Angiogenesis, Metastasis, and the Cellular Microenvironment

Clinical Implications of the Influence of Ehm2 on the Aggressiveness of Breast Cancer Cells through Regulation of Matrix Metalloproteinase-9 Expression

Hefen Yu¹,², Lin Ye¹, Robert E. Mansel¹, Yuxiang Zhang², and Wen G. Jiang¹

Abstract

Ehm2, a member of NF2/ERM/4.1 superfamily, has been indicated in disease progression and metastasis of prostate cancer. However, its function and implication in malignancies remain largely unknown. The present study aimed to examine the role of Ehm2 in breast cancer. We first constructed a hammerhead ribozyme transgene to knock down Ehm2 expression in breast cancer cells. The effect on growth, cell matrix adhesion, motility, and invasion following knockdown of Ehm2 was then investigated using in vitro models. Reduction of Ehm2 had inhibitory effects on in vitro growth and invasion of breast cancer cells. Flow cytometric analysis showed that knockdown of Ehm2 induced apoptosis. Knockdown of Ehm2 also significantly decreased matrix metalloproteinase 9 mRNA and protein levels, as well as the corresponding enzymatic activity, and consequently led to a reduction of the invasion. The expression pattern of Ehm2 in a cohort of breast specimens (normal, n = 33; cancer, n = 127) was analyzed using both quantitative real-time PCR and immunohistochemical staining. Increased expression of Ehm2 in breast cancer was seen at both mRNA and protein levels. Higher levels of Ehm2 transcripts were correlated with disease progression, metastasis, and poor prognosis. Disease-free survival of the patients with lower levels of Ehm2 was 135.8 (95% confidence interval, 125.1-146.5) months, significantly longer compared with 102.5 (95% confidence interval, 78.7-126.4) months of patients with higher levels of Ehm2 expression (P = 0.039). Taken together, increased Ehm2 expression correlates with poor prognosis and metastasis. Ehm2 may promote the invasive ability of breast cancer cells via regulation of matrix metalloproteinase 9.

Introduction

Malignant behavior of a tumor comes from a complex network of interactions between tumor cells, tumor microenvironment, and the organism that contains the tumor (1). Depicting the network of simultaneous events that take place within both tumor cells and tumor stroma becomes more important to push forward the frontiers of cancer research. Metastatic disease accounts for ~90% of all cancer-related deaths (2). The process of cancer metastasis consists of linked sequential steps, including detachment, invasion, intravasation, circulation, adhesion, extravasation, and growth in distant organs. Each of these steps may be promoted by a specific group of factors. Proteins involved in mediating cell migration and attachment have been identified, and many of these proteins contain highly conserved protein interaction domains. One such family of proteins contains a 4.1 protein/ezrin/radixin/moesin (FERM) domain, which functions as a protein docking surface with the cytoplasmic tail of transmembrane proteins such as CD44 (3).

FERM domain proteins belong to the NF2/ERM/4.1 superfamily, which have a conserved molecular structure, called FERM domain. The FERM domain consists of three subdomains that form a cloverleaf structure with lobes of ~100 amino acids each (F1, F2, and F3) and each lobe representing a compactly folded structure itself (3-5). FERM domains mediate protein-protein interactions, and two different interactions have been described. First, FERM domains can mediate intermolecular interactions, usually allowing docking with the cytoplasmic tails of transmembrane proteins. The best-characterized FERM domain–mediated interactions are those of the ERM family of proteins. The ERM proteins bind via their FERM domains to the cytoplasmic domains of transmembrane proteins, e.g., CD44 (3). Other examples of FERM domain–mediated intermolecular interactions...
include binding of the talin FERM domain to the β subunit of integrins and the interaction of the FERM domains of Janus-activated kinases with the γc and gp130 subunits of cytokine receptors (6, 7). Second, FERM domains function in either intramolecular or homophilic intermolecular interactions. One example is that the FERM domains of the ERM proteins bind intramolecularly to a site within the COOH terminus (3). This interaction obscures the CD44 binding site within the FERM domain and an actin binding site in the COOH terminal tail. Regulation of this intramolecular interaction modulates FERM protein--mediated intermolecular interactions. The FERM domain of focal adhesion kinase interacts with the COOH terminal catalytic domain, and this interaction represses its catalytic activity (8).

FERM domain proteins can be activated in two ways. One is the phosphorylation of threonine residues in the COOH terminal domain (9), and the other is the binding of phosphoinositides to the FERM domain (10). Both change the conformation of the protein and expose target protein binding sites in the FERM domain. Most FERM domain proteins contain one or more functional components in the COOH terminal half of the protein that encode an actin or spectrin binding domain, a protein tyrosine phosphatase activity, PDZ domain, or even a Rho GEF function (11). Activated ezrin, for example, binds to the cytosolic tail of the membrane protein CD44 through its FERM domain and to actin molecules through an actin binding domain, thereby tethering actin filaments to the plasma membrane (3).

Ehm2 (expressed in high metastatic cells), belonging to the NF2/ERM/4.1 superfamily, was first discovered in 1996, and its gene was cloned in 2000 (12, 13). It was expressed in high, but not in low, metastatic murine melanoma cells (12). It was proposed that altered expression levels of Ehm2 were likely to be linked to one or more steps of cancer metastasis through regulating inter- actions between cell surface transmembrane proteins and cytoskeletal proteins (12). Human Ehm2 has been shown to be regulated by androgen in a human fibrosarcoma cell line model studying steroid-regulated cytoskeletal reorganization (14). Chauhan et al. did tissue expression analysis of the human Ehm2 gene and found that there are two Ehm2 protein isoforms (isoform 1 and isoform 2). Isoform 1 is brain-specific, containing 913 amino acids, whereas isoform 2 exists in testes, prostate, and breast. In comparison with isoform 1, isoform 2 misses 382 amino acids at the carboxy terminal (15). A recent study has shown that Ehm2 was overexpressed in prostate cancer and may enhance disease progression and metastasis (16). However, the biological function of Ehm2 and its role in many other cancers remain unknown.

In this study, we examined the expression of Ehm2 in human breast cancer specimens and cell lines. We found that Ehm2 was highly expressed in breast cancer and cell lines, and its high expression was correlated with metastasis and poor prognosis. An Ehm2 knockdown cell model using hammerhead ribozymes was used to study the function of Ehm2 in vitro.

Materials and Methods

Materials and cell culture

Breast cancer cell lines were purchased from the European Collection of Animal Cell Cultures. Cells were routinely cultured in DMEM/Ham’s F12 with L-glutamine (PAA Laboratories) supplemented with streptomycin, penicillin, and 10% FCS (PAA Laboratories), in an incubator at 37.0°C, 5% CO2, and 95% humidity. Polyclonal goat anti-human Ehm2, polyclonal mouse anti-human matrix metalloproteinase 9 (MMP9), and monoclonal mouse anti-human glyceraldehyde-3-phosphate dehydrogenase antibodies were purchased from Santa Cruz Biotechnologies, Inc. Unless stated, other materials and reagents were purchased from Sigma-Aldrich Ltd.

Human breast specimens

A total of 160 breast samples was obtained from breast cancer patients (33 were background normal breast tissues, and 127 were breast cancer tissues). These tissues were collected immediately after mastectomy and snap frozen in liquid nitrogen, with approval of the local ethical committee. Background normal mammary tissues were removed from the same patients. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumor deposits. The median follow-up for the cohort was 120 months (June 2004). The relevant information is provided in Table 1.

Immunohistochemical staining of Ehm2 in breast specimens

Frozen sections of breast tumors and background tissues were cut into 6-μm sections using a cryostat (Leica Microsystems Ltd.). The sections were mounted on super frost plus microscope slides, fixed in a 1:1 mixture of acetone and methanol for 20 minutes, and air-dried. The sections were stored at –20°C. Staining for each molecule was conducted on all the slides at the same time in a single batch to avoid variance in experimental conditions. The sections were first placed in Optimax wash buffer (BioGenex) for 5 to 10 minutes to rehydrate. Sections were incubated for 20 minutes in a blocking solution that contained 10% horse serum and probed with the primary antibody (goat anti-human Ehm2) at a concentration of 1:100 for 60 minutes. The dilution chosen here was based on an evaluation test, during which the antibody was tested over a range of dilution from 1:10 to 1:1,000. Primary antibodies were omitted in the negative controls. Unbound primary antibody was then removed by washing the sections four times in wash buffer. A universal secondary antibody (Vectorstain ABC kit, Vector Laboratories, Inc.) was then applied for 30 minutes at room temperature. Following washings, the avidin-biotin complex (Vector Laboratories) was then applied to the sections followed by extensive...
wash. Diaminobenzidine chromogen (Vector Laboratories) was then added to the sections that were incubated in the dark for 5 minutes. Sections were then counterstained in Gill’s hematoxylin and dehydrated in ascending grades of ethanol before clearing in xylene and mounting with a coverslip.

Knockdown of Ehm2 in MCF-7 cells using the ribozyme transgenes

Antihuman Ehm2 hammerhead ribozymes were designed based on the secondary structure of human Ehm2 mRNA using Zuker’s RNA mFold program (17). The ribozymes were cloned into a mammalian expression pEF6/V5-His-TOPO plasmid vector (Invitrogen Ltd.). Ribozyme transgenes and control plasmid vectors were then transfected into MCF-7 cells, respectively. After intense selection using blasticidin, the transfectants were verified for the success of the knockdown before being used in the following experiments.

RNA isolation and reverse transcription PCR

RNA was isolated using Total RNA Isolation Reagent (ABgene). Reverse transcription was done using the DuraScript reverse transcription-PCR (RT-PCR) kit, followed by PCR using a REDTaq ReadyMix PCR reaction mix. Primer sequences are shown in Table 2. Cycling conditions were 94°C for 5 minutes, followed by 36 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds.

### Table 1. Transcript levels of Ehm2 and MMP9 in breast cancer

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>n</th>
<th>Transcripts (copies/μL)</th>
<th>Ehm2</th>
<th>MMP9</th>
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<tr>
<td>Tissue sample</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Normal</td>
<td>33</td>
<td>6.9 ± 5.7</td>
<td>11.6 ± 7.4</td>
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<tr>
<td>Tumor</td>
<td>127</td>
<td>20.6 ± 3.5</td>
<td>127.5 ± 98.9</td>
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</tr>
<tr>
<td>NPI</td>
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<tr>
<td>1 (NPI score &lt; 3.4)</td>
<td>72</td>
<td>11.8 ± 6.7</td>
<td>207.0 ± 189.0</td>
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<tr>
<td>2 (NPI score = 3.4-5.4)</td>
<td>39</td>
<td>35.5 ± 11.0</td>
<td>56.2 ± 52.8</td>
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</tr>
<tr>
<td>3 (NPI score &gt; 5.4)</td>
<td>16</td>
<td>40.3 ± 5.6</td>
<td>20.3 ± 12.9</td>
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<td>Tumor grade</td>
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<tr>
<td>1</td>
<td>24</td>
<td>8.0 ± 1.4</td>
<td>9.4 ± 7.4</td>
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</tr>
<tr>
<td>2</td>
<td>44</td>
<td>49.5 ± 5.5</td>
<td>4.4 ± 2.3</td>
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</tr>
<tr>
<td>3</td>
<td>59</td>
<td>20.1 ± 6.1</td>
<td>258.0 ± 205.0</td>
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<td>TNM staging</td>
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<td>I</td>
<td>73</td>
<td>32.0 ± 3.3</td>
<td>210.0 ± 189.0</td>
<td></td>
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<tr>
<td>II</td>
<td>42</td>
<td>13.4 ± 2.2</td>
<td>5.5 ± 4.5</td>
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<tr>
<td>III</td>
<td>8</td>
<td>47.0 ± 5.4</td>
<td>0.6 ± 0.6</td>
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<tr>
<td>IV</td>
<td>4</td>
<td>104.7 ± 54.0</td>
<td>0 ± 0</td>
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<td>Survival status</td>
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<tr>
<td>Disease-free</td>
<td>92</td>
<td>18.6 ± 4.42</td>
<td>16.3 ± 6.2</td>
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<tr>
<td>With metastases</td>
<td>13</td>
<td>64.0 ± 25.6</td>
<td>1,829.0 ± 1,541.0</td>
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<tr>
<td>With local recurrence</td>
<td>6</td>
<td>14.5 ± 3.2</td>
<td>33.3 ± 33.1</td>
<td></td>
</tr>
<tr>
<td>Died of breast cancer</td>
<td>16</td>
<td>65.4 ± 5.1</td>
<td>1.5 ± 0.7</td>
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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Q-PCR, quantitative PCR.

### Table 2. Primer sequences used in current study

<table>
<thead>
<tr>
<th>Molecular</th>
<th>Sense primers (5’-3’)</th>
<th>Antisense primers (5’-3’)</th>
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<td>Ehm2</td>
<td>gaaattggaccctgctttag</td>
<td>acgagtgtcattgtgcctt</td>
</tr>
<tr>
<td>Ehm2 (Q-PCR)</td>
<td>aaaggcctaggtgtt</td>
<td>actgaacgtcagctacagagtcgaggaactgagag</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ggtgctcttcactcgta</td>
<td>gagcctgtgctgcatagctt</td>
</tr>
<tr>
<td>GAPDH (Q-PCR)</td>
<td>ctgagctgctgcttgagc</td>
<td>actgaacgtcagctacagagtcgatgctt</td>
</tr>
<tr>
<td>MMP9</td>
<td>aactagcggctggaacaag</td>
<td>attcagctgccttgtcagc</td>
</tr>
<tr>
<td>MMP9 (Q-PCR)</td>
<td>aactagcggctggaacaag</td>
<td>actgaacgtcagctacagagtcgatgctt</td>
</tr>
</tbody>
</table>

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Q-PCR, quantitative PCR.
This was followed by a final 10-minute extension period at 72°C. The products were visualized on 2% agarose gel stained with ethidium bromide.

Real-time quantitative PCR

The assay was based on the Amplifiouor technology, and primers were designed by Beacon Designer software, which included complementary sequence to universal Z probe (Intergen, Inc.). Primer sequences are shown in Table 2. Each reaction contained half of the 2x concentrated Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer (which has the Z sequence), 10 pmol of FAM-tagged universal Z probe (Intergen), and cDNA (equivalent to 50 ng RNA). The quantitative PCR was carried using an IcyclerIQ (Bio-Rad), which is equipped with an optical unit that allows real-time detection of 96 reactions. The following conditions were used in the reaction 94°C for 12 minutes, 60 cycles of 94°C for 15 seconds, 55°C for 40 seconds (the data capture step), and 72°C for 20 seconds. The levels of the transcripts were generated from an internal standard that was simultaneously amplified with the samples.

Western blot analysis

The protein concentration in cell lysates was determined using the DC Protein Assay kit (Bio-Rad) and an ELx800 spectrophotometer (Bio-Tek). Equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose sheets. Proteins were then probed with the respective primary antibodies and corresponding peroxidase-conjugated secondary antibodies. Protein bands were visualized using the Supersignal West Dura system (Pierce Biotechnology, Inc.) and photographed using an UVTech imager (UVTech, Inc.).

In vitro cell growth assay

A standard procedure was used as previously described (18, 19). Cells were plated into a 96-well plate (2,500 cells per well). Cell growth was assessed after 1, 3, and 5 days. Crystal violet was used to stain cells, and absorbance was determined at a wavelength of 540 nm using a spectrophotometer (Bio-Tek, ELx800).

In vitro invasion assay

This was done according to an established procedure (20). Transwell inserts with 8-μm pore size were coated with 50 μg Matrigel (BD Matrigel Basement Membrane Matrix) and air-dried. The Matrigel was rehydrated before use. Twenty thousand cells were added to each well. After 96 hours, cells that had migrated through the matrix to the other side of the insert were fixed in 4% formalin, stained with 0.5% (w/v) crystal violet, and counted under a microscope.

Wounding assay

Two hundred fifty thousand cells were seeded into each well of a 24-well plate and allowed to reach subconfluence (21). The monolayer of cells was then scraped with a fine gauge needle. Migration of cells into the wounding space was recorded using a time-lapse video recorder and analyzed using the Optimas 6.0 motion analysis.

Cell-matrix adhesion assay

This procedure was previously described (20): 40,000 cells were added in each well of a 96-well plate, which was precoated with the Matrigel (5 μg/well). After 40 minutes of incubation, nonadherent cells were washed off using balanced salt solution buffer. The remaining adhered cells were fixed, stained, and then counted.

Flow cytometric analysis of apoptosis

All cells, including those floating in the culture medium, were harvested after a period of incubation. The apoptotic population of the cells was determined using Vybrant Apoptosis Assay kit (Invitrogen), in which FITC-labeled Annexin V was used to identify apoptotic cells (22). Briefly, the cells were washed in cold PBS and resuspended in 1× Annexin-binding buffer at a density of 1 x 10^6 cells/mL after centrifugation. Five microliters of FITC Annexin V and 1 μL of the propidium iodide working solution (100 μg/mL) were added to 100 μL of the cell suspension. Following 15 minutes of incubation at room temperature, 400 μL of 1× Annexin-binding buffer were added and mixed gently, and the samples were kept on ice. The stained cells were immediately analyzed using a flow cytometer (CyFlow SL) and FlowMax software package (Partec GmbH).

Gelatin zymography assay

MMP9 activity was assayed as described previously (23). Briefly, cells were grown to 70% confluence, washed twice with 1× balanced salt solution and once with serum-free DMEM, and incubated in serum-free medium. After 24 hours, conditioned medium was collected. Protein samples were prepared in nonreducing sample buffer containing 0.625 mmol/L Tris-HCl, 10% glycerol, 2% SDS, and 2% bromphenol blue. Proteins (40 μg/lane) were separated using SDS-PAGE on gels containing 1 mg/mL gelatin (Bio-Rad). Gels were renatured for 1 hour at room temperature in a solution containing 50 mmol/L Tris-HCl (pH 7.6), 5 mmol/L CaCl2, and 2.5% Triton X-100. Gels were incubated overnight at 37°C in a buffer containing 50 mmol/L Tris-HCl (pH 7.6) and 5 mmol/L CaCl2. The gel was stained with Coomassie blue. The brightness of clear bands, where MMP9 was located and gelatin was degraded, was analyzed using densitometry.

Statistical analysis

Statistical analysis was done using the Minitab statistical software package (version 14). Nonnormally distributed data were assessed using the Mann-Whitney test, whereas the two-sample t test was used for normally distributed data. Kaplan-Meier survival analysis and cyclooxygenase hazardous proportion analysis were done using SPSS statistical software (version 12, SPSS, Inc.). Differences were considered to be statistically significant at P < 0.05.
Results

The expression of Ehm2 in breast cancer

The expression of Ehm2 was examined in breast cancer cell lines and a cohort of breast tumor tissues. The expression of Ehm2 mRNA in breast cancer cell lines was determined using RT-PCR. Ehm2 was highly expressed in the three breast cancer cell lines (Fig. 1A). We quantified Ehm2 transcript levels in the breast specimens (tumor, n = 127; background, n = 33) using real-time quantitative PCR (all values are displayed as mean Ehm2 transcript copies/μL of cDNA from 50 ng total RNA). In comparison with normal breast tissues, elevated levels (P = 0.05) of Ehm2 were seen in the tumor specimens (Fig. 1B). Ehm2 immunochemical staining was observed in the human breast tissue sections. Ehm2 was expressed at higher level in breast cancer tissue (Fig. 1C, right) compared with its staining in the normal mammary tissue (Fig. 1C, left), which was consistent with the Ehm2 mRNA expression. Staining intensity analysis using Image J software further confirmed higher expression of Ehm2 in breast cancer (Fig. 1C, bottom). These results together suggested that Ehm2 is highly expressed in breast cancer and may play a role in the progression of breast cancer.

Correlation of Ehm2 expression with histologic type, grade, lymph node involvement, and tumor-node-metastasis staging

To assess the relation of Ehm2 expression with disease progression, Ehm2 transcript levels in the breast cancer samples were analyzed against histologic type, grade, nodal status, and tumor-node-metastasis (TNM) staging (Table 1). A higher level of Ehm2 transcripts (27.9 ± 4.3 copies/μL) was seen in ductal breast cancer, which was the most common
type of breast cancer, compared with lobular (10 ± 7.1) and other types of breast cancer (12.9 ± 14.6 copies/μL; Fig. 2A). In relation to the histologic grade of tumor cells, the moderately differentiated grade 2 (49.5 ± 5.5 copies/μL) tumors had significantly increased levels of Ehm2 compared with well-differentiated grade 1 tumors (8.0 ± 1.4 copies/μL, grade 1 versus grade 2, \( P = 0.0195 \)), whereas Ehm2 expression in the poorly differentiated grade 3 tumors was 20.1 ± 6.1 copies/μL (\( P = 0.25 \) versus that of grade 1; Fig. 2B). An increased level of Ehm2 transcript was revealed in node-positive tumors being 37.9 ± 16.0 copies/μL (\( P = 0.11 \)) compared with its levels in tumors without lymph node involvement (11.8 ± 6.7 copies/μL; Fig. 2C). The expression of Ehm2 was increased in the advanced breast cancer according to TNM stage grouping, particularly in TNM4, which were 104.7 ± 54.0 copies/μL, in comparison with that of TNM1 (32.0 ± 3.3 copies/μL), although this was not statistically significant (Fig. 2D). Using the cyclooxygenase’s model of multivariate analysis for nodal status, TNM stage, tumor grade, estrogen receptor (ER) status, and Ehm2, it was also found that Ehm2 was not an independent prognostic factor.

Prognostic relevance and clinical outcomes of Ehm2 in breast cancer

The prognostic potential of Ehm2 expression was firstly examined in accordance with the Nottingham prognostic index (NPI) of the patients. The NPI 1 group (NPI score < 3.4; \( n = 48 \)), NPI 2 group (NPI score = 3.4-5.4; \( n = 32 \)), and NPI 3 group (NPI score > 5.4; \( n = 13 \)) represent patients with good, moderate, and poor prognosis, respectively. Our data showed that patients with a moderate and poor prognosis (NPI 2 and NPI 3, respectively) had a tendency of increased levels of Ehm2 when compared with that of the NPI 1 group patients with good prognosis. The transcript levels of Ehm2 were 35.5 ± 11.0 copies/μL in NPI 2 group and 40.3 ± 5.6 copies/μL in NPI 3 group (\( P = 0.22 \) and \( P = 0.17 \)) when compared with that of NPI 1 group (11.8 ± 6.7 copies/μL), respectively (Fig. 3A).

Regarding the clinical outcomes, Ehm2 transcript levels seemed to be increased in patients with poor prognosis (62.6 ± 7.3 copies/μL), including those with local recurrence and metastases and those who died of breast cancer (\( P = 0.23 \)) compared with that of patients who remained disease-free (18.6 ± 4.4 copies/μL; Fig. 3B and C).

The Kaplan-Meier survival model was used to analyze the disease-free survival status of patients with breast cancer. It was found that patients with lower Ehm2 transcript levels had a longer overall survival (135.8 months; 95% confidence interval, 125.1-146.5; \( P = 0.039 \)) compared with those with high levels (102.5 months; 95% confidence interval, 78.7-126.4; Fig. 3D).

Knockdown of Ehm2 within breast cancer cell lines reduces cell growth and invasion

To study the role of Ehm2 in breast cancer metastasis and progression, we developed an Ehm2 knockdown
breast cancer cell line using anti-Ehm2 hammerhead ribozyme transgenes. We confirmed that Ehm2 ribozyme transgenes had successfully knocked down the expression of Ehm2 in the MCF-7 breast cancer cells (Fig. 4). A reduction of Ehm2 mRNA was revealed in Ehm2 knockdown cells (MCF-7\(^{\Delta Ehm2}\)) compared with MCF-7 wild-type and empty vector transfectants (MCF-7\(^{pEF}\)) control cells using RT-PCR (Fig. 4A). This was further confirmed using real-time quantitative PCR, which showed 80% knockdown of Ehm2 transcripts in MCF-7\(^{\Delta Ehm2}\) cells (Fig. 4B). In line with PCR results, Western blot analysis showed a decrease of protein production of Ehm2 after knockdown (Fig. 4C and D).

Using this Ehm2 knockdown cell line, we analyzed the general cell functions including growth, adhesion, invasion, and migration. Compared with MCF-7\(^{pEF}\) control, Ehm2 knockdown resulted in a significant reduction of cell growth. The absorbance of MCF-7\(^{\Delta Ehm2}\) cells at day 5 was 1.32 ± 0.09 (\(P < 0.001\)) compared with that of MCF-7\(^{pEF}\) cells (0.91 ± 0.04; Fig. 5A). Invasion assay data showed that knockdown of Ehm2 in MCF-7 cells resulted in a dramatic reduction of their invasiveness (\(P < 0.0001\) versus \(pEF\) control; Fig. 5B). Moreover, Ehm2 knockdown had no obvious affect on migration and adhesion of MCF-7 cells (Fig. 5C and D).

Ehm2 reduces apoptosis of breast cancer cells

To investigate whether apoptosis is involved in the inhibitory effect of Ehm2 knockdown on the growth of breast cancer cells, we determined the proportion of apoptotic cells using a flow cytometer. As shown in Fig. 6, there was an obvious shift in the cell population toward

**FIGURE 3.** Ehm2 and clinical outcomes and prognosis. A, Ehm2 and NPI. The NPI 1 group (NPI score < 3.4; \(n = 48\)), NPI 2 group (NPI score = 3.4-5.4; \(n = 32\)), and NPI 3 group (NPI score > 5.4; \(n = 13\)) represented patients with good, moderate, and poor prognosis, respectively. Ehm2 transcript levels were increased in the patients with moderate and poor prognosis in comparison with that of NPI 1 group patients. B, Ehm2 and clinical outcomes. Ehm2 expression was significantly decreased in patients with local recurrence or in patients who died of breast cancer compared with that of disease-free patients. C, Ehm2 expression was increased in patients with poor prognosis including those who had local recurrence and metastases and who died from the disease compared with that of disease-free patients. D, the lower level of Ehm2 expression in primary breast tumors correlates with longer disease-free survival. * \(P < 0.05\).
apoptosis in the Ehm2 knockdown cells, which is 24.1% in the MCF-7 Ehm2 knockdown cells, in comparison with MCF-7 pEF controls (13.7%).

Regulation of MMP9 expression and its activity by Ehm2

Invasive potential correlates with the ability of cancer cells to degrade the extracellular matrix. We analyzed several proteins associated with extracellular matrix degradation using Western blot and found that the protein level of MMP9 was dramatically decreased in MCF-7ΔEhm2 cells (Fig. 7B). Using gelatin zymography, it was further shown that supernatant from MCF-7ΔEhm2 cells exhibited a decreased MMP9 activity compared with pEF control (Fig. 7A). We further examined mRNA levels of MMP9 using RT-PCR, which was also significantly decreased in MCF-7 Ehm2 knockdown cells (Fig. 7C). This result suggests that Ehm2 can regulate the transcription of MMP9 and in turn affect the protein level and activity of MMP9. The transcript level of MMP9 in the same breast cancer cohort was also determined using quantitative PCR being shown in Table 1. Using Pearson’s rank correlation analysis, we found that MMP9 transcript levels were significantly correlated with Ehm2 expression (coefficient = 0.450, P < 0.05).

Discussion

In the present study, we showed for the first time that Ehm2 was highly expressed in breast cancer, and its higher expression was correlated with breast cancer metastasis and poor prognosis. We further show that knockdown of Ehm2 induces apoptosis potential and decreases the in vitro invasive properties of MCF-7 breast cancer cells.

The Ehm2 gene was originally identified by differential display analysis as a gene that was upregulated in highly metastatic clones of K-1735 and B16 murine melanoma cells (13). The Drosophilla orthologue of Ehm2, called Yurt, has been shown to be required for epithelial cell migration during embryogenesis, suggesting that its mammalian homologue may play a role in cancer metastasis by altering cell migratory properties (24). Wang et al. have reported that Ehm2 was expressed at higher levels in prostate cancer, and the higher expression was associated with the
progression of the disease (16). These indirect observations are consistent with our findings in breast cancer. Our study assessed the association of Ehm2 expression to clinical aspects of breast cancer through quantitatively determining its transcript levels in a cohort of breast cancer. Higher levels of Ehm2 transcripts were revealed in breast cancer tissues compared with normal background mammary tissues. Immunohistochemical staining also showed elevated Ehm2 expression in breast cancer. Ehm2 expression also seemed to be higher in patients with lymphatic metastases. In addition, a similar increase was also observed in more advanced diseases according to the TNM staging and tumor grade. It suggests that Ehm2 may be a metastasis-related factor and contributes to disease progression.
Structural analysis of Ehm2 revealed a FERM domain, which is highly conserved among different species and has been shown to be involved in the linkage of cytoplasmic proteins to the membrane. The remaining COOH terminal amino acids of the Ehm2 protein showed no significant homology to other known proteins (13, 16). A high level of sequence homology between the human and the mouse Ehm2 proteins leads to the prediction that this gene is highly conserved among different species (15). The FERM domain is located at the NH2 terminus in the majority of FERM-containing proteins (11); however, the FERM domain of Ehm2 is located near the center of the primary structure. It is noted that the FERM domain is also located at the center of a novel protein, bal, that is preferentially expressed in aggressive lymphomas (25, 26). These studies suggest that several proteins of the NF2/ERM/4.1 superfamily could be involved in the regulation of proliferation and metastatic potential.

In line with the clinical implication of Ehm2 in breast cancer, the current in vitro studies showed that Ehm2 was involved in the regulation of proliferation and metastatic potential of breast cancer cells. A reduction of cell growth was seen in the cancer cells after the loss of Ehm2 expression, suggesting that Ehm2 may contribute to the development and progression of breast cancer through promoting growth of cancer cells. The subsequent apoptosis analysis showed an increase of apoptotic population in breast cancer cells after knockdown of Ehm2. Taken together, it suggests that Ehm2 may promote growth of breast cancer cells via its antiapoptotic function. However, the mechanism of Ehm2-regulated apoptosis requires further investigation.

Increasing evidence suggests that MMP, a family of multidomain, zinc-containing neutral endopeptidases, contributes to the formation of a microenvironment that promotes tumor growth during early stages of tumorigenesis (27, 28). MMPs have traditionally been considered proteolytic enzymes for extracellular matrix (ECM) components, although it is now recognized that MMPs also cleave “nonmatrix” molecules, including growth factors, growth factor binding proteins, cytokines, chemokines, adhesion, and death receptors as well as other proteinases, modifying their biological activity (29-33). These MMP degradative activities may augment or reduce proliferation, survival, and migration of both tumor and stromal cells. The cleavage of the ECM by MMPs facilitates the invasion of tumor cells as well as the release of ECM-bound growth factors (e.g., of insulin-like growth factors and fibroblast growth factors). MMP-induced release of biological mediators from the ECM surrounding a tumor may thus constitute a system by which neoplastic and stromal cells communicate.

MMP9, also known as gelatinase B, has been associated with tumor progression in multiple studies (31, 34-37). In particular, several analyses show that expression of MMP9 is a prognostic indicator in breast cancer patients (36, 38, 39). As a type IV collagenase, it has been regarded as a critical enzyme for destruction of the basement membrane, the first barrier for cancer invasion. It is also well characterized for its activity during metastasis (27, 40). To examine whether MMP9 is involved in the reduced invasiveness of breast cancer cells after knockdown of Ehm2, we determined the expression of MMP9 in the same cells. A significant decrease of MMP9 expression was seen in the Ehm2 knockdown cells at both mRNA and protein level with a reduction of relevant enzymatic activity. It suggested an association between Ehm2 and MMP9 expression, which was further confirmed in examining the transcripts of these two molecules in a breast cancer cohort. In the current study, MMP9 expression was associated with the expression of Ehm2 in both mRNA and protein level with a reduction of relevant enzymatic activity. It suggested an association between Ehm2 and MMP9 expression, which was further confirmed in examining the transcripts of these two molecules in a breast cancer cohort. In the current study, MMP9 expression was associated with the expression of Ehm2 in both breast cancer tissues and cell lines, which was profound in regulating invasive capacity of the cancer cells. Furthermore, alternative methods to transcript analysis should be used in analyzing MMPs given the enzymatic nature of MMP. Indeed, previous studies have shown a raised level of MMP9 in the circulation of patients with cancer, including breast cancer using zymography (41, 42). However, whether the effect on invasion by Ehm2 is MMP9 dependent and how
Ehm2 regulates MMP9 are currently under investigation in the host laboratory.

In summary, we characterized a pattern of high expression of Ehm2 in breast cancers, and this was correlated with metastasis and poor clinical outcomes. Using a hammerhead ribozyme-targeted Ehm2 knockdown MCF-7 cells, we found that Ehm2 may be an antiapoptotic factor and can promote tumor metastasis by increasing invasion. Further study elucidated that interfering with the expression of Ehm2 inhibits the invasive potential of cancer cells by regulating the transcription of MMP9. We are currently studying how Ehm2 regulates the transcription of MMP9.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank the Albert Hung Foundation and Cancer Research Wales for supporting our study. Dr. H. Yu is a recipient of Cardiff University China Medical Scholarship.

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Received 05/05/2010; revised 08/31/2010; accepted 09/04/2010; published OnlineFirst 10/05/2010.

References
11. Baines AJ. A FERM-adjacent (FA) region defines a subset of the 4.1 superfamily and is a potential regulator of FERM domain function. BMC Genomics 2006;7:85.
32. McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor


Molecular Cancer Research

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Mol Cancer Res  Published OnlineFirst October 5, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-10-0186

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