	Recommendation	Comments
	hours. Samples should be sliced at 5-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of NBF to allow adequate tissue penetration. If tumor comes from remote location, it should be bisected through the tumor on removal and sent to the laboratory immersed in a sufficient volume of NBF. Cold ischemia time, fixative type, and time the sample was placed in NBF must be recorded.	
	As in the ASCO/CAP HER2 guideline, storage of slides for more than 6 weeks before analysis is not recommended.	
	Time tissue is removed from patient, time tissue is placed in fixative, duration of fixation, and fixative type must be recorded and noted on accession slip or in report.	
Optimal internal validation procedure	Validation of any test must be done before test is offered. See separate article on testing validation (Fitzgibbons et al[3]).	
	Validation must be done using a clinically validated ER or PgR test method.	
	Revalidation should be done whenever there is a significant change to the test system, such as a change in the primary antibody clone or introduction of new antigen retrieval or detection systems.	
Optimal internal QA procedures	Initial test validation. See separate article on testing validation (Fitzgibbons et al[3]).	
	Ongoing quality control and equipment maintenance.	
	Initial and ongoing laboratory personnel training and competency assessment.	
	Use of standardized operating procedures including routine use of external control materials with each batch of testing and routine evaluation of internal normal epithelial elements or the inclusion of normal breast sections on each tested slide, wherever possible.	
	Regular, ongoing assay reassessment should be done at least semiannually (as described in Fitzgibbons et al[3]). Revalidation is needed whenever there is a significant change to the test system.	
	Ongoing competency assessment and education of pathologists.	
Optimal external proficiency assessment	Mandatory participation in external proficiency testing program with at least two testing events (mailings) per year.	
	Satisfactory performance requires at least 90% correct responses on graded challenges for either test.	Unsatisfactory performance will require laboratory to respond according to accreditation agency program requirements.
Optimal laboratory accreditation	On-site inspection every other year with annual requirement for self-inspection.	Reviews laboratory validation, procedures, QA results and processes, and reports.
		Unsuccessful performance results in suspension of laboratory testing for ER or PgR.

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; IHC, immunohistochemistry; QA, quality assurance; NBF, neutral buffered formalin; ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; HER2, human epidermal growth factor receptor 2.

Table 2

ER Expression by Original LBA and Retrospective IHC Versus Benefit From Endocrine Therapy (Selected Trials)

Reference	No. of Patients (eligibility)	Intervention (outcome)	Original Assay (cutoff)	Retro-spective Assay (cutoff)	Assay Concordance	Outcome According to Biomarker	Comments
McCarty et al[4]	Pop A, n = 62 (early stage); Pop B, n = 72 (early stage); Pop C, n = 23 (MBC)	Endocrine Rx (Pop C)	LBA (≥ 20 fmol/mg)	H222 Sp γ Pop (score 75)	Pop A = specificity, 89% and sensitivity, 95%; Pop B = specificity, 94% and sensitivity, 88%	Objective clinical response: specificity, 89%; sensitivity, 93%	Among the original reports describing IHC correlation with LBA and with response to endocrine Rx
Barnes et al[5]	170 patients; 74% ER positive by LBA	First-line TAM in MBC (51% response rate)	LBA; 74% ER positive (≥ 20 fmol/mg); response rate, 58%	IHC with ER 1D5 antibody; 31% to 69% ER positive (various IHC scoring methods); response rate, 64% to 69%	137 (81%) of 170	Responses in 72% of ER/PgR positive and 61% of ER positive/PgR negative; IHC superior for predicting duration of response	All 8 IHC scoring methods useful
Harvey et al[6]	1,982 patients	26% received endocrine Rx and 13% received combined chemoendocrine Rx	LBA (positive if ≥ 3 fmol/mg)	IHC with 6F11 (Allred score > 2 or 1% to 10% weakly positive cells)	71% of all tumors were ER positive by IHC (86% concordance with LBA)	Multivariate analysis of patients tested by LBA showed ER status determined by IHC better than by LBA at predicting better DFS	This study was based on samples prepared in an unconventional manner (see text for details)
Elledge et al[7]	205 patients with blocks (original n = 349, all ER positive by LBA)	SWOG 8228, TAM 10 mg twice a day (n = 56) or 10 mg/m^2 twice a day (n = 149)	LBA (positive if ≥ 3 fmol/mg)	IHC with ER-6F11 antibody (Allred score)	185 (90%) of 205 were IHC positive	Overall response rate of 56% if LBA positive and 60% if IHC positive; significant correlation between IHC ER and response (ER negative, 25%; intermediate, 46%; and high, 66%) and time to Rx failure (ER negative, 5 months; intermediate, 4 months; and high, 10 months)	In low ER by LBA (< 50 fmol/mg), response rate of 25% if IHC negative and 63% if IHC high
Thomson et al[8]	332 patients (premenopausal patients with stage II disease); 81% had tumor assayed for ER by LBA	Adjuvant OA v CMF chemotherapy	LBA originally done in 270 patients or 81% (negative if < 20 fmol/mg with 2 categories, or negative if 0–4 fmol/mg with 4 categories)	IHC done in 236 patients (or 71%; quick score)	Spearman's rank correlation coefficient, 0.55	Significant interaction between IHC quick score and Rx with OA more beneficial for patients with positive quick score, whereas patients with quick score of 0 had significantly higher risk of death with OA	Original trial = better outcome with OA if ER > 20 fmol/mg v with CMF if ER < 20 fmol/mg
Regan et al[9]	571 patients [premenopausal (IBCSG trial VIII)] and 976 patients [postmenopausal with node-negative disease	IBCSG trial VIII (none, CMF, goserelin, or CMF → goserelin); IBCSG trial IX (TAM or CMF → TAM)	55% patients had LBA (positive if ≥ 20 fmol/mg) and 45% had ELISA	IHC with 1D5 antibody (present if > 0% stained cells and positive if ≥ 10% stained cells)	Concordance of 88% (κ = 0.66) in postmenopausal patients	HR similar for association between DFS and ER status (all patients) or PgR status (postmenopausal patients) as determined by the various methods	

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Reference	No. of Patients (eligibility)	Intervention (outcome)	Original Assay (cutoff)	Retro-spective Assay (cutoff)	Assay Concordance	Outcome According to Biomarker	Comments
	(IBCSG trials VIII and IX)]						

Abbreviations: ER, estrogen receptor; LBA, ligand-binding assay; IHC, immunohistochemistry; Pop, population; MBC, metastatic breast cancer; Rx, therapy; TAM, tamoxifen; PgR, progesterone receptor; DFS, disease-free survival; SWOG, Southwest Oncology Group; CMF, cyclophosphamide, methotrexate, and fluorouracil; OA, ovarian ablation; IBCSG, International Breast Cancer Study Group; ELISA, enzyme-linked immunosorbent assay.

Table 3

Well-Validated Assays for Evaluating Estrogen Receptor and Progesterone Receptor in Breast Cancer by Immunohistochemistry

Reference	Primary Antibody	Cut Point for "Positive"
Estrogen receptor		
Harvey et al, 1999[6]	6F11	Allred score ≥ 3 (1% to 10% weakly positive cells)
Regan et al, 2006[9]; Viale et al, 2007[13]; Viale et al, 2008[14]	1D5	1% to 9% (low) and ≥ 10% (high)
Cheang et al, 2006[15]	SP1	≥ 1%
Phillips et al, 2007[16]	ER.2.123 + 1D5 (cocktail)	Allred score ≥ 3 (1% to 10% weakly positive cells)
Dowsett et al, 2008[17]	6F11	H score > 1 (≥ 1%)
Progesterone receptor		
Mohsin et al, 2004[10]	1294	Allred score ≥ 3 (1% to 10% weakly positive cells)
Regan et al, 2006[9]; Viale et al, 2007[13]; Viale et al, 2008[14]	1A6	1% to 9% (low) and ≥ 10% (high)
Phillips et al, 2007[16]	1294	Allred score ≥ 3 (1% to 10% weakly positive cells)
Dowsett et al, 2008[17]	312	≥ 10%

Table 4

IHC ER/PgR Testing Interpretation Criteria

Review controls (external standard and internal normal breast epithelium if present). If not as expected, the test should be repeated and not interpreted.
Provide an interpretation of the assay as receptor positive, receptor negative, or receptor uninterpretable.
Positive interpretation requires at least 1% of tumor cells showing positive nuclear staining of any intensity.
Receptor negative is reported if < 1% of tumor cells show staining of any intensity.
Receptor uninterpretable is reported if the assay controls are not as expected or the preanalytic or analytic conditions do not conform to the guideline and there is no tumor cell staining in the absence of normally stained intrinsic epithelial elements.
Report the percentage of cells with nuclear staining using either estimation or quantitation. Quantitation may be done either by image analysis or manually.
Entire slide should be reviewed to assess the tumor-containing areas. Cytology samples with limited tumor cells and little tumor staining must have at least 100 cells counted.
Report an average intensity of tumor cell nuclei recorded as strong, moderate, or weak.
A score may be provided if the scoring system is specified.
Quantitative image analysis is encouraged for samples with low percentages of nuclear staining or in cases with multiple observers in the same institution. It is also a valuable way to quantify intensity and assure day-to-day consistency of control tissue reactivity.
If cytoplasmic staining occurs, repeat assay or perform on another sample.
Reject sample if normal ducts and lobules do not show obvious staining of some cells with variable intensity in the presence of totally negative tumor cells.
Reject sample if there are obscuring artifacts such as decalcification of sample or staining only of necrotic debris.
In samples with DCIS only, the type of DCIS should be mentioned and the DCIS may be scored for ER/PgR; in patients with invasive disease and DCIS, ER/PgR should be reported only for the invasive component. DCIS staining pattern may also be provided in a comment
The ER and PgR results should fit the clinical profile of the patient being evaluated: Consider the type of invasive cancer and the grade of the cancer in interpretation; some cancer types like lobular, mucinous, and tubular carcinoma are almost always strongly ER positive and only rarely ER negative.

Abbreviations: IHC, immunohistochemistry; ER, estrogen receptor; PgR, progesterone receptor; DCIS, ductal carcinoma in situ.

Table 5

Elements to Be Included in Accession Slip for ER and PgR Assays

Patient identification information
Physician identification
Date of procedure
Clinical indication for biopsy
Specimen site and type of specimen
Collection time
Time sample placed in fixative
Type of fixative
Fixation duration

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor.

Table 6

Reporting Elements for ER and PgR IHC Assays

Patient identification information*
Physician identification*
Date of service*
Specimen site and type*
Specimen identification (case and block number)*
Fixative
Cold ischemia time (time between removal and fixation)
Duration of fixation
Staining method used
Primary antibody and vendor
Assay details and other reagents/vendors
References supporting validation of assay (note: most commonly, these will be published studies performed by others that the testing laboratory is emulating)
Status of FDA approval
Controls (high protein expression, low-level protein expression, negative protein expression, internal elements or from normal breast tissue included with sample)
Adequacy of sample for evaluation
Results*
Percentage of invasive tumor cells exhibiting nuclear staining†
Intensity of staining: strong, medium, or weak
Interpretation:
Positive (for ER or PgR receptor protein expression), negative (for ER or PgR protein expression), or uninterpretable Internal and external controls (positive, negative, or not present)
Standard assay conditions met/not met (including cold ischemic time and fixation parameters)
Optional score and scoring system
Comment: Should explain reason for uninterpretable result and or any other unusual conditions, if applicable; may report on status any DCIS staining in the sample; should also provide correlation with histologic type of the tumor; may provide information about laboratory accreditation status

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; IHC, immunohistochemistry; FDA, US Food and Drug Administration.

*Report should contain these elements as a minimum. Other information must be available in the laboratory for review and/or appear on the patient accession slip.

†There is no recommendation in this guideline concerning whether specimens containing only ductal carcinoma in situ should be tested for ER/PgR.

Table 7

CAP Laboratory Accreditation Elements Requiring Documentation

Validation of test method before reporting patient results
Use and following of standard operating procedures with appropriate elements and sign-offs
Qualifications, responsibilities, and training of personnel involved in testing
Proper labeling of samples and reagents
Proper storage and handling of samples and reagents
Equipment calibration, maintenance, QC, and remedial action; proficiency testing performance and corrective actions when 100% not achieved
Internal QA plan for entire testing process, evidence that it is followed, and identified problems monitored and resolved effectively
Quality of tests for interpretation
Ongoing competency assessment of technologists and pathologists*
Report adequacy and quality, including required dates and times
Recordkeeping for entire test process and record retention
Accurate, timely submission of results

Abbreviations: CAP, College of American Pathologists; QC, quality control; QA, quality assurance.

*Competency assessment is monitored by periodic or continuous review of performance of those doing tests against peers. When failure is documented, remediation is undertaken.

UICC
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Nudix-type motif 2 in human breast carcinoma: a potent prognostic factor associated with cell proliferation

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Nudix-type motif 2 (NUDT2) hydrolyzes diadenosine 5′,5‴-p1,p4-tetraphosphate (Ap4A) associated with various cellular functions. Previous studies demonstrated its regulation through estrogens, suggesting possible importance of NUDT2 in breast carcinoma. NUDT2, however, has not been examined in malignant tissues. Therefore, we examined its expression and functions in breast carcinoma. Immunohistochemistry for NUDT2 was examined by invasive ductal carcinoma (IDC: $n = 145$) and pure ductal carcinoma *in situ* (DCIS: $n = 82$), and NUDT2 mRNA was examined by real-time PCR in 9 DCIS, 19 IDC and 6 non-neoplastic breast tissues. We also used T47D breast carcinoma cells in *in vitro* studies. NUDT2 immunoreactivity was detected in 78% of DCIS and 63% of IDC, and NUDT2 mRNA level was significantly higher in DCIS or IDC than non-neoplastic breast. NUDT2 status was significantly correlated with Van Nuys classification, HER2 or Ki-67 in DCIS, and with stage, lymph node metastasis, histological grade or HER2 in IDC. NUDT2 status was significantly associated with adverse clinical outcome of IDC patients and proved an independent prognostic factor. Results of transfection experiments demonstrated that proliferation activity of T47D cells was significantly associated with NUDT2 expression level according to the treatment of estradiol and/or tamoxifen. NUDT2 expression was significantly decreased by estradiol, and it was also significantly decreased in T47D cells transfected with HER2 siRNA. These findings suggest that NUDT2 is an estrogen-repressed gene and is also induced by HER2 pathways in breast carcinoma cells. NUDT2 promotes proliferation of breast carcinoma cells and is a potent prognostic factor in human breast carcinomas.

Key words: NUDT2, breast carcinoma, estrogen, Ap4A, estrogen-responsive gene

Abbreviations: Ap4A: diadenosine 5′,5‴-p1,p4-tetraphosphate; DCIS: ductal carcinoma *in situ*; ER: estrogen receptor; ERE: estrogen-responsive element; IDC: invasive ductal carcinoma; NUDT2: Nudix (nucleoside diphosphate linked moiety X)-type motif 2; PR: progesterone receptor

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Nudix (nucleoside diphosphate linked moiety X)-type motif 2 (NUDT2) is a member of a MutT family of nucleotide pyrophosphatases, a subset of the larger NUDIX hydrolase family.[1] NUDT2 hydrolyzes diadenosine 5′,5‴-p1,p4-tetraphosphate (Ap4A) to yield AMP and ATP and regulates an intracellular Ap4A level. Results of previous studies all demonstrated that Ap4A is associated with a wide variety of basic cellular functions, including protein synthesis associated with an initiation of DNA replication,[2] cell contact growth inhibition[3] and apoptosis.[4] Increased NUDT2 activity was reported in lung and breast carcinoma tissues[5] and an association of Ap4A with the cell proliferation of hepatocellular carcinoma cell line C3A was also reported.[6]

Recently, Bourdeau *et al.* performed genome-wide screen for high-affinity EREs and identified an estrogen-responsive element (ERE) in *NUDT2* gene.[7] In addition, Carroll *et al.* reported that NUDT2 expression was regulated by estrogens in MCF7 breast carcinoma cells in genome-wide analysis of estrogen receptor (ER)-binding sites.[8] These findings suggest that NUDT2 is one of estrogen-responsive genes and possibly plays an important role in estrogen-dependent breast carcinoma considering its involvement in various biological functions reported above. However, little information is available about the NUDT2 status in human malignant tissues, and biological or clinical significance of NUDT2 is therefore not

known in human breast carcinoma. Therefore, in this study, we examined NUDT2 in human breast carcinoma including ductal carcinoma *in situ* (DCIS) using immunohistochemistry and real-time PCR and performed subsequent *in vitro* studies based on the results of human breast carcinoma to further explore its mechanisms of actions.

Material and Methods

Patients and tissues

Two sets of tissue specimens were used in our study. As a first set, the specimens of invasive ductal carcinoma of human breast (IDC: $n = 145$) and pure ductal carcinoma *in situ* (DCIS: $n = 82$), which is noninvasive breast carcinoma and generally regarded as a precursor lesion of IDC, were obtained from Japanese female patients who underwent surgical treatment from 1984 to 1992 for IDC or 1990 to 2005 for DCIS in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. All the specimens had been fixed with 10% formalin and embedded in paraffin wax. The patients did not receive chemotherapy, irradiation or hormonal therapy before the surgery. Review of the charts of IDC patients revealed that 117 patients received adjuvant chemotherapy, whereas 54 patients received tamoxifen therapy and 11 patients received radiation therapy after the surgery. The clinical outcome of the IDC patients was evaluated by disease-free and breast cancer-specific survival in our study. Disease-free survival was defined as the time from surgery to the date of the first locoregional recurrence or first distant metastasis.[9] Breast cancer-specific survival was defined as the time from first diagnosis of primary breast cancer to death from the breast cancer. The mean follow-up time was 100 months (range 3–157 months) in the 145 IDC patients.

As a second set, snap-frozen specimens of pure DCIS ($n = 9$), IDC ($n = 19$) and non-neoplastic breast tissues ($n = 6$) were available for examining the mRNA expression of NUDT2 in our study. These specimens were obtained from Japanese female patients who underwent surgical treatment from 2001 to 2004 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan (45–72 years for DCIS and 42–86 years for IDC) and stored at −80°C for RNA isolation. The non-neoplastic breast tissues were obtained from 6 of the 19 IDC patients who underwent total mastectomy (51–72 years), and these were distant breast tissues from the IDC associated with no significant pathological abnormalities. These 28 patients did not receive any neoadjuvant therapy. Informed consent was obtained from all the patients before their surgery and examination of specimens used in our study.

Research protocols for our study were approved by the Ethics Committee at Tohoku University School of Medicine and Tohoku Kosai Hospital.

Immunohistochemistry

Mouse monoclonal antibody for NUDT2 (4A4-3C3) was purchased from Abnova (Heidelberg, Germany). Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR) (MAB429) and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA) and DAKO (Carpinteria, CA), respectively. Rabbit polyclonal antibody for HER2 (A0485) was obtained from DAKO.

A Histofine Kit (Nichirei, Tokyo, Japan) that uses the streptavidin-biotin amplification method was used in our study. Human tissue of the stomach was used as a positive control for NUDT2 antibody (http://www.proteinatlas.org/normal_unit.php?antibody_id=4684&mainannotation_id=694370), and normal mouse or rabbit IgG was used instead of the primary antibody as a negative control for immunohistochemistry.

NUDT2 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 NUDT2 status. Immunoreactivity for ER, PR and Ki-67 was detected in nuclei of carcinoma cells. ER, PR and Ki-67 immunoreactivity was evaluated in more than 1,000 carcinoma cells for each case, and subsequently, the percentage of immunoreactivity, *i.e.*, labeling index (LI), was determined.[10] Cases with ER LI or PR LI of more than 10% were considered ER- or PR-positive breast carcinoma according to a report by Allred *et al.*[11] HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and moderately or strongly circumscribed membrane staining of HER2 in more than 10% carcinoma cells was considered positive.

An association between NUDT2 status and clinicopathological factors of the patients was evaluated using a one-way ANOVA and a Bonferroni test or a cross table using the chi-square test. Disease-free survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Breast cancer-specific survival was also estimated by the Kaplan-Meier method, using death with evidence of the breast carcinoma as the endpoint. Univariate and multivariate analyses were evaluated by a proportional hazard model (COX). p values of less than 0.05 were considered significant in our study. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC).

Real-time PCR

Total RNA was extracted from breast carcinoma tissues or cultured cells using TRIzol reagent (Invitrogen Life Technologies, Gaitherburg, ND), and a reverse transcription kit (Superscript II Preamplification system) (Gibco-BRL, Grand Island, NY) was used in the synthesis of cDNA.

The LightCycler System (Roche Diagnositics GmbH, Mannheim, Germany) was used to semiquantify the mRNA expression levels by real-time PCR.[12] The primer sequences used in our study are as follows: NUDT2 (Genbank accession number: NM_001161): FWD: 5′-GGCATTCATCACTGGACTC-3′ (cDNA position 458–475) and REV: 5′-CCTCAATAATGGTCAGCTGG-3′ (cDNA position 543–562), HER2 (M11730): FWD: 5′-CTGCCTCCACTTCAACCACA-3′ (cDNA position 912–931) and REV: 5′-TCCCACGTCCGTA

GAAAGGT 3′ (cDNA position 1,039–1,058) and ribosomal protein L 13a (RPL13A: NM_012423): FWD: 5′-CCTGGAG GAGAAGAGGAAAGAGA-3′ (cDNA position 487–509) and REV: 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′ (cDNA position 588–612), and 40 amplification cycles were performed in our study. PCR products were subsequently purified and subjected to direct sequencing to verify amplification of the correct sequences. Negative control experiments lacked cDNA substrate to check the possibility of exogenous contaminant DNA.

To determine the quantity of target cDNA transcript, cDNAs of known concentrations for target genes, and the housekeeping gene RPL13A were used to generate standard curves for real-time quantitative PCR. The Ct (cycle threshold) values were used to calculate the gene-specific input mRNA amount according to the calibration curve method. The mRNA level in each case was represented as a ratio of RLP13A and was evaluated as a ratio (%) compared with that of each control.

Cell line and chemicals

T47D and MCF7 human breast carcinoma cell lines, which express ER, were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan), respectively, and cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). Estradiol and tamoxifen were obtained from Sigma-Aldrich, and an ERα agonist propyl-pyrazole-triol (PPT),[13] ERβ agonist diarylpropionitrile (DPN),[13] and pure ER antagonist ICI 182,780[14] were all purchased from Tocris Cookson (Ellisville, MO).

Immunoblotting

The cell protein (whole cell extracts) was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). After SDS-PAGE (10% acrylamide gel), proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, England) and were then incubated with a primary antibody for NUDT2 (4A4-3C3, Abnova), HER2 (A0485, DAKO) or β-actin (AC-15, Sigma-Aldrich).

Immunointensity of specific bands was measured by LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan), and relative immunointensity of NUDT2 was evaluated as a ratio of β-actin in each sample examined in our study.

Transient transfection

The cDNA for human NUDT2 (NM_001161) was cloned through reverse transcription-PCR (RT-PCR) from MCF7 cells. A DNA fragment, which included the open-reading frame of NUDT2 and contained *Nhe* I and *EcoR* I restriction sites, was amplified using a primer pair of 5′-GGGCTAG CATGGCCTTGAGAGCATGTGG-3′ and 5′-GGGAATTCAG GCCTCTATGGAGCAAAG-3′ and inserted into pcDNA 3.1(−) vector (Invitrogen). The sequence and orientation of the constructs were confirmed by sequencing. Subsequently, the pcDNA3.1/NUDT2 vector was transiently transfected into T47D or MCF7 cells using Lipofectamine LTX (Invitrogen), according to the manufacturer's instruction. As a control, empty vector pcDNA3.1 was also transfected under the same condition in our study.

Small interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotides for NUDT2 and HER2 were generated by Silencer Predesigned siRNAs [Qiagen and Invitrogen (Tokyo, Japan), respectively] in our study. The target sequences of siRNA against NUDT2 were Hs_NUDT2_5 (si5): 5′-CAGATTTGTGAAATCGGCTCA-3′ and Hs_NUDT2_6 (si6): 5′-CCCAAAGTGGACAACAATG CA-3′, whereas those against HER2 were ERBB2-HSS103333 (si3): 5′-AAACGTGTCTGTGTTGTAGGTGACC-3′ and ERB B2-HSS103334 (si4): 5′-GAGATGACAGGTTACCTATACATC T-3′. Silencer Negative Control #1 (SNC) siRNA (Ambion, Austin, TX) was also used as a negative control in our study.

The siRNA (5 nM) was transfected using HiperFect transfection reagent (Qiagen, GmbH, Hilden, Germany) by reverse transfection method according to the manufacturer's protocol.

Cell proliferation assay and migration assay

One day after the transfection with NUDT2 siRNA in T47D cells, medium was changed to phenol red-free RPMI 1640 medium containing 10% dextran-coated charcoal (DCC)-FBS with or without estradiol (10 nM). Three days after the transfection, the status of cell proliferation of cells was measured by a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) method using Cell Counting Kit-8 (Dosin Kagaku, Kumamoto, Japan).[15]

Effect of Ap4A on the cell proliferation in T47D cells was examined according to a previous report.[16] Briefly, T47D cells were incubated with permeabilizing buffer [0.01 M EDTA, 30 mM 2-mercaptoethanol, 4 mM $MgCl_2$ and 0.01 M Tris-HCl (pH 7.8)] added an indicated concentration of Ap4A for 15 min at 4°C. The medium was subsequently changed to RPMI-1640 with 10% FBS, and cell proliferation status was measured by Cell Counting Kit-8 (Dosin Kagaku) at 3 days after the permeabilization.

Cell migration assay was performed using a 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and Chemotaxicell (8-μm pore size) (Kurabo, Osaka, Japan) according to a previous report.[16] After incubation for 72 hr at 37°C, cells on the upper surface of membrane were removed by wiping with a cotton swab, and the migration ability was evaluated as a total number of cells on the lower surface of membrane, which was counted under light microscopy.

Cell cycle and apoptosis analyses

Cell cycle fractions were determined in T47D cells at 3 days after transfection with NUDT2 siRNA using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were obtained and

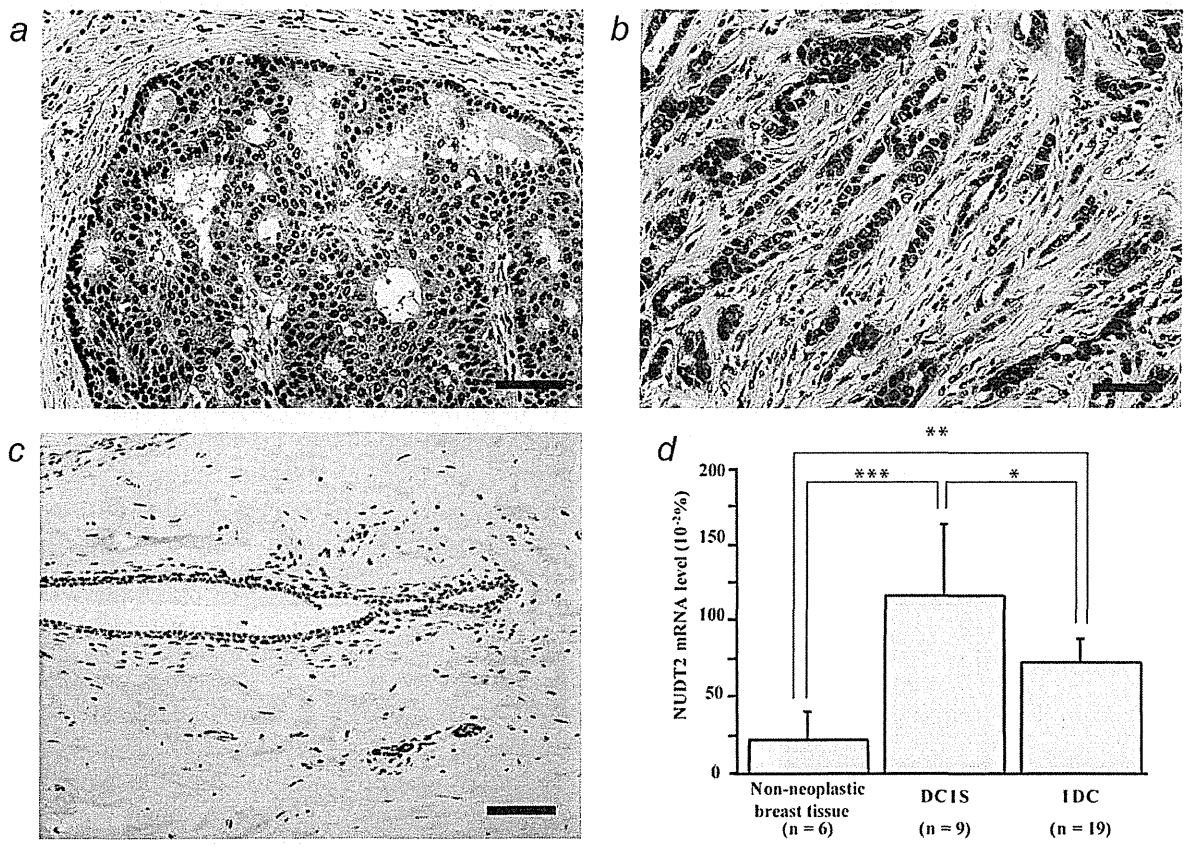


Figure 1. Expression of NUDT2 in the breast carcinoma. (*a–c*) NUDT2 immunoreactivity was detected in the cytoplasm of carcinoma cells in DCIS (*a*) and IDC (*b*). NUDT2 immunoreactivity was not detected in morphologically normal mammary glands (*c*). Bar = 50 μm, respectively. (*d*) Expression levels of NUDT2 mRNA in non-neoplastic breast, DCIS and IDC tissues by real-time PCR analysis. NUDT2 mRNA level was summarized as the ratio of RPL13A mRNA level (%), and data are presented as mean ± SD. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

processed using the Lysis II software (Becton Dickinson), and percentage of each cell cycle phase was evaluated on a DNA linear plot using the CellFit software (Becton Dickinson).

The apoptotic status of T47D cells was evaluated by Caspase-Glo3/7 Assay (Promega, Madison WI) in a 96-well plate at 3 days after the transfection with siRNA. Luminescent signal value that reflects caspase-3 activity was subsequently measured by Centro LB960 (Berthold Technologies, Bad Wildbad, Germany).[17] Fluorescence ($560_{Ex}/590_{Em}$) for cellular viability was obtained with Fluoroscan Ascent FL (Thermo scientific, Waltham, MA). Apoptosis index was calculated according to the following equation: (Luminescent signal value/cellular viability fluorescence ($560_{Ex}/590_{Em}$) value) and subsequently evaluated as a ratio (%) compared with that at 0 day after the transfection.

Luciferase assay

To measure the transcriptional activity of ER, T47D cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days after the transfection, and subsequently, 25 ng ptk-ERE-Luc plasmids and 25 ng pRL-TK control plasmids (Promega) were transiently transfected using TransIT-LT Transfection Reagents (Takara Bio, Shiga, Japan). The luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200) (ATTO) at 1 day after the treatment with estradiol (10 nM). The transfection efficiency was normalized against Renilla luciferase activity using pRL-TK control plasmids, and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.[15]

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA) in our study.[18] Briefly, T47D cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 2 days and subsequently cultured with phenol red-free RPMI 1640 medium without FBS for 1 day.

Table 1. Association between NUDT2 status and clinicopathological parameters in 82 DCIS cases

	NUDT2 status		
	+(n = 64)	−(n = 18)	p value
Patient age[1] (years)	56.2 ± 1.4	59.3 ± 2.9	0.33
Menopausal status			
Premenopausal	20 (24%)	7 (9%)	
Postmenopausal	44 (54%)	11 (13%)	0.54
Van Nuys classification			
1	12 (15%)	11 (13%)	
2	36 (44%)	7 (8%)	
3	16 (20%)	0 (0%)	**0.001**
ER			
Positive	59 (72%)	15 (18%)	
Negative	5 (6%)	3 (4%)	0.36
PR			
Positive	53 (65%)	15 (18%)	
Negative	11 (13%)	3 (4%)	0.99
HER2			
Positive	34 (41%)	4 (5%)	
Negative	30 (37%)	14 (17%)	**0.02**
Ki-67 LI[1] (%)	17.5 ± 1.3	11.7 ± 1.9	**0.03**

p values less than 0.05 were considered significant and described as boldface.
[1]Data are presented as mean ± SEM. All other values represent the number of cases and percentage.

Table 2. Association between NUDT2 status and clinicopathological parameters in 145 IDC cases

	NUDT2 status		
	+(n = 91)	−(n = 54)	p value
Patient age[1] (years)	54.2 ± 1.2	53.5 ± 1.7	0.72
Menopausal status			
Premenopausal	33 (22%)	27 (19%)	
Postmenopausal	58 (40%)	27 (19%)	0.1
Stage			
I	23 (16%)	16 (11%)	
II	42 (29%)	34 (23%)	
III	26 (18%)	4 (3%)	**0.01**
Tumor size[1] (cm)	3.6 ± 0.3	3.2 ± 0.5	0.47
Lymph node metastasis			
Positive	45 (31%)	16 (11%)	
Negative	46 (32%)	38 (26%)	**0.02**
Histological grade			
1	20 (14%)	26 (18%)	
2	41 (39%)	16 (11%)	
3	30 (21%)	12 (8%)	**0.01**
ER			
Positive	71 (49%)	41 (28%)	
Negative	20 (14%)	13 (9%)	0.77
PR			
Positive	63 (44%)	39 (27%)	
Negative	28 (19%)	15 (10%)	0.7
HER2			
Positive	31 (22%)	9 (6%)	
Negative	60 (41%)	45 (31%)	**0.02**

p values less than 0.05 were considered significant and described as boldface.
[1]Data are presented as mean ± SEM. All other values represent the number of cases and percentage.

After the treatment with estradiol (10 nM) for an indicated period or ethanol as a negative control, cells were crosslinked with 1% formaldehyde for 15 min at 37°C. Protein–DNA complex was precleaned for 1 hr with Protein G sepharose, and supernatant was incubated with or without anti-ERα antibody (6F11, Novocastra, Newcastle upon Tyne, UK). The immunoprecipitated complex obtained was then bound to Protein G sepharose for 1 hr at 4°C, and protein–DNA complex was subsequently eluted from the immunoprecipitated complex through elution buffer (1% SDS, 0.1 M $NaHCO_3$ and 10 mM DTT). Reverse protein–DNA crosslink procedure was performed by heating at 65°C for 4 hr, and DNA was purified using PCR purification kit (Qiagen). The sets of PCR primers for NUDT2 used in our study were FWD: 5′-GC TTGCTTGCAGCTAGAATATC-3′ and REV: 5′-CTCAAG CACAAAATGACATCTC-3′. PCR-amplified products were subsequently analyzed on ethidium bromide-stained 2% agarose gels.

Results

NUDT2 in human breast carcinoma tissues

NUDT2 immunoreactivity was detected in the cytoplasm of carcinoma cells in both DCIS (Fig. 1*a*) and IDC (Fig. 1*b*). The number of cases positive for NUDT2 status was 64 of 82 (78%) in DCIS and 91 of 145 (63%) in IDC. NUDT2 immunoreactivity was not detected in epithelial cells of morphologically normal glands or stromal cells present in the specimens (Fig. 1*c*).

Real-time PCR studies demonstrated that NUDT2 mRNA level was significantly higher in DCIS ($p < 0.001$ and 5.4-fold) or IDC ($p < 0.01$ and 3.4-fold) than non-neoplastic breast tissue. The NUDT2 mRNA level was also significantly higher in DCIS than IDC ($p < 0.05$ and 1.6-fold).

Association between NUDT2 status and clinicopathological factors in human breast carcinoma

Associations between NUDT2 status and various clinicopathological parameters in DCIS and IDC patients are summarized in Tables 1 and 2. In DCIS, NUDT2 status was positively associated with the Van Nuys classification[19] ($p = 0.001$), HER2 status ($p = 0.02$) and Ki-67 LI ($p = 0.03$) (Table 1). There were no significant correlations between NUDT2 status and

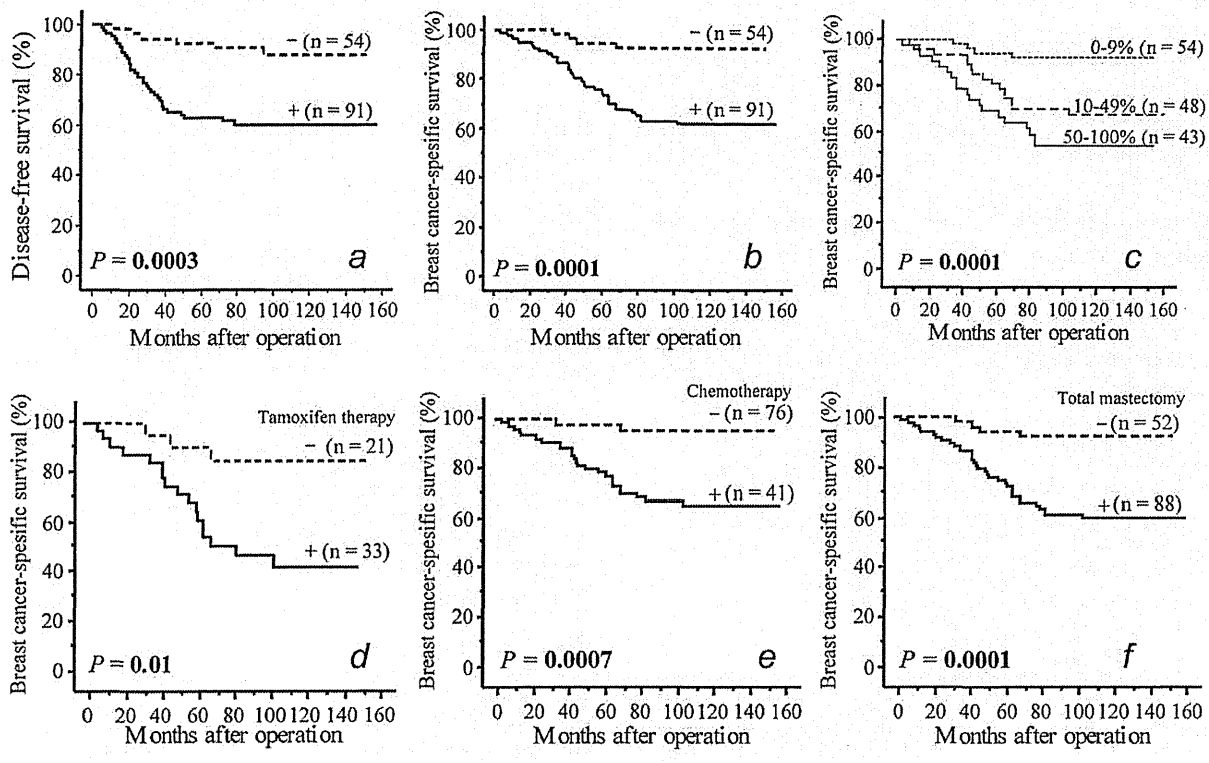


Figure 2. Disease-free and breast cancer-specific survival of 145 IDC patients according to NUDT2 status studied by Kaplan-Meier method. (*a*, *b*) NUDT2 status was significantly associated with an increased risk of recurrence ($p = 0.0003$) (*a*) and worse prognosis ($p = 0.0001$) (*b*). (*c*) Association between NUDT2 status and worse prognosis was also detected in increased rankings of positivity for NUDT2 immunoreactivity in three groups (0–9%, 10–49% and 50–100% positive cells; $p = 0.0001$). (*d–f*) NUDT2 status was significantly associated with worse prognosis in a group who received tamoxifen therapy ($n = 54$; $p = 0.01$) (*d*) or adjuvant chemotherapy ($n = 117$; $p = 0.0007$) (*e*) and who underwent total mastectomy as the surgical treatment ($n = 140$; $p = 0.0001$) (*f*). Statistical analysis was performed by the log-rank test, respectively.

other clinicopathological parameters examined in our study including patient age, menopausal status, ER and PR. On the other hand, NUDT2 status was significantly associated with clinical stage ($p = 0.01$), lymph node metastasis ($p = 0.02$), histological grade[20] ($p = 0.01$) and HER2 status ($p = 0.02$) in IDC cases, but not with patient age, menopausal status, tumor size, ER, PR and Ki-67 LI (Table 2). Positivity of HER2 status in our study (46% in DCIS and 28% in IDC) was consistent with that of a previous report.[21]

Correlation between NUDT2 status and clinical outcome of IDC patients

As shown in Figure 2*a*, NUDT2 status was significantly associated with an increased risk of recurrence ($p = 0.0003$ in the log-rank test) in 145 IDC patients examined, and results of the following multivariate analysis revealed that lymph node metastasis ($p = 0.001$), NUDT2 status ($p = 0.01$) and PR ($p = 0.01$) were independent prognostic factors for disease-free survival with relative risks over 1.0 (Table 3) in this cohort of IDC patients. Breast cancer-specific survival curve of the patients is summarized in Figure 2*b*. A significant correlation was detected between NUDT2 status and adverse clinical outcome of the 145 IDC patients ($p = 0.0001$ in the log-rank test), and the multivariate analysis revealed that lymph node metastasis ($p = 0.01$), NUDT2 status ($p = 0.004$) and histological grade ($p = 0.01$) remained independent prognostic factors for breast cancer-specific survival with a relative risk over 1.0 (Table 4).

Similar tendency was detected when the NUDT2 immunoreactivity was further categorized into three groups (0–9%, 10–49% and 50–100% positive cells) ($p = 0.0001$ for disease-free and breast cancer-specific survival (Fig. 2*c*), respectively). Fifty-four patients received tamoxifen therapy after surgery among 112 ER-positive IDC cases examined, and NUDT2 status was significantly associated with an increased risk of recurrence ($p = 0.01$) and adverse clinical outcome ($p = 0.01$) (Fig. 2*d*) in the group who received tamoxifen therapy as an adjuvant treatment. Significant association between NUDT2 status and clinical outcome of the patients was also detected in the 117 IDC patients who received adjuvant

Table 3. Univariate and multivariate analyses of disease-free survival in 145 IDC patients examined

Variable	Univariate *p*	Multivariate *p*	Multivariate Relative risk (95% CI)
Lymph node metastasis (+/−)	**<0.0001**	**0.001**	3.8 (1.8–7.9)
NUDT2 status (+/−)	**0.001**	**0.01**	3.1 (1.2–7.6)
PR (negative/positive)	**0.004**	**0.01**	2.4 (1.2–4.7)
Histological grade (3/1, 2)	**0.01**	0.37	
HER2 (+/−)	**0.01**	0.84	
Tumor size[1] (≥2.0 cm/<2.0 cm)	**0.01**	0.35	
Tamoxifen therapy (yes/no)	0.054		
ER (−/+)	0.07		
Patient age[1] (22–81 years)	0.13		
Types of surgery (others/total mastectomy)	0.60		
Menopausal status (premenopausal/ postmenopausal)	0.83		
Adjuvant chemotherapy (no/yes)	0.90		
Ki-67 LI[1] (82–2%)	0.94		

Data considered significant ($p < 0.05$) in the univariate analyses were described as boldface and were examined in the multivariate analysis. [1]Data were evaluated as continuous variables. All other data were evaluated as dichotomized variables.

Table 4. Univariate and multivariate analyses of breast cancer-specific survival in 145 IDC patients examined

Variable	Univariate *p*	Multivariate *p*	Multivariate Relative risk (95% CI)
Lymph node metastasis (+/−)	**<0.0001**	**0.01**	3.0 (1.3–6.9)
NUDT2 status (+/−)	**0.001**	**0.004**	5.0 (1.6–14.7)
Histological grade (3/1, 2)	**0.001**	**0.01**	2.5 (1.2–5.2)
Tumor size (≥2.0 cm/<2.0 cm)	**0.01**	0.54	
PR (−/+)	**0.03**	0.09	
HER2 (+/−)	**0.03**	0.26	
Tamoxifen therapy (yes/no)	**0.03**	0.50	
Adjuvant chemotherapy (no/yes)	0.09		
Ki-67 LI[1] (82–2%)	0.18		
ER (−/+)	0.22		
Menopausal status (postmenopausal/ premenopausal)	0.25		
Patient age[1] (22–81 years)	0.77		
Types of surgery (others/total mastectomy)	0.8		

Data considered significant ($p < 0.05$) in the univariate analyses were described as boldface and were examined in the multivariate analysis. [1]Data were evaluated as continuous variables. All other data were evaluated as dichotomized variables.

chemotherapy [$p = 0.004$ for disease-free and $p = 0.0007$ for breast cancer-specific survival (Fig. 2*e*)] or 140 patients who underwent total mastectomy [$p = 0.0002$ for disease-free and $p = 0.0001$ for breast cancer-specific survival (Fig. 2*f*)].

Effects of NUDT2 expression on cell proliferation in T47D breast carcinoma cells

To examine biological functions of NUDT2 in human breast carcinoma cells, we performed transient transfection with NUDT2 expression vector in T47D and MCF7 cells. NUDT2 protein level was increased according to the amount of NUDT2 expression vector transfected, and that in T47D and MCF7 cells transfected with 1 μg of NUDT2 expression vector became 3.0- and 5.5-fold, respectively, compared to the cells transfected with empty vector (1 μg) (Fig. 3*a*). Under this condition, we subsequently examined effects of overexpressed NUDT2 on cell proliferation of T47D and MCF7 cells. As shown in Figure 3*b*, the number of cells was significantly increased both in T47D and MCF7 cells transfected with NUDT2 expression vector than to those transfected with empty vector ($p < 0.05$ and 1.07-fold in T47D and $p < 0.05$ and 1.18-fold in MCF7) under the treatment with 10 nM estradiol for 2 days. Similar tendency was detected between in T47D cells transfected with NUDT2 and empty vector when these cells were treated with 5 μM tamoxifen with or without 10 nM estradiol for 2 days ($p < 0.05$, respectively) (Fig. 3*c*).

We next transfected with specific siRNA for NUDT2 in T47D cells. As demonstrated in Figure 4*a*, NUDT2 protein level was markedly decreased in T47D cells transfected with NUDT2 (si5 or si6) siRNA from 2 to 6 days after the transfection compared to that in T47D cells transfected with control siRNA(SNC). As shown in Figure 4*b*, the number of cells was significantly lower in T47D cells transfected with NUDT2 siRNA ($p < 0.001$ and 0.77-fold in si5 and $p < 0.001$ and 0.74-fold in si6) than the control cells transfected with SNC siRNA at 3 days after the transfection. Similar tendency was also detected under the treatment with 10 nM estradiol for 2 days ($p < 0.01$ and 0.89-fold in si5 and $p < 0.001$ and 0.77-fold in si6). On the other hand, treatment of Ap4A significantly inhibited the proliferation of T47D cells in a dose-dependent manner, and the cell proliferation of T47D cells treated with 100 μM Ap4A was decreased into 78% of the basal level (non-treatment with Ap4A; $p < 0.001$; data not shown). Subsequent flow cytometry analysis demonstrated that G0/G1 fraction was increased in T47D cells transfected with NUDT2 siRNA (61% in SNC, 67% in si5 and 73% in si6) (Fig. 4*c*).

No significant association was detected among these three T47D cells transfected in a migration assay ($p = 0.84$), apoptosis index ($p = 0.11$) and transcriptional activity mediated through ERE by luciferase reporter gene assays ($p = 0.41$) in our study.

Regulation of NUDT2 expression by estradiol and HER2 in T47D cells

NUDT2 has been suggested a possible estrogen-responsive gene in breast carcinoma cells,[8] but detailed regulatory mechanisms of NUDT2 expression still remain unclear. Therefore, we then examined effects of estradiol on NUDT2 expression in T47D cells. As shown in Figure 5*a*, the level of NUDT2 mRNA expression was dose dependently decreased by estradiol treatment for 3 days, and it became significant from 1 nM compared to the basal level (nontreatment) ($p < 0.05$ and 0.56-fold). Similar significance ($p < 0.01$) was detected when T47D cells were treated with 10 nM estradiol for 1 day under RPMI-1640 without FBS (data not shown). On the other hand, NUDT2 mRNA level did not significantly change when the T47D cells were treated with 5 μM tamoxifen with or without 10 nM estradiol (Fig. 5*a*). The significant suppression of NUDT2 mRNA expression was also detected by the treatment with ERα selective agonist PPT ($p < 0.001$ and 0.5-fold), but not by ERβ selective agonist DPN ($p = 0.81$ and 0.97-fold) (Fig. 5*b*). Estradiol did not significantly change the NUDT2 mRNA expression levels when the T47D cells were treated together with estradiol and a potent ER antagonist ICI 182,780 ($p = 0.23$ and 1.3-fold). Results of immunoblotting analysis also demonstrated that NUDT2 protein level was significantly ($p = 0.02$ and 0.73-fold) decreased by the treatment of 10 nM estradiol for 3 days in T47D cells (Fig. 5*c*). When we performed ChIP assay, ERα was recruited to NUDT2 promoter within 15 min after the addition of 10 nM estradiol, and the promoter occupancy was maximum at 30–45 min after the treatment (Fig. 5*d*).

NUDT2 status was positively associated with HER2 status both in DCIS and IDC tissues (Tables 1 and 2) as demonstrated above. Therefore, these results suggest possible association between NUDT2 and HER2 overexpressions in human breast carcinoma, but such findings have not been reported yet to the best of our best knowledge. Therefore, to examine this hypothesis, we transfected T47D cells with siRNA for HER2 gene. As shown in Figure 5*e*, the level of NUDT2 mRNA was significantly lower in T47D cells transfected with

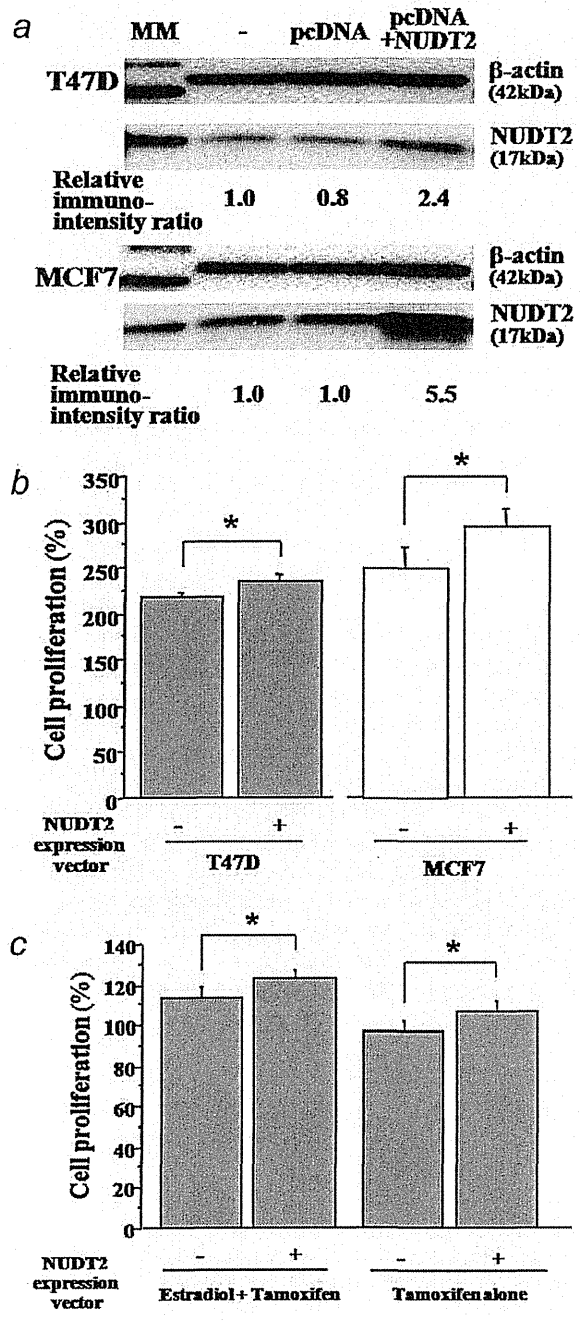


Figure 3. Effects of NUDT2 on the proliferation of breast carcinoma cells by transient transfection. (*a*) Immunoblotting for NUDT2 in T47D and MCF7 cells transfected with NUDT2 expression vector. The protein of cells was extracted at 3 days after the transfection, and 5 μg of protein was loaded in each lane. NUDT2 (lower panel) and β-actin (upper panel) immunoreactivity was detected as a specific band (approximately 17 and 42 kDa, respectively). MM: molecular marker; -: cells without transfection; pcDNA: cells transfected with empty vector (1 μg); pcDNA + NUDT2: cells transfected with NUDT2 expression vector (1 μg). Relative immunointensity ratio of NUDT2 was indicated as a ratio compared to that in the cells without transfection. (*b*, *c*) Proliferation assay of T47D (*b*, *c*) and MCF7 (*b*) cells transfected with NUDT2. After the transfection, the medium was changed to phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 1 day, and these cells were subsequently treated with estradiol (10 nM) and/or tamoxifen (5 μM) for 2 days. The cell number was evaluated as a ratio (%) compared to that at 0 day after the treatment. +: transfection with NUDT2 expression vector; -: transfection with empty vector. Data were presented as mean ± SEM ($n = 4$ independent experiments with three biological replicates), respectively. *$p < 0.05$.

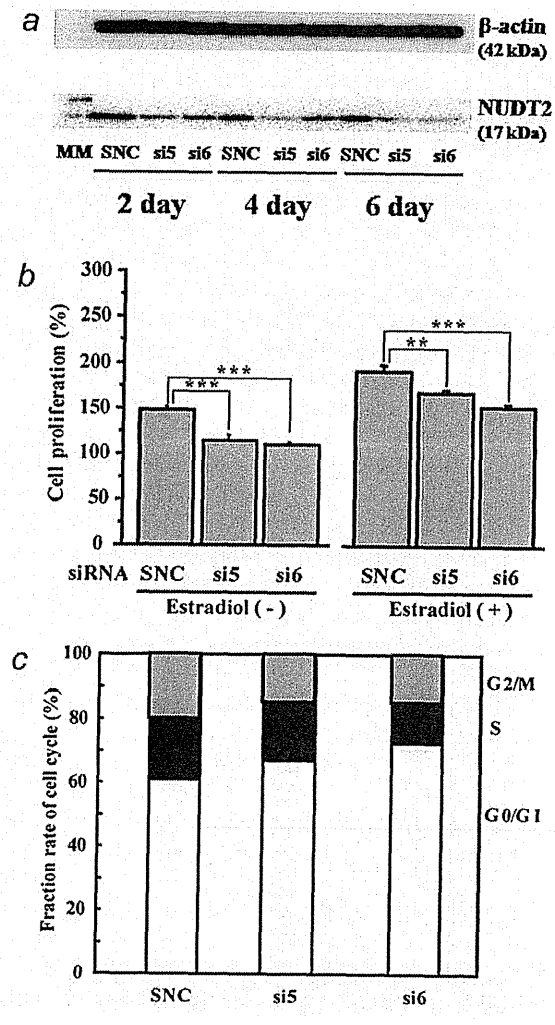


Figure 4. Effects of NUDT2 on the proliferation of T47D cells. (*a*) Immunoblotting for NUDT2 in T47D cells transfected with specific NUDT2 (si5 or si6) siRNA or control (Silencer Negative Control #1; SNC) siRNA. The protein of cells was extracted at 2, 4 or 6 days after the transfection, and 5 μg of protein was loaded in each lane. NUDT2 (lower panel) and β-actin (upper panel) immunoreactivity was detected as a specific band (approximately 17 and 42 kDa, respectively). MM: molecular marker. (*b*) Proliferation assay of T47D cells transfected with si5, si6 or SNC siRNA. After the transfection, the medium was changed to phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 1 day, and the cells were subsequently treated with or without estradiol (10 nM) for 2 days. The cell number was evaluated as a ratio (%) compared to that at 0 day after the treatment. Data were presented as mean ± SEM ($n = 4$ independent experiments with three biological replicates), respectively. $^{**}p < 0.01$ and $^{***}p < 0.001$. (*c*) Flow cytometry analysis in T47D cells transfected with si5, si6 or SNC siRNA. Cell cycle fractions were shown as follows: G0/G1 phase: open bar; S phase: closed bar; G2/M phase: gray bar.

HER2 siRNA than that transfected with SNC siRNA ($p < 0.01$ and 0.30-fold in si3 and $p < 0.05$ and 0.40-fold in si4). However, HER2 mRNA level itself was not significantly ($p = 0.92$) different between the T47D cells treated with NUDT2 (si5 and si6) and SNC siRNA.

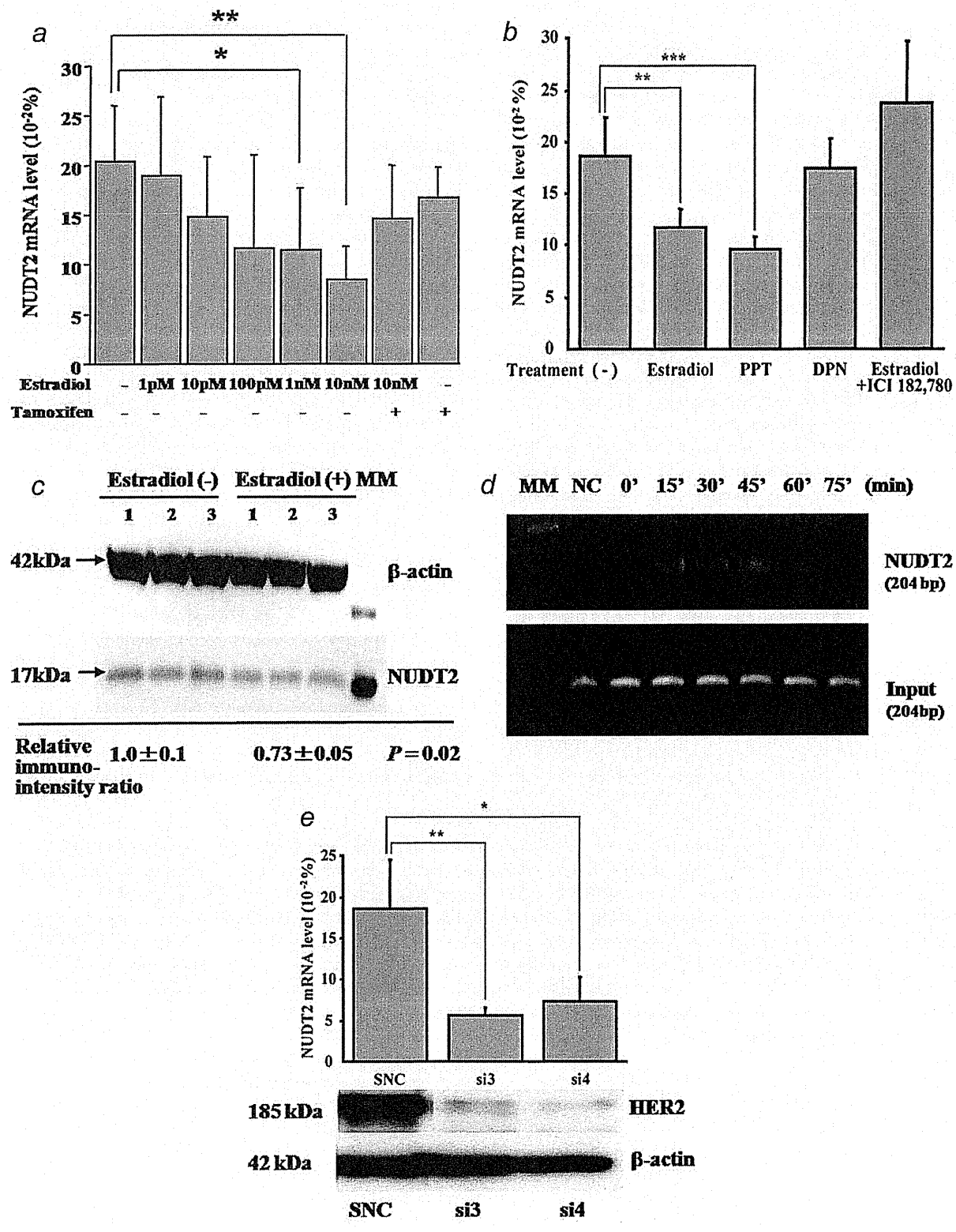
Discussion

In this study, we demonstrated that the level of NUDT2 mRNA was significantly higher in DCIS or IDC than non-neoplastic breast tissues. In addition, NUDT2 immunoreactivity was detected in carcinoma cells in 78% of DCIS and 63% of IDC cases, although NUDT2 immunoreactivity was almost negligible in morphologically normal mammary glands. The relatively wide distribution of NUDT2 detected in human breast carcinoma therefore suggests an important role of NUDT2 in human breast carcinoma.

In our study, NUDT2 status evaluated by immunohistochemistry was significantly associated with the Van Nuys classification and Ki-67 LI in DCIS, and with clinical stage, lymph node metastasis and histological grade in IDC. The Van Nuys classification has been established as a potent prognostic classification for DCIS,[19] and Ki-67 LI has also been demonstrated to be closely correlated with the S-phase fraction and mitotic index by numerous investigators.[22,23] Results of our study also demonstrated that NUDT2 status was significantly associated with an increased risk of recurrence or worse prognosis in IDC patients, and NUDT2 status was indeed an independent prognostic factor for both recurrence and prognosis following multivariate analyses. Therefore, these results all indicated an involvement of NUDT2 in the progression and/or recurrence of breast carcinoma. The clinical significance of NUDT2 in human breast carcinoma will, however, need to be further verified in a follow-up study on an independent materials.

Subsequent *in vitro* studies demonstrated that T47D cells transfected with siRNA NUDT2 significantly decreased the cell proliferation associated with increased G0/G1 fraction. Previous *in vitro* studies demonstrated that Ap4A is involved in slowing the process of replication during S-phase, which subsequently allows the opportunity of the cells to repair possible DNA damages,[24–26] and Ap4A itself interrupts the cell cycle progression when damages to the genome are detected in the cells.[27,28] Considering the fact that NUDT2 hydrolyses Ap4A, NUDT2 may promote the proliferation of breast carcinoma cells, at least in a part, through a decrement of the intracellular Ap4A level. An increased level of its expression in DCIS compared to that in IDC and non-neoplastic breast tissues suggests that NUDT2 could play a role in the development of carcinoma in the ductal epithelium of mammary glands, but further investigations are required for clarification.

Recently, Bourdeau *et al.* examined genome-wide identification of EREs in human and detected functional ERE at the promoter region of *NUDT2* gene.[7] In addition, Carroll *et al.* reported that NUDT2 mRNA expression was reduced by addition of estrogens in MCF7 breast carcinoma cells by


a
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*
NUDT2 mRNA level (10⁻²%)
30
25
20
15
10
5
0
Estradiol – 1pM 10pM 100pM 1nM 10nM 10nM –
Tamoxifen – – – – – – + +
b
***
**
NUDT2 mRNA level (10⁻² %)
Treatment (-) Estradiol PPT DPN Estradiol +ICI 182,780
c
Estradiol (-) Estradiol (+) MM
1 2 3 1 2 3
42kDa
β-actin
17kDa
NUDT2
Relative immuno-intensity ratio 1.0±0.1 0.73±0.05 P=0.02
d
MM NC 0' 15' 30' 45' 60' 75' (min)
NUDT2 (204 bp)
Input (204bp)
e
*
**
NUDT2 mRNA level (10⁻²%)
SNC si3 si4
185 kDa
HER2
42 kDa
β-actin
SNC si3 si4

genome-wide analysis of ER-binding sites.[8] Therefore, in our study, we also examined possible effects of estrogens on NUDT2 expression. NUDT2 expression was repressed up to half following estradiol in T47D cells through ERα, and subsequent ChIP assay demonstrated binding of ERα with the promoter region of *NUDT2* gene. Results of our study are consistent with those of the previous studies above, and NUDT2 is therefore considered one of estrogen-repressed genes in breast carcinoma cells. Frasor *et al.* demonstrated that a majority (~70%) of estrogen-regulated genes are downregulated, and major functional categories for these estrogen-repressed genes include enzymes, signal transduction and transcription in MCF7 cells.[29] In addition, the estrogen-mediated repression of gene expression was not detected at all in ERα-knockout mice.[30] Therefore, estrogenic functions are characterized not only by estrogen-induced genes but also by estrogen-repressed genes, and therefore, it is important to clarify biological and/or clinical significance of estrogen-repressed genes to understand estrogenic actions in its entirety in human breast carcinoma.

In our study, NUDT2 status was, however, not significantly associated with ER status in DCIS or IDC, although it was expected from the results of our *in vitro* studies described above. Jiang *et al.* reported an induction of NUDT2 expression in human fetal cardiac cells under hypoxic condition.[31] In addition, Yan *et al.* demonstrated that interferon α increased NUDT2 protein level in Huh7 human liver cells.[32] In our study of breast carcinoma patients, NUDT2 status was also positively correlated with HER2 status both in DCIS and IDC cases, and T47D cells transfected with HER2 siRNA significantly decreased the expression level of NUDT2 mRNA. Therefore, several factors other than estrogen may be involved in the regulation of NUDT2 expression in human breast carcinoma cells, which may explain that NUDT2 status was not necessarily associated with ER status in breast carcinomas examined in our study. The *in vitro* experiments conducted in our study are preliminary, and further investigations are required for clarification. However, our experiments may serve as a starting point for clarification of biological functions or regulation mechanisms of NUDT2 in the breast carcinoma.

NUDT2 status was significantly associated with an increased risk of recurrence or worse prognosis in IDC patients in our study, and similar tendency was also detected in ER-positive patients who received tamoxifen therapy as an adjuvant treatment. In addition, results of multivariate analyses demonstrated that NUDT2 status is indeed an independent prognostic factor for both recurrence and overall survival in these patients. Results of our *in vitro* study demonstrated that cell proliferation activity of T47D cells was significantly associated with NUDT2 expression level under the estradiol and/or tamoxifen treatment, and tamoxifen inhibited the estradiol-mediated suppression of NUDT2 expression in these cells. In addition, estrogen-mediated cell proliferation was detected both in NUDT2-positive and -negative T47D cells; the cell proliferation of NUDT2-positive cells without estradiol treatment was nearly equivalent to that of NUDT2-negative cells with estradiol treatment (Fig. 4*b*). However, NUDT2 did not influence the transcriptional activity of ER in T47D cells as results of the luciferase assay demonstrated. All the data above suggest that cell proliferation of breast carcinoma cells is mediated with ER and NUDT2 in different manners or in an additive manner, although the NUDT2 expression was partially suppressed by estradiol, but it awaits for further investigations for clarifying the exact mechanisms. Results of our immunohistochemical studies demonstrated no significant association between NUDT2 status and tumor size in 145 IDC cases (Table 2), but it is true that the tumor size alone does not necessarily represent the overall cell proliferation activity of breast carcinoma,[33] due to the presence

Figure 5. Regulation of NUDT2 expression in T47D cells. (*a*) Effects of estradiol and tamoxifen on NUDT2 mRNA expression level by real-time PCR analysis. T47D cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 2 days and then treated with indicated concentration of estradiol and/or tamoxifen (5 μM) for 3 days. NUDT2 mRNA level was summarized as the ratio of RPL13A mRNA level (%). Data were presented as mean ± SEM ($n = 4$ independent experiments with three biological replicates), respectively. *$p < 0.05$ and **$p < 0.01$. (*b*) Effects of ER agonists and antagonist on NUDT2 mRNA expression evaluated by real-time PCR analysis. T47D cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 2 days and then treated with estradiol (10 nM), ERα agonist PPT (10 nM), ERβ agonist DPN (10 nM) or ER antagonist ICI 182,780 (1 μM) for 3 days. Data were presented as mean ± SEM ($n =$ 3 independent experiments with three biological replicates), respectively. **$p < 0.01$ and ***$p < 0.001$. (*c*) Effects of estradiol on NUDT2 protein level by immunoblotting analysis. The cells were cultured with phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 2 days and then treated with or without estradiol (10 nM) for 3 days. A total of 5 μg of protein was loaded in each lane. The results of immunointensity ratio (NUDT2/β-actin) were presented as mean ± SEM ($n = 3$ independent experiments with three biological replicates), and a significant association ($p = 0.02$) was detected between these two groups. (*d*) Recruitment patterns of ERα on NUDT2 promoter in T47D cells treated with estradiol (10 nM) by ChIP assay. The sample before immunoprecipitation by anti-ERα antibody was also used as a control (input; lower panel). MM: molecular marker; NC: negative control sample treated with ethanol. (*e*) Effects of HER2 on NUDT2 mRNA expression by real-time PCR analysis. NUDT2 mRNA level was examined in T47D cells treated with specific HER2 (si3 or si4) or SNC siRNA 3 days after the transfection (upper panel). NUDT2 mRNA level was summarized as the ratio of RPL13A mRNA level (%). Data were presented as mean ± SEM ($n = 3$ independent experiments with three biological replicates), respectively. *$p < 0.05$ and **$p < 0.01$. The knockdown effects mediated by si3 or si4 siRNA were subsequently confirmed by immunoblotting for HER2 3 days after the transfection (lower panels).

of the amount of stromal cells in the tumor tissues. Therefore, residual carcinoma cells after surgical treatment in NUDT2-positive breast carcinomas may still have the potential to rapidly grow regardless of antiestrogen therapies such as tamoxifen, thereby resulting in an increased recurrence and poor prognosis in these patients.

In summary, NUDT2 immunoreactivity was frequently detected in the breast carcinoma in our study. NUDT2 status was significantly associated with an increased risk of recurrence or worse prognosis of IDC patients, and it was an independent prognostic factor. Results of further *in vitro* studies demonstrated that proliferation activity of T47D cells was associated with NUDT2 level according to the treatment of estradiol and/or tamoxifen. These findings suggest that NUDT2 promotes the proliferation of breast carcinoma cells by different mechanisms from estrogens, and the NUDT2 status is established as a potent prognostic factor in human breast carcinomas.

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