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PRECLINICAL STUDY

Menin, a product of the *MENI* gene, binds to estrogen receptor to enhance its activity in breast cancer cells: possibility of a novel predictive factor for tamoxifen resistance

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Abstract Multiple coactivator and corepressor complexes play an important role in endocrine processes and breast cancer; in particular, estrogen and estrogen receptor-α (ERα) promote the proliferation of breast cancer cells. Menin is a tumor suppressor encoded by *Men1* that is mutated in the human-inherited tumor syndrome multiple endocrine neoplasia type 1 (MEN1); it also serves as a critical link in the recruitment of nuclear receptor-mediated transcription. Here, we show that menin expressed in breast cancer cell line MCF-7 is colocalized with ERα and functions as a direct coactivator of ER-mediated transcription in breast cancer cells. In MCF-7 cells, coexpression of menin and estrogen-response element-luciferase induced the activity of the latter in a hormone-dependent manner. Cells knocked down for ERα exhibited impaired ERE-luciferase activity induced by menin. Mammalian two-hybrid assay and GST pull-down assays indicated that menin could interact with the AF-2 domain of ERα. These results indicate that menin is a direct activator of ERα function. Tamoxifen inhibited the binding of menin to AF-2 in mammalian two-hybrid assay, but in menin-overexpressing clones, tamoxifen suppressed ERE-luciferase activity only to the levels of nontreated wild-type MCF-7. In a clinical study with 65 ER-positive breast cancer samples—all of which had been treated with tamoxifen for 2–5 years as adjuvant therapies—menin-positive tumors had a worse outcome than menin-negative ones. These indicated that menin can function as a transcriptional

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regulator of ERα and is a possible predictive factor for tamoxifen resistance.

Keywords Menin · Estrogen receptor · Breast cancer · Tamoxifen

Introduction

Estrogen and estrogen receptor (ER) play an important role in the development of mammary glands and in the genesis, growth, and progression of breast cancer [1]. ERα is a ligand-activated nuclear receptor that regulates the transcription of estrogen-responsive genes in diverse target cells. ERα and its ligand 17-estradiol not only play a critical role in normal breast development but also have long been linked to mammary carcinogenesis, breast cancer progression, and outcomes of breast cancer patients [2]. Given the fact that 17-estradiol stimulates the growth of ER-positive breast tumors via functional ERs, endocrine therapy such as the use of antiestrogens or ovarian ablation has been established as an important part of breast cancer management [3]. Clinically, ERα is an important prognostic factor in breast cancer, and the measurement of its expression is applied to the determinants of hormone therapy in these cancer patients. At the onset, approximately 70% of breast cancers are ER positive, and up to 70% of these ER-positive cancers respond to hormone therapy [4, 5]. As with hormonal regulation, the transcriptional activity of ER is affected by a number of regulatory cofactors, including chromatin remodeling complexes, coactivators, and corepressors [6–10]. Examples of ER coactivators include members of the p160 family, namely, SRC1–3, AIB1, TRAM1, RAC3, cAMP-response element-binding protein-binding protein CBP, and p300 [11, 12].

Menin is a tumor suppressor encoded by *Men1* that is mutated in the human inherited tumor syndrome multiple endocrine neoplasia type 1 (MEN1) [13, 14]. MEN1 is an autosomal dominant disorder characterized by multiple endocrine tumors of the parathyroid glands, pancreatic islets, and anterior pituitary [15, 16]. Although the primary sequence of menin is highly conserved from fly to humans, it does not show obvious homology to known protein motifs, thereby making it difficult to elucidate its biochemical function. Recent studies have shown that menin regulates cell proliferation [17–19], apoptosis [20, 21], and genome stability [22–24]. Menin interacts with several transcription factors such as Jun D and NF-B and yeast SET1-like complex containing the mixed-lineage leukemia (MLL) protein [25–29].

Multiple coactivator and corepressor complexes play an important role in endocrine processes and breast cancer [1]. In the present study, we report that menin could function as a direct coactivator of ER-mediated transcription in breast cancer cells and that menin could be a novel predictive factor for tamoxifen resistance in clinical preliminary study. These results provide a molecular mechanism for the recruitment of ERα-menin complexes and could provide an explanation for the clinical manifestations of breast cancer.

Materials and methods

Cell culture

Human breast cancer cell line MCF-7 was obtained from the National Cancer Institute (Bethesda, MD) and maintained routinely in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 1 nM of 17β-estradiol (E2, Wako Pure Chemical Industries, Osaka, Japan), 100 units/ml penicillin G, and 100 mg/ml streptomycin. For experiments evaluating the effect of E2, cells were cultured in phenol red-free RPMI 1640 (PRF-RPMI, Gibco BRL) containing 10% FBS stripped of steroids by absorption onto dextran-coated charcoal (DCC-FBS). COS-7 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS, 1 nM of E2, 100 units/ml penicillin G, and 100 mg/ml streptomycin.

Western blot

An anti-menin antiserum was generated using a recombinant peptide (amino acid residues 443–535 of menin); the homology of this peptide sequence between humans and rat is 87% . This antibody recognized both human and rat menin as described previously [30]. The preadsorption test of the antiserum showed complete disappearance of a 67-kDa band in Western blotting. Proteins were transferred onto PVDF membranes, and the membranes were incubated with a 1:3,000 dilution of anti-menin antiserum [31]. The membranes were further incubated with a 1:3,000 dilution of goat anti-guinea pig IgG coupled to horseradish peroxidase. Immunoreactive bands were visualized by the ECL system (GE Healthcare, Buckinghamshire, UK).

Immunohistochemistry staining

Fixation, paraffin embedding, and serial sectioning were performed as described previously. The primary antibodies used were anti-menin rabbit polyclonal antibody A300-105A (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, for menin staining, after deparaffinization, the

sections were microwaved at 98°C for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval, soaked in 0.3% H_2O_2 methanol solution for 30 min, and then incubated in 4% blocking ace solution (Dainippon Sumitomo Pharma, Co., Osaka, Japan) for 60 min to prevent nonspecific staining. The primary antibody was used at a 1:250 dilution and incubated at 4°C overnight. After three washes with phosphate-buffered saline (PBS), Envision Plus solution (Dako, Kyoto, Japan) was applied for 30 min at room temperature. The sections were developed with 3,3′-diaminobenzidine (DAB; Nichirei, Co., Tokyo, Japan) and counterstained with Mayer's hematoxylin. The normal rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as a negative control to observe no staining.

Immunofluorescence staining

Formaldehyde-fixed, paraffin-embedded samples were serially cut into 4-μm-thick sections for staining. Antigens were retrieved by boiling the sections in 0.01 M citric buffer (pH 6.0) for 15 min. The sections were incubated overnight at 4°C with the primary antibodies 1D5 (Dako) for ERα and A-300 (Bethyl Laboratories, Inc.) for menin, and the final concentrations were 1:50 and 1:250 of anti-ERα and anti-menin antibodies, respectively. In all cases, isotype-matched monoclonal antibodies were used as a negative control. The sections were washed with PBS and then incubated for 1 h at room temperature in a 1:400 diluted cocktail of Alexa Fluor 488-labeled goat anti-rabbit IgG and Alexa Fluor 568-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR). The sections were finally washed with PBS and mounted. Fluorescence signals and digital images were acquired with an Olympus FluoView^{TM}FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan) and analyzed using FV10-ASW image analysis software (Version 1.5, Olympus).

Plasmid construction

The protein coding region (1,833 bp) of the human menin cDNA [18] was amplified by polymerase chain reaction (PCR) using primers that included two recognition sites of restriction enzymes (*Not*I and *Bam*HI) (ATT AGG ATC CAT GGG GCT GAA GGC CGC CCA and ATG CGG CCG CTC AGA GGC CTT TGC GCT GCC, respectively), as described previously [20]. After digestion with *Not*I and *Bam*H1, the PCR products were purified and inserted into the plasmid vector pcDNA3.1 (Invitrogen, Groningen, the Netherlands). Plasmids pCMX-GAL4 and pCMX-VP16 for mammalian two-hybrid experiments were kindly provided by Dr. K. Umesono (Kyoto University, Kyoto, Japan) together with the reporter tk-GALpx3-Luc possessing the GAL4-binding sequence [32]. An internal control plasmid for luciferase assay, pRL-TK, was purchased from Promega (Madison, WI). To construct the expression plasmids of fusion protein with GAL4 DNA-binding domain or VP16 activation domain, *Eco*RI and *Bam*HI sites were designed adjacent to the initiation and termination codons of the inserted ERα cDNA and menin cDNA, respectively [33]. Glutathione S-transferase (GST) fusion protein expression vectors were constructed as follows: full-length ERα cDNA fragments were ligated into the pGEX-2T vector (GE Healthcare). The sequences of all constructed plasmids were confirmed using ABI Prism 310 automatic DNA sequencer.

Transfection of small interfering RNA

Small interfering RNAs (siRNAs) were designed to target the following cDNA sequences: ERα scrambled siRNA, 5′-CCCUUAUCGCUCUCAGUAAGA-3′; and ERα-specific siRNA, 5′-GUCAUCGCAUUCCUUGCAAAC-3′ (Nihon Bioservice, Kanagawa, Japan). Transfection of the siRNAs was performed using siPORT Amine (Ambion, Austin, TX). At 3 days after transfection, ERα protein expression was examined by Western blot analysis.

Transfection of MCF-7 cells and luciferase reporter gene assay

The estrogen-responsive reporter plasmid ptk-ERE-Luc was constructed by insertion of estrogen response element (ERE) (59-AGC TAG GTC AGG ATG ACC TAG CTA-39) into the *Hin*dIII site of the tk-luciferase plasmid [32]. The purified reporter plasmid was transfected into MCF-7 cells (at 60% confluence) by a conventional cationic liposome transfection method (Lipofectamine, Life Technologies, Inc., Gaithersburg, MD). All assays were corrected for β-galactosidase activity, and the total amount of protein in each reaction was identical as described previously [34]. Twenty-microliter aliquots were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

Protein–protein interaction assay using the mammalian two-hybrid system

COS-7 cells were cultured in 60-mm-diameter dishes in phenol red-free Dulbecco's minimal essential medium (Gibco BRL) supplemented with 10% charcoal dextran-treated FBS. The reporter plasmid tk-GALpx3-Luc (1 mg) was cotransfected with pCMX-VP16-menin (0.1 mg) and

pCMX-GAL4-ER (0.1 mg) by the conventional cationic liposome transfection method (Lipofectamine). After 4 h incubation, the medium was replaced with fresh phenol red-free Dulbecco's minimal essential medium supplemented with 10% charcoal dextran-treated FBS and with or without 10 nM E2. The luciferase activity was measured as described previously.

Stable transfection

The menin expression vector was transfected into the cultured MCF-7 cells by the conventional cationic liposome transfection method as described previously [35]. The transfected cells were selected by the addition of G418 to the media, and clones showing high menin production were identified by Western blot analysis.

GST pull-down and in vitro binding assays

GST-ERα fusion protein was expressed in bacteria and bound to glutathione-Sepharose 4B (Pharmacia) beads as described previously [32, 36]. In vitro translation for the linearized expression vectors containing menin cDNA was performed with [^{35}S]methionine by using the TNT kit (Promega) as described previously [37]. For the GST pull-down assay, a 50% suspension of GST-protein beads (50 ml), which contained up to 1.0 mg of protein, was resuspended in the same volume of binding buffer (20 mM Tris–HCl [pH 7.5], 0.12 M NaCl, 10% [vol/vol] glycerol, 0.055% 2-mercaptoethanol, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). An aliquot (15 ml) of the in vitro translation reaction mixture was mixed with GST-protein beads and suspended for 1 h at 4°C. The beads were then washed four times with washing buffer (replacing 0.12 M NaCl in the binding buffer with 0.1 M NaCl) and resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. After electrophoresis, radiolabeled proteins were visualized with an image analyzer (BAS2000; Fuji Film, Tokyo, Japan).

Patients

Sixty-five women aged more than 37 years with breast cancer were enrolled in the study, and provided informed consent. The median age was 60 years, and the median observation period was 103 months. Tamoxifen had been administrated as adjuvant therapy and was administered to all of the patients for 2–5 years. This study was carried out according to the ethical guidelines of the Declaration of Helsinki, and specific approval was obtained from the Ethics Committee of Kagawa Medical University.

Immunohistochemical analysis

Immunohistochemical staining of sections of formalin-fixed, paraffin-embedded tissue was performed with antibodies to menin and an EnVision+, Peroxidase, Rabbit System (Dako, Kyoto, Japan). In brief, sections (thickness, 4 μm) were heated at 100°C for 16 min in 10 mM sodium citrate buffer (pH 6), subjected to paraffin removal, and rehydrated. After quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide, the sections were treated for 2 h at room temperature with 5% bovine serum albumin to block nonspecific staining. They were then incubated at room temperature first overnight with primary antibodies (5 μg/ml), and then streptavidin-biotin-conjugated secondary antibodies. 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. An immunoglobulin fraction isolated from normal rabbit serum (Dako) was used as a negative control. All sections were counterstained with Mayer's hematoxylin solution. The percentage of stained tumor cells in each section was determined independently by two observers. Menin expression was negative when no staining was detectable, and positive when staining was clearly detectable.

Statistical analysis

The data were analyzed using unpaired Student's *t*-test with one-way analysis of variance for in vitro study and Kaplan–Meier method for clinical study. The data are expressed as means ± SE for in vitro study, and statistical significance was considered at $P < 0.05$.

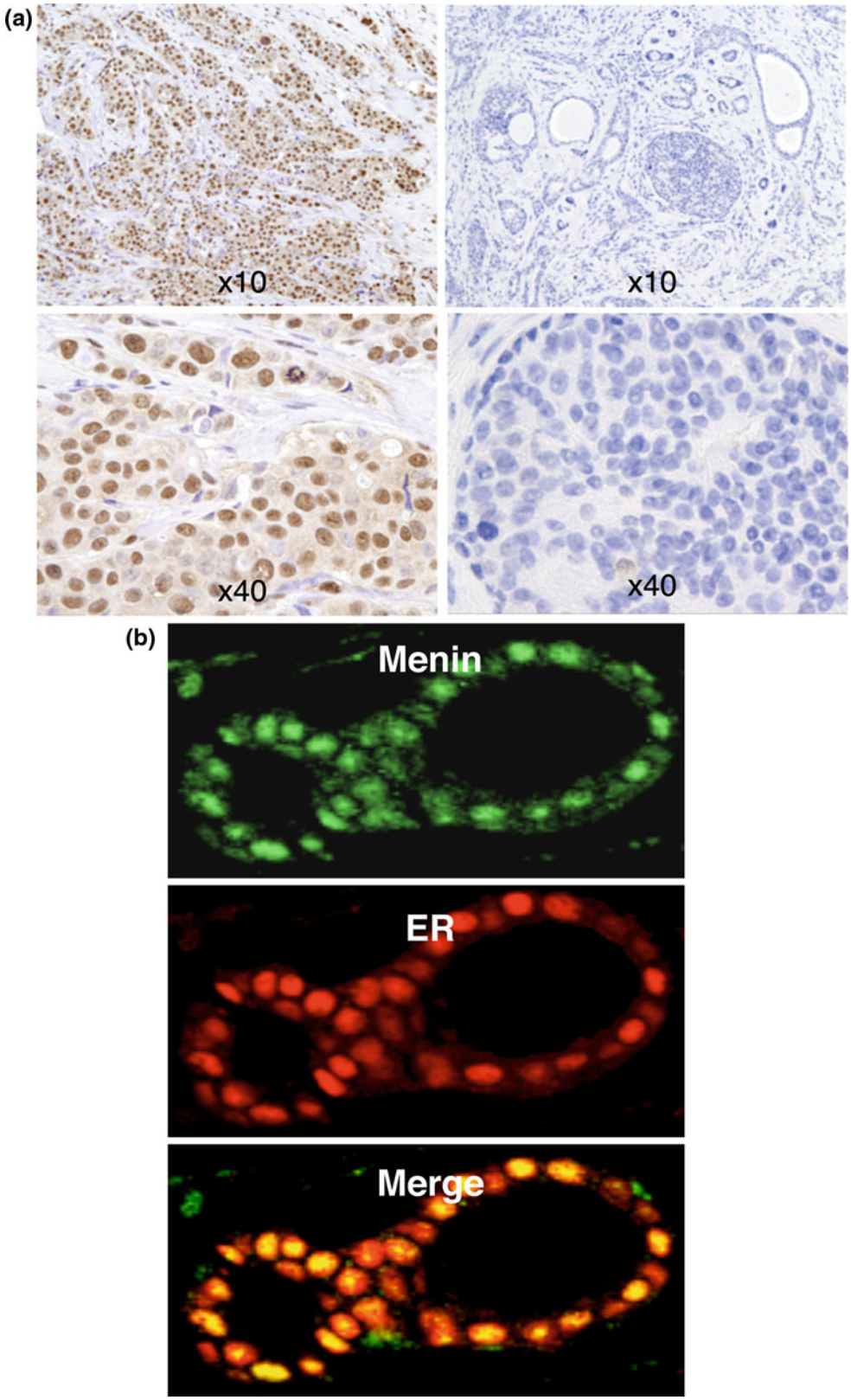
Results

Menin expression in breast cancer cells

To examine the expression of menin in breast cancer cells, immunohistochemical analysis using an anti-menin antibody was used to localize menin in the tissue samples. The results (Fig. 1a) showed strong immunostaining for menin in the breast cancer cells (left panel). The use of nonspecific IgG failed to show staining in the cells (right panel). Together, these findings show that menin is abundantly expressed in human breast cancer cells but not in the adjacent normal tissue.

Colocalization of menin and ER on breast cancer cells

To confirm the colocalization of menin and ERα on breast cancer cells, immunofluorescence analysis using a confocal laser-scanning microscope was performed. Merged images of immunofluorescence showed yellow coloration,


(a)
x10
x10
x40
x40
(b)
Menin
ER
Merge

◀ **Fig. 1** Expression of the menin protein in breast cancer cells. **a** Sections were stained with anti-menin antibody (*left panels*) and control IgG (*right panels*) as described in Materials and Methods. **b** Localization of the menin protein and ERα in breast cancer cells were observed by confocal laser microscopy. Sections were double-stained with anti-menin and anti-ERα labeled with Alexa Fluor 488 and 568, respectively, as described in Materials and Methods. The expression of the menin protein (*green*) (upper, ×64) and ERα (*red*) (middle, ×64) was observed in the nuclei of cells from a breast cancer tissue sample. Colocalization of menin and ER in the same nuclei of cells was shown by merged images (*yellow*) (lower, ×64)

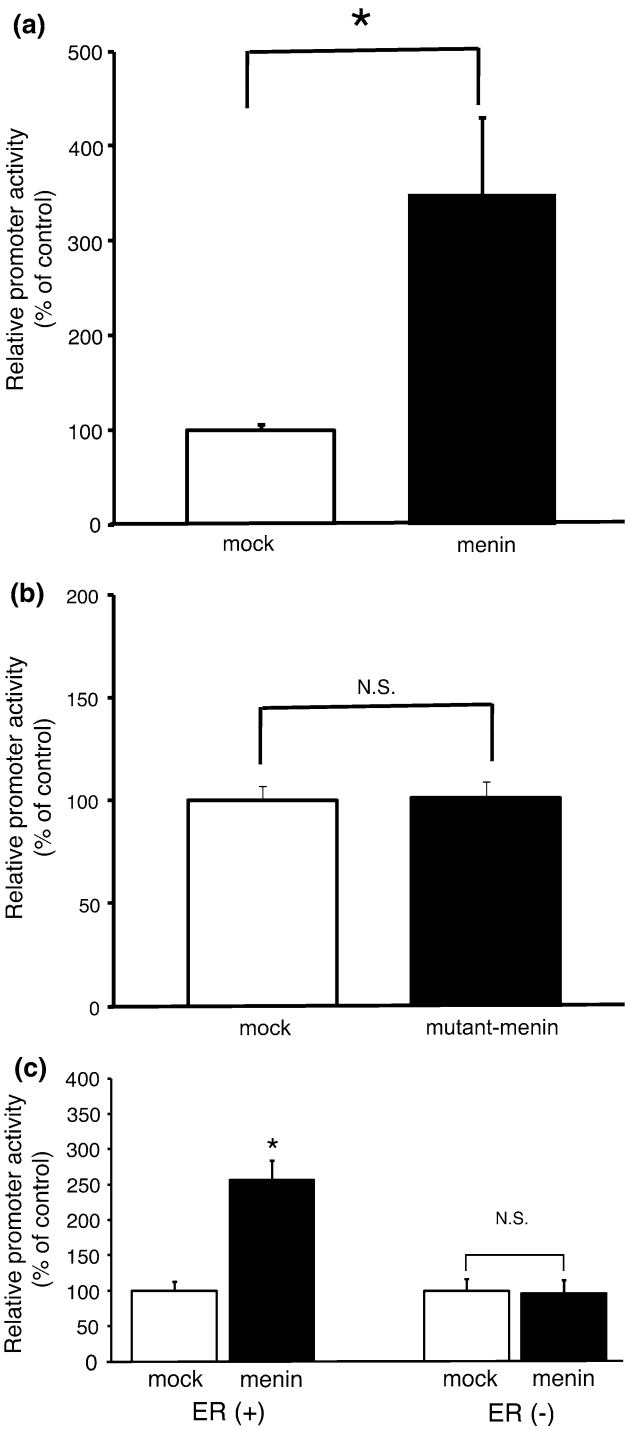
suggesting the nuclear colocalization of the menin protein, and ERα was observed in a part of breast cancer cells (Fig. 1b).

Effect of menin on ERE activity

Several lines of evidence suggested that menin is a nuclear protein and thus may affect the transcriptional activity of selected genes [20, 38]. A recent report indicated that menin is linked to estrogen receptor activation [39]. To study the biological role of menin in ER activation, we used ERα-positive breast cancer cells, namely, MCF-7 cells. MCF-7 cells were cotransfected with either a vector that overexpressed menin or an empty vector together with the reporter plasmid ptk-ERE-Luc, which possesses a perfect palindromic ERE in the front of the thymidine kinase (tk) promoter. The results (Fig. 2a) showed that the expression of menin significantly increased the luciferase activity. We have previously identified a point mutation (569 delC) in menin isolated from a Japanese patient with MEN1; this mutation caused truncation of menin (from amino acid 183–610) [40]. When MCF-7 cells were cotransfected with a vector containing mutant menin together with ptk-ERE-Luc, the luciferase activity was not affected by the expression of mutant menin (Fig. 2b). To determine whether ERα function is modulated by menin, we carried out a transient transfection experiment using ERα-null COS-7 cells. The reporter plasmid ptk-ERE-Luc and the ERα expression plasmid pCER were cotransfected into COS-7 cells with or without the menin expression vector. The results (Fig. 2c) showed that transfection of the menin expression vector enhanced ERE-luciferase activity. In COS-7 cells without transfection of the ERα expression vector, the transfection of the menin expression vector had no effect on ERE-luciferase activity (data not shown).

Menin enhances ERα function with estrogen

Next, we tested whether ERα might affect the menin-induced ERE-luciferase activity. MCF-7 cells were treated with a specific or scrambled ERα siRNA and then transfected with the menin and ERE-luciferase reporter genes. Our results showed that the ERE-luciferase activity



increased in the cells treated with the scrambled ERα siRNA and transfected with the menin expression vector (Fig. 3a), while the ERE-luciferase activity was markedly reduced in the cells treated with the ERα-specific siRNA (Fig. 3a). These findings suggest that the menin-mediated induction of the ERE-luciferase activity requires ERα. To address the role of estrogen in menin-stimulated ERE-luciferase activity, we seeded MCF-7 cells in the medium

◀ **Fig. 2** Effects of menin on ERE-luciferase activity. **a** Effect of menin on ERE-luciferase activity in MCF-7 cells. MCF-7 cells grown in 60mm dishes were cotransfected with ptk-ERE-Luc along with the menin expression vector. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.001$). **b** Effect of mutant menin on ERE-luciferase activity. MCF-7 cells were transfected with the reporter ptk ERE-Luc plus an empty (mock) or the mutant menin expression vector. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean and SEM ($n = 4$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.005$). **c** Role of ERα in menin-induced ERE-luciferase activity. The reporter plasmid ptk-ERE-Luc with {ER(+)}/without {ER(−)} the ERα expression plasmid pCER were cotransfected into COS-7 cells with or without the menin expression vector. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean and SEM ($n = 4$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.005$)

with charcoal-stripped FBS and then cotransfected with the menin and ERE-luciferase reporter genes in the absence or presence of E2. As shown in Fig. 3b, the expression of menin significantly increased the luciferase activity in the presence of E2 but not in its absence. These findings suggest that the menin-stimulated ERE-luciferase activity was estrogen dependent.

Menin activates AF-2 transcriptional activity of ERα

To determine whether ERα interacts directly with menin, protein–protein interaction of ERα and menin in vitro was performed by GST pull-down assay. As shown in Fig. 4a, the in vitro translated menin protein but not mutant menin interacted specifically with ERα. This result indicates that ERα and menin can interact directly with each other. To examine whether menin physically interacts with ERα, we evaluated the interaction in vivo by performing a mammalian two-hybrid assay. ERα has two distinct transactivation domains, namely, AF-1 and AF-2, which mediate ligand-dependent transactivation. The full-length ERα (1–596), AF-1 (1–180), AF-2 (302–553), and DBD (1–163) were each fused to the GAL4 DNA-binding domain. Cotransfection of the reporter plasmid tk-GALpx3-Luc along with expression vectors for each ER domain and VP16-menin chimeras was performed in COS-7 cells. As shown in Fig. 4b, the expression of full-length ERα increased menin-mediated luciferase activity by approximately threefold. The expression of AF-1 or DBD did not increase menin-mediated luciferase expression, but AF-2 enhanced menin-mediated luciferase expression by approximately twofold. Therefore, menin potentiated ERα transcriptional activity through its AF-2 domain.

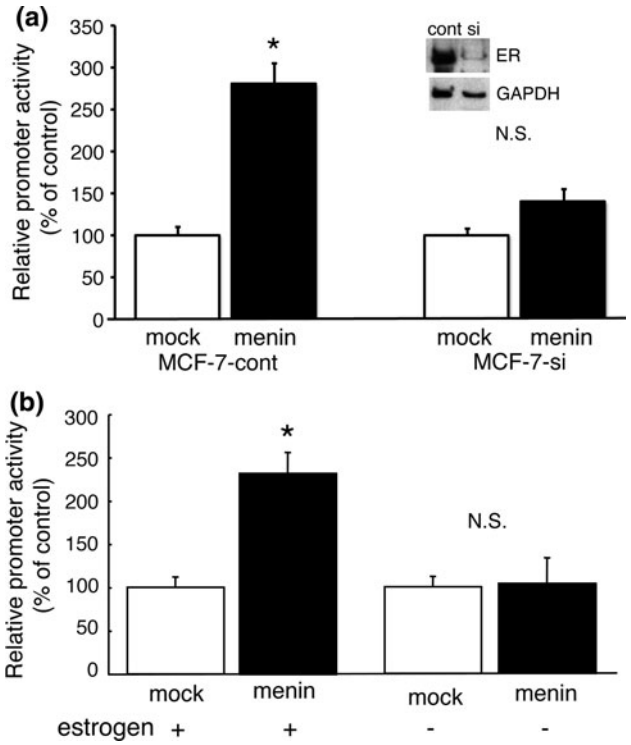
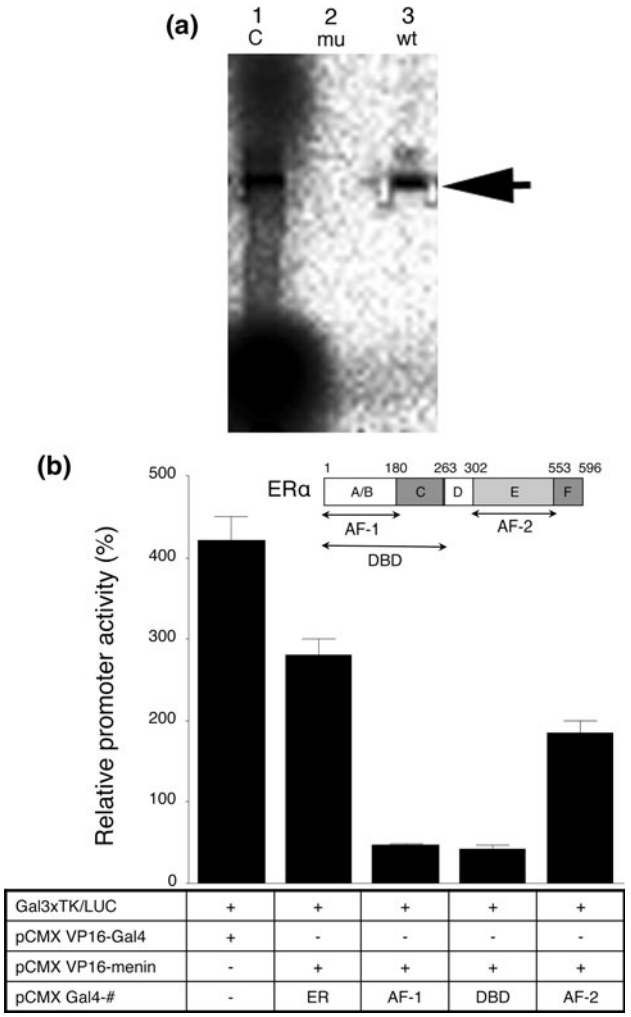


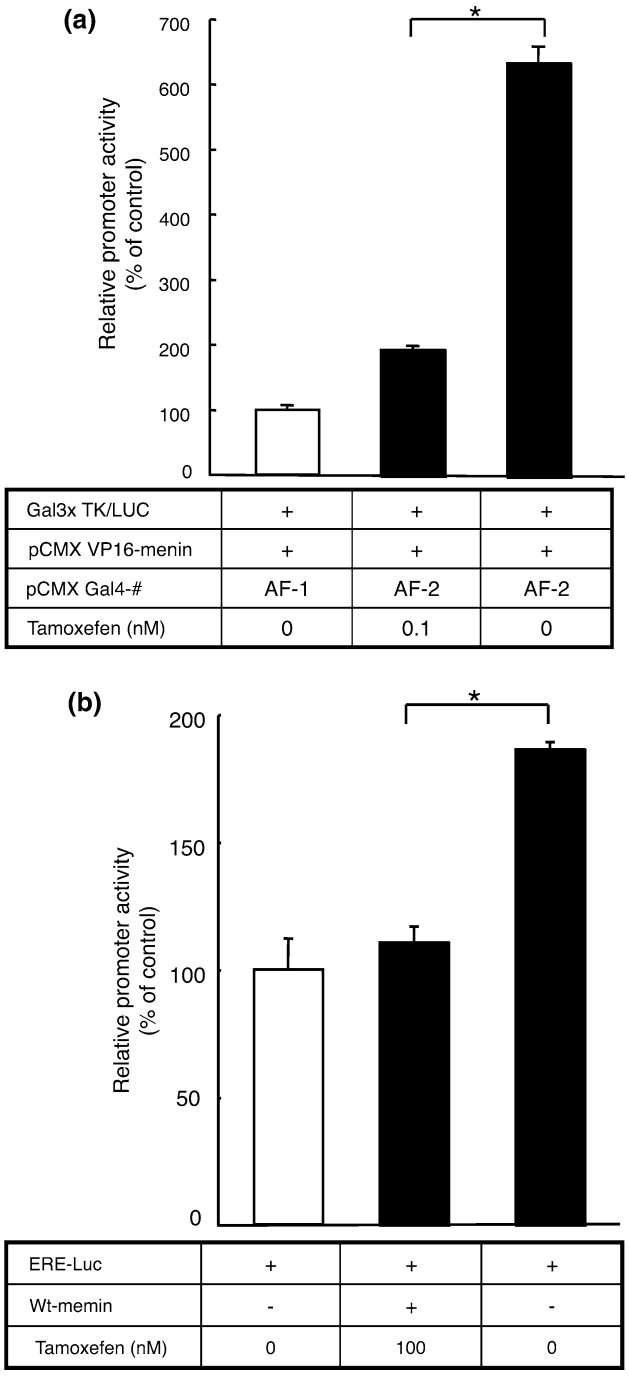
Fig. 3 Effects of ERα knockdown on menin-induced ERE-luciferase activity in MCF-7 cells. **a** ERα siRNA (MCF-7-si) or scrambled siRNA (MCF-7-cont) was transfected into MCF-7 cells with ptk-ERE-Luc along with the menin expression vector. *Top*, efficiency of siRNA on ERα (ER) expression by Western blot analysis. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.01$). **b** MCF-7 cells were seeded in the medium with charcoal-stripped FBS and then cotransfected with the menin and ERE-luciferase reporter genes in the absence or presence of 17β-estradiol. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.005$)

Tamoxifen inhibits the binding of menin to the AF-2 domain of ERα

To investigate the clinical role of menin in ERα activation, we used tamoxifen. Tamoxifen is an antiestrogen drug used extensively for adjuvant therapy of breast cancer. A previous report indicated that tamoxifen inhibits AF-2 but not AF-1 [41]. The reporter plasmid ptk-ERE-Luc and ERα expression plasmid pCER were cotransfected into MCF-7 cells with or without the menin expression vector. The results (Fig. 5a) showed that transfection of the menin expression vector enhanced ERE-luciferase activity. The cells transfected with the reporter gene and tamoxifen have



Gal3xTK/LUC	+	+	+	+	+
pCMX VP16-Gal4	+	-	-	-	-
pCMX VP16-menin	-	+	+	+	+
pCMX Gal4-#	-	ER	AF-1	DBD	AF-2



Gal3x TK/LUC	+	+	+
pCMX VP16-menin	+	+	+
pCMX Gal4-#	AF-1	AF-2	AF-2
Tamoxefen (nM)	0	0.1	0

ERE-Luc	+	+	+
Wt-memin	-	+	-
Tamoxefen (nM)	0	100	0

Fig. 4 Menin directly interacts with ERα in vitro. **a** Pull-down assay. In vitro translated and [^{35}S]Met-labeled wild-type or mutant menin protein was incubated with recombinant GST-ER. Proteins that bound to the GST fusion protein were recovered with glutathione-Sepharose 4B and resolved by SDS–PAGE after elution in SDS sample buffer. The gel was dried and visualized by autoradiography. *Lane 1* positive control, *lane 2* mutant menin, *lane 3* wild-type menin. **b** Mammalian two-hybrid assay. The full-length ERα (1–596), AF-1 (1–180), AF-2 (302–553), and DBD (1–163) were each fused to the GAL4 DNA-binding domain. Cotransfection of the reporter plasmid tk-GALpx3-Luc along with expression vectors for each ER domain and VP16-menin chimeras was performed in COS-7 cells. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.01$)

been incubated more than 24 h (Fig. 5a) compared to the cells in Fig. 4b. Tamoxifen attenuated the effect of menin on ERE-luciferase activation. Furthermore, tamoxifen inhibited the binding of menin to AF-2 in mammalian two-hybrid assay (Fig. 5b). These results indicate that menin bound to the AF-2 domain of ERα might compete with tamoxifen in breast cancer cells.

Clinical study

The clinical characteristics are summarized in Table 1. None of lymph node status, tumor size, stage, and histological grade has shown significant correlation with menin expression. Correlation of each of age and human epidermal growth factor receptor 2 (HER2) expression with menin expression was significant ($P = 0.048$ and 0.037, respectively). When the cut-off value was 50%, 9 out of 20

◀ **Fig. 5** Effects of tamoxifen on menin-induced ERα activation. **a** MCF-7 cells were cotransfected with ptk-ERE-Luc along with the menin expression vector with or without tamoxifen treatment. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.001$). **b** AF-1 (1–180) and AF-2 (302–553) were each fused to the GAL4 DNA-binding domain. Cotransfection of the reporter plasmid tk-GALpx3-Luc along with expression vectors for each ER domain and VP16-menin chimeras with or without tamoxifen treatment was performed in COS-7 cells. All assays were corrected for β-galactosidase activity, and equal total amounts of protein per reaction were used. The results are expressed as luciferase activity relative to that of control cells, which was arbitrarily set at 100. Each data point represents the mean ± SE ($n = 3$) of independent transfections. *Asterisk* denotes a significant difference ($P < 0.001$)

menin-positive patients underwent local or distant recurrence, while only 4 out of 46 menin-negative cases recurred after treated with tamoxifen ($P = 0.0025$). As shown in Fig. 6, Kaplan–Meier analysis showed that menin-negative group had more favorable disease-free survival than menin-positive group by Logrank test ($P < 0.001$).

Discussion

In the present study, we showed that the transcriptional factor menin physically interacts with ERα, leading to increased levels of estrogen-dependent transcription. The biological significance of this finding is that menin functions as a direct coactivator of estrogen.

Table 1 Correlation of menin expression and clinical features in breast cancer patients

	No. of patients	Menin-positive ratio (MR)		
		MR ≦ 50 ($n = 45$)	MR > 50 ($n = 20$)	c^2 test P
Age (years)				
≦50	19	17	2	0.048
>50	46	28	18	
Lymph node				
Negative	53	37	16	0.894
Positive	12	8	4	
Tumor size (cm)				
≦2	27	21	6	0.208
>2	38	24	14	
Stage				
I	26	20	6	0.273
II	39	25	14	
Histology				
Grade 1	22	18	4	0.153
Grade 2	17	9	8	
Grade 3	26	18	8	
HER2				
0	52	39	13	0.037
1+, 2+, 3+	13	6	8	

Numerous studies have shown a crucial role of menin in regulating gene transcription. For instance, menin interacts with a number of transcriptional factors such as JunD, NF-κB, Smad3, and homeobox-containing DNA-binding

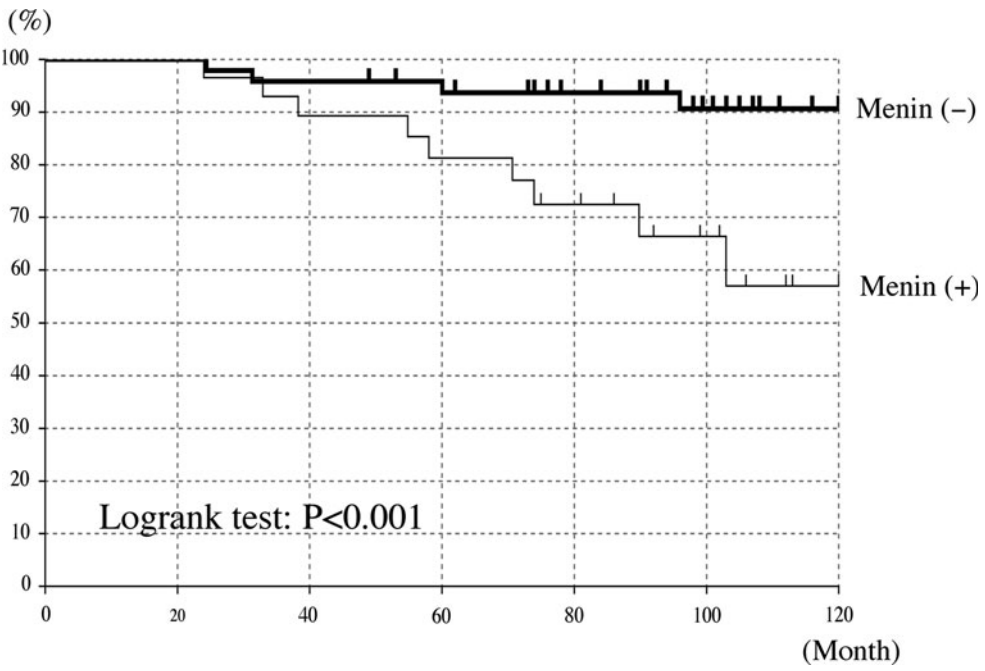


Fig. 6 Relapse-free survival by Kaplan–Meier analysis and Logrank test. Cut-off value of menin expression assessed by immunohistochemistry in breast cancer tissues was 50%

protein Pem [17] and inhibits the activities of JunD and NF-κb [25, 26]. Ectopic expression of menin inhibits promoter activity of the prolactin and insulin genes in pituitary tumor cells or insulinoma cells, respectively [20, 38]. Menin regulates gene transcription at least in part by modulating chromatin structure. Menin has been shown to associate with a protein complex containing *Drosophila trithorax*-like histone lysine methyltransferases, the *MLL* gene products, namely, MLL and MLL2, both of which are SET domain-containing methyltransferases [28, 29]. This complex contains multiple proteins that are homologous to the members of the yeast SET1 complex (COMPASS) and three mammalian SET1-like complexes, including the activating signal cointegrator 2 complex (ASCOM), the HCF-1 complex, and the MLL complex [28], which were previously found to methylate histone H3 lysine 4 (H3K4) and activate gene transcription. The menin-interacting complex isolated from mouse embryonic fibroblasts (MEF) also methylates H3K4 in vitro [28]. These results support a model that menin recruits histone methyltransferases (HMTs) and thus upregulates gene transcription. A recent report showed that menin is a transcriptional coactivator of the nuclear receptors for estrogen and vitamin D (VD). Activation of the endogenous estrogen-responsive TFF1 (*pS2*) gene results in promoter recruitment of menin and in elevated trimethylation of H3K4. Knockdown of menin reduces both activated TFF1 (pS2) transcription and H3K4 trimethylation [39]. In the present study, we also showed that menin functions as a direct coactivator of ERα.

The MEN1 syndrome is diverse in its clinical manifestations; furthermore, nuclear receptors perform many functions in the affected organs in MEN1 patients. For example, elevated levels of parathyroid hormone (PTH) are very common in these patients [30, 40]. In the normal situation, activation of VD receptor (VDR) will inhibit the production and release of PTH. Menin is a regulator of ER and VDR function, and inactivation of menin leads to the disruption of ER-mediated transcription [39]. Prolactinomas are also a common manifestation of MEN1 [38]. ERα has a direct effect on prolactin production in the pituitary gland and is expressed in pituitary adenomas [42].

Several lines of evidence revealed that coregulatory proteins, coactivators, and corepressors modulate the transactivation function of nuclear receptors via protein–protein interaction [43]. Among these coactivators, CBP-300/p300 [44], AIB1 [45], and MDM2 [32] were found to interact with ERα, resulting in an enhanced estrogen-dependent transcription activity. In our mammalian two-hybrid assay, ERα interacted with the menin protein (Fig. 4b). The pull-down assay using GST-ERα with the in vitro translated menin protein showed the capability of their interaction (Fig. 4a). This result is consistent with a recent report in which an almost identical pull-down assay was performed [39]. Our experiments could not clarify whether the interaction between ERα and menin in the living cells is ligand dependent or independent because both in vivo and in vitro assays used the exogenously expressed chimera proteins in poor physiological condition. However, at least, the presence of E2 seems to be a more preferable condition for their interaction. Moreover, the ERE-luciferase reporter assay in COS-7 cells showed that menin enhanced the ligand-dependent transcriptional activity of ERα but did not affect the ligand-independent one (Fig. 2c). This observation is consistent with the finding that the AF-2 domain of ERα, the component for ligand-binding and ligand-dependent activation, is responsible for the interaction with menin (Fig. 4b). All these findings indicate that the interaction between ERα and menin must be important for the biology of breast cancer cells when they respond to E2 for growth.

Transcriptional activation by ERα is mediated by two distinct activation domains, namely, the constitutively active AF-1 located in the N terminus of the receptor and the ligand-activated AF-2 located in the C terminus. AF-1 activity is usually weaker than AF-2 activity, but the two activation domains function synergistically in ERα. A large number of proteins have been identified recently that interact with and regulate the transcriptional activity of ERα by modifying the activity of the ligand-inducible activation domain AF-2 [46–48]. These proteins are believed to facilitate transcription by remodeling chromatin structure and stabilizing the transcription preinitiation apparatus at target genes [49, 50]. Tamoxifen is a selective estrogen receptor modulator used clinically to treat ER-positive breast cancers. Among the corepressors reported to affect tamoxifen action, only one has been suggested to show ERα and tamoxifen specificity [51]. This corepressor, termed RTA, was shown to interact with the N-terminal domain of ERs. With regard to coactivators, in addition to the p160 coactivators, one coactivator that affects tamoxifen activity has been reported, but there is no information on ERα or tamoxifen specificity of this coactivator [52]. In the present study, we demonstrated that menin can bind to AF-2, and tamoxifen inhibits the binding of menin to ERα, but tamoxifen suppressed ERE-luciferase activity only to the levels of nontreated wild-type MCF-7 in menin-overexpressing clones. Antiestrogen therapy with tamoxifen is often initially efficient, but eventually, most tumors become refractory to the antiestrogen treatment. On the basis of the results of this study, it can be hypothesized that the expression of menin in breast cancer cells might be associated with resistance to the antiestrogen therapy; however, a large number of clinical studies will be required to confirm this hypothesis.

The discovery of a second ER form and its therapeutic implications sparked great interest. Both the original ERα

and the more recently identified ERβ subtypes bind and respond similarly to many physiological and pharmacological ligands. The current published data on tumors and patient profiles show that the majority of ER-positive breast tumors contain both ERα and ERβ and that a small population of tumors contains only ERβ [53]. Several studies suggest that ERβ modifies the responses of ERα in breast cancer cells and is generally antiproliferative. This may be because ERβ is less active transcriptionally and constrains ERα activity through heterodimers and/or because the heterodimers or ERβ homodimers may have distinct beneficial activities [53]. In this study, we showed that menin can function as a transcriptional regulator of ERα, further investigations will be needed to determine the role of menin on the activity of ERβ.

On the basis of the results of this study, we further performed menin immunohistochemistry on 65 ER-positive breast cancer samples, all of which had been treated with tamoxifen for 2–5 years. As shown in Fig. 6, menin-positive tumors had a worse clinical outcome than tumors that were menin negative. Recently, Hortado et al. reported that the paired box 2 gene product (*PAX2*) might be a predictive factor for tamoxifen. *PAX2* was reported to bind to *HER2* gene and repress *HER2* expression at transcriptional levels, resulting in up-regulation of tamoxifen responses, when *PAX2* formed a tertiary complex with estrogen receptor and estrogen or tamoxifen [54]. In our clinical study, *HER2* expression of menin-positive tumors was significantly higher than that of menin-negative tumors, suggesting the possibility that the induction of tamoxifen resistance by menin might come from the interaction with *PAX2*, considering from the higher expression of *HER2* in menin-positive tumors.

It is well recognized now that cleavage of the basement membrane and the extracellular matrix (ECM) by cancer cell proteinases is a prerequisite for malignant cells to invade tissues and spread through the body [55]. Matrix metalloproteinases play a highly significant and proven role in these processes [56, 57]. Recent report indicated that ERα can be implicated with signaling pathways and therefore induce cell proliferation and affect the expression of ECM molecules [58]. The effects of estrogen linked to the menin-mediated ERα activation on the expression of ECM molecules deserve further investigations.

In summary, our findings show that menin can function as a transcriptional regulator of ERα. Menin activates ERα activity by binding to the AF-2 domain of ERα; furthermore, because tamoxifen insufficiently inhibits menin-mediated ERα activation, menin is a possible predictive factor of tamoxifen therapy. Furthermore, our clinical study showed that menin-positive tumors had a worse outcome than menin-negative ones. Taken together, menin can function as a predictive factor for tamoxifen resistance.

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