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Nucleobindin 2 in human breast carcinoma as a potent prognostic factor

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It is well-known that estrogens immensely contribute to the progression of human breast carcinoma, but their detailed molecular mechanisms remain largely unclear. In this study, we identified nucleobindin 2 (*NUCB2*) as a gene associated with recurrence based on microarray data of estrogen receptor (ER)-positive breast carcinoma cases (n = 10), and subsequent *in vitro* study showed that *NUCB2* expression was upregulated by estradiol in ER-positive MCF-7 cells. However, *NUCB2* has not yet been examined in breast carcinoma, and its significance remains unknown. Therefore, we further examined the biological functions of *NUCB2* in breast carcinoma using immunohistochemistry and *in vitro* studies. *NUCB2* immunoreactivity was detected in carcinoma cells in 77 of 161 (48%) breast cancer cases, and positively associated with lymph node metastasis and ER status of the patients. In addition, *NUCB2* status was significantly associated with an increased risk of recurrence and adverse clinical outcome of the patients using both univariate and multivariate analyses. Results of siRNA transfection experiments showed that *NUCB2* significantly increased cell proliferation, and migration and invasion properties in both MCF-7 and ER-negative SK-BR-3 cells. These results suggest that *NUCB2* is upregulated by estrogens and plays an important role, especially in the process of metastasis, in breast carcinomas. *NUCB2* status is considered a potent prognostic factor in human breast cancer. (*Cancer Sci* 2012; 103: 136–143)

Breast cancer is one of the most common malignancies in women. Estrogens play an important role in the progression of breast cancer through an interaction with ER, and ER is positive in approximately two-thirds of breast carcinoma cases. The great majority of ER-positive breast carcinomas respond to endocrine therapy such as tamoxifen and aromatase inhibitors, but it is also true that some of these carcinomas acquire clinical resistance to endocrine therapy.[1,2]

Estrogen receptor activates the transcription of various target genes in a ligand-dependent manner by binding EREs located in the promoter region. Various estrogenic functions are characterized by the expression patterns of these genes, which make it extremely important to examine the expression and roles of estrogen-responsive genes to obtain a better understanding of estrogenic actions such as progression, recurrence, and resistance to endocrine therapy.[3] Various estrogen-responsive genes have been identified in breast carcinoma,[4,5] but their detailed clinical significance and/or function remain unclear in a great majority of these genes. Therefore, in this study, we first studied the expression profiles of genes containing ERE in ER-positive breast carcinoma tissues based on microarray data, and identified *NUCB2* as a possible gene associated with recurrence in these patients.

Nucleobindin 2 has a characteristic constitution of functional domains, such as a signal peptide, a Leu/Ile rich region, two Ca^{2+} binding EF-hand domains separated by an acidic amino acid-rich region, and a leucine zipper,[6,7] and has a wide variety of basic cellular functions.[8–10] However, to the best of our knowledge, *NUCB2* has not yet been studied in breast carcinoma. Therefore, we examined *NUCB2* in breast carcinoma using immunohistochemistry and *in vitro* studies to explore its clinical and biological significance.

Materials and Methods

Patients and tissues. Two sets of tissue specimens were evaluated in this study. As a first set, 10 specimens of ER-positive breast carcinoma were obtained from women (age range, 48–74 years) who underwent surgical treatment in 2001 or 2002 in the Department of Surgery, Tohoku University Hospital (Sendai, Japan). All patients received tamoxifen therapy after surgery. The status of recurrence was evaluated whether the first locoregional recurrence or distant metastasis was detected within the follow-up time after surgery (mean, 80 months; range, 37–204 months) or not. These specimens were stored at −80°C for microarray analysis.

As a second set, 161 specimens of invasive ductal carcinoma of human breast were obtained from women who underwent surgical treatment between 1984 and 1997 in the Department of Surgery, Tohoku University Hospital. The patients did not receive chemotherapy, irradiation, or hormonal therapy before the surgery. Review of the charts revealed that 125 patients received adjuvant chemotherapy, 66 patients received tamoxifen therapy, and 12 patients received radiation therapy following surgery. The clinical outcome of the patients was evaluated by disease-free and breast cancer-specific survival. The mean age was 54 years (range, 22–81 years), and the mean follow-up time was 103 months (range, 3–157 months). Mitotic score and histological grade were evaluated according to a previous report.[11] All the specimens were fixed in 10% formalin and embedded in paraffin wax.

Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan).

Laser capture microdissection/microarray analysis. Gene expression profiles of breast carcinoma cells in the first set (n = 10) were examined using microarray analysis. Gene expression profile data was assembled previously.[12,13] Briefly, approximately 5000 breast carcinoma cells were laser transferred from the frozen section, and total RNA was subsequently extracted. In this study, we focused on the expression of 519 genes identified to have a functional ERE by Bourdeau *et al.*[14]

Immunohistochemistry. Rabbit polyclonal antibody for *NUCB2* and *HER2* (A0485) were purchased from Aviva Systems Biology (San Diego, CA, USA) and Dako (Carpinteria, CA, USA), respectively. Monoclonal antibodies for ER (ER1D5), PR (MAB429), and Ki-67 (MIB1) were purchased

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from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and Dako, respectively.

A Histofine Kit (Nichirei, Tokyo, Japan), which incorporates the streptavidin–biotin amplification method, was used. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin. Human tissue of the stomach was used as a positive control for NUCB2 antibody,[15] and normal rabbit IgG was used instead of the primary antibody, as a negative control of NUCB2 immunostaining.

NUCB2 immunoreactivity was detected in the cytoplasm of breast carcinoma cells, and the cases that had more than 10% of positive carcinoma cells were considered positive for NUCB2 status. Immunoreactivity for ER, PR, and Ki-67 was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case. The percentage of immunoreactivity, that is, the LI, was determined. Cases with an ER LI or PR LI of more than 10% were considered ER- or PR-positive breast carcinoma, respectively, according to a previous report.[16]

Immunoblotting. The protein of MCF-7 cells was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). Twenty micrograms of the protein (whole cell extracts) was subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond-P PVDF membrane (GE Healthcare, Chalfont St Giles, UK). Primary antibody was the same anti-NUCB2 antibody used in the immunohistochemistry (Aviva Systems Biology). Antibody–protein complexes on the blots were detected using ECL Plus Western blotting detection reagents (GE Healthcare), and the protein bands were visualized with a LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using a QuantiTect reverse transcription Kit (Qiagen, Hilden, Germany). Real-time PCR was carried out using the LightCycler System and FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The primer sequences of NUCB2 and the ribosomal protein L13A (RPL13A) were: NUCB2, 5′-AAAGAAGAGCTACAACGTCA-3′ (forward) and 5′-GTGGCTCAAACTTCAATTC-3′ (reverse); and RPL13A, 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ (forward) and 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (reverse). The NUCB2 mRNA level was calculated as the ratio of the RPL13A mRNA level.

Small interfering RNA transfection. Small interfering RNA for NUCB2 was purchased from Ambion (Austin, TX, USA). The target sequences of siRNA against *NUCB2* were as follows: si1, 5′-UAUCUUCGCACUUUCCACAGGGUGA-3′ (sense) and 5′-UCACCCUGUGGAAAGUGCGAAGAUA-3′ (anti-sense); and si2, 5′-UUGAUUAGCAUAUCUAAAUCUGUGG-3′ (sense) and 5′-CCACAGAUUUAGAUAUGCUAAUCAA-3′ (anti-sense). In addition, medium GC duplex #2 (Invitrogen) was also used as a negative control (siC). The siRNA was transfected using HiperFect transfection reagent (Qiagen).

Cell proliferation, migration, and invasion assays. MCF-7 and SK-BR-3 cells were transfected with NUCB2-specific siRNA or control siRNA in a 96-well culture plate. Three days after transfection, the cell number was evaluated using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

The cell migration assay was carried out using a 24-well plate and Chemotaxicell (8 μm pore size; Kurabo, Osaka, Japan) according to a previous report.[17] MCF-7 and SK-BR-3 cells were plated at the upper chamber, and the cells on the upper surface of the membrane were removed after incubation for 72 h. The migration ability was evaluated as an average number of cells in five middle power fields (×200) randomly selected on the lower surface of the membrane.

The cell invasion assay was carried out using a modified migration assay. The upper surface of the membrane of a Chemotaxicell was coated with 80 mg/cm^2 of Matrigel basement membrane matrix (BD Biosciences, Heidelberg, Germany), and the invasion ability was evaluated as the total number of cells on the lower surface of the membrane.

Results

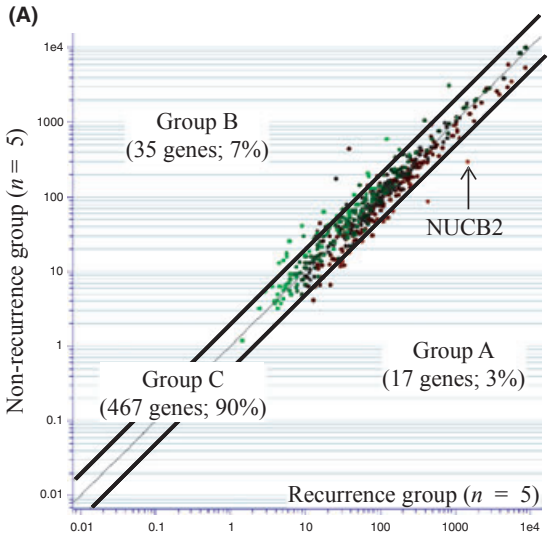
Comparison of gene expression profiles between recurrent and non-recurrent groups of breast carcinoma patients. The microarray data used in this study are available through the National Center for Biotechnology Information Gene Expression Omnibus database (accession GSE11965, http://www.ncbi.nlm.nih.gov/geo). In this analysis, when the expression ratio of a gene in the recurrence group compared to that in the non-recurrence group was more than 2.0 or <0.5, we determined that the gene was predominantly expressed in the recurrence or non-recurrence group, respectively.

As shown in Figure 1(A), of the 519 genes examined, the number of genes predominantly expressed in the recurrence group (group A) was 17 (3%); the number of genes predominantly expressed in the non-recurrence group (group B) was 35 (7%). A great majority of the genes (467 genes; 90%) had a similar expression level in each of the two groups (ratio 0.5–2.0) (group C). The lists of genes classified in group A and group B are summarized in the right panel of Figure 1(A), and in Table S1. When we carried out gene ontology enrichment analysis between groups A and B (http://cbl-gorilla.cs.technion.ac.il/), no significant enriched gene ontology term was detected. Among the genes in Group A, *NUCB2* showed the highest ratio (4.9) and expression level, indicating its possible involvement in the recurrence in ER-positive breast carcinoma patients after surgery.

The *NUCB2* gene contains functional ERE in the promoter region[14] but the regulation of *NUCB2* expression by estradiol has not been investigated in breast carcinoma cells. As shown in Figure 1(B), NUCB2 mRNA expression was significantly increased by estradiol treatment for 3 days in MCF-7 cells. However, the NUCB2 mRNA expression level was significantly lower ($P < 0.05$, and 0.3-fold) than the basal level, when the cells were treated together with estradiol (10 nM) and a potent ER antagonist ICI 182780 (1 μM). When MCF-7 cells were treated with estradiol (10 nM) and anti-estrogen tamoxifen (10 μM), the NUCB2 mRNA level was not significantly changed compared to the basal level ($P = 0.10$, and 1.5-fold). Estradiol (10 nM) time-dependently induced NUCB2 mRNA expression in MCF-7 cells (Fig. 1C).

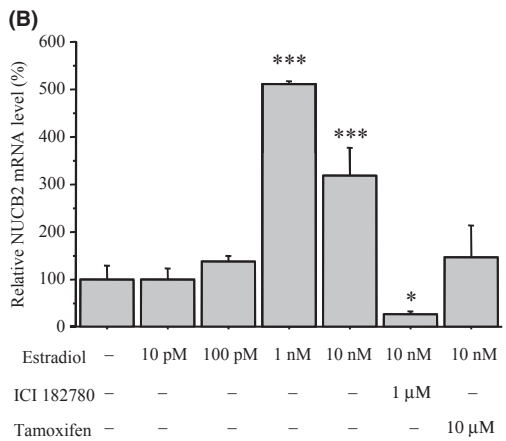
***NUCB2* immunolocalization in human breast carcinoma.** As shown in Figure 2(A), immunoblot analysis for *NUCB2* revealed a specific band (approximately 43 kDa) in MCF-7 cells, which confirmed the specificity of the anti-*NUCB2* antibody used in this study.[18] In the immunohistochemistry, *NUCB2* immunoreactivity was detected in the cytoplasm of breast carcinoma cells (Fig. 2B). *NUCB2* immunoreactivity was weakly and focally detected in the epithelial cells of morphologically normal glands (Fig. 2C), but it was negative in the stroma. In the positive control, *NUCB2* was mainly positive in the epithelium of the fundic glands in the stomach (Fig. 2D), as reported previously,[15] whereas no significant immunoreactivity was detected in the same areas of the negative control section (Fig. 2E).

Associations between *NUCB2* immunohistochemical status and various clinicopathological parameters in breast carcinomas are summarized in Table 1. Of 161 cases of breast carcinoma examined in this study, 77 (48%) were *NUCB2*-positive. *NUCB2* status was significantly associated with lymph node metastasis ($P = 0.004$) and ER status ($P = 0.002$) of the patients, whereas no significant association was detected in patients' age, menopausal status, clinical stage, tumor size,



Genes classified as Group A		
Fold†	Common name	Gene symbol
4.9	**NM_005013**	**NUCB2**
4.8	AI824012	NRIP1
3.2	NM_001557	CXCR2
3.0	X02189	ADA
2.6	NM_001874	CPM
2.5	NM_001228	CASP8
2.4	AK023540	BUB1
2.3	NM_016083	CNR1
2.3	AF109161	CITED2
2.3	NM_016580	PCDH12
2.2	NM_001256	CDC27
2.2	BC005312	HLA-DRB4
2.2	AF149096	TGFA
2.1	NM_006889	CD86
2.1	X63575	ATP2B2
2.1	X81006	TRIM31
2.1	AW157548	IGFBP5

†: Expression ratio in the recurrence group to that in the non-recurrence group.



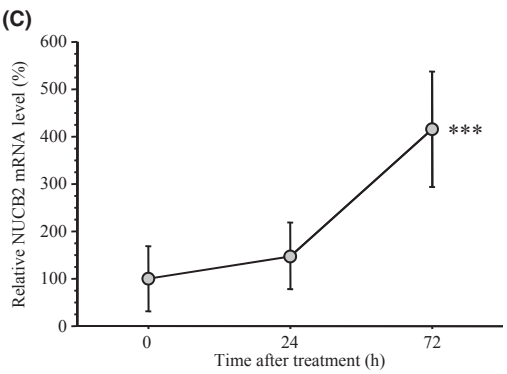


Fig. 1. Nucleobindin 2 (*NUCB2*) as an estrogen-induced gene associated with breast carcinoma. (A) Scatter plot analysis of microarray data for 519 genes containing functional estrogen-responsive element in breast carcinomas comparing the recurrence and non-recurrence group (n = 5 in each group). Genes with an expression ratio, recurrence group to non-recurrence group, of more than 2.0 or <0.5 are located outside the diagonal line, and classified as group A or group B, respectively. Genes with a ratio between 2.0 and 0.5 were classified as group C. *NUCB2* showed the highest ratio in these genes (arrow). The right panel summarizes the gene list of group A. (B,C) Effects of estradiol on NUCB2 mRNA expression. MCF-7 cells were treated with indicated concentrations of estradiol with or without (–) ICI 182780 or tamoxifen for 3 days (B) or treated with estradiol (10 nM) for the indicated period (C). The relative NUCB2 mRNA level summarized as a ratio (%) compared with the basal level (non-treatment). Data are presented as the mean ± SD (n = 3). *P < 0.05 and ***P < 0.001 versus non-treatment (left bar) (B) or 0 h (left plot) (C).

histological grade, mitotic score, PR status, *HER2* status, or Ki-67 LI. The positive association between *NUCB2* status and lymph node metastasis was significant regardless of the ER status of these cases (P = 0.02) (Table S2). *NUCB2* status was positively associated with Ki-67 LI in the ER-positive group (P = 0.02), and was positively correlated with tumor size in ER-negative cases (P = 0.03).

When immunohistochemistry was carried out in ductal carcinoma *in situ*, *NUCB2* immunoreactivity was detected in the carcinoma cells (Fig. 2F) in 7 (32%) of 22 cases. The *NUCB2* positivity was 1.5-fold higher in invasive carcinoma (48%) than non-invasive carcinoma (32%), although it did not reach a level of significance (P = 0.15).

Association between *NUCB2* status and clinical outcome. In order to thoroughly examine the association between *NUCB2* status and patient prognosis, we excluded stage IV cases and used stage I–III breast carcinoma patients (n = 141) in the following analyses. As shown in Figure 3(A), *NUCB2* status was significantly associated with an increased incidence of recurrence (P = 0.003), and the multivariate analysis revealed that lymph node metastasis (P = 0.01), ER status (P = 0.002), and *NUCB2* status (P = 0.001) were independent prognostic factors for disease-free survival with relative risks over 1.0 (Table 2).

A breast cancer-specific survival curve of the patients is summarized in Figure 3(B); a significant correlation (P = 0.0002) was detected between *NUCB2* status and adverse clinical outcome in the 141 breast carcinoma patients examined. In the univariate analysis (Table 2), lymph node metastasis (P = 0.0004), *NUCB2* status (P = 0.002), ER status (P = 0.003), histological grade (P = 0. 01), *HER2* status (P = 0.01), and tumor size (P = 0.02) were all indicated as significant prognostic variables for breast cancer-specific survival. A following multivariate analysis showed that only *NUCB2* status (P = 0.0004) and ER status (P = 0.01) were independent prognostic factors with a relative risk over 1.0, whereas lymph node metastasis

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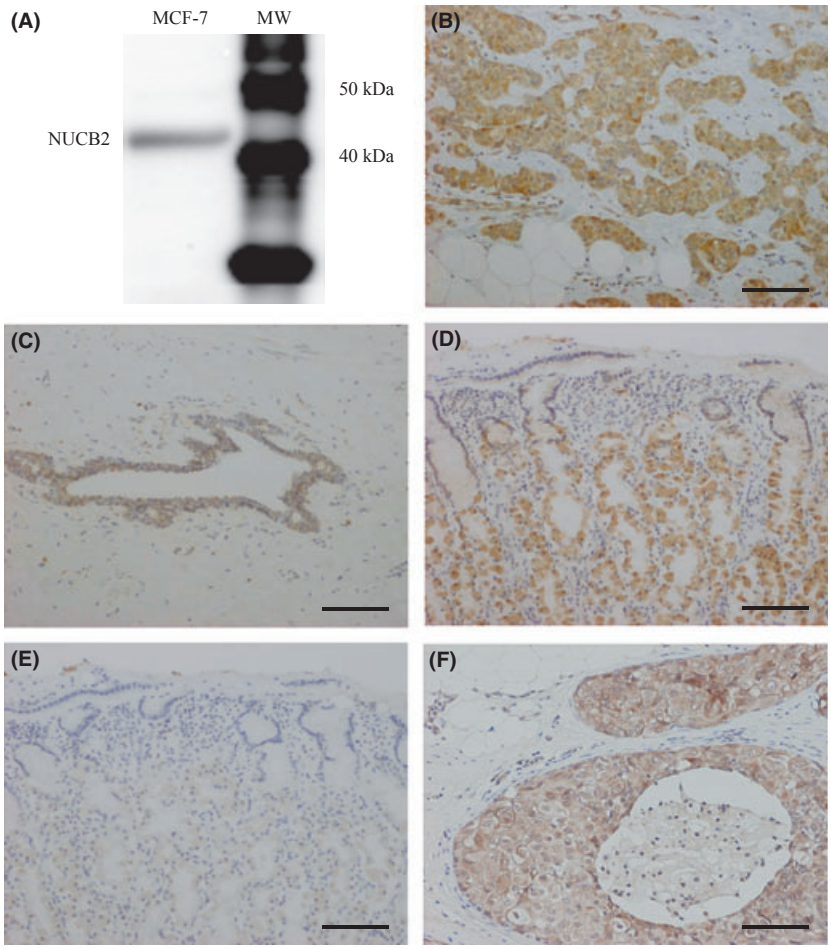

Fig. 2. Immunohistochemistry for nucleobindin 2 (*NUCB2*) in breast carcinoma. (A) Immunoblotting for *NUCB2* in MCF-7 cells. MW, molecular weight. (B) *NUCB2* immunoreactivity was detected in the carcinoma cells of invasive ductal carcinoma. (C) *NUCB2* immunoreactivity was weakly and focally detected in morphologically normal mammary glands. (D) Positive control section of *NUCB2* immunohistochemistry (gastric mucosa). (E) Negative control section of *NUCB2* immunohistochemistry (same area as Fig. 2D). (F) *NUCB2* immunoreactivity was detected in the carcinoma cells of ductal carcinoma *in situ*. Bar = 100 μm.

($P = 0.22$), histological grade ($P = 0.28$), *HER2* status ($P = 0.60$), and tumor size ($P = 0.07$) were not significant.

A similar association between *NUCB2* and worse prognosis was detected regardless of the Ki-67 status ($P = 0.03$ in cases with Ki-67 LI ≥ 10% and $P = 0.04$ in cases with Ki-67 < 10% for disease-free survival [Fig. 3C]; $P = 0.004$ in cases with Ki-67 LI ≥ 10% and *P*-value not available cases with Ki-67 < 10% because no patient died in the *NUCB2*-negative group for breast cancer-specific survival). When the 66 *NUCB2*-positive cases were further categorized into two groups according to immunointensity (++, strongly positive [$n = 16$]; +, modestly positive [$n = 50$]), no significant difference was detected between these two groups ($P = 0.60$ for disease-free survival [Fig. 3D], and $P = 0.49$ for breast cancer-specific survival).

Forty patients with stage I–III disease received tamoxifen therapy following surgery as an adjuvant treatment, and these cases were all positive for ER. *NUCB2* status was also markedly associated with an increased risk of recurrence (Fig. 3E) and worse prognosis (data not shown) in the patients who received tamoxifen therapy, although *P*-values were not available because no patient had recurrent disease or died in the group of *NUCB2*-negative cases. Significant association between *NUCB2* status and patients' clinical outcome was also detected in the 113 patients who received adjuvant chemotherapy ($P = 0.03$ for disease-free and $P = 0.002$ for breast cancer-specific survival), 38 ER-negative cases ($P = 0.0001$ for disease-free and $P < 0.0001$ for breast cancer-specific survival), or 24 cases with ER LI < 1% ($P = 0.001$ for disease-free [Fig. 3F] and $P = 0.0004$ for breast cancer-specific survival).

Effects of *NUCB2* expression on cell proliferation and invasion in breast carcinoma cells. The results of our study suggest that *NUCB2* is associated with worse prognosis of breast carcinoma patients regardless of their ER status, although *NUCB2* expression is upregulated by estrogen. In order to further examine the biological functions of *NUCB2* in human breast carcinoma, we transfected specific siRNA for *NUCB2* both in ER-positive MCF-7 and ER-negative SK-BR-3 breast carcinoma cells. The NUCB2 mRNA expression level was markedly decreased in these cells transfected with specific *NUCB2* siRNA (si1 or si2) at 3 days after transfection compared to cells transfected with control siRNA (siC). The ratio of NUCB2 mRNA level compared to that in the control siRNA was: MCF-7, 5% (si1) and 8% (si2); and SK-BR-3, 11% (si1) and 12% (si2).

As shown in Figure 4(A), the number of cells was significantly lower in MCF-7 cells transfected with *NUCB2* siRNA ($P < 0.001$ and 0.52-fold in si1, and $P < 0.001$ and 0.64-fold in si2) than in control cells transfected with siC 3 days after the transfection. A similar association was also detected in SK-BR-3 cells under the same conditions ($P < 0.001$ and 0.75-fold in si1, and $P < 0.001$ and 0.81-fold in si2). Figure 4(B) shows the

Table 1. Association between nucleobindin 2 (*NUCB2*) immunohistochemical status and clinicopathological parameters in 161 breast carcinomas

	NUCB2 status		*P*-value
	Positive (n = 77)	Negative (n = 84)	
Age† (years)	53.9 ± 1.4	54.4 ± 1.2	0.770
Menopausal status (%)			
Premenopausal	31 (19)	35 (22)	0.860
Postmenopausal	46 (29)	49 (30)	
Stage (%)			
I	18 (11)	24 (15)	0.730
II	38 (24)	43 (27)	
III	10 (6)	8 (5)	
IV	11 (7)	9 (6)	
Tumor size† (cm)	3.4 ± 0.4	3.4 ± 0.4	0.990
Lymph node metastasis (%)			
Positive	41 (25)	26 (16)	**0.004**
Negative	36 (22)	58 (36)	
Histological grade (%)			
1 (well)	20 (12)	24 (15)	0.930
2 (moderate)	30 (19)	31 (19)	
3 (poor)	27 (17)	29 (18)	
Mitotic score (%)			
1 (low)	33 (20)	43 (27)	0.570
2 (moderate)	22 (14)	21 (13)	
3 (high)	22 (14)	20 (12)	
ER status (%)			
Positive	65 (40)	53 (33)	**0.002**
Negative	12 (7)	31 (19)	
PR status (%)			
Positive	55 (34)	51 (32)	0.150
Negative	22 (14)	33 (20)	
HER2 status (%)			
Positive	22 (14)	24 (15)	0.990
Negative	55 (34)	60 (37)	
Ki-67 LI† (%)	23.6 ± 1.8	21.1 ± 2.1	0.380

†Data are presented as the mean ± SEM. All other values represent the number of cases and percentage. ER, estrogen receptor; LI, labeling index; PR, progesterone receptor. *P*-values <0.05 were considered significant, indicated in bold.

results of the migration assay. The number of migrated cells was significantly lower in both MCF-7 cells ($P < 0.001$ and 0.11-fold in si1, and $P < 0.001$ and 0.43-fold in si2) and SK-BR-3 cells ($P < 0.001$ and 0.36-fold in si1, and $P < 0.001$ and 0.31-fold in si2) transfected with *NUCB2* siRNA than in those transfected with control siRNA at 1 day (MCF-7) or 2 days (SK-BR-3) after the transfection. Moreover, the number of invaded cells was also significantly lower in the cells transfected with *NUCB2* siRNA (MCF-7, $P < 0.05$ and 0.21-fold in si1 and $P < 0.05$ and 0.29-fold in si2; SK-BR-3, $P < 0.01$ and 0.66-fold in si1 and $P < 0.001$ and 0.47-fold in si2) (Fig. 4C,D).

Discussion

Gene expression profiling is an important method to predict the likelihood of recurrence of disease in breast cancer patients,[19] in addition to conventional clinical and histopathological examination. A multigene classifier associated with recurrence has been proposed for breast carcinoma patients by several research groups,[19–21] and molecular-based diagnostic systems have been developed, such as MammaPrint[22] and Oncotype DX,[23] as well as the genomic grade index.[24] However, the selected genes vary markedly between these diagnostic systems, which may be partly due to the fact that they use different platforms for the analysis of gene expression. In addition, the biological functions have remained largely unknown in a great majority of these genes. In our present study, the results of microarray analysis revealed 17 genes that are potentially associated with recurrence in ER-positive breast carcinoma patients (group A in Fig. 1A). Among these, *IGFBP5* (insulin-like growth factor-binding protein 5) was reported to play an important role in breast carcinoma metastasis,[25,26] and is included in MammaPrint. In addition, *TGFA* (transforming growth factor α), a member of the epidermal growth factor family, is well-known to be involved in cellular proliferation and carcinogenesis.[27] The kinetochore-bound protein kinase *BUB1* (budding uninhibited by benzimidazoles 1) is a possible link to tumorigenesis.[28] *NUCB2* showed the highest expression ratio in this study, but this gene has not been listed in any multigene classifiers predicting breast carcinoma recurrence, nor has it been examined in breast carcinoma, to the best of our knowledge.

In this study, we first showed that *NUCB2* immunoreactivity was detected in 48% of breast carcinoma cases, although levels were almost negligible in morphologically normal mammary glands. *NUCB2* is known to mainly express in key hypothalamic nuclei with proven roles in energy homeostasis.[9] Moreover, recent investigations have indicated that *NUCB2* is also expressed in various human peripheral tissues, including the stomach, pancreas, reproductive organs, and adipose tissues, with relevant metabolic functions, suggesting that *NUCB2* signaling might participate in adaptative responses and in the control of body functions gated by the state of energy reserves.[29] However, *NUCB2* expression in carcinoma has only been examined in the stomach; Kalnina *et al.*[15] reported that *NUCB2* immunoreactivity was not detected in carcinoma cells in 15 gastric carcinoma cases examined. The relatively wide distribution of *NUCB2* immunoreactivity in our present study suggests that *NUCB2* plays an important role in human breast carcinoma.

Bourdeau *et al.*[14] evaluated genome-wide identification of EREs in humans, and identified a functional ERE element at 8257 bp from the most upstream mRNA 5′-end of the *NUCB2* gene. In our present study, *NUCB2* immunohistochemical status was positively associated with ER status in breast carcinoma tissue, and NUCB2 mRNA was significantly upregulated by estradiol in MCF-7 cells through ER. Therefore, *NUCB2* is considered one of the estrogen-induced genes in breast carcinoma cells. Results of our present study also indicated the presence of *NUCB2* in 12 of 43 (28%) ER-negative breast carcinoma cases; it might be the case that *NUCB2* was induced by a low or undetectable level of ER in these cases. However, it is also true that estrogen-mediated induction of NUCB2 mRNA was relatively slow in MCF-7 cells in our time-course study (Fig. 1C), suggesting that *NUCB2* expression is, at least in a part, induced by secondary responses, although the half-life of mRNA is an important factor in determining how long it takes to detect a change in the mRNA level of a specific gene.[4] In addition, *NUCB2* is expressed in various human tissues not necessarily considered targets for estrogens, as described above.[29] Therefore, other factors than estrogens might be involved in the expression of *NUCB2* in breast carcinoma cells. No information is currently available regarding the regulation mechanisms of *NUCB2* expression to the best of our knowledge, and further research is required.

Previous studies have shown that ICI 182780 possesses a greater ability to suppress estrogen-sensitive gene expression and greater antitumor activity than tamoxifen in breast carcinoma.[30] This is partly due to the fact that ICI 182780 does not have agonistic ER activity and reduces steady-state levels of ER by increasing the turnover of the protein, whereas tamoxifen does possess partial agonistic ER activity.[31] In our study, ICI 182780 was superior to tamoxifen in suppressing estradiol-mediated induction of NUCB2 mRNA in MCF-7 cells (Fig. 1B), which is consistent with previous studies.

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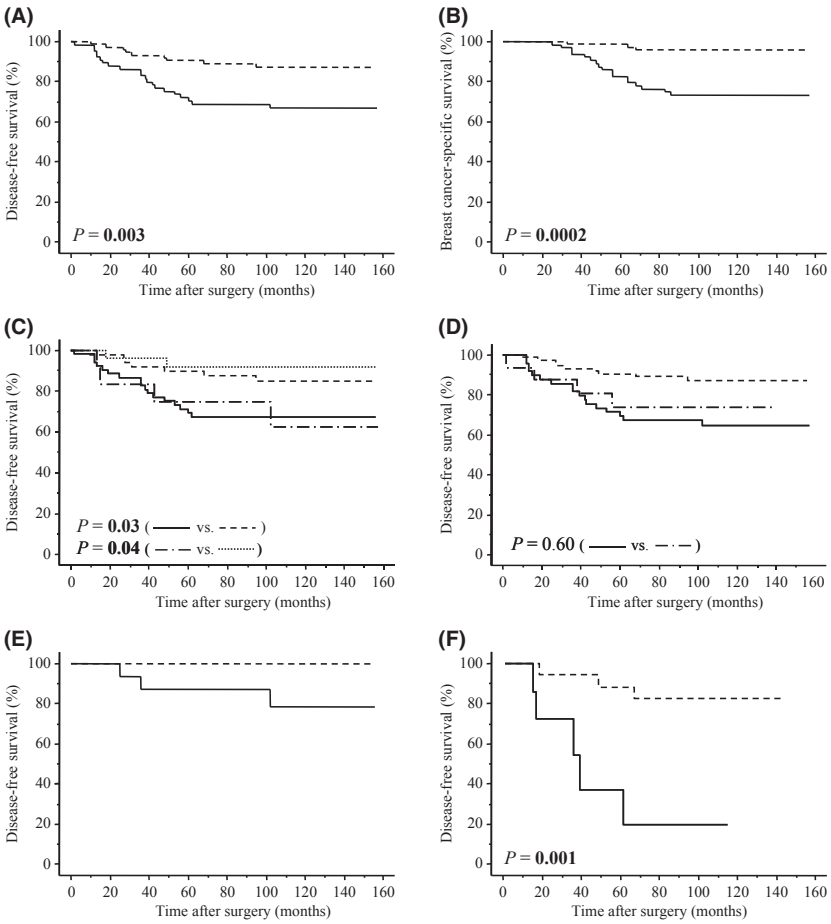

Fig. 3. Disease-free and breast cancer-specific survival of 141 breast carcinoma patients according to nucleobindin 2 (*NUCB2*) status. (A,B) *NUCB2* status was significantly associated with an increased risk of recurrence ($P = 0.003$) (A) and worse prognosis ($P = 0.0002$) (B). Solid line, positive for *NUCB2* ($n = 66$); dashed line, negative for *NUCB2* ($n = 75$). (C) Disease-free survival curve according to *NUCB2*/Ki-67 status. Solid line, positive for *NUCB2*/Ki-67 labeling index (LI) ≥ 10% ($n = 54$); dashed line, positive for *NUCB2*/Ki-67 LI < 10% ($n = 12$); dotted line, negative for *NUCB2*/Ki-67 LI ≥ 10% ($n = 50$); dot-dashed line, negative for *NUCB2*/Ki-67 LI < 10% ($n = 25$). (D) Disease-free survival curve according to *NUCB2* immunointensity. Solid line, strongly positive ($n = 16$); dashed line, modestly positive ($n = 50$); dot-dashed line, negative ($n = 75$). (E) *NUCB2* status was associated with recurrence in 40 patients who received tamoxifen therapy. *P*-value not available as there were no patients with recurrent disease in the *NUCB2*-negative group. Solid line, positive for *NUCB2* ($n = 16$); dashed line, negative for *NUCB2* ($n = 24$). (F) *NUCB2* status was significantly ($P = 0.001$) associated with recurrence in a group with estrogen receptor labeling index < 1% ($n = 24$). Solid line, positive for *NUCB2* ($n = 7$); dashed line, negative for *NUCB2* ($n = 17$).

In our present study, *NUCB2* immunoreactivity was positively associated with the presence of lymph node metastasis in breast carcinoma tissue both in ER-positive and ER-negative cases. In addition, subsequent *in vitro* studies indicated that both MCF-7 and SK-BR-3 cells transfected with siRNA *NUCB2* significantly decreased cell proliferation, and migration and invasion properties. Metastasis is considered as the major cause of treatment failure and death of carcinoma patients. It is a multistep process that involves migration and invasion of carcinoma cells, lymphogenous and/or hematogenous spread, and cell proliferation in the metastatic sites. Previous studies have shown that *NUCB2* has a wide variety of basic cellular functions, including an involvement in the energy homeostasis,[9] Ca^{2+} homeostasis,[8] and extracellular tumor necrosis factor receptor type 1 release,[10] although the biological functions have not yet been fully clarified. Results of our present study suggest that *NUCB2* plays a pivotal role, especially in the metastasis of breast carcinomas, and serve as a starting point for clarification of the biological roles of *NUCB2* in breast carcinoma. However, we could not necessarily detect a significant association between *NUCB2* status and mitotic score, Ki-67 LI, or invasion status in the clinical samples. Therefore, other factors might also play important roles in the processes of proliferation and invasion in breast carcinoma tissues.

In our present study, *NUCB2* status was also significantly associated with recurrence and worse prognosis in breast carcinoma patients, and a similar tendency was also detected in ER-positive patients who received tamoxifen therapy, or in ER-negative cases. In addition, results of multivariate analyses showed that *NUCB2* status was indeed an independent prognostic factor for both recurrence and breast cancer-specific survival. Results of our *in vitro* study indicated that tamoxifen inhibited estradiol-mediated induction of *NUCB2* expression in MCF-7 cells, but the basal expression level of NUCB2 mRNA still remained. Considering that the *NUCB2* expression level was the highest among the genes predominantly expressed in the recurrence group despite tamoxifen therapy (group A in Fig. 1A), *NUCB2* status in breast carcinoma tissues at the time of surgery might reflect the basal level of *NUCB2* as well as the level induced by estrogens in breast carcinoma cases. Therefore, residual carcinoma cells following surgical treatment in *NUCB2*-positive breast carcinomas could still have the potential to rapidly grow and/or metastasize,

Table 2. Univariate and multivariate analyses of disease-free survival and breast cancer-specific survival in patients with stage I–III breast cancer (n = 141)

Variable	Univariate	Multivariate	
	P-value	*P*-value	Relative risk (95% CI)
Disease-free survival			
Lymph node metastasis (positive/negative)	**<0.0001**	**0.0100**	3.1 (1.3–7.6)
ER status (negative/positive)	**0.0020**	**0.0020**	4.8 (1.8–13.0)
NUCB2 status (positive/negative)	**0.0100**	**0.0010**	4.6 (1.8–11.4)
HER2 status (positive/negative)	**0.0100**	0.6600	ND
Tumor size (≥2.0 cm/<2.0 cm)	**0.0200**	0.1300	ND
Histological grade (3/1, 2)	0.0900	ND	ND
Ki-67 LI (≥10%/<10%)	0.3500	ND	ND
Menopausal status (pre/post)	0.6400	ND	ND
Breast cancer-specific survival			
Lymph node metastasis (positive/negative)	**0.0004**	0.2200	ND
NUCB2 status (positive/negative)	**0.0020**	**0.0004**	12.0 (3.0–47.7)
ER status (negative/positive)	**0.0030**	**0.0100**	5.6 (1.5–20.7)
Histological grade (3/1,2)	**0.0100**	0.2800	ND
HER2 status (positive/negative)	**0.0100**	0.6000	ND
Tumor size (≥2.0 cm/<2.0 cm)	**0.0200**	0.0700	ND
Ki-67 LI (≥10%/<10%)	0.1000	ND	ND
Menopausal status (post/pre)	0.7800	ND	ND

Data considered significant (P < 0.05) in the univariate analyses are shown in bold, and were examined in the multivariate analyses.
CI, confidence interval; ER, estrogen receptor; LI, labeling index; ND, not done; *NUCB2*, nucleobindin 2; PR, progesterone receptor.

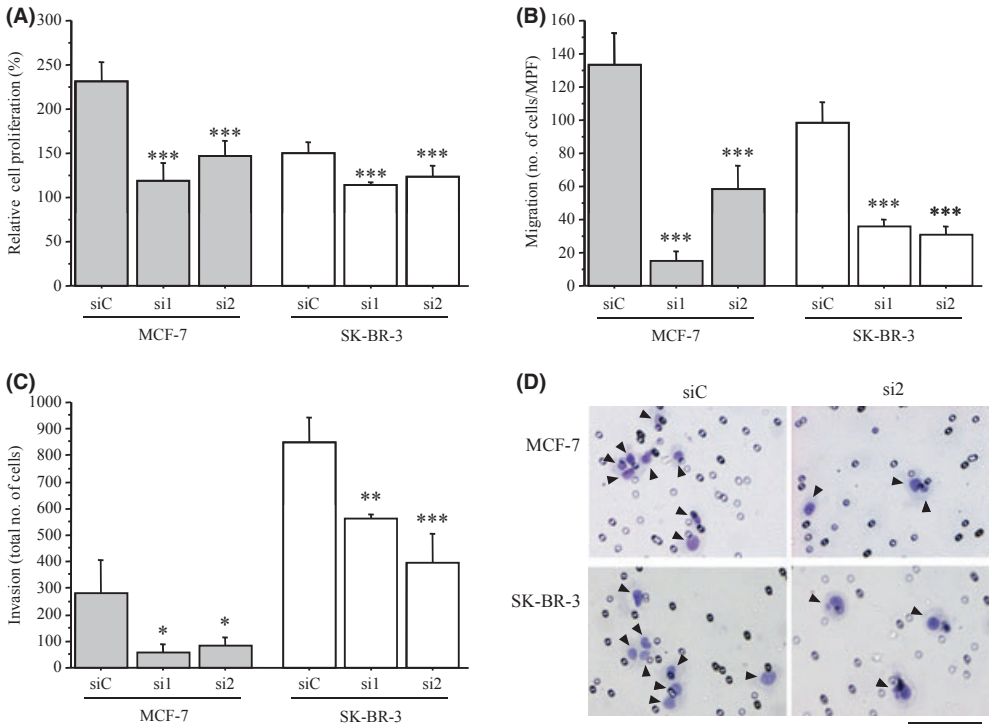

Fig. 4. Effects of nucleobindin 2 (*NUCB2*) on proliferation (A), and migration (B) and invasion (C,D) properties in breast carcinoma cells. (A–C) MCF-7 (gray bar) and SK-BR-3 (open bar) were transfected with *NUCB2*-specific siRNA (si1, si2) or control siRNA (siC). The relative cell proliferation was evaluated as a ratio (%) compared to that at 0 day after treatment (A). Migration ability was evaluated as an average number of cells in five middle power fields (MPF) (×200) on the lower surface of the membrane (B). Invasion ability was evaluated as the total number of cells (C). Data are presented as the mean ± SD (n = 6 [A]; n = 3 [B,C]). *P < 0.05; **P < 0.01; ***P < 0.001 versus control cells (left bar). (D) Representative microphotographs of results of invasion assay. Invaded carcinoma cells (arrows) were observed with 8 μm-sized pore. Nucleii stained with hematoxylin. When MCF-7 (upper panel) and SK-BR-3 (lower panel) cells were transfected with *NUCB2*-specific siRNA (si2) (right panel), the number of invading cells was decreased compared to the corresponding control (transfection with control siRNA [siC], left panel). Bar = 100 μm.

despite the fact that tamoxifen therapy partially suppresses the *NUCB2* expression level, thereby resulting in an increased recurrence and poor prognosis in these patients.

In summary, *NUCB2* was newly identified as a gene associated with recurrence in breast carcinoma patients by microarray analysis, and a subsequent *in vitro* study indicated that *NUCB2*

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expression was upregulated by estrogen in MCF-7 cells. *NUCB2* immunoreactivity was detected in 48% of breast carcinoma tissues, and was an independent prognostic factor of the patients. Results of further *in vitro* studies showed that *NUCB2* significantly increased the proliferation activity, and migration and invasion properties both in MCF-7 and SK-BR-3 cells. These findings suggest that *NUCB2* plays an important role, especially in the metastasis of breast carcinoma, and *NUCB2* status in primary breast carcinoma is reasonably considered a potent prognostic factor.

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

ER	estrogen receptor
ERE	estrogen-responsive element
LI	labeling index
NUCB2	nucleobindin 2
PR	progesterone receptor

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Genes predominantly expressed in the non-recurrence group, classified as group B.

Table S2. Association between nucleobindin 2 (*NUCB2*) status and clinicopathological parameters according to estrogen receptor status in 161 breast carcinomas.

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