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Original Article

Low HECTD1 mRNA expression is associated with poor prognosis and may be correlated with increased mitochondrial respiratory function in breast cancer

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Abstract: HECT domain E3 ubiquitin ligase 1 (HECTD1) has been reported to be a negative regulator of epithelial-mesenchymal transition and to decrease breast cancer invasion and metastasis. However, the clinical significance and detailed role of HECTD1 in breast cancer remain elusive. We investigated the role of HECTD1 in two large breast cancer cohorts at our institution and The Cancer Genome Atlas using mRNA expression and bioinformatics analysis. We also examined the prognostic significance of HECTD1 mRNA expression by multivariate analysis and HECTD1 protein expression by immunohistochemistry using our cohort. HECTD1 mRNA expression was significantly lower in breast cancer tissues compared with those in adjacent normal tissues (P<0.001). HECTD1 mRNA expression levels also differed among breast cancer subtypes. Decreased HECTD1 mRNA expression was significantly associated with aggressive tumor characteristics, including large tumor size and high histological grade. HECTD1 mRNA expression was inversely associated with mitochondrial cellular respiratory function (oxidative phosphorylation (P<0.001, FDR *q*-value <0.001) the respiratory chain complex (P<0.001, FDR *q*-value <0.001) and reactive oxygen species (P<0.001, FDR *q*-value <0.001), but not with epithelial-mesenchymal transition, in breast cancer tissues. Low expression of HECTD1 mRNA was associated with shorter disease-free survival (log-rank: P=0.013) and overall survival (log-rank: P=0.038) in breast cancer patients. Multivariate analysis also identified low HECTD1 mRNA expression level as an independent risk factor for disease-free (hazard ratio: 1.54, 95% confidence interval: 1.11-2.13, P=0.009) and overall (hazard ratio: 1.50, 95% confidence interval: 1.01-2.24, P=0.046) survival among breast cancer patients. There was no association of HECTD1 protein expression with HECTD1 mRNA expression and prognosis. In conclusion, we identified low expression of HECTD1 mRNA as an independent poor prognostic factor in breast cancer and showed that HECTD1 mRNA expression was inversely correlated with genes involved in mitochondrial cellular respiratory function in breast cancer.

Keywords: Breast cancer, HECTD1, mitochondrial respiration, prognostic factor

Introduction

Breast cancer is the most prevalent malignancy in women and its incidence is still increasing [1]. Although breast cancer mortality has decreased as a result of improvements in systemic therapy [2, 3], breast cancer remains the leading cause of cancer-related death in women worldwide. Further research is thus needed to understand the molecular mechanisms of breast cancer and to improve the prognosis of breast cancer patients.

Ubiquitination is an important post-translational enzymatic protein modification that is mediated by a three-enzyme cascade (E1, E2, and

E3) [4]. Ubiquitinated proteins are subsequently degraded by the proteasome [5, 6]. HECT domain E3 ubiquitin ligase 1 (HECTD1) negatively regulates the functions of specific target proteins through mediating their ubiquitination, leading to subsequent protein degradation. HECTD1 was previously shown to play crucial roles in the negative regulation of cell migration [7-10]. A recent report demonstrated that HECTD1 negatively regulates epithelial-mesenchymal transition (EMT), resulting in decreased invasion and metastasis in breast cancer, and HECTD1 mRNA expression was identified as a poor prognostic factor by in silico analysis using microarray data of breast cancer patients. HECTD1 protein expression was also reported to be associated with invasion, metastasis and prognosis in another dataset of mRNA expression [11].

The expression of HECTD1 mRNA was reported to be a potential prognostic factor in breast cancer; however, only publicly available microarray databases have been analyzed. Furthermore, the association between HECTD1 mRNA expression and protein expression, clinicopathological factors and subtype-specific prognosis has not been reported. Therefore, the clinical significance and detailed roles of HECTD1 in breast cancer remain elusive.

In this study, we investigated the possible role of HECTD1 in breast cancer patients by bioinformatics analysis and examined the association of HECTD1 and clinicopathological factors, including protein expression and prognosis, in two large cohorts of breast cancer patients.

Materials and methods

Patients and samples

A total of 625 consecutive invasive breast cancer tissue samples from the Nagoya City University Hospital archive (collected between 1992 and 2008) were included in this study (the NCU cohort). Among the 625 samples, 340 had only mRNA expression data available; the other 285 cases, which were collected between 2000 and 2008, had both mRNA and protein expression data available. Information of patient clinical parameters was collected from the clinical records. Histological tumor grade was estimated according to the Bloom and Richardson method proposed by Elston and Ellis (Elston and Ellis 1991). We defined disease-free survival (DFS) as the interval from the date of curative resection to the earliest occurrence of locoregional recurrence, distant metastasis or death from any cause; overall survival (OS) was defined as the interval from the date of curative resection to death from any cause. The median follow-up periods in the mRNA and protein expression cohorts were 10.2 years (range, 0.01-17.9 years) and 9.8 years (range, 0.07-17.9 years), respectively. The study protocol (70-00-0166) was approved by the Institutional Review Board of Nagoya City University Graduate School of Medical Sciences and conformed to the guidelines of the Declaration of Helsinki. Written informed consent for comprehensive research use was obtained from all patients before surgery.

The cancer genome atlas (TCGA) data acquisition

The gene expression data from RNA sequencing and protein expression data from mass spectrometry of breast cancer patients in TCGA Firehose legacy dataset were downloaded through cBioPortal (www.cbioportal.org) [12, 13] and the UCSC Genome Browser (http://genome.ucsc.edu/). Of the 1093 TCGA primary breast cancer patients with gene expression data from RNA sequencing, 3 patients and 74 patients also had mRNA expression data of the metastatic site and protein expression data from mass spectrometry, respectively.

Gene set enrichment analysis (GSEA)

GSEA was performed using data from TCGA cohort to analyze correlations with HECTD1 mRNA expression. HECTD1 mRNA expression-related gene sets were identified within the 50-hallmark gene sets [14], the Molecular Signature Database curated collection (c2) and Gene Ontology gene sets (c5).

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Breast cancer tissue samples were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C until RNA extraction. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) in accordance with

the manufacturer's protocol. The quantity of RNA extracted from breast cancer tissues was evaluated using a DS-11 Spectrophotometer (DeNovix, Wilmington, DE, USA). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol. qRT-PCR was carried out on a Step One Plus™ Real-time PCR system (Thermo Fisher Scientific). Reactions were multiplexed using the following assays: HECTD1 (FAM; Thermo Fisher Scientific) and GAPDH (VIC; Life Technologies, Waltham, MA, USA) using Fast Advanced Master Mix (Applied Biosystems, Waltham, MA, USA). The results were converted into gene expression levels using a standard curve. Target gene expression was normalized relative to levels of GAPDH mRNA, as described previously [15-17]. We determined the cut-off level for HECTD1 mRNA expression level as the median value.

Immunohistochemistry (IHC)

IHC analyses for estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor 2 (HER2) were carried out as described elsewhere [17, 18]. For HECTD1 staining, a tissue microarray was constructed using 2-mm diameter tissue samples. After deparaffinization, antigens were retrieved by heating sections at 99°C-100°C in 0.01 M citrate buffer pH 6 for 20 min. Endogenous peroxidase activity was inhibited by incubation in 3% H_2O_2 for 10 min. The slides were then incubated in Protein Block solution (DS Pharma Biomedical Co., Osaka, Japan) for 10 min to minimize nonspecific staining, followed by incubation with rabbit polyclonal anti-human HECTD1 antibody (LifeSpan BioSciences, Seattle, WA, USA) at a 1:100 dilution overnight at 4°C. HECTD1 was then detected using the EnVision system, peroxidase (Dako, Santa Clara, CA, USA) and diaminobenzidine detection (Merck KgaA, Darmstadt, Germany). HECTD1 protein expression level was scored by assessing the entire slide using the Aperio ImageScope system (Leica Biosystems, Vista, CA, USA). Cytoplasmic staining intensity (0, 1+, 2+, or 3+) was determined for each cancer cell, and an H-score was assigned using the formula [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)] [19, 20]. We determined the cut-off level for HECTD1 H-score as the median.

Statistical analyses

HECTD1 mRNA expression levels were compared using Mann-Whitney and pairwise Mann-Whitney tests with Bonferroni's adjustment. Spearman's rank correlation coefficient (r) was used to analyze correlations between HECTD1 mRNA expression levels and HECTD1 H-score and protein level. Associations between clinicopathological factors and HECTD1 mRNA expression levels were assessed by Student's t-, χ^2, and Fisher's exact probability tests. Survival analyses were performed using Kaplan-Meier curves with the log-rank test. Univariate and multivariate analyses were carried out using Cox proportional hazards regression models, and a total of 69 missing data points were estimated using multiple imputation. A *P*-value <0.05 was considered significant. All statistical analyses were performed with R software version 4.1.2 (https://www.R-project.org/) and Bioconductor version 3.11 (http://bioconductor.org/). GSEA was performed using the Java GSEA implementation version 4.2.1 and MSigDB version 7.4.

Results

HECTD1 mRNA was downregulated in breast cancer tissues

We first investigated if HECTD1 mRNA expression levels are altered in breast cancer tissues by analyzing 114 matched tumor and adjacent normal tissues in TCGA cohort. We found that HECTD1 mRNA expression levels were significantly lower in the breast cancer tissues compared with those in the adjacent normal tissues (*P*<0.001) (**Figure 1A**). We also investigated the difference of HECTD1 mRNA expression between three matched primary and metastatic breast cancer tissues in TCGA cohort. We found that HECTD1 mRNA expression tended to be lower in metastatic cancer tissue than in primary breast tissue, but there was no statistical significance, which may be because of the small sample size (**Figure 1B**).

HECTD1 expression differed among breast cancer subtypes

We next examined if HECTD1 mRNA expression levels are different among breast cancer subtypes in the NCU and TCGA cohort. HECTD1 mRNA expression levels were significantly low-

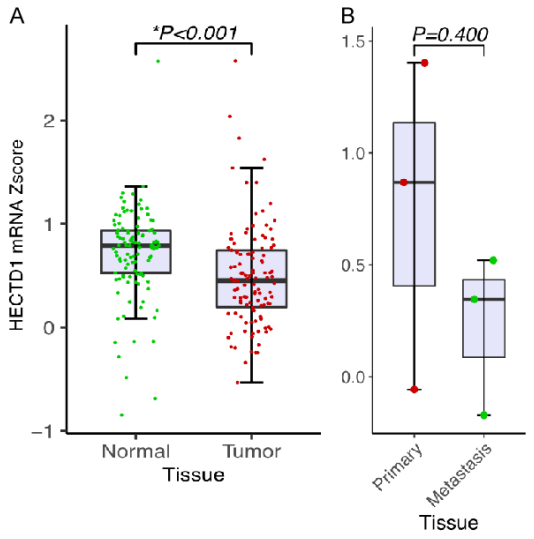

Figure 1. HECTD1 mRNA expression levels in 114 matched breast cancer tissues and adjacent normal tissues (A) and 3 matched primary and metastatic breast cancer tissues (B) from patients in TCGA cohort. The Z scores of mRNA expression were determined for each sample by comparing the mRNA expression of a gene with its distribution in a reference population showing typical expression of the gene. Vertical lines represent minimum and maximum values; lines within each box represent the median; boxes indicate first to third quartiles; outliers are plotted as individual points. *P<0.05, based on Mann-Whitney test. *HECTD1* HECT domain E3 ubiquitin ligase 1; *TCGA* The Cancer Genome Atlas.

er in ER-negative tumors compared with levels in ER-positive tumors (P<0.001), lower in PgR-negative tumors compared with levels in PgR-positive tumors (P<0.001), and lower in HER2-positive tumors compared with levels in HER2-negative tumors (P<0.001) in the NCU cohort (**Figure 2A**). Lower HECTD1 mRNA expression levels in ER-negative and PgR-negative breast cancers compared with those in the respective counterparts were also found in the TCGA cohort (P<0.001 and P<0.001, respectively), but HECTD1 mRNA expression was not associated with HER2 status in TCGA cohort (**Figure 2B**). We further stratified the patients into four subtypes based on the IHC findings: luminal (ER-positive and/or PgR-positive, HER2-negative), luminal-HER2 (ER-positive and/or PgR-positive, HER2-positive), HER2 (ER-negative, PgR-negative, HER2-positive), and triple-negative (ER-negative, PgR-negative, HER2-negative). HECTD1 mRNA expression levels were significantly lower in the HER2 and triple-negative subtypes compared with those in the luminal subtype in the NCU cohort (P=0.001 and P<0.001, respectively) (**Figure 2A**), but were only significantly lower in the triple-negative subtype compared with those in the luminal subtype in the TCGA cohort (P<0.001) (**Figure 2B**).

HECTD1 mRNA level was negatively associated with aggressive tumor characteristics

On the basis of the downregulation of HECTD1 mRNA in tumor tissues (**Figure 1**) and the previous reports showing that HECTD1 suppressed EMT [10, 11], we investigated the association between HECTD1 mRNA expression and tumor aggressiveness. HECTD1 mRNA expression was significantly lower in larger tumors (P= 0.009) and in higher grade tumors (Grade 3 vs. Grade 1; P<0.001, Grade 3 vs. Grade 2; P< 0.001, Grade 2 vs. Grade 1; P=0.042) in the NCU cohort (**Figure 3A**). However, HECTD1 mRNA expression was not associated with tumor size in TCGA cohort and was not associated with lymph node metastasis in the NCU or TCGA cohort (**Figure 3A**, **3B**). We also investigated the associations between HECTD1 mRNA expression levels and other patient demographics in the NCU cohort. High HECTD1 mRNA expression was associated with a higher proportion of invasive lobular carcinomas compared with low HECTD1 expression (P=0.009) (**Table 1**). However, because of the small number of lobular carcinomas, we could not confirm statistical significance. There was no significant association between HECTD1 mRNA expression and other examined factors, including age, sex or menopausal status.

HECTD1 mRNA was inversely correlated with genes involved in mitochondrial cellular respiratory function

HECTD1 was shown to negatively regulate EMT [11]. We therefore investigated the relationship between HECTD1 mRNA expression and EMT using TCGA cohorts. In contrast to the previous report [11], our GSEA results revealed no correlation between HECTD1 mRNA expression and the EMT gene set (**Figure 4A**). In contrast, we found that the mitochondrial gene set, mitochondrion gene set (P<0.001, FDR *q*-value <0.001) and especially the respiratory-related gene sets for oxidative phosphorylation (OXPHOS) (P<0.001, FDR *q*-value <0.001), the respiratory chain complex (P<0.001, FDR *q*-value <0.001) and the reactive oxygen species (ROS) pathway (P<0.001, FDR *q*-value <0.001)

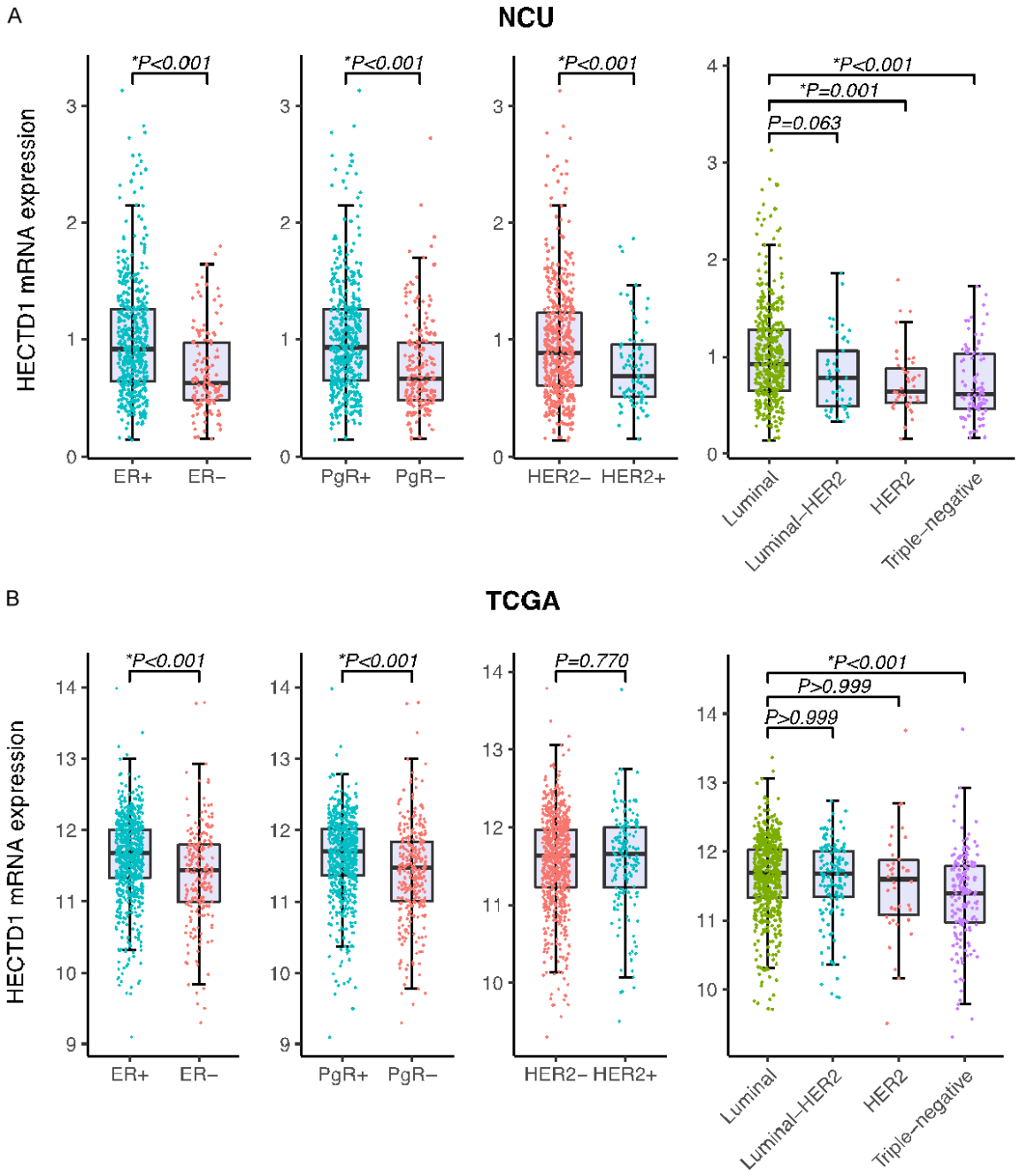

Figure 2. HECTD1 mRNA expression levels in relation to breast cancer subtype in the NCU (n=625) (A) and TCGA cohort (n=1093) (B). Vertical lines represent minimum and maximum values; lines within each box represent the median; boxes indicate first to third quartiles; outliers are plotted as individual points. *P<0.05, based on Mann-Whitney test. *HECTD1* HECT domain E3 ubiquitin ligase 1; *NCU* Nagoya City University; *TCGA* The Cancer Genome Atlas.

were inversely correlated with HECTD1 mRNA expression in TCGA cohort (**Figure 4B**). These findings thus indicated that HECTD1 mRNA might be directly or indirectly involved in the negative regulation of mitochondrial cellular respiratory function in breast cancer.

Low HECTD1 mRNA expression was associated with poor prognosis in breast cancer

We analyzed the impact of HECTD1 mRNA expression on the survival of breast cancer patients. Patients with HECTD1 mRNA low-

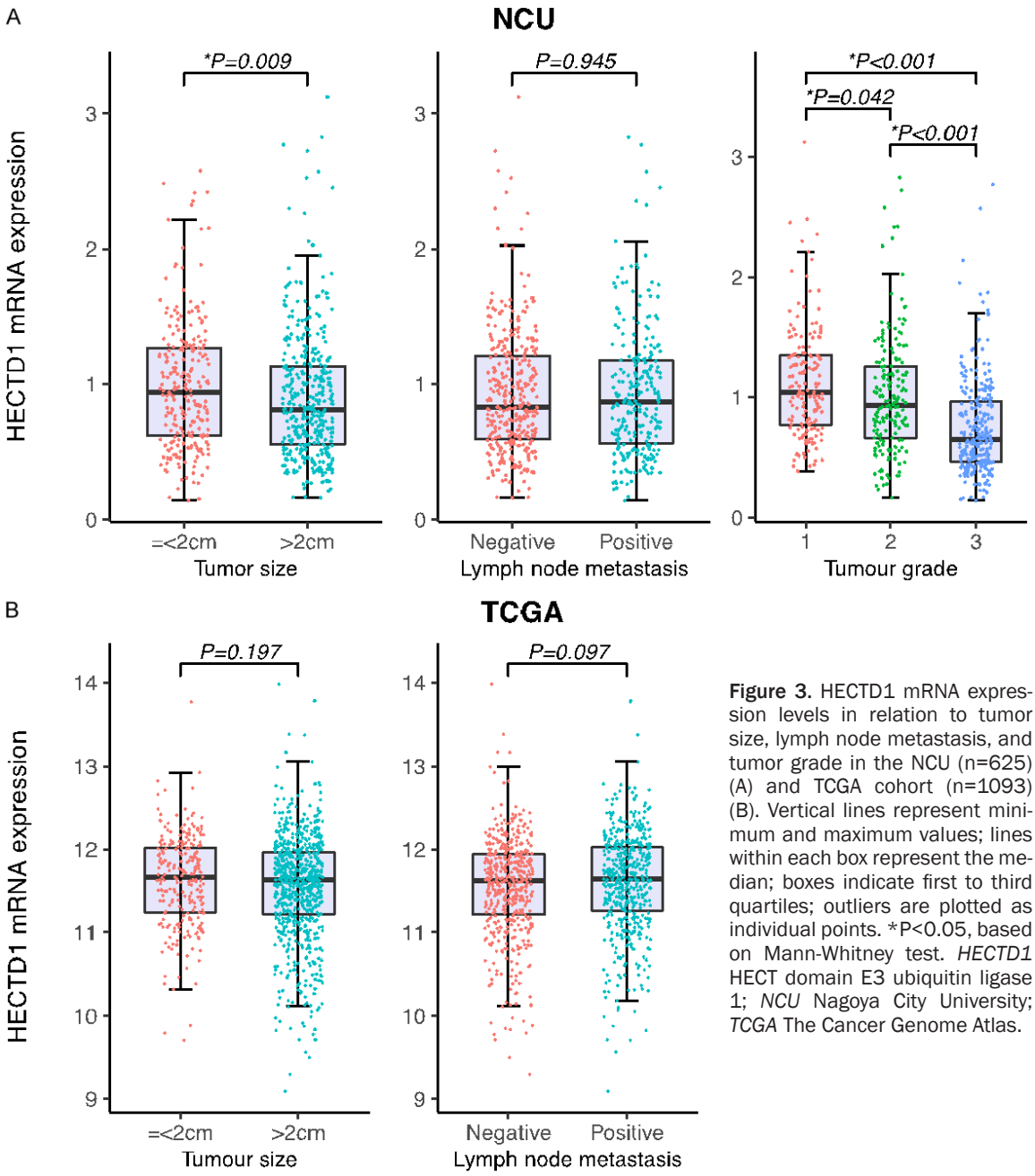

Figure 3. HECTD1 mRNA expression levels in relation to tumor size, lymph node metastasis, and tumor grade in the NCU (n=625) (A) and TCGA cohort (n=1093) (B). Vertical lines represent minimum and maximum values; lines within each box represent the median; boxes indicate first to third quartiles; outliers are plotted as individual points. *P<0.05, based on Mann-Whitney test. *HECTD1* HECT domain E3 ubiquitin ligase 1; *NCU* Nagoya City University; *TCGA* The Cancer Genome Atlas.

expressing tumors had significantly shorter DFS and OS compared with patients with HECTD1 mRNA high-expressing tumors (P= 0.013 and P=0.038, respectively) in the NCU cohort (**Figure 5A**). Univariate analyses showed that larger tumor size (DFS, P=0.032; OS, P=0.002), lymph node metastasis (DFS, P< 0.001; OS, P<0.001), higher histological grade (DFS, P=0.003; OS, P=0.002) ER-negativity (DFS, P=0.004; OS, P=0.005), HER2-positivity (DFS, P=0.015; OS, P=0.011) and low HECTD1 mRNA expression level (DFS, P=0.014; OS, P=0.039) were all significantly associated with shorter DFS and OS (**Table 2**). Multivariate analyses identified low HECTD1 mRNA expression level as an independent factor associated with lower DFS (hazard ratio (HR): 1.54, 95% confidence interval (CI): 1.11-2.13, P=0.009), together with lymph node metastasis (P< 0.001) and ER status (P=0.009) (**Table 1**). Low HECTD1 mRNA expression was also an independent prognostic factor for poor OS (HR:

Table 1. Association between HECTD1 mRNA expression and clinicopathological characteristics of breast cancer patients in the NCU cohort

	All patients n (%)	HECTD1 mRNA expression		
		High n (%)	Low n (%)	*P* value
Patients	625	313	312	
Mean ± SD, (years)	56.2 ± 13.3 (25-94)	57.2 ± 13.2 (28-92)	55.3 ± 13.3 (25-94)	0.067
Gender				0.249
Male	2 (1)	0	2 (1)	
Female	623 (99)	313 (100)	310 (99)	
Menopausal status				0.416
Pre	261 (42)	127 (41)	134 (43)	
Post	357 (57)	186 (59)	171 (55)	
Unknown	7 (1)	0	7 (2)	
Histology				0.009*
IDC	554 (89)	276 (88)	278 (89)	
ILC	28 (4)	21 (7)	7 (2)	
Others	43 (7)	16 (5)	27 (9)	

HECTD1 HECT domain E3 ubiquitin protein ligase 1, *SD* standard deviation, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma. **P*<0.05 was considered statistically significant.

1.50, 95% CI: 1.01-2.24, P=0.046), together with lymph node metastasis (P<0.001) and ER status (P=0.037) (**Table 2**).

Given that HECTD1 mRNA expression levels differed according to breast cancer subtype, we further investigated the impact of HECTD1 mRNA expression on patient survival in each subtype. There was no significant difference in either DFS or OS between the HECTD1 high- and low-expressing tumors in ER-positive (DFS, P=0.273; OS, P=0.388) (**Figure 5B**) and HER2-positive patients (DFS, P=0.617; OS, P=0.972) (**Figure 5D**). However, low HECTD1 expression tended to be associated with worse prognosis in ER-negative (DFS, P=0.079; OS, P=0.139) (**Figure 5C**) and triple-negative patients (DFS, P=0.123; OS, P=0.106) (**Figure 5E**).

Lack of association of HECTD1 protein expression with mRNA expression or patient survival in breast cancer

We investigated if HECTD1 mRNA expression was correlated with its protein expression levels in breast cancer. Representative images of HECTD1 protein expression detected by IHC are shown in **Figure 6A**. There was no significant correlation between HECTD1 mRNA expression levels and HECTD1 H-score in the NCU cohort (r=-0.089, P=0.134) (**Figure 6B**) or protein expression by mass spectrometry in TCGA cohort (r=0.205, P=0.079) (**Figure 6C**). In addition, there was no significant difference in DFS or OS between patients with HECTD1 high and low H-score tumors in the NCU cohort (P=0.698 and P=0.819, respectively) (**Figure 6D**).

Discussion

The results of this study revealed that HECTD1 mRNA expression levels were lower in breast cancer tissues compared with those in normal adjacent tissues. In addition, HECTD1 mRNA expression levels were different among breast cancer subtypes. We demonstrated that low HECTD1 mRNA expression was associated with aggressive tumor characteristics, such as large tumor size and high histological grade, and was an independent poor prognostic factor in breast cancer patients. We identified an inverse correlation between HECTD1 mRNA expression and genes involved in mitochondrial cellular respiratory function, which may represent a possible mechanism for these relationships.

Low protein expression of HECTD1 in breast cancer as determined by IHC has previously been reported [11]; however, HECTD1 mRNA levels in cancer have not been reported to date. Our results provide the first evidence that HECTD1 mRNA expression in breast tumor tissues is downregulated compared with that in

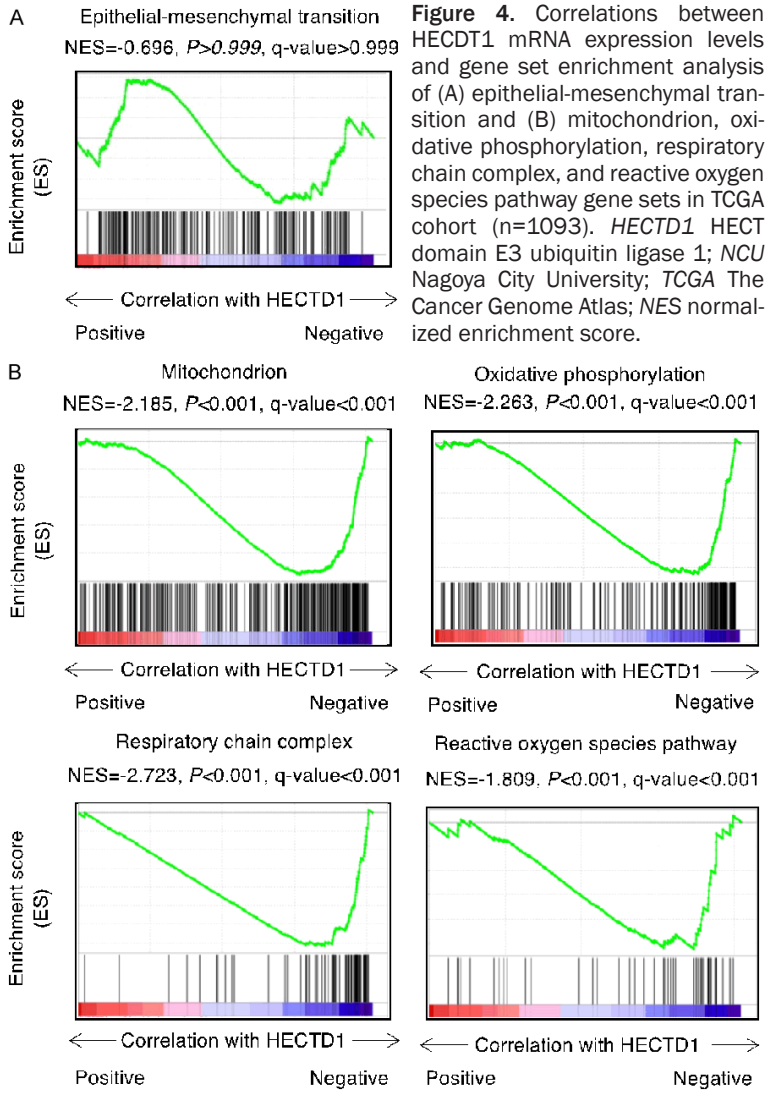

Figure 4. Correlations between HECDT1 mRNA expression levels and gene set enrichment analysis of (A) epithelial-mesenchymal transition and (B) mitochondrion, oxidative phosphorylation, respiratory chain complex, and reactive oxygen species pathway gene sets in TCGA cohort (n=1093). *HECTD1* HECT domain E3 ubiquitin ligase 1; *NCU* Nagoya City University; *TCGA* The Cancer Genome Atlas; *NES* normalized enrichment score.

adjacent normal tissues. Depletion of HECTD1 has been reported to lead to increased breast cancer cell proliferation and invasion [8, 11]. These studies are consistent with our findings that low mRNA expression of HECTD1 was associated with aggressive tumor characteristics, such as larger size and higher grade.

Breast cancer subtypes, classified by IHC analyses of ER, PgR and HER2, have different biological characteristics and prognoses. We found that HECTD1 mRNA expression varied among the breast cancer subtypes, with low expression in ER-negative, PgR-negative and HER2-positive tumors compared with that in the respective counterparts. These findings might be consistent with the fact that ER-negative, PgR-negative and HER2-positive breast cancers tend to have more aggressive features and poorer clinical outcomes [21, 22]. However, high expression of HECTD1 mRNA was associated with tumor size and HER2 negativity in the NCU cohort but not in TCGA cohort. The small differences in HER2 status and tumor size in these results may be from racial and ethnic differences that influence breast cancer characteristics [23].

To investigate the possible role of HECTD1 in breast cancer, we performed GSEA in this study. Although previous reports indicated that HECTD1 negatively regulates EMT [11], no association was shown between HECTD1 mRNA expression and EMT and lymph node metastasis in the current study results. These results imply that HECTD1 may have a different role in breast cancer other than previously reported.

While glycolysis is the dominant metabolic pathway in cancer cells, mitochondria also play a critical role in the development of some types of cancer [24]. OXPHOS, one of the typical processes of mitochondrial respiration, efficiently produces ATP via the respiratory chain complex using oxygen and generating ROS [25]. Enhanced mitochondrial OXPHOS in breast cancer was reported to be associated with cancer cell motility, distant metastasis and poor patient prognosis through high ATP production [26]. The increase in ROS associated with enhancement of OXPHOS was also reported to promote cancer cell proliferation via multiple signaling pathways [27, 28]. In the current study, unbiased GSEA identified that genes involved in mitochondrial respiratory functions such as OXPHOS, the respiratory chain complex, and ROS were inversely correlated with HECTD1 mRNA expression. To the best of our knowledge, this is the first report suggesting that HECTD1 mRNA expression may be involved in the negative regulation of mitochondrial respiratory function, resulting in

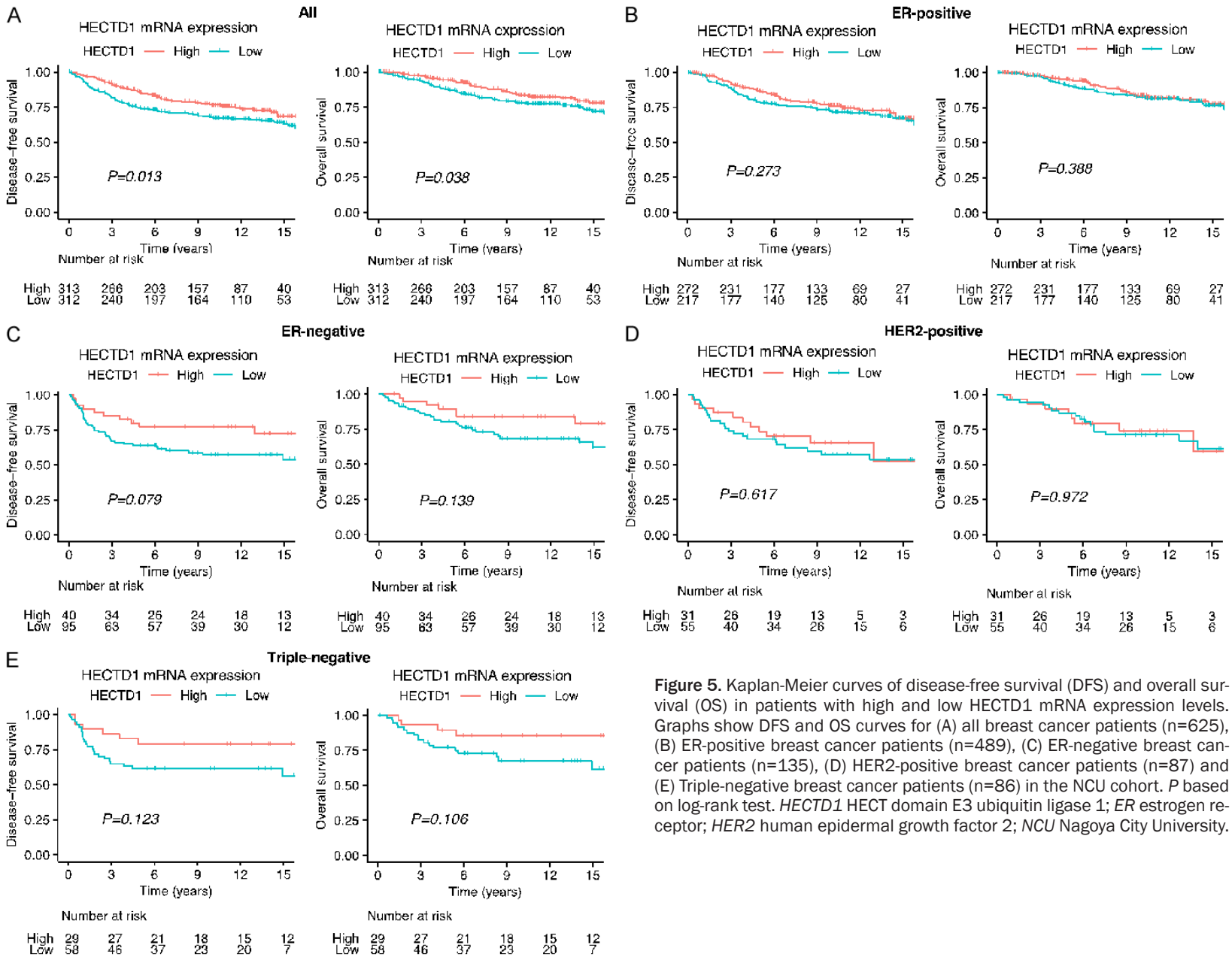

Figure 5. Kaplan-Meier curves of disease-free survival (DFS) and overall survival (OS) in patients with high and low HECTD1 mRNA expression levels. Graphs show DFS and OS curves for (A) all breast cancer patients (n=625), (B) ER-positive breast cancer patients (n=489), (C) ER-negative breast cancer patients (n=135), (D) HER2-positive breast cancer patients (n=87) and (E) Triple-negative breast cancer patients (n=86) in the NCU cohort. *P* based on log-rank test. *HECTD1* HECT domain E3 ubiquitin ligase 1; *ER* estrogen receptor; *HER2* human epidermal growth factor 2; *NCU* Nagoya City University.

Table 2. Univariate and multivariate Cox regression analyses of independent predictors of disease-free and overall survival among patients with breast cancer in the Nagoya City University (NCU) cohort

Variables	n (%)	Disease-free survival			Overall survival		
		Univariate	Multivariate		Univariate	Multivariate	
		P value	*P* value	HR (95% CI)	*P* value	*P* value	HR (95% CI)
Age (by 1 year)	625	0.233	0.194	1.01 (0.99-1.02)	0.130	0.105	1.01 (0.99-1.03)
Tumor size	624						
≤2 cm	241 (39)	0.032	0.452	1 (Reference)	0.002	0.062	1 (Reference)
>2 cm	383 (61)			1.14 (0.81-1.59)			1.54 (0.98-2.41)
Nodal status	597						
Negative	340 (60)	<0.001	<0.001*	1 (Reference)	<0.001	<0.001*	1 (Reference)
Positive	257 (40)			4.18 (2.97-5.88)			4.22 (2.77-6.43)
Grade	609						
1, 2	346 (55)	0.003	0.449	1 (Reference)	0.002	0.314	1 (Reference)
3	263 (45)			1.13 (0.82-1.57)			1.23 (0.82-1.82)
ER status	624						
Positive	489 (78)	0.004	0.009*	1 (Reference)	0.005	0.037*	1 (Reference)
Negative	135 (22)			1.66 (1.13-2.44)			1.63 (1.03-2.58)
HER2 status	602						
Negative	516 (86)	0.015	0.269	1 (Reference)	0.011	0.298	1 (Reference)
Positive	86 (14)			1.27 (0.83-1.93)			1.32 (0.79-2.20)
HECTD1 mRNA expression	625						
High	313 (50)	0.014	0.009*	1 (Reference)	0.039	0.046*	1 (Reference)
Low	312 (50)			1.54 (1.11-2.13)			1.50 (1.01-2.24)

HECTD1 HECT domain E3 ubiquitin protein ligase 1, *ER* estrogen receptor, *HER2* human epidermal growth factor receptor 2, *HR* hazard ratio, *CI* confidence interval. *P<0.05 was considered statistically significant.

reduced cancer aggressiveness and better patient survival. This hypothesis might also provide a clue to elucidate the role of HECTD1 in breast cancer and improve breast cancer patient survival.

The impact of HECTD1 mRNA expression on patient prognosis also differed by subtypes, with low HECTD1 mRNA expression levels tending to be associated with a poorer prognosis in ER-negative and triple-negative breast cancer patients but not ER-positive and HER2-positive breast cancer patients. Previous studies showed that mitochondrial respiration differs depending on the subtype of breast cancer, especially in triple-negative breast cancer, which shows enhanced mitochondrial respiration compared with other subtypes [29]. These findings suggest that HECTD1 may play different roles in the regulation of mitochondrial respiration depending on the breast cancer subtype.

The current study found no correlation between HECTD1 mRNA levels and protein levels measured by IHC in the NCU cohort or protein levels measured by mass spectrometry in TCGA cohort. Although the mechanism that regulates HECTD1 protein expression has not yet been fully elucidated, the discrepancy of HECTD1 mRNA and protein expression may also be because of mechanisms involving translational control [30, 31] and post-transcriptional modification [32, 33]. Furthermore, in this study, there was an association between HECTD1 mRNA expression and breast cancer prognosis and mitochondrial respiratory function, but no association between HECTD1 protein expression and breast cancer prognosis. These findings imply that HECTD1 mRNA is associated with breast cancer prognosis and mitochondrial respiratory function, either through a mechanism that does not involve HECTD1 protein function or through a mechanism upstream of translation into HECTD1 protein. Further research is required to investigate how HECTD1 mRNA expression regulates mitochondrial respiratory function and correlates with prognosis.

In this study, there was an association between HECTD1 mRNA expression and breast cancer prognosis and mitochondrial respiratory func-

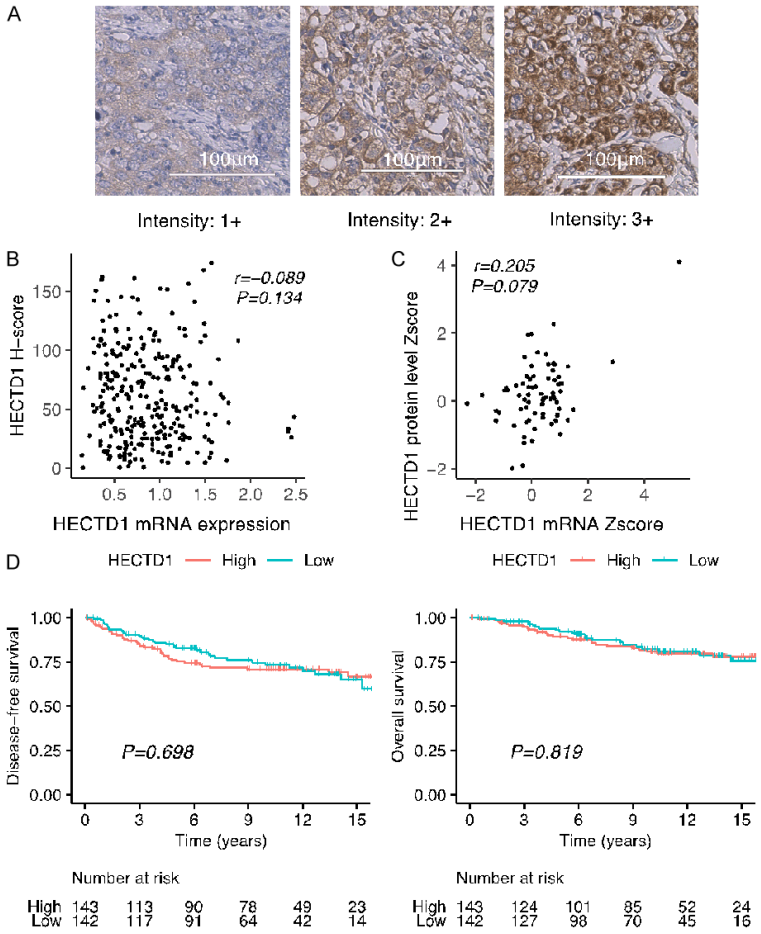

Figure 6. Representative images of HECTD1 protein expression in breast cancer tissues from the NCU cohort detected by immunohistochemistry (A). Correlations between HECTD1 mRNA and protein expression levels in the (B) NCU (n=285) and (C) TCGA cohorts (n=74) (Spearman's rank correlation coefficient (r)). The Z scores for protein in TCGA cohort were determined for each sample by comparing with all samples with protein data. (D) Kaplan-Meier curves of disease-free survival (DFS) and overall survival (OS) in patients with high and low HECTD1 protein expression levels. Graphs show DFS and OS curves for all breast cancer patients in the NCU cohort (n=285). *P* based on log-rank test. *HECTD1* HECT domain E3 ubiquitin ligase 1; *NCU* Nagoya City University; *TCGA* The Cancer Genome Atlas.

tion, but no association between HECTD1 protein expression and breast cancer prognosis. In contrast, a previous study reported that low HECTD1 protein expression was associated with shorter survival and EMT in breast cancer patients [11]. These findings imply that HECTD1 is associated with breast cancer prognosis by not only suppressing EMT via protein function as previously reported [11], but also by suppressing mitochondrial respiratory function via mRNA expression as suggested in this study. Further studies are required to investigate the mechanism by which HECTD1 mRNA, but not protein, is involved in the regulation of mitochondrial respiratory function.

This study has several limitations. First, this was a retrospective analysis at a single institution using archived materials. The breast cancer tissue samples analyzed in this study were obtained approximately 10 years ago, and therefore the possibility that the quality of these tissue samples may have affected the results of this study cannot be completely ruled out. Second, the IHC methodology for HECTD1 protein evaluation has not yet been well established. Third, the role of HECTD1 in breast cancer was only investigated using in silico analysis, and further in vitro and in vivo experimental approaches are needed to verify the role of HECTD1 in breast cancer.

Conclusion

In this study, we demonstrated for the first time that HECTD1 mRNA expression levels were different between normal adjacent tissues and primary and metastatic breast cancer tissues and were also different among breast cancer subtypes. We also newly identified that low expression of HECTD1 mRNA was an independent poor prognostic factor in breast cancer and showed that HECTD1 mRNA expression was inversely correlated with genes involved in mitochondrial cellular respiratory function in breast cancer. Further studies to elucidate the role of HECTD1 in breast cancer are warranted.

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Disclosure of conflict of interest

None.

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