frequency in patients of group A (A), and group B (B).
See Figure 1 for details of groups.

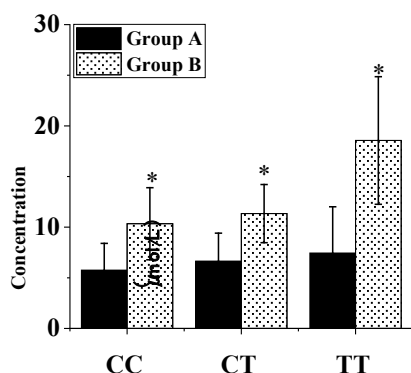


Fig. 4. Homocysteine and folic acid levels in subjects with the three genotypes.
* shows there is statistically marked differences in contrast to group A ($P < 0.05$).
See Figure 1 for details of groups.

Figure 5B revealed that the RR of CT heterozygous genotype and mutant homozygous TT genotype for RSA was 2.3 and 2.9 in turn, indicating that the genotype containing T allele had a higher RR of RSA.

DISCUSSION

The cause of RSA has not been clear up to now. In recent years, studies have pointed out that ischemic decidual villus necrosis is one of the critical causes of

abortion (Yogi *et al.*, 2018). Bhoil *et al.* (2019) found that the incidence of abnormal blood flow of placenta was high in the vaginal color ultrasound examination of patients with RSA. With the gradually deepening research

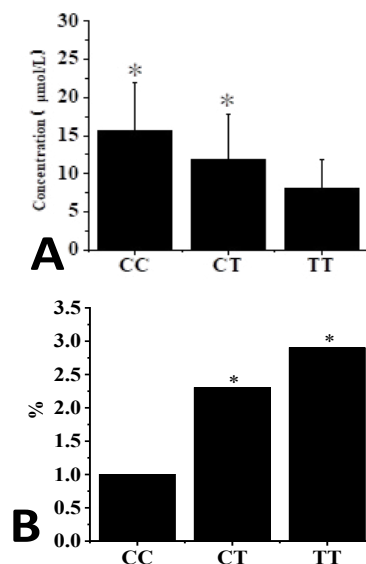


Fig. 5. Folic acid (A) and RR (B) of three genotypes in group B.
* shows there are statistically great differences in contrast to TT (A) and CC (B) genotype ($P < 0.05$).
See Figure 1 for details of groups.

The TT homozygous mutation genotype of *CDX1* in patients with 3 or more abortions was greater than that of patients with less than 3 abortions ($\chi^2 = 4.3$ and $P < 0.05$) (Fig. 6). Therefore, it predicted that patients with 3 or more abortions were more likely to have mutations in the *CDX1* gene.

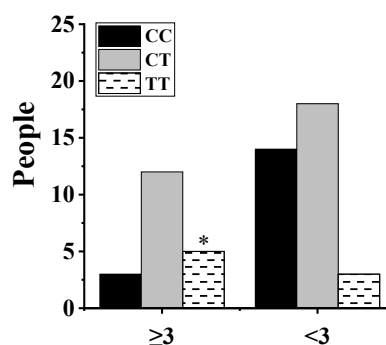


Fig. 6. Comparison on abortion frequency of different genotypes.
* shows that the difference is statistically considerable in contrast to TT genotype in patients with less than 3 abortions ($P < 0.05$).

of genetic diseases internationally, it is found that there are 3 kinds of genetic variation that are clearly associated with RSA, namely *CDX1*, V Leiden blood coagulation factor mutation, and prothrombin 29210A. However, there are big gaps in the research results (Meyer *et al.*, 2007; Rah *et al.*, 2017). V Leiden blood coagulation factor mutation and prothrombin 29210A are very rarely emerged in China's population, while high incidence of RSA may be caused by the gene expression of *CDX1*. Thus, *CDX1* gene prevailed in China was chosen for research with the population associated with RAS in China.

Avagliano *et al.* (2019) suggested that *CDX1* was a genetic risk factor for neural tube deformity and preeclampsia in the Chinese population (Avagliano *et al.*, 2019). Therefore, detection of *CDX1* mutation in women before pregnancy and targeted prophylactic treatment with folic acid and other drugs could reduce homocysteine levels in serum, decrease the rate of spontaneous abortion in early pregnancy, and drop the incidence of complications of neural tube malformation and preeclampsia during pregnancy. In this study, PCR-restriction fragment length analysis method was employed to detect the *CDX1* genotypes of 25 patients with RSA and 25 healthy workers. The results meant that the RR of CT heterozygous genotype for RSA was 2.3 and TT homozygous genotype for RSA was 2.9. This suggested that *CDX1* gene mutation was a genetic risk factor for RSA in China. The over-activation of ERK1/2 could promote the progression of cell cycle, enhance cell proliferation, and play a vital role in the proliferation, differentiation, and invasion of trophoblast cells (Hutton *et al.*, 2017). A large number of studies had shown that ERK1/2 was closely related to cell proliferation and differentiation as well as platelet production (Yu and Huang, 2010; Li *et al.*, 2018). But only phosphorylated ERK1/2 was active, and cell proliferation was reduced if the activity of ERK1/2 was dropped.

The results of this study were consistent with those of previous studies (Zhao *et al.*, 2017). It was found that TT genotype of *CDX1* had a higher RR, indicating that it was markedly associated with the increased homocysteine level and decreased folic acid level in serums. ANOVA was adopted to tested homocysteine, folic acid, and vitamin B12 in serums of subjects from group A, showing that the differences were not statistically obvious. The homocysteine level in serum was not only affected by *CDX1* gene mutation imaging, but also might be affected by other factors. The frequency of TT genotype in patients with 3 or more abortions was higher than the frequency of patients with less than 3 abortions, which was consistent with the research results of Liu *et al.* (2017). It indicated that women who had more spontaneous abortions were more likely to have *CDX1* abnormalities.

CONCLUSION

The ERK1/2 phosphorylation levels of uterine decidua and villi tissue cells in the cytoplasm were determined to investigate the genotype and allele frequency of *CDX1* gene and to detect the homocysteine, folic acid, and vitamin B12 levels in serums. The results showed that the ERK1/2 signaling pathway of uterine decidua and villi tissue cell in cytoplasm of patients with RSA would be damaged, and RSA was closely related with the homocysteine, folic acid, and vitamin B12 levels in serums. Besides, homocysteine level was associated with *CDX1* gene mutation, and patients with more than 3 abortions were more likely to have *CDX1* gene abnormalities. The shortcoming of this study was that different criteria would be applied to select different patients with abortion, resulting in different experimental results. Moreover, other mechanisms of action had not been confirmed due to the limitations of research time and funding. To sum up, it confirmed the expression mechanism of *CDX1* gene in RSA, which could lay a reliable therapeutic basis for clinical medicine.

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IRB approval

The study was carried out with the approval of Research Guidance Workshop Committee (Qingdao Women and Children's Hospital).

Ethical statement

The Medical Ethics Committee of Binzhou Medical University Hospital had approved this experiment. The patient and her family members had been informed of this experiment and signed the informed consent.

Statement of conflict of interest

The authors have declared no conflict of interest.

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