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The splice variant Ehm2/1 in breast cancer MCF-7 cell interacted with β-catenin and increased its localization to plasma membrane

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Abstract

Ehm2, belonging to FERM superfamily, is a metastasis-associated protein. However its function in cancer metastasis and the molecular mechanism is not definitely clear. Alternative splicing is an important biological step during mRNA processing and has been reported to be related with many diseases including cancers. Ehm2 has two transcript variants. Transcript variant 1(Ehm2/1) encodes isoform 1 of 518 amino acids, while transcript variant 2(Ehm2/2) encodes isoform 2 of 913 amino acids. In this study we found that Ehm2/1 was the main transcript variant in breast cancer cell line, MCF-7. Forced expression of Ehm2/1 upregulated the total protein amount but had no effect on the mRNA levels of β-catenin. The increased β-catenin was found to be dominantly located at the cell membrane. Meanwhile knockdown of Ehm2/1 in MCF-7 cells decreased the total protein amount but not the mRNA levels of β-catenin. Further results showed that Ehm2/1 interacted with β-catenin and colocalized with it at the cell membrane. E-cadherin, a partner of β-catenin in cadherin-catenin complexes, was also upregulated by overexpression of Ehm2/1 and also colocalized with it at cell membrane. Meanwhile overexpression of Ehm2/1 inhibited the migration ability of MCF-7 cells. These results suggested that Ehm2/1 may render β-catenin at the cell membrane through interacting with β-catenin and E-cadherin.

Introduction

Expressed in high metastatic cells (Ehm2), also named erythrocyte membrane protein band 4.1-like protein 4B (EBP41L4B), belonging to the FERM (Four.1 protein, ezrin, radixin, moesin) superfamily, was suggested to be linked with cancer metastasis through regulating interactions between cell surface transmembrane proteins and cytoskeletal proteins[1, 2]. However, the role of Ehm2 was not well

known. Human Ehm2 was shown to be androgen-regulated in a human fibrosarcoma cell line model of steroid-regulated cytoskeletal reorganization[3]. Wang *et al*. have reported that expression of the FGFR-4 Arg388 variant resulted in increased expression of Ehm2 in prostate epithelial cells[3, 4]. They also found that Ehm2 expression was upregulated in prostate cancer cell lines and prostate cancer tissues. Enhanced expression of Ehm2 in prostate cancer may promote disease progression and metastasis[4]. In our previous study, we showed that Ehm2 was highly expressed in breast cancer, and its higher expression was correlated with distant metastasis and poor prognosis of the patients[5]. We also showed that knockdown of Ehm2 induced apoptosis potential and decreased the *in vitro* invasive properties of MCF-7 breast cancer cells through regulating expression and activity of matrix metalloproteinase 9.

Alternative splicing is an important biological step during mRNA biosynthesis in eukaryotes and has been reported to be related with many diseases including cancers[6-10]. It is estimated that 90 % of all multi-exon genes are subjected to some form of alternative splicing[11-13]. Alternative splicing of a single gene can give rise to functionally distinct protein isoforms[14-16]. However, very little is known about whether alternate transcripts are a driving force or the result of cancer progression.

Chauhan *et al.* did tissue expression analysis of the human Ehm2 gene and found that there were two Ehm2 protein isoforms (isoform 1 and isoform 2)[3, 17]. Isoform 2, which was translated from Ehm2/2, contains 913 amino acids, whereas isoform 1 exists ubiquitously in testes, prostate and breast, produced from Ehm2/1. In comparison with isoform 2, isoform 1 misses 382 amino acids at the carboxyl terminal region containing recognizable protein motifs[3]. This maybe suggest that Ehm2 isoform 1 function as a constitutively active FERM signaling protein that is controlled by transcriptional regulation, rather than by autoregulation involving intramolecular folding of subdomains[18, 19]. As mentioned above, Ehm2 is a metastasis-associated protein in prostate cancer and breast cancer, but it is difficult to discriminate which transcript variant plays function in these processes. Thus, understanding the function of these transcript variants is necessary to determine their potential functions in breast cancer. We analyzed the levels of transcript variants of Ehm2 in breast cancer cell MCF-7 and MDA-MB-231, and found that MCF-7 cells contained dominantly transcript variant 1 of Ehm2 while MDA-MB-231 cells had both variants at relatively high level. Based on our previous result that knockdown of Ehm2 induced cell apoptosis and decreased the *in vitro* invasive properties of MCF-7 cells[5], together with dominantly expressed Ehm2/1 in MCF-7 cells, we speculated that Ehm2/1 may play functions through regulating β-catenin in MCF-7 cells. In current study we forced the expression of Ehm2/1 in MCF-7 cells and found that upregulation of Ehm2/1 may render β-catenin at the cell membrane through interaction with β-catenin and inhibit the migration ability of MCF-7 cells.

Materials and Methods

Cell cultures

MCF-7 and MDA MB-231 cells and A549 cells were purchased from the ATCC. HEK-293A cell lines were gifted by Professor Junqi He from Capital Medical University. Cells were routinely cultured in DMEM with L-Glutamine (Thermo Fisher Scientific Inc, Carlsbad, USA) supplemented with streptomycin, penicillin(Ameresco, Solon, USA) and 10% fetal bovine serum (ExCell Bio, Shanghai, China) in an incubator at 37℃, 5% CO_2 and 95% humidity.

Gene knockdown and overexpression

Knockdown of Ehm2 in MCF-7 cells was performed using transfection with anti-human Ehm2 hammerhead ribozyme, which was designed based on the secondary structure of Ehm2 transcript variant 1 generated using Zuker's RNA mFold program[20] and was cloned into a mammalian expression pEF6/V5-His-TOPO plasmid vector (Thermo Fisher Scientific Inc, Carlsbad, USA). The stable clones were established by treatment with blasticidine.

Express plasmid of Ehm2/1 (NM_018424.2) tagged with FLAG was purchased from OriGene Technologies (Cat. RC223085, Beijing, China). For overexpression of Ehm2/1, cells were transfected with pCMV-Entry and pCMV-Ehm2/1-FLAG. Stably transfectants were selected with medium containing 500 μg/ml G418.

For transfection, 1 μg of express vectors or ribozyme vectors were mixed with 4 μl of Lipofectamine® 2000 Reagent (Thermo Fisher Scientific Inc, Carlsbad, USA) and upon 20 minutes of complex formation, the liposomes were given to the cells plated in 6-well plates for the analysis of gene expression.

RNA isolation and reverse transcription PCR

Total RNA was extracted from the cell lines with the TRIzol Reagent (Thermo Fisher Scientific Inc, Carlsbad, USA). Extracted RNA was reverse transcribed into first-strand cDNA using IScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, USA). PCR was carried out using a REDTaq^{TM} ReadyMix PCR reaction mix according to the manufacturer's instructions. The specific primers were: Ehm2/1 forward, 5'-CACTTTGAGAGACTGAAGCATCTC-3' and reverse, 5'-CAACTTCTACGACA GGAATATATGC-3'; Ehm2/2 forward, 5'-CCTGTTGCGGATCATGTGAAGTG-3' and reverse, 5'-TATCAGGAAACGGGTTCATTGTATC-3'; β-catenin forward, 5'-AGGGATTTTCTCAGTCCTTC-3' and reverse, 5'-GAACCAAGCATTTTCACCAG -3'; CyclinD1 forward, 5'-GAACAGAAGTGCGAGGAGGAG-3' and reverse, 5'-AGGCGGTAGTAGGACAGGAAG-3'; GAPDH forward, 5'-GGCTGCTTTTAACT CTGGTA-3' and reverse, 5'-GACTGTGGTCATGAGTCCTT-3'. Cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s. This was followed by a final 10 min extension period at 72°C. The products were visualized on 1.2% agarose gel stained with ethidium bromide.

Immunoblotting and isolation of nuclear, cytosol and membrane proteins

Cells were lysed in lysis buffer (20 mM Tris- HCl, pH 7.4, containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 10% glycerol) containing

complete protease inhibitor cocktail(Roche Applied Science, Penzberg, Germany) for 30 min before clarification at 13,000 × g for 20 min. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher Scientific Inc, Carlsbad, USA) and an ELx800 spectrophotometer (BioTek Instruments Inc, Burlington, USA). Equal amount of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose sheets. After blocking for 1 h in 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated with the desired primary antibody overnight. The membranes were then treated with the appropriate HRP-conjugated secondary antibody (115-035-044 and 111-035-003, Jackson ImmunoResearch Inc, West Grove, USA). Protein bands were visualized using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc, Carlsbad, USA), and photographed using a LAS-3000 imager (FujiFilm, Tokyo, Japan).

The primary antibodies used to target β-catenin (ab22656), E-cadherin (ab40772), Snail (ab167609) and LaminA/C (ab108595) were from Abcam Ltd, (Cambridge, UK). Anti-β-actin (SC-130301) and anti-Ehm2 antibodies (SC-14234) were from Santa Cruz (San Diego, California, USA). Anti-FLAG antibody (F-1804) was from Sigma-Aldrich Ltd (Poole, England, UK).

Nuclear, cytosol and membrane proteins analyzed by immunoblotting were isolated using the Nucl-Cyto-Mem Preparation Kit (APPLYGEN, Beijing, China) according to the manufacturer's instructions.

Immunoprecipitation and GST pull-down assay

For immunoprecipitation, the cells were lysed in lysis buffer (20 mM Tris- HCl, pH 7.4, containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 10% glycerol) containing complete protease inhibitor cocktail. Lysates (200 μg of total protein) were incubated with 2 μg of anti-FLAG overnight and then with 20 μl of protein A-agarose beads (GE Healthcare, Chicago, USA) for 4 h at 4 °C. β-catenin, E-cadherin and FLAG were detected by incubating the blots with specific antibodies.

For GST pull-down assays, 293A cells transiently transfected with pCMV-Ehm2/1-FLAG were lysed in lysis buffer (20 mM Tris- HCl, pH 7.4, containing 150 mM NaCl, 0.5% NP-40, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 10% glycerol) containing complete protease inhibitor cocktail. β-catenin-GST fusion proteins and GST proteins (a gift from Dr. He Junqi, Capital Medical University) and glutathione–Sepharose 4B beads (GE Healthcare, Chicago, USA) were added to the lysate. After 4 h incubation at 4 °C, the beads were washed five times in lysis buffer. The obtained samples were analyzed by western blotting.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde in PBS for 10 min at RT. The fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with 2% BSA in PBS for 30 min at room temperature. Thereafter, the cells were incubated with the appropriate primary antibodies in 1% BSA in PBS with 0.2% Triton X-100 for 1.5 h at 37 °C. The primary antibody used to stain β-catenin (9581) was from Cell

Signaling Technology (Beverly, Massachusetts, USA). The primary antibodies used to stain E-cadherin (ab40772) and Ehm2/1(ab77484) were from Abcam Ltd (Cambridge, UK). Anti-FLAG antibody (F-1804) was from Sigma-Aldrich Ltd (Poole, England, UK). Next, the cells were washed three times with PBS and incubated with Alexa Fluor 488 donkey anti-mouse IgG (1423052) or Alexa Fluor 594 donkey anti-rabbit IgG (1454437)(Thermo Fisher Scientific Inc, Carlsbad, USA) in 1% BSA in PBS with 0.2% Triton X-100 for 1 h at room temperature. After three washes with PBS, coverslips were incubated with DAPI for 5 minutes at room temperature. After three washes with PBS and then rinsing in water (Milli-Q; Millipore, Billerica, USA), the coverslips were mounted with DAKO Fluorescent Mounting Medium (DAKO North America, Inc. Carpinteria, USA). Spectral image acquisition was performed using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Cell migration assay

Cell migration was assessed by wound-healing assay. 500,000 cells were seeded into each well of a 6-well plate and allowed to reach near confluence. The layer of cells was then scraped with a fine gauge needle, followed by washing once with phosphate buffered saline. The movement of cells to close the wound was recorded using an Olympus phase contrast microscope (Olympus, Tokyo, Japan) at different time. Cell migration was evaluated with Image J.

Statistical analysis

Experimental procedures were repeated independently at least 3 times. Data were expressed as means ± s.d., and statistical comparisons were made using analysis of variance. Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by the Student's t-test.

Results

1. Ehm2 transcript variants were expressed differently in different breast cancer cells and Ehm2/1 was distributed at cell membrane

To investigate the potential functions of Ehm2 transcript variants in breast cancer, we firstly analyzed the express pattern of Ehm2 transcript variants in two breast cancer cell lines. Ehm2/1 was expressed at moderate level in both cell lines although it was much higher in MDA-MB-231 cells, while Ehm2/2 was highly expressed in MDA-MB-231 cells but very low in MCF-7 cells (Fig. 1A). This result indicates that both transcript variants of Ehm2 are expressed differently and may have distinct roles in breast cancer progression. Western blot results showed that only Ehm2/1(about 58 kDa) was detected in MCF-7 cells (data not shown). Cell immunofluorescence using goat-anti-Ehm2 antibody was carried out to detect the subcellular localization of endogenous Ehm2/1, the results showed that endogenous Ehm2/1 was mainly distributed at the cell membrane (Fig. 1B, Supplementary Fig. S1), although this antibody is not very good at immunofluorescence assay.

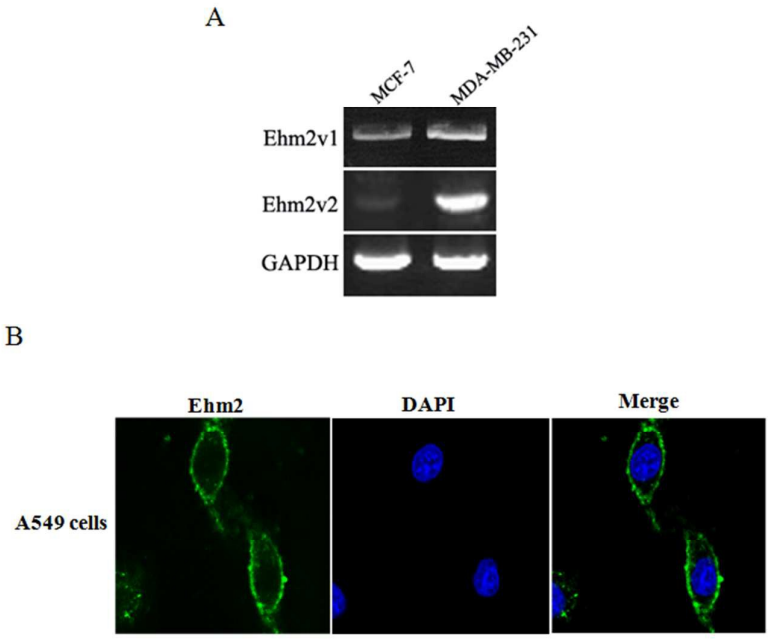

Figure 1. Expression of endogenous Ehm2 and its subcelluar distribution. (A) Ehm2 transcript variants in breast cancer cell lines were differently expressed. RNA was extracted from cell lines. Reverse transcription PCR was carried out using primers specific to Ehm2 transcript variant 1 and 2, GAPDH was used as internal control. (B) Endogenous Ehm2/1 (green) was mainly distributed along the cell membrane.

2. Changing expression Ehm2/1 in MCF-7 led to corresponding alternation of the protein levels but had no effect on mRNA levels of β-catenin

Considering that MCF-7 cells have moderate expression of Ehm2 transcript variant 1 but very low expression of Ehm2 transcript variant 2, we therefore focus on the role of Ehm2/1 in MCF-7 cells. To study the role of Ehm2/1 in MCF-7 cells, we developed Ehm2/1 overexpressed MCF-7 cell line by transfecting MCF-7 cells with pCMV-Ehm2/1-FLAG (MCF-7-Ehm2/1$^{ex)}$ and Ehm2/1 knockdown MCF-7 cell line using anti-Ehm2 hammerhead ribozyme transgenes (MCF-7-ΔEhm2/1). We verified the overexpression of Ehm2/1 using ordinary reverse-transcript PCR and western blotting (Fig. 2A and 2C, Supplementary Fig. S2). The antibody used to detect Ehm2 was raised against a peptide mapping within the internal region of Ehm2, so it can recognize Ehm2/1 and Ehm2/2. The result showed that only Ehm2/1 was detected. We also confirmed that Ehm2 ribozyme transgenes had successfully knocked down the expression of Ehm2/1 within the MCF-7 cells (Fig. 2B and 2D).

In our previous study, we found that knockdown of Ehm2 had inhibitory effects on *in vitro* growth and invasion of MCF-7 cells and significantly decreased the mRNA and protein levels of MMP9 as well as its enzymatic activities[5]. We hypothesized that Ehm2 may play functions through β-catenin, a co-transcript factor in Wnt signaling[21, 22]. Therefore we assessed the expression of β-catenin in MCF-7-Ehm2/1^{ex} and MCF-7-ΔEhm2/1 cells. Overexpression of Ehm2/1 in MCF-7 cells significantly

increased the protein levels of β-catenin ($p < 0.01$, Fig. 2A and Supplementary Fig. S3) but had no effect on its mRNA levels (Fig. 2C and Supplementary Fig. S4). In contrast, knockdown of Ehm2/1 significantly decreased the protein levels of β-catenin ($p < 0.05$, Fig. 2B and Supplementary Fig. 3) and also had no effect on its mRNA levels (Fig. 2D and Supplementary Fig. S4). These results showed that Ehm2/1 regulates the protein levels of β-catenin through other mechanism rather than transcription regulation.

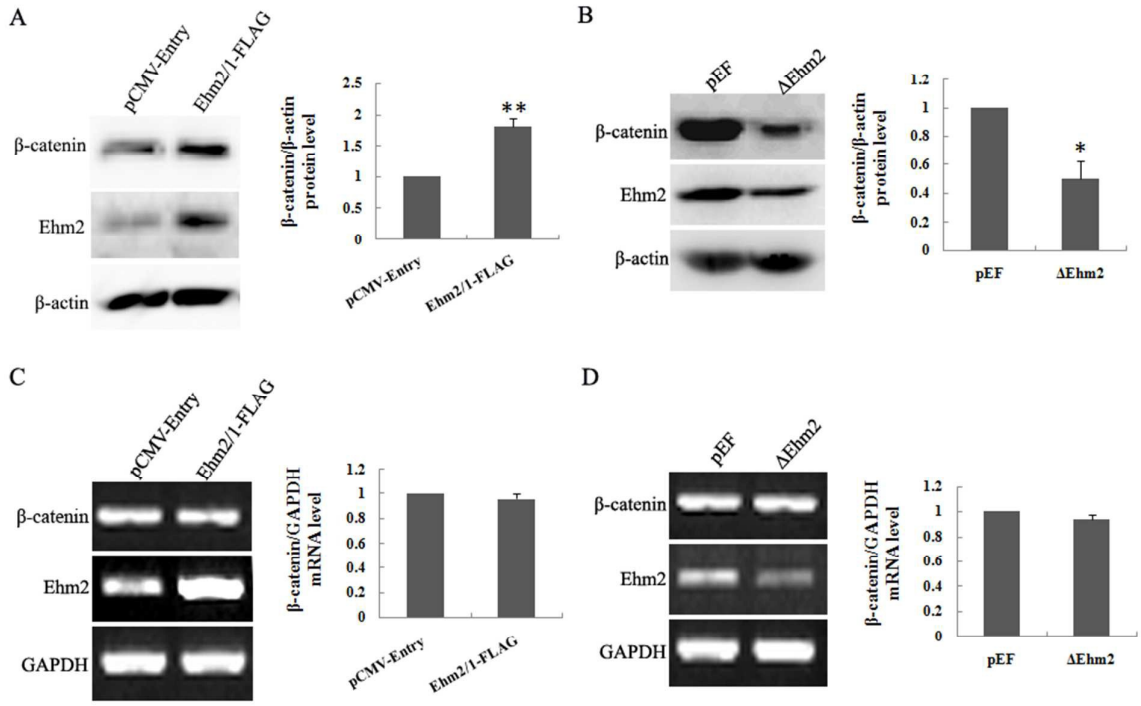

Figure 2. Effect of overexpression and knockdown of Ehm2/1 on endogenous β-catenin. (A) Overexpression of Ehm2/1 increased the endogenously expressed β-catenin protein levels. MCF-7 cells stably transfected with pCMV-Entry and pCMV-Ehm2/1-FLAG were harvested and subjected to western blotting using anti-Ehm2 and anti-β-catenin antibodies (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged. Data were shown relative to the pCMV-Entry control (set to 1, right panel). (B) Knockdown of Ehm2/1 decreased the endogenously expressed β-catenin protein levels. Cells stably transfected with pEF and Ehm2 ribozyme constructs were harvested and subjected to western blotting using anti-Ehm2 and anti-β-catenin antibodies (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged. Data were shown relative to the pEF control (set to 1, right panel). (C,D) mRNA levels of β-catenin were unaffected by expression alternation of Ehm2/1. RNA was extracted from the same cell lines as in A or B. Reverse transcription PCR was carried out using primers specific to Ehm2/1 and β-catenin (left panel) and the intensities were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the corresponding

control (set to 1，right panel). n=3, statistical significance was assessed by paired t-test in comparison to control. Error bars indicate SD, * indicates significance $p < 0.05$, * * indicates significance $p < 0.01$.

3. Ehm2/1 interacted with β-catenin

To understand the molecular mechanism how Ehm2/1 upregulates protein levels of β-catenin, we identified the interaction of Ehm2/1 with β-catenin. Firstly, the interaction between FLAG-tagged Ehm2/1 and endogenous β-catenin was detected by overexpression of Ehm2/1 in MCF-7 cells and coimmunoprecipitation assay (Fig. 3A). The result showed that FLAG coprecipitated β-catenin. The result also verified upregulation of endogenously expressed β-catenin in total lysate of Ehm2/1 overexpressed MCF-7 cells. We confirmed the interaction of Ehm2/1 with β-catenin by GST pull-down assays using GST-fused β-catenin in HEK-293A cells transiently transfected with pCMV-Ehm2/1-FLAG (Fig. 3B). Cell immunofluorescence showed that Ehm2/1 detected using tagged-FLAG antibody also mainly localized at the cell membrane and colocalized with β-catenin (Fig. 3C). These results showed that Ehm2/1 can interact with β-catenin at the cell membrane.

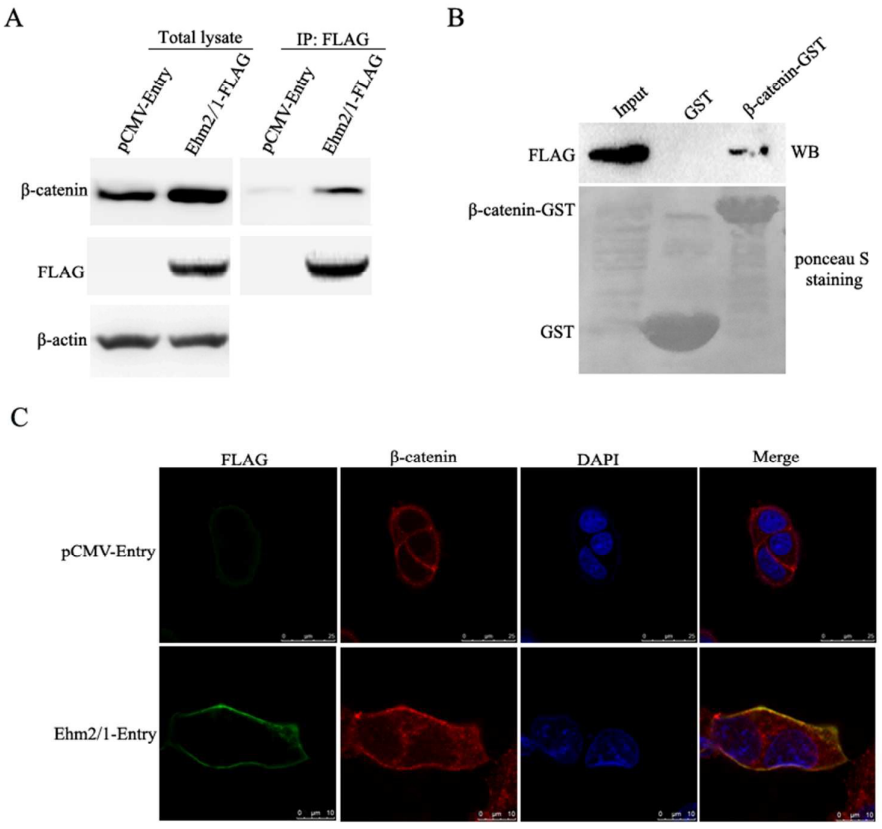

Figure 3. Ehm2 interacted with β-catenin. (A) Lysates of MCF-7 cells stably transfected with pCMV-Entry or pCMV-Ehm2/1-FLAG constructs were immunoprecipitated (IP) with anti-FLAG antibody. (top) Total lysate and

coprecipitated β-catenin were detected by immunoblotting with anti-β-catenin antibody. (middle) Total lysate and immunoprecipitated FLAG were detected with immunoblotting. (bottom) Comparable amounts of β-actin were expressed as loading control. (B) Lysates of HEK-293A cells transfected with pCMV-Ehm2/1-FLAG were examined for GST pull-down assays using GST or β-catenin-GST. Ehm2/1 was detected by immunoblotting with anti-FLAG antibody. Comparable amounts of GST and β-catenin-GST beads were used by staining the membrane using ponceau S. (C) In MCF-7 cells expressing Ehm2/1-FLAG, the Ehm2/1-FLAG staining (green) significantly overlaps with that of β-catenin (red), along the cell membrane.

4. Overexpression of Ehm2/1 increased the fraction of membraneous β-catenin but not cellular and nuclear β-catenin

β-catenin is a critical component of cadherin-based adhesion junction and also a regulatory node of the Wnt signaling pathway[23]. The result that Ehm2/1 interacted with β-catenin at the cell membrane promoted us to check the subcellular localization of β-catenin upon Ehm2/1 upregulation. We isolated the cell membrane, cytosolic and nuclear fractions and detected the levels of β-catenin in each fraction. The result showed that Ehm2/1 upregulation significantly increased the levels of β-catenin in cell membrane fraction ($p < 0.05$) and had no obvious effect on cytosolic and nuclear β-catenin (Fig. 4A), which promotes transcription of target genes, including cyclin D1, c-myc, and so on[24-26]. In fact, overexpression of Ehm2/1 in MCF-7 didn't increase the mRNA levels of cyclin D1, a downstream target gene of β-catenin (Fig. 4B), indicating nuclear localization of β-catenin was not affected after overexpression of Ehm2/1. This result showed that Ehm2/1 may render β-catenin at the cell membrane.

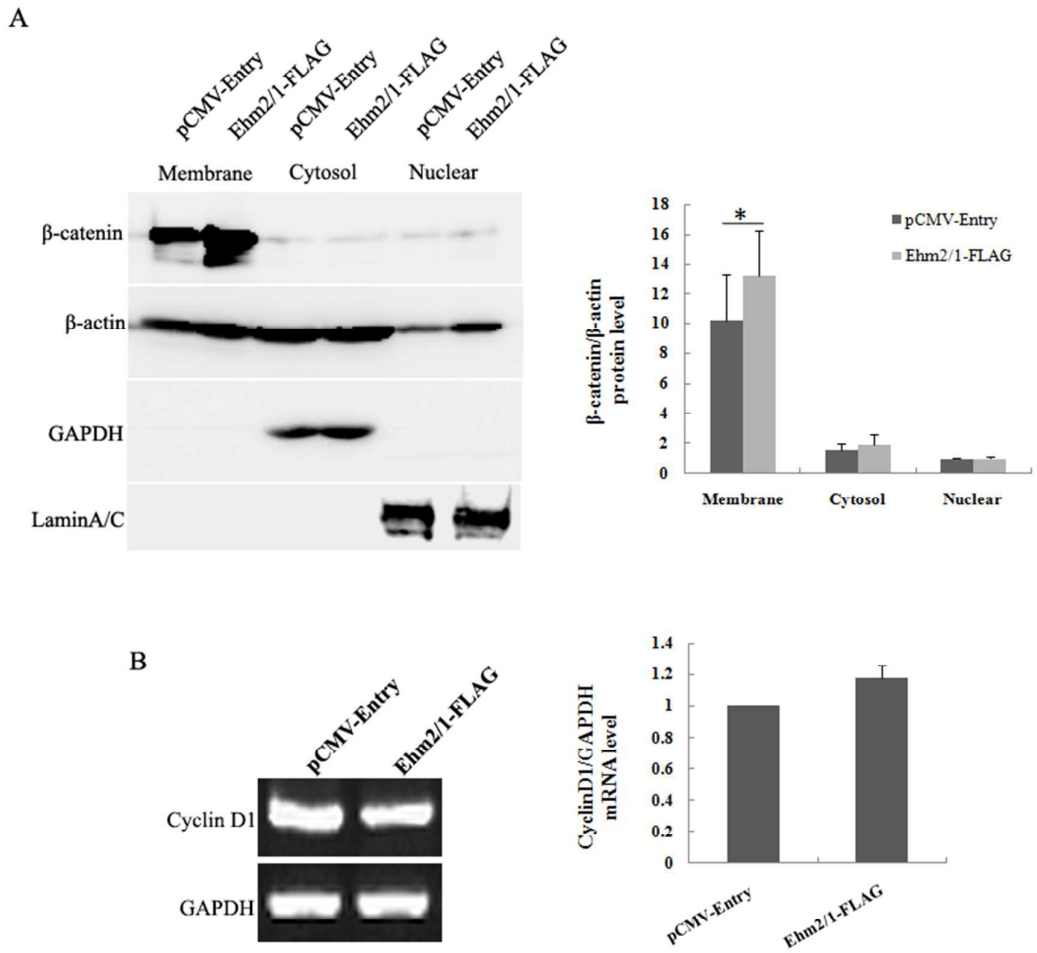

Figure 4. Overexpression of Ehm2/1 elevated cell membrane localization of β-catenin. (A) Overexpression of Ehm2/1 increased β-catenin levels in cell membrane fraction. Cells stably transfected with pCMV-Entry and pCMV-Ehm2/1-FLAG were harvested and fractioned with a kit to isolate cell membrane, cytosolic and nuclear proteins, then β-catenin was analyzed using western blotting, GAPDH was the loading control of cytoplasmic proteins, LaminA/C was used as a loading control for nuclear proteins. β-actin, a loading control for whole cell proteins, together with GAPDH and LaminA/C, was also used as a loading control for cell membrane (left panel). The immunoreactive bands of β-catenin were densitometrically quantified and normalized to the amounts of β-actin present in each sample and then averaged (right panel). (B) A target gene of β-catenin, cyclin D1 was unaffected at transcription level by overexpression of Ehm2/1. RNA isolated from cells stably transfected with pCMV-Entry and pCMV-Ehm2/1-FLAG were reverse transcripted into cDNA, then mRNA levels of cyclin D1 were detected using ordinary RT-PCR (left panel) and the intensities were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the pCMV-Entry control (set to 1，right panel). n=3, statistical significance was assessed by paired t-test in comparison to control. Error bars indicate SD, * indicates significance $p < 0.05$.

5. Overexpression of Ehm2/1 increased the protein levels of E-cadherin and inhibited cell migration

Cell membrane localized β-catenin is a member of E-cadherin/catenin complex, which forms adhesion junction. To investigate if Ehm2/1 regulates cadherin-catenin complex, we analyzed the protein levels of E-cadherin and colocalization of Ehm2/1 with E-cadherin in MCF-7-Ehm2/1^{ex} cells. E-cadherin was significantly upregulated in MCF-7-Ehm2/1^{ex} cells compared with in control cells ($p < 0.05$, Fig. 5A). Cell immunofluorescence showed that Ehm2/1 mainly distributed at the cell membrane and colocalized with E-cadherin (Fig. 5B). Adhesion junction was associated with cell migration, therefore we analyzed the migration ability of MCF-7-Ehm2/1^{ex} cells using wounding assay. The result showed that upregulation of Ehm2/1 caused an obviously decreased cell migration, although the tendency wasn't significant (Fig. 5C, $p > 0.05$), which in line with increasing distribution of β-catenin at the cell membrane. In fact, upregulation of Ehm2/1 not only decreased cell migration, but also reduced the invasion ability of MCF-7 cells ($p < 0.05$, Supplementary Fig. S5).

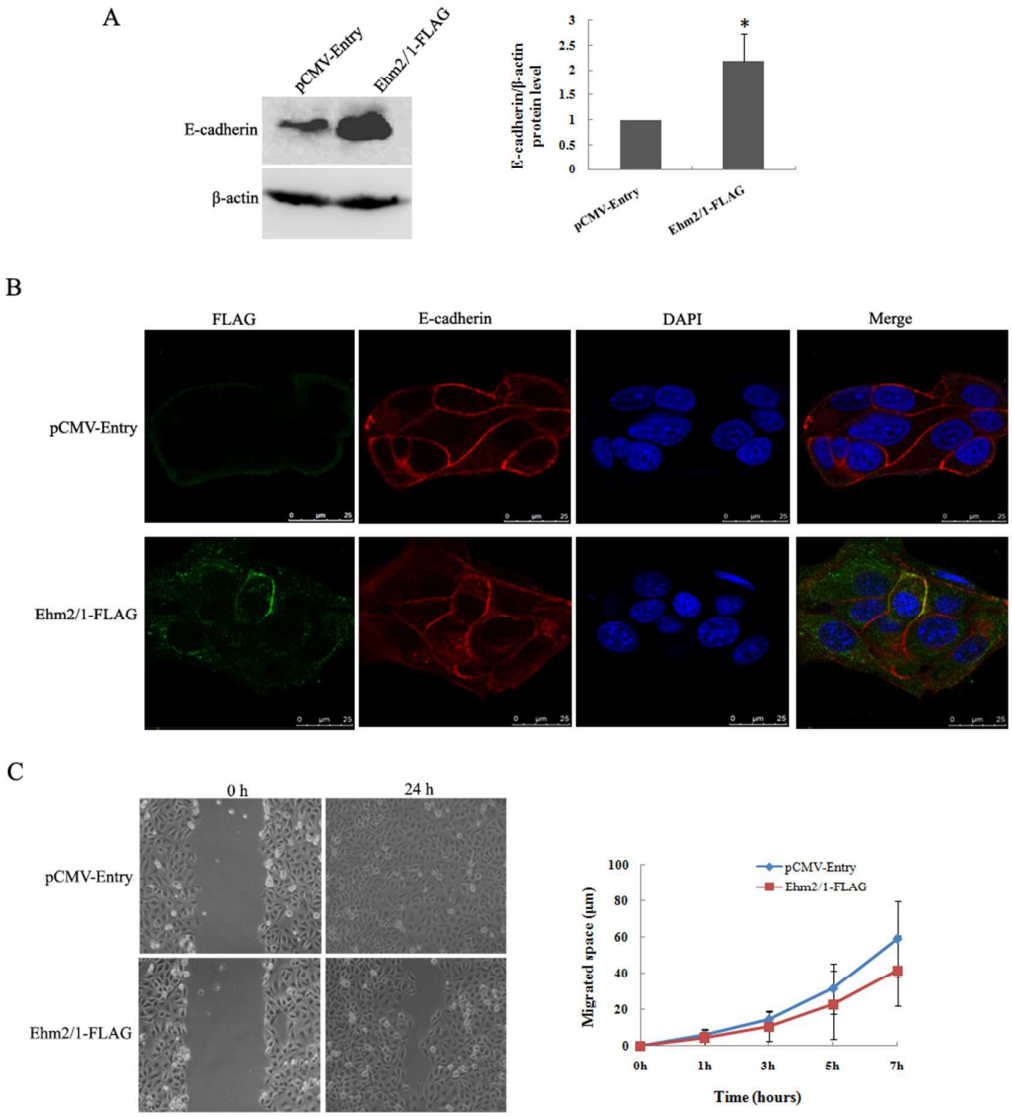

Figure 5. Effect of Ehm2/1 upregulation on endogenous E-cadherin and cell migration. (A) Overexpression of Ehm2/1 increased the endogenously expressed E-cadherin protein levels. Cells stably transfected with pCMV-Entry and pCMV-Ehm2/1-FLAG were harvested and subjected to western blotting using anti-E-cadherin antibodies (left panel) and the intensities of E-cadhesion bands were densitometrically quantified and normalized to the amounts of GAPDH and shown relative to the pCMV-Entry control (set to 1，right panel). n=3, statistical significance was assessed by paired t-test in comparison to control. Error bars indicate SD, * indicates significance $p < 0.05$. (B) In MCF-7 cells expressing Ehm2/1-FLAG, the Ehm2/1-FLAG staining (green) significantly overlaps with that of E-cadherin (red), along the cell membrane. (C) Ehm2/1 upregulation reduced cell migration compared with the pCMV-Entry group, although the difference was not significant. Shown are representative results of three independent experiments of wounding assay. Quantification of migrated space is shown in the right graph (n=3). Error bars indicate

SD.

Discussion

Ehm2/1 is generated by transcript variant 1, which lacks several exons and includes an alternate 3' terminal exon, compared to variant 2. So Ehm2/1 is shorter and has a distinct C-terminus, compared to Ehm2/2. The biological functions of Ehm2/1 and Ehm2/2 are not definitely known. In the present study, we found that MCF-7 cells mainly express Ehm2/1, which is a membrane-associated protein. One of the main findings of our present study is that Ehm2/1 interacts with β-catenin and retards it at the cell membrane. Membranous β-catenin is a critical component of cadherin-based cell-cell adhesion. Our result also confirmed that Ehm2/1 interacts with E-cadherin at the cell membrane and upregulates its protein levels.

Ehm2 is a FERM domain-containing protein, originally identified as a metastasis promoting protein in murine melanoma cells[2]. FERM domain-containing proteins have a conserved FERM domain, which mediates protein-protein interactions[27-31]. FERM domains can mediate intermolecular interactions, usually interacting with the cytoplasmic tails of transmembrane proteins[18, 32, 33]. For example, the ERM proteins bind via their FERM domains to the cytoplasmic domains of transmembrane proteins, such as CD44[18]. Murine Ehm2, also called Lulu2, is a potent activator of cortical myosin II contractile forces in epithelial cells[34]. Murine Ehm2 can interact with p114RhoGEF through its FERM domain and be recruited at cell-cell boundaries[35]. The *Drosopholia* orthologue of Ehm2, called Yurt, was reported to be a negative regulator of apical membrane size in epithelial cells[36]. Yurt was recruited to the apical membrane by Crb and can bind directly to the FDB site in the cytoplasmic tail of Crb through its FERM domain[37]. Zebrafish Moe, the sole Ehm2 molecule in the species, participates in layering of the retina and inflation of the brain ventricles as well as restricting the photoreceptor apical domain[38]. Moe also interacts with and negatively regulates Crumbs, thereby restricting apical membrane size in epithelial structures[39]. Mammalian EPB41L5, a FERM protein very closed to Ehm2 (EPB41L4B), associates with the intracellular domains of Crumbs through its FERM domain and is involved in maintaining cell polarity[40]. Like its orthologues in other species and homology in mammals, human endogenous Ehm2 was distributed at the cell membrane. Considering Ehm2/1 isn't highly expressed in MCF-7 cells and Ehm2 antibody is not quite right for cell immunofluorescence, human lung adenocarcinoma epithelial A549 cells were used for endogenous distribution assay. Exogenous overexpressed Ehm2 was also distributed at the cell membrane.

β-catenin is a critical component of cadherin-based cell-cell adhesion and also a regulatory node of the wnt signaling pathway[41]. It links cadherins indirectly to the actin cytoskeleton[42, 43]. It also interacts with the LEF/TCF family members of transcriptional activators as a critical intermediate in wnt signal transduction pathways[24, 44-47]. In this study we found that β-catenin can be regulated by Ehm2/1 and be retained at cell membrane via interacting with Ehm2/1. The supporting evidences came from (□) in extracts of those transfected MCF-7 cells FLAG-tagged

Ehm2/1 could co-immunoprecipitated β-catenin; (□) β-catenin can pull down FLAG-tagged Ehm2/1 in extracts of those transfected 293A cells; (□) FLAG-tagged Ehm2/1 co-localized with β-catenin at the cell membrane. This result was consistent with the role of 4.1R, the prototypical member of the protein 4.1 superfamily, in linking the cadherin/catenin complex to the cytoskeleton through its direct interaction with β-catenin[48, 49]. Therefore we speculated that the FERM domain of Ehm2/1 behaves similarly to those of the 4.1 and ERM proteins. But we can't make sure whether the interaction of Ehm2/1 with β-catenin is direct or indirect. EPB41L5 can bind to the C-terminal armadillo region of p120ctn through its N-terminal FERM domain[50]. In the future work we will analyze the structure basis of Ehm2/1 interaction with β-catenin, verifying the binding of N-terminal FERM domain of Ehm2/1 to armadillo region of β-catenin. Furthermore, we can't exclude the possibility of Ehm2/1 indirect interaction with β-catenin. In fact, Ehm2/1 also co-localized with E-cadherin at cell-cell contacts. Ehm2/1 has the potential ability to indirectly interact with β-catenin through binding to E-cadherin. Ezrin, forming a subfamily of conserved proteins in the band 4.1 superfamily with radixin, moesin and merlin, regulates cell-cell and cell-matrix adhesion, by interacting with cell adhesion molecules E-cadherin and β-catenin[51]. Cadherin-catenin complexes at adheren junctions can decrease cell migration and cell invasion. In our study we found that MCF-7 cells overexpressed Ehm2/1 showed decreasing tendency in cell migration, although the difference was not statistically significant. The reason why the difference was not statistically significant was that the basal E-cadherin and β-catenin levels were quite high in MCF-7 cells, we guess. Our results showed that upregulation of Ehm2/1 indeed reduced the invasion ability of MCF-7 cells significantly ($p < 0.05$). Considering the important role of Ehm2/1 in regulating cell migration and invasion through β-catenin-E cadherin axis, we speculated Ehm2/1 have negative regulation in EMT. Our results that overexpression of Ehm2/1 decreased the protein levels of an EMT marker Snail further confirmed our hypothesis (Supplementary Fig. S6). However, how Ehm2/1 is connected to E-cadherin at the molecular level and how Ehm2/1 regulates EMT remain to be elucidated.

In summary, we demonstrated here that Ehm2/1 is the principal Ehm2 protein in MCF-7 cells and is a regulator of the cadherin-catenin complexes. Elucidating the more detailed mechanisms regulating the Ehm2/1-cadherin-catenin system is a future important challenge to understand the role of Ehm2.

Acknowledgement

The work was supported by the Beijing Municipal Science & Technology Commission (no. Z151100001615039) and was also supported by Joint Research Fund on Basic and clinical medicine from Capital Medical University (12JL14).

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