Activation of the PERK-eIF2α Pathway Is Associated with Tumor-infiltrating Lymphocytes in HER2-Positive Breast Cancer

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Abstract. *Background: We evaluated endoplasmic reticulum stress and unfolded protein response (UPR) activation, as possible mechanisms for influx of tumor infiltrating lymphocytes (TILs), and the correlation between UPR activation and mammalian target of rapamycin (mTOR) pathway activation. Materials and Methods: TILs and the immunohistochemical expression of protein kinase RNA-like endoplasmic reticulum kinase (PERK), phospho-eukaryotic translation initiation factor 2α (p-eIF2α) and phosphorylated S6 (pS6) were evaluated in 447 human epidermal growth factor receptor 2 (HER2)-positive breast cancer tissues. Results: High expression of PERK, p-eIF2α and pS6 was observed in 270 (60.4%), 259 (57.9%), and 187 (41.8%) cases, respectively, and was significantly associated with a high histological grade, high numbers of TILs, peritumoral lymphocytic infiltration, and tertiary lymphoid structures in HER2-positive breast cancer tissues. Conclusion: The results suggest endoplasmic reticulum stress and UPR activation as possible mechanisms for the influx of TILs in HER2-positive breast cancer. Evaluation of PERK and p-eIF2α expression might be important in identifying targets for cancer therapies in modulating endoplasmic reticulum stress.*

Tumor-infiltrating lymphocytes (TILs) have predictive and prognostic value in human epidermal growth factor receptor 2 (HER2)-positive breast cancer, in that abundant TILs in stroma of invasive carcinoma is an independent factor for good prognosis (1-4). However, the mechanisms of TIL influx into HER2-positive breast cancer tissues have not yet been clearly defined.

The endoplasmic reticulum (EnR) is important for regulating calcium homeostasis, protein folding, and transportation in eukaryotic cells. Protein folding is very sensitive to changes in the microenvironment and can be disrupted, causing accumulation of misfolded proteins in the EnR, which is termed as EnR stress (5). EnR stress has been documented in various carcinoma types. The protein kinase RNA-like endoplasmic reticulum kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α) pathway is one of the unfolded protein response (UPR) pathways involving multiple signaling pathways that maintain the appropriate environment for EnR-protein folding (5). Under EnR stress, misfolded proteins cause the release of PERK, and activated PERK phosphorylates eIF2α. The PERK–eIF2α pathway has been reported to facilitate as well as to suppress malignant transformation in human cancer (6).

Recent work has shown that EnR stress in conjunction with UPR activation is associated with inflammatory response in cancer, wherein pro-inflammatory cytokines induce EnR stress, which in turn triggers inflammatory responses (7). The UPR pathway activates transcriptional regulators of pro-inflammatory pathways, such as nuclear factor-κB (8-10).

Furthermore, EnR stress and UPR activation are also associated with immunogenic cell death, wherein they initiate signaling pathways to cause the elimination of cancer cells by the immune system (11). However, the relationship between EnR stress, the UPR pathway, and inflammatory responses including TILs has not been studied in HER2-positive breast cancer. A recent study showed that some growth factors, independently of EnR stress, activate UPR through mammalian target of rapamycin complex 1 (mTORC1) in endothelial cells (12).

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In this study, we evaluated EnR stress and UPR activation by immunohistochemical staining of PERK and phosphorylated-eIF2α (p-eIF2α) , as possible mechanisms for the influx of TILs, and the correlation between UPR activation and phosphorylated-S6 ribosomal protein (pS6) expression, which is an indicator of mTOR pathway activation (13), in patients with HER2-positive breast cancer treated with adjuvant chemotherapy and 1 year of trastuzumab. We also analyzed the correlation of UPR activation with other clinicopathological factors, including patient survival.

Materials and Methods

Patients and tissue specimens. This study included 447 patients with HER2-positive breast cancer who underwent surgery for primary breast cancer between 2006 and 2011, at the Department of Pathology, Asan Medical Center, Seoul, Korea. Formalin-fixed, paraffin-embedded tissue samples from these patients were available for analysis as previously described (14). All patients were preoperatively chemo- and radiotherapy naive.

The expression levels of standard biomarkers, including estrogen receptor (ER), progesterone receptor (PR), and HER2, were reviewed in full sections that were immunohistochemically stained at the time of diagnosis, independently by two pathologists (H.J.L. and G.G.). The Allred scores were also calculated for ER and PR (15). HER2-overexpressing tumors were defined as those with scores of 3+ by immunohistochemistry (IHC) or by gene amplification using either fluorescence *in situ* hybridization or silver *in situ* hybridization (16). Exemption from informed consent after the de-identification of information was approved by the Institutional Review Board.

Histological evaluation. Hematoxylin and eosin (H&E)-stained slides of whole tumor sections were reviewed by two pathologists (H.J.L. and G.G.). Slides were histopathologically analyzed for the following: i) Percentage (in 10% increments) of stroma of invasive carcinoma infiltrated by TILs, and if <10% of stroma was infiltrated by TILs, 1% or 5% increments were included (all available full-sections were evaluated); ii) Peritumoral inflammatory cell infiltrate according to Klintrup criteria (17) (score 0, no inflammatory cells at the invasive margin of the tumor; score 1, mild and patchy inflammatory cells; score 2, prominent band-like inflammatory reaction; and score 3, florid cup-like inflammatory infiltrate); and iii) The presence of tertiary lymphoid structures (TLS) in adjacent tissues, including the *in situ* component (none, no TLS formation in the adjacent area to the tumor; little, TLS occupying an area of <10% of the circumference of the tumor; moderate, TLS occupying 10 to 50%; or abundant, TLS occupying over 50%) (4, 18). TILs were also divided into three groups: minimal (≤10%), moderate (20-60%), or abundant (>60%) stroma of invasive carcinoma infiltrated by TILs. Histological grade, tumor size, pT stage, pN stage, and lymphovascular invasion were analyzed. The histological grade was assessed using the modified Bloom–Richardson classification (19).

Tissue microarray construction and immunohistochemical evaluation. The tissue microarray (TMA) was constructed from formalin-fixed, paraffin-embedded tissue samples with a tissue microarrayer (Uni TMA Co., Seoul, Korea) as previously described (20). Each sample was arrayed in three 1-mm diameter cores to minimize tissue loss and account for tumor heterogeneity based on HER2 expression. TMAs were stained with an automatic immunohistochemical staining device (Benchmark XT; Ventana Medical Systems, Tucson, AZ, USA) and visualized with OptiView DAB IHC Detection Kit (Ventana Medical Systems) according to the manufacturer's instructions. Antibodies for PERK (1:200; #5683; Cell Signaling Technology, Danvers, MA, USA), p-eIF2α (1:1000; ab32157; Abcam, Cambridge, UK), pS6 (1:200; Ser240/244, #5364; Cell Signaling Technology) and HLA-ABC (1:1600; ab70328, EMR8-5; Abcam) were used. Sections of 4 μm thickness were obtained using a microtome, transferred onto silanized charged slides and dried for 10 min at room temperature, followed by 20 min in an incubator at 65°C. Sections were then cut by heat-induced epitope retrieval using Cell Conditioning 1 buffer for 32 min and incubated for 16 min with antibodies in the autoimmunostainer. Antigen–antibody reactions were visualized using Ventana OptiView DAB IHC Detection Kit (Optiview HQ Linker 8 min, Optiview HRP Multimer 8 min, Optiview H_2O_2/DAB 8 min, Optiview Copper 4 min). Counterstaining was performed using Ventana Hematoxylin II for 4 min and bluing reagent for 4 min. Finally, all slides were removed from the stainer, dehydrated, and coverslipped for microscopic examination. Negative controls consisted of identically treated histological sections with the omission of primary antibodies.

Normal pancreatic tissue was used as positive control for both PERK and p-eIF2α. The intensity of cytoplasmic expression using a four-value intensity score (0, 1, 2, and 3) (Figure 1) and the percentage of expression were evaluated separately. An immunoreactive score was generated as the product of the intensity and the percentage of positively stained cells. Immunoreactive scores were dichotomized based on mean expression for each protein. The percentages of membranous and cytoplasmic expressions of human leukocyte antigens (HLA)-ABC were evaluated. Next, the expression levels were categorized according to the following: strongly positive: 2+, expression in more than 75% of the tumor cells; weakly positive: 1+, expression in 25% to 75% of tumor cells; and negative: 0, loss of expression in more than 75% of the tumor cells, as previously described (21).

Statistical analysis. All statistical analyses were performed using SPSS statistical software (version 18; SPSS, Chicago, IL, USA). Disease-free survival (DFS) was defined as the time from start of trastuzumab to recurrence of breast cancer at any site. Chi-square tests, linear-by-linear association tests, Spearman's correlation, log-rank tests, and Cox proportional hazards regression models were used when required. All tests were two-tailed and a threshold for statistical significance was set at 5%.

Results

Clinicopathological characteristics of the study population. All 447 patients were women, and their median age at diagnosis was 49 years (range=22 to 79 years). Tumor sizes ranged from 0.1 to 8.2 cm (median=2.2 cm). Two hundred and seven cases were pT1 tumors, 225 were pT2, and 15 were pT3. In lymph-node evaluation, 161 tumors were pN0 stage, 14 were pN1mi, 162 were pN1, 61 were pN2, and 49 were pN3. The median follow-up was 54.2 months (range=25.4-118.8 months).

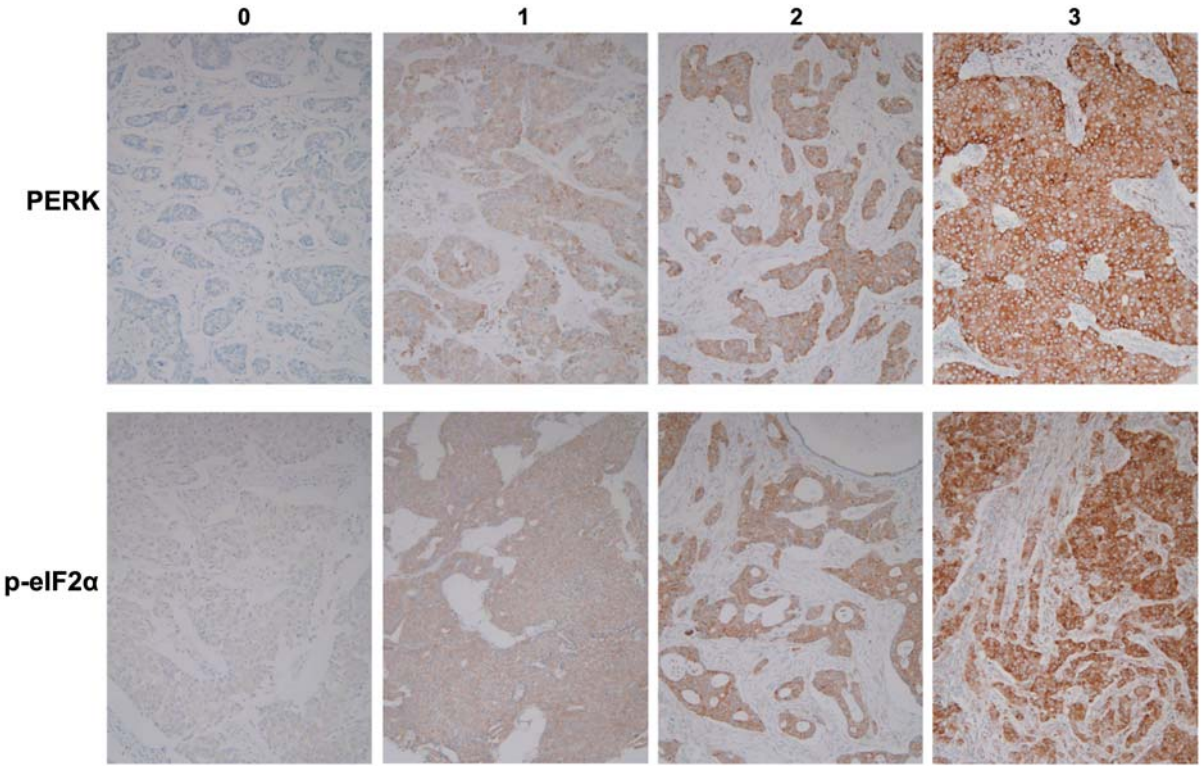

Figure 1. *Protein kinase RNA-like endoplasmic reticulum kinase (PERK) and phospho-eukaryotic translation initiation factor 2α (p-eIF2α) immunohistochemical staining intensities for cytoplasm of tumor cells for four scores (magnification ×200).*

Clinicopathological correlation of PERK and p-eIF2α expressions in HER2-positive breast cancer. The mean immunoreactive scores of PERK and p-eIF2α were 193 and 201. Immunoreactive scores were dichotomized as high or low based on the mean value. High PERK and p-eIF2α expression were observed in 270 (60.4%) and 259 (57.9%) cases, respectively. The expression of PERK was significantly correlated with that of p-eIF2α (rho=0.339, $p<0.001$). High expression of PERK was significantly associated with high histological grade, high levels of TILs (Figure 2A and D), peritumoral lymphocytic infiltration, TLS, ER negativity based on ER Allred score, strong IHC positivity for HER2 (Figure 2B), and a high level of amplification of *HER2* (Figure 2C) in HER2-positive breast cancer tissues (Table I). High expression of p-eIF2α (Figure 2E) was also associated with high histological grade and pT stage, absence of lymph node metastasis, high levels of TILs and peritumoral lymphocytic infiltration, ER negativity, strong IHC positivity for HER2, and strong HLA-ABC expression.

Correlation of PERK and p-eIF2α expression with pS6 expression. High pS6 expression was identified in 187 (41.8%) cases, and associated with high levels of TILs (Figure 2F), peritumoral lymphocytic infiltration, and TLS. pS6 expression was also significantly associated with high expression of PERK and p-eIF2α (Table II).

Prognostic significance of TILs and PERK and p-eIF2α expression. The prognostic significance of clinicopathological factors including TILs was analyzed (Table III). By univariate analysis, a lower pT stage, absence of lymphovascular invasion, and increased TILs were associated with a better disease-free survival. Neither PERK nor p-eIF2α expression levels were found to be associated with patient survival.

Discussion

In the EnR stress conditions, unfolded proteins bind to immunoglobulin heavy-chain binding protein (BIP) leading to subsequent UPR activation. The UPR activation in turn is concomitant with the activation of three linked pathways: PERK and eIF2α, activating transcription factor 6α (ATF6α), and inositol-requiring protein 1α (IRE1α)-X-box binding protein 1 (XBP1) (5). A few molecules included in these pathways have been studied in breast cancer (22-24). To the best of our knowledge, this is the first study to demonstrate that activation of the UPR pathway in HER2-positivie breast cancer tissues is associated with TILs and other clinicopathological

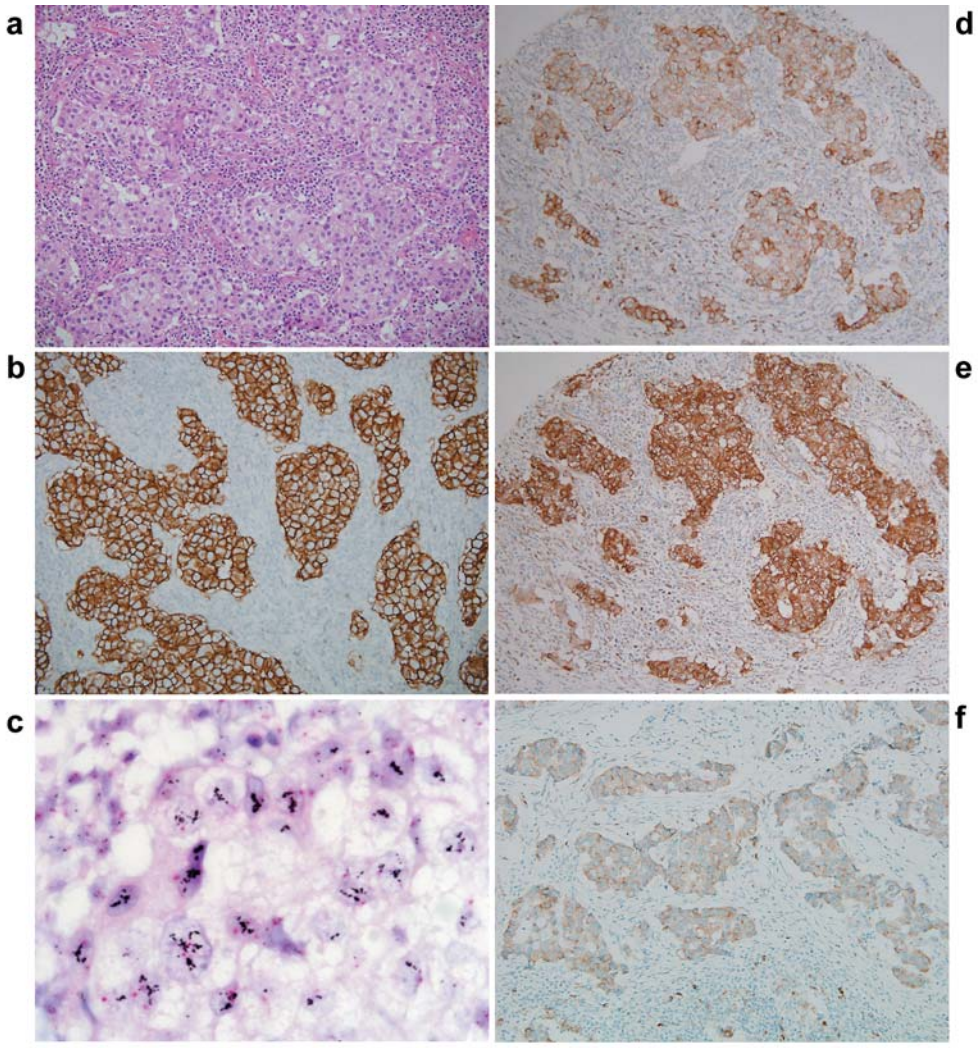

Figure 2. *High expression of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and phospho-eukaryotic translation initiation factor 2α (p-eIF2α) and phosphorylated S6 (pS6) in tumor cells. Exemplary tumor samples characterized by a high level of tumor-infiltrating lymphocytes (A, H&E; magnification ×200), strong immunopositivity for human epidermal growth factor receptor 2 (HER2) (B; magnification ×200), high level of HER2 amplification (C; silver in situ hybridization, magnification ×400), high expression of PERK (D; magnification ×200), p-eIF2α (E; magnification ×200) and pS6 (F; magnification ×200).*

factors. Although TILs have prognostic and predictive value in HER2-positive breast cancer (1-4), the mechanism of TIL influx has not been clearly defined. EnR stress has been known to induce cell death by triggering an inflammatory response (5). In this study, we suggested the activation of PERK and eIF2α as a possible mechanism of TIL influx, since high expression of PERK and p-eIF2α was significantly correlated with TILs in HER2-positive breast cancer.

Moreover, we found that high expression of PERK or p-eIF2α in HER2-positive breast cancer tissues was significantly associated with high histological grade, ER negativity, and strong IHC positivity for HER2, which are recognized as features of aggressive breast cancer. Therefore, we can assume that tumors with aggressive features might exhibit increased EnR stress and subsequently increased TIL influx.

PERK is attached to the membrane of the EnR, and PERK and eIF2α pathway can induce either cellular apoptosis or survival *via* complex downstream pathways such as growth arrest and DNA damage-inducible protein 34, ATF4, and C/EBP homologous protein (5). This may facilitate or suppress tumorigenesis depending on the context, through either cellular autophagy, translation, increased EnR protein folding and transportation, or EnR-associated protein degradation (5, 25). These complex interactions between downstream molecules might be the cause of the lack of any prognostic effect of PERK and

Table I. *Comparison of pathological variables based on the cytoplasmic expression of eukaryotic translation initiation factor 2α (eIF2α) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) in human epidermal growth factor receptor 2 (HER2)-positive breast cancer.*

	p-eIF2α			PERK		
Variable	Low (%)	High (%)	*p*-Value	Low (%)	High (%)	*p*-Value
Histological grade			<0.001			0.001
2	96 (51.1)	71 (27.4)		83 (46.9)	84 (31.1)	
3	92 (48.9)	188 (72.6)		94 (53.1)	186 (68.9)	
pT stage			0.033			0.733
1	97 (51.6)	110 (42.5)		87 (49.2)	120 (44.4)	
2	82 (43.6)	143 (55.2)		81 (45.8)	144 (53.3)	
3	9 (4.8)	6 (2.3)		9 (5.1)	6 (2.2)	
Lymphovascular invasion			0.085			0.997
Negative	100 (53.5)	159 (61.6)		103 (58.2)	156 (58.2)	
Positive	87 (46.5)	99 (38.4)		74 (41.8)	112 (41.8)	
Lymph node metastasis			<0.001			0.078
Negative	49 (26.1)	112 (43.2)		55 (31.1)	106 (39.3)	
Positive	139 (73.9)	147 (56.8)		122 (68.9)	164 (60.7)	
Tumor-infiltrating lymphocytes			0.011			0.013
≤10%	98 (52.1)	107 (41.3)		92 (52.0)	113 (41.9)	
20-60%	71 (37.8)	109 (42.1)		68 (38.4)	112 (41.5)	
>60%	19 (10.1)	43 (16.6)		17 (9.6)	45 (16.7)	
Peritumoral lymphocytic infiltration score			<0.001			0.003
0	31 (16.5)	15 (5.8)		23 (13.0)	23 (8.5)	
1	97 (51.6)	128 (49.4)		99 (55.9)	126 (46.7)	
2	47 (25.0)	89 (34.4)		44 (24.9)	92 (34.1)	
3	13 (6.9)	27 (10.4)		11 (6.2)	29 (10.7)	
Tertiary lymphoid structure in adjacent tissues			0.061			0.004
None	23 (12.2)	22 (8.5)		24 (13.6)	21 (7.8)	
Minimal	48 (25.5)	54 (20.8)		47 (26.6)	55 (20.4)	
Moderate	61 (32.4)	92 (35.5)		58 (32.8)	95 (35.2)	
Abundant	56 (29.8)	91 (35.1)		48 (27.1)	99 (36.7)	
ER Allred score			<0.001			<0.001
0-2 (negative)	80 (42.6)	157 (60.6)		56 (31.6)	181 (67.0)	
3-8 (positive)	108 (57.4)	102 (39.4)		121 (68.4)	89 (33.0)	
HER2 IHC			0.009			<0.001
2+	36 (19.1)	27 (10.4)		38 (21.5)	25 (9.3)	
3+	152 (80.9)	232 (89.6)		139 (78.5)	245 (90.7)	
HER2 amplification			0.630			<0.001
Low	43 (23.2)	55 (21.3)		55 (31.6)	43 (16.0)	
High	142 (76.8)	203 (78.7)		119 (68.4)	226 (84.0)	
HLA-ABC IHC			0.001			0.092
0	65 (36.3)	54 (20.9)		46 (27.4)	73 (27.1)	
1	33 (18.4)	52 (20.2)		41 (24.4)	44 (16.4)	
2	81 (45.3)	152 (58.9)		81 (48.2)	152 (56.5)	

ER: Estrogen receptor; IHC: immunohistochemistry; HLA-ABC: human leukocyte antigen-ABC.

eIF2α activation in this study, even though they are significantly associated with TILs.

Recently, PERK and eIF2α activation independently of EnR stress has been reported. Karali *et al*. revealed that phospholipase Cγ-mediated mTORC1 activates UPR in the absence of EnR stress (12). Loss of tuberous sclerosis complex, which leads to activation of mTOR and downstream signaling molecules, results in protein synthesis with EnR protein folding, and causes EnR stress with subsequent UPR activation (26, 27). Therefore, we evaluated the expression of pS6, a surrogate indicator of mTOR activation (13), whose expression significantly correlated with high expression of PERK and eIF2α and high level of TILs. These results support previous findings of the correlation between UPR activation and the mTOR pathway. Our results additionally indicate correlation between TILs and activation of the mTOR pathway in tumor cells.

Table II. *Comparison of variables associated with lymphocytic infiltration and expression of eukaryotic translation initiation factor 2α (eIF2α) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) based on phosphorylated S6 (pS6) expression in human epidermal growth factor receptor 2 (HER2)-positive breast cancer.*

Variable	pS6		
	Low (%)	High (%)	*p*-Value
Histological grade			0.015
2	107 (41.8)	57 (30.5)	
3	149 (58.2)	130 (69.5)	
pT stage			0.169
1	113 (44.1)	91 (48.7)	
2	131 (51.2)	93 (49.7)	
3	12 (4.7)	3 (1.6)	
Lymphovascular invasion			0.431
Negative	144 (56.2)	111 (60.0)	
Positive	112 (43.8)	74 (40.0)	
Lymph node metastasis			0.047
Negative	82 (32.0)	77 (41.2)	
Positive	174 (68.0)	110 (58.8)	
Tumor-infiltrating lymphocyte			0.006
≤10%	128 (50.0)	75 (40.1)	
20-60%	103 (40.2)	76 (40.6)	
>60%	25 (9.8)	36 (19.3)	
Peritumoral lymphocytic infiltration score			<0.001
0	34 (13.3)	10 (5.3)	
1	137 (53.5)	88 (47.1)	
2	68 (26.6)	66 (35.3)	
3	17 (6.6)	23 (12.3)	
Tertiary lymphoid structure in adjacent tissues			0.035
None	29 (11.4)	16 (8.6)	
Minimal	62 (24.2)	39 (20.9)	
Moderate	92 (35.9)	58 (31.0)	
Abundant	73 (28.5)	74 (39.6)	
p-eIF2α expression			<0.001
Low	129 (50.4)	56 (29.9)	
High	127 (49.6)	131 (70.1)	
PERK expression			<0.001
Low	129 (50.4)	46 (24.6)	
High	127 (49.6)	141 (75.4)	

Table III. *Univariate survival analyses of clinicopathological variables related to clinical outcome.*

Variable	Disease-free survival		
	Hazard ratio	95% CI	*p*-Value
Age: ≥50 *vs.* <50 years	0.593	0.303-1.194	0.146
Histological grade: 3 *vs.* 2	1.550	0.744-3.227	0.242
pT: 2–3 *vs.* 1	2.565	1.202-5.473	0.015
Lymphovascular invasion: Positive *vs.* negative	2.719	1.353-5.464	0.005
Lymph node metastasis: Positive *vs.* negative	2.130	0.927-4.892	0.075
Tumor-infiltrating lymphocytes (per 10% rise)	0.981	0.965-0.998	0.024
p-eIF2α expression: high *vs.* low	0.873	0.449-1.698	0.688
PERK expression: high *vs.* low	0.991	0.504-1.949	0.979
pS6 expression: high *vs.* low	0.638	0.312-1.302	0.217

EnR stress recently emerged as a target for cancer therapy, whereby it induces severe EnR stress, which leads to cell death. Inhibition of PERK and eIF2α activation augments EnR stress in cancer cells, inducing cancer cell death (5). In this study, 77.4% (346/447) of cases of HER2-positive breast cancers had high expression of either PERK or p-eIF2α. These cancer cells may be potential targets for specific drugs such as PERK inhibitors that induce EnR stress by inhibiting UPR activation (5).

In summary, EnR stress assessed by expression of PERK and p-eIF2α was significantly associated with TILs in HER2-positive breast cancer, and activation of PERK and eIF2α was also associated with activation of the mTOR pathway. Thus, evaluation of PERK and p-eIF2α expression in HER2-positive breast cancer might be important in identifying targets for cancer therapies modulating EnR stress.

Disclosure

There are no conflicts of interest in regard to this study.

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