Expression and subcellular localization of *menin* in human cancer cells

FENG REN[1], HONG-WEI XU[2], YU HU[1], SHUANG-HONG YAN[1], FENG WANG[1], BAO-WEI SU[2] and QI ZHAO[2]

Departments of [1]Clinical Laboratory, and [2]General Surgery, The Fourth People's Hospital of Wuxi, Wuxi, Jiangsu 214062, P.R. China

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Abstract. The aim of this study was to elucidate the expression and localization of *menin*, a protein encoded by the multiple endocrine neoplasia type I (MEN1) gene, in 13 human cancer cell lines. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the expression of the *menin* gene. The localization of the *menin* protein was detected by immunofluorescence microscopy. Western blotting was used to determine the quantity of *menin* in the nucleus, cytosol and membrane of the cells. RT-PCR revealed that *menin* was expressed in all the cell lines examined in this study. Immunofluorescence microscopy revealed that *menin* was located primarily in the nucleus. In the GES-1 (transformed human gastric epithelium), MCF-7 (breast cancer), SGH44 (brain glioma) and HeLa (cervical cancer) cell lines, *menin* was also found to be localized to the membrane, cytosol and nucleus. Moreover, in SGH44 cells more *menin* was located in the cytosol than the nucleus. Similar findings were obtained by western blotting. In the GES-1 and MKN-28 cells undergoing octreotide treatment, cytoplasmic *menin* was significantly increased compared with the control groups. Therefore, we suggest that *menin* is expressed in a number of human cancer cell lines and that the cytosolic distribution increases when the cells undergo octreotide treatment, indicating a new role for *menin*.

Introduction

Menin, encoded by the multiple endocrine neoplasia type I (MEN1) gene, is a nuclear protein which is mutated in patients with a dominantly inherited disorder characterized by the appearance of combinations of tumors in a number of endocrine tissues, including the pituitary and parathyroid glands and the pancreas (1,2), and occasionally in non-endocrine glands (3,4). The human MEN1 gene has been identified by positional cloning and is localized to chromosome 11q13. The gene consists of 10 exons and codes for an mRNA 2.8 kb in length (5). The protein product of the MEN1 gene, *menin*, is highly conserved in organisms from *Drosophila* to humans. As *menin* does not show a clear homology to any known protein motifs, it has been challenging to elucidate the mechanism by which *menin* acts as a tumor suppressor (6).

Numerous studies have revealed that *menin* is mainly located in the nucleus and possesses nuclear localization signals (NLSs) in its carboxy-terminal region (7). It has also been reported that *menin* is found in membrane and cytoplasmic fractions (8), although at lower levels than in nuclear fractions, indicating that *menin* may also play a role outside of the nucleus. Multiple lines of evidence suggest that *menin*, as a scaffold protein, interacts with cytoskeletal proteins, including glial fibrillary acid protein (GFAP), vimentin (8) and IQGAP1 (9). However, less is known concerning the expression and biochemical function of *menin* expression in non-endocrine cells. To elucide the mechanism by which *menin* functions as a tumor suppressor, in the present study, we report the detection of endogenously expressed *menin* in 13 human cancer cell lines. In particular, we further investigated the subcellular localization of *menin* in cancer cell lines.

Materials and methods

Cell lines and cell culture. The following cell lines were obtained from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China): AGS, BGC-823 and SGC-7901 (human gastric cancer), Huh7 and Hep3B2.1-7 (liver cancer), MCF-7 and MDA-MB-231 (breast cancer), SW480 (colon cancer), SGH44 (brain glioma), SKOV-3 (ovarian cancer) and HeLa (cervical cancer). The transformed human gastric epithelial cell line GES-1 was a gift from Dr Yong-Chang Chen (Jiangsu University, China). The MNK-28 cell line was a gift from Dr Xiao-Ying Li (Rui-Jin Hospital, Shanghai Jiao-Tong University School of Medicine, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% newborn calf serum (NBCS; Minhai Bio-engineering Co., Lanzhou, China) and maintained at 37˚C in a humidified atmosphere of 5% CO_2. The medium was

Correspondence to: Mr. Feng Ren, Department of Clinical Laboratory, The Fourth People's Hospital of Wuxi, 200 Huihe Road, Wuxi, Jiangsu 214062, P.R. China
E-mail: renfeng198254@163.com

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changed every two days and the cells were subcultured until reaching confluency.

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using dNTPs (1 mM), 5X reverse transcription buffer (500 mM Tris-HCL pH 8.3, 250 mM KCl, 50 mM $MgCl_2$ and 50 mM DTT), 16 units RNasin and 2.5 units of AMV reverse transcriptase (Gibco BRL, Life Technologies, Carlsbad, CA, USA). PCR was carried out in a total volume of 20 μl, containing 2 μl 10X buffer, 0.4 μl dNTP, 0.5 μl primers, 1 μl cDNA, 0.2 μl Taq enzyme and 15.4 μl H_2O (Shenergy Biocolor Biological Science and Technology Co., Shanghai, China). The specific primers were: *menin* gene, forward 5'-GCCTGGGTAGTGTTTGGGC-3' and reverse 5'-CACAGCGCATGTATGATCCTT-3', product of 452 bp; β-actin, forward 5'-TGCGTGACATTAAGGAGAAG-3' and reverse 5'-GCTCGTAGCTCTTCTCCA-3', product of 247 bp. Following an initial denaturation step of 5 min at 95˚C, 30 cycles of amplification for the primer pairs were carried out. Each cycle included a denaturation step of 30 sec at 95˚C, annealing for 1 min at 55˚C and an elongation step of 1 min at 72˚C, with a final extension for 5 min at 72˚C. The products were separated on 1.5% agarose gel and the relative gene expression was measured using a digital image (Perkin-Elmer, Wellesley, MA, USA). The experiments were performed in triplicate and the mean value was calculated.

Immunofluorescence microscopy. The cells grown on coverslips were fixed with freshly prepared paraformaldehyde (40 g/l in PBS) for 1 h prior to being penetrated with 0.3% Triton X-100 and blocked with 3% bovine serum albumin (BSA) in PBS. The cells were then incubated with the primary antibody at 4˚C overnight and then with the Cy3-conjugated secondary antibody for 1 h at room temperature (RT), with three washes following each incubation. The distribution of the target protein in the cells was analyzed by fluorescence microscopy.

Preparation of nuclear and cytoplasmic samples. The cells were extracted by Dounce homogenization in HEM buffer (10 mmol/l HEPES pH 7.5, 2 mmol/l EDTA, 1 mmol/l $MgCl_2$) as described previously (10). The homogenate was centrifuged at 500 x g at 4˚C for 5 min to obtain the nuclear proteins and the supernatant was centrifuged at 37,000 x g at 4˚C for 30 min. The supernatant and the pellet from the second centrifugation are referred as cytosol and membrane preparations, respectively. The membrane preparation was washed twice with HEM buffer to remove contaminating cytosol. The protein concentrations were determined and equal amounts of protein from each preparation (30 μg) were subjected to SDS-PAGE.

Western blotting. Sample proteins were run on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (PVDF, Amersham, Piscataway, NJ, USA) by electronic transfer. The membranes were blocked with 5% non-fat dried milk and then incubated with an antibody against *menin* (dilution 1:200) and GAPDH (dilution 1:5,000) overnight at 4˚C and with the secondary antibody for 1 h at RT, with three washes following each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. The bands on film were analyzed with GeneSnap/Gene Tool software from Syngene (Cambridge, UK).

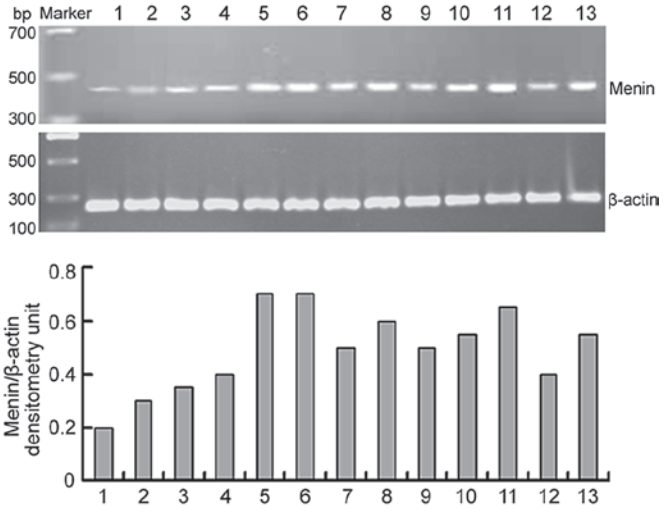

Figure 1. Expression of *menin* mRNA in 17 human cancer cell lines was detected by RT-PCR. The 452-bp human *menin*-specific sequence and a 247-bp β-actin sequence were amplified from the cDNA of cancer cell lines, separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Densitometry of *menin* transcripts was standardized to β-actin. Lane 1, AGS; lane 2, BGC-823; lane 3, SGC-7901; lane 4, MKN-28; lane 5, GES-1; lane 6, Huh7; lane 7, Hep3B2.1-7; lane 8, MCF-7; lane 9, MDA-MB-231; lane 10, SW480; lane 11, SGH44; lane 12, SKOV-3; lane 13, HeLa. RT-PCR, reverse transcription-polymerase chain reaction.

Statistical analysis. All of the numerical data are expressed as the mean ± SD. Statistical analysis was performed using SPSS 16.0 edition program (SPSS Inc., Chicago, IL, USA) for ANOVA with the Scheffé multiple comparison test. $P<0.05$ was considered to indicate a statistically significant result.

Results

Expression of menin mRNA in human cancer cells. As shown in Fig. 1, *menin* mRNA was examined in 13 human cancer cell lines. RT-PCR results revealed that *menin* was positively expressed in all the cell lines examined in this experiment. In the gastric cancer cell lines, the expression of *menin* mRNA was gradually increased from AGS, BGC-823, SGC-7901 and MNK-28 to GES-1. It was significantly higher in GES-1 than the other four gastric cancer cell lines ($P<0.05$). The expression of *menin* may be correlated with the malignancy of the cells.

Menin is generally distributed in cancer cell lines. The expression of the *menin* protein in cancer cell lines was further confirmed by immunoblotting, which revealed a 74-kDa protein band. The results also revealed that the *menin* protein was expressed in different cancer cell lines (Figs. 2 and 3). Moreover, the *menin* protein was expressed not only in the nucleus, but also in the cytosol and membrane in the GES-1, MCF-7, SGH44 and HeLa cell lines (data not shown). The expression of the *menin* protein was significantly higher in

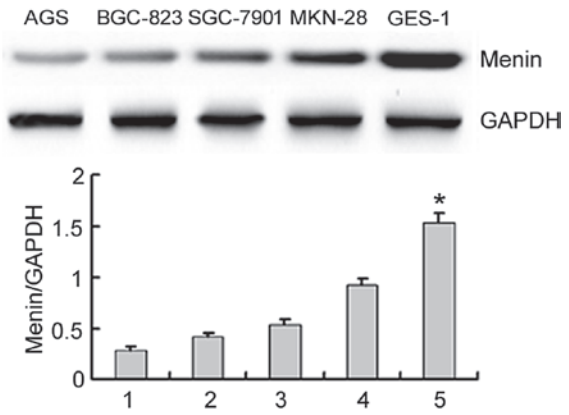

Figure 2. Expression of *menin* protein in gastric cancer and GES-1 cell lines. The expression of *menin* and GAPDH was detected by western blotting. The volume ratio of *menin*/GAPDH was calculated and presented. Each bar represents mean ± SD obtained from three independent experiments. Compared with the other four gastric cancer cell lines, the expression of *menin* in GES-1 cells was higher (*P<0.05). Bar graph: 1, AGS, 2, BGC-823; 3, SGC-7901; 4, MKN-28; 5, GES-1.

GES-1 cells than in the other gastric cancer cell lines (P<0.05; Fig. 2).

The subcellular localization of menin in different cancer cell lines. Immunofluorescence microscopy revealed that *menin* was located primarily in the nucleus. However, in the four human cancer cell lines (GES-1, MCF-7, SGH44 and HeLa), *menin* was localized not only in the nucleus, but also in the cytosol and membrane. Moreover, in the SGH44 cells more *menin* was located in the cytosol than that in the nucleus (Figs. 4 and 5).

More menin protein is distributed in the cytosol with octreotide treatment. The relative protein expression levels of *menin* in the GES-1 and MKN-28 cell lines were significantly increased in the octreotide group compared with the control group. Moreover, more *menin* protein was located in the cytosol with octreotide treatment (Fig. 6).

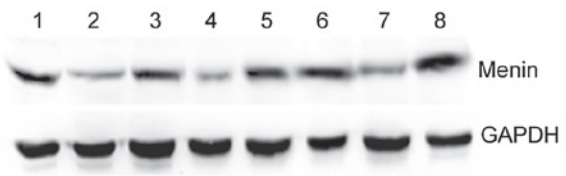

Figure 3. Expression of *menin* in human cancer cell lines. The cell extract was constructed and *menin* and GAPDH were detected by western blotting. The expression of *menin* in Hep3B2.1-7, MDA-MB-231 and SKOV-3 cells was decreased compared with the other cell lines. Lane 1, Huh7; lane 2, Hep3B2.1-7; lane 3, MCF-7; lane 4, MDA-MB-231; lane 5, SW480; lane 6, SGH44; lane 7, SKOV-3; lane 8, HeLa.

Discussion

The present study detected the expression and distribution of *menin* in 13 cancer cell lines, including AGS, BGC-823, SGC-7901 and MNK-28 (gastric cancer), GES-1 (transformed gastric epithelium), Huh7 and Hep3B2.1-7 (liver cancer), MDA-MB-231 and MCF-7 (breast cancer), SW480 (colon cancer), SKOV-3 (ovarian cancer), HeLa (cervical cancer) and SGH44 (brain glioma) cells. The results of RT-PCR, western blotting and immunofluorescence microscopy revealed that *menin* was positively expressed in all of the cell lines examined in this study. The nuclear localization of the *menin* protein was extensive and general, with a discrepancy between the gastric cancer and transformed gastric epithelial cell lines and the cancer and SGH44 (brain glioma) cell lines. Furthermore, we found that the somatostatin analog octreotide increased the expression of *menin*, particularly in the cytosol. Consistent with the findings of Mensah-Osman *et al* (11), octreotide induces *menin* expression by the suppression of PKA activation.

The MEN1 gene encodes the *menin* protein, which is thought to be involved in a number of mechanisms that are dysregulated in cancer cells, including genome stability and the regulation of gene transcription, cell proliferation and apoptosis (12-15). These diverse *menin* functions were largely attributed to the crucial role of *menin* in endocrine cells and tissues. The results of the present study support the hypothesis that the nucleus-cytoplasm-membrane treble distribution of

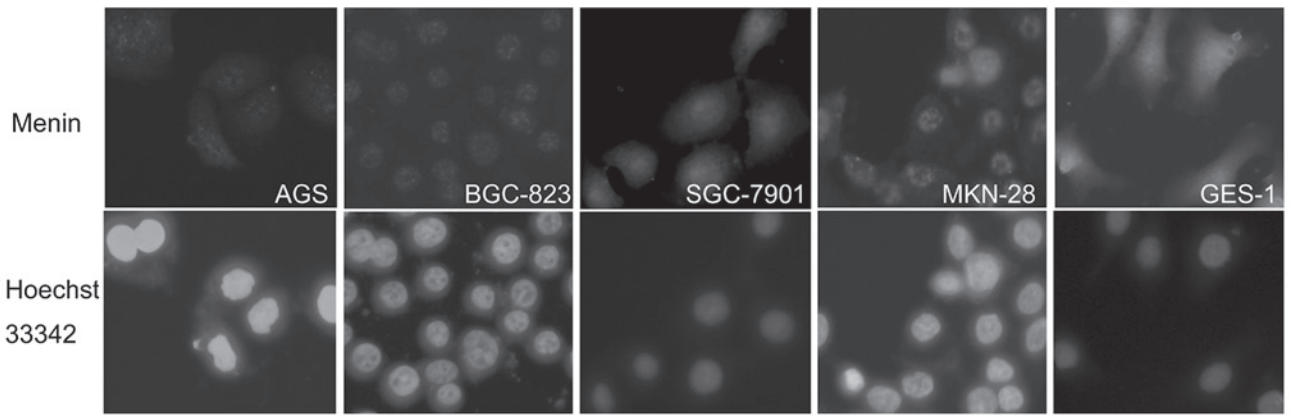

Figure 4. Nuclear localization of *menin* in gastric cancer and GES-1 cell lines. The cell lines were immunofluorescently stained with a specific fluorescence antibody against *menin* and the results showed that in all the cell lines, *menin* is located primarily in the nucleus. In the GES-1 cells, *menin* was also found to be localized to the membrane, cytosol and nucleus (original magnification, x200).

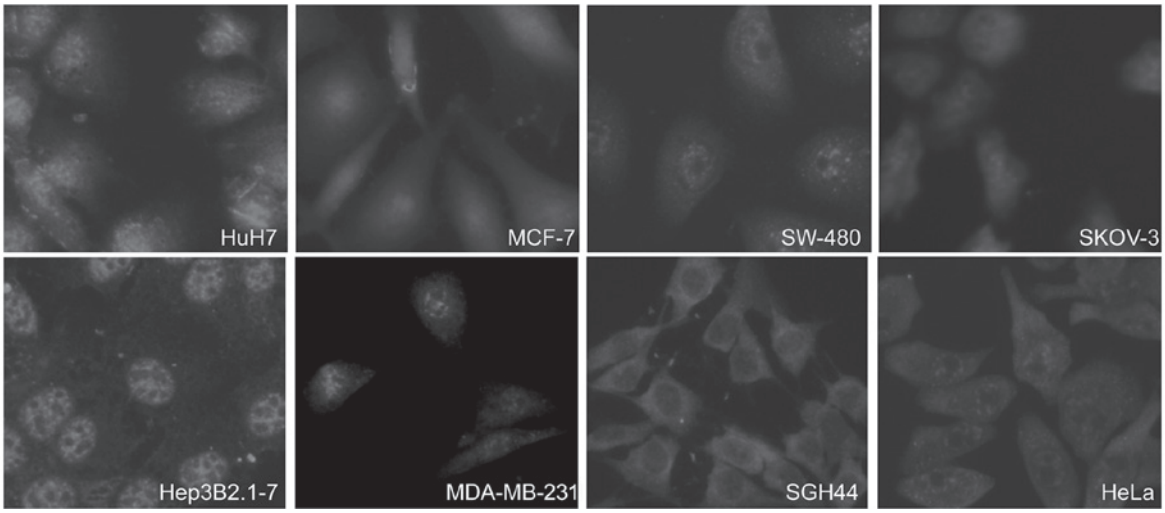

Figure 5. Localization of *menin* in different cancer cell lines. The cell lines were immunofluorescently stained with a specific fluorescence antibody against *menin* and the results showed that in all cell lines, *menin* was localized to the membrane, cytosol and mainly the nucleus. However, the expression of *menin* was higher in the cytosol than that in the nucleus in SGH44 brain glioma cells (original magnification, x200).

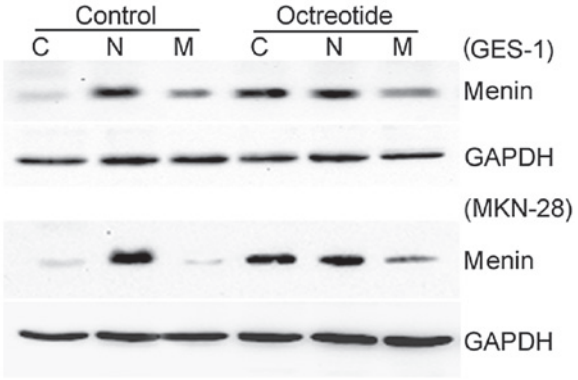

Figure 6. Expression of *menin* in GES-1 and MKN-28 cells treated with or without octreotide. Western blotting: GES-1 and MKN-28 cells were lysed and cytosolic (C), nuclear (N) and membrane (M) fractions were obtained. The expression of *menin* and GAPDH in each fraction was detected. Compared with the control, the expression of *menin* in the cytosol was significantly increased in octreatide-treated cells.

menin was a general phenomenon and that more *menin* protein was localized to the nucleus of non-endocrine cancer cells. Although the primary function of *menin* as a regulator of gene transcription, cell proliferation, apoptosis and genome stability has been determined (16,17), the role of *menin* protein in the cytoplasm-membrane localization remains unknown. These observations provide novel insights into how *menin* suppresses tumorigenesis.

Menin is predominantly located in the nucleus and contains two classic NLSs, NLS1 and NLS2 (18). Classic NLSs comprise positively charged amino acid residues which bind to a soluble transport receptor complex made up of importins α and β, causing the protein to be translocated to the nucleus (19). The nuclear localization of *menin* is thought to be necessary for its ability to regulate gene transcription, as the protein regulates gene expression by associating with chromatin and the nuclear matrix and binding double-stranded DNA (20). Whereas, the simultaneous deletion of NLS1 and NLS2 in *menin* attenuates *menin* translocation into the nucleus, on the contrary, accumulating quantities of *menin* protein are noted in the cytosol. Our results showed that *menin* was also localized to the cytosol and membrane in GES-1, MCF-7, SGH44 and HeLa cells. In particular, the level of expression of *menin* was higher in the cytosol than in the nucleus in SGH44 cells. These results suggest that *menin* NLS deletion mutants and the deletion of a stretch of amino acid residues may affect the expression and general structure of *menin*. On the other hand, *menin* has several NLSs, suggesting that its transcriptional activity may be regulated by its ability to move in and out of the nucleus (18).

Mensah-Osman *et al* (11) found that octreotide-suppressed PKA activation may markedly increase the numbers of *menin*-expressing cells and levels of *menin* mRNA and *menin* protein expression. This study also revealed that when the cells were treated with octreotide, the expression of the *menin* protein was increased and the protein was rapidly transported out of the nucleus and congregated in the cytosol and on the membrane. Generally, stimuli that promote the expression of a protein are also able to promote its intracellular movement (most are cytoplasm-to-nucleus translocations) (21,22). Octreotide may promote the reverse translocation of the *menin* protein from the nucleus to the cytoplasm. Consistent with our results, Yan *et al* (9) observed the co-localization of IQGAP1 with a non-nuclear pool of *menin* in β cell lines. The co-localization of menin at the plasma membrane was observed to be extensive, particularly at cell-cell junctions. The authors also reported that *menin* interacts with IQGAP1, a scaffold protein, reducing its interaction with GTP-Rac1 and increasing its interaction with E-cadherin/β-catenin. This suggests the existence of a *menin*-IQGAP1 pathway which influences cell migration and cell-cell adhesion in endocrine tissue. According to its new localization, our results provide new insight into some unknown functions of *menin* protein in non-endocrine cells.

Together, these results demonstrated that *menin* was positively expressed in all of the cell lines examined in this study.

The nuclear localization of the *menin* protein was extensive and general and its expression in the cytoplasm may play a more significant role in coordinating the activation and repression of gene transcription than merely targeting *menin* to the nucleus. The precise roles of *menin* in the cytoplasm and the membrane are not yet fully understood. Further detailed analysis must be carried out to establish the functions of *menin* and its role in the cytoplasm.

Acknowledgements

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