Menin promotes hepatocellular carcinogenesis and epigenetically up-regulates Yap1 transcription

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Menin is a scaffold protein encoded by the multiple endocrine neoplasia type 1 (MEN1) gene in humans, and it interacts with a variety of transcriptional proteins to control active or repressive cellular processes. Here, we show that heterozygous ablation of Men1 in female mice reduces chemical carcinogen-induced liver carcinogenesis and represses the activation of the inflammation pathway. Using ChIP-on-chip screens and ChIP assays, we find that menin occupancy frequently coincides with H3K4me3 at the promoter of many liver cancer-related genes, such as Yes-associated protein (Yap1). Increased menin and Yap1 expression in human hepatocellular carcinoma specimens was associated with poor prognosis. Our findings reveal that menin plays an important epigenetic role in promoting liver tumorigenesis, and support the notion that H3K4me3, which is regulated by the menin-mixed-lineage leukemia complex, is a potential therapeutic target in hepatocellular carcinoma.

Menin Is Up-Regulated in HCC and Is Correlated with Poor Prognosis.

To unravel the functional significance of menin in HCC, we measured the expression of menin in 89 patients’ primary HCCs. The HCC specimens exhibited robust expression and exclusive nuclear staining of menin (42.7%) compared with adjacent tissues (Fig. L4). Kaplan–Meier survival analysis showed that the 3-y overall survival (Fig. 1B, P = 0.000) and the recurrence-free survival (Fig. 1C, P = 0.002) were significantly lower in the menin(−) HCC patients than in the menin(+) patients. The serum level of alpha-fetoprotein (AFP) was dramatically elevated.

Significance

Epigenetic changes commonly occur in hepatocellular carcinoma (HCC) and are associated with aberrant gene expression. Most studies have focused on epigenetic gene-silencing events; therefore, the mechanism that promotes gene activation in HCC is not well established. We identify an epigenetic activation mechanism whereby menin promotes Yes-associated protein (Yap1) transcription, which is associated with a poor prognosis for HCC patients. Substantial overexpression of the menin-mixed-lineage leukemia complex is associated with increased histone 3 lysine 4 trimethylation at certain loci of the tumor promoter in HCC. Heterozygous ablation of multiple endocrine neoplasia type 1 (MEN1) in mice reduces diethylstilbestrol-induced development of HCC. Our findings reveal that menin plays an important epigenetic role in up-regulating Yap1 transcription, leading to liver tumorigenesis.

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This study is designed to investigate the possible tumor promoter function of menin in the liver.

Results

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To unravel the functional significance of menin in HCC, we measured the expression of menin in 89 patients’ primary HCCs. The HCC specimens exhibited robust expression and exclusive nuclear staining of menin (42.7%) compared with adjacent tissues (Fig. L4). Kaplan–Meier survival analysis showed that the 3-y overall survival (Fig. 1B, P = 0.000) and the recurrence-free survival (Fig. 1C, P = 0.002) were significantly lower in the menin(−) HCC patients than in the menin(+) patients. The serum level of alpha-fetoprotein (AFP) was dramatically elevated.

Significance

Epigenetic changes commonly occur in hepatocellular carcinoma (HCC) and are associated with aberrant gene expression. Most studies have focused on epigenetic gene-silencing events; therefore, the mechanism that promotes gene activation in HCC is not well established. We identify an epigenetic activation mechanism whereby menin promotes Yes-associated protein (Yap1) transcription, which is associated with a poor prognosis for HCC patients. Substantial overexpression of the menin-mixed-lineage leukemia complex is associated with increased histone 3 lysine 4 trimethylation at certain loci of the tumor promoter in HCC. Heterozygous ablation of multiple endocrine neoplasia type 1 (MEN1) in mice reduces diethylstilbestrol-induced development of HCC. Our findings reveal that menin plays an important epigenetic role in up-regulating Yap1 transcription, leading to liver tumorigenesis.
in the menin−(+) group compared with the menin−(−) group (Fig. 1D, P = 0.001). Further correlation analyses in both cohorts showed that the robust expression of menin was associated with the more aggressive phenotype of HCC, including tumor multiplicity and neoplasm staging (Table S1).

To explore how menin influences liver cancer cells, the HepG2 cells were stably transduced with either a control (shLuc) or shMEN1 (17). MEN1 shRNA substantially repressed HepG2 cell proliferation and colony forming activity (CFA) (Fig. 2, A–C). Conversely, ectopic expression of menin increased the CFA in HepG2 and PLC/PRF5 HCC cell lines (Fig. 2 D–F). Furthermore, reduced expression of menin significantly suppressed tumor volume and weight in two independent pairs of MEN1 shRNA knockdown (KD) HepG2 xenografts (Fig. 2 G and H and Fig. S1). Overall, these findings suggest a potential tumor-promoting function of menin in HCC.

The Essential Role of the Menin–MLL Complex in Yap1 Transcription and Function. Currently, it is poorly understood whether alterations of active histone modifications, including those of H3K4, are associated with liver tumorigenesis. We further identified the genomic occupancy of menin and two associated transcriptionally active histone modifications, H3K4me3 and H3K79me2 in HepG2, using ChIP-on-chip screens. Our data showed that menin occupied the promoter regions of thousands of human genes (Fig. S2 A and B and Dataset S1). Menin occupancy frequently coincides with H3K4me3 and H3K79me2; however, menin can also target promoters independently of H3 modifications (Fig. S2B). Menin occupancy at the HOXA clusters is extensive with broad footprints that spanned intergenic and intragenic portions of the HOXA genes in chromosome 7 (Fig. S2A), which is similar to the epigenetic-regulating features of menin in leukemia systems (5–7). Hyperexpression of HOXA genes, including HOXA5, 7, 10, and 13, was observed in a series of HCC specimens, and deregulation of HOXA13 led to cell-cycle alterations (18). Fig. S2C shows that genes (HOXA7, 9, and 13) with promoters bound by menin were correlated with higher mRNA levels in menin-overexpressing PLC/PRF5 cells (Fig. S2C). We previously found that menin represses cell proliferation and transcription of pleiotrophin (PTN) through polycomb group (PcG)-mediated H3K79me3 in lung adenocarcinoma and melanomas (19, 20). Nevertheless, the PTN mRNA level is augmented by menin in HepG2 cells (Fig. S2D), which further implies a tumor-promoting function of menin in HCC in a tissue-specific manner. Unsupervised hierarchical clustering analysis indicated that the top 26 genes with menin promoter occupancy coincide with H3K4me3 and H3K79me2 in ChIP-on-chip, and these genes are closely related with tumor development and progression, including the proto-oncogene Yap1 (Fig. S2E). These findings indicate an interesting epigenetic mechanism by which menin regulates gene expression through H3 modification in HCC.

We explored the relationship between menin and Yap1 expression. The substantial MEN1 siRNAs reduced Yap1 mRNA and protein expression in HepG2 cells (Fig. 3A), whereas the expression and nuclear localization of Yap1 were increased in menin-overexpressing HepG2 cells (Fig. 3B and Fig. S3 A and B). Following the increased total Yap1 protein level resulting from menin expression, phospho-Yap1 (Ser127) was also slightly elevated in PLC/PRF5 cells (Fig. S3C). Nevertheless, there are no obvious effects on Yap1 protein expression by menin overexpression or MEN1 siRNA KD in other types of cell lines, including MCF-7, Wilm’s, and AS49 (Fig. S3 D and E), suggesting a liver-specific function of menin in regulating Yap1. Correlated of HCC specimens, and deregulation of HOXA13 led to cell-cycle alterations (18). Fig. S2C shows that genes (HOXA7, 9, and 13) with promoters bound by menin were correlated with higher mRNA levels in menin-overexpressing PLC/PRF5 cells (Fig. S2C). We previously found that menin represses cell proliferation and transcription of pleiotrophin (PTN) through polycomb group (PcG)-mediated H3K79me3 in lung adenocarcinoma and melanomas (19, 20). Nevertheless, the PTN mRNA level is augmented by menin in HepG2 cells (Fig. S2D), which further implies a tumor-promoting function of menin in HCC in a tissue-specific manner. Unsupervised hierarchical clustering analysis indicated that the top 26 genes with menin promoter occupancy coincide with H3K4me3 and H3K79me2 in ChIP-on-chip, and these genes are closely related with tumor development and progression, including the proto-oncogene Yap1 (Fig. S2E). These findings indicate an interesting epigenetic mechanism by which menin regulates gene expression through H3 modification in HCC.

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with reduced Yap1 expression, the specific targeting of Yap1 by siRNA dramatically reduced the HepG2 CFA (Fig. 3C and Fig. S3F). Furthermore, the promoted CFA by ectopic expression of menin was reduced by the Yap1 KD in HepG2 cells (Fig. 3D and Fig. S3G).

Yap1 is an important “driver gene” for HCC (16), however the transcriptional regulation of Yap1 by critical effectors in HCC has not been defined to date. In our ChIP-on-chip analyses, we found that menin occupancy coincides with the transcriptional activation of H3 modification at Yap1 promoter loci (Fig. S2E), which raises an interesting hypothesis of whether menin regulates Yap1 transcription through epigenetic mechanisms, such as H3K4me3. First, we assessed the expression of the MLL–HMTase complex in HCC using Western blotting. Associated with immunohistochemistry (IHC) observation, menin (6/11), Ash2L (9/11), WDR5 (6/11), RbBP5 (5/11), and MLL (5/8) expression were robustly activated in HCC compared with the surrounding tissues (Fig. 3E). We further performed ChIP assays using three distinct pairs of primers for the Yap1 promoter loci (Fig. 3F). As expected, the ChIP assays clearly showed that menin bound to the Yap1 promoter, and MEN1 KD notably reduced binding of menin at Yap1 loci but not at the GAPDH locus (Fig. 3F). Further, either MEN1 or MLL KD by siRNA reduced the H3K4me3 level at Yap1 loci, especially in primer pair 1 (PP1) and PP2 (Fig. 3G). Nevertheless, although menin bound to the promoter of Yap1, there are no obvious effects on H3K4me3 levels at Yap1 loci by menin overexpression in other types of cell lines, including MCF-7, Wilm’s, and A549 (Fig. S3H and I), which further suggests the liver-specific function of menin in regulating Yap1 was dependent on H3K4 histone remodeling. In addition, the substantial KD of menin, Yap1, pSTAT3, and pAKT was increased in the livers of males and females exposed to CCl4 (Fig. S4), which further suggests the metastatic tumors came from pancreatic islets to the livers. As shown in Fig. 4C, 47.6% of the Men1+−/− female mice developed HCC; however, a significantly lower HCC incidence of only 8.33% was observed in Men1−/− female mice (P < 0.05). Furthermore, Men1+−/− dramatically reduced the tumor multiplicity and maximal tumor size in female mice (Fig. 4D and E). Compared with Men1 WT mice, the tumor–liver ratio was reduced to almost one-third in Men1+−/− female mice, and it was correlated with a slightly reduced liver–body weight ratio (Fig. 4F and G). Men1+−/− females displayed significantly less hepatic injury after DEN administration, which was determined by the reduced serum AFP, IL-6, and TGF-β (Fig. 4H–J).

Unexpectedly, all male mice developed HCC with no difference in tumor incidence (Fig. 4C) between Men1 WT and Men1+−/−. To elucidate the contribution of menin in hepatocarcinogenesis in male mice, 6-wk-old male mice were exposed to DEN. Administration of DEN resulted in the stimulation of the Akt, also known as protein kinase B (PKB), STAT3, and MAPK pathways in the liver; however, Men1+−/− reduced their activation (Fig. S4B). Further, the serum IL-6 and TNF-α concentrations up-regulated by DEN exposure were remarkably reduced in Men1+−/− male mice (Fig. S4C and D), which indicates that menin participates in the liver injury response of male mice at early stages. The carbon tetrachloride (CCl4)-induced mouse model incorporates chronic injury, inflammation, and fibrogenesis and shares several features with the microenvironment in which the majority of human HCCs arise (22). In this model, we found that expression of menin, Yap1, pSTAT3, and pAKT was increased in the livers of males and females exposed to CCl4 (Fig. S4E, lanes 1–3 and
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Heterozygous loss of Men1 promoter were revealed by ChIP assays (Fig. 5 G23) mice were injected with DEN (25 G Yap1 and (Fig. S5 E and F and Fig. S5 D and E). Furthermore, the up-regulation of IL-6 by ectopic expression of menin was reduced by the Yap1 KD in HepG2 cells (Fig. 5G), which suggests that menin regulates IL-6 expression at least partly through Yap1. Finally, the protein or mRNA expression of menin and Yap1 was increased in HL-7702 or HepG2 cells exposed to IL-6 (Fig. 5 H and J). These results point to an interesting HCC-related positive feedback loop between menin–Yap1 and IL-6.

Fig. 4. Heterozygous loss of Men1 reduces DEN-induced development of HCC. Men1+/− (n = 42) and Men1−/− (n = 23) mice were injected with DEN (25 mg/kg, i.p.) at 11 d of age and killed 8 mo after DEN injection. Shown are representative liver images (A) and H&E staining sections (B). (C) Shown are different tumor incidences in male and female mice with DEN exposure. (D–G) The compared results of tumor multiplicity, maximal tumor diameter, tumor–liver, and liver–body weight ratios between Men1+/− and Men1−/− mice. (H–J) Circulating serum levels of AFP, IL-6, and TGF-β were measured by ELISA. Data of D–J are represented as mean ± SEM. (K) Western blot analysis of menin, Yap1, pYap1 (ser127), Ash2L, RbBP5, and WDR5 in tumor (T) and normal (N) liver tissues of mice. (L) H3K4me3 ChIP assays performed with indicated Pps of mouse Yap1 promoter in female mice liver.

7–9). Nevertheless, the Men1−/− effectively reduced their effect in female mice but not in males (Fig. S4F, lanes 4–6 and 10–12). In addition, administration of DEN significantly increased the menin levels in the livers of C57BL/6 males compared with females at 4 mo (Fig. S4 F and G). These results suggest that although the function of menin is involved in inflammation response at an early stage, the heterozygous ablation of Men1 was easily compensated in liver injury and inflammation in male mice compared with female mice.

To evaluate the potential relationship of the menin–Yap1 axis and liver tumorigenesis, we collected liver tumors and surrounding tissues. Because the primary tumors resulting from DEN exposure of Men1+/− female mice were not large enough for Western blotting analysis, we used tumors from Men1+/− male mice. We detected elevated menin, Yap1, MLL, Ash2L, RbBP5, and WDR5 levels in the liver tumors (Fig. -MK), as well as pAKT, pSTAIR3, pERK1/2, and p65 (Fig. S4H). In ChIP assays, following the increase of menin at the Yap1 loci (Fig. S4I), the H3K4me3 levels increased nearly threefold in tumors compared with surrounding tissues (Fig. 4L). The H3 antibody ChIP was used as a loading control (Fig. S4I).

The Menin–Yap1 Axis Responded to CCL4-Induced Liver Inflammation. HCC promotion depends on the microenvironment, including inflammation pathways (12, 13). To determine the relationship between menin–Yap1 axis and inflammation pathways, we used CCL4-induced inflammation mouse model. Exposure to CCL4 significantly stimulated the expression of menin, MLL, and Yap1 in the livers of male C57BL/6 mice at 24 and 48 h (Fig. S4A). This response is associated with elevated mRNA levels of IL-6 and TGF-β and serum concentrations of IL-6 (Fig. 5 S A and B). An increased binding of menin and accumulation of H3K4me3 at the Yap1 promoter were revealed by ChIP assays (Fig. SB and Fig. SSC). In accord with the reduction of Men1 or Yap1 expression by siRNA, the IL-6 mRNA level was markedly reduced in primary isolated liver Kupffer cells (KCs) (Fig. 5 C and D). Yap1 KD dramatically reduced IL-6 mRNA expression in two independent pairs of Yap1 siRNA KD HL-7702 and HepG2 cells (Fig. 5 E and F and Fig. S5 D and E). Furthermore, the up-regulation of IL-6 by ectopic expression of menin was reduced by the Yap1 KD in HepG2 cells (Fig. 5G), which suggests that menin regulates IL-6 expression at least partly through Yap1. Finally, the protein or mRNA expression of menin and Yap1 was increased in HL-7702 or HepG2 cells exposed to IL-6 (Fig. 5 H and J). These results point to an interesting HCC-related positive feedback loop between menin–Yap1 and IL-6.

Yap1 Is Epigenetically Regulated by Menin and Correlated with Poor Prognosis in Human HCCs. To confirm the clinical significance of the menin–Yap1 axis, we sought to address the deregulation of Yap1 in HCCs. The HCC specimens exhibited robust expression and exclusive nuclear staining of Yap1 compared with the adjacent tissues (Fig. 6A). Kaplan–Meier survival analysis showed that the 3-y overall survival rate of patients with Yap1− (+) HCCs was significantly lower than that of patients with Yap1− (−) HCCs (Fig. 6B, P = 0.000). A significantly lower recurrence-free survival rate was found in HCC patients in the Yap1− (+) group compared with Yap1− (−) HCC patients (Fig. 6C, P = 0.000). Furthermore, serum AFP levels were dramatically higher in the Yap1− (+) group than in the Yap1− (−) group (Fig. 6D, P = 0.004). Yap1 hyperexpression was significantly associated with more aggressive phenotypes of HCC, including tumor multiplicity, vascular invasion, and neoplasm staging (Table S2). In 23.75% of HCCs, menin and Yap1 were collectively up-regulated (Fig. 6E, P = 0.014). In HCC tissue ChIP assays, the binding of menin and accumulation of H3K4me3 at the Yap1 promoter were markedly increased in HCC specimens compared with adjacent tissues (Fig. 6 F and G and Fig. S6). These results support the mechanistic and clinical significance of the menin–Yap1 axis as an effective biomarker for HCC diagnosis and prognosis evaluation.

Discussion

The findings of this study advance our knowledge of the epigenetic activation mechanism of H3K4me3 and indicate that menin plays an important, yet previously unappreciated, role in promoting the development of HCC. Menin, as a scaffold protein, lacks identifiable functional motifs, and the putative function of menin is not well defined. Menin interacts with a variety of transcription factors and is involved in a variety of cellular processes, including gene activation and repression (7). In this notion, the crystal structure shows that menin contains a deep pocket that interacts similarly with MLL1 and JUND, indicating
that the diverse functions may be largely attributed to the crucial role of menin as a core scaffold molecule (7). MLL, an epigenetic regulator, plays a critical role in acute leukemias, but the putative biological function of MLL in the liver is not well defined. Supporting our notion, HGF-MET (hepatocyte growth factor-mesenchymal epithelial transition factor) signals were reported to promote the expression of matrix metallopeptidases (MMPs), and the development of HCC through the ETS2–MLL complex mediated H3K4me3 (23). Strikingly, whole-genome sequencing analysis revealed that the MLL family of methyltransferases for H3K4 was somatically mutated in HCCs (24), which further supports the biological relevance of MLL in HCC.

In this report, we propose that the unique biological role of menin in promoting HCC depends on the oncogenic activity of MLL. Although we have identified a unique tumor-promoting action of menin that is essential for liver tumorigenesis, significant work remains to identify the functions of MLL in HCC. It is clear that better definitions of active histone modification in HCC are important steps in the design of improved therapeutic strategies. HCC is the sixth most prevalent cancer, and systemic chemotherapy has marginal activity and frequent toxic effects (10). MI-2 (menin inhibitor-2), a small molecule inhibitor that is based on the menin structure, has been shown to effectively inhibit leukemia cell proliferation by disrupting the menin–MLL complex up-regulates Yap1 transcription through H3K4me3 in HCC.
complex, highlighting the potential therapeutic application of inhibitors targeting the menin–MLL complex in human diseases (25). Similarly, considering the importance of the menin–MLL complex in controlling the aggressive nature of HCCs, we propose a unique therapeutic strategy for HCC by targeting the menin–MLL complex. Further studies will be required to evaluate the therapeutic effect of epigenetic-targeting drugs, such as MI-2, on drug-resistant, aggressive HCC.

Site-specific histone modifications are major epigenetic mechanisms for maintaining stable gene transcription, which is fundamental to the development of most cancers (26). Here, we present the profile of genomic occupancy of menin and two associated transcriptionally active histone methylation markers, H3K4me3 and H3K27me3, in liver cancer cells. These methylation markers are associated with many liver cancer-related genes, including Yap1. Notably, Hippo-dependent or -independent mechanisms of Yap1 transcription remain to be identified. Recently, Wu et al. demonstrated that the Ets family member GABP binds to the Yap promoter and activates YAP transcription in liver cancer (27). Our results indicate that menin binds to Yap1 promoter loci and up-regulates H3K4me3. The menin–MLL complex and Yap1 are constitutively activated in primary HCC patients and DEN-induced liver cancer, and they serve as critical HCC tumor promoters. Additionally, the expression pattern of the menin–Yap1 axis is strongly correlated with poor prognosis, suggesting the clinical significance of the menin–Yap1 axis as a biomarker for HCC diagnosis and prognosis evaluation.

Epidemiological surveys indicate that HCC occurs mainly in men and that men are about three to five times more likely to develop HCC than women (28). Similar sex disparity is seen in mice given DEN (29). Estrogen suppresses IL-6 production in liver KCs and reduces liver cancer risk in females but not in males (29). Our results suggest that Men1+/− mice dramatically repressed the development of HCC in female mice but not in male mice. We also observed that menin participates in the liver injury response of male mice at early stages, and menin activation was more readily compensated upon liver injury in males than in females. It is reported that menin expression, which contributes to gestational diabetes in females, is repressed by increased prolactin and progesterone (30). So we suppose that the expression of menin was regulated by estrogen only in female mice. This is likely one of the reasons for sex biases of menin in our liver cancer animal model. It will be interesting to determine whether the homozygous deletion of Men1 can repress development of HCC in males and determine how menin expression is regulated by estrogen in the liver. Conditional knockout mice that are viable and have the Men1 gene inactivated in their livers would allow further assessment of menin in liver disease processes, which would advance the understanding of the precise mechanism by which menin contributes to HCC. Here, we did not find a significant sex bias of hyperexpression of menin in HCC specimens. However, we cannot rule out the possibility that the result was limited by an insufficient number of female samples.

Altogether, we propose that augmented menin activation during chronic liver injury plays a crucial role in promoting hepatocarcinogenesis (Fig. 6F).

Materials and Methods

Human HCC Samples. The study was approved by the Xiamen University Medical Ethics Committee. Frozen and paraffin-embedded primary HCC tissues and corresponding adjacent nontumorous liver samples were obtained from the Chronic Liver Disease Biological Sample Bank, Department of Hepatobiliary Surgery, Zhongshan Hospital, Xiamen University. These samples were from male and female patients histopathologically diagnosed as having stage I–IV HCC. The demographic data and clinicopathological features of the HCC patients are listed in Tables S1 and S3. The ages of the cases ranged from 25 to 75 y. In total, we examined 89 HCC samples together with adjacent normal tissues. Sections from paraffin-embedded samples were stained with affinity-purified antimemin or anti-Yap1 (Cell Signaling) antibodies (Table S4) for IHC. The specificity of the antimemin antibody was verified in menin-null and menin-expressing cells (19). The method and procedure of IHC are as described previously (19). The expression of menin and Yap1 in livers was determined from the IHC results by three independent pathologists.

ACKNOWLEDGMENTS. We thank Dr. Francis Collins at National Human Genome Research Institute for providing the heterozygous Men1 locus (Men1+/−) mice and Dr. Xinmin Hua for critical reading of the manuscript. This research was supported by grants from the Natural Science Foundation of China (91229111 and 81272719 to G.-H.J., 81101924 to S.-H.L., and 81101763 to S.-B.G.), the Natural Science Foundation of Fujian Province (2011J06016 to G.-H.J.), and the Natural Science Foundation of Xiamen (3502220104001 to G.-H.J.).

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