GRP78 as a Novel Predictor of Responsiveness to Chemotherapy in Breast Cancer

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Abstract

The discovery of predictive factors for chemoresistance is critical for improving adjuvant therapy for cancer patients. The 78-kDa glucose-regulated protein (GRP78), widely used as an indicator of the unfolded protein response (UPR), is induced in the tumor microenvironment. *In vitro* studies suggest that GRP78 confers chemoresistance to topoisomerase inhibitors, such as Adriamycin (doxorubicin). Here, we report on a retrospective cohort study of 127 stage II and III breast cancer patients who were treated with Adriamycin-based chemotherapy. Archival tumor specimens were available for analysis and the relationship of GRP78 expression level to "time to recurrence" (TTR), used as a surrogate marker for drug resistance, was examined. Our data show that 67% of the study subjects expressed high level of GRP78 in their tumors before the initiation of chemotherapy and suggest an association between GRP78 positivity and shorter TTR [hazard ratio (HR), 1.78; *P* = 0.16]. Interestingly, subgroup analysis reveals that the HR for the GRP78-positive group increased significantly among patients who did not receive further taxane treatment (HR, 3.00; *P* = 0.022) and among mastectomy patients (HR, 3.33; *P* = 0.027). The HR was even stronger among mastectomy patients who did not receive further taxane treatment (HR, 4.82; *P* = 0.010). The use of GRP78 as a predictor for chemoresponsiveness and the potential interaction of GRP78 and/or the UPR pathways with taxanes warrant larger studies. (Cancer Res 2006; 66(16): 7849-53)

Introduction

Adjuvant therapy of early breast cancer improves survival; however, standard treatment strategies result in unnecessary treatment and exposure to side effects of large numbers of women who do not benefit (1). Because current adjuvant strategies are primarily based on grouped risk assessments mainly using tumor stage, histologic grade, and receptor status, there is a critical need for identification of additional, novel predictive factors for chemoresponsiveness. Adriamycin (doxorubicin), an anthracycline-inhibiting topoisomerase II, is a standard chemotherapeutic agent for adjuvant therapy of early-stage breast cancer. Despite its benefits, ~50% of patients with stage II and III disease will recur within 5 years with drug resistance as a major contributing factor (2). The 78-kDa glucose-regulated protein (GRP78), also referred to as immunoglobulin heavy chain binding protein (BiP), is a central regulator of endoplasmic reticulum function due to its roles in protein folding and assembly, targeting misfolded protein for degradation, endoplasmic reticulum Ca^{2+} binding, and controlling the activation of transmembrane endoplasmic reticulum stress sensors (3). Induction of GRP78 has been widely used as a marker for endoplasmic reticulum stress and the onset of the unfolded protein response (UPR; ref. 3). Due to its antiapoptotic property, stress induction of GRP78 represents an important prosurvival component of the evolutionarily conserved UPR (4). Recent evidence shows that the microenvironment of tumors represents physiologic endoplasmic reticulum stress, and the UPR is crucial for survival of tumor cells subjected to persistent hypoxia (5). Overexpression of GRP78 has been reported in many types of cancer cell lines and tumor biopsies, including breast cancer (6, 7). The induction of GRP78 in solid tumors can be attributed to glucose starvation stress and anoxia in poorly vascularized tumors, as well as higher glucose utilization rate of cancer cells. *In vitro* studies show that GRP78 also protects cells from chemotherapeutic agents (7). In a panel of human breast cancer cell lines, the induction of GRP78 was most prominent in the sublines resistant to topoisomerase II inhibitors (Adriamycin and etoposide/VP-16; ref. 8). Topoisomerase inhibitors stabilize the topoisomerase-DNA complexes, resulting in DNA breakage and triggering the apoptotic cascade, including BAX and caspase-7 activation. Recent studies showed that GRP78 conferred resistance against Adriamycin- and etoposide-mediated apoptosis in cancer cells, at least in part, through inhibition of BAX and caspase-7 activation (7, 9, 10). The strong *in vitro* link between GRP78 overexpression and the development of resistance to topoisomerase-targeted drugs suggests that the overexpression of GRP78 within tumors may be predictive of resistance to Adriamycin in breast cancer patients. Here, we report the results of a retrospective cohort study of 127 stage II or III breast cancer patients. Our data provide the first evidence that GRP78 expression level may be a predictive factor of breast cancer patient's responsiveness to Adriamycin-based chemotherapy.

Materials and Methods

Study subjects. From 1989 to 1999, 432 female patients with stage II or III invasive breast cancer were treated in the University of Southern California (USC)/Norris Cancer Hospital (Los Angeles, CA), among whom 209 patients were treated with Adriamycin-based adjuvant chemotherapy. Demographic and clinical information were abstracted from hospital records. Tumor samples collected before the initiation of chemotherapy for 127 of the 209 patients were available for immunohistochemical staining. This study was approved by the USC Institutional Review Board (IRB). A waiver of informed consent was justified and granted by the IRB consistent with the waiver criteria of the common rule.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Immunohistochemical staining of GRP78 and evaluation. Five-micron sections of paraffin-embedded formalin fixed tissues were stained for GRP78 using anti-GRP78 H129 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (Supplementary Data; ref. 11). Plasma cell staining was used as internal positive controls. The negative control was a sample within each batch, in which the primary antibody was omitted. Immunohistochemically stained slides from each subject were reviewed by a pathologist (P.N.) who was blinded to all clinical data. Staining was graded for intensity of staining (1, weak; 2, moderate; 3, strong) and percentage of cells stained (1, 0 to <10%; 2, 10 to <50%; 3, 50-100%). The overall index of GRP78 expression was determined based on the previous two variables: positive when both scores were 2 or above; negative otherwise. To examine the reader reproducibility of GRP78 immunohistochemistry evaluation, a random sample of 31 slides was chosen and reevaluated by the same pathologist, without knowledge of the previous results. The κ coefficient was used to evaluate the agreement between two evaluations (12). The κ coefficient was 0.73 [95% confidence interval (95% CI), 0.50–0.98], indicating substantial agreement according to the Landis-Koch criterion (13).

Statistical analyses. The measure of outcome, time to recurrence (TTR), was calculated from start of chemotherapy until the date of documented recurrence. For patients who had not experienced a recurrence at the time of last follow-up (death or last contact at the hospital or with the treating physician), TTR was censored at the date of last follow-up. Associations between demographic and clinical characteristics (listed in Supplementary Data) and GRP78 expression were evaluated using contingency tables and Pearson's χ^2 or Fisher's exact test. The association between TTR and GRP78 expression or other potential prognostic factors was evaluated using Kaplan-Meier plots and Cox Proportional hazards model (14). All *P*s reported are two sided and are based on the likelihood ratio test associated with the Cox model. Inspection of the hazards suggested that the assumption of constant proportional hazards was not well satisfied; analyses were repeated with the log-rank test and nearly identical hazard ratio (HR) estimates and *P*s were obtained. For simplicity, we have reported all results based on the Cox model.

To assess whether the association between GRP78 and TTR was independent of other prognostic factors, two approaches were used: (*a*) stratification by each prognostic factor and (*b*) stratification by quintiles of a propensity score (see Supplementary Data; ref. 15). In post hoc examination, the relationship between GRP78 and TTR according to treatment modalities (types of chemotherapy, surgery, and radiation) was evaluated. The test of interaction was done by introducing an interaction term into the Cox model.

Results

Patient characteristics. In general, patients with tumor specimens available for GRP78 analysis were not substantially different in all major prognostic factors compared with those without available tumor specimens, with the exception that patients with available specimens were more likely to have undergone a mastectomy. When the associations between TTR and each of the patient and tumor characteristics were examined, as expected, tumor stage of T_3 or T_4, lymph node involvement, and high tumor grade were all associated with higher hazards of recurring. However, only the association with high tumor grade reached statistical significance at the 0.05 level (Supplementary Table S1).

GRP78 expression in breast cancer patients. As an essential chaperone protein, GRP78 is expressed constitutively at varying basal levels in most cell types. In the current study, for simplicity, the tumors were classified into "GRP78-negative" or "GRP78-positive" groups based on the overall index of intensity of staining and the percentage of cells stained. Thus, the negative group included tumors that stained weakly and/or with limited stained areas, whereas positive tumors reached or exceeded the staining criterion. The specificity of the antibody against GRP78 was confirmed by Western blot of human cell lysates, as well as immunohistochemical staining of paraffin sections of established tissue culture cell lines that expressed differential level of GRP78 (Supplementary Fig. S1). Further, plasma cells express high levels of GRP78, which facilitates immunoglobulin chain assembly (16). All subject samples contained plasma cells on their slides and their generally uniform high level immunoreactivity with the anti-GRP78 antibody conveniently served as internal positive controls (Supplementary Fig. S1). Representatives of GRP78-negative and GRP78-positive tumors are shown in Fig. 1. As expected for an endoplasmic reticulum protein, GRP78 staining was primarily in the perinuclear/cytoplasmic region. Among the 127 patients, 85 (67%) showed positive staining of GRP78, which was consistent across all subsets of patients, except subsets by tumor type (histology), where the numbers within categories were very small (Table 1).

Association between GRP78 and TTR. Among the 127 study subjects who received Adriamycin-based therapy, the GRP78-positive group showed an increased likelihood of recurring (HR, 1.78; 95% CI, 0.77-4.14; Fig. 2*A*; Table 2). Although this trend does

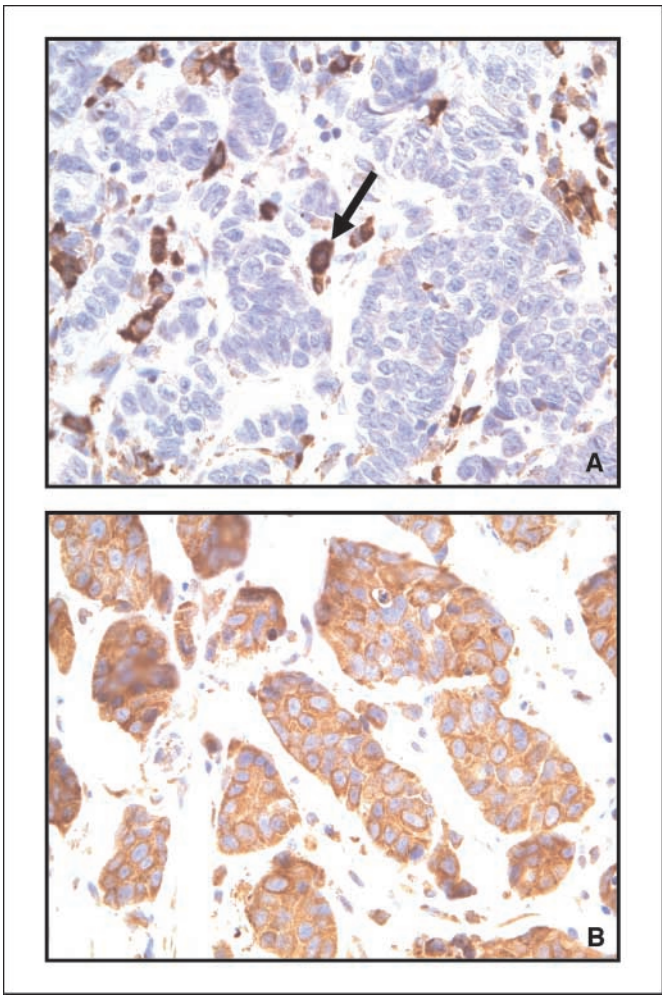

Figure 1. Photomicrographs of immunohistochemical staining of GRP78. Magnification, ×400. *A,* negative staining for GRP78 in neoplastic cells of an infiltrating ductal carcinoma. *Arrow,* plasma cells stain intensely. *B,* intense staining (3+) for GRP78 in neoplastic cells of an infiltrating ductal carcinoma.

not achieve statistical significance (P = 0.16), the observed HR is very close to the value stipulated in the design (1.70 or 70% increase). Adjustment for each patient and tumor characteristic did not substantially change the results (Supplementary Table S2). In a multivariable analysis, the magnitude of the association remained the same even after adjusting for tumor stage, lymph node status, and grade using the propensity score.

Post hoc analyses of GRP78 staining and TTR in subsets of patients by the treatment modalities revealed two strong and interesting trends (Table 2). First, the HR for the GRP78-positive group increased significantly among patients treated with Adriamycin-based chemotherapy who did not receive further treatment with taxane (paclitaxel or docetaxel; HR, 3.00; P = 0.022; also see Fig. 2*B*). The interaction (differences in the two HRs depending on addition of taxanes) was statistically significant (P = 0.012). Further, the adjustment for tumor stage, lymph node status, and grade (using propensity scores) did not change the results. In agreement with taxane treatment exerting an opposing trend, among patients treated with Adriamycin combined with or followed by a taxane, positive GRP78 seemed to have a lower risk of recurrence with borderline significance (HR, 0.15; P = 0.072).

Second, when stratified by type of surgery (segmental mastectomy versus mastectomy), a positive association between GRP78 expression and TTR was observed among patients who underwent mastectomy (HR, 3.33; P = 0.027; also see Fig. 2*C*). The interaction between GRP78 expression and surgery type with regard to TTR was borderline significant (P = 0.078). However, after adjustment for tumor stage, lymph node status, and grade (using propensity scores), the interaction was not statistically significant (P = 0.28), and the association among patients with mastectomy was reduced by 25% (HR, 2.53; P = 0.11). When evaluating the patients who had mastectomy and did not receive a taxane, the association between positive GRP78 and TTR was stronger (HR, 4.82; 95% CI, 1.12-20.87; P = 0.010; Fig. 2*D*) and remained statistically significant after adjustment for tumor stage, lymph node status, and grade (HR, 3.77; 95% CI, 0.85-16.66; P = 0.041). Most patients who had mastectomy did not receive radiation therapy, whereas all but one patient who had segmental mastectomy received radiation therapy. Stratification by radiation therapy yielded similar results as with stratification by type of surgery (data not shown).

Discussion

GRP78 has been implicated as a major player in cancer progression by its role in protecting cancer cells from apoptosis, promoting metastasis, and allowing dormant cancer cells to resist Adriamycin toxicity (9, 10, 17). The retrospective study reported here supports the *in vitro* findings and provides new insight into the relationship between GRP78 induction and chemoresponsiveness. In tumor specimens collected before adjuvant therapy, ~65% expressed high level of GRP78. This agrees with a previous study, which showed the same percentage of breast tumors that exhibited overexpression of GRP78 mRNA (6). Collectively, these suggest that, although the tumor microenvironment has been shown to induce the UPR and GRP78 expression, tumors arising from a subset of patients are unable to induce GRP78 to high level. Although the mechanism for the negative phenotype remains to be determined, our study provides the first evidence that the difference in GRP78 level in tumors can be exploited to predict response to Adriamycin-based chemotherapy among stage II and III breast cancer patients.

Table 1. Association between GRP78 expression and patient characteristics

Patient characteristics	Number of patients	GRP78 expression	
		% Positive*	P †
Total	127	67	
Menopausal status			
Premenopause	66	64	0.41
Postmenopause	61	70	
Histology			
Infiltrating ductal carcinoma	115	66	0.045
Infiltrating lobular carcinoma	10	90	
Others ‡	2	0	
Stage			
T_1 (≤2 cm)	44	68	0.94
T_2 (>2, ≤5 cm)	68	68	
T_3, T_4 (>5 cm or inflammatory)	11	73	
T_x (cannot be measured)	4	(25)	
Lymph node status			
Negative	21	57	0.30
Positive	106	69	
Lymphovascular invasion			
Negative	77	66	0.84
Positive	50	68	
ER/PR status§			
−/−	27	63	0.62
−/+, +/−, or +/+	97	68	
Unknown	3	(66)	
HER-2/neu status			
Negative	76	66	0.96
Positive	23	65	
Unknown	28	(71)	
Grade‖			
1+2	52	65	0.84
3	52	67	
Unknown	11	(64)	
Chemotherapy			
Adriamycin based¶	102	67	0.90
Taxanes added**	25	68	
Surgery type and radiation therapy			
Segmental, radiated	34	62	0.45
Segmental, not radiated	1	0	
Mastectomy, radiated	22	73	
Mastectomy, not radiated	70	68	

*Percent of subjects with GRP78-positive staining.
†Based on χ^2 test, except for histology and surgery type and radiation therapy, for which P is based on Fisher's exact test. Excludes patients with unknown status.
‡Others include medullary carcinoma and papillary carcinoma.
§Estrogen receptor/progesterone receptor status.
‖Limited to infiltrating ductal carcinoma.
¶Adriamycin with one or more of cyclophosphamide, 5-fluorouracil, or methotrexate.
**Adriamycin-based chemotherapy followed by or combined with taxanes.

This study further reveals a potentially significant interaction between GRP78 and taxanes with regard to the resistance to Adriamycin-based chemotherapy. In the subset of patients treated with Adriamycin-based chemotherapy without a taxane, the

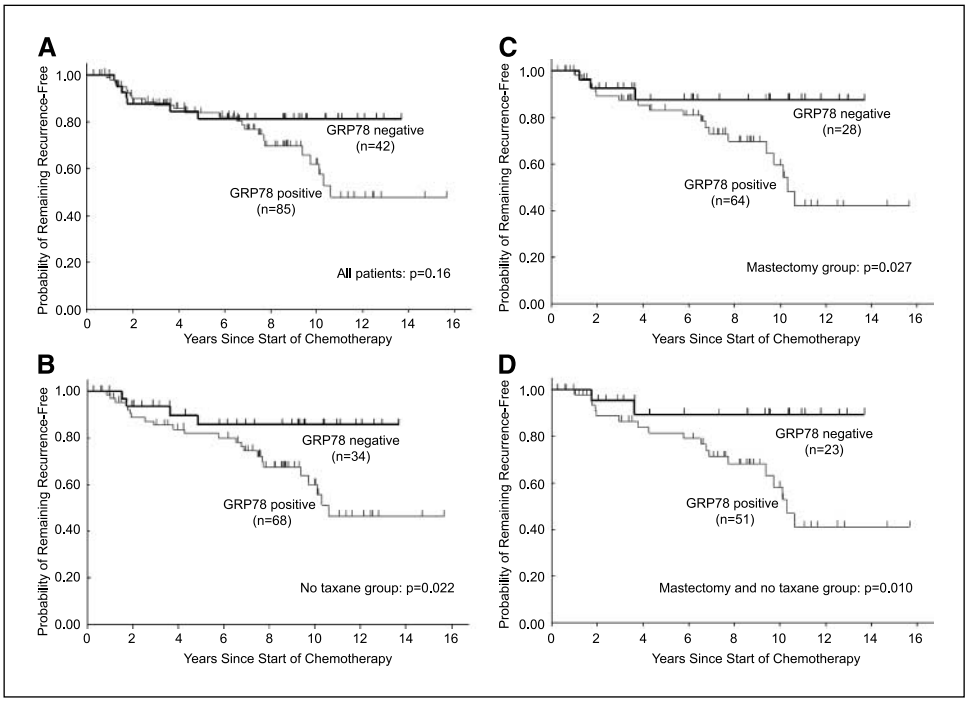

Figure 2. Probability of remaining recurrence-free according to GRP78 expression in patients treated with Adriamycin-based adjuvant chemotherapy. *A,* all 127 patients. *B,* subset of 102 patients who did not receive taxanes (paclitaxel or docetaxel) as part of the Adriamycin-based regimen. *C,* subset of 92 patients who underwent mastectomy. *D,* subset of 74 patients who underwent mastectomy and did not receive taxanes as part of the regimen.

association between GRP78 positivity and higher risk of recurrence attained statistical significance, suggesting a strong association between GRP78, or the underlying UPR, and chemoresistance. In contrast, GRP78 positivity seems to be associated with higher responsiveness to chemotherapy in patients who received both Adriamycin and taxane treatment. Although the observed effect in the taxane group was derived from a relatively small number of patients, it suggests that taxanes may diminish, or even reverse, the

Table 2. Relative risk of recurrence associated with GRP78 expression

Treatment characteristics	GRP78	*n*	Events*	Univariate analysis		Multivariable analysis†	
				HR (95% CI)	*P*‡	HR (95% CI)	*P*‡
Overall analysis							
Total subjects	Negative	42	7	1		1	
	Positive	85	24	1.78 (0.77-4.14)	0.16	1.76 (0.74-4.17)	0.18
Subgroup analyses							
Chemotherapy							
Adriamycin based§	Negative	34	4	1		1	
	Positive	68	23	3.00 (1.04-8.70)	0.022	3.00 (1.02-8.84)	0.026
Taxanes added‖	Negative	8	3	1		1	
	Positive	17	1	0.15 (0.016-1.46)	0.072	0.24 (0.020-3.00)	0.24
P for interaction (GRP78 and chemotherapy)					0.012		0.012
Surgery type							
Segmental mastectomy	Negative	14	4	1		1	
	Positive	21	5	0.74 (0.20-2.77)	0.66	0.53 (0.11-2.54)	0.42
Mastectomy	Negative	28	3	1		1	
	Positive	64	19	3.33 (0.98-11.30)	0.027	2.53 (0.73-8.75)	0.11
P for interaction (GRP78 and Surgery)					0.078		0.28

*Number of recurrences.
†Stratified analysis using propensity score (based on tumor stage, lymph node status, and grade) divided into quintiles.
‡*P*s from likelihood ratio test based on Cox model.
§Adriamycin with one or more cyclophosphamide, 5-fluorouracil, or methotrexate.
‖Adriamycin-based chemotherapy followed by or combined with taxanes.

effect of GRP78 and/or the UPR on Adriamycin resistance. Taxanes, through prevention of polymerization of new microtubules, block endoplasmic reticulum elongation and movement, which is required for maintenance of its unique subcellular structure (18). Further, in breast cancer cells, taxanes increased nuclear localization of the transcription factor YB-1, which represses transcription of the *GRP78* gene (19, 20). Thus, apparently, taxanes can alter or interfere with GRP78 function as well as UPR protective pathways, such as inhibition of translation and degradation of misfolded proteins, by disrupting the endoplasmic reticulum structure and inhibiting GRP78 transcription. Further, GRP78 and the UPR may play different roles in different types of cancer and treatment regimens (4, 7).

We observed a strong association between GRP78 and TTR among mastectomy patients. However, the heterogeneity in the association by surgery type was reduced when adjusted for tumor stage, lymph node status, and grade. Therefore, the type of surgery could have been a proxy of intrinsic tumor characteristics. Alternatively, it could have been a reflection of radiation therapy: chemoresistant breast cancer cells were subsequently killed by radiation, masking the role of GRP78 in chemoresistance. However, the effect of radiation therapy is likely limited to the breast, favoring the former view. In conclusion, this study suggests that GRP78 may represent a novel biomarker for prediction of chemoresponsiveness in breast cancer patients. These results warrant confirmation in larger clinical studies and in other types of cancer.

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