Clinicopathological Significance of TARBP2, APP, and ZNF395 in Breast Cancer

Ryoko Oi[1,2], Hirotaka Koizumi[1], Ichiro Maeda[1], Akira Noguchi[1], Shinobu Tatsunami[3], Tsuguo Iwatani[2], Hisanori Kawamoto[2], Koichiro Tsugawa[2] and Masayuki Takagi[1]

[1]Department of Pathology, St. Marianna University School of Medicine, Kawasaki, Japan. [2]Division of Breast and Endocrine Surgery, Department of Surgery, St. Marianna University School of Medicine, Kawasaki, Japan. [3]Unit of Medical Informatics, Faculty of Education and Culture, St. Marianna University School of Medicine, Kawasaki, Japan.

ABSTRACT: The double-stranded RNA-binding protein TARBP2 has been suggested to act as an upstream regulator of breast cancer metastasis by destabilizing transcripts of the possible metastasis suppressors amyloid precursor protein (APP) and ZNF395. We examined this hypothesis by immunostaining of TARBP2, APP, and ZNF395 in 200 breast cancer specimens using tissue microarrays and analyzed the relationships between expression levels and clinicopathological parameters and prognosis. Increased TARBP2 overexpression was associated with shorter overall survival and disease-free survival, and increased but not reduced APP expression correlated with lower overall survival and disease-free survival. ZNF395 expression levels had no prognostic value, but reduced expression correlated with reduced lymph node metastasis. There was no significant relationship between TARBP2 overexpression and reduced APP and/or ZNF395 expression. Patients with tumors with higher TARBP2 or APP expression had unfavorable prognoses. Although reduced ZNF395 expression was significantly related to reduced lymph node metastasis, further studies are needed to clarify the role of TARBP2/APP/ZNF395 in breast cancer.

KEYWORDS: breast cancer, metastasis, TARBP2, amyloid precursor protein, ZNF395, immunohistochemistry

Introduction

Breast cancer is the most common invasive cancer in women worldwide.[1] Despite earlier diagnosis and the development of various treatments, such as chemotherapy, radiation, and hormonal and molecularly targeted therapies, the prognosis of breast cancer patients with distant metastasis remains poor.

Cancer metastasis involves a multistep process in which cell populations with enhanced metastatic capacity initiate specific molecular mechanisms, which in turn modulate gene expression levels.[2] The systematic characterization of metastasis-suppressive and metastasis-promoting microRNAs has highlighted the potential role of posttranscriptional regulatory mechanisms in cancer metastasis.[3,4] TARBP2 is a double-stranded RNA-binding protein implicated in microRNA processing. Goodarzi et al[5] recently showed that TARBP2 was overexpressed in breast cancer cells and acted as an upstream regulator of tumor suppressor genes. The authors identified two transcripts that were directly bound by TARBP2, with potential roles in suppressing metastatic progression in human breast cancer. Both the following genes were related to neurodegeneration: the amyloid precursor protein (*APP*) gene encodes a protein linked to Alzheimer's disease and *ZNF395* is associated with Huntington's disease.[5]

Using lung colonization assays in mice, the authors showed that TARBP2 played a direct role in promoting metastasis by destabilizing *APP* and *ZNF395*. They also found that patients with primary breast tumors with reduced expression levels of *APP* and *ZNF395* had significantly lower survival rates than those with primary breast tumors with higher levels of *APP* and *ZNF395*.[5]

Despite these initial findings by Goodarzi et al, subsequent reports have provided conflicting results regarding the tumor promoter or suppressor roles of TARBP2, APP, and ZNF395. Overexpression of TARBP2 has been shown in many cancers, such as breast carcinoma, prostate carcinoma, and malignant lymphoma, but its downregulation has also been noted in some tumors, including colorectal and urothelial carcinomas.[6] In contrast to the results of Goodarzi et al, Takagi et al[7] showed a significant link between increased APP expression and shorter survival in breast cancer patients. In addition, APP expression was increased in breast cancer cell lines with higher metastatic potential.[8] ZNF395 was overexpressed in various types of cancers, such as osteosarcoma, malignant melanoma, and gastric carcinoma,[9] and its high expression has been associated with poorer prognosis in patients with osteosarcoma and other cancers.[10] Further studies are therefore required to resolve these inconsistent results.

In this study, we aimed to analyze the immunohistochemical expression of TARBP2, APP, and ZNF395 proteins in human breast cancers and analyze the relationships between these expression levels and various clinicopathological parameters and survival rates in patients with breast cancer.

Materials and Methods

Patients and tissues. The study protocol was approved by the Human Ethics Review Committee of St. Marianna University School of Medicine (No. 3212). Patients gave their informed consent to participate in the research. This research complied with the principles of the Declaration of Helsinki. A total of 200 specimens of invasive breast carcinoma of no special type[1] were obtained from female patients (mean age 56 years, range 27–87 years) at St. Marianna University Hospital from 2005 to 2007. The first-line clinical treatment in each patient was surgery, followed by adjuvant endocrine therapy, adjuvant chemotherapy, and radiation therapy in 154, 84, and 133 patients, respectively. The mean follow-up time was 90 months (range 1–132 months). All the specimens were fixed in 10% formalin and embedded in paraffin wax. Tumor estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) statuses were determined by immunostaining, as described previously,[7] and their intrinsic subtypes were determined according to the 2011 St Gallen surrogate definition.[11]

Tissue microarray. Hematoxylin–eosin-stained sections were reviewed by two independent and experienced pathologists who selected representative normal and cancerous tissues in each specimen. Two 2.0-mm tissue cores per case were obtained from archival paraffin blocks using a manual tissue arrayer with accessory (KIN-2; Azumaya) and set side by side in a tissue microarray (TMA) (6 × 8 matrix) (Fig. 1).

**Immunohistochemistry.** Paraffin sections (3 µm thick) were cut from TMA blocks, dewaxed in xylene, and rehydrated in ethanol. The sections were incubated in citrate buffer (pH 9.0) for 40 minutes in a water bath at 95°C and then incubated overnight at 4°C with Human Protein Atlas (HPA) antibodies raised against TARBP2 (HPA051181, 1:400), APP (HPA001462, 1:400), and ZNF395 (HPA049382, 1:50) (all Sigma-Aldrich). The specificities of the antibodies have been described elsewhere.[12] The anti-TARBP2, -APP, and -ZNF395 antibodies recognize most of the proteins coded by the splice variants of the following each gene: *TARBP2*-001, -006, -009, -016; *APP*-001, -002, -003, -004, -015, -016, -201, and -017 and *ZNF395*-001, -002, -003, -006, -007, and -008.[12] The sections were then incubated with peroxidase-labeled antirabbit secondary antibody (Simple Stain MAX PO; Nichirei), followed by color development with 3′-diaminobenzidine tetrahydrochloride.

After immunostaining, we evaluated the expression levels of TARBP2, APP, and ZNF395 in breast cancers by comparing the staining intensities in the tumor cells with those in paired nontumor samples in adjacent cores (Fig. 1). Occasional immunostaining in stromal and/or inflammatory cells was morphologically distinguishable from that in cancer and normal tissues. Staining intensities were classified into the following three groups: reduced (level 1), unchanged (level 2), and overexpressed (level 3). When heterogeneous staining was seen in tumor and/or normal tissues, the areas showing the strongest intensity were evaluated. TMA immunoreactivity was evaluated independently by two pathologists who were blinded to the pathological data. Discrepancies were resolved by simultaneous reexamination of the slides by both investigators.

Statistical analysis. The associations between TARBP2, APP, and ZNF395 immunoreactivities and clinicopathological factors were evaluated using Student's t-tests or cross-tables using χ^2 tests. Overall survival (OS) and disease-free survival (DFS) were calculated from the date of initial surgery to death and relapse, respectively. OS and DFS curves were generated according to Kaplan–Meier analysis, and statistical significance was calculated using the log-rank test. The data were computed using the JMP software (version 12.2.0; SAS Institute Inc.). The threshold for significance was $P < 0.05$.

Results

Immunohistochemical expression of TARBP2, APP, and ZNF395 in breast cancer. Immunoreactivities for TARBP2, APP, and ZNF395 were detected in the cytoplasm of breast cancer cells and normal glands (Fig. 2). Consistent with previous reports,[12,13] TARBP2 and ZNF395 nuclear immunoreactivities were detected in some cases (data not shown). However, only cytoplasmic staining was observed in subsequent analyses, because the hematoxylin counterstain often interfered with the immunostaining intensity in the nuclei. We classified the expression levels of TARBP2, APP, and ZNF395 as reduced, unchanged, or overexpressed, as described in the "Materials and methods" section.
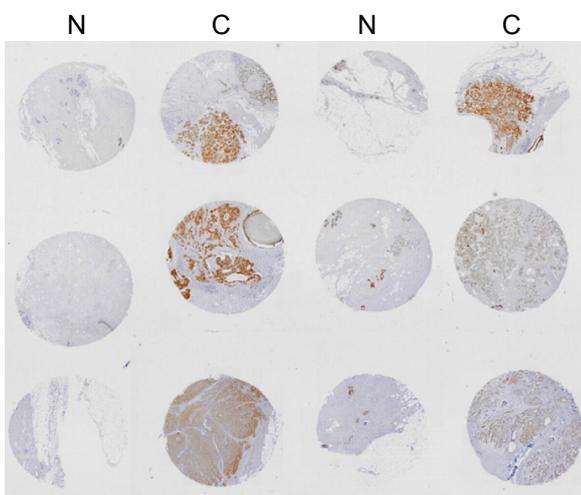
Figure 1. Part of tissue microarray with TARBP2 immunostaining. Normal (N) and cancer (C) cores from same specimen were examined together. Bar, 1 mm.
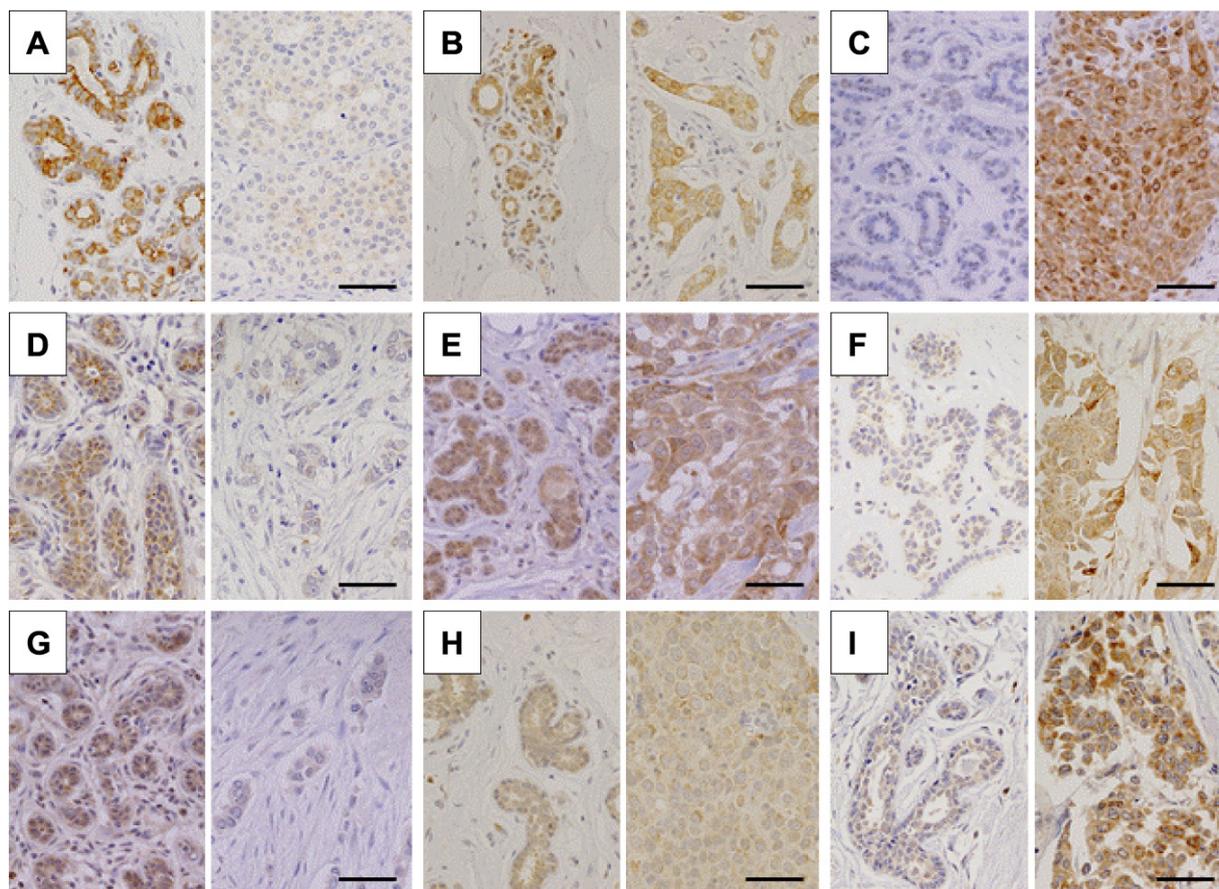
Figure 2. Immunohistochemical expression of TARBP2, APP, and ZNF395 in breast cancer. Figure shows representative immunostaining results for TARBP2 (**A–C**), APP (**D–F**), and ZNF395 (**G–I**) with expression level 1 (**A**, **D**, and **G**), level 2 (**B**, **E**, and **H**), and level 3 (**C**, **F**, and **I**). Left and right columns show normal and cancer tissues, respectively, in all figures. Bar, 50 µm.

The staining groups are summarized in Table 1. Among 200 breast cancers, TARBP2 overexpression (level 3) was seen in 60 (30.0%), while reduced expression levels (level 1) of APP and ZNF395 were detected in 48 (24.0%) and 46 (23.0%) cases, respectively.

Association between TARBP2 overexpression and clinicopathological characteristics of breast cancer. TARBP2 overexpression (level 3) was found in 60 of the 200 breast cancers (30.0%) (Table 1). We evaluated its correlation with various clinicopathological parameters in the breast cancer cases (Table 2). TARBP2 overexpression was significantly associated with PR positivity ($P = 0.003$) but showed no significant correlation with other parameters, including clinical stage, pathological tumor factor, lymph node metastasis, histological grade, ER status, HER2 status, and intrinsic subtype.

The Kaplan–Meier survival curves of OS and DFS according to TARBP2 overexpression are shown in Figure 3. Breast cancer patients with TARBP2 overexpression (level 3) had significantly shorter OS ($P = 0.019$) and DFS ($P = 0.046$) than those without overexpression (levels 1 and 2) (Fig. 3A and B). We also performed these analyses in the subgroups of patients with ER-positive ($n = 169$) or ER-negative tumors ($n = 31$). TARBP2 overexpression was significantly correlated with shorter OS ($P = 0.004$) but not with DFS ($P = 0.052$) in the ER-positive group (Fig. 3C and D), while there is no significant relationship between TARBP2 overexpression and OS or DFS in the ER-negative group (data not shown).

Associations between reduced APP and ZNF395 expression and clinicopathological variables in breast cancer patients. Downregulation of APP and ZNF was

Table 1. Expression levels of TARBP2, APP, and ZNF395 in 200 breast cancers.

	EXPRESSION LEVELS, *n* (%)		
	1	2	3
TARBP2	73 (36.5)	67 (33.5)	60 (30.0)
APP	48 (24.0)	86 (43.0)	66 (33.0)
ZNF395	46 (23.0)	99 (49.5)	55 (27.5)

Note: Columns 1, 2, and 3 represent reduced, unchanged, and overexpressed levels, respectively (see "Materials and methods" section).
Abbreviation: APP, amyloid precursor protein.

Table 2. Association between TARBP2 overexpression and clinicopathological parameters in breast cancer.

	TARBP2 EXPRESSION LEVELS, *n* (%)		*P*-VALUE
	1, 2 (*n* = 140)	3 (*n* = 60)	
Age (mean)	54.8	57.6	0.17
Menopausal status			
Premenopausal	50 (35.7)	18 (30.0)	0.43
Postmenopausal	90 (64.3)	42 (70.0)	
cStage			
I	78 (55.7)	34 (56.7)	0.24
II	59 (42.1)	22 (36.7)	
III	3 (2.1)	4 (6.6)	
pT			
pT1	81 (57.9)	39 (65.0)	0.34
pT2–4	59 (42.1)	21 (35.0)	
Lymph node metastasis			
Positive	68 (48.6)	32 (53.3)	0.54
Histological grade			
1	31 (22.1)	11 (18.3)	0.98
2	59 (42.1)	35 (58.3)	
3	50 (35.8)	14 (23.4)	
ER status			
Positive	118 (84.3)	51 (85.0)	0.89
PR status			
Positive	87 (62.1)	50 (83.3)	0.003*
HER2 status			
Positive	20 (14.3)	8 (13.3)	
Subtype			
Luminal A	112 (80.0)	47 (78.4)	0.86
Luminal B	9 (6.4)	5 (8.3)	
HER2 type	9 (6.4)	5 (8.3)	
Triple negative	10 (7.2)	3 (5.0)	

Note: *P-value < 0.05 was considered significant.
Abbreviations: pT, pathological T factor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

previously reported to induce breast cancer metastasis in mice.[5] We therefore examined the correlations between reduced expression levels of these proteins and various clinicopathological parameters and prognosis in breast cancer patients. Reduced APP and ZNF395 expression levels (level 1) were detected in 48 (24.0%) and 46 (23.0%) cases, respectively (Table 1). Reduced APP expression was significantly associated with higher histological grade ($P = 0.004$) and ER positivity ($P = 0.042$) (Table 3), and reduced ZNF395 expression was correlated with a reduced lymph node metastasis ($P = 0.043$) (Table 4). In contrast to the previous report,[5] reduced APP expression (level 1) was significantly correlated with higher OS ($P = 0.021$) (Fig. 4A) but not with DFS (Fig. 4B), and reduced ZNF395 expression was not correlated

with either OS or DFS (Fig. 4C and D). Reduced expression levels of APP and ZNF395 showed no significant relationships with OS and DFS in either ER-positive group or ER-negative group (data not shown).

Comparison of survival rates between patients with reduced and increased APP/ZNF395 expression levels. We further compared survival rates between patients with simultaneously reduced expression levels of APP and ZNF395 ($n = 20$) and those with concurrent overexpression of both proteins ($n = 26$). Although there was no significant correlation between APP/ZNF395 expression levels and OS ($P = 0.064$) (Fig. 5A), DFS was significantly lower in the APP/ZNF395 overexpression group ($P = 0.020$) (Fig. 5B). Additional analyses according to ER status were not performed in this category because of small sample numbers.

Correlation between TARBP2 overexpression and reduced APP and ZNF395 expression. Among the 60 breast cancers with TARBP2 overexpression, reduced expression levels of APP and ZNF395 were found in 6 (10.0%) and 11 (18.3%) cases, respectively. These frequencies were not significantly different from those in breast cancers without TARBP2 overexpression ($n = 140$, $P = 0.151$) (Table 5). Simultaneous reduction in APP and ZNF395 was less frequent in breast cancers with TARBP2 overexpression (3/60, 5.0%) than in those without TARBP2 overexpression (19/140, 13.6%), although this difference was not statistically significant ($P = 0.075$) (Table 6). There was also no significant correlation between overexpression of TARBP2 and overexpression of APP or ZNF395 ($P > 0.95$) (Supplementary Table 1), reduced expression of TARBP2 and reduced expression of APP or ZNF395 ($P = 0.54$) (Supplementary Table 2), and reduced expression of TARBP2 and overexpression of APP or ZNF395 ($P = 0.44$) (Supplementary Table 3).

Association between APP or ZNF395 overexpression and clinicopathological characteristics of breast cancer. Patients without APP reduction (Fig. 4A) or those with simultaneous APP/ZNF395 overexpression (Fig. 5B) showed significantly lower survival rates, and we therefore examined the correlations between APP and ZNF395 overexpression and clinicopathological characteristics of breast cancer separately. APP overexpression was significantly associated with higher clinical stage ($P = 0.023$), ER negativity ($P < 0.001$), PR negativity ($P < 0.001$), HER2 positivity ($P = 0.047$), and HER2- and triple-negative subtypes ($P < 0.001$), whereas ZNF395 overexpression correlated with HER2 positivity ($P = 0.049$) (Tables 7 and 8). The Kaplan–Meier survival curves showed significantly shorter OS ($P = 0.032$) and DFS ($P < 0.001$) in breast cancer patients with APP overexpression (Fig. 6A and B), but no such correlation was found in patients with ZNF395 overexpression (data not shown). There were also significant correlations between APP overexpression and lower OS ($P = 0.028$) and DFS ($P = 0.005$) in the ER-positive group (Fig. 6C and D), but no such correlation in the ER-negative group (data not shown).
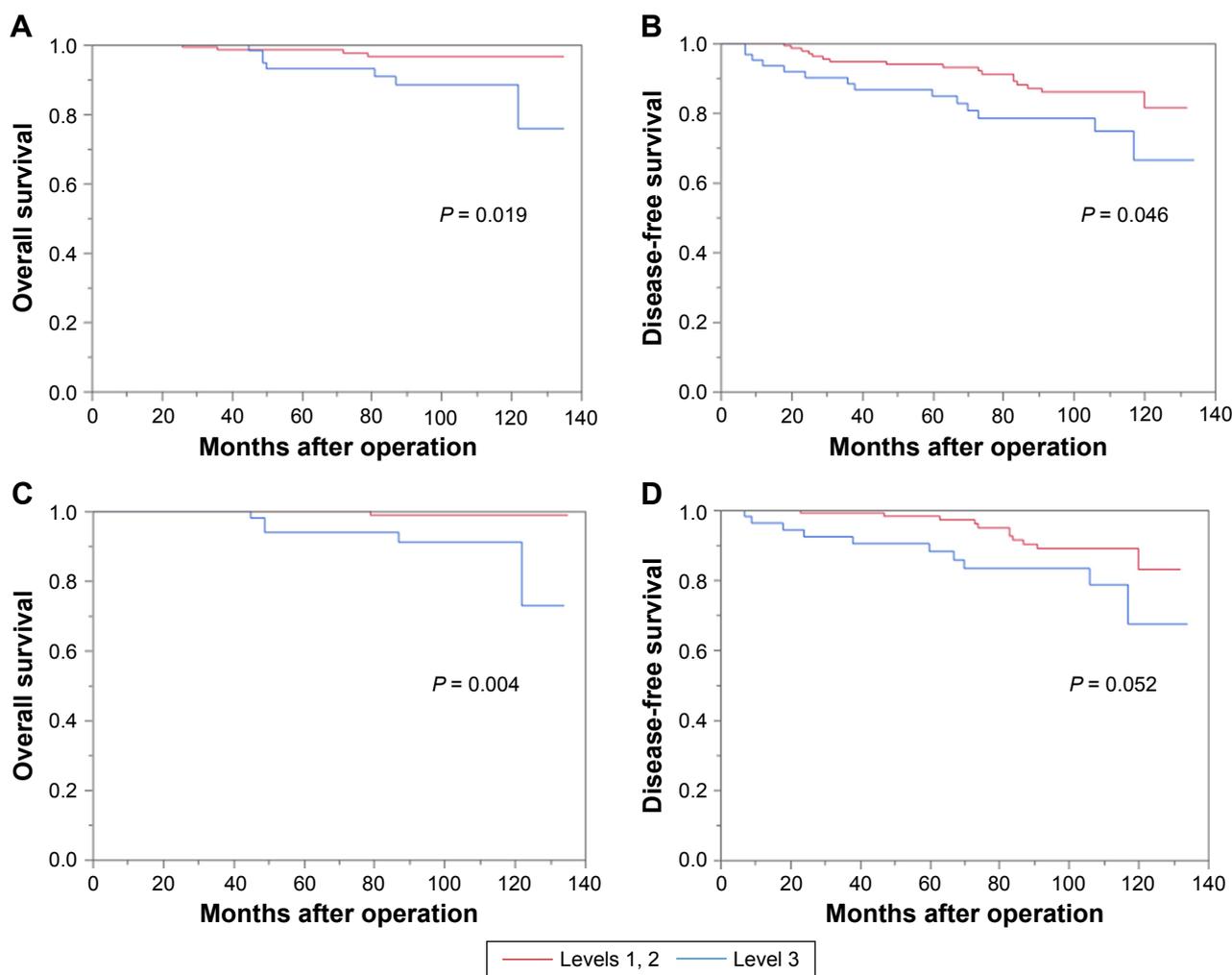
Figure 3. Correlation between TARBP2 overexpression and patient prognosis. Figure indicates overall survival (**A** and **C**) and disease-free survival (**B** and **D**) for all patients (**A** and **B**) or patients with ER-positive tumors (**C** and **D**) according to TARBP2.

Discussion

In this study, we investigated the immunohistochemical expression of TARBP2, APP, and ZNF395 in 200 breast cancers using TMA and analyzed the relationships between these expression patterns and various clinicopathological characteristics in breast cancer patients. Overexpression of TARBP2 and APP was significantly associated with some clinicopathological parameters and lower survival rates, whereas ZNF395 overexpression was correlated with HER2 positivity but not with patient prognosis. Reduced expression of APP was significantly associated with higher histological grade and ER positivity, and reduced expression of ZNF395 was associated with reduced lymph node metastasis. However, there was no significant association between reduced APP or ZNF395 expression and lower survival rates.

We classified tumor expression of TARBP2, APP, and ZNF395 into three levels based on immunostaining results (reduced, unchanged, and overexpressed), using normal glands in the same specimens as internal controls. This method had two advantages. First, conventional grading of

immunostaining intensity (eg, weak, moderate, and strong) is subjective and susceptible to variations in preanalytical variables, such as specimen size, fixation delay, time in fixative, and the duration of paraffin-block storage,[14] while our method using paired normal and cancer tissues for each case may overcome these problems. Second, we aimed to assess increased as well as reduced protein expression levels,[5] and this could not be achieved using conventional methods.

We found a correlation between TARBP2 upregulation and poor prognosis in breast cancer patients, thus supporting the proposed role of TARBP2 in breast cancer progression.[5,13] Overexpression of TARBP2 has also been observed in many other cancers, including prostate cancer, cutaneous malignant melanoma metastasis, malignant B-cell lymphoma, and adrenocortical carcinoma.[6] Although several in vitro and in vivo studies using mouse models have also demonstrated TARBP2-induced tumorigenesis,[15] the exact mechanisms underlying this phenomenon remain obscure. Although TARBP2 has been shown to mediate cell growth during viral infection via inhibition of double-stranded RNA-dependent

Table 3. Association between reduced APP expression and clinicopathological parameters in breast cancer.

	APP EXPRESSION LEVELS, n (%)		P-VALUE
	1 (n = 48)	2, 3 (n = 152)	
Age (mean)	55.6	55.6	0.99
Menopausal status			
Premenopausal	15 (31.2)	53 (34.9)	0.64
Postmenopausal	33 (68.8)	99 (65.1)	
cStage			
I	31 (64.6)	81 (53.3)	0.17
II	17 (35.4)	64 (42.1)	
III	0	7 (4.6)	
pT			
pT1	26 (54.2)	96 (63.2)	0.31
pT2–4	22 (45.8)	56 (36.8)	
Lymph node metastasis			
Positive	22 (45.8)	79 (52.0)	0.45
Histological grade			
1	10 (20.8)	32 (21.1)	0.004*
2	14 (29.2)	80 (52.6)	
3	24 (50.0)	40 (26.3)	
ER status			
Positive	45 (93.8)	124 (81.6)	0.042*
PR status			
Positive	33 (68.8)	94 (61.8)	0.38
HER2 status			
Positive	4 (8.3)	24 (15.8)	0.091
Subtype			
Luminal A	42 (87.5)	117 (77.0)	0.13
Luminal B	4 (8.3)	10 (6.6)	
HER2 type	0	14 (9.2)	
Triple negative	2 (4.2)	11 (7.2)	

Note: *P-value < 0.05 was considered significant.
Abbreviations: APP, amyloid precursor protein; pT, pathological T factor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 4. Association between reduced ZNF395 expression and clinicopathological parameters in breast cancer.

	ZNF395 EXPRESSION LEVELS, n (%)		P-VALUE
	1 (n = 46)	2, 3 (n = 154)	
Age (mean)	57.4	55.1	0.30
Menopausal status			
Premenopausal	13 (28.3)	55 (35.7)	0.34
Postmenopausal	33 (71.7)	99 (64.3)	
cStage			
I	25 (54.3)	87 (56.5)	0.79
II	20 (43.5)	61 (39.6)	
III	1 (2.2)	6 (3.9)	
pT			
pT1	26 (56.5)	94 (61.0)	0.58
pT2–4	20 (43.5)	60 (39.0)	
Lymph node metastasis			
Positive	17 (37.0)	83 (53.9)	0.043*
Histological grade			
1	13 (28.3)	29 (18.8)	0.23
2	17 (37.0)	77 (50.0)	
3	16 (34.8)	48 (31.2)	
ER status			
Positive	41 (89.1)	128 (83.1)	0.32
PR status			
Positive	29 (63.0)	99 (64.3)	0.87
HER2 status			
Positive	6 (13.0)	22 (14.3)	0.83
Subtype			
Luminal A	37 (80.4)	122 (79.2)	0.83
Luminal B	4 (8.7)	10 (6.5)	
HER2 type	2 (4.3)	12 (7.8)	
Triple negative	3 (6.5)	10 (6.5)	

Note: *P-value < 0.05 was considered significant.
Abbreviations: pT, pathological T factor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

protein kinase, this pathological phenomenon is not directly linked to normal cell physiology.[16–18] In addition to its role as a protein kinase inhibitor, TARBP2 is also known to function as a Dicer cofactor. Dicer is a key component in the biogenesis of microRNAs, which negatively regulates their complementary target mRNAs.[19] Although TARBP2 was suggested to stimulate microRNA processing by increasing the substrate affinity to Dicer,[20] Kim et al[21] recently showed that TARBP knockout in human cells had no effect on Dicer stability or microRNA abundance. These findings highlight the importance of a Dicer-independent role of TARBP2 in regulating gene expression (discussed below). Such mechanism might also account for TARBP2-mediated tumor cell

growth via degradation of the mRNAs encoding various tumor suppressor genes.

The recent study by Goodarzi et al[5] implicated the TARBP2/APP/ZNF395 pathway in the metastatic progression of breast cancer. Using in vitro and in vivo experiments in mice, they showed that TARBP2 promoted metastasis by destabilizing transcripts of the possible metastasis suppressor genes, *APP* and *ZNF395*. The authors demonstrated a TARBP2-dependent and Dicer-independent pathway of mRNA destabilization and suggested the existence of a novel posttranscriptional regulatory network, whereby TARBP2 binding of structural hairpins contained in the *APP* and *ZNF395* transcripts may lead to their destabilization.
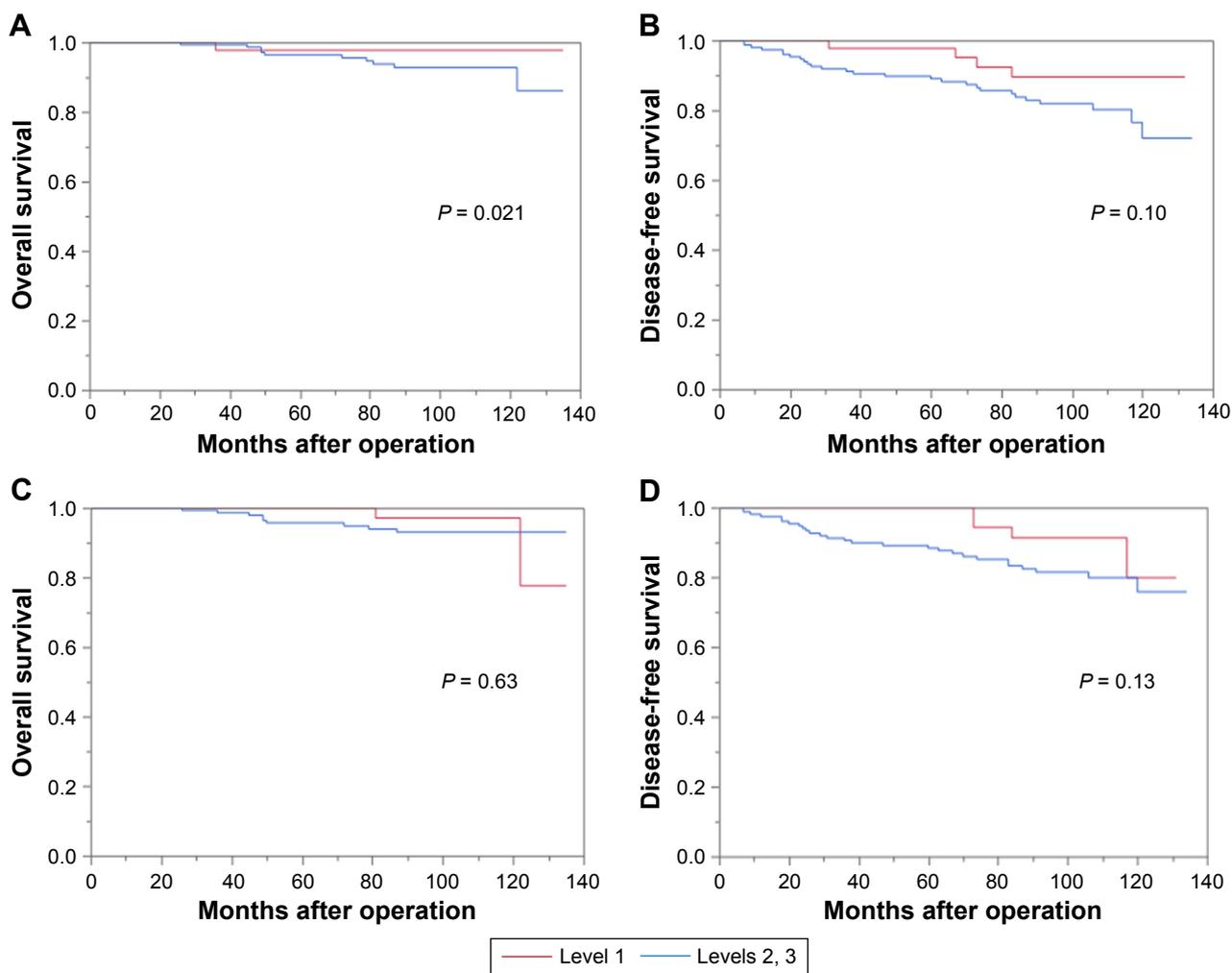
Figure 4. Correlation between reduced APP or ZNF395 expression and patient prognosis. Figure shows overall survival (**A** and **C**) and disease-free survival (**B** and **D**) of breast cancer patients according to APP (**A** and **B**) and ZNF395 (**C** and **D**).
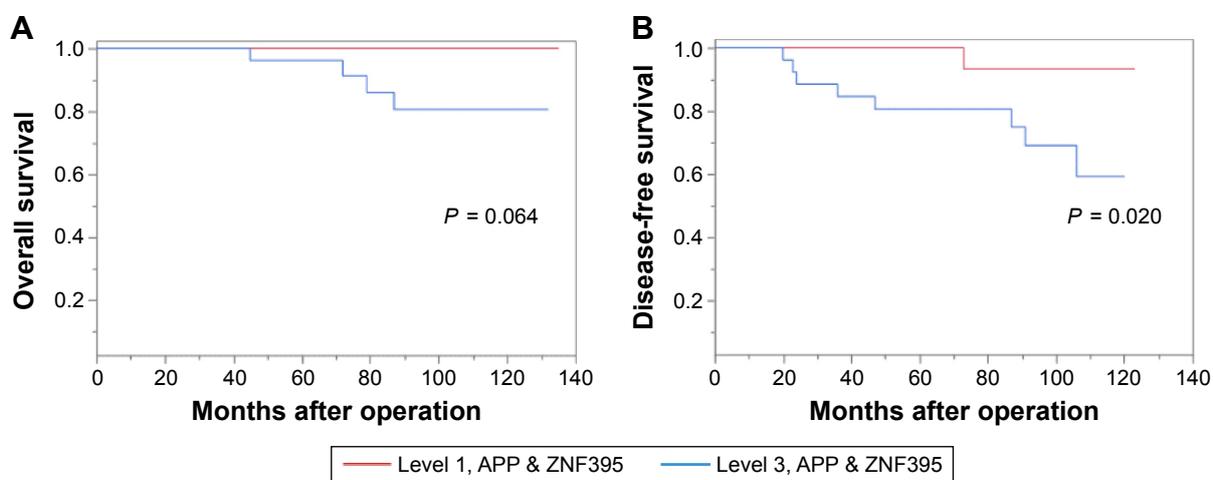

Figure 5. Correlations between reduced and increased APP/ZNF395 expression and patient prognosis. Figure demonstrates overall survival (**A**) and disease-free survival (**B**) of breast cancer patients according to both APP and ZNF395. Red and blue lines indicate patients with simultaneous reduced and increased APP and ZNF395 expression, respectively.

Table 5. No significant association between TARBP2 overexpression and APP or ZNF395 reduction in breast cancer.

	TARBP2 LEVELS, n (%)	
	1, 2 (n = 140)	3 (n = 60)
APP, level 1	40 (28.6)	6 (10.0)
ZNF395, level 1	35 (25.0)	11 (18.3)

Note: $P = 0.151$, χ^2 test.
Abbreviation: APP, amyloid precursor protein.

They also showed an increased *TARBP2* expression in human breast cancers that metastasized (stage IV) and correlations between simultaneous low expression of *APP* and *ZNF395* and reduced DFS at the mRNA level.[5] Another recent study reported a similar link between higher *APP* expression and longer DFS in breast cancer.[22] Although we confirmed a link between TARBP2 overexpression and poor prognosis, we found conflicting results in that patients with simultaneous overexpression of APP and ZNF395 had significantly lower DFS (discussed below). Moreover, the incidence of APP and/or ZNF395 reduction in TARBP2 overexpressing tumors did not differ significantly from that in the control group. We were, therefore, unable to confirm the hypothesis proposed by Goodarzi et al, which stated that overexpressed TARBP2 led to reduced APP and ZNF395 expressions, thereby causing metastatic progression of breast cancer.

APP, which has previously been implicated in Alzheimer's disease, is a membrane protein that is proteolytically cleaved to yield soluble products (such as soluble amyloid-a peptide).[23] However, whether APP functions as a tumor promoter or suppressor in breast cancer remains unclear. In contrast to recent reports,[5,22] we found a link between higher APP expression and shorter OS and DFS. Takagi et al[7] reported a similar relationship between increased APP expression and shorter DFS in ER-positive breast cancer patients. We also noted significant correlations between APP overexpression and several clinicopathological parameters, including higher clinical stage, ER negativity, and HER2- and triple-negative subtypes. Intriguingly, Lim et al[8] showed that APP expression was increased in breast cancer cell lines with higher metastatic potential and that knockdown of *APP* in cancer cells retarded cell proliferation in vitro and in vivo, implying close correlations between APP expression and tumor cell growth, metastasis,

Table 6. No significant association between TARBP2 overexpression and simultaneous APP and ZNF395 reduction in breast cancer.

	TARBP2 LEVELS, n (%)	
	1, 2 (n = 140)	3 (n = 60)
APP & ZNF395, level 1	19 (13.6)	3 (5.0)
APP & ZNF395, levels 2, 3	121 (86.4)	57 (95.0)

Note: $P = 0.075$, χ^2 test.
Abbreviation: APP, amyloid precursor protein.

Table 7. Association between APP overexpression and clinicopathological parameters in breast cancer.

	APP EXPRESSION LEVELS, n (%)		P-VALUE
	1, 2 (n = 134)	3 (n = 66)	
Age (mean)	54.9	57.1	0.27
Menopausal status			
Premenopausal	48 (35.8)	20 (30.3)	0.38
Postmenopausal	86 (64.1)	46 (69.7)	
cStage			
I	83 (61.9)	28 (42.4)	0.023*
II	48 (35.8)	34 (51.5)	
III	3 (2.2)	4 (6.1)	
pT			
pT1	84 (62.7)	36 (54.5)	0.26
pT2–4	50 (37.3)	30 (45.4)	
Lymph node metastasis			
Positive	61 (45.5)	38 (57.6)	0.10
Histological grade			
1	29 (21.6)	13 (19.7)	0.93
2	63 (47.0)	31 (47.0)	
3	42 (31.3)	22 (33.3)	
ER status			
Positive	124 (92.5)	46 (69.7)	<0.001*
PR status			
Positive	87 (64.9)	29 (44.0)	<0.001*
HER2 status			
Positive	14 (10.4)	14 (21.2)	0.039*
Subtype			
Luminal A	117 (87.3)	42 (63.6)	<0.001
Luminal B	9 (6.7)	5 (7.6)	
HER2 type	5 (3.7)	9 (13.6)	
Triple negative	3 (2.2)	10 (15.2)	

Note: *P-value < 0.05 was considered significant.
Abbreviations: APP, amyloid precursor protein; pT, pathological T factor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

and progression in breast cancer. In addition to breast cancer, increased expression of APP has been detected and correlated with increased cancer cell proliferation in several other cancer types.[24–27] Overall, these results support a possible role for APP in breast cancer progression, though recent studies have suggested an opposite function.[5,22] These previous two studies and ours differed in terms of the evaluation methods of APP expression. The microarray-based analyses measured *APP* mRNA expression levels in breast cancer tissues and classified them as either high-expression group or low-expression group, according to values above or below the median.[5,22] In contrast, we classified tumors according to APP protein expression levels, determined by immunohistochemistry, as reduced,

Table 8. Association between ZNF395 overexpression and clinicopathological parameters in breast cancer.

	ZNF395 EXPRESSION LEVELS, *n* (%)		*P*-VALUE
	1, 2 (*n* = 145)	3 (*n* = 55)	
Age (mean)	55.4	56.1	0.44
Menopausal status			
Premenopausal	47 (32.4)	21 (38.2)	0.44
Postmenopausal	98 (67.6)	34 (61.8)	
cStage			
I	83 (57.2)	28 (50.9)	0.14
II	59 (40.7)	23 (41.8)	
III	3 (2.1)	4 (7.3)	
pT			
pT1	84 (57.9)	36 (65.5)	0.33
pT2–4	61 (42.1)	19 (34.5)	
Lymph node metastasis			
Positive	75 (51.7)	24 (43.6)	0.30
Histological grade			
1	29 (20.0)	13 (23.6)	0.85
2	69 (47.6)	25 (45.5)	
3	47 (32.4)	17 (30.9)	
ER status			
Positive	122 (84.1)	47 (85.5)	0.81
PR status			
Positive	83 (57.2)	35 (63.6)	0.78
HER2 status			
Positive	16 (11.0)	12 (21.8)	0.049*
Subtype			
Luminal A	120 (82.7)	39 (70.9)	0.07
Luminal B	6 (4.1)	8 (14.5)	
HER2 type	10 (6.9)	4 (7.3)	
Triple negative	9 (6.2)	4 (7.3)	

Note: *P-value < 0.05 was considered significant.
Abbreviations: pT, pathological T factor; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

unchanged, and overexpressed, using paired normal tissues as internal controls. There are advantages and disadvantages associated with both methods in terms of assessing the molecular expression levels. The presence of an *unchanged* group in our analysis may at least partly explain the difference in survival results between the previous[5,22] and present studies, given that high- and low-expressing tumors according to the RNA-based analyses may have been intermixed in the *unchanged* group according to our criteria. Alternatively, different proportions of intrinsic subtypes of breast cancer in the study populations may have affected the survival analysis results. Fernandez-Nogueira et al showed that *APP* was differentially overexpressed in basal-related tumors (basal-like and/or *HER2*-enriched phenotypes) using the GOBO database, which included 30.5%

of such tumors.[22,28] The frequencies of these tumors in Goodarzi et al[5] cohorts were uncertain. In our study, HER2-positive and triple-negative tumors accounted for 13.5% of all breast cancers, and these two subtypes were significantly associated with APP overexpression, in line with the results of Fernandez-Nogueira et al.[22] Although molecular and immunohistochemical subtypes are not equivalent,[1] this difference in the prevalence of basal-related tumors among the distinct cohorts might have affected the results of the survival analyses. Further prognostic analyses of APP expression status in breast cancer patients at both the mRNA and protein levels would help to clarify this discrepancy.

ZNF395 is a poorly characterized transcription factor involved in the transcriptional activation of the gene encoding the Huntington's disease protein, huntingtin.[29] In addition to breast cancer, ZNF395 is expressed in various types of cancers, including osteosarcoma, synovial sarcoma, malignant melanoma, lung carcinoma, gastric carcinoma, and pancreatic carcinoma.[9] Although a previous study[5] suggested a possible link between high ZNF395 expression and favorable prognosis in breast cancer, its expression has been associated with poorer prognosis in the Ewing's sarcoma family of tumors and in osteosarcoma.[10] We found no correlation between ZNF395 expression levels and patient prognosis, but there was a significant association between reduced ZNF395 expression and reduced lymph node metastasis. The latter finding may be inconsistent with the TARBP2/APP/ZN395 hypothesis for breast cancer metastasis,[5] though lymphatic spread is not the only means of cancer metastasis.

In conclusion, we have demonstrated a significant correlation between TARBP2 and APP overexpression and poorer prognosis in breast cancer, while ZNF395 expression levels had no prognostic value. In contrast to previous results,[5] we found no apparent correlation between TARBP2 overexpression and reduced expression of APP and/or ZNF395. Further studies are therefore needed to clarify the roles of APP and ZNF395 in human breast cancer.

Author Contributions
Conceived and designed the experiments: RO and HK. Analyzed the data: RO, HK, IM, AN, ST, TI, and HKawamoto. Wrote the first draft of the article: RO and HK. Contributed to the writing of the article: RO, HK, and AN. Agreed with the article results and conclusions: KT and MT. Jointly developed the structure and arguments for the article: AN and TI. Made critical revisions and approved the final version: RO, HK, IM, AN, ST, TI, HKawamoto, KT, and MT. All authors reviewed and approved the final article.
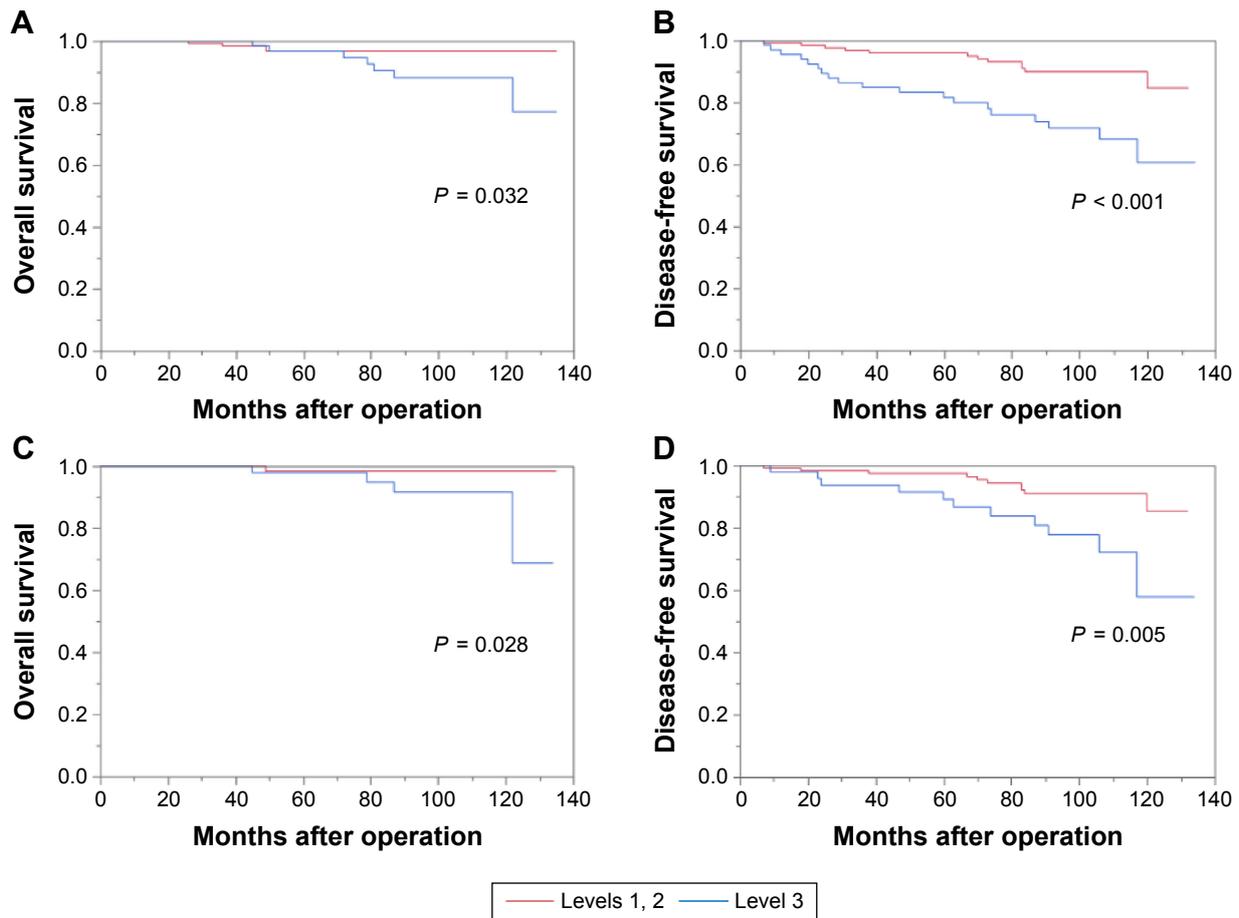
Figure 6. Correlation between APP overexpression and patient prognosis. Figure shows overall survival (**A** and **C**) and disease-free survival (**B** and **D**) for all patients (**A** and **B**) or patients with ER-positive tumors (**C** and **D**) according to APP.

Supplementary Materials

Supplementary table 1. No significant association between overexpression of TARBP2 and overexpression of APP or ZNF395 in breast cancer.

Supplementary table 2. No significant association between reduced expression of TARBP2 and reduced expression of APP or ZNF395 in breast cancer.

Supplementary table 3. No significant association between reduced expression of TARBP2 and overexpression of APP or ZNF395 in breast cancer.

REFERENCES

1. Lkhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ, eds. *WHO Classification of Tumours of the Breast*. 4th ed. Lyon: IARC; 2012.
2. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;28:518–524.
3. Pencheva N, Tran H, Buss C, et al. Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis. *Cell*. 2012;151:1068–1082.
4. Png KJ, Halberg N, Yoshida M, Tavazoie SF. A microRNA regulon that mediates endothelial recruitment and metastasis by cancer cells. *Nature*. 2011; 481:190–194.
5. Goodarzi H, Zhang S, Buss CG, Fish L, Tavazoie S, Tavazoie SF. Metastasis-suppressor transcript destabilization through TARBP2 binding of mRNA hairpins. *Nature*. 2014;513:256–260.
6. Yu X, Li Z. The role of TARBP2 in the development and progression of cancers. *Tumour Biol*. 2016;37:57–60.
7. Takagi K, Ito S, Miyazaki T, et al. Amyloid precursor protein in human breast cancer: an androgen-induced gene associated with cell proliferation. *Cancer Sci*. 2013;104:1532–1538.
8. Lim S, Yoo BK, Kim HS, et al. Amyloid-beta precursor protein promotes cell proliferation and motility of advanced breast cancer. *BMC Cancer*. 2014; 14:928.
9. Tsukahara T, Nabeta Y, Kawaguchi S, et al. Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. *Cancer Res*. 2004;64:5442–5448.
10. Yabe H, Tsukahara T, Kawaguchi S, et al. Overexpression of papillomavirus binding factor in Ewing's sarcoma family of tumors conferring poor prognosis. *Oncol Rep*. 2008;19:129–134.
11. Goldhirsch A, Wood WC, Coates AS, et al. Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol*. 2011;22:1736–1747.
12. Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347:1260419. The Human Protein Atlas from www.proteinatlas.org
13. Lin X, Wu M, Liu P, et al. Up-regulation and worse prognostic marker of cytoplasmic TARBP2 expression in obstinate breast cancer. *Med Oncol*. 2014;31:868.
14. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med*. 2011;135:537–543.
15. Lee JY, Kim H, Ryu CH, et al. Merlin, a tumor suppressor, interacts with transactivation-responsive RNA-binding protein and inhibits its oncogenic activity. *J Biol Chem*. 2004;279:30265–30273.
16. Benkirane M, Neuveut C, Chun RF, et al. Oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with RNA-dependent protein kinase PKR. *EMBO J*. 1997;16:611–624.

17. Francois C, Duverlie G, Rebouillat D, et al. Expression of hepatitis C virus proteins interferes with the antiviral action of interferon independently of PKR-mediated control of protein synthesis. *J Virol*. 2000;74:5587–5596.

18. Kim Y, Lee JH, Park JE, Cho J, Yi H, Kim VN. PKR is activated by cellular dsRNAs during mitosis and acts as a mitotic regulator. *Genes Dev*. 2014;28:1310–1322.

19. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol*. 2014;15:509–524.

20. Chendrimada TP, Gregory RI, Kumaraswamy E, et al. TRBP recruits the dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*. 2005;436: 740–744.

21. Kim Y, Yeo J, Lee JH, et al. Deletion of human tarbp2 reveals cellular microRNA targets and cell-cycle function of TRBP. *Cell Rep*. 2014;9:1061–1074.

22. Fernandez-Nogueira P, Bragado P, Almendro V, et al. Differential expression of neurogenes among breast cancer subtypes identifies high risk patients. *Oncotarget*. 2016;7:5313–5326.

23. Kuhn PH, Wang H, Dislich B, et al. ADAM10 is the physiologically relevant, constitutive alpha-secretase of the amyloid precursor protein in primary neurons. *EMBO J*. 2010;29:3020–3032.

24. Itoh H, Kataoka H, Koita H, et al. Establishment of a new human cancer cell line secreting protease nexin-II/amyloid beta protein precursor derived from squamous-cell carcinoma of lung. *Int J Cancer*. 1991;49:436–443.

25. Meng JY, Kataoka H, Itoh H, Koono M. Amyloid beta protein precursor is involved in the growth of human colon carcinoma cell in vitro and in vivo. *Int J Cancer*. 2001;92:31–39.

26. Hansel DE, Rahman A, Wehner S, Herzog V, Yeo CJ, Maitra A. Increased expression and processing of the Alzheimer amyloid precursor protein in pancreatic cancer may influence cellular proliferation. *Cancer Res*. 2003;63:7032–7037.

27. Takayama K, Tsutsumi S, Suzuki T, et al. Amyloid precursor protein is a primary androgen target gene that promotes prostate cancer growth. *Cancer Res*. 2009;69: 137–142.

28. Ringner M, Fredlund E, Hakkinen J, Borg A, Staaf J. GOBO: gene expression-based outcome for breast cancer online. *PLoS One*. 2011;6:e17911.

29. Tanaka K, Shouguchi-Miyata J, Miyamoto N, Ikeda JE. Novel nuclear shuttle proteins, HDBP1 and HDBP2, bind to neuronal cell-specific cis-regulatory element in the promoter for the human Huntington's disease gene. *J Biol Chem*. 2004;279:7275–7286.