Abstract. Alternative splicing provides additional genomic complexity by producing multiple mRNAs and protein variants from any given gene. Splice variants have been identified in a large variety of cancer genes, suggesting that widespread aberrant and alternative splicing may be a consequence or even a cause of cancer. Human ortholog of mammalian enabled (hMena), a family of enabled/vaso-dilator-stimulated phosphoproteins (Ena/VASP), is an actin regulatory protein involved in the regulation of cell motility. hMena has been shown to have several splice variants, including the hMenaINV isoform, expressed in invasive cancer cells, and the epithelial-specific isoform, hMena11a. We assessed the relative mRNA expression of hMena splice variants in 50 cases of invasive ductal breast carcinoma of no special type (IDC-NST) and 45 cases of ductal breast carcinoma in situ (DCIS) with special reference to non-neoplastic breast epithelial tissues. The samples were dissected from their respective regions by laser microdissection. Our results confirmed previous reports that hMenaINV expression is augmented during tumor progression, while hMena11a is downregulated. Furthermore, simultaneous expression of hMena 11a and hMena INV was found only in malignant lesions, while their expression was hardly detected in normal breast tissue and benign proliferative breast lesions. These results indicate that the higher relative expression of hMena11a compared with hMena INV may predict malignant transformation in breast epithelial cells, and, furthermore, a reversal of expression of hMena11a and hMenaINV may dictate the state of cancer progression. Here, we demonstrate that determination of hMena11a and hMenaINV expression could be a useful biomarker for predicting malignant behavior in breast epithelial lesions, and show that their relative expression is linked to adverse prognostic factors. Although the biological activity of the majority of alternatively spliced isoforms and their contribution to cancer biology has yet to be determined, their elucidation will have a large impact on therapeutic strategies for cancer.

Introduction

Breast cancer is one of the most common malignancies among women, with approximately 232,340 new cases of invasive breast cancer and 39,620 breast cancer deaths predicted to occur among US women in 2013 (1). Despite early diagnosis through screening programs and aggressive therapeutic strategies, the age-standardized mortality rate still remained at 14.1 per 100,000 individuals in 2008 (2). Therefore, precise prediction of prognosis is important because advanced therapy is necessary for high-risk patients. To predict prognosis, clinicians evaluate various parameters, such as classical histological features and hormone receptors. Recently, the expression of various molecular markers such as HER2, TP53, Ki-67, and EGFR has contributed to improvements in the prediction of prognosis in individual patients.

The formation of distant metastasis is the main cause of morbidity and mortality in patients with cancer. The invasion or metastasis of cancer cells involves multiple steps, including dissociation from the primary tumor, invasion into the surrounding stroma, and intravasation into the surrounding vascular systems (3). Each step requires a complex network involving gene activation or repression. It has recently been shown that epithelial-mesenchymal transition (EMT), a mechanism important for embryonic development, plays a critical role during malignant transformation (4,5). A functional hallmark of the EMT program is considered the acquisition of the ability to migrate and invade the extracellular matrix as a single cell. Furthermore, it has also been indicated that in molecules such as fibroblast growth receptor 2 (FGFR2), alternative splicing of pre-messenger RNAs, in which one of two mutually exclusive exons are included, results in differential ligand binding specificity of the receptor during EMT (6) and contributes

Relative expression of hMena11a and hMenaINV splice isoforms is a useful biomarker in development and progression of human breast carcinoma

NORIYUKI TANAKA1,2, HIROSHI YOSHIDA1,2, YOSHI SUZUKI2 and KENICHI HARIGAYA1,3

1Department of Molecular and Tumor Pathology, Chiba University Graduate School of Medicine, Chuo-ku, Chiba 260-8670; 2Department of Pathology, Asahi General Hospital, Chiba 289-2515; 3Laboratory of Medicine, Chiba Rosai Hospital, Ichihara, Chiba 290-0003, Japan

Received May 3, 2014; Accepted June 26, 2014

DOI: 10.3892/ijo.2014.2591

Correspondence to: Dr Kenichi Harigaya, Laboratory of Medicine, Chiba Rosai Hospital, 2-16 Tatsumidaihigashi, Ichihara, Chiba 290-0003, Japan
E-mail: harigaya@faculty.chiba-u.jp

Key words: hMena, ena/VASP, splicing isoform, breast, invasive ductal carcinoma
to the morphological conversion accompanying EMT (7). Accordingly, in malignant breast neoplasms, expression of the splice variants of pre-messenger RNAs promoting EMT leads to progression of the neoplasm, and ultimately result in poor prognosis (8). Human ortholog of mammalian enabled (hMena), a member of the Ena/Vasodilator-stimulated phosphoprotein (VASP) family, is a key molecule in cell migration. It regulates actin filament dynamics, and protects the filaments from capping proteins at their barbed ends and also reduces branching density (9,10). Recently, hMena has been shown to have multiple splice variants in tumor cells; two of the best characterized isoforms are hMena invasive (hMenaINV), expressed exclusively in invasive tumor cells, and hMena11a, an epithelial-specific isoform expressed in primary breast carcinomas and downregulated in invasive tumor cells (11-13). The exact regulation of hMena is also unclear; however, Warzecha et al reported CD44 and hMena transcripts undergo changes in splicing in vitro during EMT (7).

The aim of this study was to evaluate whether the expression of hMena isoforms hMena11a and hMenaINV differ in non-invasive and invasive breast cancer using breast surgical specimens and cancer cell lines. We hypothesized that the expression of different hMena isoforms could be a useful biomarker of malignancy and invasive or metastatic potential.

### Materials and methods

**Patients and tumor tissue.** Archival specimens collected between 2005 and 2012, and 50 patients with invasive ductal carcinoma of no special type (IDC-NST) (Fig. 1A), 45 patients with ductal carcinoma in situ (DCIS) (Fig. 1B) and 10 patients with intraductal papilloma of the breast (Fig. 1D) were enrolled in the study. Non-neoplastic duct tissue was obtained from each specimen to serve as a control (Fig. 1C). All patients were surgically treated with either breast-conserving lumpectomy or modified radical mastectomy and axillary lymph node dissection at Asahi General Hospital (Chiba, Japan). All samples were collected with approval from the ethics committee of Asahi General Hospital. The clinicopathological characteristics of each patient are shown in Tables I and II.

The primary tumor specimens were fixed in 10% buffered formalin and embedded in paraffin using standard tissue processing methods. Pathological tumor staging was determined using the current tumor-node-metastasis system (UICC). Diagnoses and histology were confirmed by two pathologists (Noriyuki Tanaka and Kenichi Harigaya) who reviewed the specimens and cancer cell lines. We hypothesized that the expression of different hMena isoforms could be a useful biomarker of malignancy and invasive or metastatic potential.

**Laser capture microdissection (LCM).** RNA isolation and cDNA synthesis from FFPE sections. Ten micrometer-thick membrane sections (Leica Microsystems) were prepared from formalin-fixed paraffin-embedded (FFPE) tissue using standard protocols. The slides were air-dried and stained with toluidine blue. Laser dissection was performed using a laser capture microdissection microscope (Leica AS LMD; Leica Microsystems, Wetzlar, Germany) with a pulsed 337 nm UV laser according to the manufacturer’s protocols. The size of each dissected specimen was ≥2 mm². Total RNA was purified from dissected tissue using an RNeasy FFPE kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocols. cDNA was synthesized from the extracted total RNA with Primerscript reverse transcript reagent (Takara) according to the manufacturer’s protocols.

**Real-time PCR analysis.** Semi-quantitative real-time polymerase chain reaction (PCR) was then performed using the ABI PRISM 7000 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) thermalycler with PreMix ExTaq reagent (Takara), as previously described (14). The primers were as follows: MENA (sense, 5'-GCCGCAATATACAGTGCCTG-3'; antisense, 5'-TCCCCAGCACAGGTTTAGAGG-3'), MENAINV (sense, 5'-TTTGTGACACAGGTCTATGATTCC-3'; antisense, 5'-CTTCGCTGTGAACTCATCCT-3'), MENA (sense, 5'-CCATGATGCATGCCTTAGAA-3'; antisense, 5'-TCCTGGTGAGCTAACCTGG-3'), GAPDH (sense, 5'-AGC CACATCGCTCGACAC -3'; antisense, 5'-GGCCAATACGACCAAAATCC-3'). The TaqMan Probes (UPL, Roche) used for each variant were as follows: hMena (TaqMan no. 34), hMena11a (TaqMan no. 26), hMenaINV (TaqMan no. 26), and GAPDH (TaqMan no. 60). The PCR protocol consisted of 30 sec at 95°C followed by 60 cycles of 5 sec at 95°C and 31 sec at 60°C. The relative expression level of hMena variants was calculated by the comparative threshold cycle (CT) method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control (15). All experiments were performed in triplicate and the results were expressed as mean ± SD.

**Cell culture.** Human breast cancer cell lines MDA-MB231, BT549, MDA-MB468, MCF-7, T47D, ZR75-1 and human cervical cancer cell line HeLaS3 were purchased from American Type Culture Collection (Rockville, MD, USA). All cell lines except HeLaS3 were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 10% fetal bovine serum. HeLaS3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Nicui) supplemented with 10% calf serum. All cell lines were maintained under 5% CO₂ at 37°C.

**Antibodies.** Anti-E-cadherin antibody (BD610182) was purchased from BD Biosciences (San Jose, CA, USA). Anti-vimentin antibody (sc-6260) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-actin antibody (A 2066) was purchased from Sigma (St. Louis, MO, USA). Primary polyclonal antibodies against hMena variants were raised in rabbits against amino acid sequences AQSKV TATQD STNLR CIFC (hMenaINV) and RDSPR KQIV FDNRS YDLSH (hMena11a). The obtained antibodies were affinity-purified using each immunizing peptide.

**Western blotting.** Whole cell lysates were prepared with ice-cold RIPA buffer (50 mM Tris-HCl pH 7.5, 1% nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid) containing 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM DTT, 1 mM NaVO₄ and 0.5 mM PMSF. The supernatants were recovered as total cell lysates following centrifugation. Aliquots of the cell lysates (50 µg of protein) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA).
Primary antibodies bound to their antigens on the membranes were detected using appropriate HRP-conjugated secondary antibodies (Amersham Bioscience, Piscataway, NJ, USA) and a Super Signal chemiluminescence detection system (Pierce, Rockford, IL, USA) or the Lumi-LightPLUS western blotting substrate (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

**Statistical analysis.** Data are summarized in bar graphs. Bars represent the mean and whiskers the standard deviation (SD). Statistical analysis was performed using Microsoft Excel (Microsoft Corp., Seattle, WA, USA). Paired-sample t-tests, $\chi^2$ test, or Kruskal-Wallis tests were used as appropriate. Differences were considered statistically significant when the P-value was $<0.05$.

**Results**

$h\text{Mena}^{INV}$ and $h\text{Mena}^{11a}$ splice variant mRNA was expressed in invasive ductal carcinoma of no special type (IDC-NST). We

<table>
<thead>
<tr>
<th>Table I. Clinicopathological characteristics of IDC-NST cases (total n=50).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (max)</td>
</tr>
<tr>
<td>&lt;2.5 cm</td>
</tr>
<tr>
<td>&gt;2.5 cm</td>
</tr>
<tr>
<td>Lymph node status</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Molecular subtype</td>
</tr>
<tr>
<td>Luminal A</td>
</tr>
<tr>
<td>Luminal B</td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>WHO histological grade</td>
</tr>
<tr>
<td>Grade 1</td>
</tr>
<tr>
<td>Grade 2</td>
</tr>
<tr>
<td>Grade 3</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Table II. WHO histological grades of DCIS cases (n=45).</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCIS histological grade (WHO)</td>
</tr>
<tr>
<td>Low grade</td>
</tr>
<tr>
<td>Intermediate grade</td>
</tr>
<tr>
<td>High grade</td>
</tr>
</tbody>
</table>

DCIS, ductal carcinoma in situ.
sought to determine whether the mRNA expression of hMena splice isoforms hMenaINV and hMena11a is altered in different IDC-NST lesions [invasive areas (IA-IDC-NST, Fig. 1A) and non-invasive intraductal lesions (NIDL-IDC-NST, Fig. 1B)] compared with corresponding non-neoplastic duct epithelium (Fig. 1C) (50 cases). Fig. 2A demonstrated that an increase in hMenaINV mRNA expression was found in the different IDC-NST lesions IA-IDC-NST and NIDL-IDC-NST, while no detectable expression was observed in non-neoplastic duct epithelia. More intense augmentation of hMenaINV mRNA expression was demonstrated in IA-IDC-NST than NIDL-IDC-NST (P<0.05). We further examined whether there were any differences in hMenaINV mRNA expression among three different subtypes of IDC-NST according to the WHO classification (16) of histological grading (tubule formation, nuclear pleomorphism, and mitotic accounting).
or molecular phenotype (luminal A, luminal B, and basal subtypes according to the presence or absence of estrogen receptor, progesterone receptor, or Her2 protein). Fig. 2B showed that higher expression of hMenaINV mRNA was significantly detected in IA-IDC-NST lesions of grade 3 breast carcinomas compared with either those of grade 1 tumors (P<0.05) or those of grade 2 tumors (P<0.05). Additionally, no significant differences in hMenaINV mRNA expression were found in NIDL-IDC-NST lesions of the three different subgroups. Furthermore, a significant increase in hMenaINV mRNA expression was found in IA-IDC-NST lesions of three different molecular phenotypes; basal subtype expressed higher levels of hMenaINV mRNA than either luminal A subtype, or luminal B subtype (P<0.05; Fig. 2C). However, we did not find any statistically significant difference between luminal B and basal subtypes. In NIDL-IDC-NST lesions, there was no statistically significant difference in hMenaINV mRNA expression among the three different molecular phenotypes. Taken together, it would be reasonable to conclude that higher hMenaINV mRNA expression is found in more aggressive histological and molecular subtypes of IA-IDC-NST lesions. Furthermore, our data suggest that no statistically significant difference in hMenaINV expression was found in NIDL-IDC-NST lesions, but increased expression was found in IA-IDC-NST in accordance with tumor progression. It is well known that determination of these subtypes reflect patient prognosis (17), and, therefore, our results are comparable with a previous report that showed increased expression of hMenaINV could confer a potent pro-metastatic phenotype when expressed in breast cancer cells (18,19). Our results also showed that levels of hMenaINV expression also tended to be increased in non-invasive ductal carcinoma in clinical breast carcinoma specimens according to histological grade, but this was not statistically significant. These results indicate that the measurement of hMenaINV mRNA in IA-IDC-NST but not NIDL-IDC-NST may be useful in predicting patient prognosis from histological samples of IDC-NST. It should be stressed that mutually-exclusive alternative hMena mRNA splicing would not necessarily be observed only in invasive breast carcinoma cells in clinical specimens and that cell sheets of non-invasive intraductal lesions in IDC-NST produced significant amounts of hMenaINV mRNA.

Expression of hMena splice isoform mRNA is increased in breast ductal carcinoma in situ. Several lines of evidence indicate that molecular expression of hMenaINV is strictly regulated during breast carcinoma development and is predominantly found in the non-invasive stage of breast carcinoma (12,20). However, our results showed that both hMena splice isoforms were dramatically increased in the different IDC-NST lesions, IA-IDC-NST (Fig. 2A) and NIDL-IDC-NST (Fig. 2D). Additionally, our results also revealed that the expression of hMenaINV is further augmented in cells at the invasive front, while hMena11a is downregulated in cells at the invasive front. It should be stressed that cell sheets of NIDL-IDC-NST produced hMena11a mRNA as well as significant amounts of hMenaINV mRNA in clinical breast carcinoma specimens. This would reflect whether some populations of invasive breast carcinoma cells are intermingled in non-invasive intraductal lesions of IDC-NST or some cell populations of non-invasive intraductal lesions could produce both hMenaINV and hMena11a mRNA simultaneously. Nevertheless, there has been no evidence indicating that both the INV and 11a exons are included in the Mena mRNA at the same time or expressed at high levels within the same cell (21).

Next, we examined the expression of hMena11a mRNA at the afore-mentioned lesions of IDC-NST. The expression of hMena11a mRNA was hardly found in non-neoplastic breast duct epithelium but was dramatically increased in the different lesions of IDC-NST, as in the case of hMenaINV mRNA. Fig. 2D shows that significant expression of hMena11a mRNA was consistently found in NIDL-IDC-NST as well as IA-IDC-NST in contrast to non-neoplastic duct epithelia, while the relative expression in IA-IDC-NST was downregulated to approximately 85% that of the level in NIDL-IDC-NST. This reduction reflects previous reports that Mena11a is downregulated in invasive tumor cells (11). Compared with the elevation in hMenaINV mRNA expression in accordance to adverse histological grade, hMena11a mRNA expression did not change among the three subgroups classified according to WHO histological grades or molecular phenotypes, as shown in Fig. 2E and F. Rather, grade 3 subtype of both NIDL-IDC-NST and IA-IDC-NST tended to have increased hMena11a mRNA expression, although this was not statistically significant. Our results indicated that significant hMena11a mRNA expression was found in different NIDL and IA lesions of IDC-NST but was decreased in IA-IDC-NST, which is supposed to undergo tumor progression. However, our results also showed that hMena11a mRNA expression was not downregulated in different lesions with either histological or molecular tumor progression. Accordingly, our results do not necessarily coincide with previous reports in vitro and in vivo that hMena11a is downregulated in invasive tumor cells (11,20). Collectively, our results showed that cell sheets of non-invasive intraductal lesions in IDC-NST produced significant amount of hMena11a mRNA as well as hMenaINV mRNA. This would reflect whether some populations of invasive cells are intermingled in intraductal lesions of NIDL-IDC-NST or some populations of non-invasive intraductal lesions could produce both hMenaINV and hMena11a mRNA simultaneously. Nevertheless, there has been no evidence indicating that both the INV and 11a exons are included in the Mena mRNA at the same time or expressed at high levels within the same cell (21).
showed that among three different breast carcinoma lesions, cells in DCIS always showed the highest level of hMena\(^{11a}\) mRNA expression, followed by those in NIDL-IDC-NST. Cells of IA-IDC-NST consistently showed the lowest levels of hMena\(^{11a}\) mRNA expression among the three different lesions. Conversely, the expression of hMena\(^{INV}\) mRNA was reversed and increased in cells of NIDL-IDC-NST, and showed the highest expression levels in cells of IA-IDC-NST (Fig. 3F). These results indicated that the expression of hMena\(^{11a}\) suppressed metastatic potential and hMena\(^{INV}\) expression promoted tumor progression. Previous reports showed that Mena\(^{INV}\) is exclusively expressed in invasive tumor cells in \textit{in vitro} and \textit{in vivo} (18,23). Nevertheless, our results indicate that cells in non-invasive breast duct carcinoma produce Mena\(^{11a}\) mRNA as well as Mena\(^{INV}\) mRNA in clinical breast carcinoma samples.

Expression of hMena splice isoforms in cancer cell lines. To validate our results, we examined the expression of hMena splice isoforms in several non-invasive and invasive cancer cell lines by western blotting. Fig. 4 shows the expression of hMena splice variants in several breast cancer cell lines. Seven cancer cell lines, including four E-cadherin-positive and vimentin-negative cell lines and three E-cadherin-negative and
vimentin-positive cell lines were used. The E-cadherin-positive cell lines are epithelial and show non-invasive phenotypes. All four lines expressed hMena11a protein. However, the E-cadherin-positive and vimentin-negative non-motile breast cancer cell line, MDA-MB468, simultaneously expressed both hMenaINV and hMena11a protein but not hMenaINV protein. These results suggest that a proportion of the non-invasive carcinoma cells could express hMenaINV as well as hMena11a in cancer cells is not strictly regulated during tumor progression.

Whole hMena isoform expression appears not to differ between different neoplastic or non-neoplastic breast epithelial lesions. To examine the expression of whole hMena mRNA expression in different non-neoplastic and neoplastic breast epithelial lesions, we investigated 210 foci (non-neoplastic lesions: n=105; papilloma: n=10; DCIS: n=45, IDC-NST: n=50). Fig. 5 showed that levels of whole hMena mRNA expression were not statistically different among the non-neoplastic and neoplastic breast epithelial foci used in this study.

Our data resulting from semi-quantitative mRNA expression in microdissected samples of clinical surgical specimens are compatible with the mRNA expression of hMenaINV and hMena11a splice variants in previous studies (18,24) using xenograft models; however, whole hMena expression levels were different from our results. These researchers claimed that there was 3-4-fold augmented expression of whole hMena during tumor progression. Although we could not determine the cause of this discrepancy, we speculate that it stems from different model systems; we investigated clinical human breast cancer samples while the previous report used xenograft models.

Discussion

The Ena/VASP family of proteins is an important regulator of actin cytoskeleton dynamics involved in cell motility. Changes in the cellular actin network play a role in malignant transformation and tumor progression. Previous reports have shown that hMena variant hMena11a is predominantly overexpressed in tumor cell lines expressing epithelial phenotypes, while hMenaINV was shown to be overexpressed in tumor cell lines expressing invasive phenotypes (18,22). Furthermore, expression of ERSP1 and ERSP2 induces the inclusion of the hMena11a exon, through which cancer cells undergo morphological changes into an adhesive form of epithelial-like cells. Loss of their expression induces the inclusion of the hMenaINV exon, resulting in EMT and rendering cancer cells motile (25). In our study, however, we showed that simultaneous expression of hMena11a and hMenaINV is found either in non-invasive or invasive carcinoma lesions using FFPE breast cancer tissue from clinical surgical specimens. In contrast, their expression was hardly detected in normal breast tissue and benign proliferative breast lesions. These results indicate that the higher relative expression of hMena11a compared with that of hMenaINV may predict malignant transformation in breast epithelial cells, and, furthermore, a reversal in the expression of hMena11a and hMenaINV may dictate the state of cancer progression. Based on these results, we suggest that differential regulation of hMena11a and hMenaINV splice variant expression during tumor progression is not performed in a mutually exclusive switch-on and switch-off manner. Furthermore, using cancer cell lines, we have shown that MDA-MB468 intermediate expresses both splice variants,
and HeLaS3 cells undergoing EMT (vimentin-positive and E-cadherin negative) express only hMena11a without hMenaINV. Through our studies, it appears that the molecular mechanism is sometimes promiscuous during malignant transformation or progression, and it seems that the invasive potential of cancer cells is hard to predict from the expression of a single splicing isofrom such as hMenaINV.

In conclusion, we have demonstrated that determination of hMena11a and hMenaINV expression could be a useful biomarker in malignant transformation and progression in breast epithelial lesions and that their relative expression is linked to adverse prognostic factors. Further studies may provide insights into the understanding of the nature of cancer initiation and progression, and improve diagnosis of non-invasive and invasive ductal carcinomas.

Acknowledgements

A portion of these data were presented at the 101st and 102nd annual meeting of the Japanese Society of Pathology in Tokyo and Sapporo. We are grateful to Asahi General Hospital for surgical specimens, and T. Shida, T. Umemiya, K. Azuma and K. Takaoka for technical help. This study was supported by Grants-in-Aid for Scientific Research 15390122 and 22390074 K. Takaoka for technical help. This study was supported by the Japan Society for the Promotion of Science (to K. Harigaya).

References