

# THE INTERACTIONS BETWEEN GPR30 AND THE MAJOR BIOMARKERS IN INFILTRATING DUCTAL CARCINOMA OF THE BREAST IN AN ASIAN POPULATION

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## SUMMARY

**Objective:** G-protein-coupled receptor 30 (GPR30) has been reported to be a novel estrogen receptor  $\alpha$  (ER $\alpha$ ) *in vitro*. Therefore, the interactions among GPR30, ER $\alpha$ , progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu), and their prognostic utilities in the infiltrating ductal carcinoma (IDC) of the breast were evaluated.

**Materials and Methods:** Messenger RNA (mRNA) levels of GPR30, ER $\alpha$ , PR and HER-2/neu in the tumor samples of 118 Taiwanese IDC patients and 27 non-tumor mammary tissues were measured via quantitative polymerase chain reaction analyses. The correlations of GPR30 mRNA levels with clinical parameters, i.e. tumor/non-tumor, ER $\alpha$ , PR, HER-2/neu, age, lymph node metastasis, lymph-vascular invasion, grade, stage and patient survival, were assessed by using appropriate statistical analyses.

**Results:** GPR30 expression was observed to be lower in IDC ( $p < 0.001$ ) than in non-tumor mammary tissues. Importantly, GPR30 mRNA level was positively correlated with that of ER $\alpha$  ( $p = 0.001$ ) and PR ( $p = 0.001$ ) but not correlated with that of HER-2/neu when they were analyzed as continuous variables. However, lower GPR30 was noticed in tumors with HER-2/neu protein overexpression. GPR30 expression was not correlated with age, lymph node metastasis, lymph-vascular invasion, grade and stage in IDC. GPR30 expression was not an independent prognostic factor for patient survival.

**Conclusion:** GPR30 expression is downregulated in IDC. GPR30 is preferentially co-expressed with ER and/or PR but is lowly expressed in HER-2/neu(+) tumors. The correlation of GPR30 expression with clinical parameters, including patient survival, was not evident in this cohort. [*Taiwan J Obstet Gynecol* 2007;46(2):135-145]

**Key Words:** estrogen receptor  $\alpha$ , G-protein-coupled receptor 30, human epidermal growth factor receptor-2, mRNA, progesterone receptor

## Introduction

Breast cancer is one of the most common cancers in the world. Its incidence has increased quickly during the past decade in Taiwan [1]. Breast cancer has a known association with the steroid hormone estrogen

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[2]. Estrogen receptor  $\alpha$  (ER $\alpha$ ) status is very important in the clinical management of breast cancer patients, both as a prognostic factor and as a target of adjuvant hormone therapy.

Carmeci et al [3] identified a gene, G-protein-coupled receptor 30 (GPR30), whose expression at mRNA level is preferentially associated with ER $\alpha$  expression in breast cancer cell lines, as well as in primary breast cancer. Based on its nucleotide and amino acid sequences that share homology with the G-protein-coupled receptor superfamily [3–5], GPR30 is thought to be G-protein-coupled receptor. Using a breast cancer cell model, Filardo et al [6] found the dual regulatory action mediated via GPR30. It recognizes estrogen as the ligand, and GPR30 activates the G-protein coupled signaling cascade that regulates both growth factor receptor and ER signaling transduction pathways. GPR30-mediated *c-fos* gene expression in response to estrogen action is via an ER $\alpha$ -independent mechanism in breast cancer cells [7]. GPR30 is found mainly involved in non-genomic signaling of estrogen [8].

GPR30 has been reported previously as a functional membrane estrogen receptor that may contribute to normal estrogen physiology as well as pathophysiology [9], but this particular function was found to be insignificant in MCF-7 cell line [10].

Based on the evidence accumulated for GPR30, the clinical impact of GPR30 in breast cancer is worth investigating.

## Materials and Methods

### *Surgical specimens*

One hundred and eighteen patients with primary infiltrating ductal carcinoma (IDC) of the breast, who underwent surgery between 1998 and 2005 at National Taiwan University Hospital (NTUH), were included in this study. The tumor specimens were excised, collected and snap-frozen in liquid nitrogen immediately. Then, they were immersed in liquid nitrogen for long-term storage. All patients had given informed consent according to guidelines approved by the Institutional Review Board of NTUH. For comparison, 27 non-tumor mammary tissue samples were also collected. Clinical data were retrieved from their medical records. Immunohistochemical examination for ER $\alpha$ , progesterone receptor (PR) and HER-2/neu, using paraffin-embedded breast cancer specimens, was performed routinely by pathologists.

### *RNA isolation*

Total RNA was isolated from the stored specimen using Trizol reagent (Life Technologies, Rockville, MD,

USA) and subsequently purified using RNeasy Mini Kit (Qiagen, DE, USA). Purified RNA was quantified at OD<sub>260 nm</sub> by a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and qualitatively analyzed by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA samples with RNA integrity number for RNA quality ranged between 6 and 10 were used for quantitative reverse transcription polymerase chain reaction (Q-PCR) analysis.

### *Relative quantification of mRNA by Q-PCR*

The basic rationale for using the following method has been documented by Bookout et al [11]. For Q-PCR analysis, total RNA ( $\leq 5 \mu\text{g}$ ) was reversely transcribed using MMTV reverse transcriptase (Promega, Madison, WI, USA). Five genes, GPR30, ER $\alpha$ , PR, human epidermal growth factor receptor-2 (HER-2/neu) and TATA box-binding protein (TBP), were chosen for quantification by Q-PCR analysis using commercially available Assays-on-Demand probe-primer sets (Applied Biosystems, Foster City, CA, USA). TBP, a housekeeping gene, was selected as the reference gene because it is constitutively expressed in both mammary tissue and breast tumors. The primer IDs (Applied Biosystems, Foster City, CA, USA) designated as Hs00172183\_m1, Hs00170433\_m1, Hs00173506\_m1, Hs00174860\_m1 and Hs00427620\_m1 were for amplification of cDNA-PR, HER-2/neu, GPR30, ER and TBP, respectively. Each sample mix was run in duplicate and consisted of cDNA generated from the sample being investigated.

Briefly, each 10  $\mu\text{L}$  reaction contained 2  $\mu\text{L}$  of Master Mix, 0.5  $\mu\text{L}$  of AOD probe-primer solution, 1.5  $\mu\text{L}$  of nuclease-free water, and 6  $\mu\text{L}$  of cDNA, generated as described above. The polymerase chain reaction parameters were 95°C for 10 min, 50 cycles of 95°C for 10 seconds, and 60°C for 15 seconds. At the end of each cycle, the data were automatically analyzed by the system, and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler software (Roche Applied Science, Penzberg, Germany) calculated the threshold cycle (C<sub>p</sub>), which is defined as the fractional cycle number at which the fluorescence reaches the baseline.

We defined  $\Delta\text{Cp}$  ( $\Delta\text{Cp} = \text{Cp}_{\text{target gene}} - \text{Cp}_{\text{TBP}}$ ) as either the level for a gene of interest relative to an endogenous reference RNA or the normalized mRNA expression level for the gene of interest. The normalized mRNA expression level for the gene of interest in this text was further expressed as the negative value of  $\Delta\text{Cp}$ . This was for the purpose of making a positive correlation between the numerical values and their corresponding mRNA levels.

### Data analysis

The RNA yield for our normal mammary tissue (non-tumor part) was very low. Therefore, for Q-PCR analysis, a minimum number of samples ( $n = 27$ ) were studied, based on the method described by Kuehl [12].

Box plots [13] were used for conveying location and variation information in our Q-PCR data sets, particularly for detecting and illustrating location and variation changes between different groups of data. A box was drawn between the 25<sup>th</sup> and 75<sup>th</sup> percentile of data, and the parallel line inside the box indicates the sample median. Possible extreme values for each data set were also labeled. The observation was considered to be extremely small/large if the value was more than 1.5 times the interquartile range (interquartile range = the height of the box) away from the box.

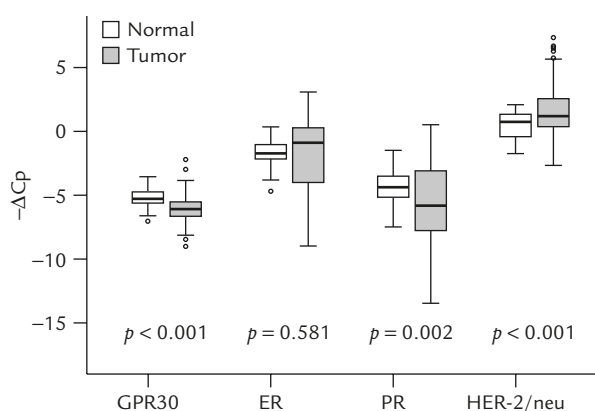
The correlation between the GPR30 mRNA level and the clinical index was analyzed by applying the two-sample  $t$  test and analysis of variance (ANOVA) to dichotomous and multichotomous indices, respectively. The Pearson's correlation coefficient used to find the linear relationships between mRNA levels of GPR30 and continuous clinical variables.

ANOVA,  $t$  test and Cox proportional hazards regression model were used to test the significance of GPR30 and/or others as the measure of patient survival.

## Results

### Relative mRNA levels of ER $\alpha$ , GPR30, PR and HER-2/neu in IDC of the breast and non-tumor mammary tissues

The median values of mRNA levels of GPR30, ER $\alpha$ , PR and HER-2/neu of IDC as represented by  $-\Delta\text{Cp}$  were  $-6.1$ ,  $-0.9$ ,  $-5.9$  and  $1.2$ , respectively (Figure 1). The



**Figure 1.** Box plots for pairwise mRNA levels of estrogen receptor  $\alpha$  (ER $\alpha$ ), G-protein-coupled receptor 30 (GPR30), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu) in non-tumor/tumor tissues.

middle 50% of the data for GPR30 showed a small variation among breast cancer patients.

Both GPR30 and HER-2/neu did have a few extremely small/large values, which are marked as open circles. For non-tumor mammary tissue samples, the median values of GPR30, ER, PR and HER-2/neu mRNA levels were  $-5.3$ ,  $-1.8$ ,  $-4.4$  and  $0.7$ , respectively. The middle 50% of the data for GPR30 showed a similar pattern of variation in both non-tumor and tumor parts. GPR30 and PR expression were significantly higher in normal mammary tissue than in tumor tissues ( $p < 0.001$ ,  $p = 0.002$ , respectively) (Figures 1 and 2). In contrast, HER-2/neu expression was significantly lower in non-tumor parts than in the tumor parts ( $p < 0.001$ ), while there was no difference in ER expression between tumor and non-tumor tissues ( $p = 0.581$ ).

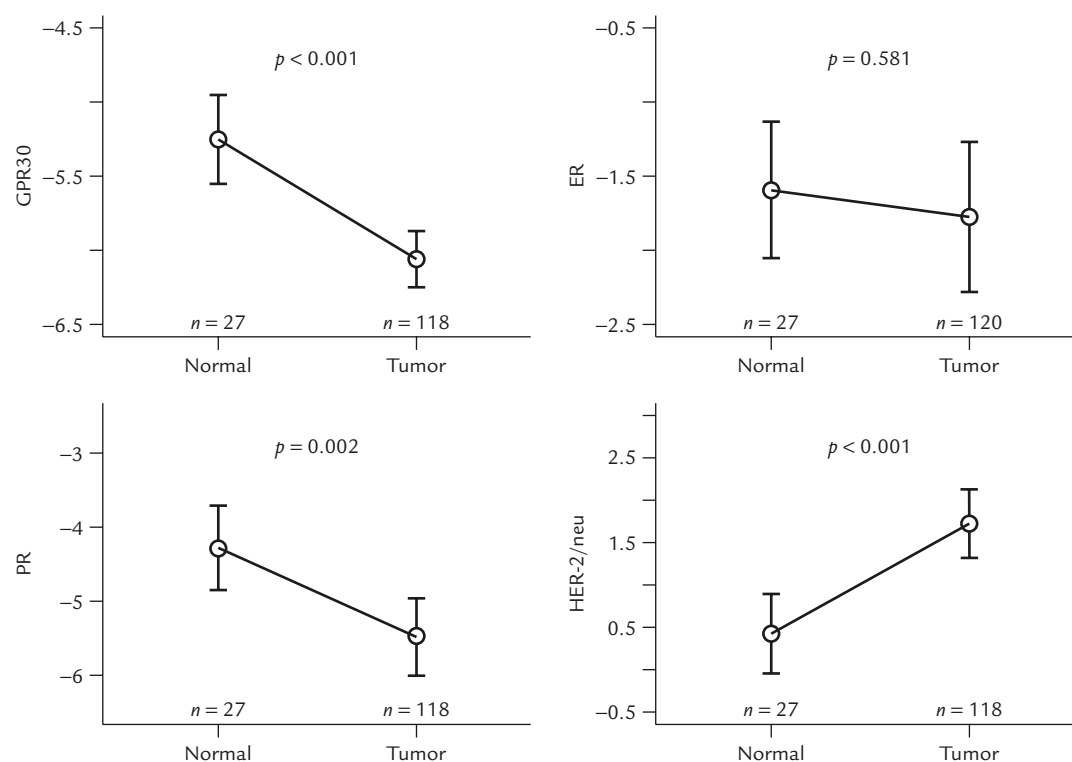
### GPR30 expression vs. ER, PR and HER-2/neu expression

The expression level of GPR30 was significantly higher in ER(+) breast cancer than ER(-) breast cancer ( $p < 0.001$ ) (Figure 3A). Similarly, the expression level of GPR30 was significantly higher in PR(+) breast cancer than PR(-) breast cancer ( $p = 0.028$ ). In contrast, GPR30 expression level was lower in HER-2/neu positive breast cancer than in HER-2/neu negative breast cancer ( $p = 0.004$ ). When analyzed as continuous variables, the expression of GPR30 was positively correlated with the expression of ER $\alpha$  ( $p = 0.001$ ) and PR ( $p = 0.001$ ) at mRNA level (Figure 3B). In this series, there existed significantly positive correlations between GPR30 and ER ( $r = 0.29$ ,  $p = 0.001$ ), GPR30 and PR ( $r = 0.304$ ,  $p = 0.001$ ), and PR and ER ( $r = 0.638$ ,  $p < 0.001$ ).

On the contrary, HER-2/neu mRNA levels were negatively correlated with the GPR30 mRNA levels but the correlation was statistically insignificant ( $r = -0.056$ ,  $p = 0.544$ ), though HER-2/neu expression demonstrated a significantly negative correlation with ER ( $r = -0.241$ ,  $p = 0.008$ ) and PR ( $r = -0.215$ ,  $p = 0.02$ ) (Figure 3C). For immunohistochemistry (IHC) categories, HER-2/neu mRNA level was significantly higher in ER(-) breast cancer than ER(+) breast cancer ( $p = 0.032$ ). It was also significantly higher in PR(-) breast cancer than PR(+) breast cancer ( $p = 0.014$ ). As the control of this analysis, HER-2/neu mRNA level was significantly higher in HER-2/neu protein overexpressing breast cancer than in breast cancer without HER-2/neu overexpression ( $p < 0.001$ ) (Figure 3D).

### GPR30 expression against clinical parameters

There was no significant difference in GPR30 expression in terms of lymph-vascular invasion, lymph node metastasis, age, tumor size, grade and stage (all  $p$ 's  $> 0.05$ )



**Figure 2.** Univariate analyses of G-protein-coupled receptor 30 (GPR30), estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2/neu) mRNA levels in non-tumor/tumor tissues. The vertical lines indicate the 95% confidence intervals.

(Figure 4). The results of univariate analyses for the ER, PR and HER-2/neu expression vs. clinical indices are shown in Figures 5 to 7.

#### **Multivariate analysis of expression of GPR30 and associated biomarkers**

Multiple regression using Cox proportional hazards regression model showed that PR had the lowest relative risk for death with a significance of  $p = 0.003$  as compared with those of GPR30, ER and HER-2/neu (Table).

#### **Survival analyses of expression of GPR30 and associated biomarkers**

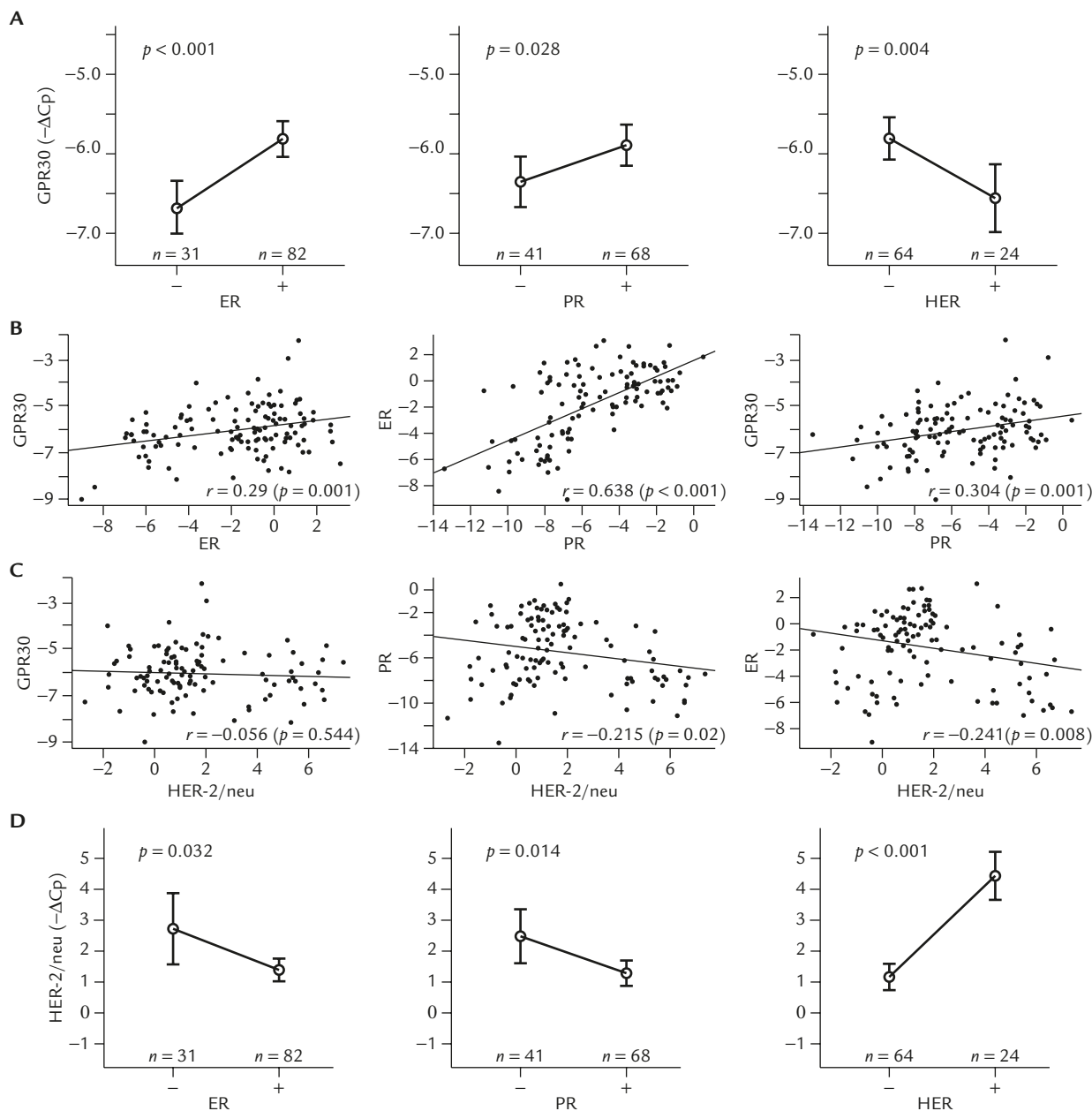
For survival study, the subjects were divided into two groups: either higher or lower than the mean  $-\Delta C_p$  of GPR30 ( $-6$ ), ER ( $-1.8$ ) and PR ( $-5.5$ ) in 118 IDCs. Using the cut-off point of  $-6$ , GPR30 did not serve as a significant measure of patient survival ( $p = 0.383$ ) (Figure 8A). On the contrary, both mean expression levels for ER and PR were significant discriminators of patient survival ( $p = 0.003$  and  $p < 0.001$ , respectively). Like GPR30, HER-2/neu mean expression level was also not an effective discriminator of patient survival in this series ( $p = 0.830$ ). The effects of different combinations of ER $\alpha$  vs. GPR30 and PR vs. GPR30 on patient survival are shown in Figures 8B and 8C. It is clear that when

an ER $\alpha$  or a PR mRNA level was fixed (above or below mean), there was no significant difference in patient survival resulting from changes in GPR30 expression levels. Similar effect of GPR30 expression on patient survival was seen when it was co-expressed with ER protein or PR protein as determined by IHC (Figures 9 and 10).

## **Discussion**

In this report, we intended to explore the roles of ER $\alpha$ , PR and GPR30 by studying gene expression of ER $\alpha$ , PR and GPR30 in breast cancer tissues and normal mammary glands. Also, the relationships among GPR30 expression and other breast cancer biomarkers, such as ER $\alpha$ , PR, HER-2/neu, were studied. Statistical analysis of the correlations between GPR30 expression and clinical indices, including patient survivals, were conducted. The same analyses were applied to ER $\alpha$ , PR and HER-2/neu. The functional end-point for GPR30, ER $\alpha$ , PR and HER-2/neu was patient survival.

To study the GPR30 mRNA levels, Q-PCR analysis was applied to quantify their relative mRNA levels. There are two splicing variants of GPR30 as identified by others [4]. Therefore, we used the primers for Q-PCR analysis of GPR30 to detect both splicing variants. Likewise, the primers for quantifying mRNA of PR were



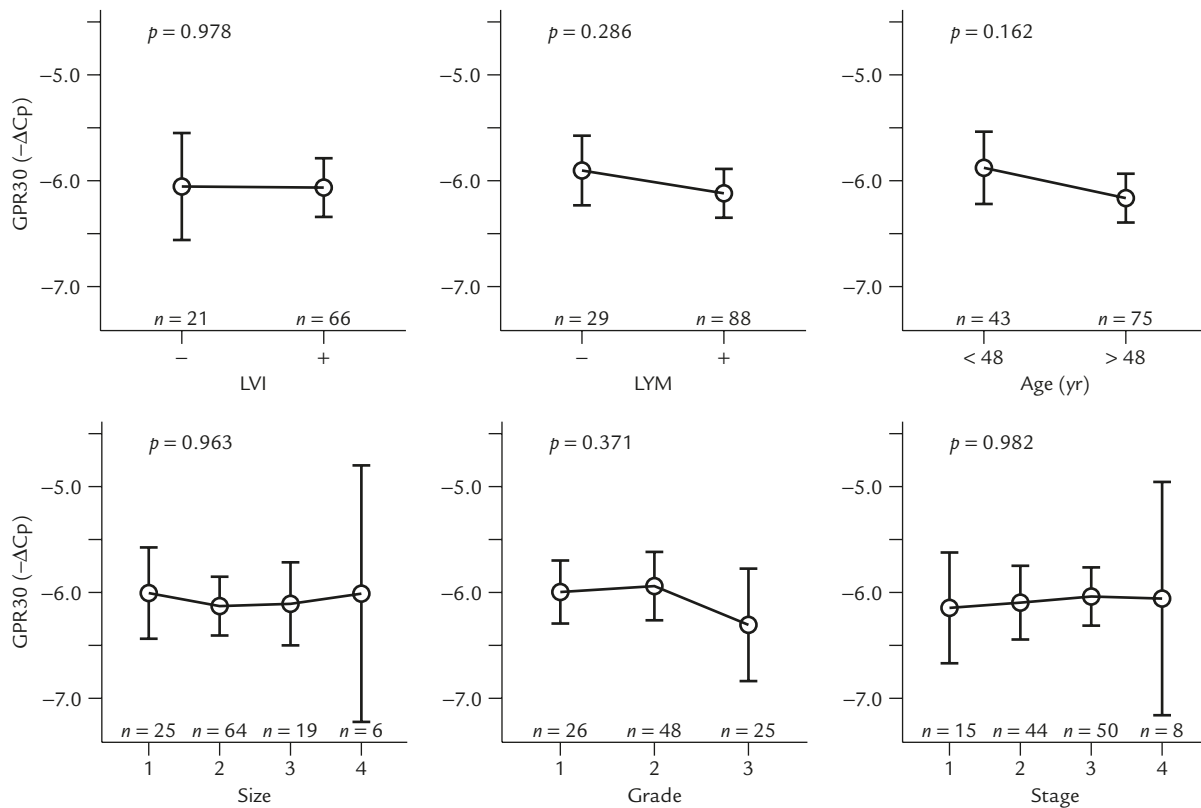
**Figure 3.** Univariate analysis of the expression levels for G-protein-coupled receptor 30 (GPR30) vs. estrogen receptor  $\alpha$  (ER $\alpha$ ), progesterone receptor (PR) or human epidermal growth factor receptor-2 (HER-2/neu). (A) Univariate analyses of GPR30 mRNA level against ER, PR and HER-2/neu (immunohistochemistry [IHC] status) in breast cancer specimens. (B) Scatter plots of the correlation between mRNA levels of GPR30 and those of ER $\alpha$ , PR and HER-2/neu in breast cancer specimens. (C) Scatter plots of the correlation between HER-2/neu mRNA levels and those of ER $\alpha$ , PR and HER-2/neu in breast cancer specimens. (D) Univariate analyses for HER-2/neu mRNA levels against ER $\alpha$ , PR and HER-2/neu (IHC status) in breast cancer specimens.

designed to detect both PR-A and PR-B. However, the primers used were to only detect full-length cDNA of ER $\alpha$  and HER-2/neu.

#### **Expression of GPR30, ER $\alpha$ , PR and HER-2/neu in tumor vs. non-tumor mammary tissues**

Although GPR30 protein levels have been found to be different in normal mammary tissues and breast tumors [8], there is no research report that compares GPR30

mRNA levels in breast cancers with those in normal mammary tissues. We observed a significant difference in GPR30 mRNA levels between tumor and non-tumor mammary tissue ( $p < 0.001$ ). In the non-tumor mammary tissue, the GPR30 mRNA levels were significantly higher than those in breast cancer tissue (Figure 1). This finding showed a similar trend with the results provided by immunohistochemistry stain of GPR30 protein [14] but at mRNA level.



**Figure 4.** Univariate analyses of G-protein-coupled receptor 30 (GPR30) mRNA levels against clinicopathologic variables in breast cancer specimens. The sample number being analyzed for each category was based on the availability of samples with the records of their clinicopathologic information. Some of the samples were missing of a few records for their clinicopathologic features. The vertical lines indicate the 95% confidence intervals.

The variations in GPR30 expression levels among breast cancer samples were smaller than the remaining three biomarkers: ER $\alpha$ , PR and HER-2 (Figure 1). The variation of ER $\alpha$ , PR and GPR30 mRNA levels were also smaller in non-tumor mammary samples as compared with those in the tumor part. Overall, our results showed that the expression levels of GPR30 and PR in non-tumor mammary tissue were significantly higher than those in breast cancer ( $p < 0.001$  for GPR30,  $p = 0.002$  for PR) (Figures 1 and 2) while tumor and non-tumor samples revealed no significant difference in the expression levels of ER ( $p = 0.581$ ). This suggests the possible transcriptional silencing for GPR30 and PR during tumorigenesis. These are quite interesting findings. Although ER $\alpha$  and PR were found to be upregulated in breast tumor samples via IHC stain [15], selective epigenetic silencing of ER $\alpha$  and PR genes in breast cancer also have been observed [16,17]. Whether ER $\alpha$  in breast cancer samples is upregulated or not needs further investigation. The IHC stain for both ER $\alpha$  and PR in our tumor and non-tumor samples also confirmed that ER $\alpha$  and PR protein levels can be reflected by their corresponding mRNA levels (data not shown). HER-2/neu expression was upregulated in breast cancer

samples in our setting, consistent with the findings of others [15].

#### **Correlation between GPR30 and ER, PR and HER-2/neu expression in breast cancer**

Linear regression analysis revealed statistically significant correlation between GPR30 and ER $\alpha$  at mRNA level in breast cancer samples ( $p = 0.001$ ) (Figure 3B). Correlation was also significant between GPR30 and PR ( $p = 0.001$ ) (Figure 3B). However, this relationship did not apply between HER-2/neu and GPR30 ( $p = 0.544$ ) (Figure 3C). If examined in the IHC category (Figure 3A), GPR30 expression level was significantly higher in ER(+) cancer than that in ER(-) cancer ( $p < 0.001$ ). Also, PR(+) breast cancers had higher GPR30 expression than PR(-) breast cancers ( $p = 0.028$ ). By using IHC, Filardo et al [14] reported the close association between GPR30 and ER but not between GPR30 and PR. In our study, GPR30 expression levels were significantly associated with ER and PR in both Q-PCR and IHC data. Also, we demonstrated the tight correlation between ER and PR ( $p < 0.001$ ) (Figure 3B). These observations strongly support our finding that GPR30 is positively associated with both ER and PR in breast cancer samples.

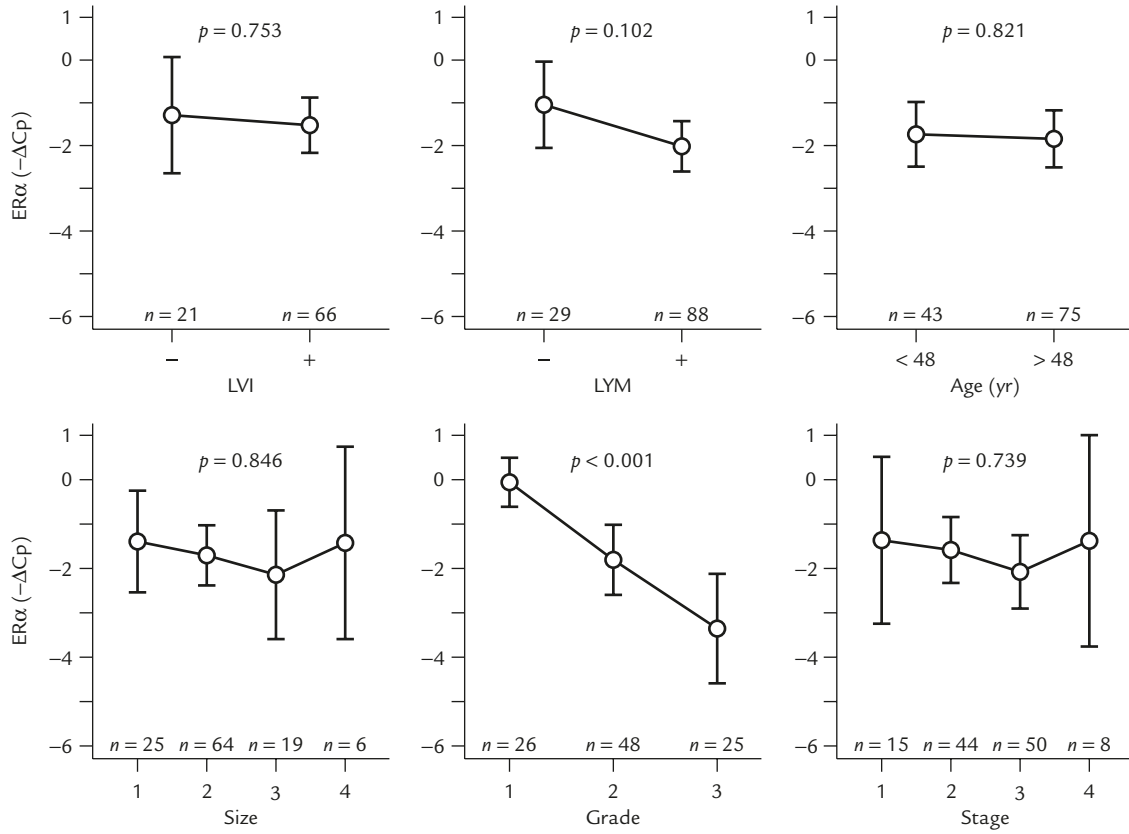


Figure 5. Univariate analysis of estrogen receptor α (ERα) status vs. clinical parameters.

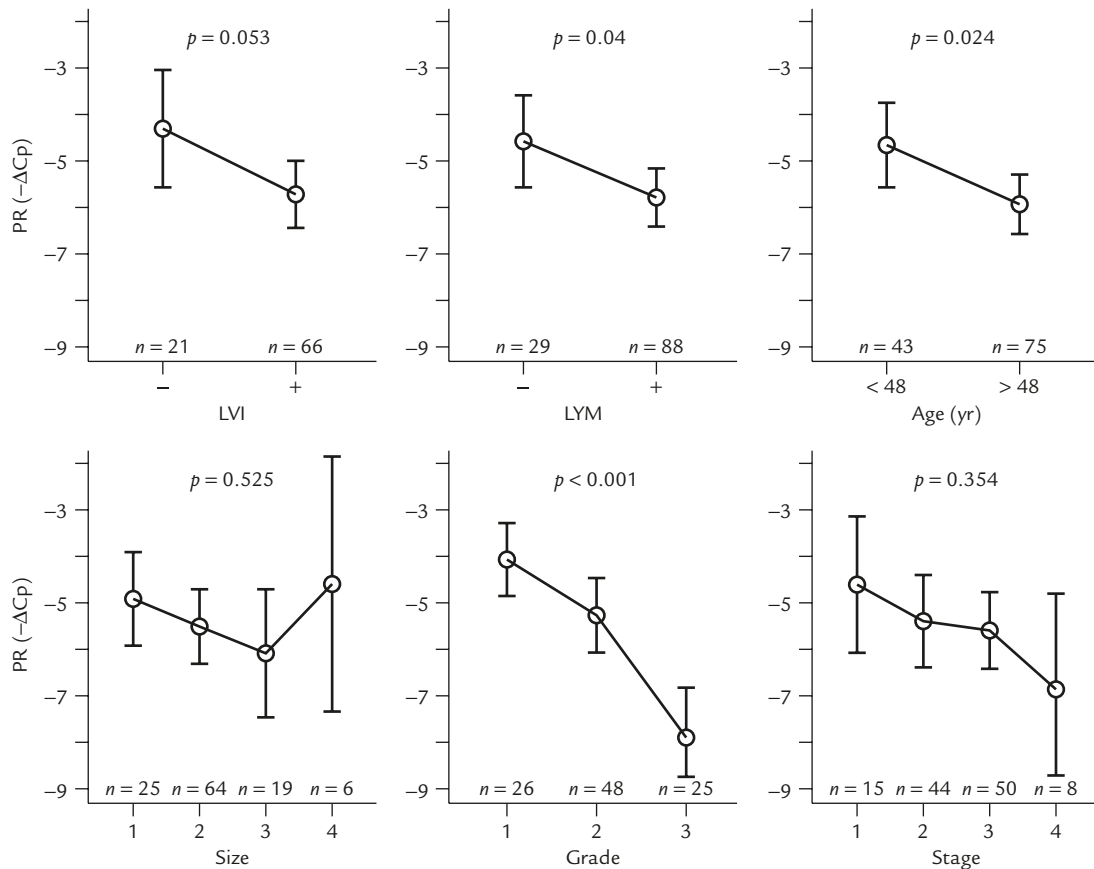


Figure 6. Univariate analysis of progesterone receptor (PR) status vs. clinical parameters.

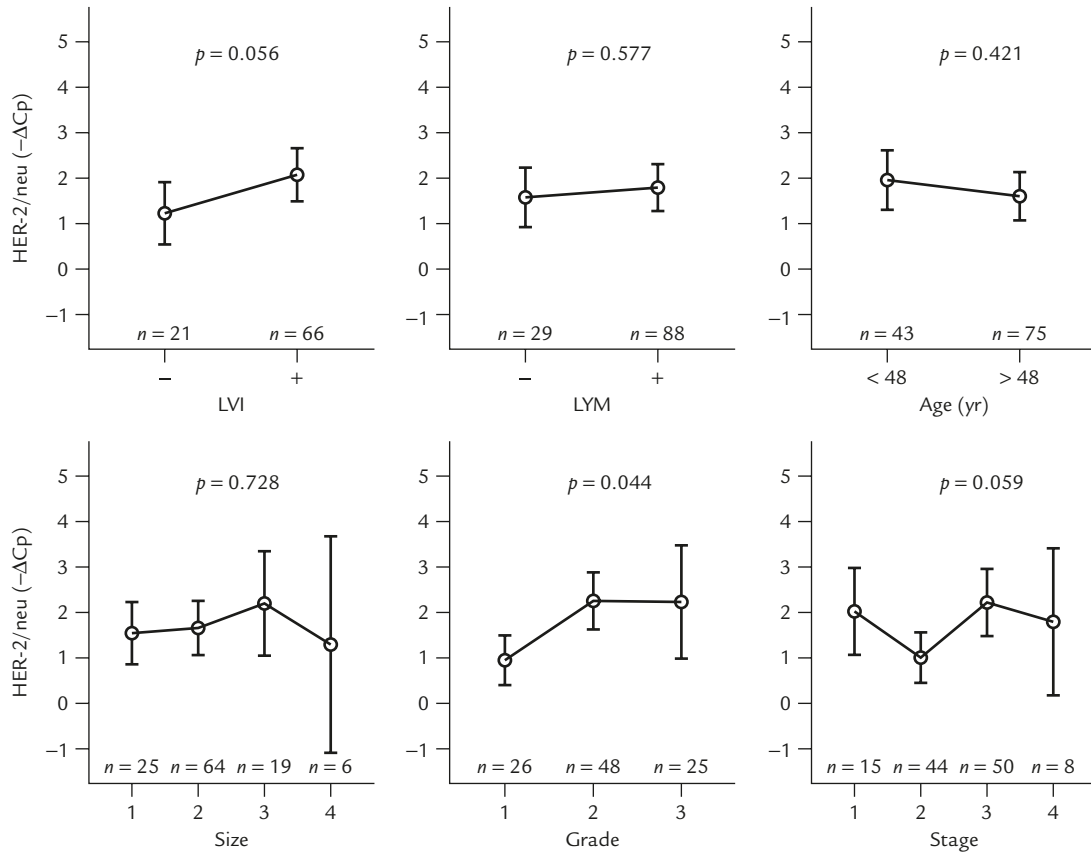


Figure 7. Univariate analysis of human epidermal growth factor receptor-2 (HER-2/neu) status vs. clinical parameters.

Table. Analysis results of Cox proportional hazards regression model

Variable	RR	95% CI	p
GPR30	1.146	(0.743, 1.767)	0.540
ER	0.943	(0.779, 1.141)	0.550
PR	0.735	(0.601, 0.899)	0.003
HER-2/neu	0.969	(0.826, 1.138)	0.700

RR = relative risk; CI = confidence interval; GPR30 = G-protein-coupled receptor 30; ER = estrogen receptor; PR = progesterone receptor; HER-2/neu = human epidermal growth factor receptor-2.

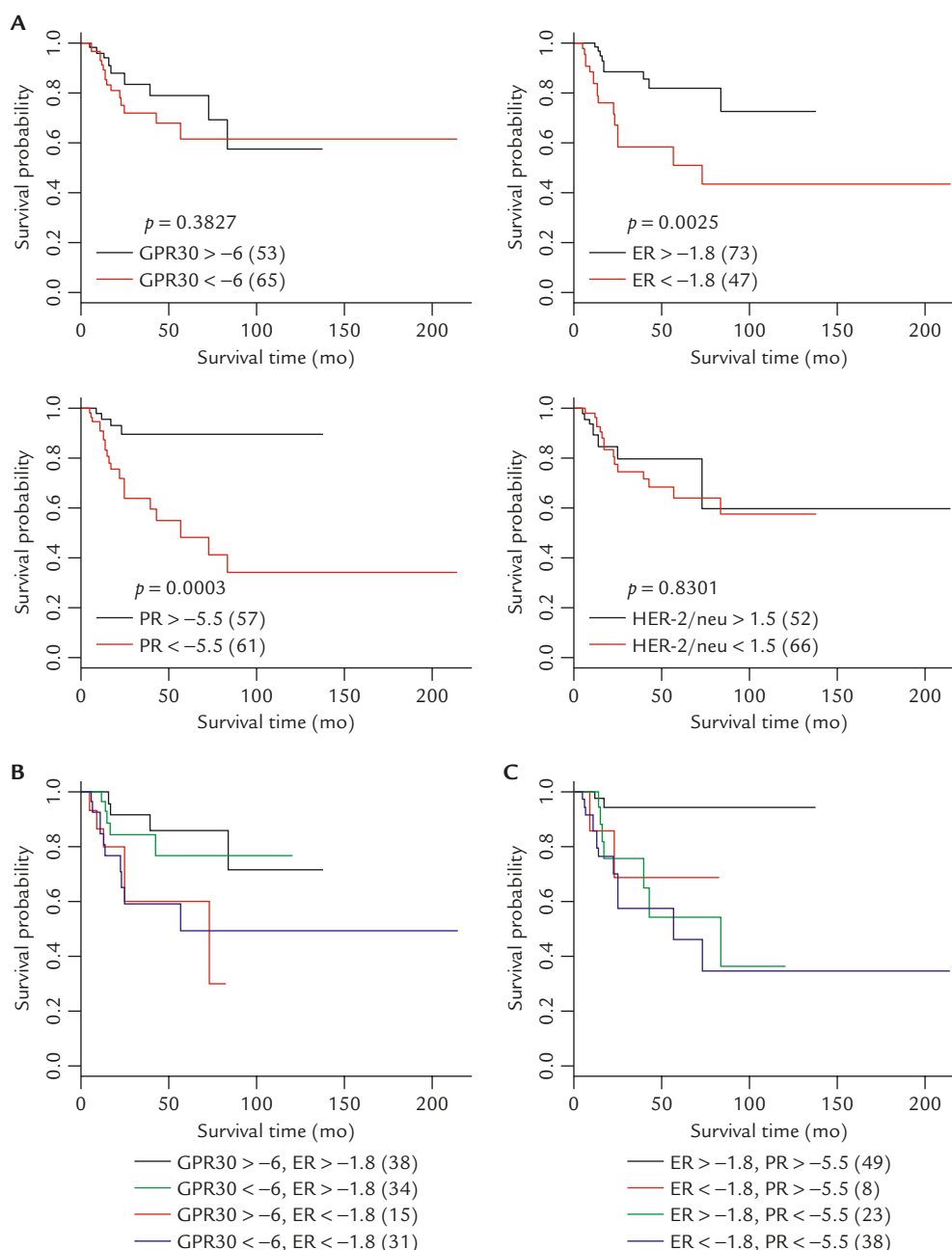
However, breast cancers with HER-2/neu overexpression as examined by IHC had significantly lower GPR30 mRNA levels than breast cancers without HER-2/neu overexpression ( $n = 88, p = 0.004$ ) (Figure 3A). This finding is contradictory to that of Filardo et al [14]. By using IHC, Filardo et al concluded that in breast cancer samples, GPR30 protein levels are positively correlated with HER-2/neu protein levels. They observed in 143 cases that GPR30(+) tumors presented higher HER-2/neu expression score than GPR30(-) tumors ( $p = 0.038$ ). Although our series revealed the inverse association between ER and HER-2/neu (Figures 3C and 3D), our observation for the trend of gene expression between GPR30 and

HER-2/neu is clearly different to that of Filardo et al [14]. This difference may be caused by the methodologic differences in assessing GPR30, since the scoring of GPR30 using IHC is not well established like those of ER, PR, and HER-2/neu. The relationship between GPR30 and HER-2/neu expression in breast cancer awaits further investigation, since this link might be important in understanding the role of the much-touted EGFR pathway in breast cancer. Filardo et al [14] has attributed the sensitivity of ER(-) breast cancer to estrogen.

**Roles of GPR30 expression in breast cancer**

The roles of GPR30 were examined by studying its differential expression in six clinical categories. By such univariate analyses, we have observed that GPR30 expression level in the breast cancer samples did not have a significant correlation with clinical indices including patient age, tumor size, tumor grade, clinical stage, lymphovascular infiltration and lymph node metastasis. There are very limited reports concerning the clinical implications of GPR30 in breast cancer. Filardo et al [14] described the association of GPR30 with tumor size and the presence of metastatic disease in breast cancer but no association between GPR30 and age, grade or lymph node involvement.

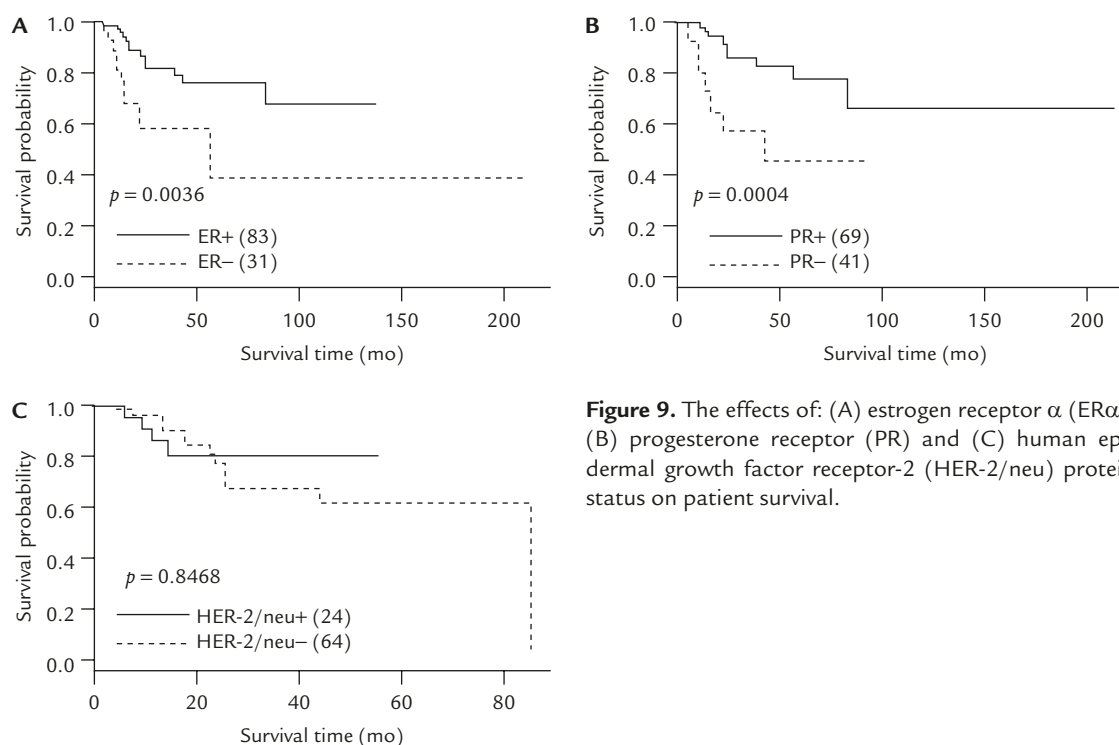




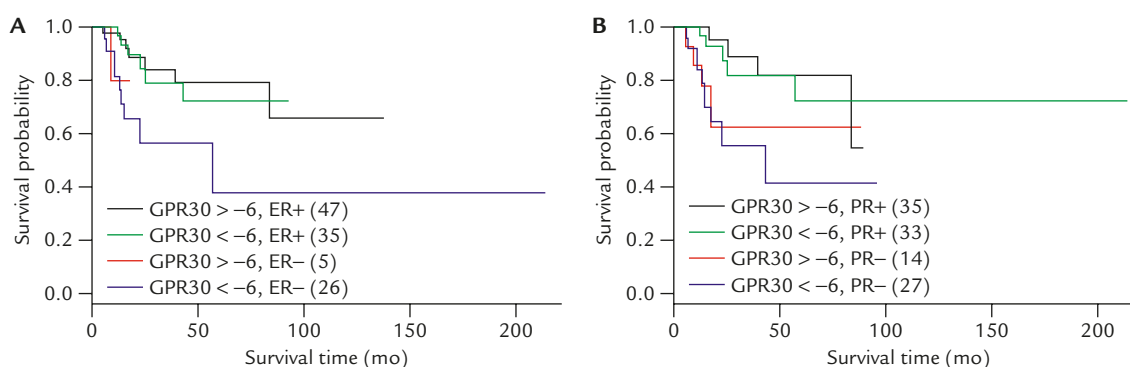
**Figure 8.** Survival analysis of G-protein-coupled receptor 30 (GPR30) and/or its associated clinical parameters in infiltrating ductal carcinoma. (A) The effects of estrogen receptor  $\alpha$  (ER $\alpha$ ), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2/neu) and GPR30 mRNA levels (higher or lower than the group mean) on patient survival curves. (B) The co-expression of ER and GPR30 on patient survival curves. The results of pairwise comparisons are: GPR30 > -6, ER > -1.8 vs. GPR30 < -6, ER > -1.8 ( $p = 0.519$ ); GPR30 > -6, ER < -1.8 vs. GPR30 < -6, ER < -1.8 ( $p = 0.905$ ); and GPR30 > -6, ER > -1.8 vs. GPR30 < -6, ER < -1.8 ( $p = 0.020$ ). (C) The co-expression of PR and GPR30 on patient survival curves. The results for pairwise comparisons are: GPR30 > -6, PR > -5.5 vs. GPR30 < -6, PR > -5.5 ( $p = 0.329$ ); GPR30 > -6, PR < -5.5 vs. GPR30 < -6, PR < -5.5 ( $p = 0.882$ ); and GPR30 > -6, PR > -5.5 vs. GPR30 < -6, PR < -5.5 ( $p = 0.003$ ).

In our series, for different tumor size groups (< 1 cm,  $n = 25$ ; 1–2 cm,  $n = 54$ ; 2–3 cm,  $n = 19$ ; > 3 cm,  $n = 6$ ), there was no difference in the expression level of GPR30 as determined by Q-PCR. Since there were very few metastatic diseases in our series, we have no information about the association of GPR30 with breast

cancer metastasis. Generally, our finding, in terms of the association of GPR30 with various clinical indices, is quite similar to that of Filardo et al [14]. It seems quite clear that GPR30, either at mRNA or protein level, has no evident association with the conventional clinical indices of breast cancer.



**Figure 9.** The effects of: (A) estrogen receptor  $\alpha$  (ER $\alpha$ ), (B) progesterone receptor (PR) and (C) human epidermal growth factor receptor-2 (HER-2/neu) protein status on patient survival.



**Figure 10.** Survival analyses for the co-expression status of (A) G-protein-coupled receptor 30 (GPR30) and estrogen receptor  $\alpha$  (ER $\alpha$ ); and (B) GPR30 and progesterone receptor (PR). The results for pairwise comparisons are: GPR30 >-6, ER+ vs. GPR30 <-6, ER+ ( $p = 0.874$ ); GPR30 >-6, ER- vs. GPR30 <-6, ER- ( $p = 0.730$ ); GPR30 >-6, ER+ vs. GPR30 <-6, ER- ( $p = 0.015$ ); and GPR30 >-6, PR+ vs. GPR30 <-6, PR+ ( $p = 0.894$ ); GPR30 >-6, PR- vs. GPR30 <-6, PR- ( $p = 0.775$ ); GPR30 >-6, PR+ vs. GPR30 <-6, PR- ( $p = 0.005$ ), respectively.

### Role of GPR30 on patient survival

GPR30 was not an independent prognostic factor, based on the Cox regression analysis (Table). The survival analysis also showed that GPR30 alone was not a determinant for patient survival (Figure 8A). In this study, we found that unless it was co-expressed with ER and/or PR, GPR30 was not a measure for survival (Figures 8B, 8C and 9).

### GPR30 in other cancers and biologic processes

GPR30 is structurally distinct from membrane ER $\alpha$ . It is involved in estrogen signaling through the non-genomic pathway [6,8]. Breast cancer is not the only

disease involve the estrogen signaling, more and more cancers and biologic processes are found to involve the estrogen signaling pathway. For example, the role of estrogen signaling pathway in lung cancer has attracted increasing attention in the research community [18]. Moreover, estrogen signaling pathway has been reported to be involved in brain conditions such as aging, cognition and neuroprotection [19–21]. Dhandapani et al [20] has proposed that a non-classical estrogen receptor may underlie some of the neuroprotective effects of E2. GPR30, although overshadowed by ER and PR in the setting of breast cancer, may play some undiscovered role in the estrogen signaling pathway in

brain and other biologic processes, just like the non-classical estrogen receptor proposed by Dhandapani et al [20]. Thus, the continuing study of GPR30 may be worthwhile and may eventually benefit the clinical management of breast cancer by offering more in-depth understanding of the still mysterious estrogen signaling pathway.

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