Relative expression of hMena^{11a} and hMena^{INV} splice isoforms is a useful biomarker in development and progression of human breast carcinoma

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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/vasodilator-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 hMena^{INV} isoform, expressed in invasive cancer cells, and the epithelial-specific isoform, hMena^{11a}. 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 hMena^{INV} expression is augmented during tumor progression, while hMena^{11a} 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 hMena^{11a} compared with hMena^{INV} may predict malignant transformation in breast epithelial cells, and, furthermore, a reversal of expression of hMena^{11a} and hMena^{INV} may dictate the state of cancer progression. Here, we demonstrate that determination of hMena^{11a} and hMena^{INV} 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.

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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

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 ($hMena^{INV}$), expressed exclusively in invasive tumor cells, and $hMena^{11a}$, 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 $hMena^{11a}$ and $hMena^{INV}$ 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 hematoxylin-eosin (H&E)-stained slides prepared from the paraffin blocks.

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 mm^2. 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 Primescript 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) thermal cycler with PreMix ExTaq reagent (Takara), as previously described (14). The primers were as follows: *MENA* (sense, 5'-GCGCAGAATATCAAGTGCTG-3'; antisense, 5'-TCCCAGCACAGAGTTTAGAGG-3'), $MENA^{11a}$ (sense, 5'-TTTTGACAACAGGTCCTATGATTC-3'; antisense, 5'-CTTCCGTCTGGACTCCATTG-3'), $MENA^{INV}$ (sense, 5'-CCATGATGCATGCCTTAGAA-3'; antisense, 5'-TCCTGG GTAGCAGTAACCTTG-3'), human *GAPDH* (sense, 5'-AGC CACATCGCTCAGACAC-3'; antisense, 5'-GCCCAATACGA CCAAATCC-3'). The TaqMan Probes (UPL, Roche) used for each variant were as follows: hMena (TaqMan no. 34), $hMena^{11a}$ (TaqMan no. 26), $hMena^{INV}$ (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; Niccui) supplemented with 10% calf serum. All cell lines were maintained under 5% CO_2 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 ($hMena^{INV}$) and RDSPR KNQIV FDNRS YDSLH ($hMena^{11a}$). 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 $NaVO_4$ 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).

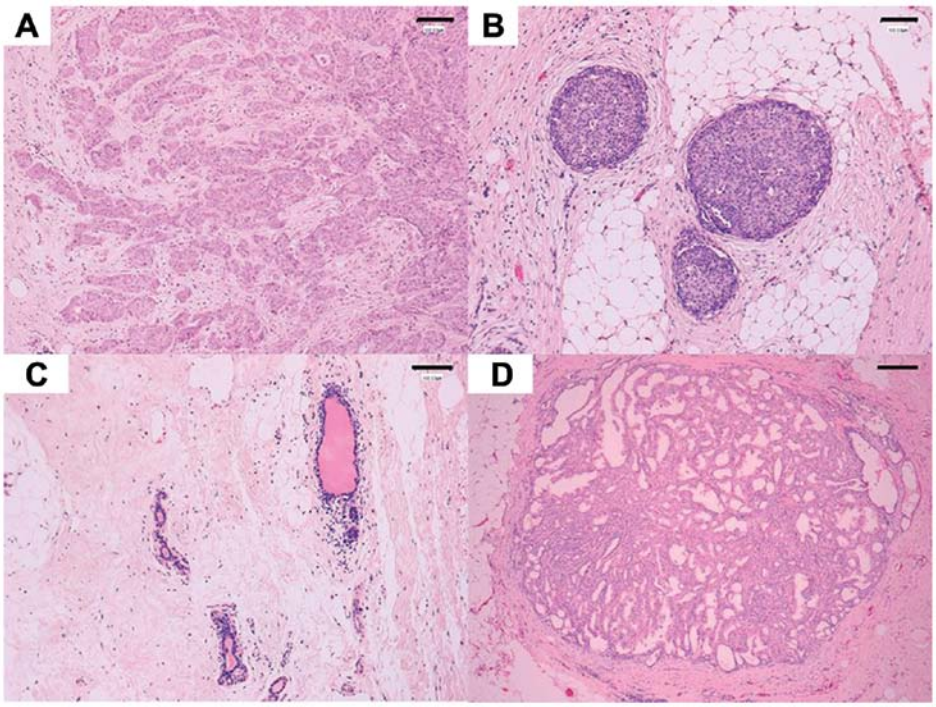

Figure 1. Representative histological images of breast tissue used in this study. (A) Invasive ductal carcinoma of no special type (IDC-NST). Samples were taken from the peripheral regions the tumors (tumor fronts), where tumor cells break off from the main tumor to form small structures that invade into the surrounding stroma. (B) Ductal carcinoma *in situ* (DCIS). Samples were taken from intraductal lesions identified using H&E staining. For lesions exhibiting comedo necrosis, care was taken not to include the necrotic areas. (C) Normal breast tissue. Samples were taken from each case analyzed in the study. Tissue containing ductal structures was used in the study. Lobular structures were not included. (D) Intraductal papilloma. Samples were taken from intraductal lesions exhibiting papillary growth but lacking nuclear atypia, abnormal mitosis and other features characteristic of DCIS. Each image was taken from hematoxylin-eosin (H&E) stained slides at x40 magnification. Bar represents 100 μm.

Table I. Clinicopathological characteristics of IDC-NST cases (total n=50).

	No. of cases (%)
Tumor size (max)	
<2.5 cm	24 (48)
>2.5 cm	26 (52)
Lymph node status	
Positive	15 (30)
Negative	35 (70)
Molecular subtype	
Luminal A	17 (34)
Luminal B	28 (56)
Basal	5 (10)
WHO histological grade	
Grade 1	17 (34)
Grade 2	18 (36)
Grade 3	15 (30)

IDC-NST, invasive ductal carcinoma of no special type. WHO, World Health Organization.

Table II. WHO histological grades of DCIS cases (n=45).

DCIS histological grade (WHO)	No. of cases (%)
Low grade	23 (51)
Intermediate grade	15 (33)
High grade	7 (15%)

DCIS, ductal carcinoma *in situ*.

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, χ^2 test, or Kruskal-Wallis tests were used as appropriate. Differences were considered statistically significant when the P-value was <0.05.

Results

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

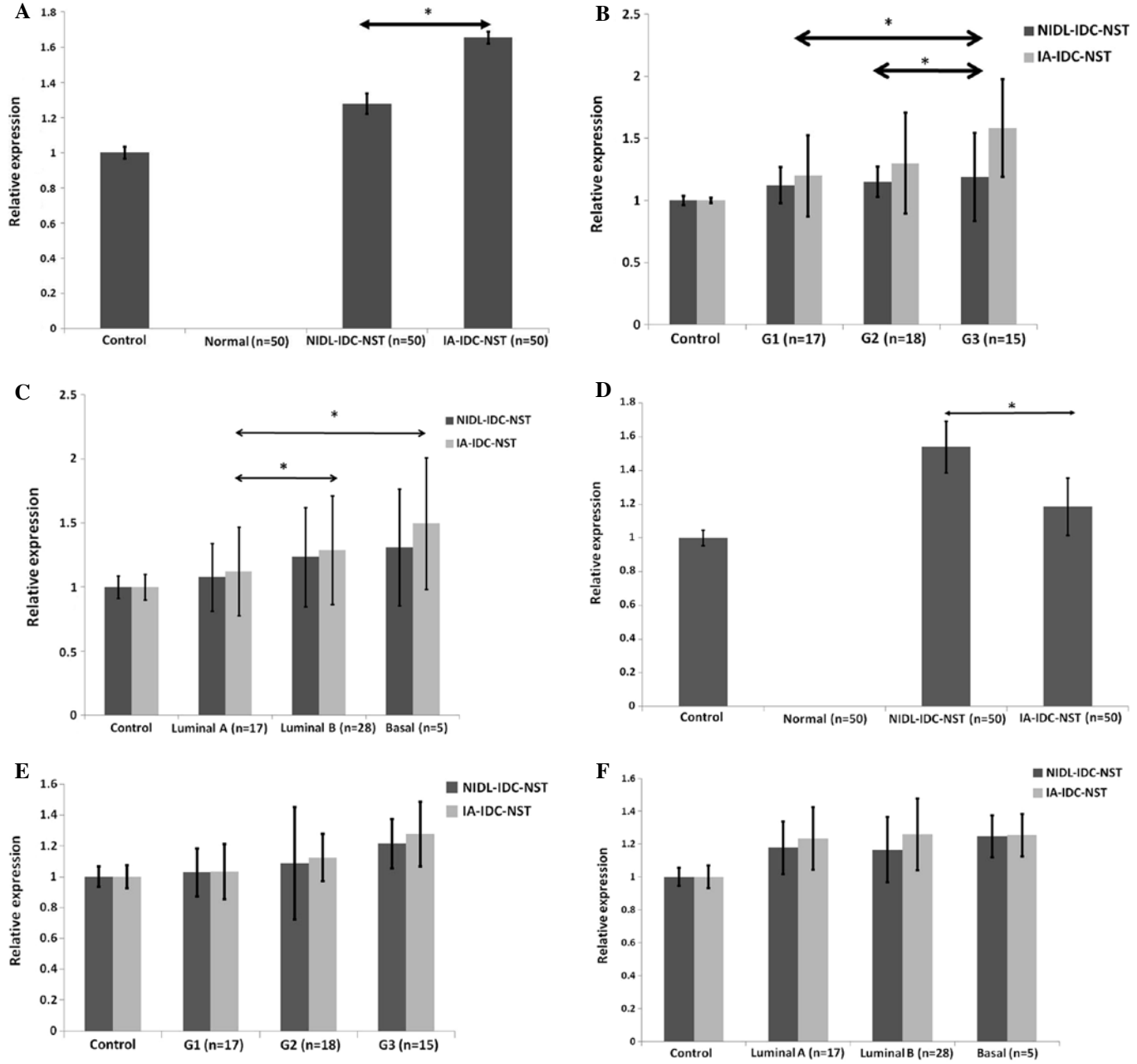

Figure 2. Analysis of hMena^{INV} and hMena^{11a} mRNA expression in invasive ductal carcinoma of no special type (*P<0.05; control, GAPDH). (A) Analysis of hMena^{INV} in IDC-NST with comparison between intraductal lesions and invasive fronts. Compared with normal breast tissue, invasive ductal carcinoma exhibited a significant increase in hMena^{INV} mRNA expression. The invasive fronts of IDC-NST expressed more hMena^{INV} compared with intraductal lesions. (B) hMena^{11a} mRNA expression was downregulated in the invasive fronts of IDC-NST compared with intraductal lesions. (C) Analysis of hMena^{INV} mRNA expression between IDC-NST of different WHO histological grades (grades 1-3) showed that hMena^{INV} mRNA expression was significantly increased in tumors of adverse WHO histological grades (G3>G1, G3>G2). However, no statistically significant difference was found between tumors of WHO histological grades 1 and 2. (D) Analysis of hMena^{11a} mRNA expression in three different histological subtypes of IDC-NST as defined by the WHO; no statistical difference was found. (E) hMena^{INV} mRNA expression was increased in luminal A compared with luminal B subtype, and between luminal A and basal subtypes. However, no statistically significant difference was found between luminal B and basal subtypes. (F) Comparison of the expression of hMena^{11a} mRNA in IDC-NST of three different WHO molecular phenotypes revealed no statistically significant difference.

sought to determine whether the mRNA expression of hMena splice isoforms hMena^{INV} and hMena^{11a} 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 hMena^{INV} 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 hMena^{INV} mRNA expression was demonstrated in IA-IDC-NST than NIDL-IDC-NST (P<0.05). We further examined whether there were any differences in hMena^{INV} 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 hMena^{INV} 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 hMena^{INV} mRNA expression were found in NIDL-IDC-NST lesions of the three different subclasses. Furthermore, a significant increase in hMena^{INV} mRNA expression was found in IA-IDC-NST lesions of three different molecular phenotypes; basal subtype expressed higher levels of hMena^{INV} 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 hMena^{INV} mRNA expression among the three different molecular phenotypes. Taken together, it would be reasonable to conclude that higher hMena^{INV} 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 hMena^{INV} 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 hMena^{INV} could confer a potent pro-metastatic phenotype when expressed in breast cancer cells (18,19). Our results also showed that levels of hMena^{INV} 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 hMena^{INV} 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 hMena^{INV} mRNA.

Next, we examined the expression of hMena^{11a} mRNA at the afore-mentioned lesions of IDC-NST. The expression of hMena^{11a} 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 hMena^{INV} mRNA. Fig. 2D shows that significant expression of hMena^{11a} 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 Mena^{11a} is downregulated in invasive tumor cells (11). Compared with the elevation in hMena^{INV} mRNA expression in accordance to adverse histological grade, hMena^{11a} 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 hMena^{11a} mRNA expression, although this was not statistically significant. Our results indicated that significant hMena^{11a} 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 hMena^{11a} 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 hMena^{11a} 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 hMena^{11a} mRNA as well as hMena^{INV} 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 hMena^{INV} and hMena^{11a} 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).

Expression of hMena splice isoform mRNA is increased in breast ductal carcinoma in situ. Several lines of evidence indicate that molecular expression of hMena^{11a} 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 hMena^{INV} is further augmented in cells at the invasive front, while hMena^{11a} is downregulated in cells at the invasive front. It should be stressed that cell sheets of NIDL-IDC-NST produced hMena^{11a} mRNA as well as significant amounts of hMena^{INV} 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 Mena^{INV} and Mena^{11a}, simultaneously. To explore these possibilities, we examined whether cell populations in ductal carcinoma *in situ* (DCIS) contain hMena splice isoform mRNA. We examined 45 cases of DCIS, 10 cases of intraductal papilloma, and 55 lesions of the corresponding non-neoplastic duct epithelia around the *in situ* carcinoma and papilloma. Fig. 3A and B showed that cells of DCIS constantly expressed significant amounts of hMena^{11a} mRNA as well as hMena^{INV} mRNA. In papilloma lesions (n=10), weak expression of hMena^{11a} was also found, but hMena^{INV} mRNA expression was not detectable. Accordingly, cells in DCIS produced increased amounts of both hMena^{INV} and hMena^{11a} mRNA in contrast to non-neoplastic epithelia. We further examined the different DCIS subtypes according to either WHO histological classification; the expression levels of hMena^{11a} and hMena^{INV} mRNA did not differ among the three different grades of DCIS lesions (Fig. 3C and D), although we did not investigate the molecular phenotypes. Our results are comparable with a recent report that showed the rate of local recurrence for low- and intermediate-grade DCIS is not significantly different from the rate of local recurrence for women with high grade DCIS (15.1%) (22). Fig. 3E

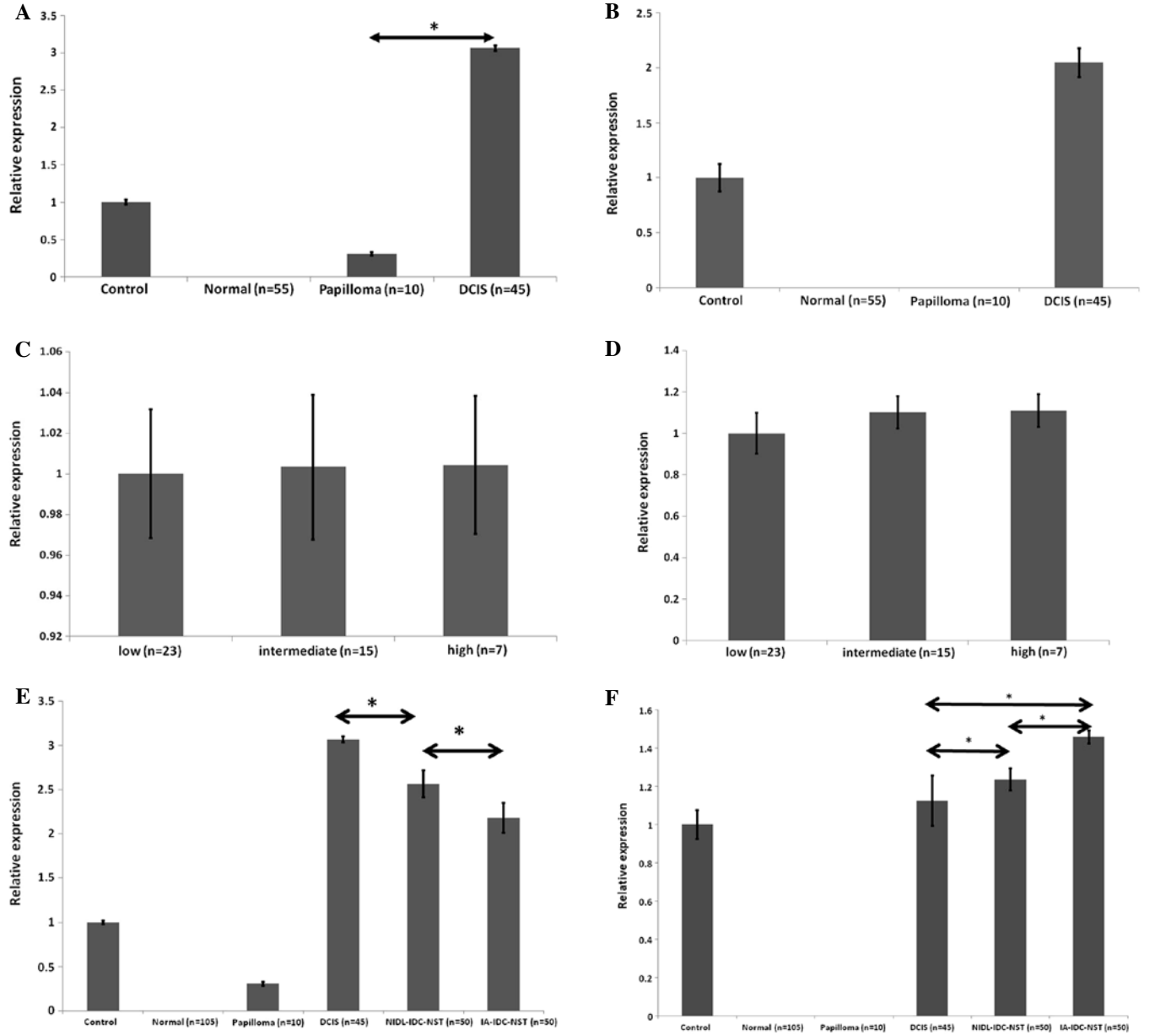

Figure 3. Expression of hMena^{11a} mRNA in benign breast lesions and DCIS (*P<0.05; control, GAPDH). (A) hMena^{11a} mRNA expression was markedly increased in DCIS lesions compared with normal breast tissue and intraductal papilloma. (B) hMena^{INV} mRNA was not detectable in benign breast lesions compared with DCIS. (C) Analysis of hMena^{11a} mRNA among DCIS lesions of different histological grades revealed no statistically significant differences. (D) Analysis of hMena^{INV} mRNA among DCIS lesions of different histological grades revealed no statistically significant differences. (E) There was a significant increase in hMena^{11a} mRNA expression between benign breast lesions and malignant breast lesions. Moreover, hMena^{11a} mRNA expression was downregulated between non-invasive intraductal lesions and invasive lesions. (F) hMena^{11a} mRNA expression was found to be increased between benign breast lesions and malignant breast lesions in a comparison of all breast study used in this study. Furthermore, we found an increase in expression when comparing non-invasive intraductal lesions and invasive lesions.

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 *in vitro* and *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

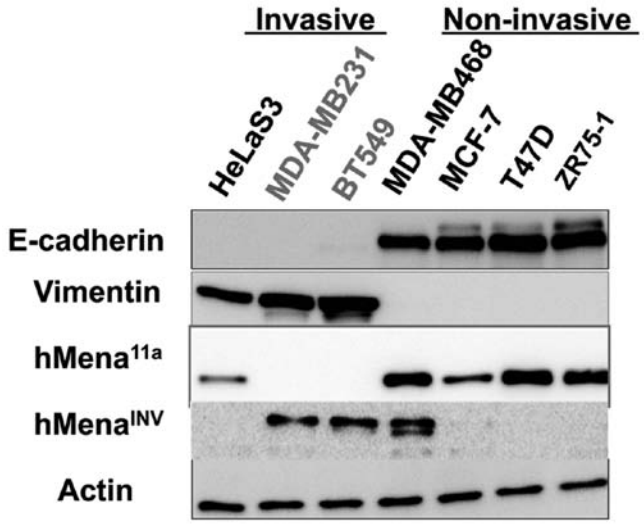

Figure 4. Expression of hMena^{11a} and hMena^{INV} mRNA in breast cancer cell lines. Western blotting of seven cancer cell lines including four E-cadherin-positive and vimentin-negative non-invasive phenotype cell lines, and three E-cadherin-negative and vimentin-positive invasive phenotype cell lines showed hMena^{11a} expression in the non-invasive phenotype cell lines. However, in one non-invasive phenotype cell line hMena^{11a} and hMena^{INV} were simultaneously expressed, and in one invasive phenotype cell lines hMena^{INV} was not found. These results suggest that the differential expression of either hMena^{INV} or hMena^{11a} in cancer cells is not strictly regulated during tumor progression.

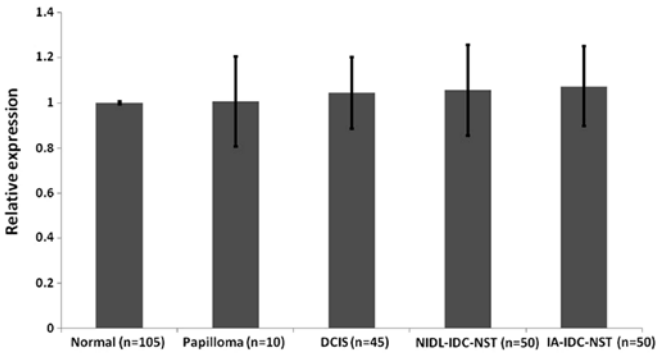

Figure 5. Expression of total hMena mRNA in various non-neoplastic and neoplastic breast lesions (*P<0.05; control, GAPDH). Analysis of total hMena mRNA expression in samples of all 105 cases used in this study (normal, 105 cases; intraductal papilloma, 10 cases; DCIS, 45 cases; IDC-NST, 50 cases) revealed no statistically significant differences.

vimentin-positive cell lines were used. The E-cadherin-positive cell lines are epithelial and show non-invasive phenotypes. All four lines expressed hMena^{11a} protein. However, the E-cadherin-positive and vimentin-negative non-motile breast cancer cell line, MDA-MB468, simultaneously expressed both hMena^{INV} and hMena^{11a}. Conversely, E-cadherin-negative and vimentin-positive HeLaS3 cells presumably undergo EMT, and were found to express hMena^{11a} protein but not hMena^{INV} protein. These results suggest that a proportion of the non-invasive carcinoma cells could express hMena^{INV} as well as hMena^{11a}, while invasive carcinoma cells such as HeLaS3 express only hMena^{11a} but not hMena^{INV}. These findings are fairly consistent with our results in clinical breast cancer samples, and suggest that a subset of non-invasive cancer cells simultaneously express hMena^{11a} and hMena^{INV}. Accordingly, our results indicate that the differential expression of either hMena^{INV} or hMena^{11a} 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 hMena^{INV} and hMena^{11a} 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 hMena^{11a} is predominantly overexpressed in tumor cell lines expressing epithelial phenotypes, while hMena^{INV} 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 hMena^{11a} 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 hMena^{INV} exon, resulting in EMT and rendering cancer cells motile (25). In our study, however, we showed that simultaneous expression of hMena^{11a} and hMena^{INV} 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 hMena^{11a} compared with that of hMena^{INV} may predict malignant transformation in breast epithelial cells, and, furthermore, a reversal in the expression of hMena^{11a} and hMena^{INV} may dictate the state of cancer progression. Based on these results, we suggest that differential regulation of hMena^{11a} and hMena^{INV} 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 intermediately expresses both splice variants,

and HeLaS3 cells undergoing EMT (vimentin-positive and E-cadherin negative) express only $hMena^{11a}$ without $hMena^{INV}$. 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 isoform such as $hMena^{INV}$.

In conclusion, we have demonstrated that determination of $hMena^{11a}$ and $hMena^{INV}$ 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.

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