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G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer

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Running title: GPR30 predicts tamoxifen treatment response in breast cancer

Key words: GPR30, breast cancer, tamoxifen resistance, estrogen receptor

Abstract

Recently we have shown that the new G-protein-coupled estrogen receptor GPR30 plays an important role in the development of tamoxifen resistance *in vitro*. This study was undertaken to evaluate the correlation between GPR30 and tamoxifen resistance in breast cancer patients. GPR30 protein expression was evaluated by immunohistochemical analysis in 323 patients with primary operable breast cancer. The association between GPR30 expression and tamoxifen resistance was confirmed in a second cohort of 103 patients treated only with tamoxifen. Additionally, we evaluated GPR30 expression in 33 primary tumors and in recurrent tumors from the same patients. GPR30 expression was detected in 56.7% of the breast cancer specimens investigated and it correlated with overexpression of HER-2 ($p=0.021$), EGFR ($p=0.024$) and lymph node status ($p=0.047$). In a first cohort, survival analysis showed that GPR30 was negatively correlated with relapse-free survival (RFS) only in patients treated with tamoxifen (tamoxifen with or without chemotherapy). GPR30 expression was associated with shorter RFS (HR=1.768; 95% CI, 1.156 – 2.703; $p=0.009$). In a subset of patients treated only with tamoxifen, multivariate analysis revealed that GPR30 expression is an independent unfavorable factor for RFS, (HR=4.440; 95% CI, 1.408-13.997; $p=0.011$). In contrast, GPR30 tended to be a favorable factor regarding RFS in patients who did not receive tamoxifen. In 33 paired biopsies obtained before and after adjuvant therapy, GPR30 expression significantly increased only under tamoxifen treatment ($p=0.001$). GPR30 expression in breast cancer independently predicts a poor RFS in patients treated with tamoxifen.

Introduction

Tamoxifen is the most frequently used anti-hormonal drug for treatment of women with hormone-dependent breast cancer. Tamoxifen treatment is very effective in tumors expressing estrogen receptors (ER) and significantly reduces the mortality of breast cancer patients [1;2]. Many patients with ER-positive breast cancer benefit from antihormonal treatment, but unfortunately, almost all patients eventually acquire resistance to these drugs. Several mechanisms underlying tamoxifen resistance have been proposed [3]. The lack of response or acquired resistance to antihormonal drugs remains a major clinical issue.

Recently, we have found that G-protein-coupled receptor GPR30 plays an important role in the development of tamoxifen resistance in breast cancer MCF-7 cell line [4]. GPR30 has been claimed to be a new membrane-bound estrogen receptor involved in the rapid nongenomic effects of estrogen [5]. It has been demonstrated that GPR30 mediates the proliferative effects of estrogen in breast cancer cells, endometrial cancer cells and ovarian cancer cells [6]. Moreover, ER antagonists such as tamoxifen and fulvestrant act as agonists for GPR30 and stimulate cell proliferation and growth [7-11] [6]. Long-term tamoxifen treatment of MCF-7 cells increased the E2-stimulated up-regulation of GPR30 and its translocation from endoplasmic reticulum to the cell surface [4]. GPR30 converts tamoxifen to a growth stimulator because its ability to act as an agonist for GPR30. In our experiments, EGFR transactivation via GPR30 was an important mechanism by which MCF-7 cells developed resistance to endocrine therapy [4]. It has been shown that crosstalk between steroid receptors and growth factor receptor pathways plays a key role in the development of resistance to endocrine therapy in breast cancer [12].

To verify these findings in breast cancer patients, we investigated the expression of GPR30 and EGFR in breast cancer tissue and compared it to established clinicopathological parameters and clinical outcome in terms of relapse free survival (RFS) and overall survival (OS). To investigate the expression of GPR30 during breast cancer progression, we compare it in 33 primary tumors and in their corresponding relapsed or metastasized tissues.

Materials and methods

Immunohistochemistry

GPR30 expression was analyzed as previously described [13]. Sections of formalin-fixed and paraffin-embedded breast cancer specimens or corresponding recurrent lesions (3.0µm thick) were mounted on SuperFrost Plus glass slides (Menzel, Braunschweig, Germany) and dried overnight. A Benchmark XT (Ventana, Unterhaching, Germany) conducted the immunostaining. The slides were incubated with affinity-purified rabbit antibody against GPR30 (SP4677P; Acris antibodies, Herford, Germany) diluted 1:500 and against EGFR (Dako, Hamburg, Germany) diluted 1:500 for 32 min at 37°C, after antigen retrieval with Protease I (Ventana) for 10 min. The reactions were visualized by DAB detection. The slides were counterstained with hematoxylin and cover slipped after being embedded in mounting medium.

Evaluation of GPR30 staining results

GPR30 expression was classified as already described [13], according to the following grading system: staining extensity was categorized as 0 (no positive cells), 1 (<10% positive cells), 2 (10-50% positive cells), or 3 (>50% positive cells), and staining intensity was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The individual categories were multiplied to give a total immunohistochemical score (IHC). IHC score ranged between 0 and 9. The GPR30 cut-off was determined using the GPR30 expression in normal breast tissue surrounding the invasive breast cancer and is in agreement with the fact that cancer-associated fibroblasts show a robust functional GPR30 response [8]. GPR30-positive

expression was defined for tumors that showed an IHC ≥ 3 . Representative examples of different GPR30 expression are shown in Figure 1. The specificity of the GPR30 peptide antibody was tested in cell lysates prepared from human embryonic kidney HEK-293 cells, human breast cancer MDA-MB231 and MCF-7 cells (Supplementary Fig. 1). GPR30 antibody detected a band with an estimated molecular weight of 42kDa only in MCF-7 cells. In HEK-293 and MDA-MB231 who do not express GPR30 [7;14;15] there was no detectable band. The transfection of MCF-7 cells with GPR30 anti-sense oligonucleotides as already described [4], reduced the expression of GPR30 significantly, resulting in a reduced intensity of the detected specific band (Supplementary Fig. 1). These data suggest that GPR30 antibody specifically detect the GPR30 protein.

Evaluation of EGFR expression

EGFR expression was determined as already described [16]. For assessment of EGFR expression, a following scoring system was used: 0, no staining or unspecific staining; 1, weak and incomplete expression of more than 10% of the tumor cells; 2, moderate and complete staining of more than 10% and 3, strong and complete staining of more than 10% of the cells. The staining was complete in a case of circumferential staining of the entire cell surface. The opposite was considered as incomplete. Representative examples of EGFR expression are shown in Figure 1f,g,h.

Patients and tissue samples

The data of 384 patients with primary invasive breast cancer, who had been admitted to the Department of Obstetrics and Gynecology, Otto-von-Guericke University, Magdeburg, Germany from 1999 to 2005, were selected by retrospective analysis. Exclusion criteria included a previous history of adjuvant anti-hormonal or cytostatic therapy, primary non-operable tumor, no available archive material for detection of GPR30 expression and incomplete follow-up data. Three hundred twenty three patients were eligible for analysis. There was no significant difference between the final data set of 323 patients and the original group of 384 patients in terms of the patient and tumor characteristics. Patients underwent either modified radical mastectomy (n=175, 54.2%) or breast conservation surgery (n=148, 45.8%) in combination with axillary lymph node dissection. Adjuvant chemotherapy was given to 159 (49.2%) patients mainly cyclophosphamide/methotrexate/5-fluoruracil (CMF) or epirubicin/cyclophosphamide (EC). Two hundred sixty eight (82.9%) patients received adjuvant hormonal therapy. The median age at the time of primary diagnosis was 63 years (range 29-97 years), with 69 (21.4%) pre-menopausal and 254 (78.6%) post-menopausal. Outcome was measured as relapse-free survival (RFS) and overall survival (OS), according to the International Union Against Cancer (IUCC) criteria [17]. The follow-up was performed at the first recurrence of disease, patient death, or was based on the last available data in patient's registry. The median follow-up time of the study population was 51 months (range 1-134 months).

For validation of the results in the first cohort, we used a second cohort including 103 patients treated only with tamoxifen after surgery. The median age at the time of primary diagnosis for the second cohort was 67 years (range 37-97 years), 15

(14.6%) of whom were premenopausal and 88 (85.4%) were postmenopausal. The median follow-up time of the study population was 51 months (range 2-118 months).

The main prognostic factors assessed in this study were patient age, menopausal status, estrogen receptor (ER), progesterone receptor (PR), HER-2 status, tumor size, tumor grading, histological type, and lymph node status. The tumors had been previously characterized immunocytochemically for their ER, PR and HER-2 status. Histological grade had also been assessed at the time of diagnosis from hematoxylin/eosin sections. The patient's characteristics are summarized in Table 1.

Statistical analysis

The statistical calculations were performed using SPSS Version 17.0 (SPSS, Chicago, IL, USA). An association between GPR30 expression and the tumor variables was evaluated using the χ^2 test or Fisher's exact test. RFS and the OS were chosen as endpoints in this study. RFS analysis took into account those who died of breast cancer-specific death or had a recurrence of disease as a primary event. Patients who died of other causes or patients lacking follow-up data were censored. Survival was calculated using the Kaplan-Meier method. The equality of survival curves was tested by the log rank test. Univariate Cox proportional hazard regression analysis was used to identify significant prognostic factors. The prognostic significance was evaluated by multivariate analysis. The statistical analyses were two sided and p-values of <0.05 were considered statistically significant. The expression of GPR30 in primary tumors (PT's) and their corresponding recurrent lesions was analyzed using nonparametric paired analysis, and was performed with the Wilcoxon signed rank sum test.

Results

GPR30 and EGFR protein expression

According to the inclusion criteria, from the 384 patients obtained from our database, 323 patients were eligible for analysis. Patients were considered positive for GPR30 if they had an IHC score of at least 2. GPR30 expression was observed predominantly on the plasma membrane and in the cell cytoplasm (Fig. 1). Figure 1 shows examples of GPR30 immunostaining of breast cancer tissue with negative staining (Fig. 1a), slightly positive staining (Fig. 1b), moderately positive (Fig. 1c) and strongly positive (Fig. 1d) cytoplasmic immunostaining. However, in 41 of 323 (12.7%) cases, GPR30 was expressed also in the nucleus, as demonstrated in Figure 1e.

One hundred eighty three of the 323 (56.7%) paraffin-embedded breast cancer specimens available for evaluation of GPR30 expression were classified as positive. All of the surrounding non-tumor mammary tissues included in the tissue specimens were GPR30-positive.

EGFR expression was evaluated in 292 cases. EGFR overexpression was observed in 46 of 292 (15.8%) cases investigated (Table1). EGFR expression correlated with GPR30 expression ($p=0.024$, Table 2) and histological grading ($p=0.009$), but did not correlate with HER-2 overexpression ($p=0.447$, data not shown). EGFR overexpression was inversely correlated to the ER and PR status (data not shown). Moreover, EGFR status was a poor predictor for DFS and OS in the whole cohort of patients (data not shown).

GPR30 protein expression and tumor characteristics

The results of GPR30 immunohistochemistry were compared with the prognostic parameters age, menopausal status, tumor size, metastases, nodal status, ER, PR, HER-2 and EGFR (Table 2). GPR30 immunostaining correlated significantly with HER-2 expression ($p=0.021$), EGFR expression ($p=0.024$) and lymph node status ($p=0.047$). About 70% of the tumors overexpressing HER-2 and EGFR demonstrated moderate or strong GPR30 expression (Table 2). Similarly, the GPR30-expression was higher in PR positive breast specimens, but without reaching a significant level. GPR30 expression was more often detected in cases of lymph node-negative tumors. There was no correlation between the expression of GPR30 and other tumor characteristics (Table 2).

GPR30 and survival

During the follow-up time (51 months median follow-up, range 1-134 months), there were 118 (36.5%) breast cancer relapses, 93 (78.8%) of which occurred under endocrine therapy. There was no significant difference in OS between GPR30-positive and -negative patients (data not shown). However, among all cases, the GPR30 positivity was associated with a significantly decreased RFS (Fig. 2a, $p = 0.024$). Patients with GPR30 positive tumors receiving tamoxifen with or without chemotherapy, had poorer RFS than GPR30-negative patients. The 5-year RFS was 70.7% for GPR30-negative patients and 59.6% for GPR30-positive patients ($p=0.030$). Univariate analysis revealed that GPR30 expression was a prognostic factor for poor DFS (HR, 1.533; 95% CI, 1.051-2.235; $p=0.026$) (Table 3). After adjustment for menopausal status, tumor size, lymph node status, tumor grading,

HER-2 status, EGFR status, ER status and PR status, multivariate analysis rendered GPR30 as an independent, unfavorable prognostic factor (HR, 1.768; 95% CI, 1.156-2.703; $p=0.009$) (Table 3). Among the patients who did not receive tamoxifen as adjuvant therapy, GPR30 seemed to be a favorable factor of RFS (5-year RFS: 66.7% vs 74.2% for negative and positive cases, respectively) (Fig. 2b). For patients who did not received tamoxifen, GPR30 negativity was associated with poor RFS. However, this association was not significant ($P = 0.204$).

Kaplan-Meier survival analysis was subsequently done for GPR30-positive patients in relation to the tamoxifen treatment (Fig. 2c). The first group consisted of GPR30-positive patients who received only tamoxifen, the second group of GPR30-positive patients treated with aromatase inhibitors or tamoxifen followed by an aromatase inhibitor. Tamoxifen treatment was associated with a significantly poorer RFS compared to the patients who received aromatase inhibitors ($p=0.001$; Fig. 2c). The 5-year RFS 50.4% in patients receiving tamoxifen and 74.2% in patients receiving aromatase inhibitors or tamoxifen followed by an aromatase inhibitor respectively ($p=0.002$). This finding shows that GPR30 expression is associated with poor RFS only in patients treated with tamoxifen.

To verify this observation, the influence of GPR30 on tamoxifen resistance was tested in a second cohort of 103 patients, who received only tamoxifen as adjuvant therapy. GPR30 expression was significantly associated with poorer RFS (Fig. 2d) with a 5-year RFS 58.5% vs. 86.8% for GPR30-positive and GPR30-negative patients, respectively ($p=0.002$). Multivariate analysis revealed that GPR30 expression remains an independent unfavorable factor regarding RFS (HR, 4.440; 95% CI, 1.408-13.997, $p=0.011$) after adjustment for menopausal status, tumor size, lymph node status, tumor grading, PR status, HER-2 status, and EGFR status (Table

4). These findings suggested again, that GPR30 is associated with poor RFS in patients treated with tamoxifen.

Change in GPR30 expression between PT and corresponding recurrent tissue

In vitro we have recently found that GPR30 cell surface expression is increased after continuous tamoxifen treatment [4]. To verify these results *in vivo* we compared the GPR30 expression score between recurrent tissues and their corresponding PTs. Forty six patients with recurrent breast cancer were investigated. The GPR30 score was obtained in 33 of them. The sites of recurrence included 22 locoregional and 11 distant metastatic lesions. In these lesions, GPR30 expression was evaluated using the above described score and was compared with GPR30 expression in the primary tumor (PT). Twenty-one of these patients had been treated with tamoxifen, 7 with aromatase inhibitor and 5 had received chemotherapy only. GPR30 expression in PTs was determined before the adjuvant therapy. Representative examples of GPR30 expression in PT and corresponding metastases are shown in Figure 1i,j,k,l.

In 12 tumors having recurred during treatment with aromatase inhibitor and/or chemotherapy (Fig. 3a), GPR30 expression was increased in 8 (66.7%) cases, decreased in 1 (8.3%) case and it remained unchanged in 3 (25%) cases (Z value, -1.658; $p=0.114$). The mean IHC score in this group of patients was 2.67 in PT and 4.42 in the recurrent lesions ($p=0.087$, Fig. 3b). However, in 21 tumors treated with tamoxifen, a significant increase in the GPR30 expression score was observed (Z value, -3.212; $p=0.001$). Thus the IHC score had increased in 16 of 21 (76.2%), was unchanged in 2 of 21 (9.5%), and decreased in only 3 of 21 (14.3%) (Fig. 3c). The

mean IHC score for GPR30 was 3.57 and 6.33 in PT and the recurrent lesions, respectively ($p=0.001$, Fig. 3d).

Discussion

In this study we investigated the expression of GPR30 in breast cancer and compared it with the RFS and OS in a cohort of 323 patients with invasive breast cancer. This study provides new insights into the mechanisms of acquired tamoxifen resistance and the pivotal role of GPR30.

GPR30-positive staining was observed in 183 of 323 (56.7%) invasive breast cancers. In these series, GPR30 expression was associated with poorer RFS but not with OS. Interestingly, GPR30 was associated with a poorer RFS only in patients treated with tamoxifen in the adjuvant setting. In contrast, in a group of patients not having received tamoxifen after operation, GPR30 expression seemed to be even a favorable factor regarding RFS. However, this effect did not reach a significant level. These findings support our recent *in vitro* results, since GPR30 was associated with an increased resistance of MCF-7 breast cancer cells to tamoxifen [4]. Thus, GPR30 expression is associated with an increased risk of development of tamoxifen resistance.

In this study, we observed a significant correlation between GPR30 and HER-2 and EGFR, two members of a family of four structurally related tyrosine kinase receptors. Amplification of HER-2 and EGFR is one of the most common genetic alterations associated with breast cancer progression. About 70% of the HER-2- and EGFR-positive tumors scored positive for GPR30, revealing a highly significant correlation between GPR30 and both receptors ($p=0.021$ and $p=0.024$, respectively). It is in agreement with a very recent investigation of two independent research groups who found that GPR30 significantly correlates with HER-2 expression in breast cancer patients [18;19]. A correlation between GPR30 and EGFR expression has been reported for endometrial cancer patients [20]. This correlation may be of pivotal

impact for tumor cells, since GPR30 can activate EGFR and make the tumor growth independent of ER [21]. Moreover, GPR30 expression is up-regulated in estrogen receptor-negative and estrogen receptor-positive cells by epidermal growth factor [22;23] and could be a good explanation of our finding that GPR30 expression correlates significantly with EGFR expression. In this context, the EGF-induced GPR30 up-regulation can be a milestone in GPR30- induced tamoxifen resistance.

The role of cross-talk between ER and growth factor receptors causing endocrine therapy resistance in breast cancer cells is well established [12]. An amplification and overexpression of HER-2 gene and increased protein expression occurs in about 12-25% of the human breast cancers [24-26]. However, the role of EGFR and HER-2 in the development of tamoxifen resistance *in vivo* is controversial. This association was very recently confirmed in large clinical trials [27-29], whereas in other studies, there was no significant correlation [30;31]. Multivariate analysis demonstrated that GPR30, HER-2 and EGFR are independent unfavorable predictors of RFS under tamoxifen therapy. GPR30 possibly supports the growth of tumors resistant towards tamoxifen by cross-talk with growth factor receptor signaling pathways, which has been observed *in vitro* [4], and will make the tumor growth independent of ER signaling [12]. Since tamoxifen acts as an agonist for GPR30 and leads to stimulation of cell proliferation and growth in many cell culture models [7-11] the growth of GPR30-positive breast cancer may be rather stimulated than inhibited by tamoxifen. The GPR30/growth factor receptor cross-talk is followed by phosphorylation of MAPK and Akt [4;7]. Thus, MAPK and Akt can further stimulate transcription of different genes (even ER), leading to cell growth proliferation. Then, again, the phosphorylated ER can further up-regulate GPR30,

establishing a vicious circle [4]. Blocking of GPR30/EGFR signaling is a potential target to circumvent the tamoxifen resistance in breast cancer cells.

Prolonged tamoxifen treatment leads to an increased cell surface expression of GPR30 [4] and also to clonal selection of GPR30-positive tumor cells. We found that GPR30 expression is significantly increased in tamoxifen resistant tumor tissues. In contrast, GPR30 expression was not changed between the primary and recurrent tissue if aromatase inhibitor or chemotherapy was used. Since aromatase inhibitors and chemotherapy both reduce the amount of endogenous estrogen without altering GPR30 expression, we can assume that estrogen does not interfere with GPR30, while tamoxifen does. GPR30 expression may change under tamoxifen therapy. Moreover, GPR30-positive patients treated with aromatase inhibitors or tamoxifen followed by aromatase inhibitor demonstrated a longer RFS as compared with the GPR30-positive patients treated with tamoxifen alone. Therefore, GPR30-positivity might define a group of (postmenopausal) patients who benefit more from estrogen deprivation, in terms of aromatase inhibitor therapy, than from tamoxifen. The primary favorable effect of GPR30 expression on RFS in patients, who did not received tamoxifen, could be good explained by very recent results by Ariazi et co-workers who have find that GPR30 agonist inhibits proliferation of breast cancer cells [32].

In this study we did not observe a correlation between GPR30 and PR and ER, which is in contrast to other recent studies [18;19;33]. The authors have found a significant correlation between GPR30 and ER and PR. Indeed, in our population, the GPR30 immunostaining observed was stronger in ER- and PR-positive tumors, but this correlation did not reach significant levels. This supports the hypothesis that GPR30 and ER signaling are different and independent as already described in

some cellular systems (for review [34]). The fact that 45 of 91 (49.5%) of ER-negative tumors are GPR30-positive demonstrates again that GPR30 does not depend of ER [5;6;34]. GPR30 positivity in ER-negative tumors represents a new potential target for endocrine therapy.

In conclusion, this study indicated that GPR30 expression correlated significantly with EGFR and HER-2 expression, and was predictive for development of tamoxifen resistance. GPR30-positive tumors are less likely to benefit from tamoxifen therapy. Estrogen deprivation or blocking of GPR30/growth factor receptor cross-talk may be an alternative way to prevent development of tamoxifen resistance. A large number of patients treated with tamoxifen have to be further investigated in prospective clinical trials to confirm these findings.

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Figure legends:

Fig. 1 Paraffin-embedded breast tumor tissue immunostained with GPR30 or EGFR antibodies. GPR30 immunostaining of breast cancer tissue showing negative (a), weak (b), moderate (c) or strong positive cytoplasmic staining (d). e) GPR30 positive staining in cytoplasm and nucleus. EGFR staining of breast cancer tissue showing no staining (f), moderate and complete staining (g) and strong and complete staining (h). GPR30 weak staining in primary breast cancer (i) and corresponding skin metastasis showing strong GPR30 staining (j). k) Example of GPR30 moderate staining in PT and GPR30 strong expression in the corresponding liver metastasis after tamoxifen (l). Original magnification: x 200.

Fig. 2 Kaplan-Meier plots for relapse-free survival according to the GPR30 status. a) Relapse-free survival in the whole patient group. b) Relapse-free survival in the patient's cohort treated with aromatase inhibitors or tamoxifen followed by aromatase inhibitors. c) Relapse-free survival in GPR30 positive patient treated with tamoxifen only (TAM) or aromatase inhibitor and tamoxifen switched to aromatase inhibitor (AI/Switch). d) Relapse-free survival in the patient's cohort treated with tamoxifen only. The log rank test was used to calculate the *p*-value.

Fig. 3 GPR30 expression in 33 matched tissue from primary tumor (PT) and corresponding relapsed/metastasized tissue (Met). Paired change (a) and quantitative (b) comparison in GPR30 expression between PT and Met in patients who did not receive tamoxifen therapy; Matched change (c) and quantitative (d) comparison in GPR30 expression between PT and Met under tamoxifen therapy.

Table 1. Patient's characteristics.

Characteristic	Cohort 1		Cohort 2	
	N	%	N	%
Total	323		103	
Age (range)	63(29-97)		67(37-97)	
GPR30				
negative	140	43.3	38	36.9
positive	183	56.7	65	63.1
Menopausal status				
premenopausal	69	21.4	15	14.6
postmenopausal	254	78.6	88	85.4
Tumor size, cm				
≤2	167	51.7	73	70.9
>2	156	48.3	30	29.1
Lymph node status				
negative	205	63.5	92	89.3
positive	118	36.5	11	10.7
Histological typ				
Ductal	254	78.6	77	74.8
Lobular	55	17.0	19	18.4
Other	14	4.3	7	6.8
Histologic grade				
1	33	10.2	20	19.4
2	182	56.3	66	64.1
3	108	33.4	17	16.5
ER status				
negative	91	28.2	13	12.6
positive	232	71.8	92	87.4
PR status				
negative	150	46.4	34	33.0
positive	173	53.6	69	67.0
HER-2 status				
negative	252	78.0	88	85.4
positive	71	22.0	15	14.6
EGFR status				
negative	246	76.2	73	70.9
positive	46	14.2	10	9.7
missing	31	9.6	20	80.6

Operative therapy				
Breast conserving	148	45.8	51	49.5
Mastectomy	175	54.2	52	50.5
Adjuvant therapy				
Chemotherapy	159	49.2	0	
Tamoxifen	191	59.1	103	
Aromatase inhib.	77	23.8	0	
No treatment	6	1.9	0	100

Table 2. GPR30 and tumor characteristics.

Characteristic	N of Patients	GPR30				p
		negative		positive		
		N	%	N	%	
Total	323	140	43.3	183	56.7	
Age (range)		64 (33-87)		62 (29-97)		0.587
Menopausal status						
premenopausal	69	27	39.1	42	60.9	0.494
postmenopausal	254	113	44.5	141	55.5	
Tumor size, cm						
≤2	167	68	40.7	99	59.3	0.369
>2	156	72	46.2	84	53.8	
Lymph node status						
negative	205	80	39.0	125	61.0	0.047
positive	118	60	50.8	58	49.2	
Histological typ						
Ductal	254	116	45.7	138	54.3	0.118
Lobular	55	17	30.9	38	69.1	
Other	14	7	50.0	7	50.0	
Histologic grade						
1	33	14	42.4	19	57.6	0.140
2	182	71	39.0	111	61.0	
3	108	55	50.9	53	49.1	

ER status						
negative	91	46	50.5	45	49.5	
positive	232	94	40.5	138	59.5	0.107
PR status						
negative	150	74	49.3	76	50.7	
positive	173	66	38.2	107	61.8	0.055
HER-2 status						
negative	252	118	46.8	134	53.2	
positive	71	22	31.0	49	69.0	0.021
EGFR status						
negative	246	120	48.8	126	51.2	
positive	46	14	30.4	32	69.6	0.024

Table 3. Uni- and multivariate analysis regarding RFS.

Prognostic factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
GPR30						
positive vs negative	1.533	1.051-2.235	0.026	1.768	1.156-2.703	0.009
Menopausal status						
pre vs post	0.714	0.476-1.072	0.104	0.836	0.527-1.324	0.444
Tumor size						
>2 vs ≤2cm	2.224	1.528-3.238	<0.0001	1.790	1.172-2.734	0.007
Lymph node status						
positive vs negative	2.048	1.427-2.939	<0.0001	1.974	1.308-2.979	0.001
Histologic grade						
3 vs 1 and 2	1.950	1.356-2.803	<0.0001	1.659	1.062-2.591	0.026
PR status						
positive vs negative	0.622	0.433-0.894	0.010	0.795	0.497-1.270	0.337
ER status						
positive vs	0.599	0.410-0.873	0.008	0.981	0.593-1.623	0.941

negative

HER-2 status
positive vs
negative

1.888 1.275-2.795 0.002 1.566 1.014-2.417 0.043

EGFR status
positive vs
negative

27.10.1905 1.360-3.324 0.001 1.506 0.944-2.402 0.86

Table 4. Multivariate analysis of RFS in cohort 2.

Fatcor	HR	95% CI	p
GPR30			
positive vs negative	4.440	1.408-13.997	0.011
Menopausal status			
pre vs post	1.749	0.373-8.195	0.478
Tumor size			
>2 vs ≤2cm	1.323	0.548-3.196	0.534
Lymph node status			
positive vs negative	0.787	0.251-2.472	0.682
Histologic grade			
3 vs 1 and 2	2.779	0.943-8.193	0.064
PR status			
negative vs positive	1.319	0.537-3.243	0.546
HER-2 status			
positive vs negative	3.120	1.097-8.873	0.033
EGFR status			
positive vs negative	4.201	1.373-12.853	0.012

Figure 1

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Fig 1

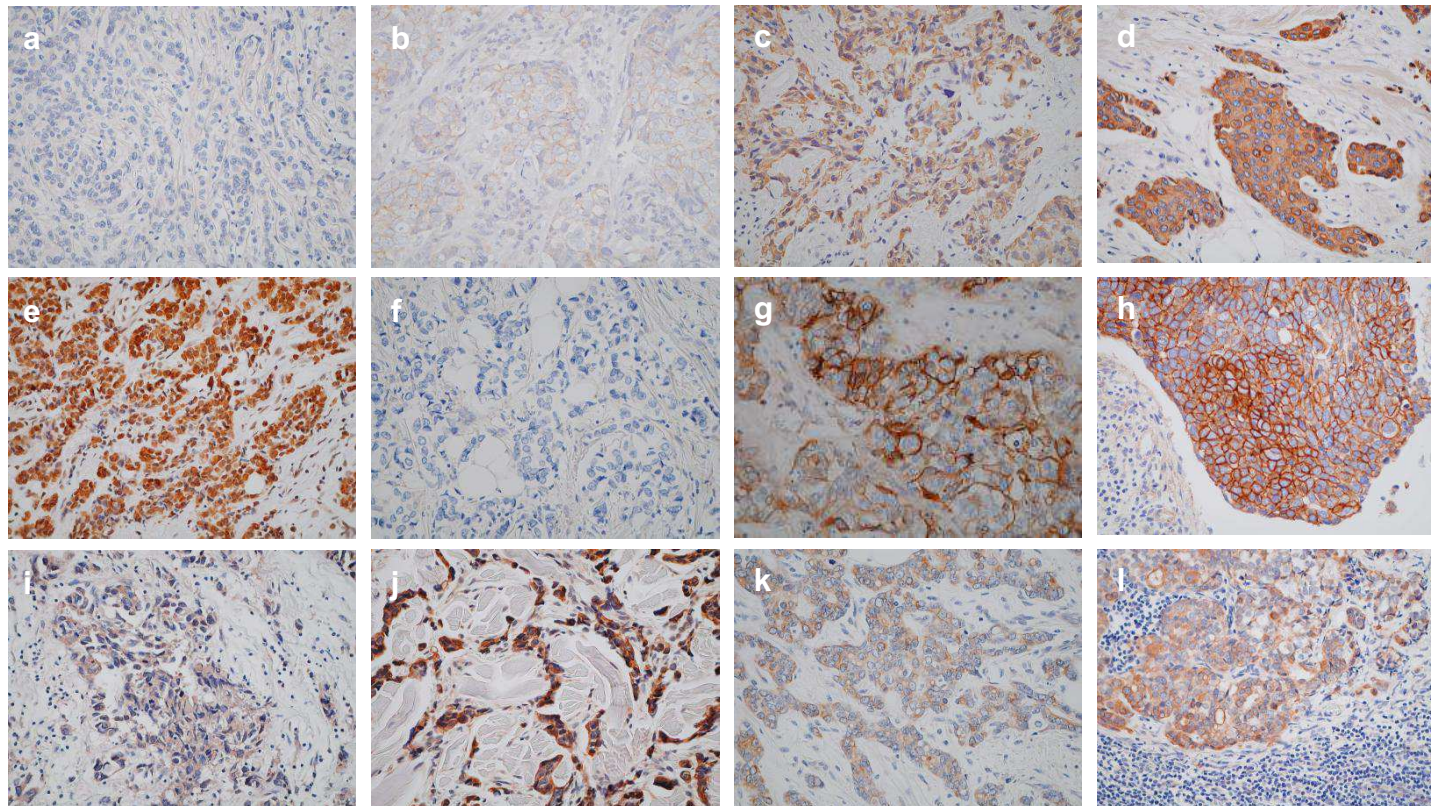


Figure 2

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Fig 2

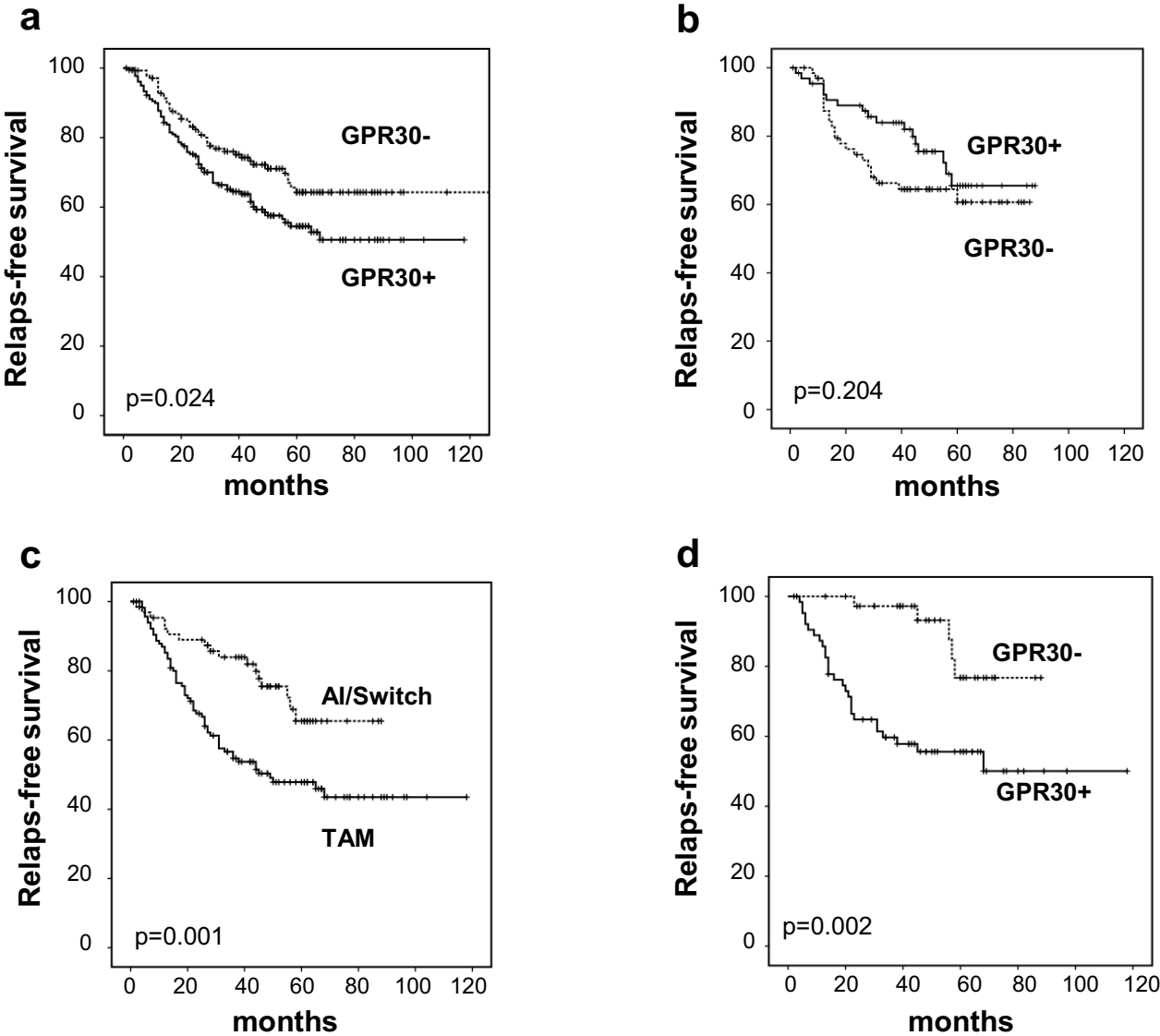


Figure 3

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