Clinical laboratory

A facility for the biologic, microbiologic, serologic, chemical, immunohematologic, hematologic, biophysical, cytologic, pathologic, or other examination of materials derived from the human body for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of, human beings. These examinations also include procedures to determine, measure, or otherwise describe the presence or absence of various substances or organisms in the body. Facilities only collecting or preparing specimens (or both) or only serving as a mailing service and not performing testing are not considered laboratories (42CFR493.2).

US Food and Drug Administration (FDA)–cleared test

A test that has been cleared by the FDA after analysis of data showing substantial performance equivalence to other tests being marketed for the same purpose. Such tests typically follow the 510(k) approval route (21CFR807).

FDA-approved test

A test that is classified as a class III medical device and that has been approved by the FDA through the premarket approval process (21CFR814.3).

Laboratory modified test

An FDA-cleared or FDA-approved test that is modified by a clinical laboratory, but not to a degree that changes the stated purpose of the test, approved test population, specimen type, specimen handling, or claims related to interpretation of results.

Laboratory developed test (LDT)

A test developed within a clinical laboratory that has both of the following characteristics: is performed by the clinical laboratory in which the test was developed and is neither FDA cleared nor FDA approved.

Note: All laboratory modified tests are, by definition, LDTs. An LDT may or may not use analyte-specific reagent, RUO, or IUOs; the type of reagents and devices used does not affect whether a test is classified as an LDT. A laboratory is considered to have developed a test if the test procedure or implementation of the test was created by the laboratory performing the testing, irrespective of whether fundamental research underlying the test was developed elsewhere or reagents, equipment, or technology integral to the test was purchased, adopted, or licensed from another entity.

Validation of a test

Confirmation through a defined process that a test performs as intended or claimed.

Note: There is no universally acceptable procedure for validating tests. The process for validating tests must take into account the purpose for which a test is intended to be used, claims made about the test, and the risks that may prevent the test from serving its intended purpose or meeting performance claims. Even FDA-approved and FDA-cleared tests require limited revalidation in clinical laboratories (a process often referred to as verification) to establish that local implementation of the test can reproduce a manufacturer's validated claims. Tests that use reagents or equipment that have not been validated (such as RUOs or IUOs) typically pose increased risks that require more extensive validation, as do tests used in more loosely controlled settings. The determination of whether a test has been adequately validated requires professional judgment.

Table A1

Panel Members

Panel Member	Institution
M. Elizabeth H. Hammond, MD, FCAP, Co-Chair	Intermountain Healthcare, University of Utah School of Medicine, UT
Antonio C. Wolff, MD, FACP, Co-Chair	The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, MD
Daniel F. Hayes, MD, Co-Chair	University of Michigan Comprehensive Cancer Center, University of Michigan Health System, MI
D. Craig Allred, MD, FCAP, Steering Committee Member	Washington University School of Medicine in St Louis, MO
Mitch Dowsett, PhD, Steering Committee Member	Royal Marsden Hospital, United Kingdom
Sunil Badve, MD	Eastern Cooperative Oncology Group, Indiana University, IN
Robert L. Becker, MD, Ex-Officio	US Food and Drug Administration, Center for Devices and Radiological Health, Office of In Vitro Diagnostic Device Evaluation and Safety
Patrick L. Fitzgibbons, MD, FCAP	St. Jude Medical Center, CA
Glenn Francis, MBBS, FRCPA, MBA	Princess Alexandra Hospital, Australia
Neil S. Goldstein, MD, FCAP	Advanced Diagnostics Laboratory, MI
Malcolm Hayes, MD	University of British Columbia, Canada
David G. Hicks, MD, FCAP	University of Rochester, NY
Susan Lester, MD	Brigham and Women's Hospital, MA
Richard Love, MD	Ohio State University, OH
Lisa McShane, PhD	National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis, MD
Keith Miller, MD	UK NEQAS, United Kingdom
C. Kent Osborne, MD	Baylor College of Medicine, TX
Soonmyung Paik, MD	National Surgical Adjuvant Breast and Bowel Project, PA
Jane Perlmutter, PhD, Patient Representative	Gemini Group, MI
Anthony Rhodes, PhD	University of the West of England, Bristol, UK NEQAS
Hironobu Sasano, MD	Tohoku University School of Medicine, Japan
Jared N. Schwartz, MD, PhD, FCAP	Presbyterian Hospital, NC
Fred C.G.J. Sweep, PhD	Radboud University, Nijmegen, the Netherlands
Sheila Taube, PhD	ST Consulting, Glen Echo, MD
Emina Emilia Torlakovic, MD, PhD	Royal University Hospital, Saskatoon, Canada
Giuseppe Viale, MD, FRCPath	European Institute of Oncology, and University of Milan, Italy
Paul Valenstein, MD, FCAP	St. Joseph Mercy Hospital, Ann Arbor, MI
Daniel Visscher, MD	University of Michigan, Ann Arbor, MI
Thomas Wheeler, MD, FCAP	Baylor College of Medicine, TX
R. Bruce Williams, MD, FCAP	The Delta Pathology Group, Shreveport, LA
James L. Wittliff, MD, PhD	University of Louisville, KY
Judy Yost, MA, MT (ASCP), Ex Officio	CMS, Division of Laboratory Services (CLIA), MD

Abbreviations: UK NEQAS, United Kingdom National External Quality Assessment Service; CMS, Centers for Medicare and Medicaid Services; CLIA, Clinical Laboratory Improvement Act.

Verification of a test

An abbreviated process through which a clinical laboratory establishes that its implementation of an FDA-approved and FDA-cleared test performs in substantial conformance to a manufacturer's stated claims.

Analytic validity

A test's ability to accurately and reliably measure the analyte (measurand) of interest. The elements of analytic validity include the following, as applicable.

- Accuracy. The closeness of agreement between the average value obtained from a large series of measurements and the true value of the analyte. Note: Technically, the term accuracy refers to the measure of the closeness of a single test result to the true value, not the average of multiple results. The definition of accuracy used here is what metrologists call trueness of measurement and describes the popular (but technically incorrect) meaning of the word accuracy.
- Precision. The closeness of agreement between independent results of measurements obtained under stipulated conditions (the International Organization of Standardization, 1993).
- Reportable range. For quantitative tests, the span of test result values over which the laboratory can establish or verify the accuracy of the instrument or test system measurement response and over which results will be reported. For semiquantitative, binary, and nominal/categoric tests, the reportable range is all of the values that can be reported by the test system (eg, 2+, 3+, "positive," "negative," *Escherichia coli, Staphylococcus aureus*).
- Analytic sensitivity. For quantitative tests (including semiquantitative tests), analytic sensitivity is the lowest amount of analyte (measurand) in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value. For qualitative tests (binary and nominal/categoric tests), analytic sensitivity is the proportion of instances in which the analyte/measurand/identity is correctly detected, within a stated CI.

Table A2

Invited Guests to Open Session December 2008 Panel Meeting

Invited Guests	Affiliation
Steven Shak, MD	Genomic Health, Redwood City, CA
Kenneth J. Bloom, MD	Clarient, Aliso Viejo, CA
Patrick Roche, PhD	Ventana Medical Systems, Tucson, AZ
Allen M. Gown, MD	PhenoPath Laboratories, Seattle, WA
David L. Rimm, MD, PhD	Yale University, New Haven, CT
Hadi Yaziji, MD	Ancillary Pathways, Miami, FL
Richard Bender, MD	Agendia, Huntington Beach, CA
Roseanne Welcher	Dako, Glostrup, Denmark

- Analytic specificity. Ability of a measurement procedure to measure solely the measurand/analyte.

Note: Analytic validity is expressed in the context of a defined set of test conditions (including standard operating procedures and permissible specimen types) and an

ongoing quality management regimen (including, as applicable, ongoing quality control, periodic assay recalibration, and external proficiency testing or alternative external testing). If the test conditions or quality management regimen changes, the analytic validity of a test may change.

Clinical validity

A test's ability to detect or predict a disorder, prognostic risk, or other condition or to assist in the management of patients. The elements of clinical validity include the following, as applicable.

- Clinical sensitivity (clinical detection rate). The proportion of individuals with a disorder, prognostic risk, or condition who are detected by the test.
- Clinical specificity. The proportion of individuals without a disorder, prognostic risk, or condition who are excluded by the test.
- Reference limits. A value or range of values for an analyte that assists in clinical decision making. Reference values are generally of two types—reference intervals and clinical decision limits. A reference interval is the range of test values expected for a designated population of individuals. This may be the central 95% interval of the distribution of values from individuals who are presumed to be healthy (or normal). For some analytes that reflect high-prevalence conditions (such as cholesterol), significantly less than 95% of the population may be healthy. In this case, the reference interval may be something other than the central 95% of values. A clinical decision limit represents the lower or upper limit of a test value which a specific clinical diagnosis is indicated specified course of action is recommended.
- Clinical utility. The clinical usefulness of the test. The clinical utility is the net balance of risks and benefits associated with using a test in a specific clinical setting. Clinical utility does not take into consideration the economic cost or economic benefit of testing and is be distinguished from cost-benefit and cost-effectiveness analysis. Clinical utility focuses entirely on the probabilities and magnitude of clinical benefit and clinical harm that result from using a test in a particular clinical context.

  Note 1: The qualities listed in this appendix represent the primary performance measurements that are used describe the clinical capabilities of a test. Other measures of clinical validity may be applicable in particular circumstances.

  Note 2: Clinical validity is expressed in the context of defined test population and a defined testing procedure. If the test population changes (eg, a change in the prevalence of disease) or the testing procedure changes, the clinical validity of a test may change.

Table 1

Summary of Guideline Recommendations for ER and PgR Testing by IHC in Breast Cancer Patients

	Recommendation	Comments
Optimal algorithm for ER/PgR testing	Positive for ER or PgR if finding of ≥ 1% of tumor cell nuclei are immunoreactive	These definitions depend on laboratory documentation of the following: **1** Proof of initial validation in which positive ER or PgR categories are 90% concordant and negative ER or PgR categories are 95% concordant with a clinically validated ER or PgR assay.[3] **2** Ongoing internal QA procedures, including use of external controls of variable ER and PgR activity with each run of assay, regular assay reassessment, and competency assessment of technicians and pathologists. **3** Participation in external proficiency testing according to the proficiency testing program guidelines. **4** Biennial accreditation by valid accrediting agency.
	Negative for ER or PgR if finding of < 1% of tumor cell nuclei are immunoreactive in the presence of evidence that the sample can express ER or PgR (positive intrinsic controls are seen)	
	Uninterpretable for ER or PgR if finding that no tumor nuclei are immunoreactive and that internal epithelial elements present in the sample or separately submitted from the same sample lack any nuclear staining	
Optimal testing conditions	Large, preferably multiple core biopsies of tumor are preferred for testing if they are representative of the tumor (grade and type) at resection.	Specimen should be rejected and testing repeated on a separate sample if any of the following conditions exist: **1** External controls are not as expected (scores recorded daily show variation). **2** Artifacts involve most of sample. Specimen may also be rejected and testing repeated on another sample if: **1** Slide has no staining of included normal epithelial elements and/or normal positive control on same slide. **2** Specimen has been decalcified using strong acids. **3** Specimen shows an ER-negative/PgR-positive phenotype (to rule out a false-negative ER assay or a false-positive PgR assay). **4** Sample has prolonged cold ischemia time or fixation duration < 6 hours or > 72 hours and is negative on testing in the absence of internal control elements.
	Interpretation follows guideline recommendation.	Positive ER or PgR requires that ≥ 1% of tumor cells are immunoreactive. Both average intensity and extent of staining are reported. Image analysis is a desirable method of quantifying percentage of tumor cells that are immunoreactive. H score, Allred score, or Quick score may be provided. Negative ER or PgR requires < 1% of tumor cells with ER or PgR staining. Interpreters have method to maintain consistency and competency documented regularly.
	Accession slip and report must include guideline-detailed elements.	
Optimal tissue handling requirements	Time from tissue acquisition to fixation should be as short as possible. Samples for ER and PgR testing are fixed in 10% NBF for 6 to 72	

	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	

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
global cancer control

IJC
International Journal of Cancer

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

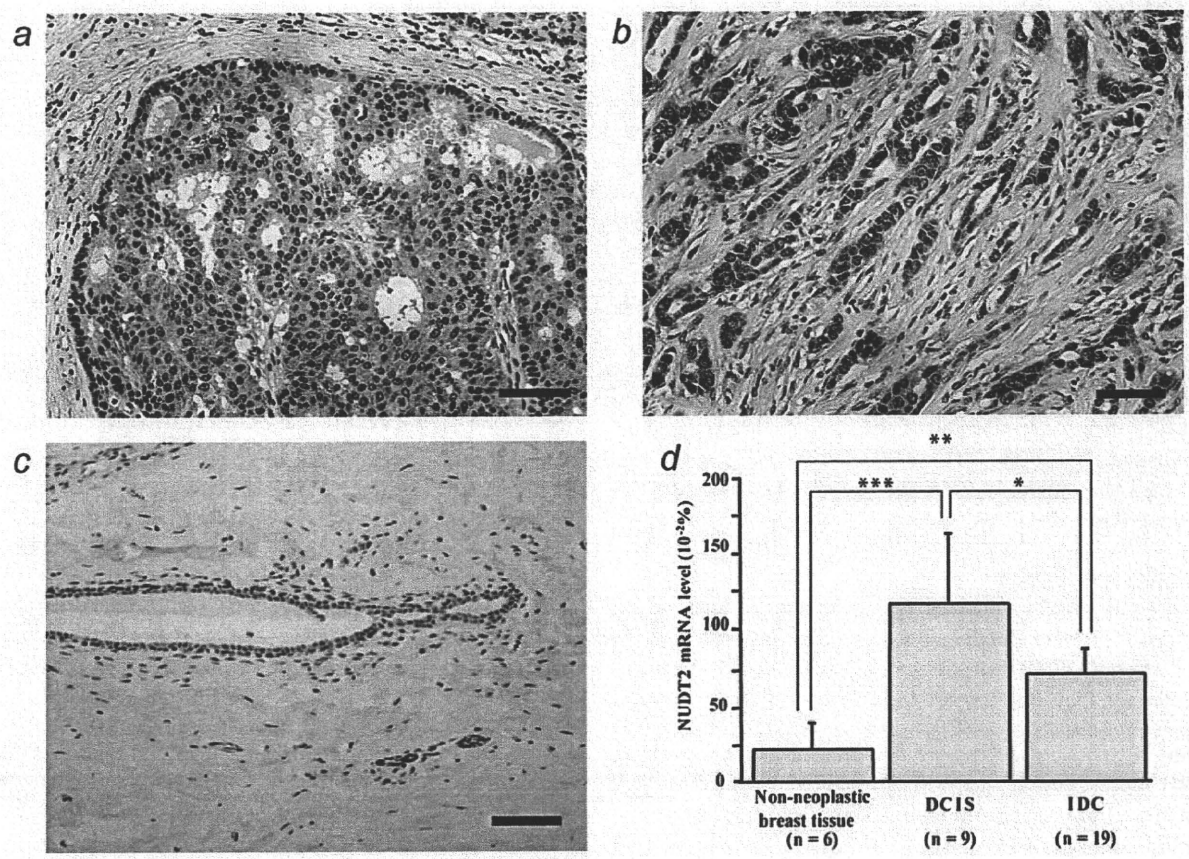

Figure 1. Expression of NUDT2 in the breast carcinoma. (*a–c*) NUDT2 immunoreactivity was detected in the cytoplasm of carcinoma cells in DCIS (*a*) and IDC (*b*). NUDT2 immunoreactivity was not detected in morphologically normal mammary glands (*c*). Bar = 50 μm, respectively. (*d*) Expression levels 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

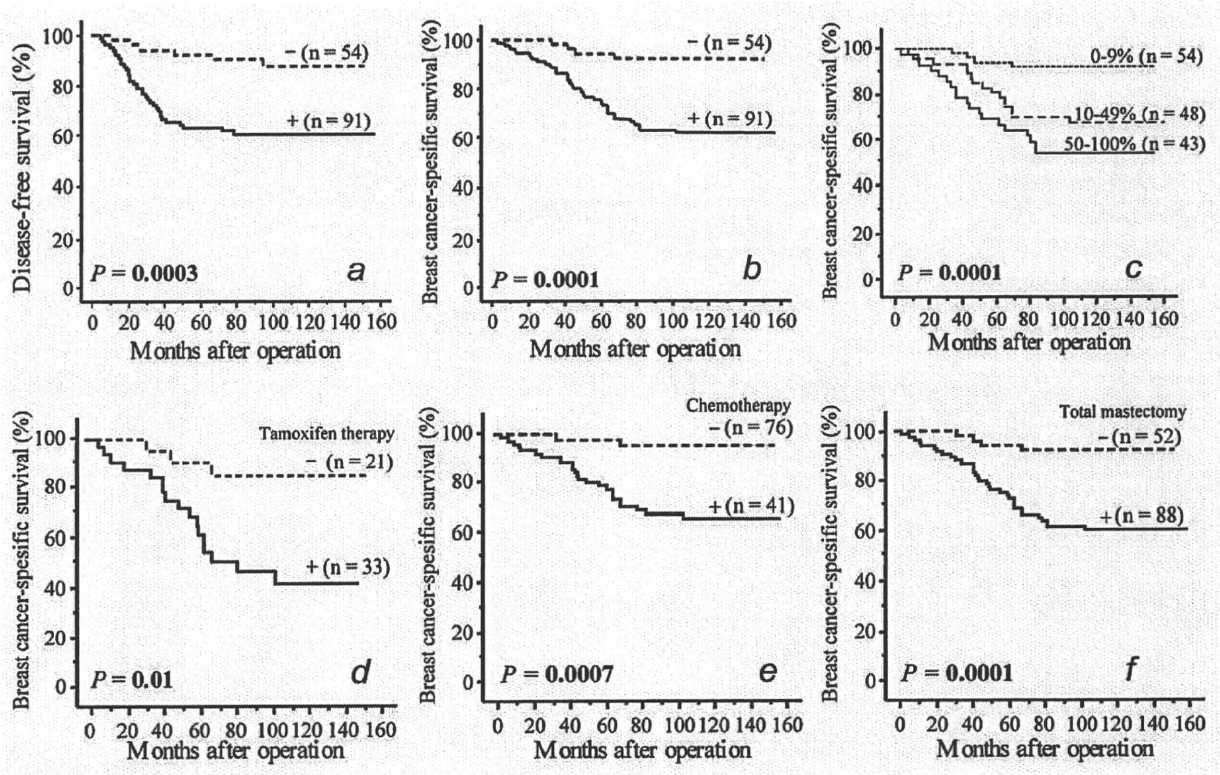

Figure 2. Disease-free and breast cancer-specific survival of 145 IDC patients according to NUDT2 status studied by Kaplan-Meier method. (*a*, *b*) NUDT2 status was significantly associated with an increased risk of recurrence ($p = 0.0003$) (*a*) and worse prognosis ($p = 0.0001$) (*b*). (*c*) Association 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.