Table 2

Heritability estimates for severity of neuropathy captured by SNPs in subsets of the GO Axonogenesis set

	Heritability Estimates					Pathway Characteristics		
GO Axonogenesis Children	V(G)/V(p)[1]	SE	P[2]	Padj[3]	Empirical P[4]	# Genes	Size (Mb)	#SNPs
Axonal Fasciculation	0.000	0.025	0.5	1	0.999	15	2.89	922
Peripheral Neuron Axonogenesis	0.005	0.010	0.3	1	0.203	2	0.13	15
Axon Guidance	0.000	0.019	0.5	1	0.999	362	57.51	699
Axonogenesis in Innervation	0.011	0.015	0.2	1	0.146	3	0.15	19
Axon Regeneration	0.000	0.013	0.5	1	0.999	29	3.31	314
CNS Neuron Axonogenesis	0.051	0.031	0.020	0.2	0.028	26	6.32	935
Axon Extension	0.097	0.050	0.020	0.2	0.003	70	8.88	1,862
Regulation of Axonogenesis	0.130	0.059	0.009	0.09	0.001	104	20.85	3,239
Collateral Sprouting	0.012	0.019	0.3	1	0.26	13	3.10	396
Axon Target Recognition	0.000	0.010	0.5	1	0.999	4	0.27	34

[1] Heritability was estimated for sets of SNPs within ±10 kb of genes in children (subsets) of the GO Axonogenesis set.

[2] P-value from GCTA. Software upper limit for p-value is 0.5; maximal values are noted as 1.

[3] P-value corrected for ten observations.

[4] P-value from permutation analysis.

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CYP2D6 Genotype and Adjuvant Tamoxifen: Meta-Analysis of Heterogeneous Study Populations

MA Province[1], MP Goetz[2], H Brauch[3], DA Flockhart[4], JM Hebert[5], R Whaley[5], VJ Suman[6], W Schroth[3], S Winter[3], H Zembutsu[7], T Mushiroda[8], WG Newman[9], M-TM Lee[10], CB Ambrosone[11], MW Beckmann[12], J-Y Choi[13], A-S Dieudonné[14], PA Fasching[12,15], R Ferraldeschi[9], L Gong[5], E Haschke-Becher[16], A Howell[17], LB Jordan[18], U Hamann[19], K Kiyotani[8], P Krippl[20], D Lambrechts[21], A Latif[9], U Langsenlehner[20], W Lorizio[22], P Neven[23], AT Nguyen[4], B-W Park[24], CA Purdie[18], P Quinlan[25], W Renner[20], M Schmidt[3,26], M Schwab[27], J-G Shin[28,29], JC Stingl[30], P Wegman[31], S Wingren[31], AHB Wu[32], E Ziv[22], G Zirpoli[11], AM Thompson[25], VC Jordan[33], Y Nakamura[7], RB Altman[5,34], MM Ames[35], RM Weinshilboum[35], M Eichelbaum[3], JN Ingle[36] and TE Klein[5]; on behalf of the International Tamoxifen Pharmacogenomics Consortium

The International Tamoxifen Pharmacogenomics Consortium was established to address the controversy regarding cytochrome P450 2D6 (*CYP2D6*) status and clinical outcomes in tamoxifen therapy. We performed a meta-analysis on data from 4,973 tamoxifen-treated patients (12 globally distributed sites). Using strict eligibility requirements (postmenopausal women with estrogen receptor–positive breast cancer, receiving 20 mg/day tamoxifen for 5 years, criterion 1), CYP2D6 poor metabolizer status was associated with poorer invasive disease–free survival (IDFS: hazard ratio = 1.25; 95% confidence interval = 1.06, 1.47; $P = 0.009$). However, *CYP2D6* status was not statistically significant when tamoxifen duration, menopausal status, and annual follow-up were not specified (criterion 2, $n = 2,443$; $P = 0.25$) or when no exclusions were applied (criterion 3, $n = 4,935$; $P = 0.38$). Although *CYP2D6* is a strong predictor of IDFS using strict inclusion criteria, because the results are not robust to inclusion criteria (these were not defined *a priori*), prospective studies are necessary to fully establish the value of *CYP2D6* genotyping in tamoxifen therapy.

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Tamoxifen, the pioneering antiestrogenic medicine targeted to the tumor estrogen receptor (ER), is used successfully for long-term adjuvant therapy in breast cancer.[1,2] Extensive analyses of clinical trials demonstrate a major increase in patient survivorship in ER-positive patients. In this age of personalized medicine, any opportunity to improve response rates with tamoxifen should be rigorously investigated. Tamoxifen is considered a prodrug, given that hepatic cytochrome P450 2D6 (CYP2D6) metabolizes tamoxifen to metabolites (4-hydroxy tamoxifen and 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen)) that exhibit significantly greater potency in terms of ER-binding affinity[3] and suppression of estradiol-stimulated cell proliferation.[4] CYP2D6-mediated metabolism is the rate-limiting enzymatic step for the formation of endoxifen, the most abundant active metabolite.

There has been great inconsistency among studies that have reported the association of known genetic and drug factors influencing CYP2D6 enzyme activity with tamoxifen efficacy. Therefore, the International Tamoxifen Pharmacogenomics Consortium (ITPC) was conceived, and researchers were invited to submit their data—both published and unpublished data sets regarding *CYP2D6* genetic variants and clinical outcomes in women treated with tamoxifen in the adjuvant breast cancer setting—to allow a meta-analysis of the potential associations between *CYP2D6* and clinical outcomes.

RESULTS

The ITPC comprises 12 research projects from nine countries and three continents that contributed clinical and genetic data for a total of 4,973 breast cancer patients treated with tamoxifen. In **Table 1**, we show the sample size by site and criteria. Further details for each site are shown in **S3c and S5 online**. We reported preliminary analyses of these collected cohorts before complete curation by pooling the data from each site.[5] For our meta-analyses, three detailed criteria, which ranged from the most restrictive (criterion 1) to the most inclusive (criterion 3), were defined before final curation (see **S4** online). In brief, criterion 1, derived from the NCCTG 89-30-52 clinical trial, consisted of postmenopausal women with surgically resected nonmetastatic invasive ER-positive breast cancers who received adjuvant tamoxifen monotherapy at a dose of 20 mg/day for an intended duration of 5 years, and were followed at least annually for recurrence. In addition, analysis of at least *CYP2D6*4* was required (detailed in **S4a** online). Criterion 2 included criterion 1 but allowed both pre- and postmenopausal patients who had received any duration of tamoxifen; moreover, annual follow-up was not required. Criterion 3 included all samples not excluded by any exclusion test for missing data or data inconsistencies (least restrictive). Patient characteristics according to each criterion are provided in **Table 2**.

The meta-analysis results combining the hazard ratio (HR) estimates (and the corresponding standard errors (SEs)) from each site are shown for all three criteria groups and both clinical outcomes in **Table 3**. For each of the six clinical outcome/criteria groups, we give the combined meta-analysis estimate across all 12 sites, its SE, and the results of two statistical tests: a test of the significance that the meta-HR differs from 1 and a test of "homogeneity of the estimates" across sites (a significant value for the latter test indicates that there is more variability than the derSimonian and Laird random-effects model can reasonably accommodate, suggesting that the meta-estimate and its associated *P* value are suspect). As can be seen for invasive disease–free survival (IDFS), the meta-analyses for criteria 2 and 3 are nearly significantly heterogeneous, whereas there was no indication of heterogeneity for criterion 1 ($P = 0.899$). For patients meeting criterion 1, the meta-HR for IDFS was 1.25 (95% confidence interval = 1.06, 1.47), and for breast cancer–free interval, it was 1.27 (95% confidence interval = 1.01, 1.61). These are both statistically significant, at $P = 0.009$ and $P = 0.04$, respectively. However, for the criterion 2 ($P = 0.25$) and criterion 3 ($P = 0.38$) subsets, the *CYP2D6* HR was not significant for either outcome.

In **Figure 1**, we show the individual HRs for each site for subjects meeting criterion 1, assuming an additive genetic model for *CYP2D6* (coded 0 = extensive metabolizer (EM), 1 = intermediate metabolizer (IM), and 2 = poor metabolizer (PM)) as estimated from a Cox proportional-hazards model using additional risk covariates to predict clinical outcome. Corresponding figures for criteria 2 and 3 are provided in **S6** online. (Note that the list of covariates used in the Cox models included age at primary diagnosis, menopause status at diagnosis, metastatic disease at primary diagnosis, maximum tumor dimension, number of positive nodes, grade, smoking status, ER and progesterone receptor status, intended tamoxifen dose and duration, systemic therapy before surgery, chemotherapy, radiation treatment, adjuvant aromatase inhibitor therapy, and additional hormone therapy. The specific set of covariates used for each site was chosen from this list so as to retain at least 70% of the patients from that site; hence, the exact set of covariates used differs in each site's Cox model. Moreover, several of these covariates were used as inclusion/exclusion items in the basic definitions of the three basic criteria subset groups and thus became irrelevant for those analyses.)

Table 1 Sample size by site and criteria

	Site (*N*)												
Criterion	1	2	3	4	5	6	7	8	9	10	11	12	Total
1	0	70	124	60	212	243	0	847	5	222	179	34	1,996
2	0	127	208	98	212	304	0	898	10	289	228	69	2,443
3	174	320	282	265	214	391	801	1,140	165	516	397	270	4,935
Total	174	320	282	267	214	423	801	1,140	165	519	398	279	4,973

Table 2 Baseline patient and tumor characteristics

Characteristic	Criterion 1 (1,996 patients)		Criterion 2 (2,443 patients)		Criterion 3 (4,935 patients)	
Age at diagnosis, years: data reported in binned ages						
Median	[65–69]		[60–64]		[60–64]	
Range	55 (ages 41–95)		75 (ages 21–95)		76 (ages 21–96)	
Menopausal status —no. (%)	Menopausal status	By age	Menopausal status	By age	Menopausal status	By age
Premenopausal (age ≤ 50)	0 (0.0%)	54 (2.7%)	241 (9.9%)	414 (16.9%)	607 (12.3%)	1,207 (24.5%)
Postmenopausal (>50)	1,688 (84.6%)	1,922 (96.3%)	1,714 (70.2%)	1,997 (81.7%)	3,267 (66.2%)	3,642 (73.8%)
Not available	308 (15.4%)	20 (1.0%)	488 (20.0%)	32 (1.3%)	1,061 (21.5%)	86 (1.7%)
Tumor size—no. (%): maximum dimension of tumor reported (if multiple tumors, largest one is ≤2 cm)						
≤2 cm	1,071 (53.7%)		1,327 (54.3%)		2,303 (46.7%)	
>2 cm	752 (37.7%)		882 (36.1%)		2,182 (44.2%)	
Unknown	173 (8.7%)		234 (9.6%)		450 (9.1%)	
Nodal status—no. (%): number of positive nodes						
Zero nodes	1,243 (62.3%)		1,531 (62.7%)		2,423 (49.1%)	
1–3 nodes	407 (20.4%)		461 (18.9%)		1,281 (26.0%)	
4–9 nodes	103 (5.2%)		111 (4.5%)		438 (8.9%)	
> 9 nodes	43 (2.2%)		45 (1.8%)		185 (3.7%)	
Not available	200 (10.0%)		295 (12.1%)		608 (12.3%)	
Grading—no. (%): 0.5 to 1.49 considered G1, 1.5 to 2.49 G2, etc.						
G1	249 (12.5%)		317 (13%)		456 (9.2%)	
G2	1,148 (57.5%)		1,324 (54.2%)		1,965 (39.8%)	
G3	330 (16.5%)		398 (16.3%)		838 (17.0%)	
Unknown	269 (13.5%)		295 (12.1%)		1,676 (34.0%)	
ER status—no. (%)						
ER-positive	1,996 (100.0%)		2,443 (100.0%)		4,675 (94.7%)	
ER-negative	0 (0.0%)		0 (0.0%)		158 (3.2%)	
Unknown	0 (0.0%)		0 (0.0%)		102 (2.1%)	
PgR status—no. (%)						
PgR-positive	1,479 (74.1%)		1,847 (75.6%)		3,634 (73.6%)	
PgR-negative	273 (13.7%)		302 (12.4%)		665 (13.5%)	
Unknown	244 (12.2%)		294 (12.0%)		102 (2.1%)	
Radiotherapy—no. (%): radiation therapy						
Yes	1,138 (57.0%)		1,412 (57.8%)		2,868 (58.1%)	
No	720 (36.1%)		842 (34.5%)		1,507 (30.5%)	
Unknown	244 (12.2%)		189 (7.7%)		560 (11.3%)	
CYP2D6 metabolizer status						
Extensive	893 (44.7%)		1,077 (44.1%)		2,286 (46.3%)	
Intermediate	985 (49.3%)		1,230 (50.3%)		2,311 (46.8%)	
Poor	118 (5.9%)		136 (5.6%)		244 (4.9%)	
Unknown	0 (0.0%)		0 (0.0%)		94 (1.9%)	
CYP2D6 metabolizer types						
EM/UM	17 (0.9%)		23 (0.9%)		49 (1.0%)	
IM/UM	2 (0.1%)		2 (0.1%)		4 (0.1%)	
EM/EM	874 (43.8%)		1,052 (43.1%)		2,233 (45.2%)	
PM/UM	7 (0.4%)		7 (0.3%)		12 (0.2%)	

Table 2 Continued on next page

Table 2 Continued

Characteristic	Criterion 1 (1,996 patients)	Criterion 2 (2,443 patients)	Criterion 3 (4,935 patients)
EM/IM	327 (16.4%)	407 (16.7%)	693 (14.0%)
EM/PM	496 (24.8%)	616 (25.2%)	1,230 (25.1%)
IM/IM	64 (3.2%)	94 (3.8%)	174 (3.5%)
IM/PM	91 (4.6%)	106 (4.3%)	192 (3.9%)
PM/PM	118 (5.9%)	136 (5.6%)	244 (4.9%)
Unknown	0 (0.0%)	0 (0.0%)	94 (1.9%)
DNA source			
Blood	996 (49.9%)	1,344 (55.0%)	2,513 (50.9%)
Tumor—Frozen	431 (21.6%)	500 (20.5%)	1,575 (31.9%)
Tumor—FFPE	569 (28.5%)	598 (24.5%)	659 (13.4%)
Normal—FFPE	0 (0.0%)	0 (0.0%)	174 (3.5%)
Unknown	0 (0.0%)	1 (0.0%)	14 (0.3%)

CYP2D6, cytochrome P450 2D6; EM, extensive metabolizer; ER, estrogen receptor; FFPE, formalin-fixed–paraffin-embedded; IM, intermediate metabolizer; PgR, progesterone receptor; PM, poor metabolizer; UM, unknown metabolizer.

Table 3 Meta-analyses of CYP2D6 HRs on clinical outcome in inclusion/exclusion criteria subsets

	IDFS				BCFI			
	Meta-estimates		*P* value		Meta-estimates		*P* value	
	HR	95% CI	Homog[a]	Association[b]	HR	95% CI	Homog[a]	Association[a]
Criterion 1	1.25	(1.06,1.47)	0.899	0.009	1.27	(1.01,1.61)	0.858	0.041
Criterion 2	1.17	(0.90,1.52)	0.055	0.249	1.21	(0.889,1.65)	0.130	0.224
Criterion 3	1.07	(0.92,1.26)	0.099	0.382	1.10	(0.868,1.35)	0.114	0.352

BCFI, breast cancer–free interval; Homog, homogeneity; HR, hazard ratio; IDFS, invasive disease–free survival; ITPC, International Tamoxifen Pharmacogenomics Consortium.

[a]The homogeneity *P* value tests the hypothesis that the individual ITPC site estimates meet the statistical random-effects modeling assumptions of the meta-analysis. A significant value indicates that there is significant heterogeneity among the sites, which casts doubt on the "combinability" of the studies for that parameter and on the validity of the corresponding association test. [b]The association *P* value tests the hypothesis that the combined meta-analysis estimate of the HR is significantly different from the null hypothesis value of HR = 1.

Site-specific product-limit estimates of the three CYP2D6 metabolizer status genotype groups (EM, IM, and PM) are shown in **Figures 2** and **3** for criterion 1 patients. Sites 1 and 7 had no subjects who met inclusion/exclusion for criterion 1. The corresponding figures for patients meeting criteria 2 and 3 are shown in **S6** online. As seen in **Figure 2**, for IDFS sites, 3, 5, and 8 show a strong significant effect in the direction expected by the known pharmacokinetic effects of *CYP2D6* on endoxifen exposure, namely, a poorer clinical response for the IM and/or PM genotype groups. Other sites show a trend in the expected direction between the IM and EM groups, but the much smaller PM group is often inconsistent with the expectation, and the separation in the three survival curves is not strong enough to reach statistical significance (e.g., sites 6 and 12). For some sites, there is no hint of any significant difference (e.g., sites 2, 4, 10, and 11), and for one of these, site 2, the direction of effect is exactly opposite than expected. There is a danger in overinterpreting such "trends" (either in favor or against expectation) when there is no statistically significant difference, because some level of site-to-site variation is to be expected. The key question is not whether such variation exists but whether it centers over the null hypothesis or over the alternative; this is the question that the meta-analysis is designed to answer. However, these simple product-limit survival curves show great study-to-study heterogeneity, which complicates both the analyses and the interpretation. We have similar heterogeneous results for the breast cancer–free interval outcome, shown in **Figure 3**. The corresponding figures in **S6** online show a similar pattern for the subsets of patients meeting criteria 2 and 3, although the heterogeneity seems to be even more pronounced as the exclusion criteria are loosened. This is not a surprising result, considering that the criteria themselves impose a certain level of homogeneity.

DISCUSSION

Prospective pharmacology studies consistently demonstrate that *CYP2D6* genetic variants are associated with variable plasma concentrations of endoxifen.[4,6] Endoxifen exposure is related to duration of tamoxifen use and dose, wherein an increase in the tamoxifen dose (from 20 to 40 mg daily) significantly increases endoxifen exposure in patients with reduced or null CYP2D6 metabolism but not in CYP2D6 EMs.[7] However, coadministration of CYP2D6-inhibiting drugs[4] reduces CYP2D6 enzyme activity, and nonadherence to tamoxifen is more commonly observed in patients with normal or increased CYP2D6 metabolism.[8]

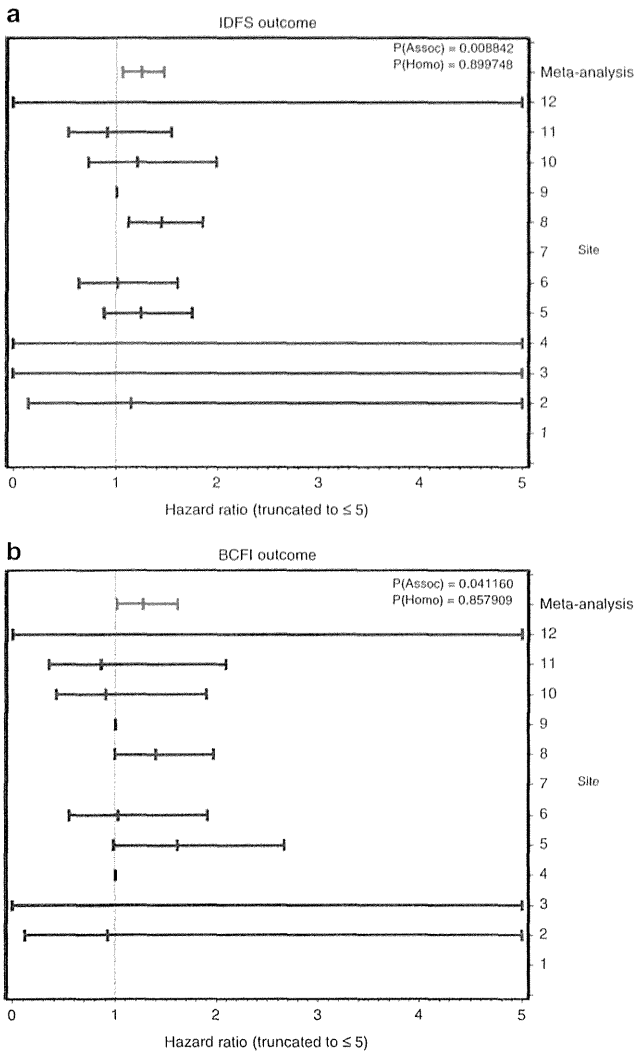

Figure 1 Individual site estimates of hazard ratios of *CYP2D6* genotype on clinical outcome, along with the meta-analyses for the criterion 1 subset. (**a**) Invasive disease–free survival (IDFS) outcome. (**b**) Breast cancer–free interval (BCFI) outcome.

Despite the consistent pharmacogenetic effects of *CYP2D6* on endoxifen exposure, there is considerable controversy regarding the validity of *CYP2D6* as a predictor of tamoxifen outcome.[9,10] Although recent secondary analyses from the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial and the Breast International Group (BIG) 1-98 study[11,12] did not demonstrate an association between *CYP2D6* and tamoxifen outcome, these studies provoked criticism due to concerns regarding genotyping error and the analysis of small subsets of the main trials.[13–16]

By contrast, a secondary analysis from another large prospective adjuvant tamoxifen trial, the Austrian Breast and Colorectal Cancer Study Group 8 (ABCSG 8), demonstrated that for women treated with 5 years of adjuvant tamoxifen at a dose of 20 mg/day, CYP2D6 PMs had a statistically significant higher odds of recurrence or death as compared with CYP2D6 EMs, and CYP2D6 PMs/IMs and PMs/EMs tended to exhibit a higher odds of recurrence as compared with patients without the PM alleles. However, this effect was not observed for patients who had switched to anastrozole, a drug not metabolized by CYP2D6. These data suggest that the effects of *CYP2D6* genotype may be masked if patients receive a shorter duration of tamoxifen or other active drugs besides tamoxifen, which alter the hazard for recurrence.[17]

We approached the tamoxifen controversy by performing a global meta-analysis of available clinical and *CYP2D6* genetic data of tamoxifen-treated breast cancer patients. All groups from across the world with both published and unpublished *CYP2D6* data were invited to participate. We initially presented a pooled analysis of these data,[5] in which we found no association between *CYP2D6* and IDFS. Following this presentation, we developed a new analysis plan (not defined before the initial negative presentation), which included the following: (i) articulation of three criteria to analyze the data according to the quality of the genetic and clinical data, (ii) additional curation to obtain missing clinical and genetic data, and (iii) a new statistical analysis plan, which applied a random-effects meta-analysis strategy instead of a pooled analysis strategy. Notably, Criterion 1 is most stringent, requiring strict control for as many pharmacologic factors as possible known to affect endoxifen exposure, which include use of tamoxifen monotherapy, genotyping of multiple *CYP2D6* alleles for accurate CYP2D6 phenotype assignment, use of one tamoxifen dose (20 mg), and intended duration of tamoxifen use for 5 years. In addition, eligibility for this cohort was restricted to women with invasive ER-positive status, postmenopausal breast cancer, and the requirement for annual follow-up, parameters required in any prospective clinical trial and that were requirements of criterion 1 (patients who were knowingly not followed were excluded from criterion 1), but not from criteria 2 and 3. These factors may have contributed to the substantial increase in heterogeneity comparing criterion 1 with criteria 2 and 3. However, it should be noted that these criteria impose a certain bias because the majority of negative studies submitted to the ITPC were observed in criteria 2 and 3.

In general, a substantial number of subjects comprising criterion 3 had misclassification of the predicted drug metabolism phenotype due to the lack of a comprehensive coverage of loss-of-function alleles.[18,19] More than 20 loss-of-function alleles out of 100 known *CYP2D6* genetic variants contributed to a frequency of ~8% of PMs in a population of European descent. Limiting the analysis to the most common such allele, *CYP2D6*4*, as was frequently done in the older published literature, will result in misclassification of 35% of PMs, thereby falsely assigning the undetected PMs to the EM or IM groups. Notably, 871/1,996 patients comprising criterion 1 had optimal CYP2D6 phenotype assessment obtained by AmpliChip genotyping, and this may have contributed to the robustness of criterion 1 results, which demonstrated an association between *CYP2D6* and tamoxifen treatment outcome (breast cancer–free interval: HR = 1.27, 95% confidence interval = 1.01–1.61).

The ITPC intended to perform a global study including several thousand patient samples; however, the majority of the subjects were not comprehensively genotyped because DNA was not of sufficient quality. We performed a subgroup analysis using patient samples for which full coverage of alleles by the

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

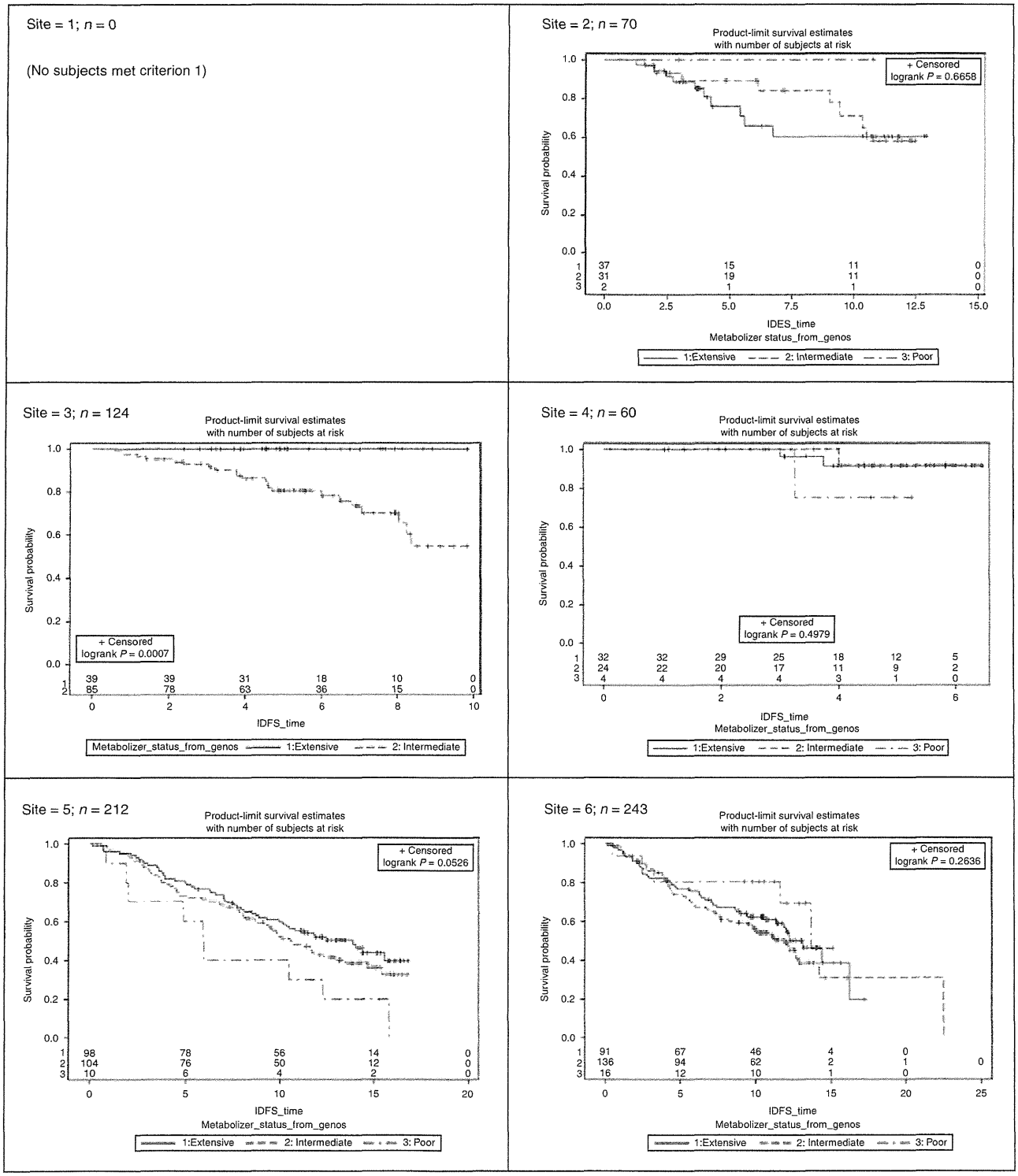

Figure 2 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = invasive disease–free survival (IDFS)).

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

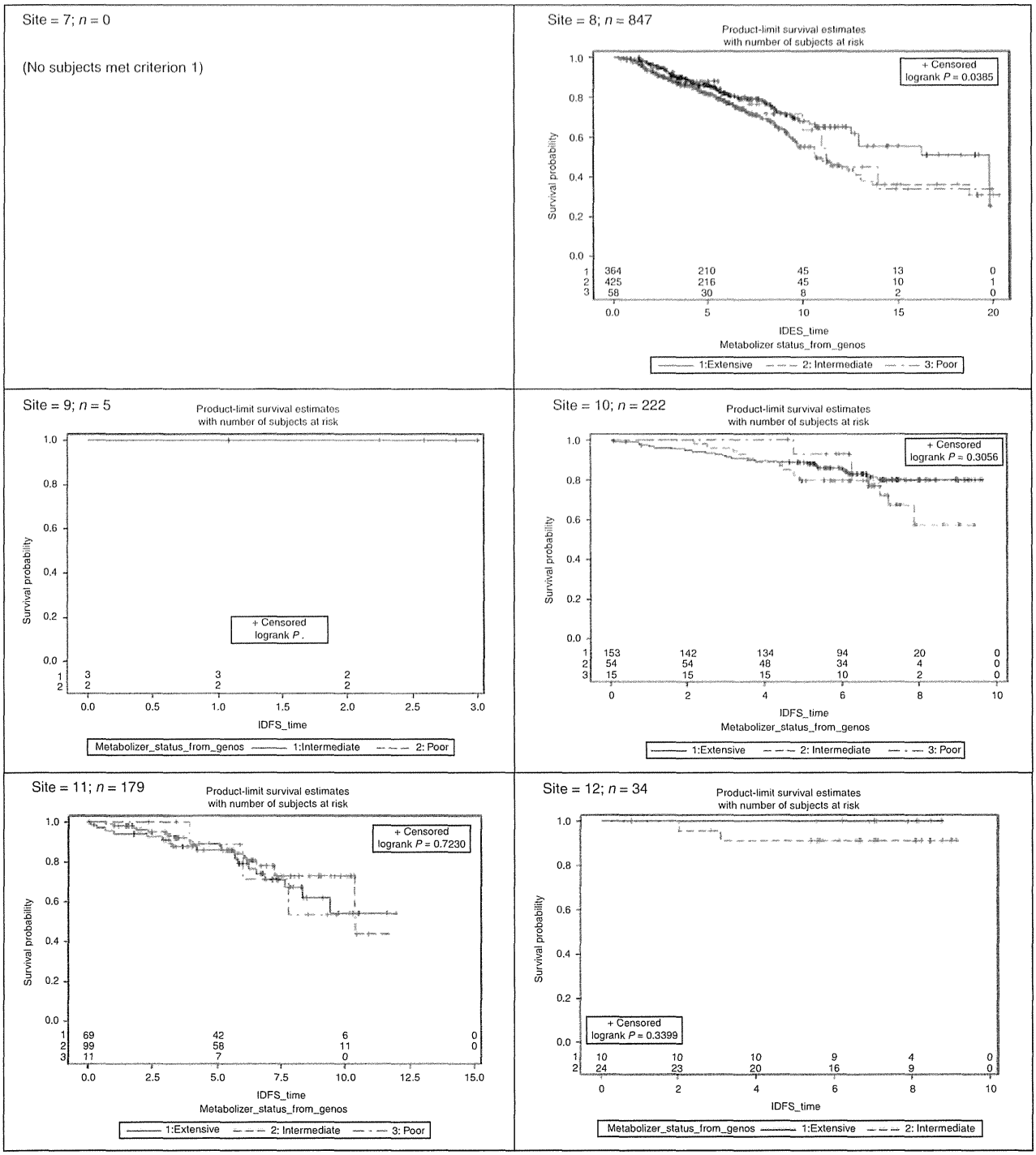

Figure 2 Continued

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

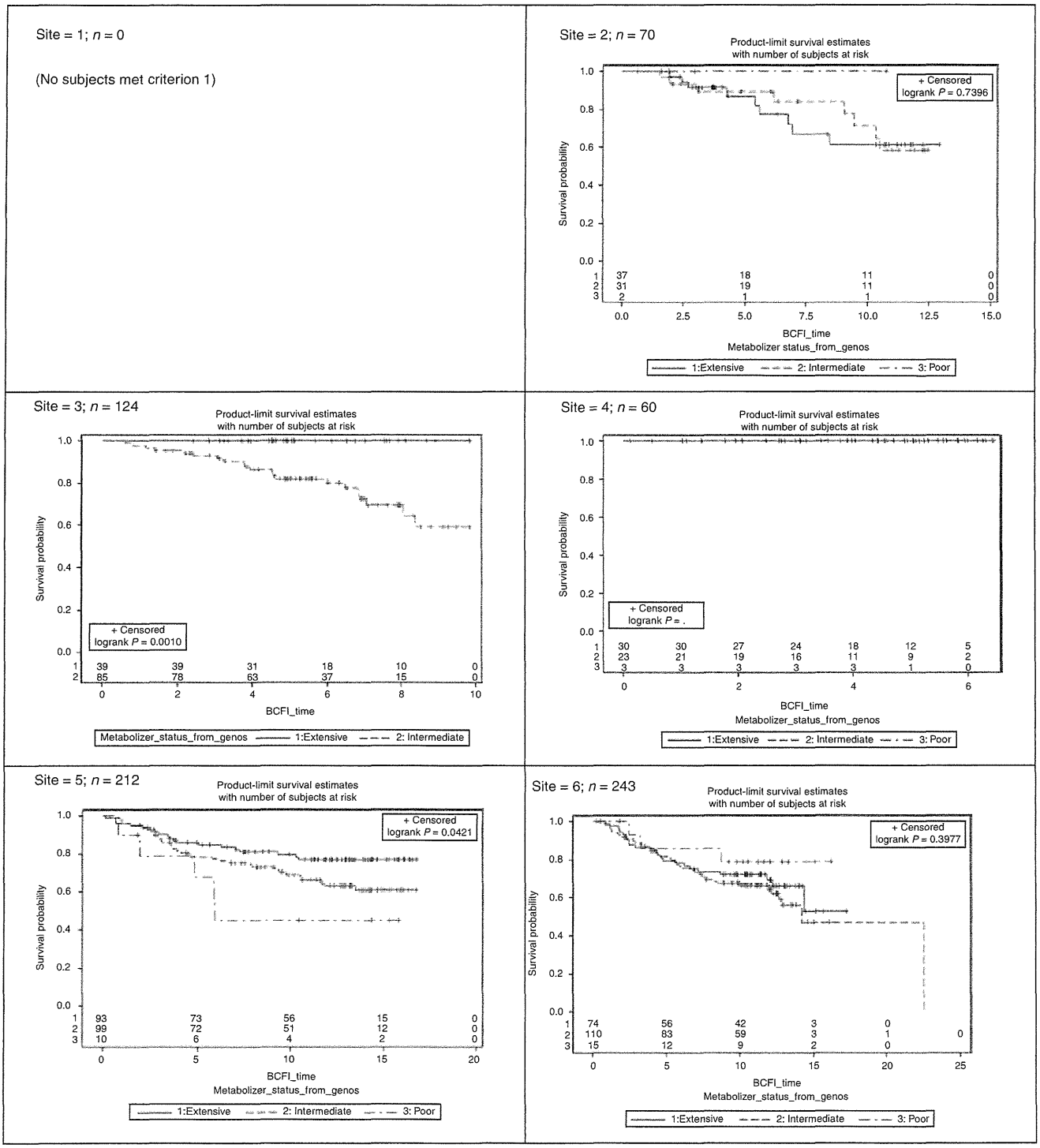

Figure 3 Site-specific effects of CYP2D6 metabolizer status on clinical outcomes for subjects meeting inclusion criterion 1 (outcome = breast cancer–free interval (BCFI)).

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

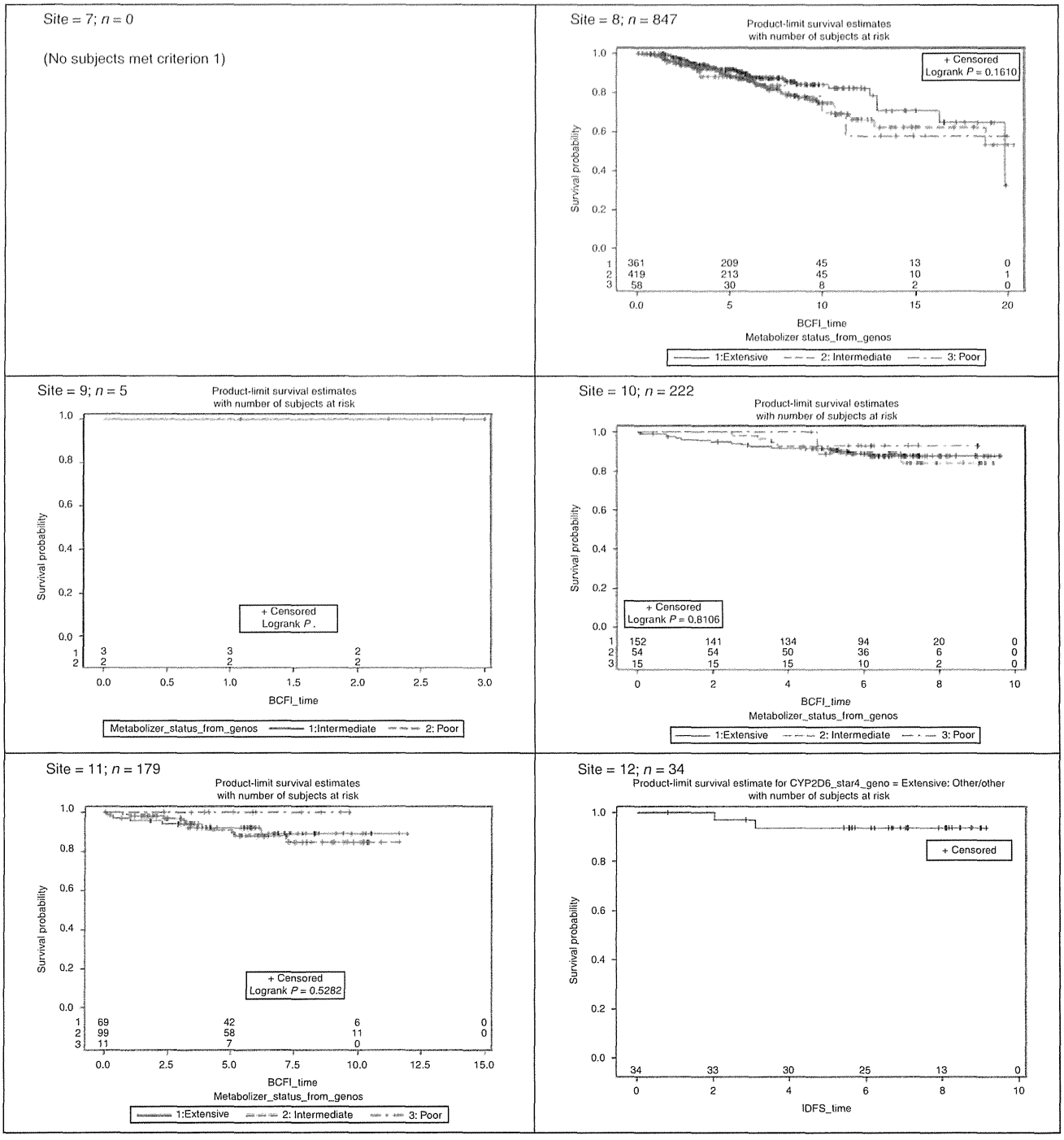

Figure 3 Continued

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

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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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RESEARCH ARTICLE

The effect of IGF-I receptor blockade for human esophageal squamous cell carcinoma and adenocarcinoma

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Abstract Insulin-like growth factor-I receptor (IGF-IR) signaling is required for carcinogenicity and tumor development, and this pathway has not been well studied in human esophageal carcinomas. Esophageal cancer is one of the human cancers with the worst prognosis and has two main histologies: squamous cell carcinomas (ESCC) and adenocarcinoma (EAC). Previously, we have reported that detection of the IGF axis may be useful for the prediction of recurrence and poor prognosis of ESCC. We have also shown the successful therapy for several gastrointestinal cancers using recombinant adenoviruses expressing dominant negative IGF-IR (ad-IGF-IR/dn). The aim of this study is to develop potential targeted therapeutics to IGF-IR and to assess the effect of IGF-IR blockade in both of these types of esophageal cancer. We determined immunohistochemical expression of IGF-IR in a tissue microarray. We then assessed the effect of IGF-IR blockade on signal transduction, proliferation, apoptosis, and motility. Ad-IGF-IR/dn, a tyrosine kinase inhibitor, BMS-536924, and adenovirus expressing shRNA for IGF-IR were used. IGF-IR expression was common in both tumor types but not in normal tissues. IGF-IR was detected in metastatic sites at similar levels compared to the primary site. IGF-IR inhibition suppressed proliferation and colony formation in both cancers. IGF-IR blockades up-regulated both stress- and chemotherapy-induced apoptosis and reduced migration. Although IGF-IR/dn blocked ligand-induced activation of Akt-1 mainly, BMS-536924 effectively blocked both activation of Akt and MAPK. The IGF axis might play a key role in tumor progression of esophageal carcinomas. The IGF-IR targeting strategies might thus be useful anticancer therapeutics for human esophageal malignancies.

Keywords Dominant negative · EAC · ESCC · IGF-IR · TKI

Abbreviations

ad-IGF-IR/482st	Adenovirus expressing IGF-IR /482st
ad-IGF-IR/950st	Adenovirus expressing IGF-IR/950st
ad-shIG F-IR	Adenovirus expressing short-hairpin IGF-IR
des(1–3)IGF-I	NH_2 terminally truncated IGF-I
dn	Dominant negative
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
ERK	Extracellular signal-regulated kinase
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IGF-IR	IGF-I receptor
IGF-IR/482st	Truncated IGF-IR of 482 amino acid long

Yasushi Adachi, Hirokazu Ohashi, and Arisa Imsumran contributed equally to this work.

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IGF-IR/950st	Truncated IGF-IR of 950 amino acid long
IGF-IR/dn	Dominant negative form of IGF-IR
InsR	Insulin receptor
mAb	Monoclonal antibody
PI3-K	Phosphatidylinositide 3-kinase
TKI	Tyrosine kinase inhibitor

Introduction

Esophageal cancer is one of the cancers with the worse prognosis worldwide [1]. At the time of diagnosis, more than half of patients have either unresectable tumors or metastatic ones. Even after a curative-intent surgical operation, the 5-year survival is still limited [2], and the therapy for unresectable esophageal carcinomas is typically minimally effective. Therefore, we must aim to seek new therapeutic options for this disease. The main types of human esophageal tumor are squamous cell carcinoma (ESCC) and adenocarcinoma (EAC).

Recently, advances in molecular research have brought new therapeutic strategies, including small molecule tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAb), into clinical testing. One group of new targets is the tyrosine kinase receptors. The insulin-like growth factor (IGF) family is a promising candidate [3, 4]. Agents targeting the IGF-I receptor (IGF-IR) pathway are moving into the clinic. Toward that end, we have studied this pathway in esophageal cancers.

IGF-IR is a heterotetramer of two α- and two β-chains [5]. Binding of the ligands IGF-I and IGF-II to IGF-IR causes receptor autophosphorylation and activates multiple signaling pathways, including ras/extracellular signal-regulated kinase (ERK) and the phosphatidylinositide 3-kinase (PI3-K)/Akt-1 axes [6]. Activation of IGF-IR is regulated by multiple factors, including IGF binding proteins (IGFBP) and IGF-2 receptor [7–9]. Elevation of serum IGF-I increases the risk of developing several cancers [10], and IGF-IR is essential for both malignant transformation and progression [3, 4]. Reduction of IGF-IR can induce apoptosis in tumors but produces only growth slowing in untransformed cells, suggesting that it might be an excellent target for therapeutic intervention [3]. IGF-IR knockout mice are viable (though physically small), indicating that relatively normal development and differentiation can occur in its absence [11]. These findings suggest a potential basis for tumor selectivity in therapeutic applications.

Human esophageal epithelial cells express IGF-IR, and IGF-I can stimulate both DNA synthesis and proliferation in these cells [12–14]. Salivary IGF-I continuously bathes the esophageal lumen and is in a free form (not bound to IGFBP, unlike the serum pool), which could enhance its binding ability to receptors on the esophageal mucosal cells [15]. These data indicate that the IGF/receptor may play important roles in homeostasis and esophageal premalignancy [14].

Both IGF-IR and IGFs are overexpressed in esophageal cancer tissues compared to normal ones [16–18]. In addition, IGFBP3 and an IGF-IR antibody suppress cancer cell proliferation [19, 20]. However, the role of the IGF axis in esophageal cancer has not been adequately studied. We reported previously that expression of IGF-IR and IGF-II were detected in 60 and 50% of ESCC, respectively, and were associated with invasion depth, metastasis, advanced tumor stage, and recurrence [21]. Patients with ESCC expressing both IGF-IR and IGF-II had a significantly shorter survival rate than those expressing either alone or neither in both single and multivariate analysis. Dominant negative for IGF-IR (IGF-IR/dn) suppressed proliferation and up-regulating chemotherapy-induced apoptosis through blocking ligand-induced Akt activation in an ESCC cell line, TE-1 [21].

In addition, there is a strong positive association between visceral obesity (metabolic syndrome) and risk of EAC, and the IGF axis is speculated to relate to both obesity and EAC [22]. IGF-IR expression in resected EAC was significantly higher in viscerally obese patients than in those of normal weight. Disease-specific survival was longer in patients with IGF-IR-negative EAC than in those with IGF-IR-positive tumors [23]. Thus, there are several lines of evidence that the IGF axis may play an important role in EAC.

There are several possible approaches to blocking IGF-IR signaling with therapeutic intent [24], including blocking the ligand or receptor using mAbs [25, 26] or TKIs [27, 28]. All of these are complicated by the high homology of this receptor to the insulin receptor (InsR). An approach that is intrinsically specific for IGF-IR is to use dominant negative or soluble IGF-IR receptor approaches to specifically inhibit the function of the wild-type receptor [29, 30]. We have constructed two different adenoviruses expressing IGF-IR/dn (ad-IGF-IR/dn) [31–34]. Ad-IGF-IR/482st encodes a truncated extracellular domain of IGF-IR (without the transmembrane domain) and thus produces a secreted protein that affects neighboring cells in addition to the transduced cells (a bystander effect). Another ad-IGF-IR/950st encodes a receptor that lacks the tyrosine kinase domain and thus remains on the membrane of the transduced cells to form non-functional receptor complexes. We have reported that ad-IGF-IR/dn may be a useful therapeutic strategy against several gastrointestinal tumors [21, 31, 32, 34, 35]. We have also reported that the adenoviral vector-based approach to express a short-hairpin inhibitory RNA of IGF-IR (ad-shIGF-IR) induced effective IGF-IR silencing in gastrointestinal cancers as manifested by effective blockade of the downstream pathway of IGF-IR and antitumor effects [36]. A dual targeting TKI for IGF-IR/InsR, BMS-536924, may have an advantage compared to a single targeting TKI,

as transformed cells can also use insulin receptor activation of similar signaling pathways for proliferation in addition to IGFR signals [35, 37].

In order to evaluate the expression of IGF-IR in EAC and in metastatic sites of ESCC, we analyzed an esophageal cancer tissue microarray immunohistochemically. To assess IGF-IR blockade for both esophageal cancers, histologies ESCC and EAC, we used several strategies including IGF-IR/dns, shIGF-IR, and BMS-536924.

Methods

Materials, cell lines, and recombinant adenovirus vectors

Anti-Akt1(c-20), anti-ERK1(K-23), anti-phospho-ERK1(E-4), ant-IGF-I(G-17), and anti-IGF-IRβ(2C8) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-Akt(Ser473) was from Cell-Signaling Technology (Beverly, MA, USA). Anti-IGF-IR(Ab-4) was from Oncogene Research Products (Cambridge, MA, USA) and anti-IGF-II was from Peninsula Laboratories (San Carlos, CA, USA). PI3-K inhibitors, wortmannin and LY294002, p38-MAPK inhibitor SB203580, cisplatin (CDDP), and 5-fluorouracil (5-FU) were purchased from Sigma (St. Louis, MO, USA), and MEK1 inhibitor PD98059 was from Cell Signaling. Recombinant human IGF-I and IGF-II were purchased from R&D systems (Minneapolis, MN, USA) and des(1–3)IGF-I from GroPep (Adelaide, Australia). All human esophageal cancer cell lines (Fig. 1) were obtained from the Japanese Cancer Collection of Research Bioresources Cell Bank (Tokyo, Japan), Riken Bioresource Center Cell Bank (Tsukuba, Japan), and European Collection of Cell Cultures (Salisbury, UK). Cells were passaged in RPMI1640 and DMEM, both with 10% fetal bovine serum.

Recombinant adenoviruses expressing IGF-IR/dn (482 and 950 amino acids long, IGF-IR/482st and IGF-IR/950st, Ad-IGF-IR/482st and Ad-IGF-IR/950st, respectively) were generated as described previously by homologous recombination [31]. Recombinant adenovirus vectors expressing shIGF-IR (ad-shIGF-IR) were generated as described previously [38]. An adenovirus expressing β-galactosidase was used as a control (ad-LacZ). Scrambled shRNA adenovirus (ad-Scr) is another control that has a short hairpin sequence but no specific target, also as described previously.

BMS-536924 was kindly provided by Bristol-Myers Squibb (New York, NY, USA). Stock solution was prepared in DMSO and stored at −20°C.

Immunohistochemical analysis

The paraffin-embedded esophageal tissue microarray (ES208) was purchased from US Biomax (Rockville, MD, USA). After deparaffinization, endogenous peroxidase activity was blocked. Antibodies were applied after blocking with normal goat serum. Sections were incubated with the anti-rabbit secondary antibody (Santa Cruz Biotechnology) and a strep tavidin-HRP followed by exposure to the diaminobenzidine tetrahydrochloride substrate (Dako). The sections were counterstained in Mayer's hematoxylin and mounted. Immunostaining signals were scored by two independent observers. Semiquantitative scores were given as the score of the percentage of positive cells plus the score of the staining intensity. The scoring criteria of the percentage of positive cells were as follows: score 0, 0–5% positive cancer cells; score 1, 6–25%; score 2, 26–50%; score 3, 51–75%; score 4, 76–100% positive. The intensity score was given as follows: score 0, no staining; score 1, weak/equivocal; score 2, moderate; score 3,

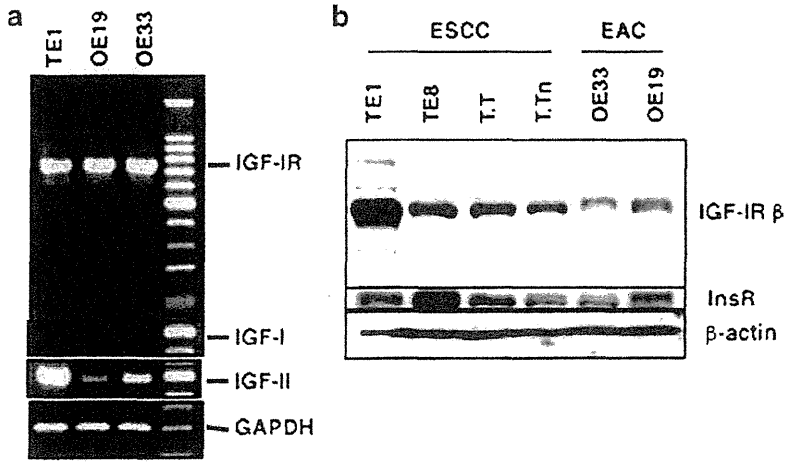

Fig. 1 The expressions of IGF-axis in esophageal carcinoma cell lines. **a** RT-PCR revealed that three cells express mRNAs of IGF-II and IGF-IR but not IGF-I. **b** Western blotting showed that two EAC and four ESCC cells express both IGF-IR and InsR

strong staining. The final scores were from 0 to 7 and four or more were considered positive.

Reverse transcription PCR

Total RNA from cells was isolated by the acid guanidinium thiocyanate–phenol–chloroform method. Primer sets for the amplification of IGF-I cDNA sequences were 5′-CACTGT CACTGCTAAATTCA-3′ and 5′-CTGTGGGCTTGTTGAAA TAA-3′ [39]. Primers for IGF-II cDNA were 5′-AGTCGATGC TGGTGCTTCTCA-3′ and 5′-GTGGGCGGGGTCTTGG GTGGGTAG-3′ [40]. Primers for IGF-IR were 5′-ATTGAG GAGGTCACAGAGAAC-3′ and 5′-TTCATATCCTGTTTT GGCCTG-3′ [40]. Randomly primed cDNAs were prepared from 1 mg of total RNA by M-MLV reverse transcriptase (Takara, Japan) and amplified by PCR. For amplification of these sequences, 35 cycles of PCR was programmed as follows: 94°C, 30 s; 60°C, 30 s; 72°C, 30 s.

Western blotting

Cells were cultured in serum-free medium for 24 h and then stimulated with 20 ng/ml IGF-1 or 10 nM insulin. Cell lysates were prepared as described previously [31]. Equal aliquots of lysate (100 μg) were separated by 4–20% SDS-PAGE and immunoblotted onto polyvinylidene Hybond-P membrane (Amersham, Arlington Heights, IL, USA). Analysis was performed using the indicated antibodies, and bands were visualized by ECL (Amersham).

Assessment of the effect on in vitro cell growth

Tumor cells were grown to 70% confluence in six-well plates and infected with adenovirus. The number of cells was then assayed by Trypan blue staining.

Four thousand cells were seeded into the wells of a 96-well plate, and each was infected with adenovirus or control. Cell growth was measured using WST-1 reagent (Roche, Basel, Switzerland) as described previously [21].

In vitro tumorigenicity

Anchorage-independent growth was assessed by soft agar clonogenicity assays. Briefly, cells were detached and plated in 0.2% agarose with 1% underlay (2×10^4 cells/5-cm dish). After 1 week, media were added over the soft agar. The medium overlay was changed after 1 week. Colonies greater than 125 μm were counted after 3 weeks using a calibrated graticule.

Colony forming activity was assessed by plating 3×10^3 per plate on 60-mm culture dishes and incubated for 24 h. The cells were then treated with BMS-536924 and were incubated for 14 days. After air-drying, cells were fixed with methanol and stained with Giemsa solution. Colonies containing 50 cells or more were counted.

Measurement of apoptosis

The DNA fragmentation assay was performed as follows: low molecular weight DNA was extracted with 0.5% Triton X-100, 10 nM EDTA, and 10 mM Tris–HCl, pH 7.4, treated with 400 μg/ml RNase A and then proteinase K for 1 h at 37°C, ethanol-precipitated, and subjected to 1% agarose gel electrophoresis. The gels were stained with 1 μg/ml ethidium bromide. Early apoptosis was quantified by staining with Annexin-V-FITC according to the manufacturer's protocol (BD Biosciences) and measured by flow cytometry. Cells undergoing apoptosis showed an increase in Annexin-V binding but excluded propidium iodide. TUNEL assays were performed with in situ apoptosis detection kit (Takara) following the manufacturer's protocol. Caspase-3 colorimetric protease assay was performed following the manufacturer's protocol (Caspase-3 Colorimetric Protease Assay Kit; MBL). In brief, 3×10^6 cells were lysed in 100 μl of chilled cell lysis buffer, and total cell lysates (100 μg) were incubated with 4 mM VETD-pNA Substrate (200 μM final concentration) at 37°C for 1 h. Caspase-3 activity was measured by colorimetric reaction at 405 nm.

First, cancer cells infected with Ad-IGF-IR/dns or Ad-LacZ were induced with 10 mJ/cm^2 UV light. To assess the efficacy of IGF-IR/dn on chemotherapy-induced apoptosis, tumor cells were treated for 24 h with 1 mM 5-FU or 50 μM cisplatin.

Migration assay

Wounding assays were performed using a modification of the procedure described by Pennisi et al. [41]. Briefly, six-well chambers were prepared by scratching registration marks onto the slide surface. TE1 cells (infected with adenoviruses) were plated, grown normally for 48 h, and starved overnight. Cells were cut with a cell scraper, and five images were captured

Table 1 Summary of immunohistochemical expression of IGF-IR

	IGF-IR (+)		
Normal esophageal mucosa	0/7	0%	
Esophageal carcinoma	31/57	54%	$p = 0.0111$ (Fisher)
	IGF-IR (+)		
Squamous cell carcinoma	23/34	68%	
Primary sites	15/23	65%	
Metastasized sites	8/11	73%	
Lymph node	6/9	67%	
Skin	2/2	100%	
Adenocarcinoma	8/22	36%	
Adenosquamous carcinoma	0/1	0%	

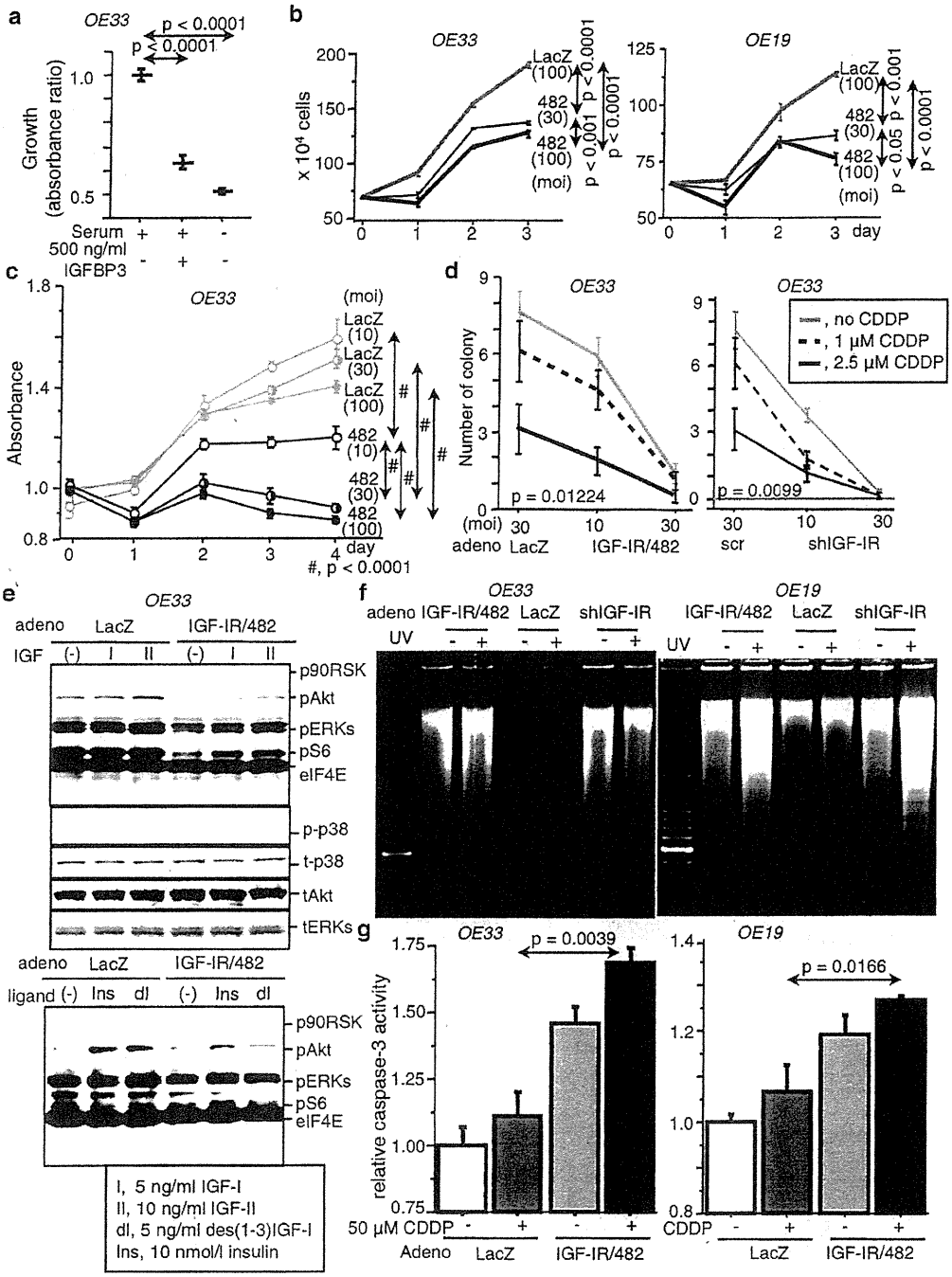

Fig. 2 The effect of IGF-IR on EAC cells. **a** WST-1 assay showed cell growth of OE33, 48 h of culture with/without IGFBP3. **b** Trypan blue assay showed the number of viable cells. **c** WST-1 assay revealed cell proliferation of adenoviruses-infected OE33. **d** Colony formation assays showed the effect of IGF-IR/dn and cisplatin on colony formation. **e** OE33 was stimulated for 5 min with ligands in serum-free medium. Western blotting showed signal transduction. **f** DNA fragmentation assay detected UV-induced apoptosis. **g** Cells were treated for 24 h with cisplatin. Then, caspase-3 assays were performed

along the cut surface on an Olympus IX-71S1F-2 microscope (Tokyo, Japan) using a ×20 objective. Additional images were captured 24 h later. For each experiment, the number of migrating cells was counted by two independent observers [41].

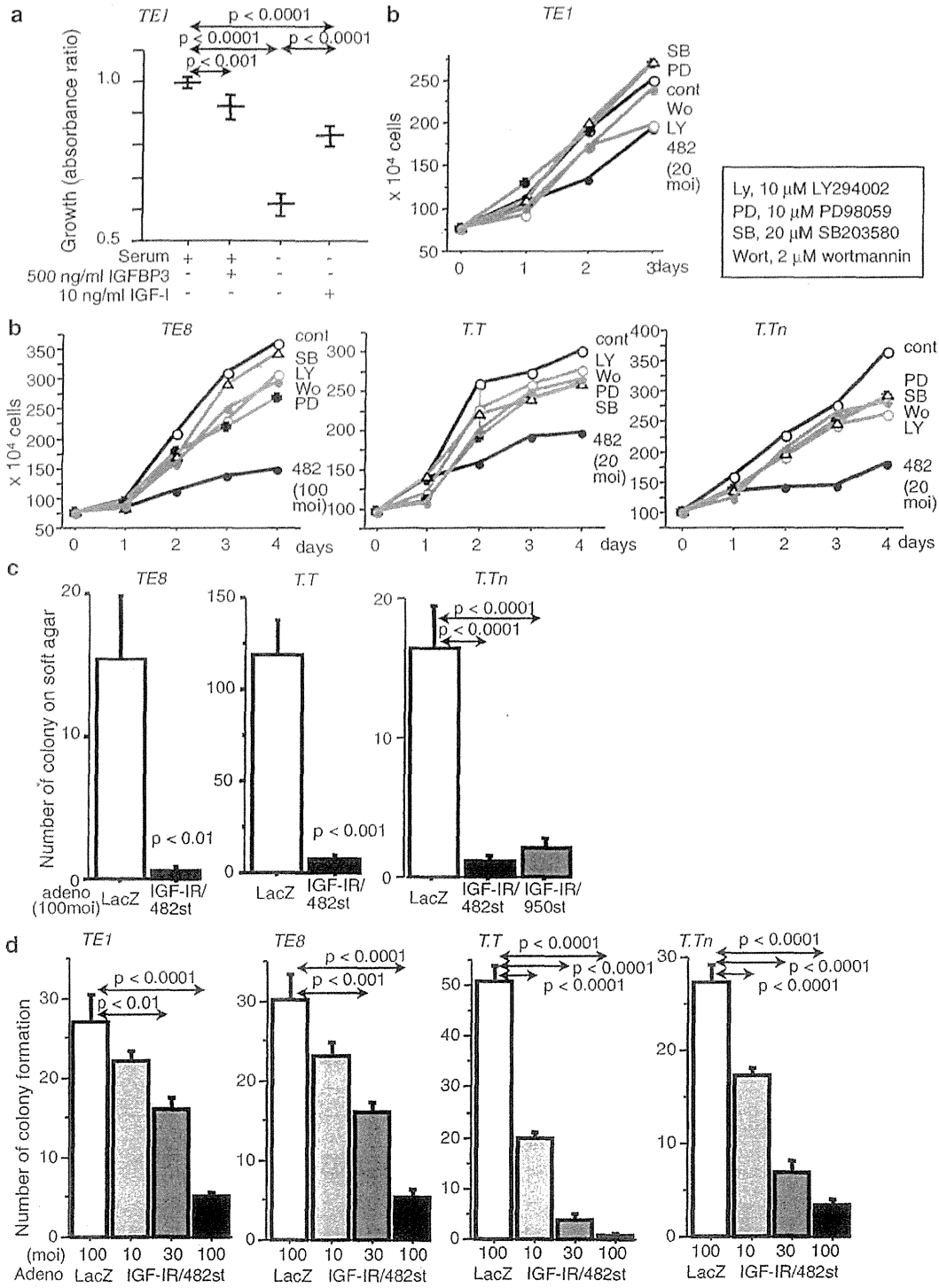

Fig. 3 The effect of IGF-IR on the growth of ESCC. **a** WST-1 assay showed cell growth after 48 h of culture. **b** Trypan blue assay showed the viable cell number of ESCC cells with several inhibitors or IGF-IR/482st. **c** Soft agar assays detected that ad-IGF-IR/dns blocked colony formation. **d** Colony formation assay showed the effect of IGF-IR/482st on colony number

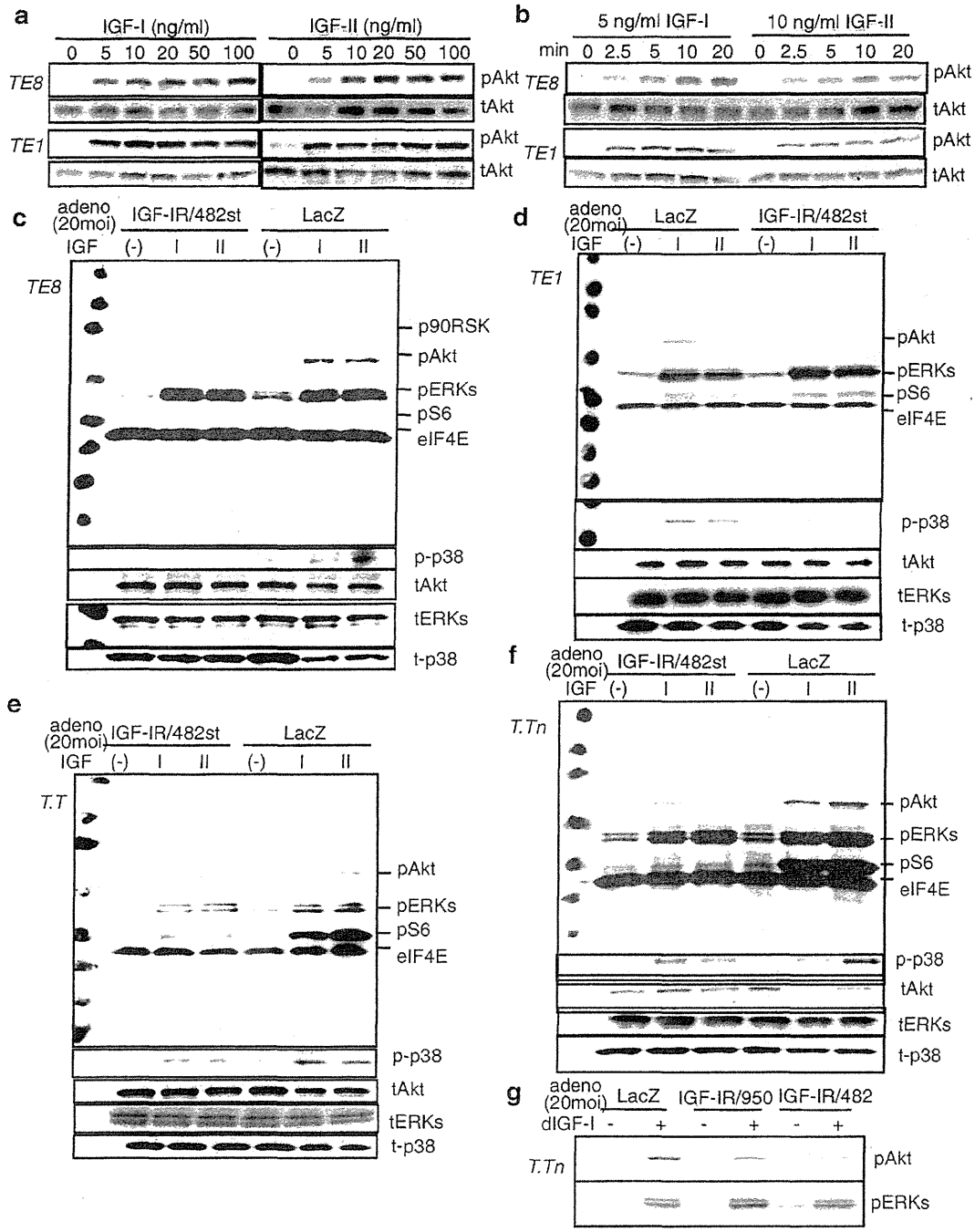

Fig. 4 The down-stream signals from IGF-IR by Western blotting. **a** Both TE8 and TE1 cells were stimulated for 5 min with IGFs, and then whole cell lysates were extracted. **b** Both cells were stimulated from 0 to 20 min with IGFs. **c**–**f** Four cell lines infected with adenoviruses were stimulated for 5 min with IGFs. **g** Adenoviruses-infected T.Tn cells were stimulated for 5 min with 5 ng/ml des(1–3)IGF-I