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Original Article

Role of MAPK/ERK signal pathway in recurrent miscarriage patients by case-control analysis

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Abstract: Objective: Recurrent spontaneous abortion (RSA) refers to two or more consecutive spontaneous abortions, and its morbidity is about 1%-1.8% of fertile women. It was to study the role of MAPK/ERK signal pathway in the recurrent miscarriage patients by case-control analysis; Methods: It collected 46 RSA patients, without growing of early embryos, which were constituted in patients group. Matched gestational time and women age, it used the normal Pregnant woman at same period as control group. The mRNA expression of were detected by RT-PCR, the protein expression of were detected by western blot. Calculated its value and found that difference; Result: It could be found the average ERK1/2 expression in tissues of VCT, EVCT and DSC were lower in the RAS patients than that of the control group ($P<0.05$). The expression of protein ERK and P-ERK was consistent with RNA testing results, under western blot testing. ERK and PERK were at lower expression in villi and deciduas, which suggested it had relationship with recurrent miscarriage; Conclusion: The expression of ERK1/2 mRNA and the relative protein expression of ERK in RSA pathway were lower than normal pregnancy, which indicated MAPK/ERK signal pathway participated in the maintenance of normal pregnancy; the decreased expression of ERK is closely related to RSA disease.

Keywords: Recurrent spontaneous abortion, ERK, MAPK, expression

Introduction

Recurrent miscarriage, habitual abortion, or recurrent pregnancy loss (RPL) is three or more consecutive pregnancy losses. Infertility differs because it is the inability to conceive. In many cases the cause of RPL is unknown. After three or more losses, a thorough evaluation is recommended by American Society of Reproductive Medicine [1]. About 1% of couples trying to have children are affected by recurrent miscarriage [2, 3].

There are various causes for recurrent miscarriage, and some are treatable. Some couples never have a cause identified, often after extensive investigations [4, 5]. About 50-75% of cases of Recurrent Miscarriage are unexplained. A common feature of immune factors in causing recurrent pregnancy loss appears to be a decreased maternal immune tolerance towards the fetus [6, 7].

In sometimes, Natural Killer Cells, a type of white blood cell, are present in uterine tissue. High levels of these cells may be linked to RPL but high numbers or the presence of these cells is not a predictor of pregnancy loss in women who have not have had a miscarriage [7].

However, earlier studies that perhaps paternal sharing of HLA genes would be associated with increased pregnancy loss have not been confirmed.

The MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell [8-10].

The signal starts when a signaling molecule binds to the receptor on the cell surface and ends when the DNA in the nucleus expresses a protein and produces some change in the cell, such as cell division. The pathway includes many proteins, including MAPK (mitogen-activated protein kinases, originally called ERK, extracellular signal-regulated kinases), which communicate by adding phosphate groups to a

neighboring protein, which acts as an “on” or “off” switch [9, 11].

When one of the proteins in the pathway is mutated, it can become stuck in the “on” or “off” position, which is a necessary step in the development of many cancers. Components of the MAPK/ERK pathway were discovered when they were found in cancer cells. Drugs that reverse the “on” or “off” switch are being investigated as cancer treatments [2, 11].

Three of the many proteins that are phosphorylated by MAPK. One effect of MAPK activation is to alter the translation of mRNA to proteins. MAPK phosphorylates 40S ribosomal protein S6 kinase (RSK). This activates RSK, which, in turn, phosphorylates ribosomal protein S6. Mitogen-activated protein kinases that phosphorylate ribosomal protein S6 were the first to be isolated [13, 14].

MAPK regulates the activities of several transcription factors. MAPK can phosphorylate C-myc. MAPK phosphorylates and activates MNK, which, in turn, phosphorylates CREB. MAPK also regulates the transcription of the C-Fos gene. By altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for the cell cycle. The 22q11, 1q42, and 19p13 genes are associated with schizophrenia, schizoaffective, bipolar, and migraines by affecting the ERK pathway.

Since, Recurrent spontaneous abortion (RSA) refers to two or more consecutive spontaneous abortions, and its morbidity is about 1%-1.8% of fertile women. Pathogen of RSA is complex, including chromosome, anatomy infection, immune, endocrine etc. Besides, there are still 50% of RSA of unknown etiology. Compared to normal pregnancy, throphblast invasion ability of RSA patients is significantly decreased, and the specific mechanism is not clear. Previous studies have proved that, MAPK/ERK signal pathway is closely related to embryo implantation during early pregnancy. Many intra-cellular signal molecules produce biological effect through the phosphoralation of ERK1/2 of MAPK. The active form of ERK1/2 phosphorylation exists only in placenta before 12 weeks of gestation, suggesting that it plays a major role in early pregnancy. Inactivation of ERK1/2 results in a decreased in cell proliferation and cell number reduced. This study was to investigate the difference of RSA protein expression between normal pregnancy and RPL patients, and analyze the signal pathway of RSA.

Material and method

Patients and diagnose

It collected 46 RSA patients, from March to September, 2014, which were pregnant again but their early embryos couldn’t grow. They were constituted in patients group. Their average gestational time was 8.32±1.73 weeks. The pregnant women were at mean age of 29.37±3.65 years. All patients had a history of spontaneous abortions for more than 2 times before.

Case exclusion

The pregnant women with embryo chromosome abnormalities, anatomy, infection, endocrine and autoimmune diseases were excluded in this study.

Diagnostic criteria were both of clinical finding with no clear cause of abortion, and examination by B ultrasound which confirmed embryos were stop growing, with no primitive heart tube pulse.

Control group

At the same period in same hospital, it chooses 100 outpatients with normal pregnant women as the control group. Their average gestational time was 8.74±1.38 weeks. The pregnant women were at mean age of 30.54±3.15 years.

Total RNA extraction

Total RNA including mRNA and microRNA from fresh tissue and pathological paraffin tissue were extracted following the instruction of the miRNeasy Mini kit (Qiagene, Germany). The concentration and purity of RNA were measured as absorbance value at 280 nm and 260 nm (A) and the ratio of A260/A280 ratios with value of between 1.8 and 2.1 was used for subsequent experiments.

Microarray hybridization

All isolated RNA samples were used to synthesize cDNA, and double-stranded cDNA was

Table 1. The sequence of primers of each gene

Genes	Primers' sequence (5'-3')	size (bp)
ERK1/2	F: CTCAAGCCTTCCAACCTC	380
	R: TTCCACGGCACCTTATTT	
β-actin	F: TACACCGCTACCAGTTCGCCAT	270
	R: TCTCCATGTCGTCCCAGTTGGT	

Table 2. Clinical comparison of two groups of indicator

	Age	Gestational time
Recurrent miscarriage	29.37±3.65	8.32±1.73
Control group	30.54±3.21	8.74±1.38

labeled and hybridized to the Microarray (Arraystar, Rockville, MD). After hybridization and washing, processed slides were scanned with the Axon GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA).

Reverse transcription polymerase chain reaction

Complementary DNA synthesis was performed using RT2 First Strand Kit (Takara, JP) and real time PCR was accomplished using the SYBR Green PCR Master Mix (Promega, USA) on Bio-Rad IQ5.0 (Bio-Rad PCR system). The primers sequences for ERK1/2 are in **Table 1**. Based on the instruction of reverse transcription kit (TAKARA), reverse transcription reaction mixture was prepared (2XmiRNA RT buffer 10 μL; 0.1% BSA 2 μL; miRNA reverse transcriptase mixture 2 μL; RNA quantification to 0.5 μg, plus Rnase-free water to the total volume of reaction solution 20 μL) with reaction at 37°C for 60 min, at 85°C for 5 s. The synthesized cDNA were stored at -20°C.

SDS-PAGE and Western blot

The samples of tissues cells suspension were harvested and centrifuged at 1000 g for 10 min (Thermo, USA). The supernatant was discarded, and the precipitation was re-suspended in lysis buffer (Beyotime, China), while adding 1% proteinase inhibitor cocktail (Sigma-Aldrich; Missouri), 25 mM NaF, and 1 mM Na3VO4.

The mixture was frozen to -80°C and thawed four times. The mixture was centrifuged at 10000 g for 30 min at 4°C (Thermo, USA). The supernatant containing protein of ERK was collected and analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE). Then transferred the proteins of antibody onto a polyvinylidene difluoride (PVDF) membrane (Millipore; California).

The membrane was blocked with 5% BSA Tris-HCl buffered saline and 0.05% Tween-20 for 2 h at 25°C. It incubated with primary antibodies of mouse anti ERK monoclonal antibody and secondary antibodies of rabbit anti mouse monoclonal antibodies. It calculated the WB Protein bands with GADPH as internal standard using an enhanced chemiluminescence system (ECL, Bestbio, China).

Summary it as that: A total collection of 46 patients with RSA (test group) and 100 normal pregnancies in the same period (control group) of early pregnancy. Cultured villus and deciduas of the two groups to isolate villous cytotrophoblasts (VCT), extravillous cytotrophoblasts (EVCT) and dccidual stromal cells (DSC). The pregnancies were monitored using ultrasound scanning, fetal karyotyping and placental analysis. Villous cytotrophoblasts were isolated from normal and trisomic placenta and cultured to investigate ERK secretion in vitro (n=10). Reserve the expression of ERK1/2 mRNA and the ERK protein content by reverse transcriptase chain reaction (RT-PCR) and Western blot respectively.

Statistical analysis

All of the data are represented as the mean ± SD (X±s) of three or more independent experiments. If the data were homogenous, an analysis of variance, Student-Newman-Keulsa and Pearson's correlation were used. If the data are not homogenous, a Kruskal-Wallis and Games-Howell test, as well as a Spearman's correlation analysis, were used. All of the analyses were carried out using the SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). Values less than 0.05 were considered to be statistically significant.

Results

General information

Through investigation, there was no significant difference between patients group and control

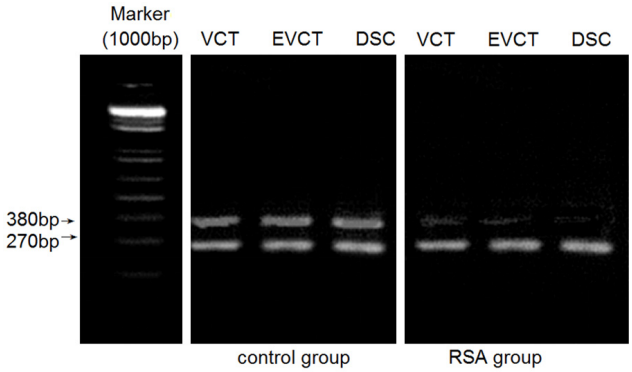

Figure 1. The image 1.5% Agarose gel electrophoresis to test mRNA of ERK in three kinds of tissues between RSA and control group.

Table 3. Analyzed the difference of ERK1/2 expression in different tissues of VCT, EVCT and DSC between these two groups

	VCT	EVCT	DSC
Recurrent miscarriage	0.43±0.07	0.47±0.06	0.29±0.04
Control group	0.74±0.11	0.79±0.08	0.46±0.03

P<0.05.

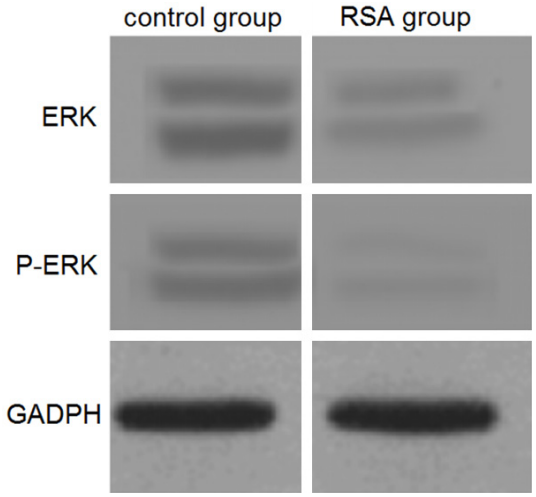

Figure 2. Grayscale of Western blot results for target proteins between the two groups.

group in gestational time and ages, which is illustrated in **Table 2**.

Made a case-control study of risk factors for recurrent miscarriage patients. Firstly, the percentage of recurrent miscarriage cases and rural patients was significantly higher in the case group than in the control group (P<0.01). After genetic background investigation, Second, family history were monitoring for gene analysis. Recurrent miscarriage indicated that genetic was an important risk factor, accounting for 83.78% of the RSA cases.

Results of RT-PCR

It tested the ERK1/2 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) technology, with three kinds of tissues samples (VCT, EVCT and DSC). Under electrophoresis conditions in 1.5% agarose gel, it taken the RT-PCR production image of ERK in three kinds of tissues between RSA and control group in **Figure 1**. After calculated grayscale value of the Electrophoresis gel images. Their average value were that, VCT with 0.43±0.07; EVCT with 0.47±0.06; DSC with 0.29±0.04. But, the average value of control group were that CT with 0.74±0.11; EVCT with 0.79±0.09; DSC with 0.46±0.03. It could be found the average ERK1/2 expression in tissues of VCT, EVCT and DSC were lower in the RAS patients than the control group (P<0.05) (**Table 3**).

Result of Western blot

After calculated grayscale value of western blot image (the original image was showed in **Figure 2**), it analyzed both ERK and P-ERK expression, which listed in **Table 4**. The expression of protein ERK and P-ERK were consistent with RNA testing results. It was at lower expression for RSA patients than control. That differences were with statistically significant (P<0.05).

ERK and PERK were at lower expression in villi and deciduas, which suggested it had relationship with Recurrent miscarriage. Furthermore, histogram were draw to analysis the expression difference in P-ERK and ER between them, which showed in **Figure 3**.

Discussion

Antiphospholipid syndrome or antiphospholipid antibody syndrome (APS or APLS), or often also Hughes syndrome, is an autoimmune, hypercoagulable state caused by antiphospholipid antibodies. APS provokes blood clots (thrombosis) in arteries and veins as well as pregnancy-related complications such as miscarriage, stillbirth, preterm delivery, and severe preeclampsia [1-4].

Table 4. The protein expression of ERK and P-ERK between thes two groups

	ERK	P-ERK
Recurrent miscarriage	0.543±0.032	0.511±0.059
Control group	0.715±0.041	0.674±0.038

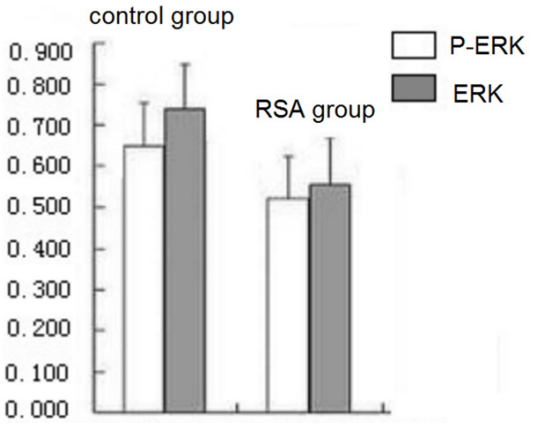

Figure 3. Histogram was drawn to analyze the expression of P-ERK and ER between two groups, according to the value of the grayscale'.

The long-term prognosis for APS is determined mainly by recurrent thrombosis, which may occur in up to 29% of patients, sometimes despite antithrombotic therapy.

Around 15% of the women who have recurrent miscarriages have high levels of antiphospholipid antibodies [3, 15]. Women who have had more than one miscarriage in the first trimester, or a miscarriage in the second trimester, may have their blood tested for antibodies, to determine if they have antiphospholipid syndrome. Women diagnosed with antiphospholid syndrome generally take aspirin or heparin in subsequent pregnancies, but questions remain due to the lack of high quality trials [16, 17].

A common feature of immune factors in causing recurrent pregnancy loss appears to be a decreased maternal immune tolerance towards the fetus. Women with a history of recurrent miscarriage are at risk of developing pre-eclampsia in later pregnancies.

Recurrent miscarriage in itself is associated with later development of coronary artery disease with an odds ratio of approximately 2.

MAP3K3 directly regulates the MAPK8/JNK and extracellular signal-regulated protein kinase (ERK) pathways by activating SEK and MEK1/2 respectively [12]. On the other hand, MAP3K7 (TAK1) participates in regulation of transcription by transforming growth factor-beta (TGF-beta) [13, 18]. By detecting its expression difference between RSA patients and normal pregnancy.

It found expression of ERK were lower in RSA patients than normal pregnancy, which suggested that MAPK/ERK signaling pathway were involved in maintaining normal pregnancy. The lower expression of ERK is closely related to RSA, but whether it had other compensation mechanisms, and what would be impacted in the downstream of signaling pathway should be more study in future.

There were three kinds of tissues used in this study, which were VCT, EVCT and DSC. Furthermore, it found VCT, EVCT of ERK1/2 mRNA expression levels were higher than DSC in RSA patients. It could make conclusion the ERK1/2 were mainly present in the trophoblast cells, which was consistent with other publication [10, 19].

As for the most important eukaryotic cell signaling pathways, the relationship between MAPK/ERK signaling pathway and pregnancy are inseparable. That might provide a new direction or ideas for the treatment of recurrent abortion. To interfere with the decreased expression of ERK in the invasion of trophoblast cells could be used to treat RSA.

In conclusion, the expression of ERK1/2 mRNA and the relative protein expression of ERK in RSA pathway were lower than normal pregnancy, which indicated MAPK/ERK signal pathway participated in the maintenance of normal pregnancy; the decreased expression of ERK is closely related to RSA disease.

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Disclosure of conflict of interest

None.

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References

[1] van den Boogaard E, Vissenberg R, Land JA, van Wely M, van der Post JA, Goddijn M, Bisschop PH. Significance of (sub) clinical thyroid dysfunction and thyroid autoimmunity before conception and in early pregnancy: a systematic review. Hum Reprod Update 2011; 17: 605-19.

[2] Kaandorp SP, Goddijn M, van der Post JA, Hutten BA, Verhoeve HR, Hamulyák K, Mol BW, Folkeringa N, Nahuis M, Papatsonis DN, Büller HR, van der Veen F, Middeldorp S. Aspirin plus heparin or aspirin alone in women with recurrent miscarriage. N Engl J Med 2010; 362: 1586-96.

[3] Yamada H, Takeda M, Maezawa Y, Ebina Y, Hazama R, Tanimura K, Wakui Y, Shimada S. A high dose intravenous immunoglobulin therapy for women with four or more recurrent spontaneous abortions. ISRN Obstet Gynecol 2012; 2012: 512732.

[4] Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 2011; 75: 50-83.

[5] Chan P, Möller A, Liu MC, Sceneay JE, Wong CS, Waddell N, Huang KT, Dobrovic A, Millar EK, O'Toole SA, McNeil CM, Sutherland RL, Bowtell DD, Fox SB. The expression of the ubiquitin ligase SIAH2 (seven in absentia homolog 2) is mediated through gene copynumber in breast cancer and is associated with a basal-like phenotype and p53 expression. Breast Cancer Res 2011; 13: R19.

[6] Lee J, Roh KB, Kim SC, Lee J, Park D. Soy peptide-induced stem cell proliferation: involvement of ERK and TGF-β1. J Nutr Biochem 2012; 23: 1341-51.

[7] Vetterkind S, Poythress RH, Lin QQ, Morgan KG. Hierarchical scaffolding of an ERK1/2 activation pathway. Cell Commun Signal 2013; 11: 65.

[8] Campbell PM. Oncogenic Ras pushes (and pulls) cell cycle progression through ERK activation. Methods Mol Biol 2014; 1170: 155-63.

[9] Waseem T, Duxbury M, Ashley SW, Robinson MK. Ghrelin promotes intestinal epithelial cell proliferation through PI3K/Akt pathway and EGFR trans-activation both converging to ERK 1/2 phosphorylation. Peptides 2014; 52: 113-21.

[10] Wang SC, Tang ChL, Piao HL, Zhu R, Sun Ch, Tao Y, Fu Q, Li DJ, Du MR. Cyclosporine A promotes in vitro migration of human first-trimester trophoblasts via MAPK/ERK1/2-mediated NF-κB and Ca2+/calcineurin/NFAT signaling. Placenta 2013; 34: 374-80.

[11] Varfolomeev E, Vucic D. Intracellular regulation of TNF activity in health and disease. Cytokine 2016; S1043-4666: 30489-6.

[12] Aguado A, Galán M, Zhenyukh O, Wiggers GA, Roque FR, Redondo S, Peçanha F, Martín A, Fortuño A, Cachofeiro V, Tejerina T, Salaices M, Briones AM. Mercury induces proliferation and reduces cell size in vascular smooth muscle cells through MAPK, oxidative stress and cyclooxygenase-2 pathways. Toxicol Appl Pharmacol 2013; 268: 188-200.

[13] Robert G, Jullian V, Jacquel A, Ginet C, Dufies M, Torino S, Pottier A, Peyrade F, Tartare-Deckert S, Bourdy G, Deharo E, Auberger P. Simalikalactone E (SkE), a new weapon in the armamentarium of drugs targeting cancers that exhibitconstitutive activation of the ERK pathway. Oncotarget 2012; 3: 1688-99.

[14] Forbes K. IFPA Gabor Than Award lecture: molecular control of placental growth: the emerging role of microRNAs. Placenta 2013; 34 Suppl: S27-33.

[15] Marlin R, Duriez M, Berkane N, de Truchis C, Madec Y, Rey-Cuille MA, Cummings JS, Cannou C, Quillay H, Barré-Sinoussi F, Nugeyre MT, Menu E. Dynamic shift from CD85j/ILT-2 to NKG2D NK receptor expression pattern on human decidual NK during thefirst trimester of pregnancy. PLoS One 2012; 7: e30017.

[16] Amato MP, Portaccio E. Fertility, pregnancy and childbirth in patients with multiple sclerosis: impact of disease-modifying drugs. CNS Drugs 2015; 29: 207-20.

[17] HoWangYin KY, Loustau M, Wu J, Alegre E, Daouya M, Caumartin J, Sousa S, Horuzsko A, Carosella ED, LeMaoult J. Multimeric structures of HLA-G isoforms function through differential binding to LILRB receptors. Cell Mol Life Sci 2012; 69: 4041-9.

[18] Williams Z. Inducing tolerance to pregnancy. N Engl J Med 2012; 367: 1159-61.

[19] Empson M, Lassere M, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. Cochrane Database Syst Rev 2005; CD002859.