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Enabled homolog shown to be a potential biomarker and prognostic indicator for breast cancer by bioinformatics analysis

Abstract

Background: Human enabled homolog (ENAH; also known as human ortholog of mammalian enabled, hMENA) is a member of the enabled/vasodilator-stimulated phosphor protein family that regulates fibroblast movement and nervous system development. The ENAH over-expression promotes breast cancer (BC) cell invasion and metastasis.

Methods: We studied *ENAH* mRNA expression in various tumors and normal tissues using the ONCOMINE database, and in an array of cancer cell lines using Cancer Cell Line Encyclopedia data. We also investigated the prognostic value of *ENAH* expression in patients with BC using Kaplan-Meier plots.

Results: Compared with normal tissues, *ENAH* expression levels were markedly elevated in BC. We identified a correlation between low *ENAH* and superior relapse-free survival (RFS) of patients with BC; specifically, those with ER(-), HER-2(+), Grade 3 and wild-type TP53 subtypes. Additionally, a correlation was detected between low *ENAH* and prolonged overall survival of patients with luminal B disease.

Conclusion: *ENAH* is a potential biomarker and important prognostic factor in BC.

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Breast cancer (BC) is the most common malignant tumor in women and its incidence is increasing annually. According to the World Health Organization, an average of 1.2 million women worldwide suffers from, and half a million die from, BC each year, equating to one woman dying of BC every minute [1].

Invasion and metastasis (both systemic and hematologic) are leading causes of death in patients with BC [2,3]. Approximately 10%–15% of patients with BC develop metastatic disease within three years of diagnosis; however, occult metastases can occur after as long as 10 years. Consequently, most patients with BC remain at risk of metastatic disease for the rest of their lives [4,5].

Breast cancer etiology is multifactorial, and includes gene mutations, virus infections and environmental factors. The detection of genes associated with BC is valuable for guiding treatment and predicting prognosis. Over-expression of enabled homolog (*ENAH*; also known as human ortholog of mammalian-enabled, *hMENA*), can promote BC infiltration and metastasis [6]. The *ENAH* is a member of the enabled/vasodilator-stimulated phosphor protein (Ena/VASP) family [7–9], members of which function in various actin-dependent processes, including fibroblast movement, nervous system development and tumor cell migration.

The *ENAH* is either not expressed or barely expressed in normal breast tissue, while its levels increase on BC development and elevate further in higher grade malignancies [10], promoting BC cell invasion and metastasis. The *ENAH* enhances calcium uptake and promotes cancer cell adhesion in metastatic sites, facilitating tumor metastasis [11]. Together with *ENAH*, epidermal growth factor (EGF) is important for tumor cell adhesion, transfer, apoptosis suppression and tumor angiogenesis, and is associated with increased lung metastasis risk [12–15].

Malignant cells are characterized by continuous growth, which is associated with cell proliferation and apoptosis dysregulation. The *ENAH* connects multiple signaling pathways [16–18], promoting signal transmission and facilitating tumor cell infiltration and migration. Regulation of the cytoskeleton protein, actin, is impaired during BC metastasis [19]. The *ENAH* regulates actin, participates in numerous actin-dependent processes, and can promote BC cell migration through actin regulation [20,21]. Moreover, *ENAH* supports nutrient supply for tumor cell differentiation and growth by promoting tumor blood vessel formation, which facilitates metastasis.

Studies had shown that *ENAH* over-expression is involved in human colorectal carcinoma, gastric cancer (GC) and hepatocellular carcinoma. A recent study demonstrated that *ENAH* over-expression in GC was associated with AJCC staging, depth of invasion and OS and *ENAH* silencing inhibited the proliferation and metastasis of GC cells. This suggests that *ENAH* is a new target for GC treatment [22].

Although *ENAH* is known to promote BC cell invasion and metastasis, its clinical significance has yet to be comprehensively analyzed in BC subtypes, including those positive for estrogen receptor (ER) or human epidermal growth factor receptor 2 (HER-2). Currently, there is no effective method for prediction of BC metastasis. In this study, by biological data mining, we provide the first evaluation of

Analysis Type by Cancer	Cancer vs. Normal		Outlier	
Bladder Cancer		2	2	2
Brain and CNS Cancer	5	3	6	4
Breast Cancer	6		9	17
Cervical Cancer	1		2	2
Colorectal Cancer	8		7	10
Esophageal Cancer	4		4	3
Gastric Cancer			2	4
Head and Neck Cancer	4		6	5
Kidney Cancer			5	4
Leukemia	2		14	12
Liver Cancer	6		5	3
Lung Cancer		2	3	10
Lymphoma	3		10	11
Melanoma			4	4
Myeloma			4	5
Other Cancer	1		8	3
Ovarian Cancer		1	3	4
Pancreatic Cancer			5	1
Prostate Cancer	1	1	2	5
Sarcoma	2		6	7
Significant Unique Analyses	43	9	96	110
Total Unique Analyses	393		852	

FIGURE 1. Expression patterns of *ENAH* mRNA in different tumor types. Datasets showing significant over-expression (red) or under-expression (blue) of *ENAH* mRNA in cancer vs. normal tissue. Threshold values were: $p < 1e-4$; fold change ≥ 2 ; and GENE RANK, top 10%. Cell color indicates the percentage level of *ENAH* expression relative to that in the comparison control group. Numbers in cells represent the number of cancer vs. normal analyses that reached the threshold from among the total of 393 studies, and that of outlier analyses from among the total of 852 studies.

FIGURE 2. ENAH mRNA analysis in breast cancer using information from the ONCOMINE database. The expression levels of ENAH mRNA in normal tissues (1) and breast cancer (2) were compared using box plots of gene expression data. Threshold values were: $P < 1e-4$; fold change ≥ 2 ; and GENE RANK, top 10%.

FIGURE 3. Comparisons of analyses of ENAH mRNA expression levels in the ONCOMINE database. (A) Comparisons of ENAH mRNA over-expression in BC vs. normal tissue. Threshold values were: $P < 1e-4$; fold change ≥ 2 ; and GENE RANK, top 10%. (B) Comparisons of ENAH mRNA over-expression based on outlier analysis of BC data

ENAH expression levels in BC compared with normal tissues and analyze their clinical significance. This information will support further study of *ENAH* as a potential biomarker for prediction of BC metastasis.

Materials and methods

ONCOMINE analysis

The *ENAH* mRNA expression was evaluated in a variety of cancers by analysis of data from the ONCOMINE database (www.oncomine.org); a publicly accessible online tumor database created to promote cancer data mining. We used the Student's t-test for comparisons between cancer specimens and normal control tissues, and outlier analysis to evaluate the significance of *ENAH* over-expression. A comparative analysis was conducted to comprehensively analyze BC subsets. Threshold values for significance were as follows: p value, < 0.0001 ; fold change, ≥ 2 ; and GENE RANK, top 10%.

Cancer Cell Line Encyclopedia (CCLE) analysis

The CCLE project (https://portals.broadinstitute.org/ccle_legacy/home) has conducted in-depth genetic and pharmacological studies on numerous human cancer models

and developed a computational analysis method to facilitate transformation of cell line genomic data into cancer patient stratification. The CCLE project provides open access to genomic data, analysis tools, and permits visualization of data from approximately 1,000 cell lines. The expression levels of *ENAH* were analyzed using CCLE data from various tumor and human tissue/organ cell lines.

Kaplan-Meier survival analysis

The prognostic value of *ENAH* expression in BC samples was assessed using the Kaplan Meier-plotter platform (<http://kmplot.com/analysis/>), which generates relapse-free survival (RFS) and overall survival (OS) curves, to analyze the correlation between *ENAH* expression and clinical survival. Kaplan-Meier survival curves were plotted and log-rank P values, hazard ratio (HRs), and 95% confidence intervals calculated, using bioinformatics evaluation methods.

FIGURE 4. Cancer Cell Line Encyclopedia (CCLE) analysis indicates that ENAH expression is elevated in breast cancer cell lines. (A) Box plots of ENAH mRNA expression levels in a variety of cancer cell lines. (B) Scatter plot of ENAH mRNA expression levels in a various cancer cell lines.

FIGURE 5. Prognostic value of ENAH in various breast cancer subtypes. (A) Low ENAH expression was associated with superior relapse-free survival (RFS) in all breast cancer patients. (B)/(C) Low expression of ENAH was associated with longer RFS in the ER(-) subgroup, but not in the ER(+) subgroup. (D) Low ENAH expression was associated with longer RFS in the Grade 3 subgroup. (E)/(F) Low ENAH expression was associated with longer RFS in the HER-2(+) subgroup, but not the HER-2(-) subgroup. (G)/(H) Low ENAH expression was associated with longer RFS in the TP53 wild-type, but not in the TP53 mutated, subgroup.

FIGURE 6. Prognostic value of ENAH for overall survival of patients with breast cancer subtypes. (A)–(D) Low ENAH expression levels were associated with longer overall survival in the luminal B subgroup, but not in other tumor subgroups.

Results

The ENAH expression levels in various tumors

Data from 393 studies that measured ENAH mRNA expression were collected from the ONCOMINE database (Figure 1). Among these studies, 52 demonstrated statistically significant differences in ENAH expression in cancer compared with normal tissue, with 43 showing elevated and nine showing reduced levels. In studies of female-specific tumors, no ovarian cancer investigation demonstrated high ENAH expression, while one indicated reduced expression; one study of cervical cancer demonstrated high ENAH expression, while none showed reduced levels; and among BC studies, six demonstrated elevated ENAH expression, with none generating evidence for reduced levels.

According to outlier analysis of ONCOMINE data to identify tumor types sharing particularly high or low ENAH levels, nine BC studies demonstrated high ENAH expression, while 17 were consistent with low levels.

The ENAH mRNA over-expression was detected in samples from patients with BC and in BC cell lines

Analysis of ONCOMINE database data revealed that ENAH mRNA levels were significantly higher in BC samples compared with normal tissue. In the Ma Breast 4 dataset (ductal *in situ* BC), ENAH transcription was increased by 2.412-fold in BC compared with normal tissues ($p = 2.70 \times 10^{-6}$) (Figure 2A). In the Richardson Breast 2 dataset (ductal BC), ENAH expression was increased by 2.012-fold in BC compared with normal tissues ($p = 1.55 \times 10^{-5}$) (Figure 2B). Data from The Cancer Genome Atlas (TCGA) database showed that ENAH expression in cancer samples was significantly increased (2.63- to 3.40-fold) in four BC subtypes (invasive lobular, invasive, invasive ductal, mixed lobular and ductal) compared with normal tissues ($p < 8.28 \times 10^{-5}$) (Figure 2C–F).

A comparative analysis of the abovementioned studies ($n =$ six studies; $n = 706$ total BC samples) from the ONCOMINE database demonstrated that, overall, BC was associated with high ENAH expression (median rank = 944.5, $p = 4.92 \times 10^{-5}$) (Figure 3A). Moreover, outlier analysis of multiple studies ($n = 16$ studies; $n = 1,951$ total BC samples) in the ONCOMINE database indicated that high ENAH expression may be particularly associated with BC subtypes (Figure 3B).

In the CCLE database, ENAH expression levels varied in human cell lines, with the highest levels in neuroblastoma and the lowest in multiple myeloma cell lines. The ENAH mRNA levels in BC cell lines were higher than those in ovarian cancer

and non-small cell lung cancer cell lines, among others (Figure 4A–B).

Low ENAH expression predicts superior RFS in patients with ER(-), HER-2(+), Grade and TP53 wild-type BC

Next, we evaluated the prognostic value of ENAH expression in BC using the Kaplan Meier-plotter database. Low ENAH mRNA levels were associated with longer RFS in all patients with BC ($n = 3,955$) (HR = 1.27, $p = 0.0029$) (Figure 5A). Subgroup analysis revealed that low ENAH expression was significantly associated with superior RFS in the ER(-) BC subtype (HR = 1.59, $p = 0.0065$, $n = 1,214$) (Figure 5B); however, no significant difference in RFS was identified in the ER(+) subtype ($n = 2,565$) (Figure 5C), suggesting that ENAH expression has prognostic value for patients with ER(-) BC. Our data indicate that the adverse prognosis associated with ER(-) status (hormone receptor-positive cancers are much more responsive to hormone therapy) may be related to ENAH upregulation.

Our analysis also demonstrated that elevated ENAH mRNA was associated with reduced RFS in patients with HER-2(+) BC (HR = 2.26, $p = 0.0035$, $n = 416$) (Figure 5F). No such association was identified for patients with HER-2(-) subtype tumors (HR = 1.16, $p = 0.32$, $n = 1,456$) (Figure 5E), suggesting that determination of HER-2 status, combined with ENAH levels, may have superior value for prediction of BC infiltration and metastasis.

Moreover, RFS was closely associated with ENAH expression in patients with Grade 3 BC (HR = 1.44, $p = 0.022$, $n = 1,090$); low expression was associated with significantly prolonged RFS (Figure 5D). In the TP53 wild-type BC subtype, RFS was significantly associated with ENAH mRNA expression, with high levels indicating significantly inferior RFS (HR = 3.4, $p = 0.007$, $n = 363$) (Figure 5H). Similar results were not obtained for the TP53 mutant BC subtype ($n = 232$) (Figure 5G), suggesting that the molecular function of TP53 may be significantly influenced by ENAH.

The ENAH levels predict OS in patients with luminal B BC

The OS of patients with BC (all tumors, $n = 1,402$) was not significantly associated with ENAH mRNA level ($p > 0.05$); however, in patients with luminal B subtype tumors, ENAH levels were significantly associated with OS (HR = 2.03, $p = 0.045$); low expression was associated with prolonged OS (Figure 6D). No association was identified for other BC subtypes (Figure 6A–C and E).

Discussion

There is currently no reliable method to predict the risk of metastatic disease in BC, which is the main cause of death from this type of cancer. The ENAH has been shown to be important for tumor cell adhesion, metastasis, apoptosis inhibition and tumor angiogenesis [3,4,23]. In invasive tumor cells, ENAH increases sensitivity to EGF and, consequent, cell mobility [2,4,10]. The ENAH up-regulation increases the invasiveness of EGF-stimulated tumor cells, providing potential opportunities to evaluate metastasis risk. Our study demonstrated that *ENAH* expression was significantly elevated in BC tumors compared with normal tissue and that this change is clinically significant, indicating that *ENAH* is a potential prognostic biomarker for BC.

The ENAH has not been detected in normal mammary epithelial cells but is over-expressed in high-risk benign lesions and in primary and metastatic tumors, and ENAH over-expression has been associated with various phenotypes related to adverse prognoses [10]. In our study, we found that low *ENAH* expression was significantly associated with superior RFS in ER(-) BC, indicating a possible oncogenic effect of ENAH in hormone-insensitive BC. This is consistent with the findings of Di Modugno et al., which showed a negative correlation between ER status and ENAH expression level in invasive cancer, with ER levels significantly lower in lesions expressing ENAH [24].

In the present study, we also found that, in patients with HER-2(+) BC, high *ENAH* levels were associated with significantly inferior RFS, suggesting a synergistic effect of ENAH and HER-2 on patient survival. The ENAH and HER-2 expression were positively correlated in BC, and both were associated with axillary lymph node metastasis and tumor, node and metastasis (TNM) stage, but not patient age or pathological type [25]. The ENAH has been postulated as a possible downstream effector of the ErbB receptor family, indicating that it is a potential new prognostic indicator for BC progression [24].

Our study also revealed that *ENAH* expression was associated with survival in patients with Grade 3 BC, and that low *ENAH* was associated with superior OS in patients with luminal B BC; further evidence is required to confirm these associations. Our data also demonstrated that low *ENAH* was significantly associated with superior RFS, specifically in patients with TP53 wild-type BC. Toyoda et al. reported that ENAH expression was associated with BC progression, which was also somewhat affected by the interaction between ENAH and p53 in the tumor cell matrix, with the possibility of

wild-type p53 inhibiting ENAH expression [26]; this warrants further investigation.

In summary, ENAH was significantly over-expressed in BC tumors compared with normal tissue. In the BC subgroups, ER(-), HER-2(+), Grade 3 and wild-type TP53, low *ENAH* was significantly associated with superior RFS, while it was associated with prolonged OS only in patients with luminal B subtype BC. Our study is the first to predict that higher stage malignancy and wild-type TP53 may be important factors that influence the biological function of ENAH. Hence, *ENAH* mRNA expression level is a potential new prognostic indicator for BC progression. These findings are based exclusively on database mining and require further verification in clinical studies.

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