Role of a Novel Splice Variant of Mitotic Arrest Deficient 1 (MAD1), MAD1β, in Mitotic Checkpoint Control in Liver Cancer

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Abstract

Loss of mitotic checkpoint contributes to chromosomal instability, leading to carcinogenesis. In this study, we identified a novel splicing variant of mitotic arrest deficient 1 (MAD1), designated MAD1β, and investigated its role in mitotic checkpoint control in hepatocellular carcinoma (HCC). The expression levels of human MAD1β were examined in hepatoma cell lines and human HCC samples. The functional roles of MAD1β in relation to the mitotic checkpoint control, chromosomal instability, and binding with MAD2 were assessed in hepatoma cell lines. On sequencing, MAD1β was found to have deletion of exon 4. It was expressed at both mRNA and protein levels in the nine hepatoma cell lines tested and was overexpressed in 12 of 50 (24%) human HCCs. MAD1β localized in the cytoplasm, whereas MAD1α was found in the nucleus. This cytoplasmic localization of MAD1β was due to the absence of a nuclear localization signal in MAD1α. In addition, MAD1β was found to physically interact with MAD2 and sequester it in the cytoplasm. Furthermore, expression of MAD1β induced mitotic checkpoint impairment, chromosome bridge formation, and aberrant chromosome numbers via binding with MAD2. Our data suggest that the novel splicing variant MAD1β may have functions different from those of MAD1α and may play opposing roles to MAD1α in mitotic checkpoint control in hepatocarcinogenesis. [Cancer Res 2008;68(22):9194–201]

Introduction

Chromosomal instability and DNA aneuploidy have been observed in almost all major types of human cancers including hepatocellular carcinoma (HCC; ref. 1). One of the causes for chromosomal instability is defective mitotic checkpoint control in cells. Accurate chromosome segregation depends on precise regulation of mitosis by the mitotic checkpoint, also known as spindle assembly checkpoint. This checkpoint monitors the status of kinetochore-microtubule attachment and delays the onset of anaphase until all kinetochores have formed stable bipolar connections to the mitotic spindle. Components of the mitotic checkpoint include mitotic arrest deficient (MAD) proteins MAD1, MAD2, and MAD3/BUBR1 (2); budding uninhibited by benzimidazole (BUB) proteins BUB1 and BUB3 (3); and other checkpoint proteins such as CDC20 and MPS1 (4, 5). When a single kinetochore is unattached, it is sufficient to generate the "wait" signal that prevents the transition of cells from metaphase to anaphase (6). When all kinetochores have been attached to the microtubules, this signal allows the onset of anaphase by ubiquitination of CDC20 and subsequent dissociation of checkpoint proteins from the APC/C^{cdc20} complex (7, 8). When the kinetochore is unattached to the microtubules, the MAD1-MAD2 complex localizes to the kinetochore; this can enhance the binding of MAD2 to CDC20 on the APC/C^{cdc20} complex (9), resulting in formation of a mitotic checkpoint complex consisting of BUBR1, CDC20, BUB3, and MAD2 (10–14).

This mitotic checkpoint complex is 3,000-fold more efficient than MAD2 alone in activating the mitotic checkpoint (10, 13, 15, 16). MAD1 is a key protein in this complex. Its NH_2-terminal coiled-coil domain is required for its kinetochore localization, and overexpression of the MAD1 mutant containing the MAD2-binding site but lacking the NH_2-terminal coiled-coil domain resulted in disruption of the mitotic checkpoint (17, 18). Depletion of MAD1 protein by RNA interference resulted in failure of MAD2 to localize to the kinetochore, impairment of the mitotic checkpoint, and aneuploidy (9, 19). MAD1 was reported to be inactivated by the Tax oncoprotein of the human T-cell leukemia virus type 1 (HTLV-1), and this resulted in multinucleated cells as seen in HTLV-1–transformed cells (18). In addition, haploinsufficiency of MAD1 in heterozygous knockout mice exhibited genomic instability with development of various neoplasms including lung carcinoma and HCC (20). MAD1 mutation is, however, uncommon in human cancers including leukemia, prostate and breast cancers, and glioblastoma (21–23).

In the present study, we have identified a novel MAD1 isoform, which we named MAD1β. Accordingly, the original MAD1 isoform was renamed MAD1α. The MAD1β isoform has deletion of exon 4 and is derived from alternative splicing of pre-mRNA. Intracellularly, MAD1α and MAD1β differentially localized to the nucleus and cytoplasm, respectively. Mechanistically, MAD1β was found to interact with MAD2, leading to retention in the cytoplasm. Enforced expression of MAD1β in a stable cell line abrogated mitotic checkpoint response and caused aneuploidy. Our work revealed another level of complexity in the regulation of mitotic checkpoint in mammalian cells. We proposed that MAD1α and MAD1β might serve opposing roles in mitotic checkpoint control in hepatocarcinogenesis.

Materials and Methods

Cell lines and clinical samples. Hepatoma cell lines, including HepG2, Hep3B, PLC/PRF/5, HLE, Huh7, BEL7402, and SMMC7721, and immortalized normal liver cell line, LO2, were maintained in DMEM high glucose (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). Other hepatoma cell lines, SNU182 and SNU449, were grown in RPMI 1640 (Life Technologies) supplemented with 1 mmol/L sodium pyruvate and 10% FBS. HEK293 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS. In addition, 50 pairs of human HCC and

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their corresponding nontumorous liver tissues from patients with HCC resected between 1992 and 2001 at Queen Mary Hospital were randomly selected for study. Among the 50 HCC patient samples, 40 (80%) were patients with chronic HBV infection. The patient tissue samples were collected according to approved research guidelines.

Plasmids. Full-length MAD1α cDNA was amplified from the pGEX-MAD1 plasmid (18) and subcloned into pEYFP-C1, Flag/pcDNA3.1$^+$, and Myc/pcDNA3.1$^+$ vectors. To obtain the full-length MAD1β cDNA, the NH_2 terminus of MAD1β (1–53 amino acids) was amplified from LO2 cDNA with replacement of the NH_2 terminus of MAD1α to generate full-length MAD1β/pEYFP-C1 plasmid. Full-length MAD1β cDNA was subcloned into Flag/pcDNA3.1$^+$ and Myc/pcDNA3.1$^+$ vectors, respectively. A series of MAD1 truncation mutants were made: exon 4 (51–97 amino acids of MAD1), MAD1ΔC1 (1–100 amino acids of MAD1), MAD1ΔC2 (1–137 amino acids of MAD1, with deletion of exon 4), MAD1ΔN1 (100–718 amino acids of MAD1), MAD1-Ex4mut (with change of three amino acids, from KRAR to LLAL at positions 79–82 of MAD1); they were subcloned into pEYFP-C1 and Flag/pcDNA3.1$^+$ vectors, respectively. In addition, MAD1-Ex4mut was also subcloned into Myc/pcDNA3.1$^+$ vector. MAD1β-Mut (MAD1β with deletion of 12 amino acids at the corresponding position between codons 540 and 551 of MAD1, a putative MAD2-binding domain; ref. 9) was synthesized and subcloned into Flag/pcDNA3.1$^+$ and Myc/pcDNA3.1$^+$ vectors, respectively. Full-length MAD2 cDNA was amplified from LO2 cDNA and subcloned into pEGFP-C3 and Flag/pcDNA3.1$^+$ vectors, respectively.

RNA extraction and semiquantitative reverse transcription-PCR. Total RNA was isolated from 50 pairs of HCC and their corresponding nontumorous liver tissues and from 9 hepatoma cell lines, using Trizol (Invitrogen) according to the manufacturer's protocol. PCR primers (5′-CACCATGGTTTTATCCACCC-3′ and 5′-GCATCCAAGTTCTGCTGACA-3′) were used to amplify MAD1 cDNA. β-Actin was used as a reference for the amount of cDNA added in PCR reaction.

Generation of MAD1β and MAD1α stably expressing cell lines. LO2 and SMMC7721 cell lines were transfected with Flag-tagged MAD1β, MAD1β-Mut, MAD1α, or Flag/pcDNA3.1$^+$ vector with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably expressing cells were selected with G418 at 800 μg/mL (for LO2 cells) or 400 μg/mL (for SMMC7721 cells) for 14 d. Those selected stably expressing clones were maintained in growth medium with G418 at 100 μg/mL.

Measurement of mitotic index. The detailed protocol was previously described (24). Mitotic indices were determined by evaluating the percentages of cells with chromosomal condensation per total number of viable cells at 24 h after treatment with nocodazole or colcemid. At least 300 cells were counted in each experiment and the experiment was done at least thrice.

Cell synchronization and DNA morphologic analysis in mitotic cells. Cells were enriched at mitotic phase with serum starvation overnight and incubation in culture medium containing 5 mmol/L thymidine for 16 h, followed by replacement with normal culture medium for 12 to 14 h. The cells were fixed according to a previously described protocol (24). The number of cells showing chromosome bridges was assessed in at least 200 mitotic cells in each cell line. The experiment was done at least twice.

Metaphase spread and chromosome counting. Cells were treated with 100 ng/mL colcemid for 4 h before harvest. Cells were then harvested by trypsinization, treated with 75 mmol/L of KCl at 37°C for 20 min, and fixed in ice-cooled fixative [methanol/acetic acid, 1:1 (v/v)]. A drop of fixed cells was allowed to fall from a height of around 20 cm onto glass slide. The air-dried slides were then submerged in Giemsa solution diluted with Milli-Q water, followed by brief washing once, and allowed to air-dry before microscopic observation. Chromosome counting was done on at least 40 cells for each cell line.

Coimmunoprecipitation assay and Western blot analysis. For general Western blot analysis, cells were lysed in NET (50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA) buffer with 1% NP40 and complete EDTA-free protease inhibitor cocktail (Roche) and separated in SDS-PAGE gel for Western blot analysis. Immunodetection was done using mouse anti-Flag (1:10,000; Sigma), mouse anti–β-actin (1:10,000; Sigma), mouse anti–α-tubulin (1:1,000; Sigma), rabbit anti-MAD1 (1:2,000; Bethyl Laboratories, Inc.), or mouse anti-MAD2 (1:1,000; Transduction Laboratories) antibodies. For coimmunoprecipitation assay, HEK293 cells were transfected with plasmids containing cDNA of Myc- and Flag-tagged fusion proteins, respectively. Cells were lysed in NET buffer supplemented with 0.1% Triton X-100 and protease inhibitor cocktail. Cell lysates were incubated with 1 μg of mouse anti-Myc (Santa Cruz Biotechnology, Inc.) antibody with addition of protein G-Sepharose. Unbound protein was washed away from the beads before electrophoresis. The mouse anti-Myc (1:1,000) and mouse anti-Flag (1:10,000) antibodies were used as primary antibodies for Western blot analysis.

Results

Identification of MAD1β and expression in hepatoma cell lines. To examine the mRNA expression pattern of MAD1 in hepatoma cells, we performed semiquantitative reverse transcription-PCR (RT-PCR) using a pair of primers flanking exon 3 and exon 5 of the gene, based on the complete cDNA sequence of MAD1 (GenBank no. AF123318), on nine hepatoma cell lines and the immortalized normal liver LO2 cell line. We observed that an additional PCR product of 249 bp in size was amplified in the same reaction in all of the nine hepatoma cell lines and LO2 (Fig. 1*A*). On sequencing, this PCR product corresponded to the MAD1 mRNA but without exon 4, likely to be a result of alternative splicing. Moreover, on Western blot analysis with anti-MAD1 antibody, two protein bands were detected in the hepatoma cell lines and LO2 cell line (Fig. 1*B*). Furthermore, in Western blot analysis with anti-MAD1 antibody, all the cell lines showed the presence of MAD1β at the protein level. Similar amounts of MAD1 protein were observed in these cell lines but MAD1β was present at various levels (Fig. 1*B*). We have named this novel isoform MAD1β and the original MAD1 isoform was renamed MAD1α.

Expression of MAD1β in human HCC samples. To investigate the abundance of both mRNA and protein of MAD1α and MAD1β in human HCC samples, we used the same primer set and antibody used on the cell lines for semiquantitative RT-PCR and Western blot analysis, respectively. Of the 50 human HCC samples, 12 (24%) showed expression of MAD1β mRNA in the tumor tissues but not in their corresponding nontumorous liver tissues; 6 cases had no detectable MAD1β mRNA in both tumors and nontumorous tissues; and the remaining cases showed similar expression levels of MAD1β in both tumors and nontumorous liver tissues (Fig. 1*C*). We attempted to perform quantitative PCR to further assess the mRNA expression levels, but no appropriate probes could be designed and obtained using various softwares including the Primer Express Software v2.0 (Applied Biosystems). In Western blot analysis, both MAD1α and MAD1β proteins were detected in the representative samples of both HCCs and their corresponding nontumorous liver tissues (Fig. 1*D*).

Subcellular localization of MAD1β in hepatoma cells. To investigate the subcellular localization of the two isoforms of MAD1 in hepatoma cells, the EYFP-tagged full-length MAD1α and MAD1β proteins were overexpressed in HLE cells. MAD1α localized predominantly in the nucleus, and MAD1β mainly in the cytoplasm. In addition, with a panel of truncation forms of EYFP-fused MAD1 proteins, we observed that whenever exon 4 was lacking, the mutant proteins localized in the cytoplasm. In contrast, truncated proteins encoded by exon 4 alone or by exon 4 and other exons localized in the nucleus. In particular, the MAD1-Ex4mut protein, in which a putative nuclear localization signal sequence (KRAR) located at 79 to 82 amino acids of MAD1α was mutated to LLAL, adopted a cytoplasmic localization (Fig. 2). These results

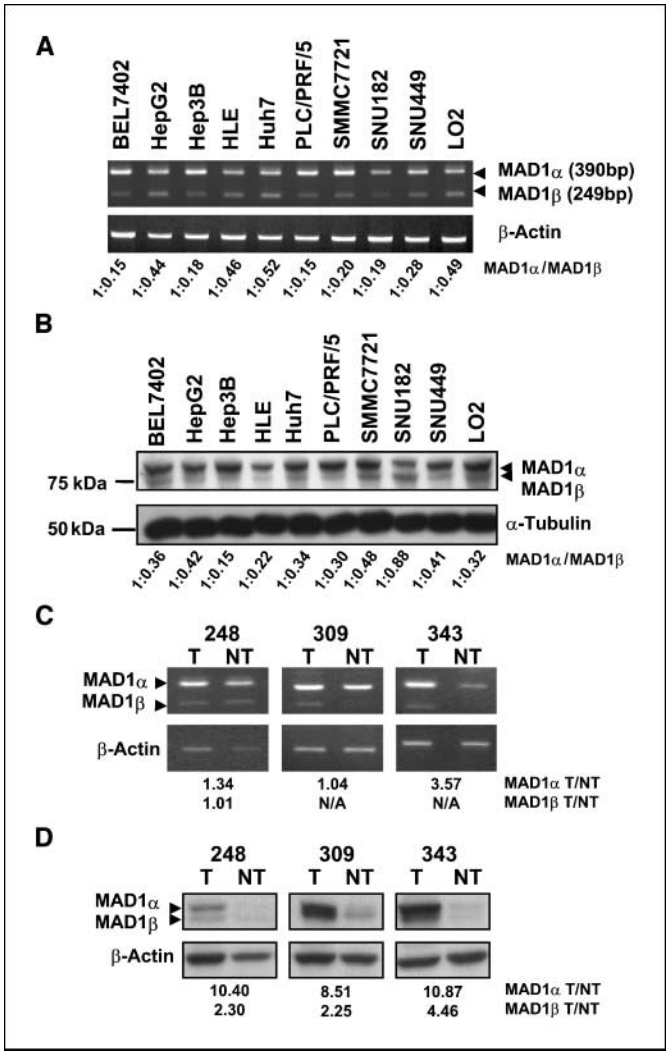

Figure 1. Identification of the novel MAD1 isoform MAD1β in hepatoma cell lines and human HCC samples. *A,* RT-PCR analysis of MAD1 in hepatoma cell lines showed the presence of MAD1β transcripts in all hepatoma cell lines screened. *B,* Western blot analysis with anti-MAD1 antibody detected two protein bands representing MAD1α and MAD1β in the hepatoma cell lines and LO2 cell line. The relative mRNA and protein levels of MAD1α and MAD1β in each hepatoma cell line are depicted in *A* and *B*. *C,* representative results of mRNA expression of MAD1 in HCC tumors (*T*) and their corresponding nontumorous liver tissues (*NT*). Case 248 showed the presence of both MAD1α and MAD1β in both tumor and nontumorous liver tissue, whereas cases 309 and 343 had MAD1β transcript in the tumors and only minimal amounts in their nontumorous liver tissues. *D,* results of protein expression of MAD1 in representative HCCs (*T*) and their corresponding nontumorous liver tissues (*NT*). The relative abundance of mRNA and protein levels of MAD1α and MAD1β in the tumors and their corresponding nontumorous livers is listed in *C* and *D*. *N/A,* data not available because MAD1β could not be detected in the samples.

suggest that exon 4 of MAD1α codes for a nuclear localization signal peptide, which is responsible for nuclear localization.

Ectopic expression of MAD1β led to mitotic checkpoint impairment. The role of MAD1β in mitotic checkpoint was investigated in the immortalized normal liver cell line LO2 and HCC cell line SMMC7721. These two cell lines had competent mitotic checkpoints, which we reported previously (24). The Flag-tagged MAD1β plasmid was stably transfected into these two cell lines and the level of overexpression was confirmed by Western blot analysis (Fig. 3*A*). Of the MAD1β stably expressing cell lines, both LO2 (LB24) and SMMC (SB18) clones showed a significant reduction of mitotic indices ranging from 19% to 63%, as compared with the corresponding vector control cells (Fig. 3*B*). This indicates a significant loss of mitotic checkpoint competence induced by MAD1β.

Ectopic expression of MAD1β led to aberrant chromosomal numbers. To assess the role of MAD1β in chromosomal instability, the chromosome numbers of individual cells in the MAD1β stably expressing LO2 and SMMC7721 cell lines and the parental cells were compared. The LO2 parental cells had chromosome numbers ranging between 46 and 64, whereas the vector control cells LV1

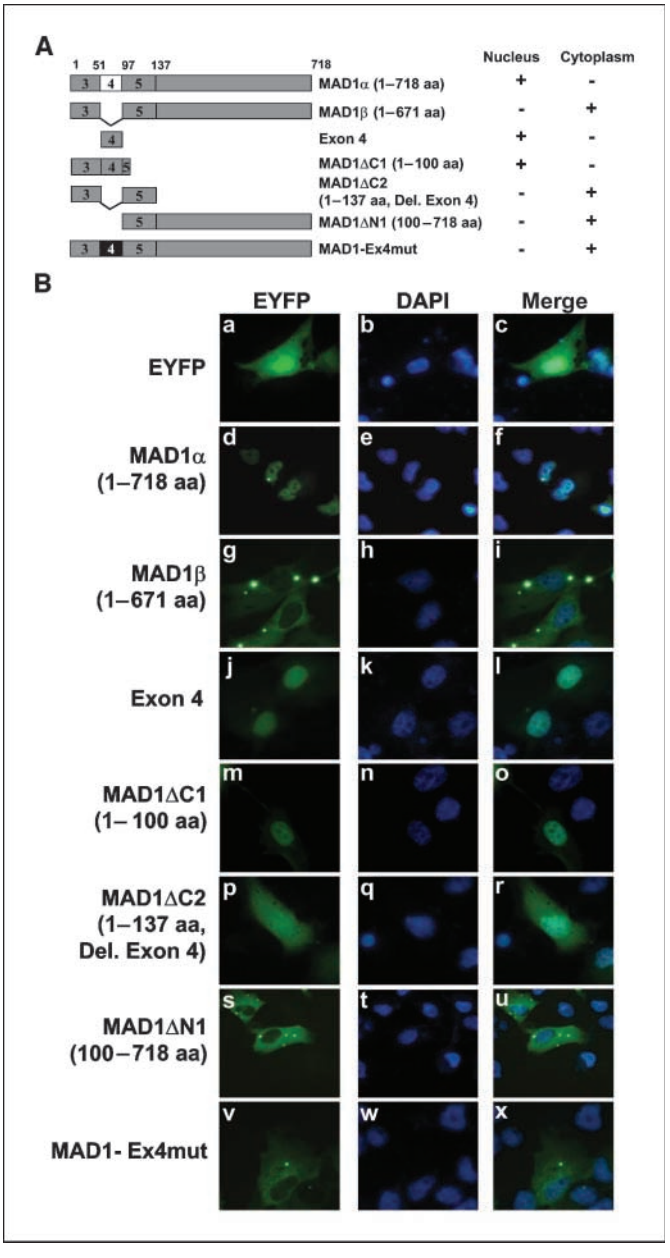

Figure 2. Subcellular localization of the different truncation and mutated forms of EYFP-tagged MAD1 proteins in HLE cells. *A,* schematic diagram showing the different constructs of MAD1 (full-length MAD1α, full-length MADβ, different truncated constructs of MAD1, and mutated forms of MAD1 cDNA) cloned into pEYFP-C1 vector. *aa,* amino acids. *B,* only the fusion proteins with wild-type exon 4 of the MAD1 cDNA [full-length MAD1 (*c*), exon 4 (*l*), and MAD1ΔC1 (*o*)] localized in the nucleus, whereas the other fusion proteins [full-length MAD1β (*i*), MAD1ΔC2 (*r*), MAD1ΔN1 (*u*), and MAD1-Ex4mut (*x*)] localized in the cytoplasm. *DAPI,* 4′,6-diamidino-2-phenylindole.

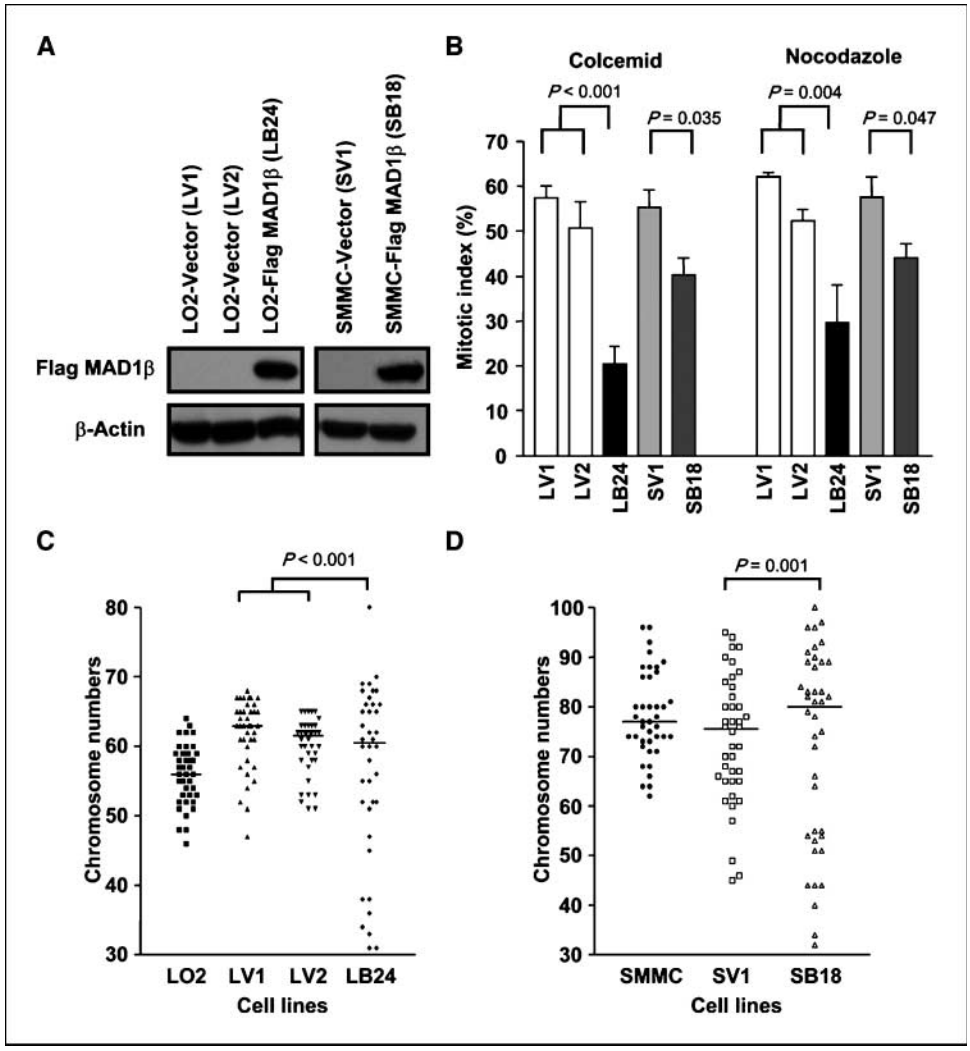

Figure 3. Characterization of MAD1β stably expressing LO2 and SMMC7721 hepatoma cells. *A,* the protein levels of exogenous Flag-tagged MAD1β protein in LO2 and SMMC7721 cell lines were determined by Western blot analysis with anti-Flag antibody. *B,* after treatment with either colcemid or nocodazole, the MAD1β stably expressing LO2 (*LB24*) and SMMC7721 (*SB18*) cell lines showed significantly lower mitotic indices as compared with the corresponding vector control cells of LO2 (*LV1* and *LV2*) and SMM7721 (*SV1*), respectively. *C* and *D,* chromosome numbers of the MAD1β stably expressing cells (*LB24* and *SB18*), corresponding vector control (*LV1*, *LV2*, and *SV1*), and parental LO2 (*C*) and SMMC7721 cell lines (*D*).

and LV2 had chromosome numbers ranging from 47 to 68. In contrast, the MAD1β-overexpressing LO2 cell line (LB24) had widely varying chromosome numbers between 31 and 80, as compared with the vector control ($P < 0.001$, Levene's test; Fig. 3*C*). Similarly, the parental and vector control of SMMC7721 cell lines had chromosome numbers mainly ranging from 62 to 96 and from 45 to 95, respectively, whereas the MAD1β-overexpressing SMMC7721 cell line (SB18) had widely varying chromosome numbers ranging between 32 and 100, as compared with the vector control ($P = 0.001$, Levene's test; Fig. 3*D*). The data indicate that there was a significant change of chromosome numbers suggestive of chromosomal instability in those MAD1β stably expressing cells.

MAD1β physically bound MAD2 and changed localization of MAD2 from nuclear to cytoplasmic. MAD1 is one of the binding partners of MAD2, and the interaction of MAD2 with MAD1 is crucial for MAD2 localization to the nucleus (25). To characterize the role MAD1β in mitotic checkpoint control, we generated a MAD1β mutant, named MAD1β-Mut, in which the putative MAD2-binding domain was deleted. As shown by *in vivo* coimmunoprecipitation assay, we observed that this MAD1β-Mut lost its MAD2-binding ability, as compared with the wild-type MAD1, MAD1β, and MAD1-Ex4mut proteins in which the MAD2-binding domain was intact (Fig. 4*A*). This indicates that MAD1β can interact with MAD2 via the MAD2-binding domain.

Moreover, overexpression of MAD1β altered the subcellular localization of exogenous MAD2 protein from nuclear to cytoplasmic, in contrast to the nuclear colocalization of MAD1 and MAD2 (Fig. 4*B, II* and *III*). However, overexpression of MAD1β-Mut, in which the putative MAD2-binding domain was deleted, could not alter the subcellular localization of exogenous MAD2 in cells, similar to when MAD2 alone was expressed (Fig. 4*B, I* and *IV*). On the other hand, overexpression of MAD1-Ex4mut, in which the putative nuclear localization signal domain was mutated but with intact MAD2-binding domain, changed the localization of MAD2 from nuclear to cytoplasmic (Fig. 4*B, V*). This indicates that MAD1β can interact with MAD2 via the MAD2-binding domain, which is critical in changing the subcellular localization of MAD2 and sequestering it in the cytoplasm.

MAD1β expression was associated with reduced MAD2 protein and induced mitotic checkpoint impairment and chromosomal instability via binding with MAD2. The Flag-tagged MAD1β and MAD1β-Mut with putative MAD2-binding domain deleted were stably expressed in LO2 cells, and respective clones were generated: MAD1β (clones 7, 17, and 20), MAD1β-Mut (clones 3, 6, and 13), and vector alone (clones 4 and 5). For the three

MAD1β-expressing LO2 cell lines, all exhibited significantly reduced mitotic indices, and the reduction ranged from 5% to 44% at 24 hours after drug treatments as compared with the vector control cells ($P < 0.001$ and $P = 0.001$, after nocodazole and colcemid, respectively; Fig. 5*A*). This reduction in mitotic indices indicates mitotic checkpoint incompet. In contrast, the three MAD1β-Mut–expressing cell lines (in which the putative MAD2-binding domain was deleted) and the two MAD1α-expressing cell lines had significantly less reduction in mitotic indices than did MAD1β-expressing cells after either nocodazole or colcemid treatment (Fig. 5*A*). Although there was some slight reduction in the MAD1α- and MAD1β-Mut–expressing cell lines, the mitotic indices were somewhat similar to those in the vector control cells. In addition, chromosome bridge formation was significantly more frequently observed in the MAD1β stably expressing cell line as compared with the MAD1β-Mut, MAD1α, or vector control LO2 cell lines (Fig. 5*B*; $P < 0.001$ or $P = 0.001$).

In the metaphase spreading analysis conducted with the established stably expressing cell lines, the MAD1β-expressing cell lines had widely ranging chromosome numbers, from 38 to 117, as compared with the vector control cell lines, with chromosome numbers ranging from 47 to 65 ($P < 0.001$, Levene's test; Fig. 5*C*). In

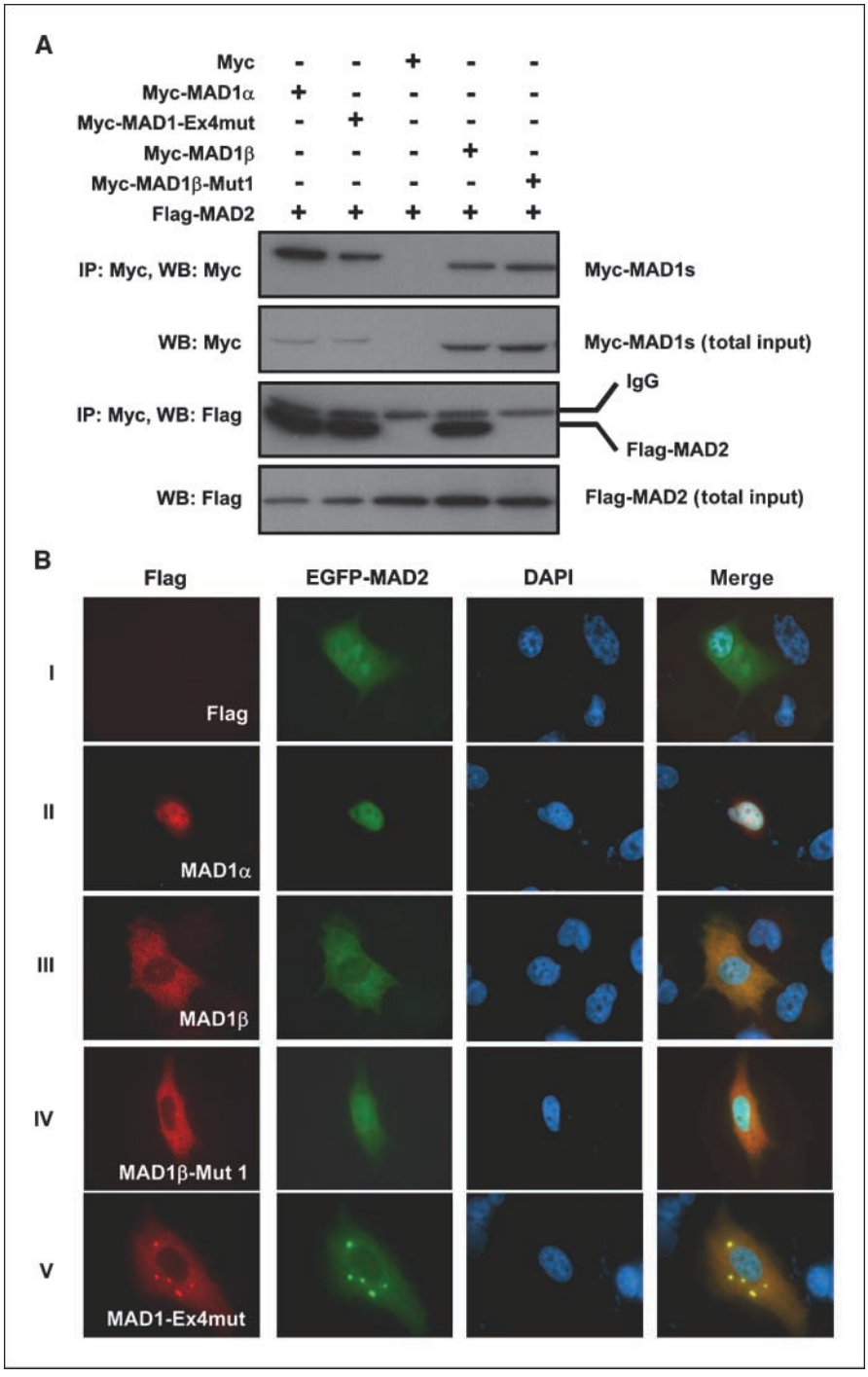

Figure 4. *A,* physical binding of Myc-tagged full-length MAD1α, MAD1β, and different mutants of MAD1α and MAD1β with Flag-tagged MAD2 protein was shown with coimmunoprecipitation assay. Among the different forms of MAD1 proteins, all but the MAD1β-Mut protein with deletion of the MAD2-binding domain coimmunoprecipitated with MAD2. *B,* alteration of subcellular localization of exogenous EGFP-tagged MAD2 on coexpression with Flag-tagged full-length and different mutated forms of MAD1α and MAD1β in HLE cells.

contrast, the MAD1β-Mut–expressing cells lost this wide range of chromosome numbers (from 41 to 73) as compared with the MAD1β-expressing cells ($P < 0.001$, Levene's test). Moreover, MAD1α-expressing cells had a relatively narrow range of chromosome numbers (from 41 to 93) as compared with MAD1β-expressing cells ($P = 0.007$, Levene's test). To gain further insight on the mechanism that induces the chromosomal instability, we examined the MAD2 protein level in these stable clones. Interestingly, with Western blot analysis, the MAD1β-expressing cell lines exhibited a significant reduction of the endogenous MAD2 protein level as compared with the vector control. In contrast, there was no reduction in the endogenous protein level of MAD2 in both the MAD1β-Mut and MAD1α stably expressing cells (Fig. 5*D*). The result suggests that expression of MAD1β was associated with significant reduction in steady-state amount of intracellular MAD2. Overall, our data suggest that MAD1β induces mitotic checkpoint impairment and chromosomal instability.

Discussion

In mammalian cells, the MAD1-MAD2 protein complex localizes in the nucleus, binds to the unattached kinetochores, and activates the mitotic spindle checkpoint, preventing cells from undergoing improper chromosomal segregation (18, 26). In this study, we have identified a novel isoform of MAD1, which we named MAD1β. This isoform lacks the exon 4 of MAD1α and its mRNA transcript was ubiquitously expressed in all hepatoma cell lines tested. The MAD1β isoform localized primarily in the cytoplasm instead of the nucleus. In addition, when we mutated a putative nuclear localization signal sequence within exon 4 of MAD1α from KRAR to LLAL (MAD1-Ex4mut), MAD1α was relocalized from the nucleus to the cytoplasm. This suggests that the nuclear localization signal sequence is essential for the nuclear localization of MAD1α protein. Interestingly, a previous report suggested that the first 320 amino acids of the MAD1 protein consisted of a kinetochore binding domain (27). It remains to be determined if this kinetochore binding domain of MAD1 is also absent in MAD1β, which lacks the 51 to 97 amino acids.

We further characterized the functional role of MAD1β in mitotic checkpoint control by stably overexpressing it in two mitotic checkpoint competent cell lines, LO2 and SMMC7721 (24). We observed significantly lower mitotic indices and severe chromosome aberrations in MAD1β-overexpressing LO2 and SMMC7721 cell lines. These results indicate that MAD1β induces mitotic checkpoint incompetence and chromosomal instability.

Importantly, we showed that MAD1β physically bound to MAD2 and this interaction was associated with reduced endogenous MAD2 protein level in cells. Using a MAD2-binding domain deletion mutant of MAD1β, we showed that the interaction between MAD1β and MAD2 is important for the MAD1β-induced mitotic checkpoint incompetence, formation of chromosomal bridges, and chromosomal instability. Moreover, overexpression of MAD1β changed the nuclear subcellular localization of exogenous MAD2 protein by retaining the MAD2 protein in the cytoplasm. This was in contrast to the previous finding that MAD1α brought exogenous MAD2 into the nucleus (25). Although enforced expression of MAD1α in LO2 cell line resulted in a slight reduction of the mitotic index and induction of chromosome aberration, it did not cause a reduction in the level of the endogenous MAD2 protein in cells. Overall, our results indicate that MAD1β physically binds to MAD2 and changes the nuclear localization of MAD2 to cytoplasm. Thus, MAD1α and MAD1β may have different, or even opposing, functions in cells.

Furthermore, our data have shown that MAD1β reduced the total MAD2 protein in cells, and this might in turn lead to mitotic checkpoint inactivation. Reduction of MAD2 protein expression was associated with mitotic checkpoint incompetence in HCC and other cancer cell lines (24, 28, 29). This reduction of MAD2 protein by MAD1β overexpression is intriguing and worth pursuing. We observed that this reduction in MAD2 protein level was not due to alteration in MAD2 transcription because the MAD2 mRNA levels in the MAD1β-overexpressing and vector control cells remained similar (data not shown). Another possible explanation for the reduction in MAD2 protein level was posttranslational modification. The physical interaction between MAD1β and MAD2 may lead to instability of the MAD2 protein in cells. MAD1 and MAD2 have been shown to localize at nuclear pore complexes at the interphase of mammalian cells (30) and at unattached kinetochores during prometaphase. One of the possible mechanisms for MAD2 degradation may be mislocalization from the nucleus to cytoplasm by the MAD1β protein, and this may trigger MAD2 degradation. Using a proteasome inhibitor, MG132, to assess proteosome-dependent degradation in MAD1β stably expressing cells, our preliminary result suggests that this proteosome-dependent degradation may not affect the MAD2 protein level (data not shown). Thus far, the exact mechanism of MAD2 stability in the cell is largely unknown and further investigation is needed. On the other hand, it has also been reported that overexpression of other putative spindle checkpoint silencers such as CMT2 and FAT10 could induce inactivation of the mitotic checkpoint in mammalian cells. These silencers can bind to MAD2 and lead to mitotic checkpoint inactivation (31, 32). Therefore, it is intriguing that MAD1β may serve as a negative regulator, similar to CMT2, in the mitotic spindle checkpoint activation, and further studies are required.

MAD1β mRNA was expressed in all of the nine hepatoma cell lines tested. In the human HCC samples, 24% of the cases showed overexpression of MAD1β mRNA in the tumor tissues as compared with their corresponding nontumorous tissues. The reason for this overexpression of MAD1β in this portion of human HCC is not clear. Moreover, more than 50% of human HCC cases showed MAD1β mRNA expression in both tumors and nontumorous tissues. This indicated that MAD1β itself might have a function in normal cells and this needs to be further investigated. Overexpression of MAD1 mRNA and protein has been reported in human HCC as well as breast cancer (33). The expression of MAD1 can be activated by gain-of-function p53 mutant (p53-281G), induction of cellular proliferation (34, 35), and gain of chromosome region 7p22.3, as in small-cell lung cancer (36). On the other hand, partial down-regulation of MAD1 protein can result in spindle checkpoint inactivation, aneuploidy in cells, and enhanced tumor formation in MAD1 heterozygous knockout mice (19, 20). Accumulation of genetic alternations along the multistep process of hepatocarcinogenesis has been shown in a recent study (37); however, whether overexpression of MAD1β is an early or a late event and its importance in hepatocarcinogenesis are still largely unknown. In a recent study, ectopic expression of hepatitis B virus X protein in hepatoma cell line resulted in alteration of another mitotic checkpoint protein, MAD3/BUBR1, which then led to mitotic checkpoint impairment and chromosomal instability (38).

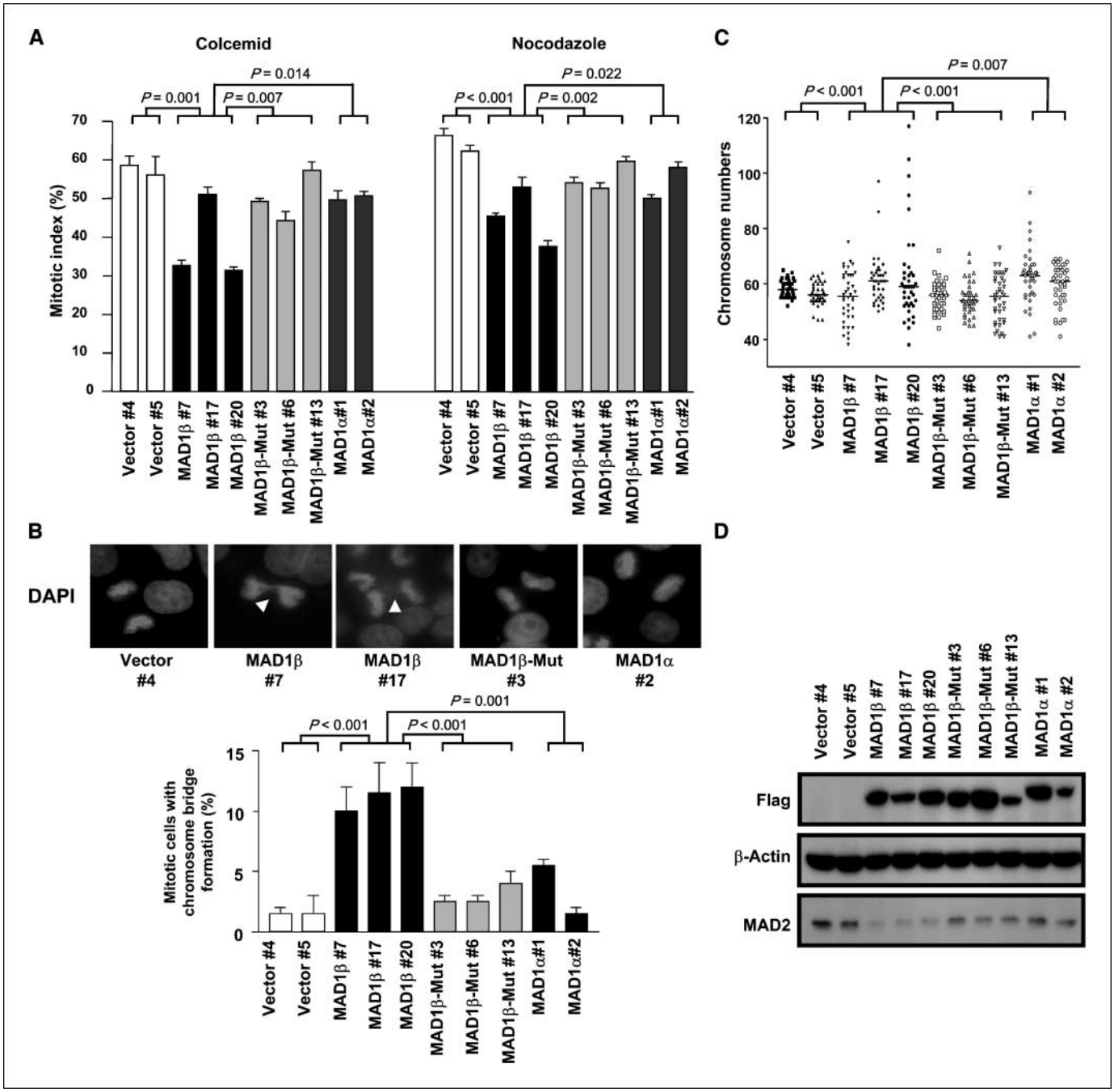

Figure 5. *A,* the mitotic indices of LO2 cells overexpressing Flag-tagged MAD1β were reduced after treatment with colcemid or nocodazole as compared with the vector control cells. *B,* in addition, 10% to 12% of mitotic cells in MAD1β stably expressing cells exhibited chromosome bridge formation (*white arrowheads*) during mitosis. Chromosome bridge formation was more frequent in the MAD1β stably expressing cells as compared with MAD1β-Mut ($P < 0.001$), MAD1α ($P = 0.001$), or vector control LO2 cell lines ($P < 0.001$). *C,* with metaphase spreading analysis, MAD1β stably expressing cell lines had chromosome numbers ranging widely between 38 and 117 as compared with MAD1β-Mut, MAD1α, and vector control cells. *D,* Western blot analysis showed significant reduction of endogenous MAD2 protein level in MAD1β stably expressing LO2 cells as compared with the vector control. This reduction of endogenous MAD2 protein level was not seen in both MAD1β-Mut– and MAD1α-expressing cell lines.

Therefore, overexpression of MAD1β might be one of many pathways that result in genetic alterations in the progression of hepatocarcinogenesis. Taken together, these results suggest that steady amounts of MAD1 and other mitotic checkpoint proteins are important for spindle checkpoint control in cells.

Although the role of MAD1β in mitotic checkpoint has been shown in this study, other queries on MAD1β remain to be clarified. MAD1β mRNA was overexpressed in nearly all of our tumorous tissues and hepatoma cell lines tested but was absent in some of nontumorous liver tissues tested. MAD1β is derived from exon skipping during splicing of pre-mRNA. Transcription of MAD1α and MAD1β is driven by the same promoter. Mutations in the splice recognition sites governing the splicing or alterations in *trans*-acting splicing regulators of some genes have been found

in various cancers (39). These findings indicate that alteration of alternative splicing mechanism in normal cells may lead to alteration of protein expression patterns and, thus, alteration of the normal cell physiology. However, other transcriptional and posttranslational modifications or degradation may also be involved in the regulation of MAD1β protein level in cells.

Overall, our data suggest that a novel splicing variant of MAD1, MAD1β, serves different functions from those of MAD1α and may play opposing roles to MAD1 in mitotic checkpoint control in hepatocarcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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