Aneuploid Abortion Positively Correlate With MAD1 Overexpression and mir125b Depression

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Research

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Abstract

Background: Aneuploid is the most frequent cause of early embryo abortion, and any defect in chromosome segregation would fail to satisfy spindle assembly checkpoint (SAC) during mitosis, which could lead to the halted metaphase and aneuploid occurrence. Mitotic checkpoint complex(MCC), a complex compound of MAD1、MAD2、Cdc20、BUBR1 and BUB3, plays an important role in SAC activation. Studies have confirmed that the overexpression of MAD2 and BUBR1 can facilitate the correct chromosome segregation and embryo stability. Research identifications also proved that miR-125b negatively regulated MAD1 expression by binding to its 3'UTR. However, the expression of mir125b, MAD1 and BUB3 genes in aneuploidy embryos of spontaneous abortion has not been reported.

Methods: In this study, embryonic villi from miscarriage pregnant women were collected and divided into two groups (aneuploidy and euploidy) by HLPA and FISH analysis. The RNA levels of mir125b, MAD1 and BUB3 were detected through QRT-PCR, while Western blot was further used to analyze the protein levels of MAD1 and BUB3.

Results: SPSS 17.0 statistical analysis($P<0.05$) showed that mir125b and BUB3 were significantly down-regulated in aneuploidy group compared to the control group, MAD1 was significantly up-regulated in RNA level; Additionally, MAD1 protein level was also significantly higher while BUB3 was mildly increased in aneuploidy abortion villus. Correlation analysis revealed that the expression of MAD1 was negatively correlated with Mir125b.

Conclusion: these results suggested that aneuploid abortion was positively correlated with MAD1 overexpression which might be caused by insufficient mir125b.

Preface

Aneuploidy is a common and natural occurrence in early human embryos, which would cause disordered cell-cycle and retarded embryonic growth, even miscarriage[1]. A systematic review and meta-analysis associated with human embryos chromosome in 2011 reported that 73% of all human embryos contain aneuploid cells [2,3]. Chromosomal instability (CIN) plays a key role in aneuploidy and tumorigenesis[4]. In this context, spindle assembly checkpoint (SAC) is crucial to ensure fidelity of chromosome segregation during mitosis. Aneuploidy is known as a frequent factor from a defective mitotic checkpoint[5]. Any defect in kinetochore–spindle attachment between sister chromatids would fail to satisfy SAC, which could lead to the halted metaphase until the defect is corrected[6].

Obviously, the process of chromosome segregation during mitosis is extremely complicated, which contains a series of cascade reactions, and multiple proteins are involved. Once all chromatids are bi-oriented at metaphase, E3 ubiquitin-ligase anaphase-promoting complex/cyclosome (APC/C) catalyses the proteasomal degradation of securin, an inhibitory chaperone of separase. Activated separase then cleaves the cohesion complex required for the physical linkage of sister chromatids. Owing to loss of

cohesion, separated chromatids move to opposite spindle poles and cells enter anaphase[7,8]. Furthermore, APC/C activity depends on an adaptor-protein Cdc20 (cell division cycle 20) [9]. When SAC is 'on', APC/C remains inactive due to sequestered Cdc20 by Mad2 and BubR1/Bub3 in the form of mitotic checkpoint complex (MCC). Premature anaphase progression is thus prevented by the 'wait-anaphase' signal generated from the diffusible MCC[10].

MCC, a complex compound of MAD1、MAD2、Cdc20、BUB1 and BUB3, plays an important role in SAC activation. One of the first events in SAC activation is the recruitment of an adaptor protein Mad1 to the kinetochore. Mad1 is crucial both in Mad2-transportation from cytosol to nucleus and its kinetochore localization[6]. According to 'template' model, Mad2 exists in two states: open/free (O-Mad2),and closed (bound to either Mad1 or Cdc20 (C-Mad2)).Studies have confirmed that reduced expression of Mad2 and Bub1 proteins is associated with spontaneous miscarriages, whereas MAD2 overexpression can facilitate the correct chromosome segregation and embryo stability[11].

Many mitotic cell cycle regulators often have modified functions in meiosis important for the meiotic chromosome segregation program.A noteworthy example of a regulatory pathway with increased roles in meiosis is the spindle checkpoint.

Research shows that he spindle checkpoint protein Mad2 、Bub1 and Bub3 have a more important role in meiosis than in mitosis .Mad2 cells have enhanced chromosome mis-segregation in meiosis I and premature anaphase I onset.Loss of BUB1 or BUB3 in meiosis,causes massive chromosome missegregation in meiosis II.though there were fewer cells with massive chromosome missegregation in meiosis I, aneuploidy still occurred[12].

Research from S Bhattacharjya et.al showed that miR-125b negatively regulates MAD1 expression by binding to its 3'UTR[13]. To date, the expression of mir125b, MAD1 and BUB3 genes in aneuploidy embryos of spontaneous abortion has not been reported. This study was designed to investigate the expression of mir125b, MAD1 and BUB3 in embryonic villi cells from aneuploidy abortion embryos, and to further explore the mechanism of spontaneous abortion.

Materials And Methods

Cohort

Embryonic villi from miscarriage pregnant women(5-11 weeks of pregnancy, pregnant women aged 21-40 years old, no toxic and harmful substances contact experience) was collected and preserved at -80°C refrigerator from May 2018 to May 2019 in the Central Laboratory of Birth Defects Prevention and Control, Ningbo Women & Children's Hospital, Ningbo, Zhejiang, China. Villi tissues were obtained after operation of uterine cleanup. Based on the results of chromosomal aneuploidy (aneuploidy and euploidy) detection, 100 embryonic villi samples were divided into two groups: 50 cases with chromosomal

abnormalities (abnormal group), and 50 cases without chromosomal abnormalities (normal group). This trial was approved by the Ethics Committee.

Aneuploidy detection by HLPA

The chromosomal abnormalities of 24 chromosomes were detected by a method of modified MLPA (Multiplex ligation-dependent probe amplification) assay named HLPA (High-throughput ligation-dependent probe amplification), which was carried out using CNVplex detection kit (Genesky Technologies (Suzhou) Inc.). The main principle of this method: Using the highly specificity of ligase to perform a set of hybridization and ligation reaction on the target regions to distinguish the ploidy of the target regions. At the step of ligation, sequence tags within different length and different fluorescein (PET, VIC, NED, and FAM) were introduced to the ends of the probes and then ligated to the target regions to get ligated products of different lengths. Then, universal primers labeled with fluorescent markers were used to amplify the concatenate products by PCR. After amplification, the PCR products were separated and detected by fluorescence capillary electrophoresis, then the copy number of target regions were calculated by analyzing the peak height of electrophoretogram, the detailed workflow refer to the study Chen, S et al reported [14]. There were totally 170 (for Chr1-12 and Chr16-17, there 8 probes for each chromosome; for Chr13-15, Chr21-22 and ChrY, there 5 probes for each chromosome; for Chr18-Chr20 and ChrX, there 7 probes for each chromosome) pairs of probes targeting 24 chromosomes were designed for the aneuploidy detection. The experimental steps were as follows: First, 2 μL gDNA (30ng/μL) mixed with 1μL probe mix(10uM), 1.25 μL 4× DNA lysis buffer and 5.75 μL DNA diluent were denatured for 2 min at 98 °C and then put on ice immediately. Then, 2μL 10× ligation buffer, 0.5μL ligase and 7.5 μL double-distilled water were added to the first step products to start the ligation under the following the program: 5 cycles of 94°C for 1 min and 60°C for 3 h, then 94 °C for 2 min, and 72 °C for 10 min. Reactions were stopped by adding 20 μL of 20 mM EDTA. After ligation, 1 μL of ligation products, 10 μL 2× PCR Master Mix, 1 μL primer mix and 8 μL double-distilled water were mixed evenly to perform the multiplex PCR amplification. The PCR program was as follows: 95°C for 2 min;5 cycles of 94°C for 20 s, 62°C for 40s decreasing 1°C per cycle, and 72°C for 1.5 min; then 27 cycles of 94°C for 20 s, 57°C for 40 s, and 72°C for 1.5 min; then 68°C for 1h; Finally ,cooling the system to 4°C to stop the reaction. The last step was capillary electrophoresis and data analysis: PCR products were diluted 5-fold. 1μL diluted products mixed with 0.1μL LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA) and 8.9 μL Hi-DiTM formamide (Applied Biosystems) were denatured at 95 °C for 5 min and fluorescently labeled products were separated by on an ABI3130XL genetic analyzer (Applied Biosystems). Data were analyzed with GeneMapper software v4.1 (Applied Biosystems).

Karyotype verification by FISH

To confirm the aneuploidy detection results by HLPA, Fluorescence in situ hybridization (FISH) was performed according to Escudero, Abdelhadi, Sandalinas, & Munné, 2003[15]. The DNA probes used for this study were purchased from Beijing GP Medical Technologies, Inc., P.R. China. DLEU2:13q14, CSP18:18p11.1-q11.1, DSCR2:21q22, CSP X: Xp11.1-q11.1were used to detect chromosome 13, 18, 21, X

and Y respectively. Fixation：Embryonic villi cells were fixed in methanol/acetic acid (3:1) for FISH analyses. To prepare the slides, eight drops of fixed villi cells were placed on each. Once dried, slides were then washed twice in 2×standard saline citrate (SSC, Vysis Inc.) at room temperature, for 3 min each. Then the slides were dehydrated in ethanol series (70%, 85% and 100%) for 2 min each, and dried at room temperature in a slanted position. Denaturing：When dry, slides were placed in 5-nmol/L dithiothreitol (DDT, Sigma) and 1% Triton X-100 solution (Sigma) at 37°C for 13 min. After that, the slides were denatured in 70% Formamide (Sigma) and 2×SSC solution for 5 min at 71°C. The slides were washed again in 2× SSC and dehydrated in ethanol series. Before hybridization, the DNA probes mixed with hybridization buffer were immersed in a water-bath for 5 minutes at 72°C to denature. Hybridization：The denatured probe was then applied to each glass slide containing the fixed villi cells and was hybridized overnight at 37°C. Then, slides were washed in 0.7× SSC at 71°C for 2 minutes, dried at room temperature, and counterstained with DAPI in antifade and covered with glass coverslips for analysis.

Quantitative real-time PCR.

Total RNA was isolated using TRIZOL (Invitrogen) according to manufacturer's protocol. 5ug isolated RNA was treated with DNase (Promega, Madison, WI, USA) and 1ug of the DNase-treated RNA was used for cDNA preparation using random hexamer (Invitrogen) and MMLV-RT (Promega). For miRNAs, 200ng isolated RNA was used for cDNA preparation in a master mix containing stem-loop primers specific for the desired miRNAs (Sigma), dNTPs (Invitrogen) and MMLV-RT (Promega). Real-time PCR was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems) using power SYBR Green PCR Master Mix (Applied Biosystems). The comparative threshold cycle method (△△Ct) was used to quantify relative amounts of product transcripts with GAPDH (for mRNAs) and U6 (for miRNAs) as endogenous reference controls. Primer sets for MAD1, BUB3 and GAPDH are listed in Supplementary Table 1. Fold activation values were calculated as mean of independent experiments.

Table 1: Primers of miRNA125b,MAD1 and BUB3

Primer name	Primer sequence
MAD1	F:GCCAGAAACAAAGAGCAGACAT; R:GACCTTCAACCTGAGCGTGT
BUB3	F:GAGTGGCGAGTAGTGGAAACG; R:AGGAGACAAGCAGGAACTGG
GAPDH	F:CCTCAACGACCACTTTGTCA; R:TCTTCCTCTTGTGCTCTTGCT
miRNA125b	F:TCCCTGAGACCCTA; R:CAGTGCGTGTCGTGGAGT
U6	F:CTCGCTTCGGCAGCACA; R:AACGCTTCACGAATTTGCGT

Western blotting and antibodies

Whole cell lysates having equal protein concentrations were resolved by SDS/PAGE (8–12% gel) and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Various primary antibodies used are

mouse monoclonal Mad1 (Millipore), mouse monoclonal BUB3 (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal b-actin (Sigma). Bands were detected using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) after treating with HRP-conjugated secondary antibody (Sigma).

Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA; http://www.spss.com) was used to perform Mann-Whitney t-test in order to determine significant differences between individual groups (normal and abnormal) with respect to miR-125b□Mad1 and BUB3 expressions. Statistically significant differences were defined by two-sided $P<0.05$.

Results

Karyotype analysis

Villi tissues were obtained after operation of uterine cleanup. The chromosomal aneuploidy was detected by HLPA.50 cases with chromosomal abnormalities were listed in Table2. To confirm the aneuploidy detection results by HLPA, we further carried out FISH assay. The results were exhibited in Figure 1.

Table 2: 50 cases with chromosomal abnormalities by HLPA

Karyotype	Number of cases	Karyotype	Number of cases	Karyotype	Number of cases
46□XX□1p+	1	47□XY□+14	1	47□XX□+12	2
47□XY□+2	2	47□XX□+14	1	47□XY□+13	2
48□XX□ +2□+8	1	47□XX□+15	2	47□XX□+13	1
47□XY□+3	1	47□XY□+16	2	48□XXX□ +13	1
47□XX□+4	1	47□XX□+16	8	47□XX□+22	3
46□XY□4p-	1	47□XY□+18	3	45□XO	6
47□XY□+6	2	48□XX□ +11□+20	1	46□ XY/45,X0	1
47□XX□+10	1	47□XY□+21	2	69□XXY	1
47□XX□+11	1	47□XY□+22	2		

Quantitative real-time PCR analysis of miR125b, MAD1 and BUB3 RNA levels

The relative expression of miR125b and BUB3 were significantly down-regulated in the abnormal group compared to normal group(P<0.05), whereas MAD1 was significantly increased at RNA level (p <0.05) Fig.2. Figure2:miR125b was significantly down-regulated in the abnormal group compared to normal group(P<0.05)MAD1 gene in abnormal group was significantly up-regulated than that in normal group, and the relative expression of BUB3 gene in abnormal group was lower than that in normal group (p <0.05).

Western blotting analysis of MAD1 and BUB3 proteins

The relative expression of BUB3 protein in abnormal group was slightly up-regulated, while the relative expression of MAD1 protein in abnormal group was significantly up-regulated (Figure3). BUB3 protein in abnormal group was slightly up-regulated, while the relative expression of MAD1 protein in abnormal group was significantly up-regulated (P<0.05).

Discussion

Aneuploid is the most frequent etiology of miscarriage, and elucidating the molecular mechanism of aneuploid is important for genetic consultation from aborted women. SAC is vital to ensure fidelity of chromosome segregation during mitosis, and aneuploidy could generate a defective SAC. It is well known that MCC exert an important influence on the whole SAC course. MAD1, MAD2, Cdc20, BUBR1 and BUB3 have been confirmed to be important components for MCC. Across these proteins, MAD2 and BUBR1 overexpression can facilitate the correct chromosome segregation and embryo stability, but the expression levels of MAD1 and BUB3 in aneuploid abortion still been unknown.

Literature retrieval discovered that MiR-125b is a highly conserved miRNA that functions as a tumor suppressor or an oncogene depending upon cellular contexts. S Bhattacharjya et.al reported that miR-125b negatively regulates MAD1 expression by binding to its 3'UTR, miR-125b up- or down regulation could give rise to CIN or lead to accelerated proliferation and cell death12. Indeed, it was earlier reported that Mad1 expression was heightened in cancer cells and its high expression was correlated with cellular proliferation[16]. At the molecular level, Mad1-bound and free-Mad2 are both essential to maintain SAC. Moreover, excess MAD1 was superior to CDC20 in binding with free-MAD2, whereas CDC20 could bind to and activate APC/C. All these lead to rapid mitotic exit and SAC abolishment[17,18].

Two crucial regulators of cell cycle, cohesin and the SAC share common regulators to coordinate faithful chromosome segregation and orchestrate meiotic progression, Research show that cohesin release factor Wapl interacts with Bub3 to mediate the SAC function in oocyte meiosis I,Nevertheless,Wapl regulates Bub3 through an unknown way that requires further investigation[19].Other research shows that ,in meiosis, Bub3 is crucial for correction of attachment errors. Depletion of Bub3 results in reduced levels of kinetochore-localized Ipl1 and concomitant massive chromosome missegregation caused by incorrect chromosome-spindle attachments. Depletion of Bub3 also results in shorter metaphase I and metaphase

II due to premature localization of protein phosphatase 1 (PP1) to kinetochores, which antagonizes Ipl1-mediated phosphorylation. they propose a new role for the Bub1–Bub3 pathway in maintaining the balance between kinetochore localization of Ipl1 and PP1, a balance that is essential for accurate meiotic chromosome segregation and timely anaphase onset[12].

In this study,the relative expression of BUB3 gene in abnormal group was lower than that in normal group,but the relative expression of BUB3 protein in abnormal group was slightly up-regulated,The relationship between BUB3 gene expression□regulatory pathway and aneuploidy requires further investigation.

In this study, Mir125b was significantly decreased while the mRNA and protein levels of MAD1 were higher in abnormal group compared to normal group; Moreover, the MAD1 expression was negatively correlated with Mir125b. Taken together, we hypothesize that insufficient miR-125b increased MAD1 expression levels, which resulted in decreased free-Mad2, actived APC/C. All these dysregulation lead to SAC abolishment and accumulation of aneuploid cells in embryo, which finally cause miscarriage through child-to-mother communication mechanism.

This will help us to further explore the elaborate regulatory mechanism between miR-125b and MAD1 in aneuploid-induced spontaneous abortion.

Abbreviations

PCR: Polymerase chain reaction; QRT-PCR:Quantitative real-time PCR.; HLPA:High-throughput ligation-dependent probe amplification; FISH: Fluorescence in situ hybridization WB: Western blotting; CNV: Copy number variation; SAC:chromosome segregation would fail to satisfy spindle assembly checkpoint; MCC□ Mitotic checkpoint complex; CIN□Chromosomal instability

Declarations

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The authors thank the families for participating in this study and for providing samples and written informed consent. We declare that the preprint of abstract of this manuscript is made available in Authorea website.

Authors' contributions

Juan Zhao and Hui li contributed equally to this study. Juan Zhao designed the study, revised and edited the manuscript. Hui li analyzed the data, interpreted the results and wrote the manuscript. Tiansheng Cao design and guide the whole experiment .Haibo Li, Guangxin Chen and LiJun Du helped sample preparation and performed experiments ,Peiyan Xu and Xiaoli Zhang helped for the clinical assessment

of the patients, wrote clinical details . All authors reviewed the manuscripts and gave inputs to improve the manuscript. The author(s) read and approved the final manuscript.

The authors who contribute to this research in Guangzhou Huadu District People's Hospital and Central Laboratory of Birth Defects Prevention and Control, Ningbo Women & Children's Hospital.China.

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Availability of data and material s

The CDS sequence is mainly obtained from NCBI: https://pmlegacy.ncbi.nlm.nih.gov/gene/?term=MAD1; https://pmlegacy.ncbi.nlm.nih.gov/gene/?term=BUB3;

The DNA sequence is mainly obtained from UCSC:http://genome.ucsc.edu/cgi-bin/hgTracks; Primer design uses Primer5.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Guangzhou Huadu District People's Hospital and Central Laboratory of Birth Defects Prevention and Control, Ningbo Women & Children's Hospital, Ningbo, Zhejiang, China All the 100 patients to participate in this study signed informed consent forms .

Consent for publication

Written informed consents for publication of identifying images and the clinical details of the patients were obtained from the 100 patients to consent.

Competing interests

The authors declare no competing interests.

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Figures

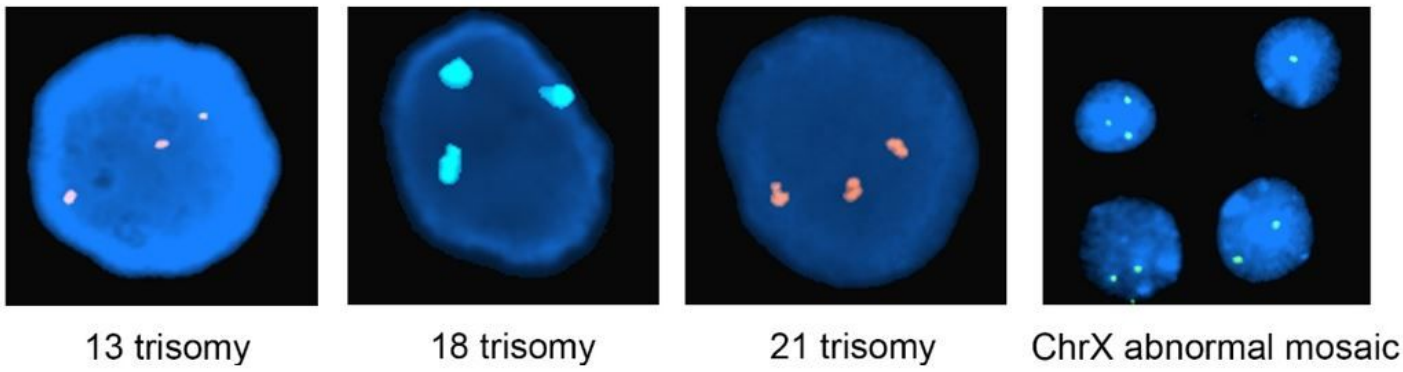

Figure 1

FISH verification of 13/18/21/X chromosomal abnormalities.

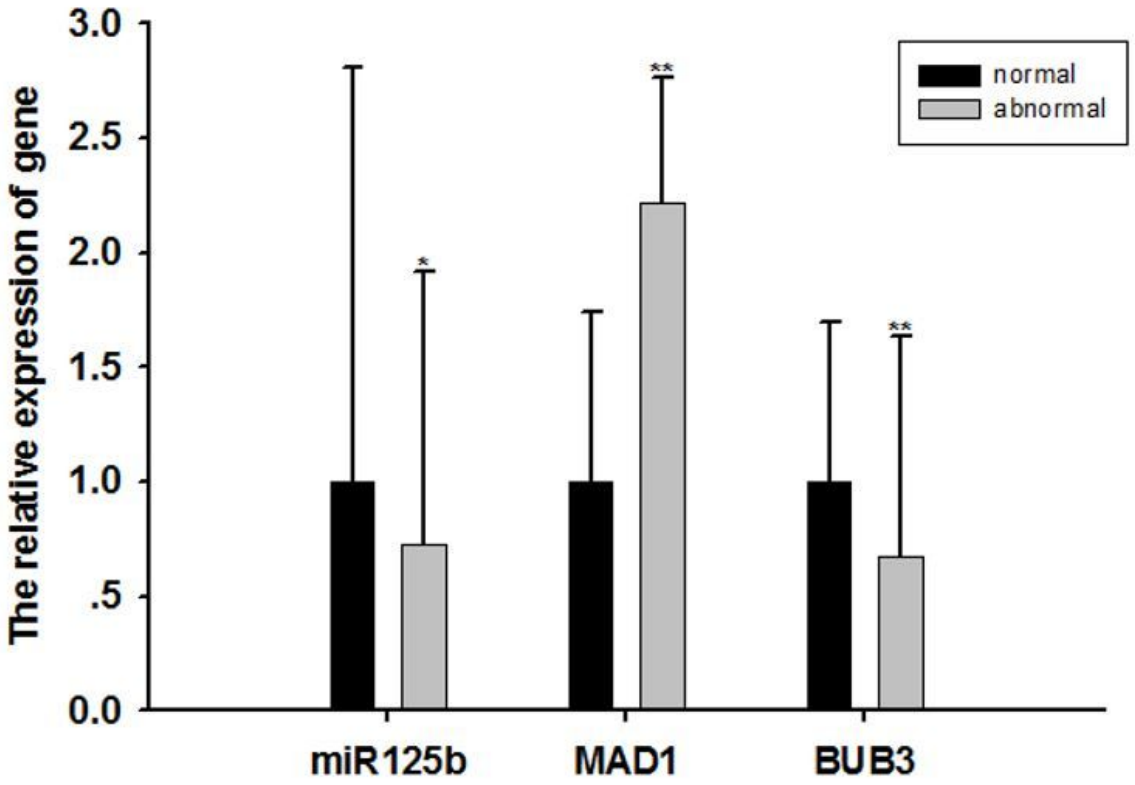

Figure 2

The relative expression of miR125b, MAD1 and BUB3

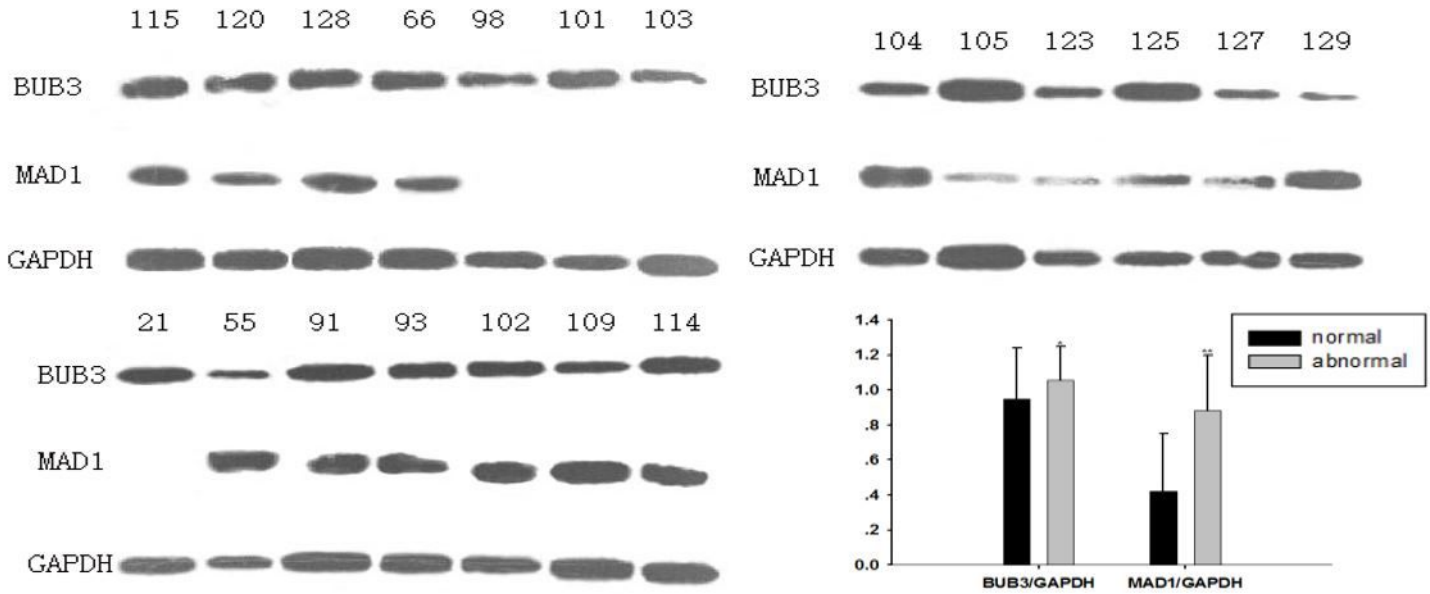

Figure 3

Western blotting analysis of the relative expression of BUB3 and MAD1

Supplementary Files

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