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Fractionated evaluation of immunohistochemical hormone receptor expression enhances prognostic prediction in breast cancer patients treated with tamoxifen as adjuvant therapy*

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Abstract: Objective: To compare the prognostic prediction between dichotomized and fractionated evaluations of hormone receptor expressions. Methods: Patients with stages I–III breast cancers, who received adjuvant tamoxifen, were enrolled. The expression of estrogen receptor (ER) and progesterone receptor (PR) was evaluated by immunohistochemistry (IHC). A fractionated score (F score), the percentage of positive-staining nuclei (0=none, 1=1%–10%, 2=11%–30%, 3=31%–50%, 4=51%–70%, and 5=71%–100%), was assigned to each case. The dichotomized scoring method defines an F score >1 as positive. The prognostic values of both scores were compared by multiple Cox's proportional hazard models of disease-free survival (DFS) and overall survival (OS). Results: Four hundred and sixteen patients with a median follow-up of 78.0 months were included. F scores for ER and PR correlated directly with DFS and OS. Although both the dichotomized and fractionated ER and PR scores were significantly associated with DFS and OS in univariate analyses, only fractionated ER and PR scores remained as independent prognostic factors of DFS and OS in the final multiple Cox's proportional hazard models. Conclusion: Fractionated IHC hormone receptor expression evaluation enhances the prognostic prediction compared with a dichotomized assessment.

Key words: Breast cancer, Fractionated evaluation, Estrogen receptor, Progesterone receptor
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1 Introduction

In breast cancer patients, both the estrogen receptor (ER) and progesterone receptor (PR) are important prognostic and predictive markers (Ravdin *et al*., 1992; Elledge *et al*., 2000; Esteva and Hortobagyi, 2004). Over the past decade, immunohistochemistry (IHC) has replaced the ligand-binding assay (LBA) for measuring ER and PR. Some studies comparing the predictive values of these two methods found that IHC is an equal or a superior method (Reiner *et al*., 1986; Stierer *et al*., 1993; Barnes *et al*., 1996; Harvey *et al*., 1999; Mohsin *et al*., 2004; Regan *et al*., 2006). However, the definitions of ER and PR positivity by IHC were not consistent among the different studies. Using a definition of positivity as the presence of any level of staining by IHC, the Early Breast Cancer Trialists' Collaborative Group (EBCTCG, 2005) showed in a meta-analysis that tamoxifen significantly

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improved the prognosis of patients with ER positive tumors. However, in clinical practice and in some clinical trials, a cut-off of ≥10% ER and/or PR positive nuclei is often used for determining that a tumor is hormone receptor-positive and should be treated with adjuvant hormonal therapy (Ellis *et al*., 2001; Jonat *et al*., 2002; Thurlimann *et al*., 2005). The commonly applied 10% cut-off was established by Pertschuk *et al*. (1996) who demonstrated a response to treatment in patients with metastatic breast cancer.

Two recent reports by Viale *et al*. (2008) and Stendahl *et al*. (2006) showed that fractionated scoring of ER and PR, defined as assigning scores based on the fraction of cells that were stained positively for ER and PR by IHC, could enhance the prediction of a benefit from adjuvant chemotherapy and/or hormonal therapy. The study by Viale *et al*. (2008) demonstrated a gradually decreasing benefit to adding chemotherapy to endocrine therapy with increasing ER and PR expression fractions when compared with endocrine therapy alone in patients with node-negative early breast cancer. Stendahl *et al*. (2006) showed that adjuvant tamoxifen could significantly improve survival in premenopausal patients with tumors showing >75% PR positive nuclei. These two studies highlighted the predictive roles of fractionated evaluation of hormone receptors. In the present study, we assessed whether the hormone receptor status determined by fractionated IHC evaluation could enhance the prognostic significance of the predictive value compared with the traditional dichotomized evaluation.

2 Materials and methods

2.1 Patients and procedures

In this study, women with stage I, II, or III breast cancer who received tamoxifen as adjuvant therapy at the National Taiwan University Hospital (NTUH) between 1992 and 2000 were recruited. Breast cancer staging was conducted according to the American Joint Committee on Cancer (AJCC) criteria (the 6th Ed.). Patients' demographic information was collected from a genetic epidemiology study on the association between selected genetic polymorphisms and breast cancer in 482 patients, which was carried out at the same hospital (Huang *et al*., 2008). To avoid bias caused by the uncertain treatment effects of tamoxifen in tumors with weak ER and/or PR expression, 43 patients who did not receive adjuvant tamoxifen were excluded. In addition, 23 patients whose archival tumor tissues were not available for fractionated scoring of ER and PR expression were also excluded. Therefore, a total of 416 patients were enrolled in the present analysis. Clinical data, including staging, type of surgery, adjuvant chemotherapy, and hormone therapy, were extracted from medical charts. Disease status and survival outcome were obtained from medical charts, hospital cancer registry records, and the National Death Certificate Registry system if a patient was lost to follow-up. The data managers of our hospital cancer registry usually contact each patient every 6 months. Additionally, all deaths in Taiwan are registered through the household registration offices. These death certificates are coded by the Department of Health, Executive Yuan, and have been computerized since 1979 (Lu *et al*., 2000). The survival data in this study were updated to Feb. 5, 2005.

Histological slides were reviewed by one pathologist, Dr. H.C. Lien, who was blinded to the patient information. Histological grade was categorized as grades I, II, and III according to the Nottingham modification of the Scarff-Bloom-Richardson criteria (Elston and Ellis, 1991), except for invasive lobular and mucinous carcinomas. The status of ER and PR in tumors was determined by IHC performed on formalin-fixed (fixation time 6–8 h), paraffin-embedded tissue sections (thickness 4 μm) in the Central Pathology Laboratory at NTUH. Ventana Benchmark system (Ventana Medical Systems Inc., Tucson, AZ, USA) and prediluted antibodies (anti-ER Clone 6F11 and anti-PgR Clone 16) were used. The reviewing pathologist assigned a six-point fractionated score called "F score" to each case after estimating the percentage of positive-staining tumor cells (0=none, 1=1%–10%, 2=11%–30%, 3=31%–50%, 4=51%–70%, and 5=71%–100%). A percentage of 10% (i.e., F score=1) was chosen as the cut-off value to dichotomize the assessments of ER and PR into positive versus negative.

2.2 Statistical analysis

Statistical analyses were performed with SAS software (version 9.1.3, SAS Institute Inc., Cary, NC,

USA) and S-PLUS software (version 7.0.4, Insightful Corporation, Seattle, WA, USA). A two-sided $P \leq 0.05$ was considered statistically significant. The dichotomized ER and PR scores are binary variables, whereas the fractionated ER and PR scores (i.e., F scores) are ordinal variables. The recruited subjects were followed from the date of diagnosis to the one of death or last contact. The disease-free survival (DFS) time was defined as the duration from diagnosis to either the confirmation of disease recurrence, including local, regional, and distant recurrences, or death from breast cancer (if the time of recurrence was missed), except for contralateral breast cancer. The overall survival (OS) time was defined as the duration between diagnosis and death from any cause. The dependent variables evaluated were age, histological type, histological grade, tumor size, axillary lymph node status, AJCC stage, dichotomized ER, dichotomized PR, fractionated ER, fractionated PR, and neoadjuvant and/or adjuvant chemotherapy. Survival curves were estimated with the Kaplan-Meier method. The association between each of the categorical variables and survival was analyzed by log-rank test. Multivariate analysis was performed using Cox's proportional hazard models to predict the hazard rates of DFS and OS, respectively. The time lag from the time of recurrence to each time at which a death from any cause occurred was computed for each patient and was included in the Cox's proportional hazard model of OS as a time-dependent covariate to falsify the hypothesis that earlier recurrence leads to earlier death. For patients whose breast cancer had not recurred at the time of death, this time lag was set to zero.

Basic model-fitting techniques for (1) variable selection, (2) assessment of goodness-of-fit (GOF), and (3) regression diagnostics were used in our regression analysis to assure the quality of the analysis results. In the stepwise variable selection procedure, all the significant univariates and non-significant covariates were considered and both the significance levels for entry (SLE) and for stay (SLS) were set to 0.15 or larger. The adjusted generalized R^2 and the Grønnesby-Borgan GOF test were examined to assess the GOF of the fitted Cox's proportional hazard model. However, the value of the adjusted generalized R^2 for Cox's proportional hazard model is usually low. Larger P values in the Grønnesby-Borgan GOF test indicate better fit. Regression diagnostics, including verification of proportional hazard assumption, residual analysis, detection of influential cases, and check for multi-collinearity, were performed. Based on the fitted final Cox's regression models for DFS and OS, estimated covariate-adjusted survival curves were plotted for personalized prediction of the patient's prognosis.

3 Results

A total of 416 patients with a median age of 49 (range: 25–81) years were included. The representative immunohistochemical image of each F score of ER is shown in Fig. 1. The distribution of each F score of ER and PR expression is shown in Table 1. Using the dichotomized definition of an F score >1 as positive, 216 (63%) and 257 (62%) cases were ER^+ and PR^+, respectively. The clinical and pathological characteristics of the patients are summarized in Table 2 by dichotomized ER and PR status. The tumor histology consisted of infiltrating ductal carcinoma (95%), infiltrating lobular carcinoma (2%), mucinous carcinoma (3%), and others (1%) including 2 medullary carcinomas and 2 invasive papillary carcinomas. The frequency of ER^+ and/or PR^+ status was heterogeneous among tumors of different sizes, axillary lymph node statuses, histologic types, and grades. Specifically, ER^+ was associated with a higher probability of a tumor size ≤2 cm and PR^+ was associated with higher probability of absence of axillary lymph node involvement. All of the lobular carcinomas and mucinous carcinomas were ER^+. Tumors with histologic grades I and II were more likely to be ER^+ or PR^+, whereas tumors with histologic grade III

Table 1 Distribution of ER and PR fractionated scores

ER score	PR score						Total
	0	1	2	3	4	5	
0	77	15	6	4	5	21	128
1	10	15	2	0	0	0	27
2	0	0	6	1	5	6	18
3	7	1	4	3	2	4	21
4	8	1	0	4	10	5	28
5	22	3	10	11	20	128	194
Total	124	35	28	23	42	164	416

Table 2 Patients' clinical and pathological characteristics by dichotomized ER and PR expression

	All cases (*n*=416)	ER, No. (%)			PR, No. (%)		
		Positive (*n*=261)	Negative (*n*=155)	*P*-value	Positive (*n*=257)	Negative (*n*=159)	*P*-value
Age				0.0922			0.0926
<36 years	26 (6%)	15 (6%)	15 (10%)		14 (6%)	16 (10%)	
36–50 years	204 (49%)	135 (52%)	65 (42%)		132 (51%)	68 (43%)	
>50 years	186 (45%)	111 (43%)	75 (48%)		111 (43%)	75 (47%)	
Tumor size				0.0057			0.1190
≤2 cm	167 (40%)	120 (46%)	47 (30%)		111 (43%)	56 (35%)	
3–5 cm	216 (52%)	124 (48%)	92 (59%)		130 (51%)	86 (54%)	
>5 cm	33 (8%)	17 (7%)	16 (10%)		16 (6%)	17 (11%)	
Axillary lymph node				0.9399			0.0271
None	232 (56%)	148 (57%)	84 (54%)		158 (61%)	74 (47%)	
1–3	96 (23%)	59 (23%)	37 (24%)		53 (21%)	43 (27%)	
4–9	54 (13%)	34 (13%)	20 (13%)		29 (11%)	25 (16%)	
≥10	34 (8%)	20 (8%)	14 (9%)		17 (7%)	17 (11%)	
AJCC stage				0.0445			0.0198
I	114 (27%)	82 (31%)	32 (21%)		82 (32%)	32 (20%)	
II	209 (50%)	121 (46%)	88 (57%)		125 (49%)	84 (53%)	
III	93 (22%)	58 (22%)	35 (23%)		50 (19%)	43 (27%)	
Histologic subtype				0.0006			0.3523
Ductal	391 (94%)	237 (91%)	154 (99%)		238 (93%)	153 (96%)	
Lobular	8 (2%)	8 (3%)	0 (0%)		5 (2%)	3 (2%)	
Mucinous	13 (3%)	13 (5%)	0 (0%)		11 (4%)	2 (1%)	
Others	4 (1%)	3 (1%)	1 (1%)		3 (1%)	1 (1%)	
Histologic grade				<0.0001			<0.0001
I	103 (25%)	78 (30%)	25 (16%)		79 (31%)	24 (15%)	
II	164 (39%)	115 (44%)	49 (32%)		113 (44%)	51 (32%)	
III	98 (24%)	33 (13%)	65 (42%)		37 (14%)	61 (38%)	
Not available	51 (12%)	35 (13%)	16 (10%)		28 (11%)	23 (15%)	
Neoadjuvant or adjuvant chemotherapy				0.3577			0.3098
Yes	262 (63%)	160 (61%)	102 (66%)		157 (61%)	105 (66%)	
No	154 (37%)	101 (39%)	53 (34%)		100 (39%)	54 (34%)	

were more likely to be ER^- or PR^-. The expression of ER and PR was not significantly associated with the use of neoadjuvant and/or adjuvant chemotherapy.

3.1 Univariate survival analyses of prognostic factors

The median follow-up time was 78.0 (range: 74.7–81.3) months. As listed in Table 3, the univariate analysis showed that age, tumor size, axillary lymph node status, AJCC stage, dichotomized ER and PR, histologic grade, and use of neoadjuvant or adjuvant chemotherapy were significantly associated with DFS and OS. Specifically, age <36 years, larger tumor size, higher AJCC stage, negative ER, negative PR, more axillary lymph node involvement, higher tumor grade, and use of neoadjuvant and/or adjuvant chemotherapy were prognostic factors for a poor outcome (Table 3). The estimated DFS and OS curves according to the F scores of hormone receptors for all patients are shown in Figs. 2a and 2b), respectively. Univariate linear trend analysis also revealed that higher ER and PR IHC scores were significantly associated with favorable DFS (P=0.003 and P<0.001, respectively) and OS (P<0.001 and P<0.001, respectively).

3.2 Multivariate survival analyses of prognostic factors

The results of multivariate analyses by fitting Cox's proportional hazard models for DFS and OS are listed in Table 4. Fractionated ER, age <36 years, ≥10 positive axillary lymph nodes, histologic grade III, and AJCC stage were independent prognostic factors for DFS. Fractionated PR, 1–9 positive axillary lymph nodes, ≥10 positive axillary lymph nodes, tumor size, histologic grade III, and the time lag between recurrence and the time of death from any

Table 3 Univariate analyses of clinical and pathological variables in patients with stages I–III breast cancers

	N (%)	DFS			OS		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age				0.0129			0.0191
<36 years	26 (6%)	1.00			1.00		
36–50 years	204 (49%)	0.41	0.23–0.76		0.40	0.20–0.77	
>50 years	186 (45%)	0.50	0.28–0.91		0.48	0.25–0.92	
Tumor size				<0.0001			<0.0001
≤2 cm	167 (40%)	1.00			1.00		
3–5 cm	216 (52%)	1.86	1.18–2.92		2.27	1.28–4.02	
>5 cm	33 (8%)	5.43	3.02–9.79		7.99	4.04–15.84	
Axillary lymph nodes				<0.0001			<0.0001
None	232 (56%)	1.00			1.00		
1–3	96 (23%)	1.98	1.17–3.35		1.92	0.99–3.70	
4–9	54 (13%)	3.81	2.22–6.53		4.67	2.49–8.75	
≥10	34 (8%)	12.08	7.19–20.30		11.81	6.47–21.55	
AJCC stage				<0.0001			<0.0001
I	114 (27%)	1.00			1.00		
II	209 (50%)	3.23	1.53–6.84		4.10	1.45–11.62	
III	93 (22%)	11.11	5.27–23.40		16.43	5.90–45.80	
ER expression				0.0090			0.0001
Positive	261 (63%)	1.00			1.00		
Negative	155 (37%)	1.65	1.13–2.42		2.35	1.50–3.69	
PR expression				<0.0001			<0.0001
Positive	257 (62%)	1.00			1.00		
Negative	159 (38%)	2.12	1.44–3.13		2.67	1.68–4.22	
Histologic subtype				0.7717			0.8421
Ductal	391 (94%)	1.00			1.00		
Lobular	8 (2%)	1.09	0.27–4.44		0.75	0.10–5.40	
Mucinous	13 (3%)	1.03	0.33–3.23		0.87	0.21–3.55	
Others	4 (1%)	NA	NA		NA	NA	
Histologic grade				0.0072			0.0010
I	103 (25%)	1.00			1.00		
II	164 (39%)	2.07	1.14–3.77		1.88	0.89–3.99	
III	98 (24%)	2.88	1.55–5.36		3.75	1.78–7.88	
Not available	51 (12%)	1.84	0.85–3.98		1.98	0.79–5.00	
Neoadjuvant or adjuvant chemotherapy				0.0010			0.0013
Yes	262 (63%)	1.00			1.00		
No	154 (37%)	0.49	0.31–0.75		0.43	0.25–0.73	

HR: hazard ratio; CI: confidence interval; NA: not available

Table 4 Multivariate analyses of disease-free survival and overall survival using Cox's proportional hazard models

Covariate	Estimate	Standard error	Wald chi-square	Hazard ratio	*P* value
Disease-free survival (month)[1]					
Fractionated ER	−0.13960	0.05628	6.1530	0.870	0.0131
Fractionated PR	−0.10107	0.05869	2.9655	0.904	0.0851
Age <36 years	0.67577	0.29191	5.3592	1.966	0.0206
Lymph node ≥10	1.35863	0.27997	23.5496	3.891	<0.0001
Histology grade III	1.32154	0.61828	4.5687	3.749	0.0326
AJCC stage	0.91674	0.18495	24.5675	2.501	<0.0001
Overall survival (month)[2]					
Fractionated PR	−0.21830	0.06066	12.9512	0.804	0.0003
Lymph node 1–9	0.81272	0.30782	6.9707	2.254	0.0083
Lymph node ≥10	1.56278	0.33739	21.4549	4.772	<0.0001
Histology grade III	0.68063	0.25213	7.2875	1.975	0.0069
Tumor size (cm)	0.32670	0.16657	3.8469	1.386	0.0498
Neoadjuvant or adjuvant chemotherapy	0.61693	0.35181	3.0750	1.853	0.0795
Time lag since recurrence	0.07444	0.00775	92.2451	1.077	<0.0001

[1]Cox's proportional hazard model: n=416, adjusted generalized R^2=0.2486; [2]Cox's proportional hazard model: n=416, adjusted generalized R^2=0.3971

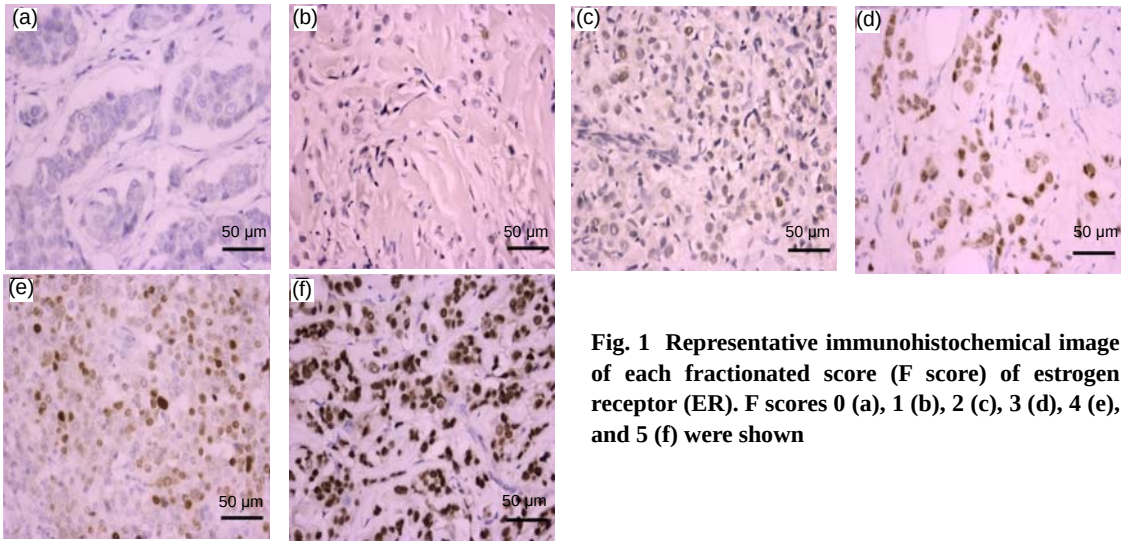

Fig. 1 Representative immunohistochemical image of each fractionated score (F score) of estrogen receptor (ER). F scores 0 (a), 1 (b), 2 (c), 3 (d), 4 (e), and 5 (f) were shown

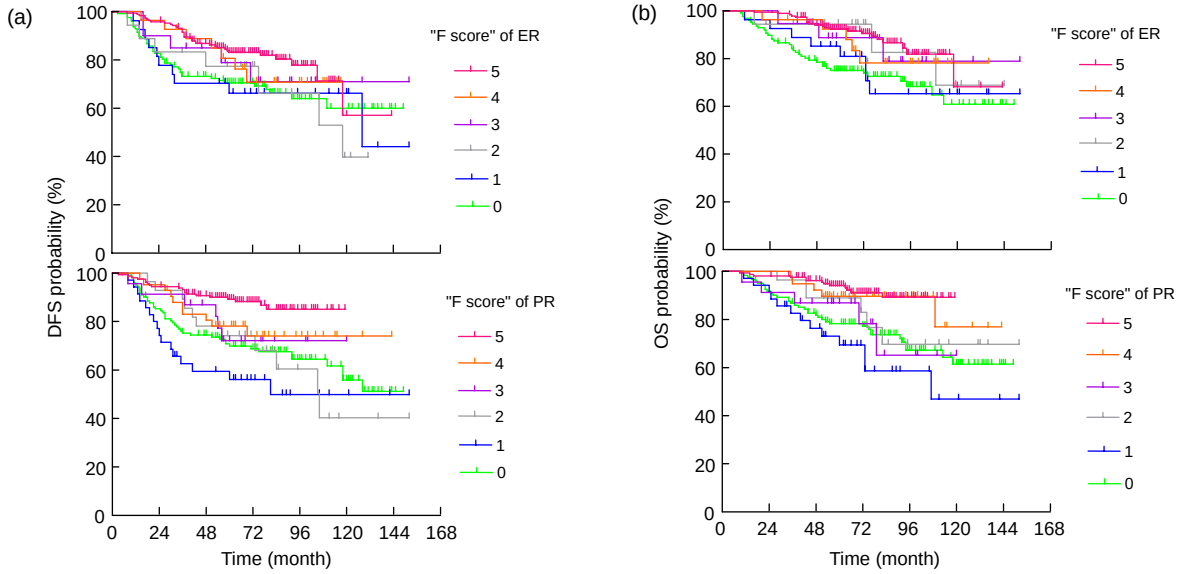

Fig. 2 Disease-free (a) and overall (b) survival curves (unadjusted analysis) by estrogen receptor (ER) and progesterone receptor (PR) IHC score

cause were independent prognostic factors for OS. Longer lag time implied an earlier recurrence. Neither dichotomized ER nor dichotomized PR was an independent prognostic factor for DFS or OS at the completion of the stepwise variable selection procedure. Moreover, we could draw covariate-adjusted survival curves for the prognostic prediction of patient's DFS or OS under any given condition. As an illustration, the estimated covariate-adjusted DFS curves by six-point fractionated ER scores for a young woman (aged <36 years, AJCC stage II breast cancer, histologic grade I or II, fractionated PR score 0, and ≥10 involved axillary lymph nodes) are shown in Fig. 3.

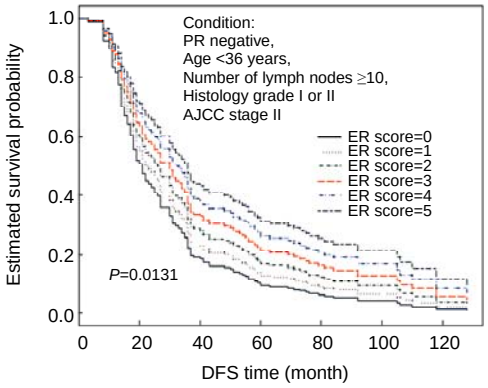

Fig. 3 Covariate-adjusted survival curves for disease-free survival (DFS) time based on a fitted multiple Cox's proportional hazard model (n=416)

4 Discussion

In this study, we found that fractionated ER and PR were predictors of DFS and OS, respectively, both independently and after competing with the dichotomized ER and dichotomized PR in a stepwise variable selection procedure. We concluded that implementing a fractionated evaluation of ER and PR enhances the prognostic correlation when compared with the dichotomized assessment.

All of the patients included in this analysis received adjuvant tamoxifen, including a substantial proportion (26%) of patients with ER^-/PR^- breast cancer. This provided us with a suitable platform to examine the effect of ER and PR expression on survival. By examining the Kaplan-Meier estimates of DFS curves stratified by fractionated ER and PR scores (Fig. 2a), the best cut-off points of dichotomized evaluation of ER and PR positivity were more than 10% positive nuclei (F score>1). Dichotomized ER and PR expression was also highly associated with favorable DFS and OS in the present study, which provides evidence to support the criteria commonly used in clinical practice and some clinical trials in the adjuvant setting (Ellis *et al*., 2001; Jonat *et al*., 2002; Thurlimann *et al*., 2005).

In addition to the prognostic factors identified by our univariate analyses for DFS and OS, which include age, tumor size, number of axillary lymph nodes, and AJCC stage, dichotomized ER and PR are well-recognized prognostic factors for breast cancer survival (Cianfrocca and Goldstein, 2004). The present study provides empirical evidence to show that earlier recurrence leads to an earlier death in breast cancer patients. Although this result is biologically sound, most of the previous studies analyzed time to recurrence (DFS) and death (OS) separately. The causal relationship between recurrence and death should be pursued further in the future, because it may shed light on improvements of the prognostic predictions in such patients. Moreover, we found that fractionated ER was an independent prognostic factor of DFS, and fractionated PR was an independent prognostic factor of OS after adjusting for the effect of time lag since recurrence. Since time to recurrence was also a prognostic factor for death, both fractionated ER and fractionated PR could predict OS, but fractionated PR had a direct prognostic effect on OS. The finding that PR added significant prognostic value beyond that obtained with ER alone was consistent with recent studies (Banerjee *et al*., 2004; Arpino *et al*., 2005; Liu *et al*., 2010). Fractionated ER and PR remained in the final multiple Cox's proportional hazard models after competing with the dichotomized ER and PR in the stepwise variable selection procedure. Thus, a fractionated evaluation of ER and PR enhanced the prognostic prediction when compared with the dichotomized assessment.

ER analysis using LBA (as fmol/mg of protein) revealed a broad range of values for ER, so there has been expectation that immunohistochemical assay for ER analysis should result in a similar distribution. However, both fractionated ER and PR in the present study were shown to display a bimodal distribution, with substantial proportions at F scores of 0 (ER: 30.8%; PR: 29.8%) and 5 (ER: 46.6%; PR: 39.4%) (Table 1). The distribution was consistent with several previous studies of ER and PR expression measured by immunohistochemical assay (Collins *et al*., 2005; Nadji *et al*., 2005; Stendahl *et al*., 2006; Badve *et al*., 2008; Viale *et al*., 2008). Despite the bimodal distribution of ER and PR expression, the present study clearly demonstrates that fractionated ER and PR scores predict a patient's prognosis more accurately than a dichotomized assessment.

The Allred, Quick and H scores, which include the evaluation of both the proportion and staining intensity of positive cells, have been used to assess IHC staining of breast tumors (Huang *et al*., 1996; Harvey *et al*., 1999; Ferrero-Pous *et al*., 2001). The Allred scores for both of ER and PR expression were reported to correlate highly with patient's survival outcome, and the predictive value of Allred score was superior to the LBA (Harvey *et al*., 1999; Mohsin *et al*., 2004). The Quick and H scores for ER and/or PR expression were shown to correlate with LBA (Huang *et al*., 1996; Ferrero-Pous *et al*., 2001) and possess similar predictive values as LBA for response to hormonal therapy (Elston and Ellis, 1998). Nevertheless, many clinical practices simply adopt a proportion score rather than the semi-quantitative scoring systems for predictive and prognostic evaluations, because the intensity interpretation is relatively subjective and some tumors have heterogeneous intensity (Horii *et al*., 2007).

The superiority of prognostic value of fractionated

assessment to dichotomized assessment may have important clinical implications. For example, the information could be used to improve risk estimation by *Adjuvant! Online* (https://www.adjuvantonline.com/breastnew.jsp), which is one of the most popular and ambitious tools to estimate the risk of breast cancer relapse and death. One of the limitations inherent in the current *Adjuvant! Online* version is that only dichotomized ER is used to estimate the risk of relapse and mortality. As illustrated in Fig. 3, the covariate-adjusted survival curves may provide some fine tuning according to the level of ER positivity and PR status to adjust the risk estimation. Of course, further population-based studies with larger sample sizes and a comparison between pure fractionated (F score) and intensity incorporated (Allred, Quick or H score) evaluations are necessary to prove that the "F score" is a simple and powerful method for risk adjustment.

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