ABSTRACT

MAD2 is localized to kinetochores of unaligned chromosomes, where it inactivates the anaphase-promoting complex/cyclosome, thus contributing to the production of a diffusible anaphase inhibitory signal. Disruption of MAD2 expression leads to defects in the mitotic checkpoint, chromosome missegregation, and tumorigenesis. However, the mechanism by which deregulation and/or abnormality of hsMAD2 expression remains to be elucidated. Here, we clone and analyze a ~0.5 kb fragment upstream of hsMAD2 and show that this fragment acts as a strong promoter. Transcriptional dysfunction of hsMAD2 is frequently observed in hepatocellular carcinoma cells, and down-regulation of hsMAD2 protein expression is correlated with transcriptional silencing of the hsMAD2 promoter by hypermethylation. These results imply a relationship between transcriptional abnormality of this mitotic checkpoint gene and mitotic abnormality in human cancers.

INTRODUCTION

The mitotic checkpoint is a highly conserved mechanism that regulates cell division and prevents cells with a perturbed spindle assembly from leaving mitosis, thereby improving the fidelity of chromosome segregation (1–2). Mitotic checkpoint proteins, such as BUB1, BUB3, BUBR1/MAD3, MAD1, and MAD2, are preferentially localized to kinetochores of unaligned chromosomes and contribute to the production of a diffusible anaphase-inhibitory signal. This signal delays anaphase by inhibiting the activity of anaphase-promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase required for the degradation of securin and subsequent activation of separase, which is necessary for separation of sister chromatids (3, 4). Defects in the mitotic checkpoint may contribute to the chromosomal instability (CIN) observed in human cancers (5–7). In support of this hypothesis, the deletion or inactivation of mitotic checkpoint proteins results in loss of checkpoint control, premature anaphase, and subsequent chromosomal instability (8–14). Interestingly, the expression of a dominant-negative mutant of BUB1 not only augments the formation of aneuploidy but also compromises apoptotic cell death in response to spindle damage (9). However, molecular analyses have revealed a rarity of genetic alterations of mitotic checkpoint proteins, despite commonly observed defects in the mitotic checkpoint in these cancers (6, 15–20).

Several lines of evidence have established that MAD2 is recruited to kinetochores via interactions with MAD1 and binds CDC20 to inhibit APC/C activity (21–23). The data suggest that MAD2 is essential in that it acts as a negative regulator of APC/C, most likely through inhibition of the production of a diffusible anaphase-inhibitory signal. This signal regulates cell division and prevents cells with a perturbed spindle assembly from leaving mitosis, thereby improving the fidelity of chromosome segregation (1–2). Mitotic checkpoint proteins, such as BUB1, BUB3, BUBR1/MAD3, MAD1, and MAD2, are preferentially localized to kinetochores of unaligned chromosomes and contribute to the production of a diffusible anaphase-inhibitory signal. This signal delays anaphase by inhibiting the activity of anaphase-promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase required for the degradation of securin and subsequent activation of separase, which is necessary for separation of sister chromatids (3, 4). Defects in the mitotic checkpoint may contribute to the chromosomal instability (CIN) observed in human cancers (5–7). In support of this hypothesis, the deletion or inactivation of mitotic checkpoint proteins results in loss of checkpoint control, premature anaphase, and subsequent chromosomal instability (8–14). Interestingly, the expression of a dominant-negative mutant of BUB1 not only augments the formation of aneuploidy but also compromises apoptotic cell death in response to spindle damage (9). However, molecular analyses have revealed a rarity of genetic alterations of mitotic checkpoint proteins, despite commonly observed defects in the mitotic checkpoint in these cancers (6, 15–20).

These observations indicate that transcriptional failure or promoter dysfunction may be responsible for changes in checkpoint protein levels and thus induce mitotic checkpoint dysfunction and chromosomal instability. Interestingly, hepatocellular carcinoma (HCC) cell lines display high frequencies of impaired mitotic checkpoints, chromosomal instability, and aneuploidy (16, 31, 32). However, alterations in mitotic checkpoint genes are not associated with HCC tumorigenesis (16), implying that other mechanisms, such as transcriptional failure or promoter methylation, may contribute to impairment of the mitotic checkpoint. The mechanisms of deregulation and/or abnormality of MAD2 expression during the development of CIN and tumorigenesis remain to be elucidated. Accordingly, we cloned the hsMAD2 promoter and investigated its activity in cancerous cell lines.

Here, we describe cloning and the characterization of the hsMAD2 promoter. Our data additionally reveal frequent transcriptional dysfunction of the hsMAD2 gene in HCC cell lines, suggesting that down-regulation of hsMAD2 mRNA expression is correlated with silencing of the promoter by hypermethylation.

MATERIALS AND METHODS

Plasmids, siRNA, and Transfection. We amplified a fragment (~2.6 kb) containing the 5′ flanking region upstream of the translation initiation start site (ATG) of hsMAD2 from 293 cell genomic DNA using the following primer pairs: 5′-CCAGATAGGTATTCGATGTTTCTCTAAAGTTG-3′ and 5′-CAGCTCGAGGCTCCACGCAAGACGAGCGCTTCAA-3′. The amplified product was digested with HindIII and XhoI and cloned into the pXP2 vector. To identify the minimal promoter region, we cloned promoter deletion constructs into pXP2 using the following restriction enzyme sites: HindIII/ApaI (~2524−552), ApaI/BglII (~552+156), HindIII/SpeI (~2524−195), and SpeI/BglII (~195+116). The ApaI/BglII clone was amplified as model, deletion of one MAD2 allele in mice resulted in defective mitotic checkpoint, premature separation of sister chromatids, and elevated rates of chromosome missegregation, even in the absence of spindle inhibitors (14). Interestingly, recent studies on the underlying mechanistic connections between genetic alteration of the hsMAD2 gene and impaired mitotic checkpoints in human cancers have not identified hsMAD2 mutations in human cancer cell lines, even those with impaired mitotic checkpoints (15, 16). However, a number of reports show that reduced hsMAD2 protein expression is correlated with the loss of checkpoint control in human cancers (24, 25). The results imply that transcriptional or post-transcriptional regulation of expression is a possible link for epigenetic alteration of hsMAD2 in human cancers.

Under both normal and pathologic conditions, genes may be inactivated through transcriptional silencing or depressed transcriptional activity. Recent studies show associations between human cancers and promoter hypermethylation (gene silencing), transcription factor dysfunction, and promoter mutation (26–29). Although impairment of the mitotic checkpoint is frequently associated with tumorigenesis, mutational inactivation of MAD and BUB family members is only observed in a small proportion of human cancers (6, 15–17, 19). Transcripts of these genes are maintained at different levels in human cancer cells, compared with neighboring normal cells (24, 25, 30). These observations indicate that transcriptional failure or promoter dysfunction may be responsible for changes in checkpoint protein levels and thus induce mitotic checkpoint dysfunction and chromosomal instability. Interestingly, hepatocellular carcinoma (HCC) cell lines display high frequencies of impaired mitotic checkpoints, chromosomal instability, and aneuploidy (16, 31, 32). However, alterations in mitotic checkpoint genes are not associated with HCC tumorigenesis (16), implying that other mechanisms, such as transcriptional failure or promoter methylation, may contribute to impairment of the mitotic checkpoint. The mechanisms of deregulation and/or abnormality of MAD2 expression during the development of CIN and tumorigenesis remain to be elucidated. Accordingly, we cloned the hsMAD2 promoter and investigated its activity in cancerous cell lines.

Here, we describe cloning and the characterization of the hsMAD2 promoter. Our data additionally reveal frequent transcriptional dysfunction of the hsMAD2 gene in HCC cell lines, suggesting that down-regulation of hsMAD2 mRNA expression is correlated with silencing of the promoter by hypermethylation.
four fragments (−552/−396, −395/−153, −152/−17, and −16/+116), which were separately inserted into pXp2. To suppress hsMAD2 expression using the RNA interference technique, we obtained the oligonucleotide sequence of hsMAD2 siRNA, 5′-GGAAGAGAUGUCGGACACAG-3′ (nucleotides 501–519), from Dharmaco RNA Technologies (Chicago, IL). Transfection of siRNAs into HCC cell lines was done by electroporation (Bio-Rad, Hercules, CA).

5′RACE Identification of Transcription Start Sites. We used the SMART RACE (5′- and 3′-rapid amplification of cDNA ends) cDNA amplification kit (BD Clontech, Palo Alto, CA) to identify transcription start sites of hsMAD2. First-strand cDNA was synthesized from human placenta total RNA, and 5′RACE was done with universal primer A and a gene-specific primer designed from the 3′ end of the hsMAD2 cDNA sequence (5′-TCAGTCTATTGACAGGAATTGGTAGGCCACC-3′). The subsequent PCR step was done with nested universal primer A and a nested gene-specific primer (5′-TGAACGAAGGCGCCTCTCCTCAGAATTGG-3′). Amplified DNA products were cloned into the pGEM-T easy vector (Promega, Madison, WI), and 20 clones were sequenced.

Luciferase Assay and Immunoblot Analysis. For the luciferase assay, HeLa cells were calcium phosphate-transfected with 9 μg of promoter construct DNA and were harvested 48 hours later with report lysis buffer (Promega). All transfections were normalized against an internal control (pcMV-β-gal). Cell debris was removed, and extracts were assayed for luciferase and β-galactosidase (β-gal) activity. For immunoblot analysis, cells were synchronized and transfected as above, harvested by scraping, washed twice in cold PBS, and lysed in lysis buffer (50 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.1% NP40, 1 mM DTT, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 50 μg/mL phenylmethylsulfonyl fluoride). Equal amounts of protein (quantitated using the Bio-Rad assay) from each sample were subjected to SDS-PAGE, transferred to nitrocellulose filters, blocked, and analyzed with anti-MAD2 (BD Transduction Lab), anti-Cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY), and anti-Actin (Sigma, St. Louis, MO) antibodies.

Establishment of Stable Cell Lines. For the generation of cell lines stably expressing the luciferase gene driven by the hsMAD2 promoter, a Zeocin-resistance gene was amplified from pVgRXX (Invitrogen, Carlsbad, CA) and inserted into the pXp2-hsMAD2 (−2524/+116) construct. HeLa cells were transfected with the hsMAD2 promoter-driven luciferase plasmid, and selected with Zeocin (400 μg/mL). The luciferase assay was used to isolate and screen 30 individual clones.

Synchronization and Flow Cytometry Analysis. To generate synchronized populations, the stable cell lines generated above were treated with 2 mM/mL thymidine for 12 hours, released, and blocked with 400 μM/mL mimosine for G2, 2 mM/mL thymidine for S, or 50 ng/mL nocodazole for the M phase. For synchronization at G2, cells arrested at S phase were released for 6 hours. Synchronized cell populations were harvested, fixed, and stained with propidium iodide for flow cytometry analysis. Samples of 10,000 cells were analyzed for DNA content on a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA). For evaluation of the mitotic index, cells were harvested, resuspended in Cytofix/Cytoperm solution (BD Pharmingen San Diego, CA), washed with Perm/Wash buffer (BD Pharmingen), and stained with a MPM2 mouse antibody (20 μg/mL; 1:250 dilution; DAKO, Carpinteria, CA) and FITC-labeled goat antimouse antibody (1 μg/mL; 1:100 dilution; IgG1; 1:100 dilution; 20 μg/mL), and subjected to flow cytometry analysis on FACscan, according to the manufacturer’s instructions. Data were presented with Cell Quest software (Becton Dickinson).

Quantitative Real-Time PCR of hsMAD2 mRNA. The RNaseaP luciferase extraction kit (Qiagen, Valencia, CA) was used to extract total RNA. A total of 1 μg of RNA was reverse transcribed and used to generate first-strand cDNA (First Strand cDNA kit, Roche, Indianapolis, IN). After quantification by fluorometry, 5 ng of cDNA was subjected to 40 cycles of real-time PCR in a LightCycler (Roche), according to the manufacturer’s instructions. Intron-spanning cDNA primers for the reference gene, GAPDH, were used to confirm adequate cDNA normalization. The primers used for hsMAD2 mRNA detection were 5′-TCTCAGAGAAGCTATCCAGGTGAA-3′ and 5′-CCAACAGTGGCAGAATTGTCA-3′.

RESULTS

Identification of the hsMAD2 Promoter and Its cis-Acting Transcriptional Elements. To investigate whether transcriptional dysfunction of MAD2 contributes to mitotic checkpoint impairment and subsequent tumor development, we identified hsMAD2 promoter sequences via a BLAST search of the human genome against the cDNA sequence of hsMAD2. A 26 Kb fragment containing the hsMAD2 5′ flanking region, upstream of the translation initiation start site (ATG) was identified. The fragment was amplified from 293 cell genomic DNA. To determine whether this fragment conferred the expected promoter activity, it was fused to luciferase cDNA, and the resulting reporter was transiently transfected (−2524 to +116, Fig. 1A) into HeLa cells. The hsMAD2 promoter conferred 225-fold higher luciferase activity, compared with background activity from the promoterless pT81 luciferase backbone vector. To further define the minimal sequences required for transcriptional initiation of hsMAD2 expression, several promoter mutants were generated by progressive deletion of nucleotides from the 3′ and/or 5′ ends. As indicated in Fig. 1B, we have identified that the minimal hsMAD2 promoter region is −395 to +116 (GenBank accession no. YA456198). Additionally, potential cis-acting regulatory elements within the optimal hsMAD2 promoter region were identified using the MatInspector V2.2 and GEMS Launcher 3.2 computer programs (http://www.genomix.de), which revealed multiple possible transcription factor binding sites, including activator protein, E2F, CCAAT, CCAAT/enhancer-binding protein, GBox, Camp response element-binding protein, nuclear factor κB, and octamer factor 1 (Fig. 1C). Analysis of the region surrounding the transcriptional start site failed to reveal a TATA box. The transcription start site was identified by 5′RACE analysis of hsMAD2 mRNA (data not shown). Next, we generated six deletion mutants of the hsMAD2 promoter, focusing on potential cis-acting transcriptional elements. As shown in Fig. 1D, disruption of the regions −90 to −69 containing E2F and octamer factor 1 motifs slightly reduced basal promoter activity. However, deletions of other transcriptional elements showed no substantial changes in their transcriptional activity, suggesting that multiple factors may be involved in the maximal activation of hsMAD2 transcription. Unexpectedly, truncation of the promoter region −59 to −26 drastically increased hsMAD2 promoter activity, indicating that this region may act as a transcriptional repression element. Thus, we isolated the hsMAD2 promoter with strong transcriptional activation activity, and we identified the minimal promoter region from −395 to +116.

Periodic Regulation of hsMAD2 Expression During the Cell Cycle. To investigate the timing of hsMAD2 expression during the cell cycle, we stably transfected HeLa cells with a luciferase gene under control of a −0.6 Kb hsMAD2 promoter fragment (−552 to +116). Eleven independent clones were established. Data from one clone, HeLa-hsMAD2-Luc, are described in this report (Fig. 2A). Populations of HeLa-hsMAD2-Luc cells were synchronized, and the cell cycle phases were confirmed by flow cytometry (Fig. 2B). Thereafter, identical sets of synchronized HeLa-hsMAD2-Luc cells were harvested for the luciferase reporter gene assay. Interestingly, luciferase activity driven by the hsMAD2 promoter was significantly decreased in cells arrested at the S phase (Fig. 2C). Reporter gene activity was recovered in G2-M populations. These results indicate periodical expression of hsMAD2 mRNA throughout the cell cycle. To further confirm the potential correlation between promoter activity and levels of endogenous hsMAD2 mRNA and protein, we compared hsMAD2 expression in synchronized HeLa-hsMAD2-Luc cells with that of cyclin A and B. Previous data indicate that cyclin A and B are induced at the S and G2 phases, respectively, and are degraded during mitosis by activated APC/C (33–36). Consistent with previous re-
ports, levels of cyclin A and B were high in synchronized HeLa-hsMAD2-Luc cells arrested at the G2-M and M phases, respectively (Fig. 2D). Under these conditions, the levels of hsMAD2 protein were increased over G2-M phases. However, it is likely that enhanced hsMAD2 expression at the M phase results from post-translational modifications, such as phosphorylation, rather than transcriptional regulation. A recent observation supports the hypothesis that MAD2 is phosphorylated in a cell cycle-dependent manner, and the phosphorylation state influences its stability during mitosis (37). It is additionally possible that the complex formation of MAD2 with MAD1 and CDC20 or multimerization of MAD2 increases the stability of the protein at the M phase (23).

Transcriptional Down-regulation of hsMAD2 Expression in Primary Hepatocellular Carcinoma Cells. In view of the observation that expression levels of hsMAD2 were maximum when cells enter the mitosis stage (Fig. 2D), we hypothesized that aberrant expression of hsMAD2 mRNA and protein are correlated with deregulation of the mitotic checkpoint and subsequent HCC tumorigenesis. To examine this possibility, we monitored the levels of hsMAD2 mRNA in primary HCC cell lines with real-time PCR. As shown in Fig. 3A, 5 of 10 primary HCC cell lines displayed significantly reduced hsMAD2 mRNA, ranging from 25 to 40% (SNU-368, -761, and -886) and from 45 to 55% (SNU-354 and -475), compared with levels from Huh7. Furthermore, a comparison of hsMAD2 mRNA and...
Actin antibodies analysis with anti-hsMAD2, anti-Cyclin A, anti-Cyclin B, and anti-synchronized cells were harvested and subjected to immunoblotting. Asynchronized and synchronized cell populations were harvested for flow cytometry (B), luciferase assay (C) and immunoblotting (D). Harvested cells were fixed and stained with propidium iodide, and samples of 10,000 cells were analyzed for DNA content on a Becton Dickinson FACSscan (B). Luciferase activity driven by the hsMAD2 promoter was analyzed in comparison with cell cycle profiles. The data reflect values from two sets of samples (C). Asynchronized and synchronized cells were harvested and subjected to immunoblotting analysis with anti-hsMAD2, anti-Cyclin A, anti-Cyclin B, and anti-Actin antibodies (D).

The corresponding protein levels revealed that HCC cell lines (SNU-354, -368, -475, -761, and -886) displayed reduced protein levels, compared with SNU-449, -739, and Huh7, in which expression was consistent (Fig. 3B). The results imply that transcriptional dysfunction of hsMAD2 is directly linked with protein expression.

hsMAD2 Expression Is Correlated with Competence of the Mitotic Checkpoint Response to Spindle Damage in HCC Cells. Inhibition of hsMAD2 expression is attributed to mitotic checkpoint defects, and it results in aberrant exit from prolonged mitotic arrest in response to checkpoint activation (24, 25). To determine whether aberrantly reduced expression of the hsMAD2 protein is associated with defects in the mitotic checkpoint response in HCC cells, we monitored the levels of histone H3 phosphorylation and accumulation of the MPM2-positive population as indicators of the mitotic phase of the cell cycle (Fig. 4). As expected, these mitotic indices activated by nocodazole treatment were dramatically reduced in all HCC cell lines transfected with hsMAD2-specific siRNA duplexes, regardless of endogenous hsMAD2 levels, in response to spindle damage. However, a comparison with MPM2-positive cells subjected to nocodazole treatment for 12 hours discloses that the sensitivity of mitotic indices is more pronounced, partly in cells with abnormally reduced expression of hsMAD2 protein, such as SNU-368, -475, -761 and -886 (23–36%; except SNU-354 cells), compared with those with relative steady-state amounts of hsMAD2 (11–17%). Therefore, in agreement with a previous report (25), our data indicate that hsMAD2 expression plays an important role in initiating and maintaining the mitotic checkpoint activation response to spindle damage in HCC cells. Next, we examined whether the reduced expression of hsMAD2 in HCC cells influences the aberrant exit of mitotic arrest in response to nocodazole treatment. Although we have only reported data obtained with SNU-739 (Fig. 5A and B, top panels), three HCC cell lines, SNU-354, -387, and Huh7, displayed similar results (data not shown). Apart from SNU-354, cell lines expressing almost normal levels of hsMAD2 protein exhibited a dramatic decrease in cyclin A levels 24 hours after microtubule inhibitor treatment. However, cyclin A levels were completely restored after inhibition of hsMAD2 expression. Interestingly, it has been known that cyclin A is destabilized when cells enter mitosis and is almost completely degraded before metaphase-to-anaphase transition, indicating that proper control of cyclin A degradation ensures chromosome segregation fidelity during mitosis (33–36). Thus, forced inhibition of hsMAD2 expression contributes to mitotic abnormality and blocks cyclin A degradation in the presence of microtubule inhibitors. In contrast, although the data shown in Fig. 5A and B (bottom panels) were obtained from SNU-761 cells, SNU-761 as well as SNU-368, -449, and -886 (data not shown) cells abolished the destabilization of cyclin A. Three cell lines, SNU-368, -739, and -886 (but not SNU-449) displayed aberrantly reduced levels of hsMAD2. However, these two groups of HCC cell lines showed the similar profiles of the accumulation of MPM2-positive cells. This finding raises the interesting possibility that three of the four HCC cell lines are adapted to express low levels of hsMAD2, resulting in loss of checkpoint sensitivity. Overall, our results support the theory that hsMAD2 expression is correlated with mitotic checkpoint competence in HCC cells. Moreover, the steady-state levels
of hsMAD2 are required for mitotic checkpoint competence and metaphase-to-anaphase transition.

**Aberrant Expression of hsMAD2 in HCC Cell Lines Is Associated with Transcriptional Silencing by Promoter Hypermethylation.** Mutations of relevant mitotic checkpoint genes are rare in various cancers tested (6, 15–20). However, aberrantly reduced expression of the MAD2 protein is associated with defective mitotic checkpoint in various tumors, including ovarian, breast, and nasopharyngeal cancers (15, 24, 25). Transcriptional down-regulation seems to correlate with hsMAD2 expression. To determine whether polymorphic base changes of the *hsMAD2* promoter region (–395 to +115) affect hsMAD2 expression in HCC cells, we did direct sequencing analyses (Fig. 6A). Eight of ten HCC cell lines exhibited no mutations in the defined *hsMAD2* promoter region. However, the two sequence variants identified in SNU-449 and -886 cells with silent polymorphic changes had no influence on the hsMAD2 mRNA levels (data not shown), indicating that promoter polymorphisms are not involved in the modulation of *hsMAD2* promoter activity.

Changes in promoter hypermethylation patterns represent an alternative to genetic lesions as causative factors for the aberrant expression of genes. However, there is little information on the promoter hypermethylation of mitotic checkpoint genes in conjunction with transcriptional regulation and impairment of mitotic checkpoint activation. To examine the possibility that DNA methylation is integrated into the transcriptional regulation of hsMAD2 expression, HeLa-Con and HeLa-*hsMAD2*-Luc cells were treated with various concentra-

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**Fig. 4.** Suppression of hsMAD2 expression results in loss of accumulation of mitotic indices in HCC cells. Ten HCC cell lines were transfected with hsMAD2 double-strand siRNA (0, 2.5, and 5 μg), as indicated, and treated with nocodazole for 12 hours. FITC-conjugated anti-MPM2 antibody was used to analyze whole cells for DNA content in a PI and MPM2-positive population. Distribution of MPM2-positive cells after nocodazole treatment was normalized to nontreated cells, as presented in Fig. 5B. Nontransfected and transfected cells were harvested for immunoblotting, using anti-phospho histone H3, anti-hsMAD2 and anti-Actin antibodies.

**Fig. 5.** Correlation between hsMAD2 expression and mitotic checkpoint sensitivity response to spindle damage. Mitotic checkpoint sensitivity of each HCC cell line after spindle damage was assessed by comparing cyclin A expression profiles. Eight HCC cell lines were transfected with hsMAD2 siRNA (0, 2.5, and 5 μg), and cultured in the absence or presence of nocodazole for 12 or 24 hours, as indicated. Nontreated and treated cells were harvested for immunoblotting with anti-Cyclin A and anti-Actin antibodies (A), and flow cytometric staining with PI and FITC-conjugated anti-MPM2 antibodies (B).
tions of the demethylation drug, 5-Aza-2'-deoxycytidine (5-Aza-2-DC; 0, 1, 2, and 5 μmol/L), for 72 hours (Fig. 6B). HeLa-Con cells containing promoterless luciferase gene treated with 5-Aza-2-DC showed no substantial changes in luciferase activity. However, HeLa-hsMAD2-Luc cells under control of the hsMAD2 promoter exhibited significantly increased luciferase activity in a concentration-dependent manner. This finding strongly indicates that hsMAD2 expression is controlled by promoter methylation. To further determine whether the deficiency in hsMAD2 expression observed in HCC cell lines is associated with promoter hypermethylation of the gene, 10 HCC cell lines were treated with the demethylating drug, and the hsMAD2 mRNA levels were determined by real-time PCR. Interestingly, 5 of the 10 HCC cell lines (SNU-368, -387, -475, -886, and THLE3) contained significantly increased hsMAD2 mRNA levels after treatment with 5-Aza-2-DC (Fig. 6C). In contrast, SNU-423, -449, and -739 expressing steady-state amounts of hsMAD2 protein displayed no apparent changes in mRNA levels after demethylation. However, SNU-354 cells containing low levels of the protein were unaffected by demethylation, implying that other genetic or epigenetic dysfunction processes are partly involved in the regulation of hsMAD2 expression in these cells. It is additionally unclear whether the control of hsMAD2 expression by DNA methylation is a universal factor for all human cancers. To confirm the recovery of hsMAD2 expression by 5-Aza-2-DC, SNU-354, -368, -387, -475, and -886 cells were cultured in the absence or presence of the agent. As shown in Fig. 6D, levels of hsMAD2 in SNU-368, -387, -475, and -886 cells were significantly enhanced after demethylation, whereas levels in SNU-354 with steady-state hsMAD2 expression (Figs. 3 and 4) remained unaffected.

Our data provide evidence that DNA methylation of the hsMAD2 gene promoter partially regulates protein levels. In summary, this study...
conclusively shows that down-regulation of hsMAD2 expression via promoter methylation potentially contributes to mitotic abnormality in HCC cells.

**DISCUSSION**

Defective mitotic checkpoints contribute to CIN commonly observed in human cancers. A number of studies show that mitotic defects are caused by inactivation of mitotic checkpoint components, leading to the premature onset of anaphase (8–14). However, mutational inactivation of mitotic checkpoint genes, such as MAD and BUB family members, is exceedingly rare in human cancers (5, 15–20). The important molecular connections among mitotic checkpoint genes, CIN and tumorigenesis are currently unknown. Previous data show that mitotic abnormalities are directly correlated with aberrantly reduced expression of MAD2 protein (25, 38), and the degree of mitotic checkpoint loss is closely associated with the incidence of CIN (9). Suppression of MAD2 protein levels by the carcinogenic compound, tetrachlorodibenzo-p-dioxin, is consistently accompanied by inactivation of mitotic checkpoint control (38). Wang et al. (25) reported decreased MAD2 expression in correlation with mitotic checkpoint defects in ovarian cancer cells and showed that recovery of MAD2 expression induced mitotic arrest in response to microtubule inhibitors. These observations indicate that MAD2 levels serve as a molecular switch for mitotic checkpoint control and monitoring of chromosomal instability. However, few cancers have been linked to mutational inactivation of the hsMAD2 gene. Nonmutational mechanisms for hsMAD2 gene inactivation may include an epigenetic process, such as DNA methylation. Aberrant promoter methylation may additionally be associated with loss of hsMAD2 gene expression. In this regard, we investigated whether the frequency and magnitude of reduced expression of mitotic checkpoint genes mechanically correlated with DNA methylation in any given tumor. Recently, Saeki et al. (16) reported that about 65% HCC cell lines exhibited an impaired mitotic checkpoint but no mitotic checkpoint gene mutations. These data further suggest that aberrant expression of hsMAD2 in human cancers is modulated by epigenetic changes, rather than by mutational inactivation. Interestingly, four of six HCC cell lines with reduced hsMAD2 expression displayed recovered hsMAD2 mRNA levels after demethylation. Thus, our study provides important evidence that mitotic checkpoint gene promoter hypermethylation is a major cause of checkpoint impairment. Consistent with this theory, some HCC cell lines with low levels of hsMAD2 showed a more substantial increase in chromosomal number instability than those displaying normal hsMAD2 expression (data not shown), suggesting that transcriptional dysfunction of this protein is associated with chromosome number instability. However, the observation that hsMAD2 mRNA expression in some HCC cell lines does not correspond with protein levels and remains unaffected by DNA demethylation raises the possibility that alterations of other components involved in mitotic checkpoint regulation induce this instability. Interestingly, expression of the BUBR1 mitotic checkpoint kinase is deregulated in these HCC cell lines, and levels of BUBR1 are correlated with chromosomal instability in response to prolonged spindle damage (39). Moreover, it is possible that a silent polymorphism in a mitotic checkpoint gene leads to changes in the biochemical properties or conformational stoichiometry of some components during tumorigenesis. Alternatively, in some cancer types or host species, post-translational degradation or alterations in hsMAD2 may induce abrogation of the mitotic checkpoint, resulting in chromosomal instability. Recently, Iwanaga et al. (40) identified a single nucleotide polymorphism in MAD1 in human cancer, which affected binding to MAD2. It is therefore likely that the function of MAD2 is additionally influenced by the relative interplay of mitotic checkpoint components.

In addition to the above hypotheses, a very recent study reveals that MAD2 is significantly overexpressed in some human cancers, particularly those in which retinoblastoma gene is inactivated, and thus E2F is constitutively activated (41). Interestingly, this aberrant overexpression of MAD2 is correlated with mitotic abnormality and chromosomal instability. In addition, MAD1 is up-regulated in human cancers, rather than down-regulated, and activated in gain-of-function p53 mutants (42). Overall, these studies suggest that not only aberrant reduction but also the elevation of the amounts of these mitotic checkpoint proteins seem to be a major cause for mitotic abnormality or result from the loss-of-mitotic checkpoint control. In support of this hypothesis, a series of studies suggest, in part, that abnormal expression, mutational structure alterations or in vivo competition of the specific mitotic checkpoint proteins lead to imbalanced complex formation, and thereby perturb the normal timing of harmonized mitotic cell cycle (9, 12, 22–23, 25, 37, 40, 43). These findings strongly indicate that stoichiometric binding and proper complex formation by sets of mitotic checkpoint proteins are important for the regulation of APC/C-Cdc20 inhibitory activity. Therefore, steady-state amounts of mitotic checkpoint proteins are essential to balance the checkpoint control.

In conclusion, our data provide evidence of frequent transcriptional dysfunction of the MAD2 gene in human cancers. Down-regulation of MAD2 expression is correlated with transcriptional silencing of the MAD2 promoter via hypermethylation. To our knowledge, this is the first study to show a relationship between transcriptional abnormality of a mitotic checkpoint gene and mitotic abnormality in human cancers.

**ACKNOWLEDGMENTS**

We thank Dr. Jae-Gahb Park and Dr. Frank McKeon for materials.

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Transcriptional Abnormality of the <i>hsMAD2</i> Mitotic Checkpoint Gene Is a Potential Link to Hepatocellular Carcinogenesis


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