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Depression of MAD2 inhibits apoptosis of gastric cancer cells by upregulating Bcl-2 and interfering mitochondrion pathway ☆

Yulei Du [1], Fang Yin [1], Changjiang Liu, Shengjuan Hu, Jun Wang, Huahong Xie, Liu Hong, Daiming Fan *

State Key Laboratory of Cancer Biology, Institute of Digestive Diseases, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China

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Abstract

Mitotic arrest deficient 2 (MAD2) is an essential component of the mitotic spindle checkpoint pathway. It was previously shown to be associated with drug resistance of tumor cells. To further explore the roles of MAD2 in responses of gastric cancer cells to chemotherapy drugs, we constructed the siRNA vectors of MAD2 and transfected them into gastric cancer SGC7901 cells to inhibit expression of MAD2. MTT assay showed that the downregulation of MAD2 increased the resistance of SGC7901 cells to spindle inhibitors and DNA damaging agents. The apoptosis rates of gastric cancer cells transfected with MAD2-siRNA were 10.7% and 10%, respectively, after treated by 1.0 μg/ml VCR and cisplatin. In contrast, the apoptosis rates of SGC7901 and SGC7901/psilencer3.1 induced by VCR were 43.2%, 38.7%; and that induced by cispaltin were 34.1%, 31.4%. The ratio of Bcl-2 to Bax was much higher in the MAD2-siRNA transfectants compared with the SGC7901/psilencer. In SGC7901/psilencer, cytochrome *c* and cleaved caspase 3 protein levels increased along with the exposure time increased. However, these protein levels of SGC7901/MAD2-siRNA had no changes during the drug treatment. These results indicate that down regulation of MAD2 could promote the drug resistance of gastric cancer cells and inhibit anticancer drugs induced-apoptosis by upregulating Bcl-2 and interfering the mitochondrion apoptosis pathway.

Keywords: MAD2; Gastric cancer; Apoptosis; siRNA; Bcl-2

Spindle checkpoint monitors a series of events to ensure accurate alignment of chromosome prior to cell division and is indispensable for chromosome stability. MAD2 is an essential component of the mitotic spindle checkpoint pathway and plays a vital role in maintaining spindle checkpoint function by generating "waiting anaphase" signal [1]. The decreased MAD2 expression has been identified in some human cancer including lung [2], breast [3], ovarian[4], and nasopharyngeal carcinomas [5]. Deletion or downregulation of MAD2 brings on chromosomal instability [6,7].

It has been reported that HTLV-1 Tax protein affects subcellular localization of Mad2 proteins in adult T-cell leukemia, leading to failure of response to mitotic checkpoint and chemoresistance to microtubule inhibitors [8]. Moreover, suppressions of Mad2 and BubR1 in paclitaxel-treated cancer cells could abolish checkpoint function, and result in paclitaxel resistance [9]. It also has been reported that MAD2 expression was correlated with cellular resistance to DNA-damaging agent cisplatin in nasopharyngeal carcinoma cell lines [10].

Our previous works indicate that the status of MAD2 is very important for determining the resistance of gastric cancer cells to anticancer drugs [11,12]. Mad2β, an alternative splicing form variant of MAD2, only expressed in the

☆ *Abbreviations:* ADR, adriamycin; MAD2, mitotic arrest deficient 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; siRNA, small interfering RNA; VCR, vincristine.

* Corresponding author. Fax: +86 29 82539041.
E-mail address: fandaim@fmmu.edu.cn (D. Fan).

[1] These authors contributed equally to this work.

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drug resistant gastric cancer cell lines SGC7901/VCR and SGC7901/ADR. Mad2β could suppress the expression levels of MAD2 and eventually result in the drug resistant phonotype of gastric cancer cells SGC7901 by reducing apoptosis [13]. Mad2β protein itself may also contribute to drug resistance of gastric cancer cells. Therefore in this study, we used siRNA techniques to downregulate the expression of MAD2 in gastric cancer cells and further explored the roles and mechanisms of MAD2 involved in apoptosis of gastric cancer cells SGC7901 induced by microtubule inhibitors and DNA damaging agents.

Materials and methods

Cell culture. The human gastric cancer cell line SGC7901 was conserved in our institute. The cells were cultured in RPMI1640 medium (Life Technology, USA) supplemented with 10% fetal calf serum in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2.

siRNA constructs and transfection. Two different MAD2-specific siRNA duplexes were designed homologous to the MAD2 mRNA consensus sequence. The first siRNA molecule, MAD2-A, (S: 5′tttgcagtcttgtcacac tcaaacattgagtgtgacaagactgctttt-3′, AS: 5′-ctagaaaaagcagtcttgtccacactcaat gtttgagtgtgacaagactg-3′), the target sequence of it was homologous to nt 311–329 of the MAD2 mRNA sequence; the second siRNA molecule, MAD2-B, (S: 5′-tttgcagtcttgtcacactcaaacatcagacagatcacagctagtttt-3′, AS: 5′-ctagaaaaagcagtcttgtccacactcaatgttcagacagatcacagcta-3′) homologous to nt 401–423 of the MAD2.

According to the manufacturer's instruction, the siRNA plasmids were transfected into SGC7901 cells using Lipofectamine™2000 reagent (Invitrogen, USA). The gastric cancer cells transfected with MAD2-A and MAD2-B were named as SGC7901/MAD2-siRNA-A and SGC7901/MAD2-siRNA-B, respectively.

RNA extraction and semi-quantitative RT-PCR. The RNA of each cell lines was extracted using Trizol (Life Technologies, USA) according to the manufacturer's protocol. DNase was used to decrease the contamination of genomic DNA. The quantity and purity of the RNA prepared from each sample were determined by UV absorbance spectroscopy. The reverse transcription reaction was performed using the First-Strand cDNA Synthesis Kit (Fermentas, USA) in a final volume of 20 μl. After incubation at 42 °C for 60 min, the reverse transcription reaction was terminated by heating at 70 °C for 10 min. The newly synthesized cDNA was amplified by PCR. MAD2 primer was (S: 5-AAGGTGAAGGTCG GAGTCAA-3; AS: 5-TGTGGTCATGAGTCCTTCCA-3). Amplication cycles were: 95 °C for 2 min, then 36 cycles at 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 50 s, followed by 72 °C for 10 min. β-Actin primer (S: 5-AGCGGGAAATCGTGCGTG-3; AS: 5-CAGGGTACATGGTGGTG CC-3) was used as an internal control. Aliquots of PCR product were electrophoresed on 1% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Western blotting analysis. Cells were washed with ice-cold PBS after trypsinized, and then centrifuged at 500*g* for 5 min at 4 °C. The pellet containing 10^6 cells was lysed in 100 μl of lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ with 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and quantified by Bradford method. One hundred micrograms of protein was separated by SDS–PAGE (12% polyacrylamide gel) and transferred to nitrocellulose membranes. Proteins bound to the membrane were stained with Ponceau S in order to confirm the identical amounts of protein being transferred. The blots were stained with first antibodies (MAD2 antibody: BD Bioscience; β-actin antibody: Sigma Chemical; Fas, FasL, Bcl-2, Bax, cytochrome *c*, cleaved caspase 3: Cell Signal Technology), followed by incubation with peroxidase-conjugated second antibody. Enhanced chemiluminescence (Amersham, Freiburg, Germany) was used for detection and developed by X-ray film.

In vitro drug sensitivity assay. The sensitivity of gastric cancer cells to anticancer drugs was evaluated with MTT assay as described [14]. Gastric cancer cells were seeded into 96-well plates (1×10^4 cells/well) and incubated overnight. And cells were treated with different concentrations of VCR, paclitaxel, ADR, and cisplatin for 72 h. Relatively inhibitory rate of cell growth by drugs was calculated according to the formula: $R = (A_2 - A_1)/A_2 \times 100\%$. R is the relatively inhibitory rate of cell growth; A_1 is the absorbance value of cells in the presence of drugs; A_2 is the absorbance value of control cells without any drug treatment. Finally, the concentration of each drug that caused a 50% reduction in number of cells (IC_{50}) was calculated.

Flow cytometry assay. The MAD2-siRNA transfected cells and control cells were seeded at a density of 3×10^4 cells/ml in 75-cm^2 flasks and treated with 1.0 μg/ml VCR or cisplatin for 24 h, respectively. The cells were harvested, fixed, and stained with propidium iodide (PI) (Sigma) in a PBS solution containing RNase A (Boehringer, Mannheim, Indianapolis, IN) for cell cycle analysis.

And the cells were stained with FITC-labeled annexin V and PI (Molecular Probes, Eugene, OR) to explore apoptosis. Apoptotic cells were measured by a FACS Calibur (Becton–Dickinson, SanJose, CA) and data analysis was performed with the standard Cell Quest software.

Mitotic index. To measure mitotic indices, at least 500 cells were counted for each slide with LSCM. Cells were cultured with 1.0 μg/ml VCR or cisplatin for 24 h. Then the cells were harvested and fixed with 4% formalin in PBS for 30 min. Cells were then attached to glass slides by centrifugation and stained with 4 V, 6-diamidino-2-phenylindole (DAPI). Mitotic indices were calculated as the percentage of mitotic cells among the total viable cells.

Statistical analysis. Data values were expressed as means ± SD. Differences were compared by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A value of $p < 0.05$ was considered significant.

Results

Downregulation of MAD2 expression in cancer cells induced by siRNA

siRNA constructs designed to target coding region of MAD2 was used to decrease the expression of MAD2 in the human gastric carcinoma cell line SGC7901. Two subclones of MAD2-siRNA transfected stable transfectants SGC7901/MAD2-siRNA-A and SGC7901/MAD2-siRNA-B were generated.

We evaluated the efficacy of siRNA transfection in the silencing of gene expression of MAD2 by using both Western blotting and reverse transcriptase-polymerase chain reaction (Figs. 1 and 2). Western blotting showed that both siRNAs MAD2-A and MAD2-B approximately

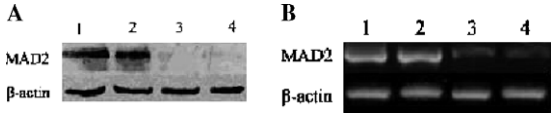

Fig. 1. Inhibition of MAD2 expression in gastric cancer cells. Four cell lines were evaluated: human gastric cancer cells SGC7901, (lane 1), SGC7901/psilencer (transfected with psilencer3.1; lane 2), SGC7901/MAD2-siRNA-A (transfected with MAD2-siRNA1; lane 3) and SGC7901/MAD2-siRNA-B (transfected with MAD2-siRNA2; lane 4). (A) Western blotting shows MAD2 protein expression was inhibited significantly in siRNA of MAD2 transfected cells. (B) RT-PCR assay shows mRNA of MAD2 in MAD2-siRNA transfectants decreased compared with that in control cells.