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Noninvasive Prenatal Diagnosis Significance of ERG Methylation as a Biomarker in Down's Syndrome

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Down's syndrome (DS) is a genetic disease with chromosome abnormality due to the increasing chromosome 21. This study focused on the clinical application value of ERG methylation level in blood of pregnant women as a biomarker in Down's syndrome.

Material/Methods: The sham group consisted of 210 nonpregnant women, the positive control group consisted of 33 women with a delivery history of DS fetus, and the negative control group consisted of 60 women with eutocia history. A combination of restriction enzyme digestion experiment and PCR was performed to examine ERG methylation levels, methylation sites, and distribution in blood of pregnant women and in chorion tissues from abortion samples. Gene sequencing was performed to determine the ERG sequence in chromosome 21. Homology between normal tissues and chorion tissues from abortion samples was analyzed with bioinformatics technology.

Results: ERG methylation in chorion tissues from 210 abortion samples at 8, 9, and 10 weeks gestational age were determined; however, no ERG methylation was determined in blood of pregnant women. Gene sequencing indicated that normal ERG sequence in chromosome 21 was in fetus chorion tissues, and these ERG sequences were aberrantly methylated. Bioinformatics result showed that homology and DNA methylation level was discrepancy in normal tissues and chorion tissues from abortion samples.

Conclusions: It was worthwhile to use ERG methylation as biomarker in noninvasive prenatal diagnosis, and ERG methylation should be applied with consent of pregnancy and her relatives.

MeSH Keywords: DNA Methylation • Pregnancy-Specific beta 1-Glycoproteins • WAGR Syndrome

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Background

With the development of medical science and progress of medical methods, prenatal diagnosis plays an important role in prenatal examination, as well as in prenatal and postnatal care. Moreover, prenatal diagnosis has great significance in protecting a fetus with a genetic defect, especially malformed fetuses [1]. China has the largest number of fetuses with genetic defects. Fetuses with genetic defects account for 5% (about 1 million) of 18 million fetuses annually, accounting for 20% of fetuses with genetic defects worldwide [2]. Down's syndrome (DS) is more common in fetuses with genetic defects [3].

Down's syndrome, also referred to as trisomy 21 syndrome, is a genetic disease with chromosome abnormality due to the increased numbers of chromosome 21 [4–6]. More than 50% of DS fetuses die of abortion, and born infants often have symptoms of growth retardation, hypophrenia, and malformation [7]. Research shows that the 21 q22 region in the long arm of chromosome 21 is the causative gene in DS fetuses, and trisomy in this region appears in patients with DS clinical symptoms [8–10]. Prenatal diagnosis is important in managing DS [11]. Accordingly, it has clinical practice value in the study of prenatal diagnosis.

Chromosome detection of amniotic fluid cells is an effective way to diagnose DS, but it has limitations, such as being a complicated process and it is time-consuming [12,13]. Noninvasive gene detection technique is a novel method for DS examination, and its theoretical basis is that blood of pregnant women contains genomic DNA of the fetus [14–16], which can help determine whether the fetus has trisomy 21 or ERG mutation by use of sequence comparative analysis based on DNA sequencing of the pregnant woman's blood [17]. It is very difficult to detect a small amount of DNA from the fetus in the blood of a pregnant woman, which makes noninvasive gene detection difficult [18]. Fortunately, epigenetics has been utilized in noninvasive gene detection to determine whether a fetus had trisomy 21 or ERG mutation and whether the fetus has DS [19]. Recent studies showed that the 21 q22 region in the long arm of chromosome 21 is methylation-modified, which provides a theoretical foundation for diagnosis of DS [20].

In the present study, we enrolled 210 women who underwent early-pregnancy abortion in the Obstetrics Department of a local hospital. The sham group consisted of 210 nonpregnant women, the positive control group consisted of 33 women who had given birth to a DS infant, and the negative control group consisted of 60 samples women with eutocia history. ERG methylation levels in blood of pregnant women and chorion tissues were examined with combination of restriction enzyme digestion experiment and PCR. Our study was focused on potential prenatal diagnosis significance and clinical application value of ERG methylation level in blood of pregnant women as a biomarker in Down's syndrome.

Material and Methods

Materials

Our study was approved by the local Ethics Committee. All pregnant women signed consent forms before examination. We enrolled 210 women with early pregnancy and abortion into the experimental groups from the Obstetrics Department in Tai'an Maternity and Child Care Hospital from August 2013 to August 2015, with different gestational ages, including 8 gestational weeks (72), 9 gestational weeks (69), and 10 gestational weeks (69). The average age was 25.8±2.77 years. All subjects had single natural pregnancy without cardiovascular disease or cancer, and no history of using an abortion-inducing drug.

The 210 nonpregnant women in the sham group, 33 women with delivery history of DS fetus, and 60 women with eutocia history were enrolled into the positive control group and negative control group, respectively. Average age was 25.3±2.77 years (ranging from 18 to 36 years). All subjects had natural pregnancies without cancer, history of cardiovascular diseases, or use of abortion-inducing drugs.

The experimental protocol has been pre-approved by the Ethical Committee of Tai'an Maternity and Child Care Hospital and written consent was obtained from all patients and healthy volunteers.

Genomic DNA extracted from samples

Small amounts of blood were extracted for pregnancy testing, and all protocols were consented to by subjects. Venous blood samples with heparin anticoagulation treatment were drawn from pregnant women, then centrifuged at 1500 g for 2 h. Supernatant was discarded after centrifugation, and genomic DNA was extracted using a routine method. Chorion tissues were collected with negative pressure method and washed with normal saline at low temperature. Chorion was extracted with use of a general-purpose microscope [21].

Restriction enzyme digestion experiment and sulfites process

We prepared 2 μg genomic DNA extracted from blood and chorion for the restriction enzyme digestion experiment (enzyme reagent kit purchased from Shanghai Biological Engineering Co., LTD) [22]. Hpa II and Msp I were used as restriction enzymes. The 20 μl reaction system was under digestion for 6 h at 37°C.

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Agarose gel electrophoresis was performed after digestion. The sulfites process was as follows: (1) Alkalis denatured. We added 3-μg DNA samples to 6 μL NaOH solution (1 mol/L) to prepare the reaction complex. Alkalis was denatured for 8 min at 37°C. (2) Sulfuration and dehydrogenation. We added 10 μL hydroquinone (20 mmol/L) and 260 μL $NaHSO_3$ (6 mol/L) into the reaction complex, then mixed complex was kept in a water bath for 12 h at 50°C in the dark. (3) Purification and desulphurization. The mixed complex of DNA samples was purified. We added 1 μL NaOH solution (6 mol/L) into the mixed complex, which was then allowed to react at room temperature for 10 min for desulphurization. (4) Precipitation recovering. We added 1 μL glycogen solution (20 mg/ml), 55 μL ammonium acetate (10 mol/L), and 500 μL absolute alcohol into the mixed complex and allowed it to stand overnight. Desiccation was performed after centrifugation at room temperature, after which we added 50 μL TE solution (pH=8.0) to dissolve mixed complex. The product was stored at –20°C.

PCR reaction

Sulfated genomic DNA was used as the template for PCR amplification. Primers were designed based on data of ERG sequence from the PubMed database [23].

ERG primers were as follows: 5'-TCCTCATATTCTCTGCCATTCG-3' (forward) and 5'-GGTCCTTCAGTCGCACTCTCAG-3' (backward).

Reference primers were as follows: 5'-CTGTATGCCCTCTGCTGTC-3' (forward) and 5'-CGATTTGTCATGCACCAC-3' (backward).

PCR reaction system was as follows: 0.5 μL template of sulfated genomic DNA, 2.5 μL 10×PCR Buffer, 1 μL dNTP mixture (2 mM), 2 μL primer (forward), 2μL primer (backward), 0.5 μL Taq DNA Polymerase, 1 μL $MgCl_2$ solution (10 mM), 14.5 μL double-distilled water.

PCR reaction procedure was as follows: 94°C, 8 min, 94°C, 50s.52°C, 50S, 28 cycles, then 72°C, 30S, and 72°C, with 5 min 4°C for storage.

Agarose gel reaction

Enzyme-digested production and PCR-reacted production were added into the agarose gel reaction (0.8% gel, electrophoresis at 120 V for 20 min) [24].

Statistical method

All results were analyzed with SPSS16.0. One-way ANOVA was performed to analyze differences. P value <0.05 was considered to be statistically significant.

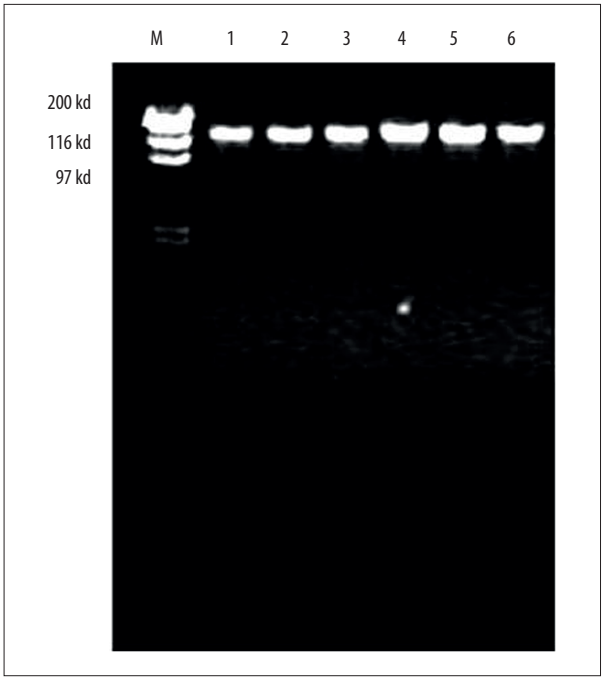

Figure 1. Results of agarose gel reaction for genomic DNA. M represents DNA marker. Ladders of genomic DNA extracted from blood (No. 1 to No. 3) and chorion (No. 4 to No. 6) are shown on the right side.

Results

Restriction enzyme digestion and related methylation level

Genomic DNA extracted from blood and chorion was analyzed (Figure 1).

Hpa II and Msp I restriction enzyme digestion was performed to detect the genomic DNA extracted from blood and chorion. As a restriction enzyme, Hpa II can recognize specific sequences of methylation sites. Hpa II cannot recognize target sequence, as the cytosine in CpG sites was methylated; therefore, amplification was completed. On the other hand, Msp I does not recognize methylation sites, and Msp I digested target sequence regardless of methylation level, and amplification cannot be completed. Ladder of amplification for β-actin reference was at 140 bp, suggesting that Hpa II digested genomic DNA thoroughly. The methylation-modified ERG sequence from chorion was 350 bp (Table 1).

PCR results of genomic DNA

PCR amplification was performed to digest genomic DNA extracted from blood and chorion. No ladder was detected at 340 bp after Msp I digestion, but there was ladder of genomic DNA extracted from chorion at 340 bp after Hpa II digestion

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Table 1. Determination of DNA methylation.

Samples	Hpa II-digested ERG amplification	Msp I-digested ERG amplification	Hpa II-digested β-actin amplification	Msp I-digested β-actin amplification	Methylation
Blood	–	–	No	No	Unmethylated
Chorion	+	–	–	–	Methylated

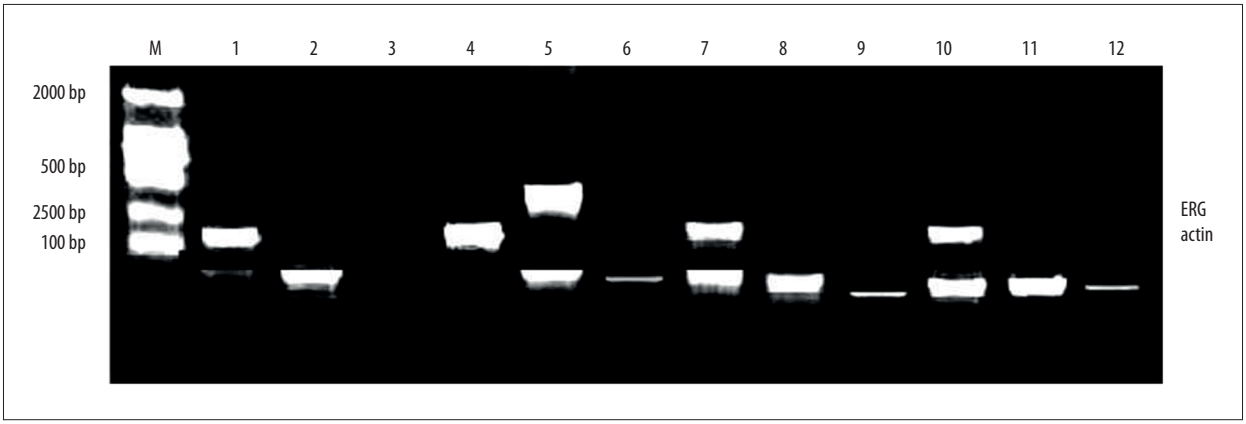

Figure 2. PCR results of genomic DNA after Msp I digestion and Hpa II digestion.

Table 2. Analysis of chorion DNA methylation at different gestational ages.

Gestational age (week)	Samples	Unmethylated	Methylated	Methylated ratio (%)
8	72	0	72	100
9	69	0	69	100
10	69	0	69	100
Total	210	0	210	100

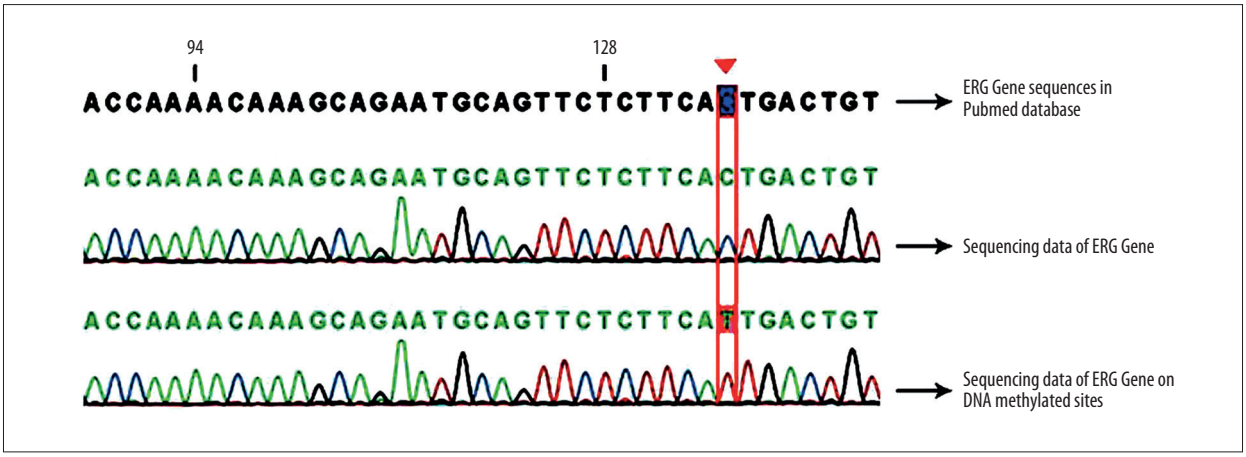

Figure 3. Results of genomic DNA sequence.

(Figure 2. lane no. 5). PCR results indicated that ERG sequence was methylated in chorion tissue, but unmethylated was detected in blood of pregnant women (Figure 2).

In Figure 2, M represents DNA marker. No.1 lane represents ERG amplification of genomic DNA extracted from blood after Hpa II digestion. No. 2 lane represents β-actin amplification

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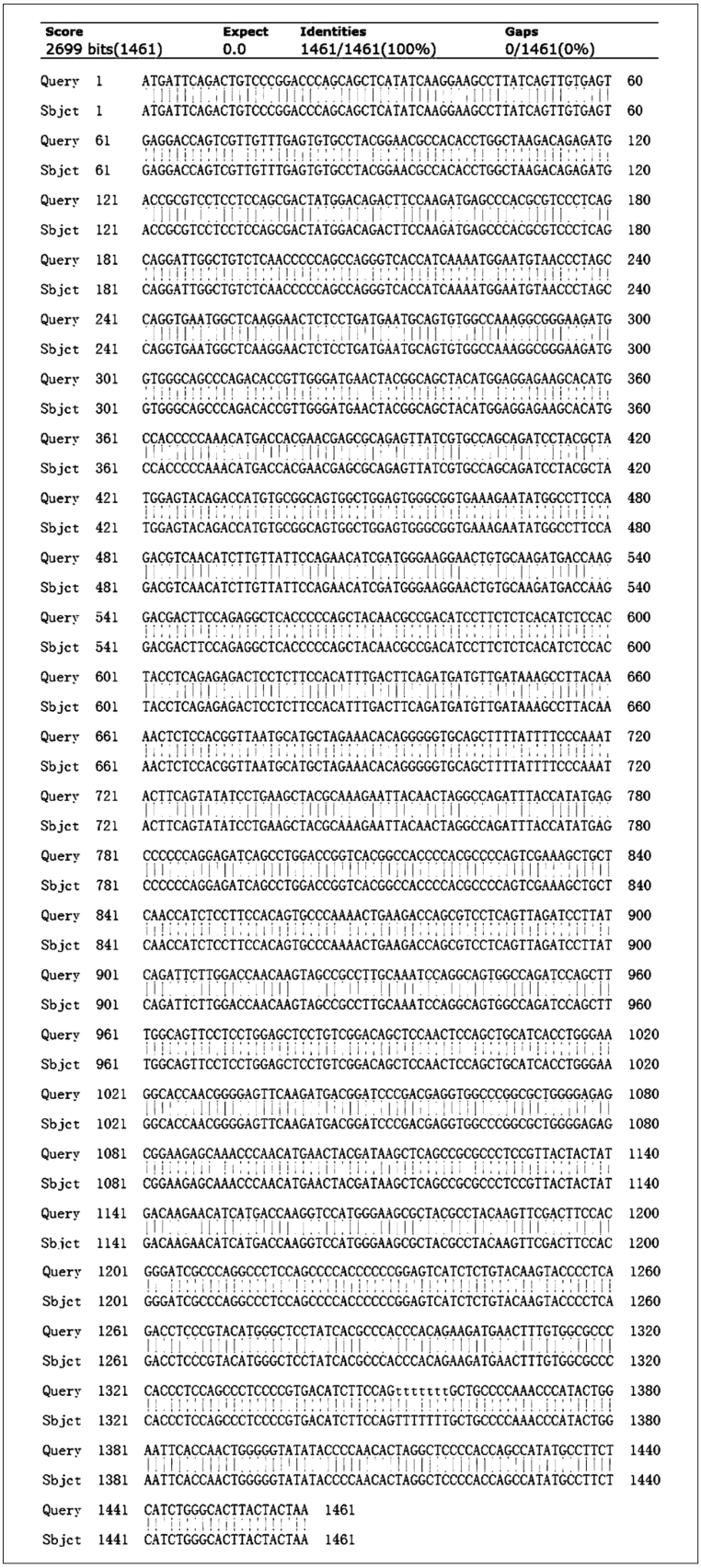

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Query  61    GAGGACCAGTCGTTGTTTGAGTGTGCCTACGGAACGCCACACCTGGCTAAGACAGAGATG  120
Sbjct  61    GAGGACCAGTCGTTGTTTGAGTGTGCCTACGGAACGCCACACCTGGCTAAGACAGAGATG  120

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Query  1441  CATCTGGGCACTTACTACTAA  1461
Sbjct  1441  CATCTGGGCACTTACTACTAA  1461
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Figure 4. Comparison of DNA sequence.

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of genomic DNA extracted from blood after Hpa II digestion. No. 3 lane represents negative control. No. 4 lane represents ERG amplification of genomic DNA extracted from chorion after Hpa II digestion. No. 5 lane represents β-actin amplification of genomic DNA extracted from chorion after Hpa II digestion. No. 6 lane represents negative control. No. 7 lane represents ERG amplification of genomic DNA extracted from blood after Msp I digestion. No. 8 lane represents β-actin amplification of genomic DNA extracted from blood after Msp I digestion. No. 9 lane represents negative control. No. 10 lane represents ERG amplification of genomic DNA extracted from chorion after Msp I digestion. No. 11 lane represents β-actin amplification of genomic DNA extracted from chorion after Msp I digestion. No. 12 lane represents negative control. Ladders below 100 bp were nonspecific bands.

Analysis of genomic DNA methylation

Genomic DNA extracted from blood and chorion at different gestational ages was tested with Hpa II digestion (Table 2). Results suggested there was no difference in ERG methylation level among subjects of different gestational ages (P>0.05).

Result of genomic DNA sequencing

DNA sequencing was performed to exam genomic DNA extracted from blood and chorion (Figure 3). In the experimental group, the ERG sequence of chromosome 21 was normal in DNA extracted from blood; however, ERG sequence in DNA extracted from chorion was different from that of the sham group.

Comparison of DNA sequence

Bioinformatics results showed that it was 100% homology discrepancy between in normal tissues and chorion tissues from abortion samples, and it existed only difference of DNA methylation level between 2 groups (Figure 4).

Discussion

Down's syndrome poses a serious threat to the health of fetuses, and prenatal diagnosis for Down's syndrome plays a vital role in prenatal and postnatal care [25]. Our study aimed to explore potential and clinical application value of ERG methylation level as a biomarker for Down's syndrome diagnosis.

There are 3 main findings in our study. (1) DNA methylation was detected in chorion tissues at 8–10 gestational weeks, while no methylation level was detected in ERG sequence from blood of pregnant women. (2) The accuracy of the EGR methylation examination was 100%. (3) ERG sequence of chromosome 21 was normal in DNA extracted from chorion, except for ERG-aberrant methylation. Bioinformatics results showed 100% homology in normal tissues and chorion tissues from abortion samples except for the discrepancy of DNA methylation level. All results suggested that the ERG gene of chorion tissues from DS children had aberrant methylation, which is consistent with previous findings [26], and indicates that there is a close correlation between ERG methylation and the incidence of Down's syndrome [27]. Examination of ERG methylation could be useful in clinical detection to determine whether a fetus has DS [28]; accordingly, it is worthwhile to use ERG methylation as the biomarker in noninvasive prenatal diagnosis.

The innovative aspect of our study was use of the digestion and PCR to diagnose DS noninvasively. Chromosome detection of amniotic fluid cells is an effective way to diagnose DS; however, it has limitations in that it is a complicated process and is time-consuming [12,13]. The noninvasive gene detection technique is a novel method for DS examination, and its theoretical basis is that the blood of pregnant women contains genomic DNA of the fetus [14–16]. Use of this technique can determine whether the fetus has trisomy 21 or ERG mutation by using sequence comparative analysis based on DNA sequencing [17].

There are some limitations in our study. (1) More high-quality, multi-center, large-sample, randomized, controlled trials are required to improve accuracy and reliability. (2) Western blot analysis was required to detect the ERG methylation in protein expression. (3) Exact methylation sites need to be determined by mass spectrometry.

Conclusions

The ERG gene of chorion tissues from Down's syndrome fetuses was aberrantly methylated. It is worthwhile to use ERG methylation as a biomarker in noninvasive prenatal diagnosis. ERG methylation should be applied with consent of the patient and her relatives.

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