AmpliChip genotyping platform was available using criterion 1 (871/1,635 AmpliChip-genotyped subjects met criterion 1). When confined to the Amplichip subjects, the estimates of the pharmacodynamic HRs for *CYP2D6* were similar to what they were for the entire set of subjects meeting criterion 1.

A major source of potential genotyping errors may be related to DNA source. *CYP2D6* is one of the most difficult genes to genotype because of the numerous polymorphisms and adjacent pseudogenes. Some platforms cannot detect the presence of the **5* deletion, particularly in DNA derived from

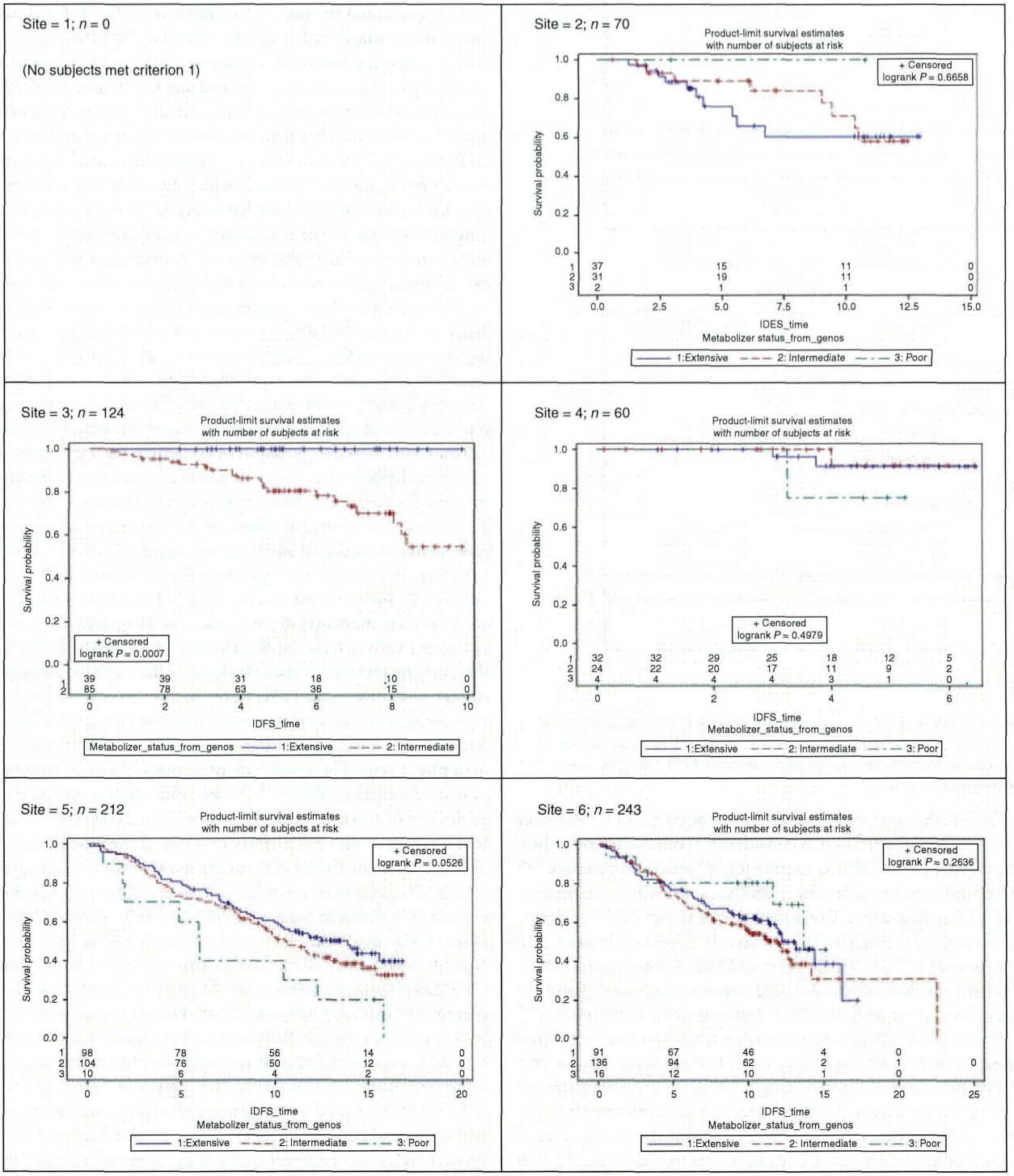

Figure 2 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = invasive disease–free survival (IDFS)).

formalin-fixed–paraffin-embedded (FFPE) tissue. However, several sites used multiple platforms to validate their genotyping data, reducing potential genotyping errors across the entire data set. Importantly, *CYP2D6* genotypes obtained from blood-derived DNA reflect the patients' germ-line genotypes, known to influence endoxifen plasma concentrations. By contrast, *CYP2D6* genotypes from tumor-derived DNA may be subject to error due to somatic mutation by loss of heterozygosity, known to affect the *CYP2D6* locus at 22q13 in up to 30% of breast tumors.[20–22] Thus, when *CYP2D6* genotype is derived from tumor samples, an excess number of homozygotes may result as a consequence of loss of heterozygosity. This form of genotyping error is revealed by Hardy–Weinberg Equilibrium (HWE) testing, as was observed in the Breast

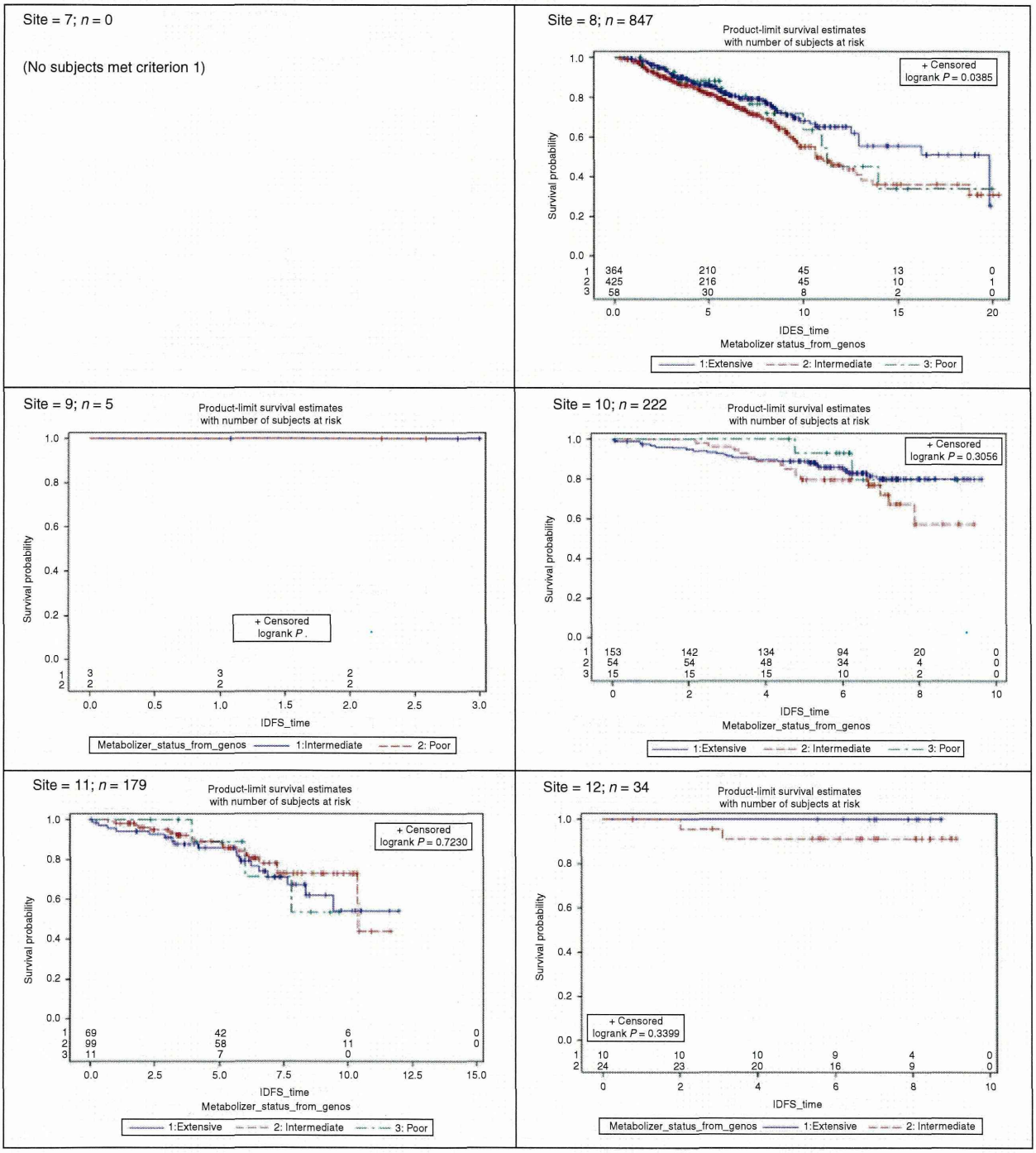

Figure 2 Continued

International Group 1-98 study, in which strong departures from HWE (to a magnitude of 10^{-92}) were observed, leading to a call for retraction of this article.[3,12,16]

For criterion 1, 49.9% of our patient DNA samples originated from blood, 21.6% from fresh-frozen tissues, and 28.5% from FFPE tissues. For criterion 2, 55.0% samples originated from blood, 20.5% were fresh-frozen tissues, and 24.5% from FFPE tissues. For criterion 3, 50.9% of DNA samples originated from blood, 31.9% from fresh-frozen tumor, 13.4% from FFPE tumor tissues, and 3.5% from FFPE normal tissue. Although we cannot exclude the presence of somatic events leading to misclassification of *CYP2D6* genotype, as evident from HWE deviation identified in data from

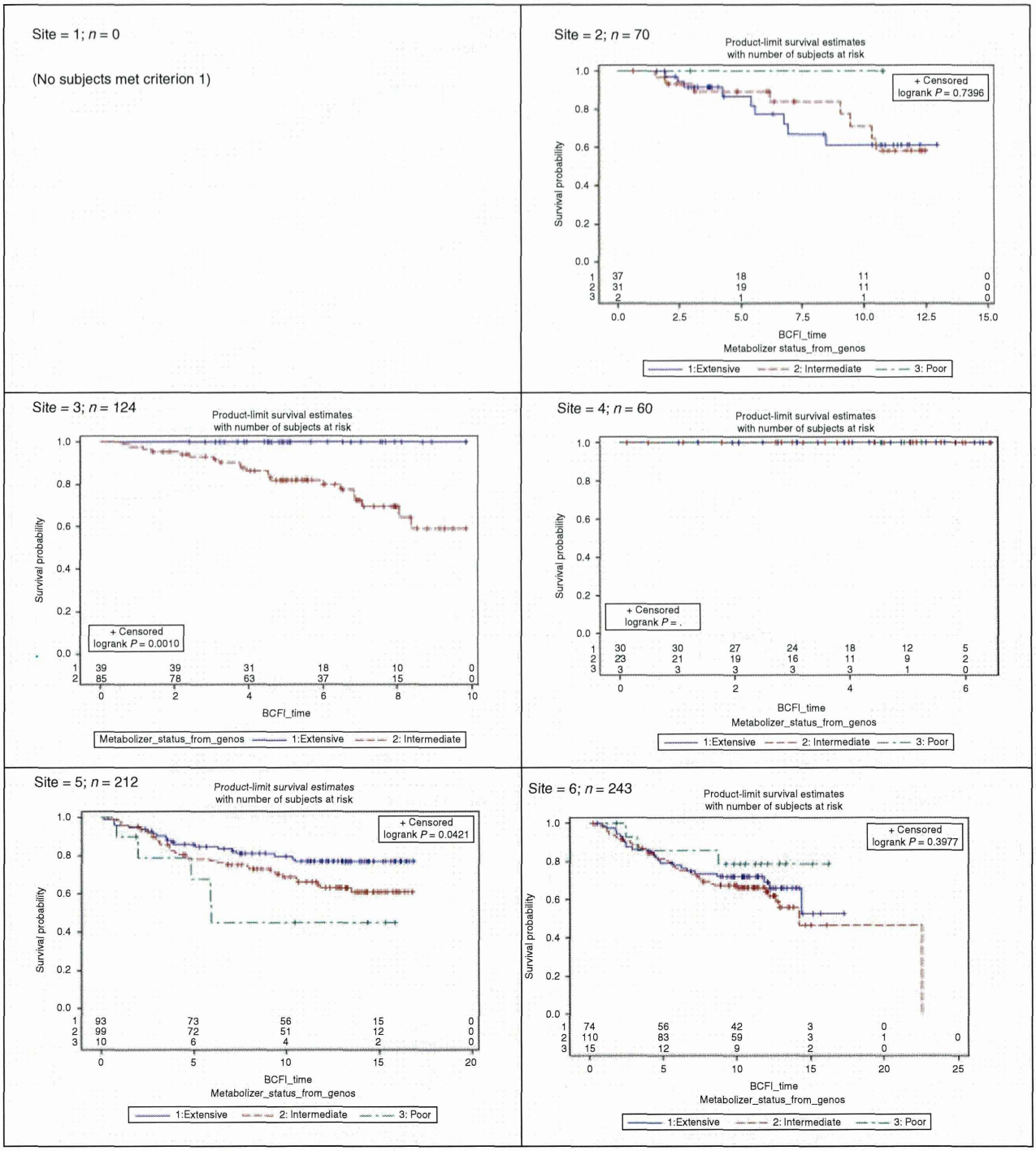

Figure 3 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = breast cancer–free interval (BCFI)).

some sites, comprehensive testing for HWE did not reveal significant violations across most sites. Moreover, the extent of deviation from HWE in the **4* allele was not associated with sites that evinced less clinical benefit from tamoxifen in patients who were assessed to be PMs in terms of their *CYP2D6* status. This suggests that genotyping errors are unlikely to be a major issue in our analyses.

Our findings are subject to the shortcomings commonly encountered when performing retrospective "biomarker" studies. In our study, most sites were unable to collect or control for the factors known to alter endoxifen exposure, including dose and duration of tamoxifen administration and patients' adherence to the regimen. Although tamoxifen adherence is increasingly recognized as a critical factor for drug efficacy,[23] most studies evaluating tamoxifen biomarkers have not controlled for adherence. Other confounders include limited *CYP2D6* allele coverage and lack of information

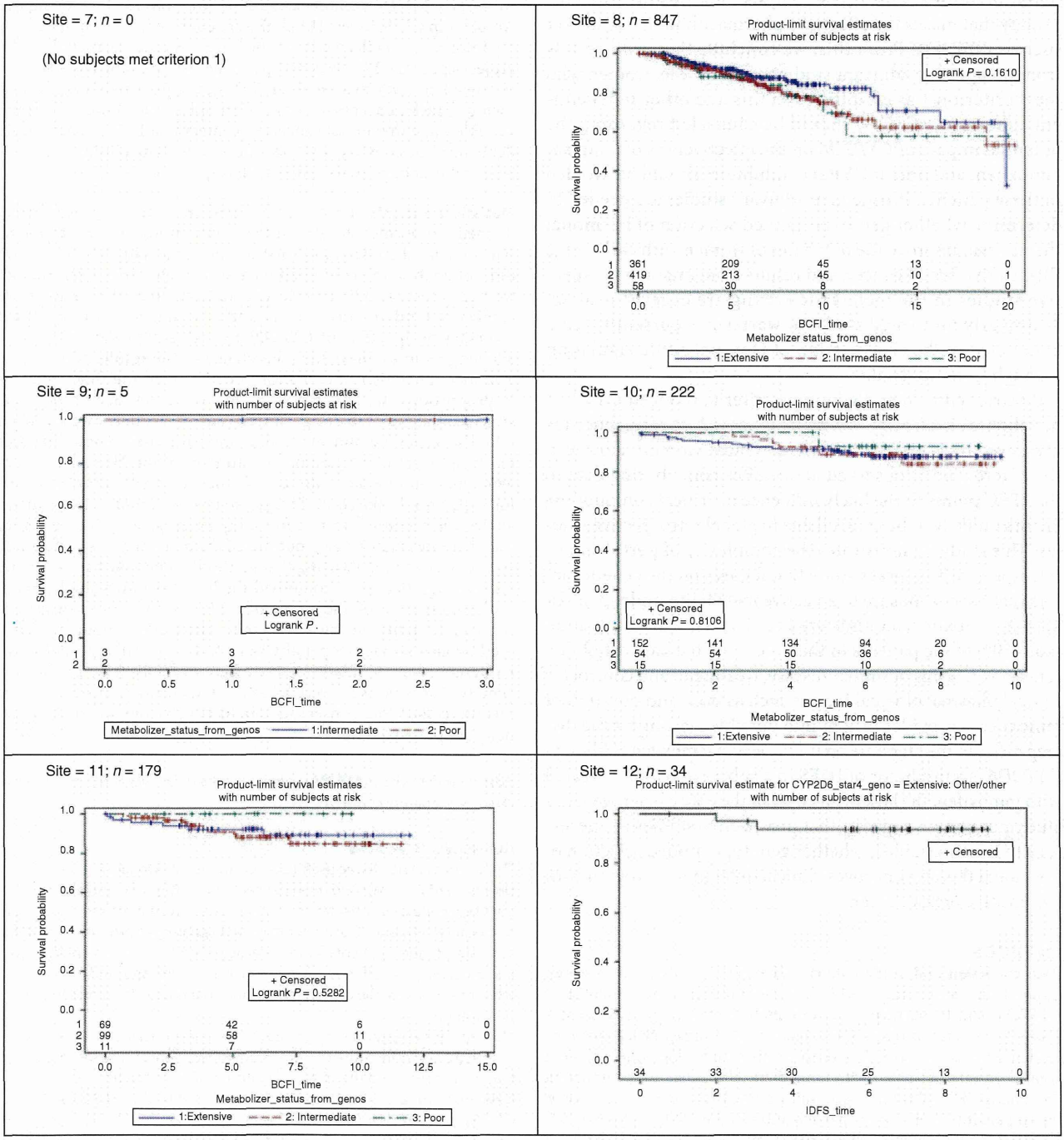

Figure 3 Continued

regarding the coadministration of CYP2D6 inhibitors, leading to potential misclassification of the CYP2D6 drug metabolism phenotype. Therefore, our meta-analysis results depend heavily on which subgroup of patients we include. If we accept that utmost precautions must be applied to avoid the distortion of results from influences derived from the aforementioned shortcomings, it follows that merely increasing the numbers of subjects without controlling the quality of input data, as done in our preliminary overview analysis,[5] may result in heterogeneity that masks the effect of a pharmacokinetic biomarker such as *CYP2D6*. From this, we conclude that until results from prospective adjuvant studies are available, women who meet criterion 1 as established in this and other independent cohorts (ABCSG 8) should be counseled regarding the potential impact of *CYP2D6* on the effectiveness of adjuvant tamoxifen, and potent CYP2D6 inhibitors should be avoided in these patients. Prospective adjuvant studies are needed to determine whether genotype-guided selection of hormonal therapy will improve the outcomes of women with early-stage ER-positive breast cancer, and results from ongoing prospective studies in the metastatic setting are eagerly awaited. A similarly motivated study on warfarin is currently being conducted in the Clarification of Optimal Anticoagulation through Genetics trial.[24]

By strict clinical and genotype criteria, reduced CYP2D6 metabolism is associated with a higher risk of recurrence (as measured by IDFS) in tamoxifen-treated women. However, the heterogeneity observed across sites contributing data to the ITPC points to the likely influence of critical confounding factors unlikely to be controllable in global retrospective studies. This study demonstrates the complexity of performing a retrospective biomarker study that focuses on the genetic factors that affect exposure to an active metabolite, endoxifen, for a drug, tamoxifen, administered for 5 years. Our observation that <50% of the patients in this study met the basic eligibility criteria—in terms of similar disease, treatment, and control for critical pharmacological factors such as dose and duration of tamoxifen—provides insight into possible reasons for the discrepancies in the literature on *CYP2D6* and tamoxifen. Although *CYP2D6* is a predictor of IDFS in a subset of patients treated with tamoxifen, the lack of an effect in the entire heterogeneous study population suggests that prospective studies are necessary to finally establish whether genotype-guided selection of hormonal therapy improves clinical outcomes of women with ER-positive breast cancer.

METHODS

Data collection and study cohorts. The ITPC invited any research group from across the world that had published or unpublished *CYP2D6* data to participate in this meta-analysis. The ITPC comprises 12 research projects for a total of 4,973 breast cancer patients treated with tamoxifen. This retrospective study does not include a control group not treated with tamoxifen. These data were curated at the PharmGKB (Pharmacogenomics Knowledge Base, http://www.pharmgkb.org). Consent for participation in the ITPC and DNA collection, *CYP2D6* genetic testing, and submission of data was obtained under local ethical review board permissions.

We collected information on clinical factors previously shown to be associated with breast cancer therapy and prognosis that were available from the information received from the sites. These data included demographic characteristics, cancer history, cancer recurrence, use of other therapies, use of concomitant medications known to affect *CYP2D6* phenotype, ER status, and classic prognostic factors such as tumor size and number of affected lymph nodes. Information was also collected regarding the presence of *CYP2D6* genetic variants (**2, *3, *4, *5, *6, *10, *17,* and **41*, categorized by their DNA sources), for which coverage of these alleles varied by site. For 1,635 subjects, *CYP2D6* variants assessable from blood DNA using the AmpliChip CYP450 test (Roche) were collected. A complete list of the information collected is detailed in **S1–S3** online, including the project-specific *CYP2D6* genotype assays used and the DNA source. Independent confirmation of *CYP2D6* genotypes was not performed owing to lack of access to subjects' samples. The clinical outcome variable was either breast cancer–free interval or IDFS, as previously defined.[25] The complete data set of genotypes and clinical variables is available at http://www.pharmgkb.org.

Statistical analysis. Because the ITPC was not a prospectively defined multicenter study with a common protocol, there is potential for considerable study-to-study heterogeneity. Therefore, we did not analyze the combined data as a single series even though we had access to individual-level data from all studies. Rather, we applied a random-effects meta-analysis strategy. This provided estimates of the effect of *CYP2D6* in each study's data separately, allowing us to examine the consistency of the results across sites. The meta-analysis is a two-stage procedure. In the first stage, we fit proportional-hazards models to the data from each of the ITPC sites separately, predicting clinical outcome after surgery from *CYP2D6* genotype and other relevant covariates. These analyses produced a set of 12 parameter estimates of the HRs of *CYP2D6* genotypes on outcome, along with their corresponding SEs (one for each site). In the second stage, we used a random-effects meta-analysis procedure[26] to test for study heterogeneity (i.e., whether the 12 studies met the assumptions of the meta-analysis sufficiently so as to be combinable using that method). When the heterogeneity was not significant, we combined the log-HRs into a single, meta-analysis estimate of the effect of *CYP2D6* on tamoxifen-treated recurrence and/or survival outcomes. The DerSimonian and Laird method also provides a penalty in its test of overall association for moderate levels of study-to-study heterogeneity (i.e., for heterogeneity that is not so severe as to be statistically significant). This method is therefore conservative in its conclusions when heterogeneity is a potential issue.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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CONFLICT OF INTEREST

A.M.T. and W.G.N. report Roche funding for genotyping H.B. and M. Schwab report that they have initiated scientific collaborations in 2009 with Roche Molecular Diagnostics and Siemens Healthcare Diagnostics Products, respectively P.A.F. and M.W.B. report Novartis research funding. M.-T.M.L. is a paid consultant of YongLin Health Foundation. R.B.A. is a founder, equity holder, and consultant for Personalis. The other authors declared no conflict of interest.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ There has been extensive controversy with regard to the association between *CYP2D6* genetic variants and the clinical outcomes of tamoxifen use.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ The ITPC was established to address this controversy and to determine the association of *CYP2D6* status with IDFS in tamoxifen-treated early-stage, ER-positive breast cancer.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ We found that *CYP2D6* genotype was associated with a higher risk of recurrence in patients meeting the strict criterion. However, the observation of substantial heterogeneity in cohorts 2 and 3 suggests that study design factors that cannot be controlled retrospectively may obscure the predictive utility of *CYP2D6* genotype. This study demonstrates the complexity of performing a retrospective biomarker study.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ Although *CYP2D6* is a predictor of IDFS in a subset of patients treated with tamoxifen monotherapy, the lack of an effect in the entire heterogeneous study population suggests that prospective studies are necessary to fully establish the value of *CYP2D6* genotyping in tamoxifen therapy.

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ELSEVIER

Experimental Hematology

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Ribosomal protein L11- and retinol dehydrogenase 11-induced erythroid proliferation without erythropoietin in UT-7/Epo erythroleukemic cells

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Q38 (Received 17 May 2014; revised 8 January 2015; accepted 11 January 2015)

Q1 **Erythropoiesis is the process of proliferation, differentiation, and maturation of erythroid cells. Understanding these steps will help to elucidate the basis of specific diseases associated with abnormal production of red blood cells. In this study, we continued our efforts to identify genes involved in erythroid proliferation. Lentivirally transduced UT-7/Epo erythroleukemic cells expressing ribosomal protein L11 (RPL11) or retinol dehydrogenase 11 (RDH11) could proliferate in the absence of erythropoietin, and their cell-cycle profiles revealed G_0/G_1 prolongation and low percentages of apoptosis. RPL11-expressing cells proliferated more rapidly than the RDH11-expressing cells. The antiapoptotic proteins BCL-XL and BCL-2 were expressed in both cell lines. Unlike the parental UT-7/Epo cells, the expression of hemoglobins (Hbs) in the transduced cells had switched from adult to fetal type. Several signal transduction pathways, including STAT5, were highly activated in transduced cells; furthermore, expression of the downstream target genes of STAT5, such as *CCND1*, was upregulated in the transduced cells. Taken together, the data indicate that RPL11 and RDH11 accelerate erythroid cell proliferation by upregulating the STAT5 signaling pathway with phosphorylation of**
Q2 **Lyn and CREB.**

Erythropoiesis, the process of production of red blood cells, consists of several stages that depend on various specific cytokines; these factors promote the differentiation and proliferation of hematopoietic stem cells into mature erythrocytes. The maturation process of erythrocytes involves many steps, including chromatin condensation, hemoglobinization, enucleation, and expulsion of certain organelles. Erythropoietin (Epo), the major growth factor in erythropoiesis, plays an essential role in proliferation and prevention of apoptosis, starting at the stage of the initial erythroid precursor.

Understanding erythroid proliferation and maturation will help to clarify the pathogenesis and prognosis of several hematologic diseases that are accompanied by anemia resulting from the abnormal production of erythroid cells. Such insights should lead to improvements in therapeutic approaches for these conditions. Most of these diseases, which include myelodysplastic syndrome and acute erythroleukemia, are still too difficult to manage, and specific treatments remain to be developed. This situation prompted us to elucidate the pivotal genes that control the growth and proliferation of erythroid cells.

To determine novel essential genes involved in this process, we performed studies using UT-7/Epo, an erythropoietin-dependent human erythroleukemic cell line [1]. Based on our previous research, we examined candidate genes with potential roles in erythroid growth and maturation by delivering genes from a human fetal

TK and TI contributed equally to this work.

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liver–derived Entry complementary DNA (cDNA) library into UT-7/Epo cells, using a lentiviral system [2,3]. After identifying eight candidate genes in a colony-forming assay, we focused on two potential candidate genes, ribosomal protein L11 (*RPL11*) and retinol dehydrogenase 11 (*RDH11*), in subsequent experiments. Here, we demonstrated that these lentivirally transduced cells could proliferate and produce fetal Hb (γ-globin) and adult Hb (β-globin) in a culture medium that lacked Epo. Moreover, during the proliferation of these erythropoietin-independent transduced cells, the STAT5 signaling pathway was significantly upregulated relative to the levels in parental UT-7/Epo cells.

Materials and methods

Cell culture conditions

The UT-7/Epo cell line [1] was cultured in IMDM (Gibco) supplemented with 10% fetal bovine serum and 1 U/mL human recombinant Epo (R&D Systems, Minneapolis, MN) at 37°C in 5% CO_2.

Screening for candidate genes in erythropoiesis

The process of screening candidate genes involved in erythropoiesis was performed as previously described [2]. In brief, 8 candidate genes with full-length insertions in transduced cells were selected from our previous report. cDNA from each gene was cloned into the pCSII-EF-RfA-IRES2-Venus lentiviral vector (kindly provided by H. Miyoshi, RIKEN, Tsukuba, Japan) using Gateway Clonase Enzyme Mix (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing. Specific lentiviral supernatant was produced from 293T cells and used to transduce UT-7/Epo cells. Cells transduced with each of the 8 lentiviruses were cultured in methylcellulose (Nacalai Tesque, Kyoto, Japan) without Epo for 1 month before analysis.

Hematopoietic colony formation assay

A total of 1×10^4 colony-derived cells were collected and seeded into 1 mL of methylcellulose using a 2.5-mL syringe and an 18G needle. The mixture of cells and methylcellulose was dispensed into 35 × 10 mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) at 1 mL per dish. Dishes were gently tilted and rotated to distribute the methylcellulose evenly, and then 3 mL of sterile water were added into an extra uncovered dish before incubation for 1 month at 37°C and 5% CO_2. Colonies in each dish were counted at day 30 and then picked, cytospun onto glass slides, and stained with May–Grunwald Giemsa solution for microscopic observation. Photographs of colonies were taken using a microscope equipped with the AxioVision software (Zeiss, Oberkochen, Germany).

Western blotting

Transduced cells, including UT-7/Epo cells, were collected at 24, 48, and 72 hours. Cells were lysed with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 1.0% NP-40. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Whole-cell extracts (5 μg/lane) were subjected to 12.5% SDS - polyacrylamide gels, and protein was transferred to PVDF membranes (Bio-Rad, Hercules, CA). The immunoreaction was performed by incubating the membrane for 1 hour at room temperature (RT) with primary antibodies as follows: mouse antihuman BCL-XL (Santa Cruz Biotechnology; dilution, 1:200), mouse anti-human BCL-2 (Santa Cruz Biotechnology; dilution, 1:200), or mouse anti–β-actin (C4, sc-47778, Santa Cruz Biotechnology; dilution, 1:1,000). Membranes were incubated at RT for 1 hour with HRP -conjugated secondary antibody: antimouse IgG Ab (sc-2005, Santa Cruz Biotechnology; dilution of 1:10,000). Antigen–antibody reactions were detected using the enhanced chemiluminescence assay (Amersham Biosciences, Piscataway, NJ). Western blots were analyzed on an LAS3000 (Fuji Film Co., Tokyo, Japan).

Gene expression analysis by quantitative RT-polymerase chain reaction (PCR)

To determine the expression of STAT5 regulated genes, RNAs were extracted from UT-7/Epo and RPL11- and RDH11-transduced cells at day 3 using the RNeasy Mini kit QIAGEN (QIAGEN, Hilden, Germany). Concentration of RNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) before proceeding to cDNA synthesis with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Expression of *PIM2* and *CCND1* was analyzed using the Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems/Life Technologies, Grand Island, NY). For detection of *PIM2*, the forward primer was 5′-TGGGCATCCTCCTCTATGAC-3′, and the reverse primer was 5′-GTACATCCTCGGCTGGTGTT-3′. For *CCND1*, the forward primer was 5′-GATCAAGTGTGACCCGGACT-3′, and the reverse primer was 5′-TCCTCCTCTTCCTCCTCCTC-3′. The PCR mixture was as follows: 10 μL Fast SYBR Green master mix (Applied Biosystems), 0.2 μL forward primer (10 μmol/L), 0.2 μL reverse primer (10 μmol/L), 1.0 μL cDNA, and 8.6 μL dH_2O. The PCR conditions were as follows: 95°C for 20 sec (holding stage); 40 cycles of 95°C for 3 sec and 60°C for 30 sec (cycling stage); and 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec (melting curve stage).

To confirm *Bcl-xL* gene expression in RPL11- and RDH11-transduced cells, quantitative RT-PCR was performed using the following primers: h*Bcl-xL* forward: 5′-CTGCCTCACTTCCTAC AAGAGC-3′ and h*Bcl-xL* reverse: 5′-CTGAGGTAGGGAAG ACCCTG-3′. In brief, RNAs were extracted from RPL11- and RDH11-transduced cells and UT7/Epo cells at 24, 48, and 72 hours before converting to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). PCR mixture was: 5 μL Fast SYBR Green master mix (Applied Biosystems), 0.1 μL Bcl-xL forward and reverse primers (10 μmol/L), or 0.1 μL GAPDH forward and reverse primers (5 μmol/L), 1.0 μL cDNA, and 3.8 μL dH_2O. The PCR was performed as above.

Cell proliferation assay

To determine the growth and proliferation of UT-7/Epo and RPL11- and RDH11-transduced cells, proliferation assays were performed using Cell Count Reagent SF (Nacalai Tesque). Briefly, each cell line was seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL culture medium, with or without Epo. After growth for 2, 4, and 6 days, 10 μL of Cell Count Reagent SF was added to each well and incubated for 1 hour at 37°C in 5% CO_2. Absorbance at 450 nm (ref. 650 nm) was recorded using a

microplate reader (Thermo Scientific). The experiments were performed in triplicate, and data were analyzed by plotting the corrected absorbance at 450 nm on the y axis and time points on the x axis.

For detection of growth factors produced in an autocrine manner, culture media from RPL11- and RDH11-transduced cells at 48 hours were collected and filtered through 0.22 μm syringe filter before used. The erythropoietin levels of these collected culture media were measured by LSI Medience Corporation (Tokyo, Japan). The UT7/Epo cells deprived of Epo were cultured with medium collected from RPL11- or RDH11-transduced cells for 2, 4, and 6 days before assessment of cell proliferation using Cell Count Reagent SF, as described above.

Determination of STAT5 signaling pathway involving in cell proliferation using STAT5 inhibitor

To determine whether STAT5 signaling pathway was involved in cell proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor (573108, Merck Millipore, Darmstadt, Germany) was added in culture medium for inhibition of cell growth [4]. Drug was dissolved with dimethyl sulfoxide (DMSO, Nacalai, Japan), diluted with medium, and used at the final concentrations of 100 and 200 μmol/L with 0.1% DMSO in cell proliferation assay.

UT7/Epo with Epo and RPL11- and RDH11-transduced cells were cultured in medium with STAT5 inhibitor at the final concentrations of 100 and 200 μmol/L for 12 hours. After washing the treated cells with PBS, cells were seeded into 96-well flat-bottom plates at 1×10^3 cells/well in 100 μL drug-free medium. Untreated cells were used as the control group. Cells were cultured until days 2, 4, and 6 before analysis using Cell Count Reagent SF as mentioned earlier.

Flow-cytometry analysis for intracellular Hb expression

UT-7/Epo and RPL11- and RDH11-transduced cells were cultured in medium with or without Epo for 2 days before analysis of intracellular Hb expression. Cells were collected and fixed with cold 0.05% glutaraldehyde for 10 min at RT. After washing with PBS-0.1% BSA, cells were permeabilized for 5 min at RT with 0.1% Triton X-100 and then blocked with PBS-BSA. Cells were incubated at RT for 15 min with diluted primary antibody in 0.1% BSA in PBS: F-APC–conjugated mouse anti-Hb (Invitrogen; dilution, 1:17) or β-PerCP-Cy5.5–conjugated mouse anti-Hb (Santa Cruz Biotechnology; dilution, 1:200). Antibody-stained cells were analyzed on a FACSCalibur (BD Biosciences) using the CellQuest software.

Cell-cycle analysis

UT-7/Epo and RPL11- and RDH11-transduced cells were seeded in 12-well plates at 2×10^5 cells/well and incubated at 37°C for 24, 48, or 72 hours in medium with or without Epo. At each time point, cells were collected, washed with PBS, and fixed with cold 70% ethanol for 10–14 hours. Cells were incubated with FITC -conjugated anti-BrdU (BD Biosciences) for 30 min, and then treated with RNase A (Nacalai Tesque) and 7-AAD (BioLegend, San Diego, CA) to exclude nonviable cells. The cell-cycle profile (i.e., the proportions of cells at G_0/G_1, S, and G_2/M phases, as well as apoptotic cells) was analyzed on a FACSCalibur with the CellQuest software.

Phosphokinase array for analysis of signaling pathways in transduced cells

To identify the signal transduction pathways activated in transduced cells, samples were analyzed using the Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN). In brief, cells were cultured with and without Epo for 12 hours, and cell lysates were prepared using the lysis buffer provided in the kit. Then, the provided membranes were blocked with Array Buffer 1 prior to incubation with cell lysates. After overnight incubation at 2–8°C, membranes were washed, and specific kinases were detected using Detection Antibody Cocktail A and B, provided in the kit. Membranes were washed and probed with Streptavidin-HRP (BD Biosciences) before being analyzed using an LAS3000 (Fuji Film Co., Tokyo, Japan). Pixel densities were measured using a transmission-mode scanner and image analysis software.

To focus particularly on STAT5 signaling pathway involved in the growth and proliferation of RPL11- and RDH11-transduced cells, STAT5 inhibitor at final concentration of 100 μmol/L was added into culture medium of all cell lines. After 12 hours, samples were prepared and assayed as above.

Immunocytochemical detection for CREB, Lyn, and JAK2 phosphorylation

To determine the phosphorylation of CREB, Lyn, and JAK2, cells were cultured with or without Epo. After 12 hours, RPL11- and RDH11-transduced cells and UT7/Epo cells were harvested, cytospun at 450 rpm for 5 min, and let dry for 2 hours at RT. Cells were fixed with 1% paraformaldehyde in PBS for 10 min at RT. After washing with ice cold PBS for 3 times, cells were permeabilized and blocked using 0.05 % Triton X-100 in 1% BSA/PBS for 30 min. Cells were then incubated with diluted primary antibody: mouse antihuman phospho-CREB (dilution 1:25, R&D Systems, UK), rabbit antihuman phospho-JAK2 (dilution 1:50, abcam, Cambridge, UK), mouse antihuman phospho-Lyn (dilution 1:25, R&D Systems, UK) in blocking buffer at 4°C overnight. After washing 3 times with PBS, cells were incubated with secondary antibody: Alexa Fluor 647 donkey antimouse (dilution 1:500; Life Technologies), Alexa Fluor 647 donkey antirabbit (dilution 1:500, Invitrogen) for 30 min at RT in the dark. Mounting and fixing were performed using VECTASHIELD with DAPI (Vector Laboratories, Inc., CA) before analysis, followed by the observation using fluorescence imaging with Olympus Ix81 Inverted Microscope ().

In addition, Hela cells treated with 200 nmol/L PMA (phorbol 12-myristate 13-acetate, Sigma) for 2 hours were used as positive control to detect CREB phosphorylation. One mmol/L of Pervanadate was prepared from Sodium orthovanadate (Sigma) and hydrogen peroxide (Nacalai Tesque) diluted with PBS as previously described [5]. Jurkat cells and Hela cells treated with 1 mmol/L Pervanadate were respectively used as positive control for JAK2 phosphorylation and Lyn phosphorylation.

Statistical analysis

Data are shown as means ± SEM. A p value <0.05 was considered to represent statistical significance.