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Double-edged role of G protein-coupled estrogen receptor 1 in breast cancer prognosis: an analysis of 167 breast cancer samples and online data sets

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Abstract: G protein-coupled estrogen receptor 1 (GPER1) is widely expressed in breast cancer; however, its prognostic significance in breast cancer patients remains controversial. In this study, expression levels of GPER1 were analyzed by using real-time polymerase chain reaction in 167 primary breast cancer samples, and overall survival (OS), recurrence-free survival (RFS), distant metastasis-free survival (DMFS), and disease-free survival (DFS) were analyzed by using Kaplan–Meier curves and multivariable Cox regression. In addition, a meta-analysis was conducted with all available online data sets found in the Web sites kmplot.com and www.prognoscan.org. The results showed that there was no significant correlation between GPER1 expression and OS, RFS, DMFS, and DFS in the total breast cancer patient population. In contrast, the meta-analysis of online data sets found that expression levels of GPER1 were slightly associated with better RFS in the total breast cancer population (P=0.021). Interestingly, higher expression of GPER1 was associated with poorer DFS in HER2-positive subtype of breast cancer (P=0.047) but with better DMFS (P=0.040) and DFS (P=0.035) in HER2-negative subtype of breast cancer. In addition, it was found that HER2 overexpression in MDA-MB-231 cell increased GPER1, which may help explain protumor effect of GPER1 in HER2-overexpressed patients. The overall results suggested that the expression of GPER1 has distinct prognostic values in HER2-positive and HER2-negative breast cancer patients.

Keywords: GPER1, breast cancer, HER2, CREB, cAMP response element-binding protein

Introduction

As breast cancer is the most commonly diagnosed disease in females in the USA[1] and the People's Republic of China,[2] it requires more attention. Increased knowledge of breast cancer revealed that it is a much more heterogeneous disease than was previously known. Classic subclassification of this cancer with estrogen receptor (ER), progesterone receptor, and HER2 may be no longer sufficient.[3] More personalized treatments that are more targeted may lead to superior efficacy and less toxicity.[4] Thus, new biomarkers and therapy targets are required.

G protein-coupled estrogen receptor 1 (GPER1), or G protein-coupled receptor 30, is a homologue of seven-transmembrane domain receptor, G protein-coupled receptor (GPCR), which was first found in the 1990s.[5] Followed by estrogen receptor-α (ERα) and ERβ, GPER1 was recognized as a new estrogen target. Later, it was found that GPER1 can activate epidermal growth factor receptor (EGFR) through matrix metalloproteinases-mediated release of heparin-binding EGF (HB-EGF).[6–8] Then, EGFR substrates mitogen-activated protein kinases (MAPKs)[8] and PI3-kinase (PI3K)[9] may be activated, followed by the activation of c-fos[10] and c-jun.[11] In addition, GPER1 can also lead to rapid activation of protein kinase A (PKA) pathway[12] and of PKA's

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downstream cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB).[13]

It was found universally that GPER1 was expressed in various cancers, including lung, prostate endometrial, ovarian, thyroid, and breast cancers,[14–18] and it can be activated by diverse ligands. Except for estrogen, ERα antagonists, tamoxifen and fulvestrant, were also found to be used as agonists of GPER1.[9,19] Other ligands include vitamins,[20] aldosterone,[21] and some environmental contaminants.[22]

Although functionally and universally involved in cancers, the role of GPER1 in prognosis remains controversial. It was reported that GPER1 plays a role in stimulating cancer cell proliferation,[23–26] and it was also reported that GPER1 functions as a tumor suppressor.[27–30] Therefore, this research, aimed to find out whether GPER1 is a tumor suppressor or a stimulator by using 167 breast cancer samples and online data sets.

Materials and methods

Patients and samples

All the 167 patients with breast cancer were diagnosed and treated with surgery from January 2009 to December 2009 in Fudan University-affiliated Shanghai Cancer Center (FDUSCC). Breast cancer samples were stored at −80°C immediately after resections. Histopathological analyses were conducted according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists by the Department of Pathology in FDUSCC. All these 167 cases were followed up for >20 months (Table S1).

RNA isolation and retro-polymerase chain reaction (PCR)

RNA was isolated with TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA, USA) by following the product protocol given by the manufacturer. Retro-PCR procedures were performed by using Bio-Rad Retro-PCR kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) with product protocol.

Real-time PCR assays

GPER1 and 18S RNA mRNA (inner reference) quantifications were performed by using Eppendorf realplex 4 with SYBR® Green from Bio-Rad.

The primers are TGCACGAGCGGTACTACGA/GATGCCATCCAGATGAGGC and CAGCCACCCGAGATTGAGCA/TAGTAGCGACGGGCGGGTGT, respectively.

Online databases

Table 1 lists all the available online data sets that could represent relationship between GPER1 expression and breast cancer prognosis and were selected under the guidelines available in kmplot.com[31] and www.prognoscan.org.[32]

Cell line and transfection

Triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 were purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, People's Republic of China. HER2 plasmid was pursued from GeneChem (Shanghai, People's Republic of China). Fugene® 6 (Hoffman-La Roche Ltd., Basel, Switzerland) was used as a transfection reagent.

Western blotting analysis

Cell lysis was carried out by using sodium dodecyl sulfate gel with 8% acrylamide. Anti-HER2 was purchased from Bethyl (Montgomery, TX, USA); anti-GPER1, anti-p-CREB, and anti-vinculin (inner reference) were purchased from Abcam (Cambridge, MA, USA); and anti-mouse and anti-rabbit were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

Statistical analysis

Kaplan–Meier (KM) plots and multivariable Cox regression analyses were conducted by using Statistical Package for the

Table 1 Cited data sets

Data set	Authors	Publication year	Citation	Data set	Authors	Publication year	Citation
E-MTAB-365	Guedj et al	2012	38	GSE2034	Wang et al	2005	39
E-TABM-158	Chin et al	2006	40	GSE20685	Kao et al	2011	41
GSE11121	Schmidt et al	2008	42	GSE20711	Dedeurwaerder et al	2011	43
GSE12093	Zhang et al	2009	44	GSE21653	Sabatier et al	2011	45
GSE12276	Bos et al	2009	46	GSE2603	Minn et al	2005	47
GSE1378	Ma et al	2004	48	GSE26971	Filipits et al	2011	49
GSE1379	Ma et al	2004	48	GSE2990	Sotiriou et al	2006	50
GSE1456-GPL96	Pawitan et al	2005	51	GSE31519	Rody et al	2011	52
GSE16391	Desmedt et al	2009	53	GSE3494	Miller et al	2005	54
GSE16446	Desmedt et al	2011	55	GSE5327	Minn et al	2007	56
GSE17705	Symmans et al	2010	57	GSE6532-GPL570	Loi et al	2007	58
GSE17907	Sircoulomb et al	2010	59	GSE7390	Desmedt et al	2007	60
GSE19615	Li et al	2010	61	GSE9195	Loi et al	2008	62

Social Sciences Version 16.0 (IBM Corporation, Armonk, NY, USA). Hazard ratios (HRs) of all the factors were achieved by using backward method in Cox regression analyses. Forest plots were performed with Stata Version 12.0 (StataCorp LP, College Station, TX, USA). Medium value was set as a cutoff in all cases. $P<0.05$ was statistically significant. *P*-values of KM plot were calculated by using log rank test.

Ethical statement

Permissions for scientific research usage of all breast cancer samples in this assay were obtained from patients before they underwent surgeries in FDUSCC. The ethics committee of FDUSCC did not require that ethical approval and informed patient consent be obtained for this study due its retrospective nature.

Results

Clinical and pathological characteristics

Among the 167 patients with breast cancer, 63.5% were aged >50 years and 36.5% were <50 years; 25.1% of patients were at T1 stage, and the remaining were at T2 or T3 stage; 45.5% of patients were lymph node positive, 40.1% were negative, and the remaining 14.4% were not informed; 75.4% of the samples were ER positive and 33.5% were HER2 positive; and 79.0% of patients underwent endocrine therapy, and 88.6% underwent chemotherapy.

ER and HER2 status might be related with GPER1 expression (*P*=0.043 and 0.090, respectively); thus, they might affect the role of GPER1 in prognosis under multivariate Cox regression analysis (Table 2).

Table 2 Clinical and pathological characteristics

Variables	Total patients	GPER1 higher	GPER1 lower	*P*-value
Total	167	84	83	
Age (years)				
>50	106 (63.5%)	56	50	
≤50	61 (36.5%)	28	33	0.389
T stage				
T1	42 (25.1%)	20	22	
T2 and T3	125 (74.9%)	64	61	0.688
N stage				
Positive	76 (45.5%)	39	37	
Negative	67 (40.1%)	28	39	0.255
Unknown	24 (14.4%)	17	7	
ER				
Positive	126 (75.4%)	69	57	
Negative	41 (24.6%)	15	26	0.043
HER2				
Positive	56 (33.5%)	23	33	
Negative	111 (66.5%)	61	50	0.090
TAM				
Yes	45 (26.9%)	25	20	
No	122 (73.1%)	59	63	0.409
Other endo[a]				
Yes	93 (55.7%)	51	42	
No	74 (44.3%)	33	41	0.188
Chemotherapy				
Yes	148 (88.6%)	75	73	
No	19 (11.4%)	9	10	0.786

Note: [a]Other endo: other endocrine therapy except for TAM.
Abbreviations: GPER1, G protein-coupled estrogen receptor 1; TAM, tamoxifen treatment.

Cox regression analysis of disease-free survival (DFS) and overall survival (OS)

In univariate Cox regression analysis, none of the characteristics presented significant relationship with DFS. While it indicated that T stage, N stage, and HER2 status were correlated with patients' OS (HR =3.16, 95% confidence interval [CI] =1.05–9.54, *P*=0.041; HR =2.19, 95% CI =1.03–4.69, *P*=0.043; HR =3.17, 95% CI =1.04–9.69, *P*=0.043, respectively). In multivariate Cox regression analysis, it was found that N stage was related to DFS (HR =3.03, 95% CI =1.04–8.87, *P*=0.043), and none was significantly correlated with OS. In both univariate and multivariate analyses, GPER1 did not present any significant relationship with DFS or OS (Tables 3 and 4).

KM analysis of GPER1

KM analysis found no significant relationship between GPER1 and breast cancer prognosis, but in HER2-overexpressed subgroup, it was found that lower expressed patients had better DFS than higher expressed patients (HR =4.47, 95% CI =0.93–21.56, log rank *P*=0.041). Conversely, in non-HER2-overexpressed or "HER2-negative" subgroup, it was found that GPER1-higher expressed patients had better distant metastasis-free survival (DMFS) and DFS (HR =0.23, 95% CI =0.05–1.07, log rank *P*=0.040); HR =0.27, 95% CI =0.08–0.99, log rank *P*=0.035, respectively; Figure 1.

Cox regression analysis of DFS and OS in HER2-overexpressed subgroup

In HER2-overexpressed subgroup, both T stage and N stage also played important roles in affecting breast cancer prognosis. In univariate Cox regression analysis, it was found that N stage were correlated with both worse DFS (HR =3.54, 95% CI =1.12–11.22, *P*=0.032) and worse OS (HR =3.17, 95% CI =1.00–10.06, *P*=0.050). In multivariate Cox regression analysis, T stage and N stage were correlated with worse DFS (HR =3.72, 95% CI =1.08–12.82, *P*=0.037; HR =8.15, 95% CI =1.05–63.47, *P*=0.045). Higher T stage was also implicated worse OS (HR =3.47, 95% CI =1.04–11.50, *P*=0.042). Besides, GPER1 was also found to be related to

Table 3 Univariate analysis of DFS and OS

Factors	DFS HR				OS HR			
	Average	Lower	Upper	*P*-value	Average	Lower	Upper	*P*-value
Age	1.02	0.98	1.06	0.317	1.05	1.00	1.10	0.072
T stage	1.90	0.81	4.47	0.139	3.16	1.05	9.54	0.041
N stage	1.38	0.86	2.23	0.183	2.19	1.03	4.69	0.043
ER	1.10	0.41	2.99	0.846	0.52	0.17	1.59	0.252
HER2	1.38	0.59	3.22	0.463	3.17	1.04	9.69	0.043
TAM	1.27	0.52	3.11	0.605	0.81	0.22	2.96	0.754
Other endo[a]	1.40	0.59	3.32	0.453	0.93	0.31	2.76	0.894
Chemotherapy	0.81	0.24	2.74	0.738	24.16	0.01	43,520.00	0.405
GPER1	0.55	0.23	1.30	0.174	0.84	0.28	2.51	0.76

Note: [a]Other endo: other endocrine therapy except for TAM.
Abbreviations: DFS, disease-free survival; ER, estrogen receptor; GPER1, G protein-coupled estrogen receptor 1; HR, hazard ratio; OS, overall survival; TAM, tamoxifen treatment.

worse DFS (HR =7.57, 95% CI =1.03–55.58, *P*=0.047) in HER2-overexpressed subgroup (Tables 5 and 6).

Meta-analysis with online data sets

Considering that there might not be enough incidents in the present research, all the possible data sets that may reflect the role of GPER1in breast cancer prognosis were searched and collected from kmplot.com and www.prognoscan.org (Table 1). By using all these available 26 online data sets and the present research, it was found that higher expressed GPER1 was slightly but significantly related to better recurrence-free survival (RFS; HR =0.91, 95% CI =0.85–0.99; Figure 2). only 14 and eight data sets were available for DMFS and OS analysis, and neither of them was significantly correlated with GPER1 expression (Figure S1). Data sets that comprise HER2 subgroups were even fewer; only three data sets were available for RFS (Figure S2) and one for DMFS and OS (data not shown).

Overexpression of HER2 in HER2-negative cell lines raised GPER1 level

In order to investigate why the role of GPER1 differed in HER2-overexpressed patients from general population, HER2-overexpressed MDA-MB-231 and MDA-MB-468 cell lines were established. It was found that in both the cell lines, HER2 overexpression led to GPER1 increase in mRNA and protein level. As a substrate of GPER1 but not of HER2, CREB's higher phosphorylation also indicated that HER2 overexpression promoted GPER1 expression and function (Figure 3).

Discussion

It was not surprised that GPER1 was related with proliferation and migration of breast cancer,[23–26] as it was reported that it can be able to active MAPKs,[8] PI3Km[9] and PKA.[12] In contrast, there were more and more newly published data showing that GPER1 might play an anti-tumorigenesis role in breast cancer,[28–30] which is also coincided with what was found in the present meta-analysis (Figure 2). In the present research, no evidence showed that GPER1 contributed to breast cancer progression in the general population (Figures 1 [left], 2, and S1) but mildly correlated with better RFS (Figure 2). It might be because that activation of GPER1 led to G2/M-phase cell cycle block with accumulation of G2-checkpoint proteins, cyclin B1 and Cdc2.[11,27,30] It might also raise p53 expression via intracellular calcium (Ca^{2+}) mobilization, leading to p53-induced cell cycle arrest.[30] The prolongation of mitotic duration that disturbs regular cell

Table 4 Multivariate analysis of DFS and OS

Factors	DFS HR				OS HR			
	Average	Lower	Upper	*P*-value	Average	Lower	Upper	*P*-value
Age	1.01	0.98	1.05	0.497	1.04	0.99	1.10	0.130
T stage	1.55	0.89	2.68	0.119	2.12	0.98	4.60	0.057
N stage	3.03	1.04	8.87	0.043	2.55	0.65	10.01	0.181
ER	0.70	0.19	2.65	0.603	0.61	0.13	2.85	0.532
HER2	1.12	0.39	3.25	0.836	1.92	0.51	7.20	0.333
TAM	1.53	0.49	4.77	0.464	0.64	0.13	3.08	0.575
Other endo[a]	2.08	0.61	7.09	0.242	1.18	0.27	5.20	0.827
Chemotherapy	0.18	0.03	1.07	0.060	N/A	N/A	N/A	N/A
GPER1	0.79	0.33	1.93	0.606	1.77	0.53	5.93	0.355

Note: [a]Other endo: other endocrine therapy except for TAM.
Abbreviations: DFS, disease-free survival; ER, estrogen receptor; GPER1, G protein-coupled estrogen receptor 1; HR, hazard ratio; N/A, not available; OS, overall survival; TAM, tamoxifen treatment.

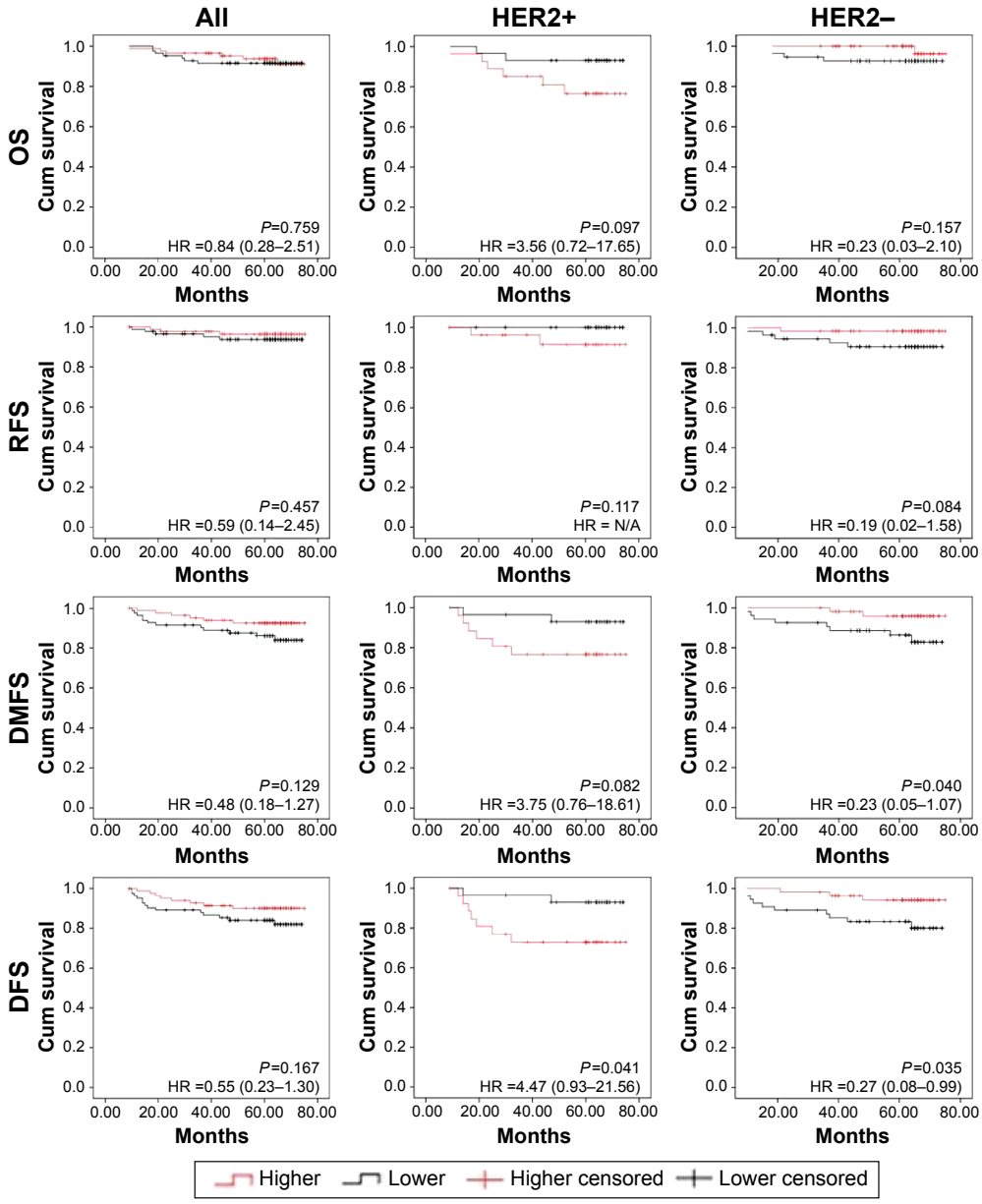

Figure 1 Kaplan–Meier analysis of OS, RFS, DMFS, and DFS of patients according to GPER1 mRNA expression.

Notes: No statistically significant difference was found in entire sample group (left column); in HER2-positive subgroup, GPER1-lower expressed patients have better DFS (middle column); in HER2-negative subgroup, GPER1-higher expressed patients have better DMFS and DFS (right column). Red lines stand for GPER1-higher expressed patients; black lines stand for GPER1-lower expressed patients. "HER2+" stands for HER2-overexpressed patients and "HER2–" stands for the remaining patients.

Abbreviations: DFS, disease-free survival; DMFS, distant metastasis-free survival; GPER1, G protein-coupled estrogen receptor 1; OS, overall survival; RFS, recurrence-free survival; Cum, cumulative.

Table 5 Univariate analysis of DFS and OS in HER2-overexpressed patients

Factors	DFS HR				OS HR			
	Average	Lower	Upper	*P*-value	Average	Lower	Upper	*P*-value
Age	1.02	0.96	1.09	0.445	1.04	0.98	1.11	0.195
T stage	2.75	2.75	0.73	0.134	1.77	0.41	7.62	0.441
N stage	3.54	1.12	11.22	0.032	3.17	1.00	10.06	0.050
ER	1.87	0.47	7.47	0.377	0.93	0.23	3.72	0.920
TAM	1.05	0.22	5.05	0.953	0.81	0.22	2.96	0.754
Other endo[a]	2.50	0.63	10.01	0.194	1.24	0.31	4.96	0.761
Chemotherapy	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GPER1	4.47	0.926	21.56	0.062	3.56	0.72	17.65	0.12

Note: [a]Other endo: other endocrine therapy except for TAM.

Abbreviations: DFS, disease-free survival; ER, estrogen receptor; GPER1, G protein-coupled estrogen receptor 1; HR, hazard ratio; N/A, not available; OS, overall survival; TAM, tamoxifen treatment.

Table 6 Multivariate analysis of DFS and OS in HER2-overexpressed patients

Factors	DFS HR				OS HR			
	Average	Lower	Upper	*P*-value	Average	Lower	Upper	*P*-value
Age	1.02	0.95	1.10	0.602	1.04	0.96	1.13	0.337
T stage	3.72	1.08	12.82	0.037	3.47	1.04	11.50	0.042
N stage	8.15	1.05	63.47	0.045	4.50	0.47	43.01	0.191
ER	1.15	0.20	6.83	0.876	0.85	0.13	5.53	0.867
TAM	0.23	0.03	1.72	0.151	0.15	0.02	1.64	0.121
Other endo[a]	1.27	0.20	8.01	0.802	0.78	0.12	5.25	0.800
Chemotherapy	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GPER1	7.57	1.03	55.58	0.047	6.63	0.93	47.02	0.059

Note: [a]Other endo: other endocrine therapy except for TAM.
Abbreviations: DFS, disease-free survival; ER, estrogen receptor; GPER1, G protein-coupled estrogen receptor 1; HR, hazard ratio; N/A, not available; OS, overall survival; TAM, tamoxifen treatment.

cycle may then lead to cell apoptosis.[28] Therefore, GPER1 may be double-edged in tumor genesis, and the anti-tumor effect might take a slight advantage in general breast cancer patient population (Figure 2).

But contrary to slight anti-tumor effect in general patient population, GPER1 seemed to be related with worse prognosis in HER2-overexpressed subgroup in the present research (Figure 1 [middle]; Table 6), and such tendency was also present in online data sets GSE17907 and E-MTAB-365, though without significance (Figure S2). It may be because that GPER1 led to activation of matrix metalloproteases as mentioned previously, resulting in the release of EGFR ligand HB-EGF.[8,18] As

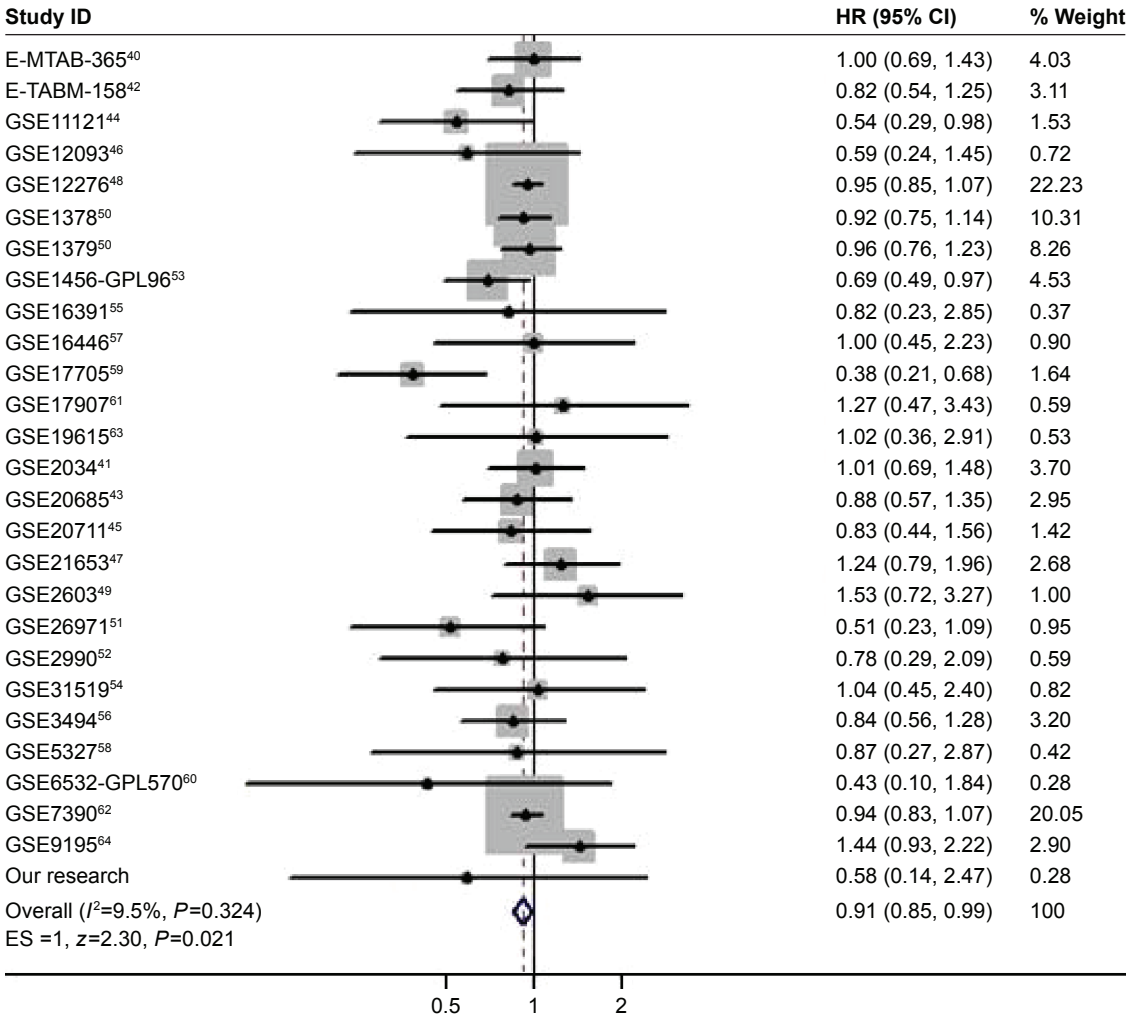

Figure 2 Forest plots of RFS analysis in online data sets combined with the present research.
Notes: The overall HR was 0.91(0.85–0.99), P=0.021. Points represent average HR, and line segments stand for 95% confidence interval. Rhombus stands for overall average HR and 95% confidence interval.
Abbreviations: ES, effect scale; HR, hazard ratio; RFS, recurrence-free survival.

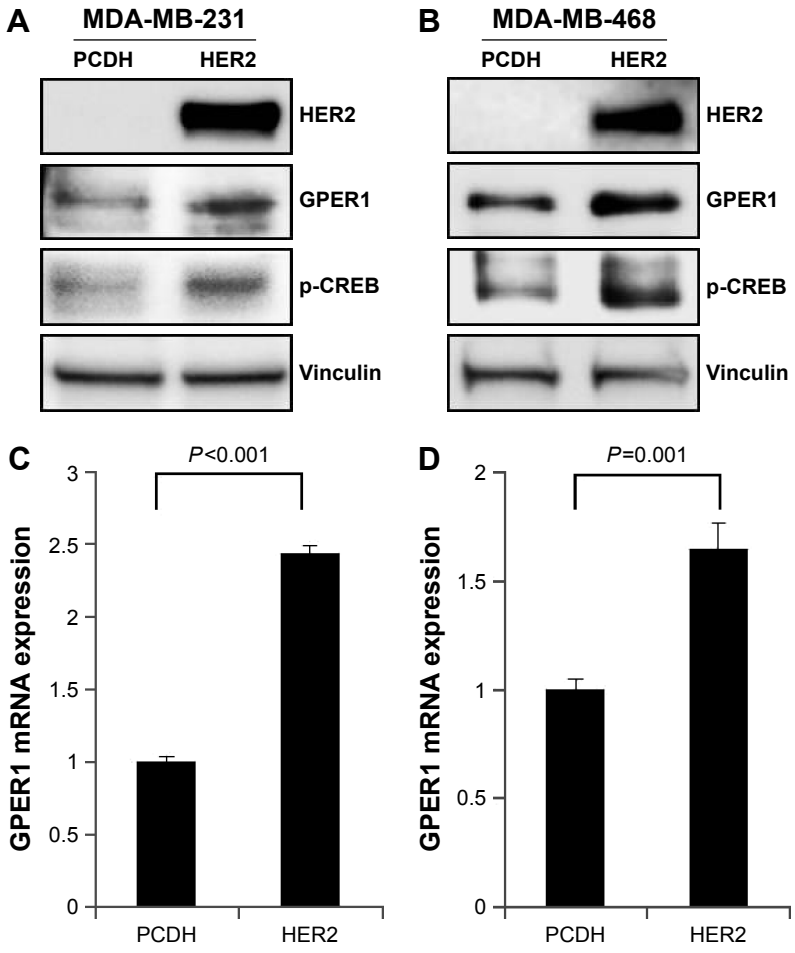

Figure 3 Overexpression of HER2 in HER2-negative breast cell lines.
Notes: Overexpression of HER2 in MDA-MB-231 (**A** and **C**) and MDA-MB-468 (**B** and **D**) raised GPER1 in protein level (**A** and **B**) and mRNA level (**C** and **D**), following with elevation of GPER1 substrate CREB phosphorylation (**A** and **B**).
Abbreviations: CREB, cAMP response element-binding protein; GPER1, G protein-coupled estrogen receptor 1.

HER2 works as heterodimers combined with other HER family members such as EGFR, GPER1-induced HB-EGF abundance might trigger EGFR/HER2 substrate progresses.[33] On the other hand, evidences showed that activation of EGFR/ERK/c-fos raised GPER1 expression,[34,35] and GPER1 was overexpressed in HER2-positive breast cancer patients.[36] Moreover, in the present study, it was shown that overexpressed HER2 led to higher GPER1 expression and function in HER2-negative cell lines (Figure 3). Thus, in HER2-overexpressed patients, activation of HER2 pathway might enhance the "protumor edge" of GPER1 and cover its weak anti-tumor effect, as evidences showed that combined use of HER2 inhibitor trastuzumab and GPER1 agonist G1 provided better inhibitory effect in HER2-positive cell lines than the use of G1 alone.[37]

Conclusion

In summary, by using 167 breast cancer samples and online data sets, the role of GPER1 was analyzed in breast cancer prognosis. In this meta-analysis, it was found that GPER1 may contribute to better prognosis in general breast cancer patients and especially in "HER2-negative" patients, but this is not the case in HER2-overexpressed patients, indicating that GPER1 plays a double-edged role in breast cancer prognosis.

Disclosure

The authors report no conflicts of interest in this work.

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