

High Expression of hsMAD2 in the Villi of Spontaneously Aborted Embryo with Chromosomal Abnormality

Zaochang Wang^{1*}, Junjie Hu^{2*}, Wang He², Humin Gong³, Weihua Xu², Zhichao Ma², Xinping Chen^{2#}

¹Clinical Laboratory, Nanda Town Hospital of Danzhou City, Danzhou, China

²Central Laboratory, Hainan General Hospital, Hainan Hospital Affiliated to The Hainan Medical College; Hainan Provincial Key Laboratory of Cell and Molecular Genetic Translational Medicine, Haikou, China

³Department of Obstetrics, Hainan General Hospital, Hainan Hospital Affiliated to The Hainan Medical College, Haikou, China

Email: #chenxinping52@126.com

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Abstract

Objective: To investigate the changes of hsMAD2 protein and gene expression levels during chromosome segregation of human embryos. **Method:** The embryos of spontaneous abortion were collected in our hospital from 2009 to 2013, the chromosomal numbers of the embryonic villi were subsequently detected by fluorescence in situ hybridization (FISH). The patients were then divided into the normal and abnormal groups based on the chromosome number. The hsMAD2 protein and gene expression levels in the villi tissues of the two embryo groups were detected by western blotting and qRT-PCR, respectively. The hsMAD2 protein and gene levels in the embryonic villus tissue of the patient were detected. **Results:** From 2009 to 2013, we collected 50 embryos from spontaneous abortion patients. The chromosome abnormality and no abnormality were 36 cases (abnormal number of 28 cases (56.0%) and chimerism in 8 cases (16.0%)) and 14 cases (28.0%), respectively. The expression of hsmad2 protein and its gene in the villi of spontaneously aborted embryo with chromosomal abnormality in the abnormal group was significantly higher than that in those without chromosomal abnormalities (0.88 ± 0.20 vs 0.61 ± 0.19 , $P < 0.05$), (23.46 ± 0.07 vs 18.35 ± 0.10 , $P < 0.05$). **Conclusion:** Abnormal number of chromosomes is closely related to spontaneous abortion. Linked, hsMAD2 factor has a card effect on the cell cycle, can block the mitotic process of cells, and play an important role in maintaining the normal separation and stability of chromosomes.

*These authors contribute equally to this work.

#Corresponding author.

Keywords

Spontaneous Abortion, Embryonic Villi, Chromosomal Abnormality, hsMAD2

1. Introduction

Spontaneous abortions account for 15% - 18% of clinically recognized pregnancies. Its occurrence is mainly related to known factors such as heredity, infection, immunity, and environment, as well as other unknown factors [1] [2]. Human embryonic development is a gradual process. After the fertilization of the ovum, the gene of the fertilized ovum is reprogrammed. After the division, the archiblast is distributed into the daughter cells, which leads to the formation of early embryos. The early embryo lacks transcriptional activity; the embryonic gene is gradually formed and activated with the development of the embryo. Chromosomal abnormalities may occur during any of these processes. Therefore, the integrity of reprogramming, continuous cleavage, and formation and activation of the embryonic genome must be maintained for normal development of the human embryo [3] [4] [5]. Mitosis is a continuous process of nuclear restitution, and a basic form of realizing histogenesis and individual development. The spindle assembly checkpoint (SAC) prevents chromosome segregation. The cell cannot enter mitosis anaphase, and continue into normal cell cycle activities, until the sister chromatid is properly connected to the mitotic spindle [6] [7]. There is no correlation between hsmad2 and chromosomal abnormalities in spontaneous abortion embryos at home and abroad. Therefore, we collected the villi tissue in spontaneous abortion patients in our hospital from March 2009 to October 2013. Compare the expression of hsmad2 protein and gene between chromosomal abnormalities and normal fetal chorionic villi, in order to explore the effect of SAC on spontaneous abortion resulting from chromosomal abnormalities.

2. Material and Methods

2.1. General Data

The 50 placental villi tissues treated for spontaneous abortion were collected for this study at the Obstetrics and Gynecology department of the Hainan Province People's Hospital between March 2009 and October 2013. These samples were collected from pregnant women with spontaneous abortion who excluded history of infection and exposure to harmful poisons, and the placentas of all patients were confirmed to have stopped developing by the b-scan ultrasonic method. This project was approved and implemented by the Ethics Committee of Hainan Provincial General Hospital, and the number is Med-Eth-Re [2021] 131.

2.2. Detection Method

2.2.1. Detection of Embryonic Villi Chromosomes by FISH

The embryonic villi were rinsed with 0.9% NaCl solution, pre-fixed, and subsequently fixed. The treated specimens were prepared into a stationary liquid, and the cell suspension stained on a glass slide, which was placed on an aging slide of 56°C for over 2 hours. The samples were rinsed, digested, and dewatered, and then subjected to *in situ* hybridization in a hybridization oven at 42°C for 17 hours; this process was repeated twice. The slides were warmed for 30 minutes, cleaned, and subsequently stained. The slides were then observed under a fluorescent microscope.

2.2.2. Detection of hsmad2 Protein by Western Blotting

One milliliter of lysis buffer was added to 200 mg embryonic villi tissues. The samples were ground to a smooth past, and incubated for 30 minutes. The supernatant was removed, and the samples centrifuged at 4°C for 5 minutes at a speed of 13,000 rpm. The supernatant was discarded again. Supernatant buffer (25 µL) was then added to 100 µL specimen that was to be tested. The samples were denatured in a 100°C water bath, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and sealed with 5% nonfat dried milk for 2 hours. The membrane was then incubated with anti-hsMAD2 and β -actin (1:1000) antibodies overnight at 4°C. Subsequently, the membrane was incubated with the corresponding secondary antibodies (1:5000) for 60 minutes at room temperature. The PVDF membrane was placed on an image scanner, and covered with a previously prepared developing-coloring solution (in the dark). The image-lab software was run in order to analyze and process, 1 minute after the reaction.

2.2.3. Detection of hsMAD2 mRNA by qRT-PCR

According to the designed primer sequences (submitted to NCBI GenBank), the length of the amplified fragment was about 100 - 300 bp. The specific primer for hsMAD2 was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences were as follows, hsMAD2-F: 5'-AGGGTTTTGAAGCCGTCTAT-3', hsMAD2-R: 5'-CGTCTGCCTTCGGTATTCT-3', GAPDH-F: 5'-CCCCTCCTCCAC-CTTTG-3', GAPDH-R: 5'-CCACCACCCTGTTGCTG-3'. The samples were soaked in 0.1% diethyl pyrocarbonate (DEPC) solution at room temperature overnight or processed at 37°C in a 1.5 mL Eppendorf tube. Tips of varying capacities (10 µL, 200 µL, and 1mL) were sterilized using a high temperature steam and autoclaved at 80°C standby. Embryonic villi tissue (300 mg) was added to 4.5 mL TRIzol extracting solution, and the mixture centrifuged at 12000 rpm after rapid grinding on ice. The supernatant was collected in another centrifuge tube, treated with 1 mL chloroform, and oscillated for 15 seconds. This was placed on ice for 5 minutes, and subsequently centrifuged again at 4°C and 12,000 rpm for 15 minutes. Again,

the supernatant was collected in another centrifuge tube; this was treated with 3 mL isopropanol, and mixed uniformly. This mixture was placed on ice for 15 minutes and centrifuged again at 12,000 rpm and 4°C for 15 minutes. The supernatant was discarded and the pellet treated with 1 mL 75% ethanol. This was centrifuged again at 7500 rpm and 4°C for 5 minutes. The ethanol solution was removed, and the pellet placed on ice for 10 minutes. The total RNA was dissolved using DEPC-H₂O, and subjected to reverse transcription. The samples were reverse transcribed using the following program: 95°C (6 minutes) → 55°C (90 seconds) → 72°C (90 seconds) → 72°C (10 minutes) → 5°C (1 hour). The real-time polymerase chain reaction (PCR) was performed in accordance with the manufacturer instructions. GAPDH and its copy number were taken as the internal reference and correction base, respectively; the cycle threshold (CT) value was obtained using the ABI7500 software (Applied Biosystems, Foster City, CA, USA). The ΔC_T value (corresponding threshold of the specimen) was obtained by subtracting the obtained C_T value from the C_T value of GAPDH of the same specimen. The ΔC_T value of the sham operation group was taken as the correction base, and the C_T gene concentration level was obtained by software calculation using the obtained $2^{-\Delta\Delta C_T}$ value.

The kits used and the operating methods of FISH detection, Wb detection and qRT-PCR detection are all provided by Lulong Biotechnology Development Co., Ltd. (Xiamen, China); And all processes are carried out in strict accordance with the manufacturer's instructions.

2.3. Statistical Analysis

The obtained data was statistically analyzed using SPSS software (v.16.0, IBM, Armonk, NY, USA). All data was presented as mean \pm standard deviation (S.D.). Significant differences were determined by analysis of variance (ANOVA). The difference was considered to be statistically significant when P-values were <0.05 .

3. Results

3.1. Comparison of General Conditions

From 2009 to 2013, we collected 50 embryos from spontaneous abortion patients. The chromosome abnormality and no abnormality were 36 cases (abnormal number of 28 cases (56.0%) and chimerism in 8 cases (16.0%)) and 14 cases (28.0%), respectively. Two groups of pregnant women were comparable as no statistical difference ($P > 0.05$) was observed (**Table 1**).

3.2. FISH Detection

Twenty-eight embryos with chromosome abnormalities were detected in embryos of 50 patients with spontaneous abortion (56%; 28/50). Eight chimeras (16%) were detected among these. The details of abnormal chromosome types are provided in **Table 2** and **Figure 1**.

Table 1. Comparison of general situation between two groups.

group	gestational age (years)	Pregnancy (second)	Gestational week (week)
Abnormal group	28.96 ± 4.59	2.41 ± 1.52	10.77 ± 2.31
Normal group	29.04 ± 4.61	2.50 ± 1.28	10.58 ± 2.42
P value	0.32	0.18	0.25
T value	3.41	3.52	4.44

Table 2. Specific distribution of chromosomal abnormal nuclei (example).

type	Abnormal chromosome number (example)	detection rate (%)
triploid	8	16
45, X	5	10
16-trisomic	4	8
18-trisomic	3	6
22-trisomic	3	6
13-trisomic	2	4
21-deficiency	1	2
21-trisomic	1	2
47, XXY	1	2

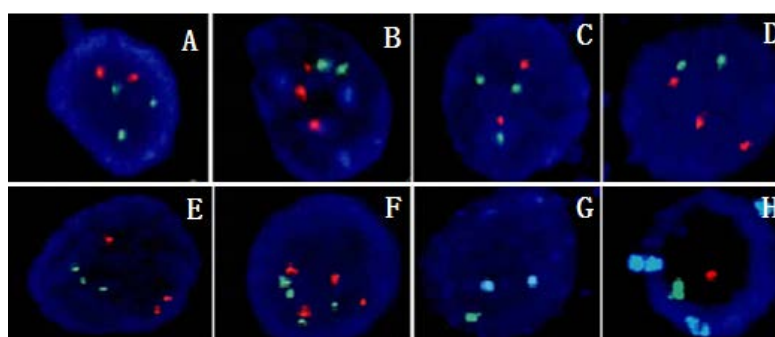


Figure 1. Fish detection of chromosomal abnormalities in different groups of embryos. Note: A, B Green fluorescent signal is chromosome 13., Red fluorescence signal is chromosome 21. C, D, E, F Green fluorescence signal is chromosome 16, red signal is chromosome 22. G, H Green fluorescence signal is x chromosome, Red fluorescence signal is y chromosome, Sky blue signal is chromosome 18. Description: 13-disome, 21-trisomic, 16-disome, 22-disome, triploid, tetraploid, X-monomer, 18-trisomic.

3.3. Comparison of Relative Expression Levels of hsMAD2 Protein in Spontaneously Aborted Embryonic Villi

The relative expression levels of the hsMAD2 protein in abnormal embryonic villi tissues was higher than those seen in the control group (Table 3 and Figure 2).

3.4. Comparison of Relative Expression Levels of hsMAD2 Gene in Spontaneously Aborted Embryonic Villi

The relative expression levels of *hsMAD2* gene was much higher in the abnormal embryo villi tissues than in the control group, as shown in Table 4 and Figure 3.

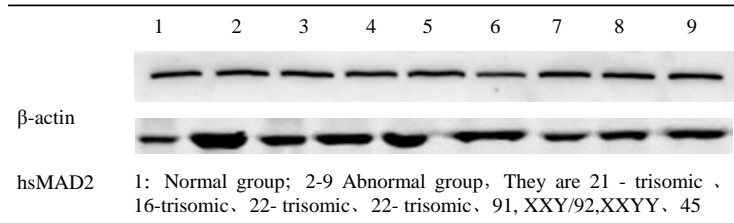


Figure 2. Comparison of the relative expression of hsMAD2 protein in two groups.

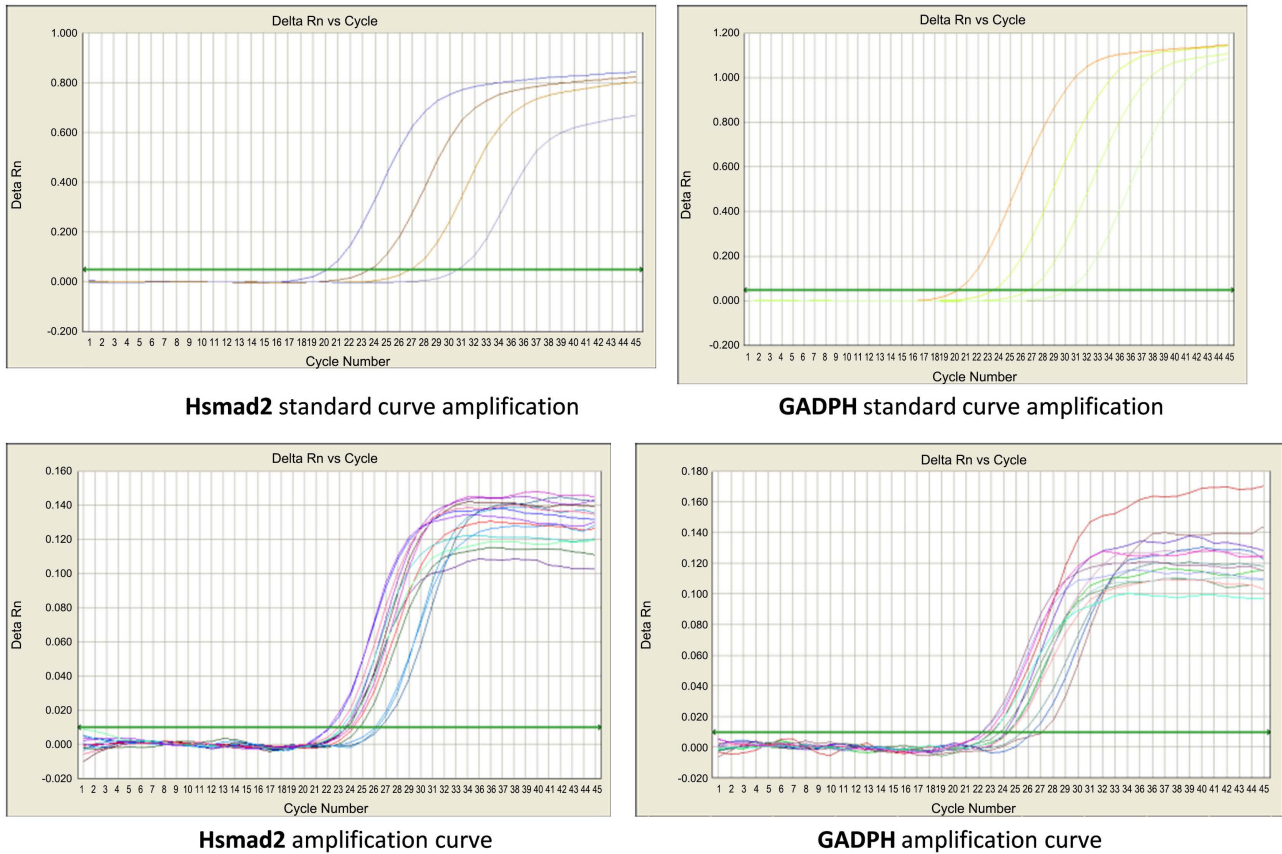


Figure 3. Comparison of relative expression of hsmad2 gene between two groups. Note: the amplification curve of internal reference is smooth and no non-specific amplification, indicating the primer synthesis and experimental system design standard.

Table 3. Comparison of relative expression of hsmad2 protein between two groups.

type	Number of examples	Protein relative expression
Abnormal group	28	0.88 ± 0.20
Normal group	53	0.61 ± 0.19
P value		0.002
T value		4.79

Table 4. Comparison of hsmad2 gene changes between two groups.

type	CT (hsMAD2)	CT (GAPDH)	2-ΔΔCT
Abnormal group	23.46 ± 0.07	21.90 ± 1.63	3.54 ± 1.01

Continued

Normal group	18.35 ± 0.10	22.89 ± 1.55	1
P value			0.019
T value			3.59

4. Discussion

Spontaneous abortion is a term used to describe an abortion caused by non-human operation. Clinical research confirms its correlation to multiple factors, including heredity and immunity. In 1983, Simoni first proposed a connection between spontaneous abortion and chromosome abnormalities; this led to a greater research focus on this subject. The latest data indicates that the rate of spontaneous abortion in pregnant women at gestational weeks < 6 is about 50%; among these 70% are believed to be a direct result of chromosomal abnormalities. On the other hand, the rates of spontaneous abortion in pregnant women at 6 - 10 and >10 gestational weeks were 15% and only 3% (50% and 5% related to chromosomal abnormality), respectively. This indicated that spontaneous abortion caused by chromosomal abnormalities takes place easily during the early stages of pregnancy [8] [9]. In this study, chromosomal abnormalities were detected in 28 out of 50 embryos (56%) obtained from patients who underwent spontaneous abortion, indicating that more than half of the embryos with chromosomal disease will be naturally eliminated and cannot survive up to birth. This morbidity rate is consistent with the results obtained in previous studies [2] [10]. After comparing pregnant women with chromosomal abnormalities and normal pregnant women, this study excluded miscarriage caused by basic factors such as gestational age, gestation time, and gestational age [11].

There are two types of chromosomal abnormalities: numerical abnormality and structural abnormality. The clinical incidence rate of numerical abnormality, especially aneuploidy, is generally higher. The occurrence of aneuploidy is mainly a result of the non-disjunction of gametes during the initial stages of fertilized ovum formation, which causes an increase or decrease in the chromosome number. Aneuploid chromosome abnormalities greatly influence the embryo; the resulting fatality rate is also very high. Therefore, it is one of the most common clinical types. The incidence rate of aneuploidy was found to be 68% in this study, which was much higher than that of polyploidy (20%) and mosaicism (12%). This data is consistent with the results obtained in previous clinical studies.

The chromosome goes through periodic changes (condensation and stretching) during the cell cycle. Therefore, chromosomal abnormalities may occur at any link of the cell cycle. The cell cycle checkpoint is a regulatory mechanism of the cell cycle. A normal cell cycle has 3 checkpoints: 1) the G1 → S stage, where one must check if the cell has developed enough for chromosome replication, or if the cell has left the cell cycle and entered the resting stage; 2) the S → G2 stage, where one can check to see if all chromosomes have been replicated; 3) a check-

point during mitosis, wherein one must confirm that all spindles are connected to the chromosomes before continuing on to mitosis. In addition, one can analyze whether the SAC makes a full impact during the spindle and chromosome docking and segregation [12] [13] [14]. Therefore, the cell cycle can be effectively monitored by detecting the SAC. *hsMAD2* is the checkpoint gene for monitoring the spindle activity performance. *MAD2* is collected in the kinetochore of the chromosome after activation. Here, its conformation changes, leading to the formation of C-*MAD2*. C-*MAD2* interacts with *Cdc20*, *BUBR1*, and *BUB3*, and forms a mitotic checkpoint complex, which provides a correct point in time for the complete docking of chromosomes during cell division. Previous studies have also reported alternative methods for the regulation of the docking and segregation of chromosomes, using methods other than the SAC. *hsMAD2* can act on mitotic kinesin-like protein 2 (*MKlp2*) during mitosis, which can induce cytokinesis. The chromosomes convey complexes driven by *MKlp2*. The ability of *MKlp2* to reload to the complexes from the centromere is restrained by *hsMAD2*, which reduces the early ineffectiveness of the SAC, as well as any increase in cytokinesis errors [15] [16]. Therefore, we attempted to detect the *hsMAD2* gene and protein expression, as an indirect means of observing the effect of the SAC, assuming *hsMAD2* to be a link between SAC and chromosome abnormality. The spindle behavior was indirectly monitored by detecting the variations in the level of *hsMAD2*.

The *hsMAD2* protein and gene expression levels were detected by western blotting and qRT-PCR; the expression of the *hsMAD2* protein and gene was higher in the embryonic villi tissues of patients with spontaneous abortion resulting from chromosomal abnormalities than those in patients without any such abnormalities. This led to the speculation that docking and segregation errors during the mitosis of fertilized ovum could result in chromosomal numerical abnormalities in the embryonic tissue. The cells with chromosomal numerical abnormalities continue the cell division. A greater amount of *hsMAD2* is provided by the organism to guarantee the correct division of ligand chromosomes into the daughter cells. This ensures that sufficient time is provided for the SAC, and that the *MKlp2* is sufficiently restrained to reload to the complex from the centromere. This facilitates the smooth transition of the cell into the mitotic anaphase. Therefore, SAC maintains the integrity of genome by increasing the concentration of *hsMAD2*.

In conclusion, our data suggests that chromosomal numerical abnormalities play a major role in spontaneous abortion. *hsMAD2* expression is higher when spontaneous abortion occurs as a result of chromosomal abnormalities. Its blocking function in mitosis plays an important role in maintaining the integrity of the chromosome. However, our research still has certain shortcomings: the number of samples is not large enough, and we should try to collect the subjects of spontaneous abortion with a large gestational week. In addition, whether there is a difference in the expression of the *hsMAD2* gene in normal people at different gestational weeks, and whether it is normal in spontaneous abortion, whether

there is a difference in the expression of hsMAD2 gene between the group and the normal unnatural abortion population.

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Conflicts of Interest

The authors declare no conflict of interest.

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