The spindle checkpoint protein MAD1 regulates the expression of E-cadherin and prevents cell migration

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Abstract. Aneuploidy is a common characteristic of human solid tumors. It has been proposed that a defect of the spindle assembly checkpoint (SAC) generates aneuploidy and might facilitate tumorigenesis. However, a direct link between the SAC proteins and tumorigenesis has not yet been elucidated. Here, we demonstrate the association of the SAC protein MAD1 with the RNA polymerase II complex and its role in gene expression. Furthermore, MAD1 binds to the E-cadherin promoter region. Knockdown of endogenous MAD1 by siRNA reduces E-cadherin expression and enhances the migration ability of non-metastatic breast cancer cells, indicating that reduced MAD1 expression is a new potential diagnostic symptom of tumor metastasis.

Introduction

Over 90% of cancer death is attributed to metastasis (1). Remodeling of the extracellular matrix and an enhanced migratory ability are critical steps in the metastatic dissemination of cancer cells (2). E-cadherin is a component of cell-cell adhesion junctions (3), and its linkage to the cytoskeleton requires peripheral membrane proteins, including α-catenin, β-catenin, vinculin, and α-actinin. In addition, β-catenin directly binds to E-cadherin (4,5). The actin cytoskeleton is also required for the association of E-cadherin with another cell adhesion protein, nectin (6). E-cadherin is thought of as a hallmark symptom of metastatic cells because the loss of E-cadherin may lead to an invasiveness of human carcinoma cells (3). Several transcriptional repressors of E-cadherin, such as the zinc-finger-containing proteins SIP-1, δEF-1, Snail, and Slug and the helix-loop-helix proteins E12/E47 and Twist, have been reported to be highly associated with tumor progression (7-10).

Abnormal chromosome number (i.e., aneuploidy) is also a common phenomenon in cancer cells. The spindle assembly checkpoint (SAC) monitors the fidelity of chromosomal segregation in mitosis to prevent aneuploidy in daughter cells. Mutations and/or reduced levels of mitotic checkpoint proteins can cause checkpoint malfunction and chromosomal instability (CIN) and thereby contribute to tumor formation (11). The heterozygous deletion of mitotic arrest-deficient protein 1 (MAD1), a component of the SAC, increases the incidence of tumors in mice (12). MAD1 has also been reported to have lower expression level in gastric tumors, hepatocellular carcinoma and renal cell carcinomas (13-16). Although it has been shown that MAD1 interacts with HDAC1 (17), the transcriptional activity of MAD1 and the role of MAD1 in tumor progression are not known. Furthermore, the direct role of SAC proteins in tumorigenesis remains to be elucidated.

Here, we demonstrate that MAD1 associates with the RNA polymerase II complex and may regulate MAD1 and E-cadherin expression. Depletion of MAD1 causes lower E-cadherin expression and enhances cell migration ability, which is a critical sign of metastatic cancer cells.

Materials and methods

Plasmid constructions and antibody production. Full-length MAD1 was cloned into phrGFP-N1 (Stratagene) and pCMV-Flag2 (Sigma-Aldrich). The E-cadherin promoter/pGL3 luciferase, E-Cad(-995/+135)-luc, plasmid was kindly provided by Liu *et al* (18).

The MAD1 promoter DNA fragment, from 35 bp downstream to 1035 bp upstream of the transcription start site, MAD1(-1035/+35), was PCR-amplified using human genomic DNA as a template, and it was cloned into the luciferase reporter vector, pGL3 (Promega). The resulting plasmid was designated as MAD1(-1035/+35)-luc.

Purified His-tagged MAD1(1-160), containing amino acid residues 1-160 of MAD1, recombinant proteins were used to generate rat polyclonal antibodies against MAD1. Rat anti-MAD1 antibodies were further purified by Affi-Gel 10 beads conjugated with GST-MAD1(1-160) recombinant proteins, and the purified antibodies were kept in 1X PBS buffer containing 50% glycerol and 2 mM 2-mercaptoethanol.

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Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed as described in a commercial manual (Millipore). Cells were fixed using 1% formaldehyde and incubated for 10 min at 37˚C. The cells were washed with cold 1X PBS and lysed with SDS lysis buffer (catalog # 20-163, Millipore) for 10 min on ice. Cell lysates were then sonicated until the genomic DNA was sheared to 200-1000 bp. After centrifuging the samples for 10 min (13,000 rpm at 4˚C), the supernatants were diluted and used to perform immunoprecipitation using 5 μg of rabbit anti-MAD1 (GeneTex), mouse anti-POLR2A antibody (GeneTex) or rabbit non-immune antibodies. The immunocomplexes were precipitated using 30 μl of protein G magnetic beads (Millipore), eluted, and treated with 2 μl of 10 mg/ml proteinase K to eliminate proteins. The precipitated DNA was purified and used for PCR amplifications. The pair of specific primers used for the E-cadherin promoter were 5'-AAAGAGTGAGCCCCATCTCCAAA-3' and 5'-TGGCTCACTAAGACCTGGGATCA-3'. The PCR reaction was expected to produce a 105-bp DNA fragment containing the region 185-290 bp upstream of the transcription start site of the E-cadherin gene.

Cell culture, transfection and RNA interference. HeLa and MCF-7 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell transfection was performed using Lipofectamine 2000, as described in the manufacturer's manual (Invitrogen). For siRNA transfection, MCF-7 cells were plated in a 6-well plate the day before the transfection. siRNA duplexes (40 pmoles) were transiently transfected into cells using oligofectamine, according to the manufacturer's manual (Invitrogen). The sequence of the MAD1 siRNA sense strand was 5'-r(CAGGCAGUGUCAGCAGAAC)dTdT-3' and that of the non-silencing siRNA sense strand was 5'-r(UUCUCC GAACGUGUCACGU)dTdT-3'.

Cell migration assay. MCF-7 cells ($3x10^5$) were plated in a 6-well dish 24 h after the siRNA treatment, and the cells were trypsinized and resuspended in 750 μl of DMEM containing 1% serum. DMEM containing 10% serum was added to a 24-well dish, the insert was placed in the well as described in the manufacturer's protocol (Corning), and $1x10^5$ of the above resuspended cells were added to the insert for 18 h. The cells were stained with crystal violet and counted under a microscope.

Western blotting and co-immunoprecipitation. To perform the immunoblot analysis, cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, and 1 mM DTT], and 60 μg of each cell lysate was resolved by 10% SDS-PAGE and transferred to a PVDF membrane. EGFP, β-tubulin, MAD2, POLR2A and E-cadherin were detected using their specific antibodies (mouse anti-securin from Zymed; mouse EGFP from Abcam; mouse anti-POLR2A from GeneTex; mouse anti-MAD2 from GeneTex; mouse anti-β-tubulin from Sigma). The proteins were visualized using a chemiluminescence-exposed (Dupont) hyperfilm (GE Healthcare).

A co-immunoprecipitation assay was performed in RIPA buffer containing protease inhibitors (Roche Diagnostics) and phosphatase inhibitors (Sigma) using protein A beads. The precipitated proteins from cell lysates were analyzed using Western blotting, as described above.

Immunofluorescence microscopy. To visualize the localization of MAD1 and the RNA polymerase II complex, rabbit anti-MAD1 and mouse anti-POLR2A antibodies were used for immunofluorescence staining. The cells were rinsed with 1X PBS buffer, fixed by 4% paraformaldehyde, and then blocked with filtered PBS containing 1% skim milk. Purified rabbit anti-MAD1 antibody and mouse anti-POLR2A antibody were diluted in filtered PBS buffer with 1% skim milk. FITC and Cy3-conjugated secondary antibodies were also diluted in filtered PBS with 1% skim milk. All of the antibody incubations were performed for 1 h at room temperature, followed by three washes with 1X PBS buffer. All of the stained cells were visualized using a confocal laser microscope (Leica TCS SP2 AOBS). Both of the images for the POLR2A and MAD1 proteins were merged by computer software provided by Leica.

Results

Ectopic overexpression of MAD1 results in a decrease in endogenous MAD1 expression. While determining the level of the GFP-MAD1 fusion protein, comprised of MAD1 fused to the C-terminus of the humanized green fluorescence protein of *Renilla reniformis*, in HeLa cells, we unexpectedly found that the level of endogenous MAD1 protein was significantly lower in the MAD1-overexpressing cells than in the mock cells (Fig. 1A). As a comparison, we also applied RNAi to deplete the endogenous MAD1 in HeLa cells using MAD1-specific siRNA duplexes. The residual level of endogenous MAD1 protein in the MAD1-depleted cells was similar to the level of endogenous MAD1 protein in the MAD1-overexpressing cells (Fig. 1A).

We speculated that it is possible that MAD1 may have a negative feedback control on its gene expression at the transcriptional level. To test this possibility, a ~1.5-kb DNA fragment, containing a region from 35 bases downstream to 1502 bases upstream of the transcriptional initiation site of human *MAD1* gene, MAD1(-1502/+35), was cloned into a luciferase (luc) reporter plasmid, as previously described (17). The MAD1(-1502/+35)-luc construct was cotransfected with a Flag or Flag-MAD1 vector into HeLa cells. Compared to the Flag vector control, the overexpression of Flag-MAD1 repressed ~40% ($p=0.007$) of the luciferase activity (Fig. 1B). This indicates that MAD1 has a role in regulating its own expression at the transcriptional level.

MAD1 associates with the RNA polymerase II complex and regulates E-cadherin expression. Because MAD1 had been reported to associate with the transcription factor HDAC1 (17) and may regulate its own expression at the transcriptional level, we determined whether MAD1 associated with the transcriptional initiation complex. Therefore, we performed a co-immunoprecipitation assay with rabbit anti-POLR2A antibody, which recognizes the largest subunit (RPB1) of the

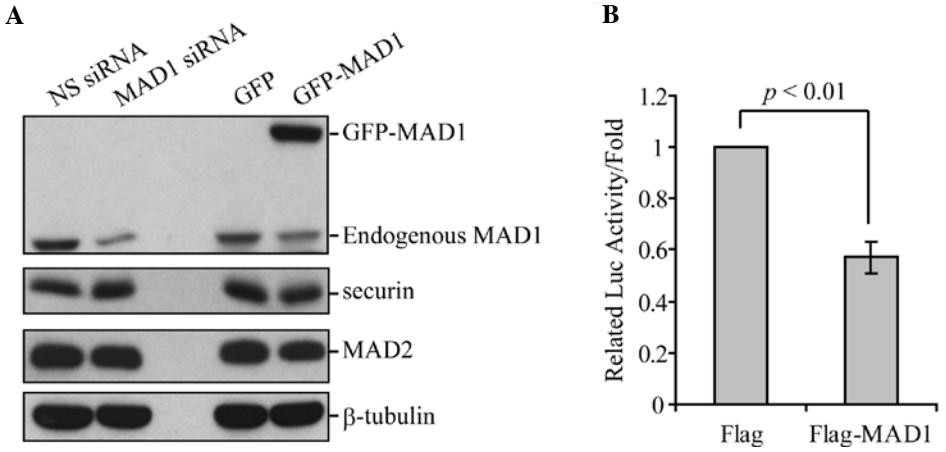

Figure 1. Overexpression of MAD1 reduces the level of endogenous MAD1 protein. (A) HeLa cells were transfected with GFP or GFP-MAD1 plasmids or NS (non-silencing) or MAD1 siRNA duplexes. After 48 h of transfection, the cells were lysed for Western blot analysis. MAD1, MAD2, securin, and β-tubulin were detected using rat anti-MAD1, mouse anti-MAD2, mouse anti-securin, and mouse anti-β-tubulin antibodies, respectively. β-tubulin was used as an internal loading control. (B) Effect of the overexpression of MAD1 on its own gene expression at the transcriptional level. Flag or Flag-MAD1 plasmid DNA was cotransfected with MAD1(-1035/+35)-luc plasmid DNA into HeLa cells for 48 h. The transfected cells were then lysed and assayed for the detection of their individual luciferase activity. The activities were normalized to the control Flag plasmids.

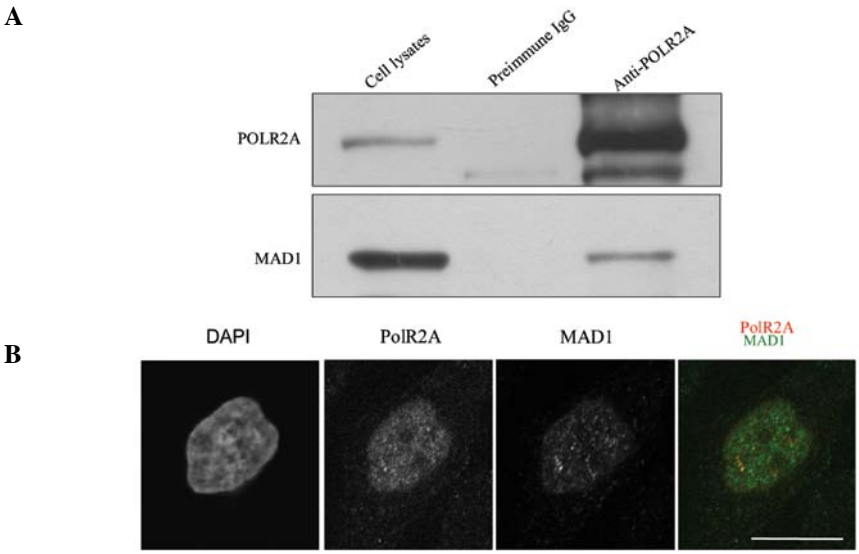

Figure 2. MAD1 associates and co-localizes with the RNA polymerase II complex. (A) HeLa cells were lysed and incubated with mouse preimmune IgG or anti-POLR2A antibodies. Proteins were precipitated with protein G beads and analyzed using Western blotting. (B) The fixed cells were simultaneously stained with rabbit anti-MAD1 and mouse anti-POLR2A antibodies, followed by Cy3-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG antibodies. Chromosomal DNA was stained with DAPI. Stained cells were visualized using confocal laser microscopy.

human RNA polymerase II complex. We found that MAD1 co-precipitated with RPB1 from HeLa cell lysates (Fig. 2A), indicating that MAD1 may associate with the RNA polymerase II complex *in vivo*.

To determine further whether MAD1 co-localized with the RNA polymerase II complex in the nucleus, rabbit anti-MAD1 and mouse anti-POLR2A antibodies were simultaneously used as probes to label MAD1 and RPB1 in HeLa cells, respectively (Fig. 2B). It was found that MAD1 co-localized with RPB1 within the nucleus. This observation further confirms the association of MAD1 with the RNA polymerase II transcription complex.

Depletion of MAD1 results in a decrease in the level of E-cadherin and enhances cell migration ability. MAD1 has been regarded as a candidate for a tumor suppressor gene, and its expression has been observed in epithelial cells and tumor cells but not in mesenchymal cells (13). E-cadherin is a well-known epithelium marker, and it had been previously elucidated that activated androgen receptor cooperates with HDAC1 to repress E-cadherin expression (18). Because MAD1 also binds to HDAC1, we sought to determine whether the down-regulation of MAD1, which is observed in many cancers (13-16), affects E-cadherin expression. We utilized RNAi to deplete MAD1 in MCF-7 cells using MAD1-specific siRNA duplexes (see Materials and methods). The E-Cad (-995/+135)-luc plasmid, the luciferase reporter gene fused to the promoter DNA fragment containing the region 995 bp upstream to 135 bp downstream of the transcription start site of the E-cadherin gene, was used to measure the effect

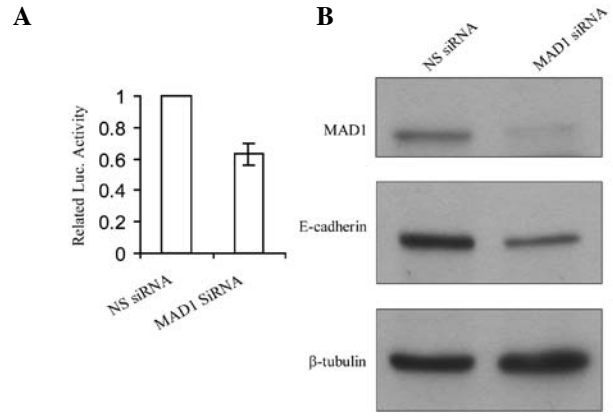

Figure 3. Knockdown of MAD1 by siRNA duplexes reduces E-cadherin expression. To test the transcriptional activity of MAD1 on the E-cadherin promoter, NS (non-silencing) or MAD1 siRNA duplexes and E-Cad (-995/+135)-luc plasmid DNA were co-transfected into MCF-7 cells. After transfection for 48 h, the cells were lysed and analyzed as described in Fig. 1B. NS or MAD1 siRNA duplexes were transfected into MCF-7 cells for 48 h. Cells were then collected and lysed for Western blot analysis. Western blotting was performed using rat anti-MAD1, mouse anti-E-cadherin, and mouse anti-β-tubulin antibodies. β-tubulin was used as an internal loading control.

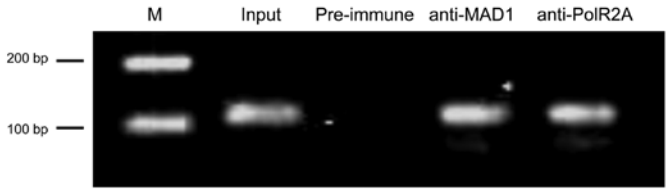

Figure 4. MAD1 binds to the E-cadherin promoter region *in vivo*. Binding of MAD1 to the E-cadherin promoter region was analyzed using the chromatin immunoprecipitation (ChIP) assay. MCF-7 chromatin extracts were sonicated and then immunoprecipitated with rabbit non-immune IgG, mouse anti-POLR2A, or rabbit anti-MAD1 antibodies, as described in Materials and methods. A mock control experiment was performed without the addition of antibodies. The precipitated POLR2A-DNA or MAD1-DNA complexes were then eluted for PCR analysis. The expected size of the E-cadherin promoter DNA fragment produced in the PCR reaction was 105 bp.

of MAD1 depletion on E-cadherin expression. Compared to the non-silencing siRNA-treated cells, the depletion of MAD1 repressed ~40% (p=0.012) of the luciferase activity (Fig. 3A). In addition, the protein level of E-cadherin was also decreased in the MAD1-depleted MCF-7 cells (Fig. 3B).

To elucidate whether the MAD1-associated transcription complex binds to the regulatory sequence of E-cadherin further, we performed a ChIP assay using anti-MAD1, anti-POLR2A or non-immune antibodies. The precipitated DNA was detected using PCR with a pair of primers specific for the E-cadherin promoter region (see Materials and methods), and the expected size of the PCR product was a 105-bp DNA fragment containing the region from 185 to 290 bp upstream of the transcriptional start site of the E-cadherin gene. Similar to the anti-POLR2A antibodies, we also detected a PCR product of 105-bp DNA fragment when using the anti-MAD1 antibodies (Fig. 4). This result indicated that the MAD1-associated transcription complex bound to the E-cadherin regulatory sequence.

The reduction of the E-cadherin protein level increases cell motility and is a hallmark symptom of metastatic cells (3,19-21). Because the depletion of MAD1 could result in a decrease in the protein level of E-cadherin in MCF-7 cells, we speculated that the loss of E-cadherin caused by the depletion of MAD1 could also enhance cell migration. We measured the cell migration ability of MCF-7 cells by the Transwell assay; MDA231 cells, which are highly metastatic breast cells, were used a positive control. We found that the MAD1-depleted MCF-7 cells had a similar migration ability as the MDA231 cells, indicating that MAD1 may regulate E-cadherin expression and thereby control cell migration (Fig. 5).

Discussion

Of the SAC component proteins, MAD1, BUB3 and CDC20 have been previously reported to interact with histone deacetylases and have been suggested to have transcriptional

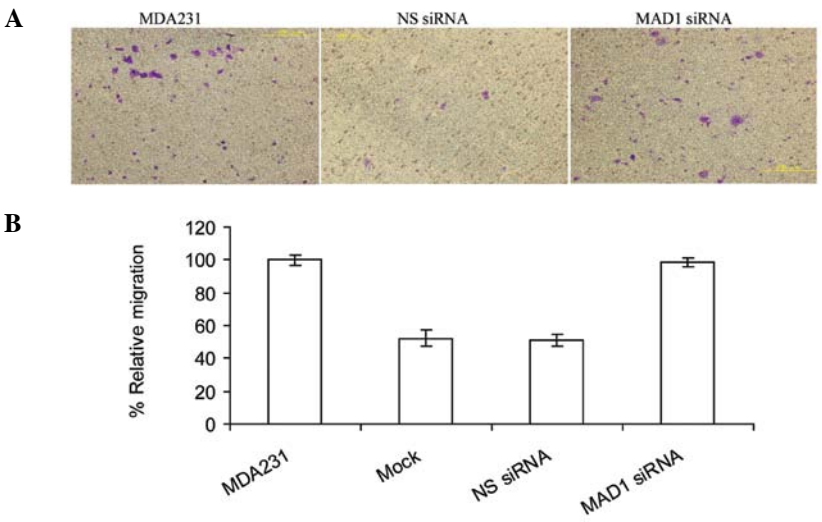

Figure 5. Reduced MAD1 levels enhance cell migration ability. (A) MCF-7 cells were mock-treated or treated with non-silencing siRNA (NS) or MAD1 siRNA duplexes for 24 h. The migration ability of mock-treated, non-silenced or MAD1-depleted MCF-7 cells was determined using the Transwell assay, as described in Material and methods. The cells were counted and analyzed under a microscope. (B) Quantification of migratory cells from (A). Highly metastatic MDA231 cells were used as a positive control. The migratory ability is presented as the relative percentage of migrating cells normalized to MDA231 cells.

repressor activity (17). Although this observation suggests the probability of multiple functions of mitotic checkpoint proteins other than the roles in spindle assembly checkpoint, the biological functions of these proteins in tumorigenesis have not been determined to date.

Here, we initially demonstrated that the SAC protein, MAD1, associates with the Pol IIA transcriptional initiation complex and thus confers transcriptional activity. It is worth noting that gastric carcinomas are commonly characterized by aneuploidy and that MAD1 is significantly down-regulated in over 50% gastric carcinoma patients, demonstrated using 2-D gel analysis (22). This observation suggests that MAD1 may directly regulate the expression of oncogenes or tumor suppressor genes and therefore affect cancer cell survival or migration ability. This implication is supported by our finding that MAD1 depletion in MCF-7 cells (breast cancer cells) promotes cell migration by down-regulating E-cadherin, a well-known tumor suppressor and a marker of the invasion of carcinoma cells (19).

A defect in SAC (spindle assembly checkpoint) is regarded as one of the major causes of aneuploidy in several cancers (23), and mutations or the dysregulated expression of spindle checkpoint proteins have also been widely observed in various tumor samples. MAD2 depletion has also been previously reported to enhance cell migration ability, but it is not clear how MAD2 exerts its effect on the migration ability of cells (24). Although the role of spindle checkpoint proteins in the cell prometaphase-metaphase transition is widely known, the direct mechanism of SAC proteins in tumorigenesis has been less well studied. Here, we demonstrate that the depletion of MAD1 may promote cancer progression by reducing the expression of E-cadherin and thus enhance cell migration ability. It will be interesting to identify whether other checkpoint proteins are also involved in tumorigenesis or cancer metastasis in future work.

Taken together, a decrease in MAD1 expression results in a lower level of E-cadherin expression, which in turn enhances cell migration ability. The detection of MAD1 expression may serve as an indication of early diagnosis in tumor metastasis; thus, exploring the underlying mechanism is another potential direction for the development of new therapeutic drugs.

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