Aberrant expression of human ortholog of mammalian enabled (hMena) in human colorectal carcinomas: Implications for its role in tumor progression

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Abstract. The human ortholog of mammalian enabled (hMena), a family of enabled/vasodilator-stimulated phosphoprotein (Ena/VASP), is an actin regulatory protein involved in the regulation of cell motility. Increasing evidence suggests that hMena over-expression is involved in human breast cancers, whereas the significance of hMena expression in colorectal carcinomas remains to be elucidated. In this study, we assessed the relative mRNA level of hMena using real-time PCR, showing that there is a statistically significant increase of hMena transcripts in matched human colorectal carcinomas and adjacent non-neoplastic colorectal epithelium (n=6, P=0.046). We also performed immunohistochemical analysis of the expression of hMena protein in 50 cases of paraffin-embedded archival colorectal tissues, and found that an elevated hMena expression is correlated to the cases with advanced TNM stages of colorectal carcinomas (P<0.001). On further inspection of immunohistochemical features of each specimen, we observed intensified hMena staining in the invasive front of colorectal carcinomas, especially in tumor budding, a transition from glandular structure to single or small clusters of cells at the invasive front. We demonstrated that there was a significantly increased hMena staining in the tumor budding as compared with more morphologically-differentiated areas of colorectal carcinomas, indicating that hMena over-expression may have a role in the initial steps of tumor invasion from primary sites. We performed *in vitro* motility assays to show that transient hMena transfection markedly enhanced the chemotactic/chemokinetic activity of HeLaS3 cells (P<0.001). Taken together, these results suggest that hMena over-expression is implicated in the progression of colorectal carcinomas by positively affecting the migratory phenotype of cancer cells.

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Key words: human ortholog of mammalian enabled, Ena/VASP, colorectal carcinoma, real-time PCR, immunohistochemistry, invasion, tumor budding, cell migration

Introduction

Colorectal carcinoma (CRC) is one of the most common causes of cancer in Western countries (1), as well as in Japan. Despite improved surgical techniques and adjuvant/neo-adjuvant chemotherapy, patients with progressive disease still have poor clinical outcomes. The five-year survival rate for patients with CRCs is about 65%, whereas the rate drops to 10% or less in patients with advanced disease (1,2). To improve treatments for CRCs, the prediction of final outcome is of great importance because more aggressive (adjuvant) treatment of high risk patients would be a rational strategy. Thus far, conventional anatomical classification (3) remains the most widely used prognostic indicator for CRCs. Nevertheless, a variety of research has focused on the expression of genes involved in invasion and metastasis (4-7), as local invasion and distant metastasis is the most frequent cause of cancer morbidity and mortality.

When a cancer cell invades or metastasizes, multiple steps are involved; dissociation from the primary tumor, invasion of surrounding stroma, and intravasation into the vascular system (8). During these steps, invasive carcinoma cells acquire a migratory phenotype associated with increased expression of several genes that regulate actin polymerization (9,10), which are also required for the processes of cell migration in normal, non-neoplastic cells including embryonic morphogenesis, wound healing and immune-cell trafficking (8). Therefore, it is critical for understanding the nature of cancer invasion and metastasis to analyze the expression of genes that regulate actin polymerization.

Cell migration involves a change of cell morphology because of a dynamic rearrangement of the actin cytoskeleton (11). In recent years, actin-associated proteins have been studied in more detail, in relation to the cell migration as well as tumor cell invasion (12). The Ena/VASP family of proteins act as a key molecule in cell migration, regulating the actin cytoskeleton by protecting actin filaments from capping

proteins at their barbed end (13,14). Recently, it has become evident that the human ortholog of mammalian enabled (hMena), one of the Ena/VASP family, may have a role in human carcinogenesis (9,15,16). In breast cancers, hMena is reported to be over-expressed (15,16), and its over-expression correlates to the poor prognostic phenotype (16). Other members of Ena/VASP family, VASP and EVL, are implicated in tumor progression (17-19). Thus, it seems to be reasonable to examine the expression of Ena/VASP in malignant neoplasms.

The aim of the present study was to examine whether the expression of the Ena/VASP protein is altered in human colorectal tumors. We assessed the expression of hMena in tumor cell lines from different types of human cancer and surgically-resected CRC specimens using real-time PCR and immunohistochemistry. We also analyzed whether the over-expression of hMena affects the migratory phenotype of cancer cells *in vitro*.

Materials and methods

Sample collection. Six fresh CRC and matched non-tumor samples were obtained from specimens surgically resected between 2005 and 2007 in JFE Kawatetsu Chiba hospital (Chiba, Japan) for semi-quantitative assay of hMena mRNA. Those samples were snap-frozen in liquid nitrogen within 10 min of arrival at the pathology facility and kept frozen until use. Fifty paraffin-embedded archival materials were provided from JFE Kawatetsu Chiba hospital and Narita Red-Cross hospital (Chiba, Japan) for immunohistochemical analysis of hMena. All the sample collections were approved by the ethics committees of those hospitals and Chiba University.

Cell culture. HeLaS3 (human cervical cancer), MCF7 (human breast cancer), MDA-MB468 (human breast cancer), PC14 (human lung squamous cell carcinoma), NCI-H69 (human small cell lung cancer), LU130 (human lung adeno-carcinoma), Colo320 (human colon adenocarcinoma) and U251 (human glioblastoma) were purchased from the American Type Culture Collection. All the cell lines, except HeLaS3, were cultured with Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% fetal bovine serum. HeLaS3 cells were grown with Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% calf serum. All the cells were maintained under 5% CO_2 at 37˚C.

Molecular cloning of hMena. To obtain human Mena cDNA, reverse transcriptase-PCR from HeLa cell mRNA was performed. A forward primer (5'-GGCACCATGAGTGA ACAGAGTA-3') and a reverse primer (5'-GCTCATAAATG TAGGGGTTTGC-3') were used. The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and their sequences were determined. The sequences of the 14 clones corresponded to hMena that has been reported in the public genome database (GenBank accession no. NM_018212).

Semi-quantitative real-time polymerase chain reaction (real-time PCR). To elucidate expression pattern of hMena mRNA in morbid cells, total RNA of cell lines or fresh frozen colorectal samples were extracted with TRIzol (Invitrogen) according to manufacturer's instruction. Prior to running real-time PCR, cDNA was synthesized from 500 ng of total RNA with PrimeScript reverse transcript reagent (Takara) according to manufacturer's instructions. Semi-quantitative real-time PCR analysis of hMena was then performed using a Prism 7000 (Applied biosystems) thermal cycler with SYBR pre-mix ExTaq reagent (Takara). Primers were as follows: hMena (sense, 5'-GCTGGTGGCTCAACTGGATTC-3'; antisense, 5'-TGCACAAGAGACATCATCCAA-3'), and human GAPDH (sense, 5'-GCACCGTCAAGGCTGAG AAC-3', antisense, 5'-TGGTGAAGACGCCAGTGGA-3'). Each PCR cycle consisted of 10 sec at 95˚C and 40 repeats (5 sec at 95˚C and 31 sec at 60˚C). The relative expression level of hMena was calculated by comparative threshold cycle (Ct) method (20).

Antibodies. The cDNA fragment encoding the N-terminal region (amino acids (aa) 1-128) of hMena was inserted into expression vector pGEX-4T-1 (Amersham Biosciences). The glutathione S-transferase (GST)-Mena fusion protein was expressed in Rosetta2 (DE3) *Escherichia coli* cells (Novagen) and purified according to the standard method. Rabbits were immunized with the purified protein (MBL Inc.). The sera were first depleted of anti-GST antibodies by chromatography on a GST column, and then the anti-Mena antibody was affinity-purified using antigen-coupled beads. Additional antibodies, a commercial anti-hMena antibody (clone 21, BD transduction laboratory) and goat polyclonal antibody against ß-actin (AbCam) were used.

Western blotting. Western blotting was performed as described previously (21-23). The primary used antibody was an anti-hMena monoclonal antibody. Blotting with anti ß-actin was used as a loading control.

Immunohistochemistry. Expression of hMena protein was assessed by indirect immunoperoxidase staining. Sections of formalin-fixed, paraffin-embedded archival tissue (5 μm thick) were harvested on MDS-coated Superfrost slides (Matsunami). Sections were de-parafinized, rehydrated and pretreated for antigen retrieval by microwave treatment for 10 min in 10 mM of citrate buffer, pH 6.0. The IgG fraction of rabbit antiserum to hMena (1:100) was used as the primary antibody. Biotinylated swine anti-rabbit Ig antiserum (1:500; Dako Cytomation) served as the secondary antibody. The immunoreaction was visualized by Vectastain ABC kit (Vector laboratories) using diaminobenzidine (Wako) as the chromogenic substrate. Sections were slightly counterstained with Mayer's hematoxylin, dehydrated, and mounted for microscopic visualization. Human cerebellum, which is known to show strong immunostaining for hMena, were included as positive controls in each staining batch. The immunoreactivity of hMena was completely eradicated by the pre-incubation with an extract of hMena-transfected colon cancer cell line. The intensity of hMena staining of colorectal tumor tissue was evaluated as scores 0 to 3+. The scoring was carried out by comparing the immunoreactivity of tumor cells and normal adjacent normal colonic epithelium. Criteria of scoring were

as follows: no staining or equal to adjacent normal epithelia, score 0; more than normal epithelia, but weak diffuse cytoplasmic staining in <10% of neoplastic cells, score 1+; moderate cytoplasmic staining in 10-70%, score 2+; strong cytoplasmic staining in >70% of the tumor cells with or without a juxtamembrane reinforce, score 3+. The histopathological characteristics and the hMena scoring of each specimen were independently assessed by two pathologists (A.T. and H.K.) in a double-blinded manner. The presence of tumor budding was determined according to the criteria proposed by Ueno *et al* (24), wherein budding is defined as an isolated single cancer cell or a cluster composed of fewer than five undifferentiated cancer cells appearing to bud from a large cancer gland at the invasive front. Each specimen demonstrated more than ten budding foci under a microscopic field using a 20x objective lens. The average scores for five glandular/budding areas in the same materials were compared to elucidate the immunohistological characteristics of hMena in the tumor budding of CRCs.

Motility assay. The full length hMena cDNA was cloned in frame with monomeric Venus, a variant YFP (a gift from Dr A. Miyawaki, Riken Brain Science Institute) as a C-terminal fusion (pVenus-hMena). A HeLaS3 cervical cancer cell line was used to investigate cell motility. pVenus-hMena was transfected to HeLaS3 cell with Lipofectamine2000 transfecting reagent (Invitrogen). After overnight incubation of cells with DMEM supplemented with 2% calf serum, motility assays were carried out using Boyden-chamber. A Boyden chamber is based on a chamber with two medium-filled compartments. Each upper/lower chamber had different concentration (2%/2%, 10%/10% and 2%/10%) of calf serum. The lower and upper chambers were separated by an 8-μm pore size polycarbonate membrane. Cells were allowed to migrate for 6 h. The membrane was fixed and stained with Meyer's hematoxylin. Cells on the upper side of the membrane were removed by cotton swabs. Cells on the lower side of the membrane were counted using a light microscope at x200 magnification. Four random microscope fields were counted, and the results were expressed as the mean ± SD.

Statistical analysis. Statistical analysis was performed using Microsoft Excel (Microsoft) and KaleidaGraph (GraphPad Software) for the PC. Unpaired/paired sample t-test, Mann-Whitney U-test, Spearman's rank correlation test, Kruskal-Wallis test or Wilcoxon's signed rank test were used as necessary. P-values ≤0.05 were considered statistically significant.

Results

hMena transcript expression profiles of human tumor cell lines. We used real-time PCR on a panel of human tumor cell lines originating from different organs to determine whether hMena mRNA is expressed in tumor cells. All data were normalized according to GAPDH mRNA. Fig. 1 reveals that the expression of hMena mRNA varies between the eight human tumor cell lines examined, with the highest level of hMena transcripts being detected in Colo320 colon cancer cell line. Similarly to a previous report (15), considerable amounts of hMena transcript is also seen in the MCF7 breast cancer cell line, whereas the HeLaS3 human cervical cancer cell line showed a very low level of hMena mRNA expression. The expression profile of hMena protein was found to be similar to that of hMena mRNA in those cell lines (data not shown).

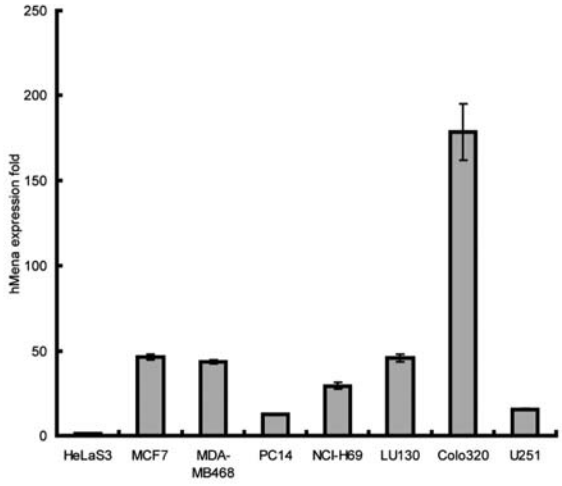

Figure 1. mRNA expression of hMena in a variety of tumor cell lines. Colo320 (colon adenocarcinoma) expressed a high amount of hMena mRNA, compared with MCF7 (breast adenocarcinoma), formerly reported to show up-regulation of hMena mRNA (15). Conversely, HeLaS3 (cervical cancer) showed a very low level of hMena mRNA. All experiments were done in triplicate, and all the values were expressed as the mean ± SE.

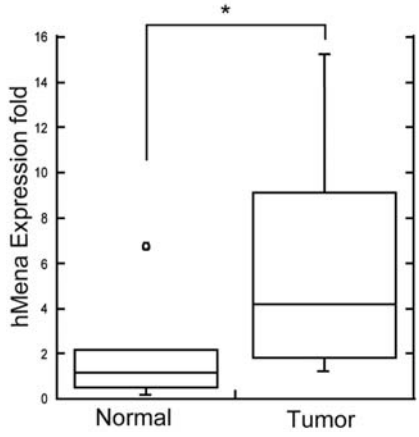

Figure 2. hMena mRNA expression in six cases of colorectal tissue. There is a significant increase of hMena transcripts in CRC as compared to matched non-neoplastic adjacent mucosa (P<0.05, Wilcoxon's signed rank test). °Extreme outlier.

Up-regulation of hMena expression in colorectal cancer tissues. As Colo320 showed the highest amount of hMena mRNA expression, we investigated whether hMena expression is altered in human CRC. To explore the differences in hMena mRNA expression between the paired cancer and non-neoplastic colorectal tissues, we examined the hMena/GAPDH mRNA ratios of the tumors to those of matched normal tissues by using comparative Ct analysis (20). We carried out real-time PCR analysis of six cases of human colorectal samples (Table I). As shown in Fig. 2, the relative expression

Table I. Clinicopathological characteristics and relative hMena mRNA expression of fresh colorectal samples derived from surgically resected specimens.

No.	Age	Sex	T	N	M	Stage	Histotype	Relative mRNA level	
								Non-neoplastic	Cancer
1	57	F	4	1	0	IIIB	Mod	1.61	15.2
2	70	M	4	2	0	IIIC	Mod	6.76	9.02
3	79	F	4	0	0	IIB	Mod	2.17	4.51
4	67	M	3	0	0	IIA	Mod	0.732	1.38
5	68	M	3	2	0	IIIB	Mod	0.51	3.86
6	74	M	1	0	0	I	Mod	0.244	1.81

F, female; M, male; Mod, moderately differentiated adenocarcinoma.

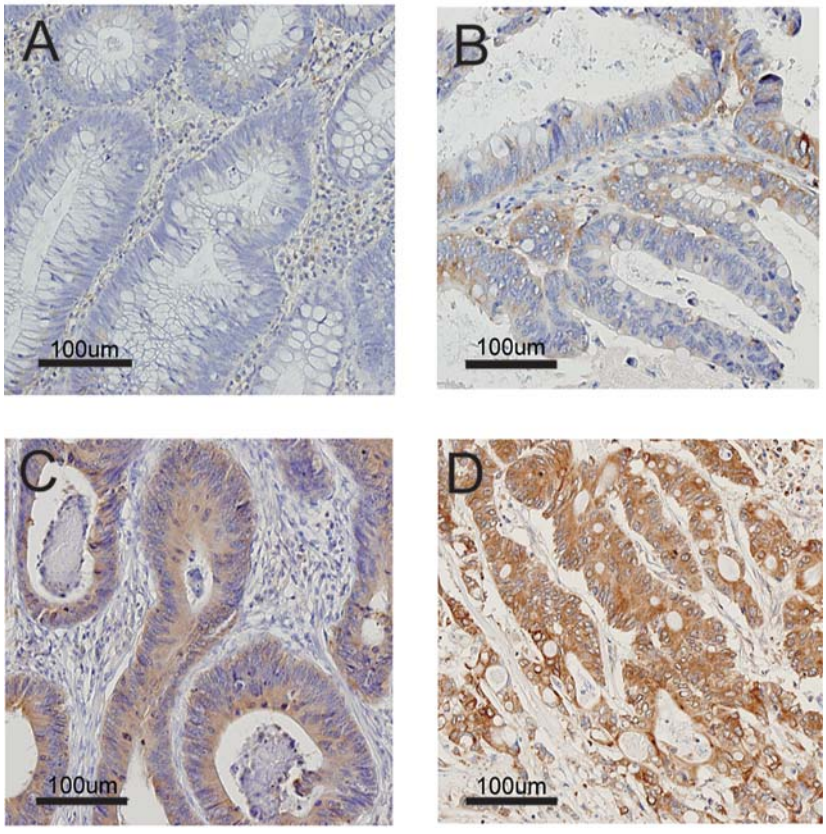

Figure 3. Immunohistochemical findings of hMena in colorectal tissues. (A) Score 0. Almost no hMena staining is observed. (B) Score 1+. Weak diffuse cytoplasmic staining in <10% of neoplastic cells. (C) Score 2+. Moderate cytoplasmic staining in 10% to 70% of the tumor cells. (D) Score 3+. Strong cytoplasmic staining in >70% of the tumor cells with juxtamembrane reinforcement.

levels of hMena ranged from 0.244 to 6.76 (median, 1.17; mean, 2.00) in non-neoplastic colorectal tissues and ranged from 1.38 to 15.2 (median, 4.18; mean, 5.96) in CRC specimens, indicating that there was a statistically significant (Wilcoxon's signed rank test, P=0.046) increase of hMena mRNA expression in CRC tissues compared with their matched normal counterparts (Fig. 2). These results indicate that the increased expression of hMena may be a marker for CRCs.

Immunohistochemistry of hMena in human colorectal tissues. To assess the expression of hMena in CRC, we examined paraffin-embedded colorectal tissues by using immunohistochemistry. We analyzed 50 cases of colorectal samples which are grouped according to the criteria defined by Tumor-Node-Metastasis (TNM) classification (25). Clinicopathological data of the cases are shown in Table II. In normal epithelium of the colon, hMena staining was almost undetectable in non-neoplastic epithelial cells. Weak to

Table II. Clinicopathological characteristics of 50 colorectal specimens served for immunohistochemical analysis.

Median age, years (range, mean)	67 (54-87, 64.91)
Non-neoplastic epithelium (3 cases)	
Inflammatory hyperplasia	3 (6%)
Adenomas (18 cases)	
Low grade dysplasia	13 (26%)
High grade dysplasia	5 (10%)
Adenocarcinomas (29 cases)	
Depth	
Tis	5 (10%)
T1	3 (6%)
T2	3 (6%)
T3	11 (22%)
T4	7 (14%)
Clinical stage	
0	5 (10%)
I	6 (12%)
IIA	5 (10%)
IIB	2 (4%)
IIIA	0 (0%)
IIIB	4 (8%)
IIIC	6 (12%)
IV	1 (2%)
Lymph node status	
Positive	11 (22%)
Negative	18 (36%)
Vascular involvement	
Positive	22 (44%)
Negative	7 (14%)
Histotype	
Well differentiated	10 (20%)
Moderately differentiated	16 (32%)
Mucinous	3 (6%)

Table III. A, hMena scores in non-neoplastic, adenomas and carcinomas of colorectal tissue.

	Score 0	Score 1+	Score 2+	Score 3+	Total
Non-neoplastic					
Hyperplasia	3	0	0	0	3
Adenomas					
Low grade	13	0	0	0	13
High grade	1	4	0	0	5
Carcinomas					
Tis	0	4	1	0	5
T1	0	1	1	1	3
T2	0	0	2	1	3
T3	0	0	5	6	11
T4	0	0	3	4	7

Cases with carcinomas were grouped according to the depth (T-factor) of tumor.

B, hMena scores of CRC, classified a criteria according to TNM staging.

TNM stage	Score 0	Score 1	Score 2	Score 3	Total
0	0	4	1	0	5
I	0	1	3	2	6
IIA	0	0	3	2	5
IIB	0	0	2	0	2
IIIA	0	0	0	0	0
IIIB	0	0	1	3	4
IIIC	0	0	1	5	6
IV	0	0	1	0	1

C, Statistical analysis between the hMena score and the clinicopathological parameters of CRC specimens.

Parameters	P-value
Carcinomas vs. adenomas	$3.7x10^{-5}$[a]
T-factor (T1 to T4)	0.00076[b]
Lymph node status (negative vs. positive)	0.0097[a]
Vascular involvement (negative vs. positive)	0.0015[a]
TNM staging (Stage 0 to Stage IV)	0.00077[b]
Metastasis (negative vs. positive)	0.67[a]
Histotype (well vs. moderately differentiated)	0.099[c]

[a]Mann-Whitney's U test, [b]Spearman's rank correlation test. [c]Kruskal-Wallis test. $P<0.05$ is considered as significant.

moderate hMena expression is found in smooth muscle, fibroblasts and intramural ganglia cells, whereas levels of expression varied and tended to be more abundant in tumor tissues than those in normal epithelia (Fig. 3). To evaluate the expression of hMena in tumor tissues, we developed an immunohistochemical scoring system of 0 to 3+ in accordance with their staining intensity of tumor cells in the most central section in colorectal tumors, where the staining intensity of hMena of tumor-adjacent normal colonic epithelium was defined as score 0. The scoring of hyperplastic epithelia (n=3) and adenomas with low grade dysplasia (n=13) were score 0, whereas adenomas with high grade dysplasia and carcinomas showed increased immunoreactivity of hMena (Table IIIA). We also examined the correlation between various clinicopathological parameters of tumors and the intensity of hMena expression. As shown in Table IIIB, advanced T stage was significantly associated with the pathological score of hMena (Spearman's rank correlation test, $P<0.001$). Primary cancer cells with lymph node metastasis showed higher scores than those without lymph

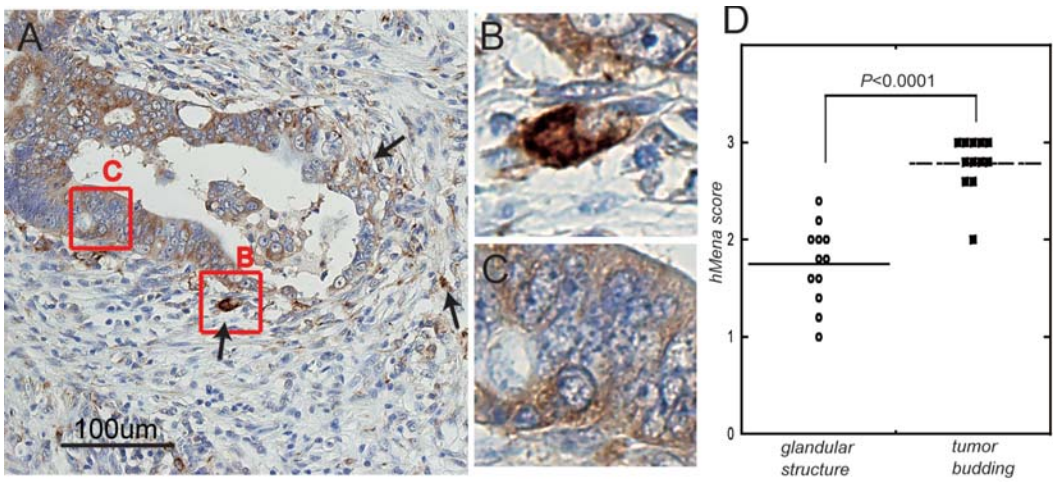

Figure 4. (A) Immunohistochemical findings of hMena at the invasive front of CRC. Arrows indicate the dissociated cancer cells derived from tumor budding. (B) Strong hMena staining with prominent juxtamembrane reinforcement is observed in focal dissociated cancer cell. (C) Differentiated glandular structure of CRC. (D) Significant increase of hMena staining intensity (P<0.0001, paired sample t-test) is observed in tumor budding.

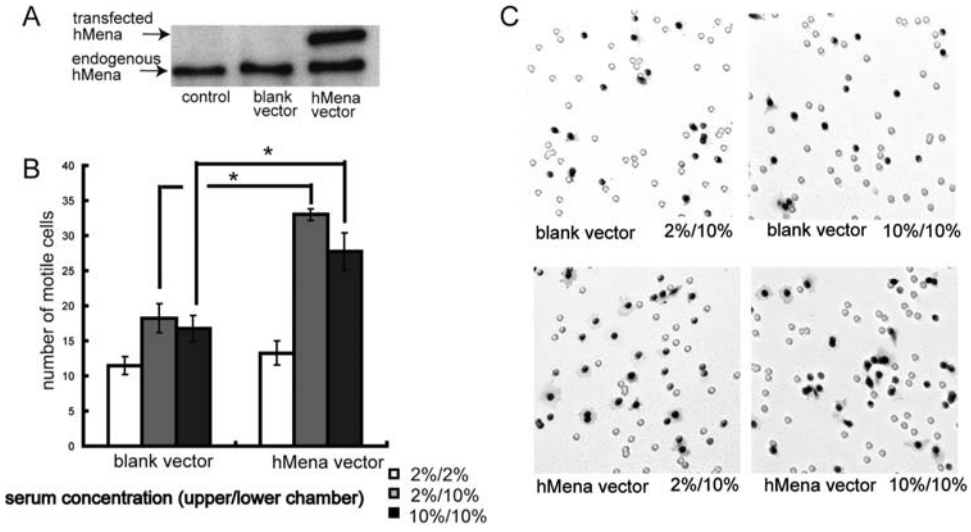

Figure 5. Transient hMena expression enhances the migratory activity of HeLaS3 cells. (A) Western blotting of hMena in HeLaS3 cells transfected with blank vector (pVenus) or hMena expression vector (pVenus-hMena). While endogenous levels of hMena are in the same level, pVenus-hMena transfected cell demonstrated a significant increase of transfected hMena. (B) Boyden chamber assay of HelaS3 cells transfected with blank vector or hMena expression vector, showing that transient transfection of hMena lead to enhanced number of motile cells (*P<0.001, unpaired sample t-test). (C) Representative images of a migration assay for migrated HeLaS3 cell expressing pVenus or pVenus-hMena. Magnification, x200.

node metastasis (Mann-Whitney's U test, P<0.01), and primary cancer cells with vascular invasion also showed a greater score than those without vascular invasion (Mann-Whitney's U test, P<0.001). Additionally, the cases with more advanced TNM stage of tumors tended to show more intense hMena staining (Spearman's rank correlation test, P<0.001). These results seem to indicate that the increased expression of hMena may be associated with CRC progression.

Enhanced expression of hMena in tumor budding. On further inspection of specimens, we noticed an uneven distribution of hMena immunoreactivity in invasive carcinomas. As shown in Fig. 4A, an intensified expression of hMena is observed at the invasive front of cancer. Especially, strong cytoplasmic/membranous hMena staining is detected at focal de-differentiated area of tumor (Fig. 4B) called tumor budding (26-28), compared with well-differentiated glandular structure of the tumor (Fig. 4C). Based on this finding, we carried out a comparison of hMena scoring between the glandular structure and the tumor budding observed in the same specimen. Twelve samples, which included prominent tumor budding, were used for this investigation. Fig. 4D demonstrates that there is a significant increase of hMena immunoreaction seen in tumor budding, compared with the differentiated cancer gland (P<0.0001, paired sample t-test). These results indicate that the increased expression of hMena may promote invasion of CRCs.

Increased expression of hMena enhances the motility of HeLaS3 cells. Based upon the immunohistochemical analysis, we hypothesized that hMena may promote cell migration of

cancer cells. Thus, an *in vitro* motility assay was performed to test this possibility. HeLaS3, which originally expressed the lowest amount of hMena in all cell lines examined, was used for this study. The motility of HeLa S3 cells transfected with either hMena (pVenus-hMena) or blank vector were examined using a Boyden chamber assay, of which upper/lower chambers contained media with the different serum concentrations (upper/lower :2%/2%, 2%/10% or 10%/10%). Media with the serum 2%/10% and 10%/10% markedly stimulated chemotaxis and chemokinesis of cancer cells respectively (29), and that with the serum 2%/2% as control. Fig. 5 shows that hMena-transfected cells, compared with control cells, showed about a 2-fold increase in the expression of hMena by Western blotting and about a 2-fold increase of both chemotaxis and chemokinesis (unpaired sample t-test, $P<0.001$). These results support the notion that over-expression of hMena induces the migratory phenotype of cancer cells and results in their invasion.

Discussion

Ena/VASP proteins are important regulatory molecules of actin cytoskeleton dynamics leading to cell motility (14,30-32). Changes of the cellular actin network play a role in malignant transformation and tumor progression (33). We postulated that increased expression of hMena may have a role in tumor progression in malignant neoplasms. In this study, we first examined the expression of hMena in tumor cell lines and found that the expression of hMena varied in cell types and remarkable over-expression was found in Colo320 cells of CRC. These results raise the possibility that the over-expression of hMena may have an important role in the behavior of CRCs. We next tested the expression of hMena in paired fresh-frozen neoplastic tissues and the corresponding non-neoplastic regions in the CRC samples by real-time PCR. All CRC tissues examined exhibited increased expression of hMena compared with non-neoplastic counterparts. These results are similar to recent reports of both breast cancer (15,16) and lung cancer (18). Accordingly, we conducted further analysis of the association between hMena expression and tumor progression in CRCs. We examined the expression of hMena in both colorectal adenomas and carcinomas by immunohistochemistry. There are significant differences of hMena expression between adenomas and carcinomas, suggesting that the expression of hMena may be altered during the malignant transformation of CRCs and have a role in colorectal cancer progression.

Clinical and pathologic variables, such as vegetating growth, tumor size, Dukes' staging (as well as Astler-Coller staging), intramural spread, tumor differentiation grade, lymph node involvement, venous involvement, distant metastasis, etc, have been discussed to predict the prognosis of patients with CRC (5,34-36). Above all, the TNM staging is regarded as the gold standard of prognostic factors in CRC, and the five-year survival rate for patients with CRCs is largely depended on TNM stage (3,5,25). We showed that over-expression of hMena is observed in the cases with advanced TNM stages. However, increasing evidence suggests that TNM staging may by itself be insufficient to predict the clinical outcome of CRC patients (37). Therefore, a variety of novel observations have been documented to develop more detailed classification parameters or molecular markers to improve the prediction of final outcome of CRC patients (4-7,38). Among them, tumor budding, a transition from glandular structure to single or small clusters of cells at the invasive front of CRCs, has attracted a lot of attention. Recent studies have shown that tumor budding is an independent risk factor of local spread, lymph node and distant metastasis, recurrence, and worse survival following curative surgery (24,39-44). In the present study, we demonstrated that a remarkable increase in staining of hMena is observed in tumor budding compared with more morphologically-differentiated structure of CRCs. This suggests that over-expression of hMena may have a role in the initial steps of tumor invasion from primary sites and might become a novel candidate for a predictor of poor outcome in CRCs.

Invasive cancer cells manifest a migratory phenotype that can lead them to respond to cues from the microenvironment that triggers tumor invasion (10). To examine the role of hMena on cell migration, we performed *in vitro* motility assay with Hela S3 cells and showed that increased expression of hMena enhances both chemotaxis and chemokinesis. These findings support the idea that hMena has an important role in cancer cell migration/invasion and raise the possibility that over-expression of hMena may augment the invasive capacity from the primary sites and promote tumor progression of CRC. However, the detailed mechanism of hMena in cell migration and invasion remains unclear. Further investigation may provide insights into the prediction of progression of CRCs and novel therapeutic strategies against CRCs.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. CA Cancer J Clin 58: 43-66, 2007.
2. O'Connell JB, Maggard MA and Ko CY: Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst 96: 1420-1425, 2004.
3. Sobin LH: TNM: principles, history, and relation to other prognostic factors. Cancer 91: 1589-1592, 2001.
4. Bosman FT: Prognostic value of pathological characteristics of colorectal cancer. Eur J Cancer 31A: 1216-1221, 1995.
5. Zlobec I and Lugli A: Prognostic and predictive factors in colorectal cancer. J Clin Pathol 212: 561-569, 2008.
6. Barozzi C, Ravaioli M, D'Errico A, *et al*: Relevance of biologic markers in colorectal carcinoma: a comparative study of a broad panel. Cancer 94: 647-657, 2002.
7. Lyall MS, Dundas SR, Curran S and Murray GI: Profiling markers of prognosis in colorectal cancer. Clin Cancer Res 12: 1184-1191, 2006.
8. Friedl P and Wolf K: Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 3: 362-374, 2003.

9. Wang W, Goswami S, Lapidus K, *et al*: Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res 64: 8585-8594, 2004.
10. Wang W, Goswami S, Sahai E, Wyckoff JB, Segall JE and Condeelis JS: Tumor cells caught in the act of invading: their strategy for enhanced cell motility. Trends Cell Biol 15: 138-145, 2005.
11. Lauffenburger DA and Horwitz AF: Cell migration: a physically integrated molecular process. Cell 84: 359-369, 1996.
12. Yamaguchi H and Condeelis J: Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta 1773: 642-652, 2007.
13. Barzik M, Kotova TI, Higgs HN, *et al*: Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. J Biol Chem 280: 28653-28662, 2005.
14. Krause M, Dent EW, Bear JE, Loureiro JJ and Gertler FB: Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol 19: 541-564, 2003.
15. Di Modugno F, Bronzi G, Scanlan MJ, *et al*: Human Mena protein, a serex-defined antigen overexpressed in breast cancer eliciting both humoral and CD8+ T-cell immune response. Int J Cancer 109: 909-918, 2004.
16. Di Modugno F, Mottolese M, Di Benedetto A, *et al*: The cytoskeleton regulatory protein hMena (ENAH) is overexpressed in human benign breast lesions with high risk of transformation and human epidermal growth factor receptor-2-positive/-hormonal receptor-negative tumors. Clin Cancer Res 12: 1470-1478, 2006.
17. Liu K, Li L, Nisson PE, Gruber C, Jessee J and Cohen SN: Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. Mol Cell Biol 19: 3696-3703, 1999.
18. Dertsiz L, Ozbilim G, Kayisli Y, Gokhan GA, Demircan A and Kayisli UA: Differential expression of VASP in normal lung tissue and lung adenocarcinomas. Thorax 60: 576-581, 2005.
19. Hu LD, Zou HF, Zhan SX and Cao KM: EVL (Ena/VASP-like) expression is up-regulated in human breast cancer and its relative expression level is correlated with clinical stages. Oncol Rep 19: 1015-1020, 2008.
20. Monney L, Sabatos CA, Gaglia JL, *et al*: Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 415: 536-541, 2002.
21. Kawana H, Tamaru J, Tanaka T, *et al*: Role of p27Kip1 and cyclin-dependent kinase 2 in the proliferation of non-small cell lung cancer. Am J Pathol 153: 505-513, 1998.
22. Kitagawa M, Oyama T, Kawashima T, *et al*: A human protein with sequence similarity to *Drosophila* mastermind coordinates the nuclear form of notch and a CSL protein to build a transcriptional activator complex on target promoters. Mol Cell Biol 21: 4337-4346, 2001.
23. Fujita Y, Kitagawa M, Nakamura S, *et al*: CD44 signaling through focal adhesion kinase and its anti-apoptotic effect. FEBS Lett 528: 101-108, 2002.
24. Ueno H, Murphy J, Jass JR, Mochizuki H and Talbot IC: Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 40: 127-132, 2002.
25. Sobin L and Wittekind C: TNM Classification of malignant tumors. Wiley-Liss, New York, 2002.
26. Kirchner T and Brabletz T: Patterning and nuclear beta-catenin expression in the colonic adenoma-carcinoma sequence. Analogies with embryonic gastrulation. Am J Pathol 157: 1113-1121, 2000.
27. Prall F: Tumour budding in colorectal carcinoma. Histopathology 50: 151-162, 2007.
28. Brabletz T, Jung A, Reu S, *et al*: Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci USA 98: 10356-10361, 2001.
29. Guan JL: Cell migration: developmental methods and protocols. In: Methods in Molecular Biology. Vol. 294. Humana Press Inc., Tatowa, NJ, 2005.
30. Bear JE, Svitkina TM, Krause M, *et al*: Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109: 509-521, 2002.
31. Loureiro JJ, Rubinson DA, Bear JE, Baltus GA, Kwiatkowski AV and Gertler FB: Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. Mol Biol Cell 13: 2533-2546, 2002.
32. Machesky LM: Sharks' teeth and dunes. Nature 417: 494-497, 2002.
33. Rao J and Li N: Microfilament actin remodeling as a potential target for cancer drug development. Curr Cancer Drug Targets 4: 345-354, 2004.
34. Crucitti F, Sofo L, Doglietto GB, *et al*: Prognostic factors in colorectal cancer: current status and new trends. J Surg Oncol Suppl 2: 76-82, 1991.
35. Horn A, Dahl O and Morild I: The role of venous and neural invasion on survival in rectal adenocarcinoma. Dis Colon Rectum 33: 598-601, 1990.
36. Ratto C, Sofo L, Ippoliti M, Merico M, Doglietto GB and Crucitti F: Prognostic factors in colorectal cancer. Literature review for clinical application. Dis Colon Rectum 41: 1033-1049, 1998.
37. Nathanson SD, Schultz L, Tilley B and Kambouris A: Carcinomas of the colon and rectum. A comparison of staging classifications. Am Surg 52: 428-433, 1986.
38. Compton C, Fenoglio-Preiser CM, Pettigrew N and Fielding LP: American Joint Committee on Cancer Prognostic Factors Consensus Conference: Colorectal Working Group. Cancer 88: 1739-1757, 2000.
39. Zlobec I, Lugli A, Baker K, *et al*: Role of APAF-1: E-cadherin and peritumoral lymphocytic infiltration in tumour budding in colorectal cancer. J Pathol 212: 260-268, 2007.
40. Ueno H, Mochizuki H, Hashiguchi Y, *et al*: Risk factors for an adverse outcome in early invasive colorectal carcinoma. Gastroenterology 127: 385-394, 2004.
41. Nakamura T, Mitomi H, Kikuchi S, Ohtani Y and Sato K: Evaluation of the usefulness of tumor budding on the prediction of metastasis to the lung and liver after curative excision of colorectal cancer. Hepatogastroenterology 52: 1432-1435, 2005.
42. Jass JR, Atkin WS, Cuzick J, *et al*: The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. Histopathology 41: 59-81, 2002.
43. Hase K, Shatney C, Johnson D, Trollope M and Vierra M: Prognostic value of tumor 'budding' in patients with colorectal cancer. Dis Colon Rectum 36: 627-635, 1993.
44. Prall F, Nizze H and Barten M: Tumour budding as prognostic factor in stage I/II colorectal carcinoma. Histopathology 47: 17-24, 2005.