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Clinical relevance of targeting the gastrin releasing peptide receptor, somatostatin receptor 2 or chemokine c-x-c motif 4 in breast cancer for imaging and therapy

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ABSTRACT

Imaging and therapy using radioligands targeting receptors overexpressed on tumor cells is successfully applied in neuroendocrine tumor patients. Since expression of the gastrin releasing peptide receptor (GRPR), somatostatin receptor 2 (SSTR2) and chemokine C-X-C motif receptor 4 (CXCR4) has been demonstrated in breast cancer, targeting these receptors using radioligands might offer new imaging and therapeutic opportunities for breast cancer patients. The aim of this study was to correlate messenger RNA (mRNA) expression of *GRPR*, *SSTR2* and *CXCR4* with clinico-pathological and biological factors, with prognosis and prediction to therapy response, to identify specific breast cancer patient groups suited for the application of radioligands targeting the receptors.

Methods:

First, we studied GRPR and SSTR2 expression in 13 clinical breast cancer specimens by in vitro autoradiography and correlated this with corresponding mRNA levels to investigate whether mRNA levels reliably represent cell surface expression. Next, *GRPR*, *SSTR2* and *CXCR4* mRNA levels were measured by RT-qPCR in 915 primary breast cancer tissues and correlated with known clinico-pathological and biological factors, disease-free, distant metastasis-free and overall survival (DFS, MFS and OS). In 224 adjuvant hormonal treatment-naïve estrogen receptor (ER, *ESR1)* positive patients who received tamoxifen as 1st line therapy for recurrent or metastatic disease, the expression levels of the receptors were correlated with progression-free survival (PFS).

Results:

Our results showed a significant positive correlation between GRPR and SSTR2 expression analyzed by in vitro autoradiography and by RT-qPCR (Rs=0.94, P<0.001 and Rs=0.73, P=0.0042, respectively). Furthermore, high *GRPR* and *SSTR2* mRNA levels were observed more frequently in *ESR1*-positive specimens, while high *CXCR4* expression was associated with *ESR1*-negative specimens. Also, high mRNA expression of *CXCR4* was associated with a prolonged DFS, MFS and OS (multivariate HR

MFS=0.76 (0.64-0.90), P=0.001), while high mRNA levels of *GRPR* were associated with a prolonged PFS after start of 1st line tamoxifen treatment (multivariate HR=0.68 (0.48-0.97), P=0.031).

Conclusion:

Our data indicate that imaging and/or therapy using GRPR or SSTR2 radioligands might especially be beneficial for *ESR1*-positive breast cancer and CXCR4 radioligands for *ESR1*-negative breast cancer.

Keywords:

Breast cancer, GRPR, SSTR2, CXCR4, PRS/PRRT

INTRODUCTION

Breast cancer is the most common cancer found in women worldwide. An estimated 1.7 million new cases were diagnosed in 2012 worldwide and 522,000 people died as a consequence of the disease, making it the fifth cause of death by cancer overall (*1*).

Multiple subtypes of breast cancer exist with different molecular characteristics such as the absence or presence of estrogen receptor (ER, *ESR1*), progesterone receptor (PR, *PGR*) and human epidermal growth factor 2 (HER2, *ERBB2*) (*2*). In the case of ER and HER2, these receptors also serve as therapeutic targets. ER-positive patients are either treated with aromatase inhibitors or ER-antagonists, most commonly tamoxifen, while HER2-positive patients are often treated with the HER2-specific monoclonal antibody trastuzumab (*2*). However, in the recurrent or metastatic setting nearly all patients acquire resistance against tamoxifen and trastuzumab after an initial response (*3,4*).

Mammography is the standard method used for breast cancer screening, in some cases supplemented with MRI and/or ultrasound (*5*). Unfortunately these methods may lead to false positive and false negative results (*6,7*). Since current imaging and particularly above-mentioned therapy options have limitations and are not always successful, new imaging and therapeutic options are urgently needed.

Peptide receptor scintigraphy and peptide receptor radionuclide therapy are methods based on targeting receptors overexpressed on tumor cells using radioligands for diagnostic and therapeutic purposes. Within nuclear medicine, radiolabeled somatostatin (SST) analogs are most widely and successfully used for the localization, treatment and evaluation of neuroendocrine tumors (*8*). These SST analogs bind to somatostatin receptors (SSTR, especially SSTR2) overexpressed on tumor cells, enabling imaging when labeled with gamma-/positron-emitters and therapy when labeled with beta- or alpha-particle emitters. Currently multiple radiolabeled SST analogs targeting SSTR2 are available and used in the clinic (*9*). In the past decade imaging of breast cancer patients using SSTR2 radioligands has been studied with

varying results (*10,11*). Currently considerable improved SSTR2-directed radiotracers as well as imaging equipment are available.

Other promising targeting radioligands for breast cancer comprise of radiolabeled gastrin releasing peptide (GRP) analogs, earlier applied for visualization and therapy of prostate cancer lesions, since significant GRP receptor (GRPR) levels are present in the majority of primary prostate cancer tissues (*12-14*). Previous studies by Reubi et al. (*15*) showed high expression of both SSTR2 and GRPR in breast cancer. SSTR2 and high density GRPR expression was found in 75% and 74% of breast cancer cases, respectively.

Moreover, CXCR4 expression has been reported in the majority of breast cancers. In a study by Salvucci et al. (*16*), where 2,022 breast cancer specimens were analyzed for CXCR4 expression using immunohistochemistry, 67% of invasive tumors showed high nuclear staining and 41% of tumors showed cytoplasmic staining (*12*). Promising radiolabeled peptide derivatives binding to CXCR4 have been synthesized to target these receptors (*17,18*). So ^{68}Ga-pentaxifor, a CXCR4 radioligand, has successfully been used in a clinical study for the imaging of multiple myeloma patients (*19*). Thus, these three promising categories of radiolabeled compounds could be of promise in breast cancer patients.

Until now little is known about the correlation between GRPR, SSTR2 and CXCR4 expression levels in breast cancer lesions and important molecular and prognostic characteristics, such as hormone receptor expression, the association of their expression with disease-free, distant metastasis-free and/or overall survival (DFS, MFS and OS, respectively) and with progression-free survival (PFS) after endocrine treatment.

In this study, we first analyzed the correlation between messenger RNA (mRNA) levels and protein expression of GRPR and SSTR2. Subsequently, we analyzed the mRNA expression of *GRPR, SSTR2* and *CXCR4* in human breast cancer specimens. The aims of this study were to correlate *GRPR*, *SSTR2* and *CXCR4* mRNA expression levels with clinico-pathological and biological factors as well as with prognosis and outcome on tamoxifen therapy, to assess the potential impact of radioligands targeting

these receptors for imaging and therapeutic purposes in breast cancer, and to thereby identify patient subgroups that potentially would benefit from application of these radiopharmaceuticals.

MATERIALS AND METHODS

Human Breast Cancer Cases

The study (MEC02·953) was approved by the Erasmus MC Medical Ethical Committee and adhered to the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands.

Primary breast cancer tissue of 915 female patients (age: 58±13 years), [684 M0 (no metastasis at diagnosis) lymph node negative (LNN), 194 M0 LNP, 24 M1 lymph node positive (LNP) and 13 patients with unknown nodal status at time of primary treatment] who visited the clinic between 1979 and 2000 were selected from the Erasmus MC fresh frozen tissue bank as described before (*20*). The inclusion criteria and the determination of clinico-pathological and biological factors are described in the Supplemental methods. Correlation of receptor expression with clinico-pathological and biological factors was initially performed in the LNN M0 patient group (n=684). A representative group of LNP tumors (n=194) was added to study the influence of positive nodal status on the correlation analyses. For prognosis we focused our analyses on the cohort of 684 systemic treatment-naive patients with LNN disease; for prediction of therapy response a cohort of 224 hormonal treatment-naive ER-positive patients who received tamoxifen as 1st line therapy for recurrent or metastatic disease was analyzed. The clinico-pathological and biological factors of the LNN M0 tumors are shown in Table 1 and for the LNN and LNP M0 patient group and the ER-positive 1st line tamoxifen treated subcohort in Supplemental Tables 1A and 1B, respectively. Patients were censored at 120 months follow-up after surgical removal of the primary tumor in the regression analysis for DFS (283 events), MFS (241 events) and OS (223 events) and at 36 months after start of tamoxifen treatment for analysis of PFS (24 events). The study design is depicted in Figure 1.

RNA Isolation, cDNA Synthesis And Quantitative Reverse Polymerase Chain Reaction

Tissue processing, RNA isolation, cDNA synthesis and quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) were performed and normalized using the delta Cq method on the average of 3 reference genes (*HMBS*, *HPRT1* and *TBP*) as described (*21*). All RNA samples that required more than 25 rounds of real-time PCR for detectable products of our 3 reference genes at a fixed input of 10 ng total RNA and at a threshold of 0.1 were considered of insufficient quality and were excluded from further analysis. Quantification of target genes was done using the following intron-spanning Taqman probe-based gene expression assays (Applied BioSystems/Life Technologies): *GRPR*, Hs01055872 m1; *SSTR2*, Hs0099356 m1 and *CXCR4*, Hs00237052 m1, according to manufacturer's instructions in a MX3000P Real-Time PCR System (Agilent). Genomic grade index (GGI), a gene expression pattern of histological tumor grade, as well as *ESR1*, *PGR* and *ERBB2* levels and status of the samples were already known based on quantification as previously described (*22-24*).

Radioligands And In Vitro Autoradiography

Peptide analogs targeting the SSTR2 and GRPR, DOTA-Tyr^3-octreotate (Mallinckrodt) and AMBA (BioSynthema), respectively, were radiolabeled with ^{111}In (Covidien), as previously described (*25*). Quenchers (10 mM methionine, 3.5 mM ascorbic acid and 3.5 mM gentisic acid) were used to prevent radiolysis (*26*). Specific activity of both radiotracers was 50 MBq/nmol. Radiometal incorporation (>99%) and radiochemical purity (>90%) were measured by instant thin layer chromatography on silica gel and high pressure liquid chromatography as previously described (*26*).

The CXCR4 radioligand, pentaxifor, available to us showed reduced receptor affinity when radiolabeled with ^{111}In and thus satisfying in vitro autoradiography studies using this compound could not be performed.

In the in vitro autoradiography assay tissue sections of 13 fresh frozen breast cancer specimens (10 µm) were incubated with 10^{-9}M ^{111}In-AMBA and ^{111}In-Octreotate for 1 h, without and with 10^{-6} M unlabeled

tracer as control for non-specific binding. H69 (SSTR2-positive, GRPR-negative) and PC3 xenografts (GRPR-positive, SSTR2-negative) were used as controls. Results were quantified using OptiQuant Software (Perkin Elmer) and the net percentage binding of added dose was calculated. The in vitro autoradiography assay and quantification of the results is described in more detail in the Supplemental methods.

Statistics

Statistical analyses are described in the Supplemental methods.

RESULTS

In Vitro Autoradiography And Correlation With mRNA Expression

Specific binding to tumor cells of the GRPR- and SSTR2-mediated radiotracers, ^{111}In-AMBA and ^{111}In-DOTA-Tyr^{3}-Octreotate, respectively, was demonstrated using in vitro autoradiography on 13 selected human breast cancer specimens with varying levels of mRNA receptor expression. Two mouse xenografts served as positive and negative control (Figure 2A). Autoradiography results were quantified and correlated with the level of mRNA expression of the respective receptors, resulting in a significant positive correlation for both GRPR (Rs=0.94, P<0.0001) and SSTR2 (Rs=0.73, P=0.0042) (Figure 2B). Furthermore, binding of the tracers was only observed on tumor cells and not on the surrounding stromal cells. We thus concluded that mRNA expression for GRPR and SSTR2 can be used as a predictor for binding of the radiotracers to tumor tissue.

Correlation Of *GRPR*, *SSTR2* And *CXCR4* mRNA Expression With Clinico-pathological And Biological factors

We focused on the 684 LNN M0 patients to study the correlation between *GRPR*, *SSTR2* and *CXCR4* mRNA levels and known clinico-pathological and biological factors. The results of the correlation analyses are shown in Table 1. To study the influence of positive nodal status on the correlation analyses a representative group of 194 LNP M0 tumors were added to the study. Results of the LNN and LNP M0 patient group are described in the Supplemental Table 1A.

A significant correlation was observed between *GRPR* mRNA levels and a smaller pathological tumor size (P=0.0014), a positive *ESR1* (P<0.001) and *PGR* status (P<0.001), a negative *ERBB2* (P<0.001) status and a favorable GGI (P<0.001).

SSTR2 mRNA expression showed a significant correlation with a positive *ESR1* (P<0.001) and *PGR* mRNA status (P<0.001), a negative *ERBB2* status (P=0.0344), favorable GGI (P<0.001) and ≤70% invasive tumor cells (P=0.002).

CXCR4 mRNA expression showed a significant negative correlation with *ESR1* (P<0.001) and *PGR* mRNA status (P<0.001), and was associated with an unfavorable GGI (P<0.001). Furthermore, *CXCR4* mRNA levels were higher in tumors with ≤70% invasive tumor cells (P<0.001).

Association Of *GRPR*, *SSTR2* And *CXCR4* mRNA Expression With Prognosis And Efficacy Of Tamoxifen Treatment

To exclude the possible confounding effect of adjuvant therapy on prognosis, the association of *GRPR*, *SSTR2* and *CXCR4* expression with prognosis was evaluated in the LNN patient group who did not receive adjuvant systemic therapy. The results of the evaluation of *GRPR*, *SSTR2* and *CXCR4* mRNA expression with DFS, MFS and OS are shown in Supplemental Table 2.

No significant associations were observed between *GRPR* and *SSTR2* mRNA expression and DFS, MFS or OS. For *CXCR4*, however, there was a significant association of its expression with a favorable DFS, MFS and OS, both when analyzed as a continuous variable and when dichotomized at the median level. For the primary endpoint MFS, the results of the multivariate analysis were HR=0.76 (0.64-0.90), P=0.001 when analyzed as a continuous variable and HR=0.71 (0.55-0.91), P=0.011, when dichotomized at the median level.

To visualize the association of the levels of *CXCR4* mRNA with MFS, Kaplan-Meier analysis was performed as function of the quartile levels of *CXCR4* mRNA (Figure 3). The results show a clear trend of quartiles with lower expression having a worse metastasis-free survival time.

In addition, *GRPR*, *SSTR2* and *CXCR4* mRNA expression levels were correlated with the efficacy of tamoxifen treatment in *ESR1*-positive patients with recurrent disease (Supplemental Table 1B). There was a significant correlation between high *GRPR* mRNA levels and prolonged PFS after start of 1st line

tamoxifen treatment, indicating that *GRPR* expression has predictive value for the efficacy of tamoxifen therapy (Figure 4, Supplemental Table 3) (25% high vs. 75% low, univariate HR=0.65 (0.47-0.91), P=0.011) and multivariate HR=0.95 (0.48-0.97), P=0.031).

DISCUSSION

We have analyzed *GRPR*, *SSTR2* and *CXCR4* mRNA expression in 915 primary breast cancer tissues and correlated mRNA expression of these receptors with clinico-pathological and biological factors, and with prognosis and prediction to therapy response, to study the relevance of the application of radioligands targeting these receptors for imaging and therapy in breast cancer patients. For this, we first successfully demonstrated in vitro binding of radiotracers for GRPR and SSTR2 to tissue sections and showed a significant positive correlation between radiotracer binding and mRNA expression, demonstrating that mRNA levels of these receptors can be used as a predictor for specific radiotracer binding. The CXCR4 radioligand, pentaxifor, available to us showed reduced receptor affinity when radiolabeled with ^{111}In for in vitro autoradiography purposes, hampering reliable in vitro autoradiography studies for CXCR4. Thus, studies correlating CXCR4 radiotracer binding and *CXCR4* mRNA expression could not be performed. However, since Philip-Abbrederis et al. (*19*) reported on detecting *CXCR4* mRNA expression in cell lines and successful in vivo imaging of corresponding xenograft models using ^{68}Ga-pentaxifor, we concluded that *CXCR4* mRNA expression can be used as well as a predictor for CXCR4 radioligand binding.

Concerning prognosis, we found no association between *GRPR* and *SSTR2* expression and DFS, MFS and OS in the M0 LNN patients. Surprisingly, we found that high *CXCR4* levels correlated with better prognosis despite its negative correlation with ER, PR and unfavorable GGI, indicating that a component of CXCR4 expression that is independent of these factors determines good outcome.

Other studies on CXCR4 expression in breast cancer have associated CXCR4 expression with poor patient survival (*16*). The discrepancy in study outcome might be explained by the fact that in our study we analyzed mRNA expression of the receptors (independent of receptor localization), while in the study by Salvucci et al. (*16*) tissue microarrays were analyzed by immunohistochemistry and nuclear and

cytoplasmatic CXCR4 staining were analyzed separately. In agreement with our study Salvucci et al. (*16*) reported more cytoplasmic CXCR4 staining in ER-negative (54%) compared to ER-positive tumors (38%). Furthermore, we found that high *GRPR* expression was of modest predictive value for increased time to progression on tamoxifen treatment, suggesting GRPR radioligands to be useful to monitor tumor response to treatment with tamoxifen. Recently, preclinical ^{68}Ga-AMBA PET imaging in a mouse model also demonstrated feasibility for monitoring tumor response after treatment with tamoxifen (*27*).

For the association with clinico-pathological and biological characteristics analyzed in the M0 LNN patients, we observed a significant positive correlation between *GRPR* and *SSTR2* expression and *ESR1*- and *PGR*-positive tumors. In line with our findings, significant positive correlation between SSTR2 and ER expression was reported previously (*28*), while van den Bossche et al. (*29*) reported estrogen-mediated regulation of SSTR2 expression in breast cancer cell lines. Because *ESR1* and *PGR* positivity correlates with breast cancer of the luminal subtype (*2*), tumors of this subtype could benefit most from GRPR and/or SSTR2-mediated imaging and/or therapy. Moreover, *ESR1*-negative tumors showed very low to no *GRPR* expression and thus patients with *ESR1*-negative primary tumors are likely not suited for the application of GRPR radioligands. Since *ESR1*- and/or *PGR*-positive tumors account for 75% of the breast cancer tumors (*2*), GRPR and SSTR2-mediated imaging and therapy might be of benefit for the larger part of the breast cancer patient population.

Concerning therapy, GRPR or SSTR2 radioligands can especially be of benefit for patients with *ESR1*-positive tumors who have progressed on various lines of endocrine treatment, since nearly all patients with recurrent disease become resistant against current anti-estrogen treatments (*4*).

Previous studies we performed on GRPR and SSTR2 expression in human breast cancer specimens showed GRPR expression in 48/50 (*30*) and SSTR2 expression in 26/53 (data not published) of the specimens analyzed by in vitro autoradiography, emphasizing that GRPR and SSTR2-mediated imaging and therapy could be applied in a large group of breast cancer patients.

Contrary to *GRPR* and *SSTR2*, high *CXCR4* mRNA expression was correlated with *ESR1*- and *PGR*-negative tumors, associated with breast cancer of the basal like subtype (*2*), indicating that particularly these tumors might be suitable for CXCR4-mediated imaging and/or therapy. Especially patients with triple negative tumors might particularly benefit from CXCR4-mediated therapy, since effective therapy options for this aggressive subtype of breast cancer are scarce. Differences in *CXCR4* expression between *ESR1*- and *PGR*-negative and *ESR1*- and *PGR*-positive patients were less pronounced than for *GRPR* and *SSTR2*. *ESR1*- and *PGR*-positive patients should therefore not be ruled out for CXCR4-mediated imaging and/or therapy.

Except for the presence of the receptors, for the selection of patients for imaging and/or treatment with radioligands, also the density of GRPR, SSTR2 and CXCR4 might determine the target of choice. In a study by Reubi et al. (*15*), amongst other receptors, GRPR and SSTR2 expression in 77 breast cancer tissues were analyzed using in vitro autoradiography. Results showed that high density GRPR expression was observed in 50/77 tumors compared to 14/77 tumors with high density SSTR2 expression. Similarly, in our previous work we found homogenous GRPR expression in 56% of the breast cancer specimens analyzed (*30*), while homogenous SSTR2 expression was seen in 29% only (data not published).

One of the benefits of targeted imaging and therapy using GRPR, SSTR2 and CXCR4 radioligands is the possibility to upfront select patients that could benefit from this using one of the radioligands. For this either frozen material from breast cancer biopsies can be used to perform in vitro autoradiography using radioligands, or formalin fixed paraffin embedded material for immunohistochemistry, or both to perform RT-qPCR, to identify patients suited for imaging and/or therapy.

There are however also limitations to our study. Firstly, mRNA was used as a surrogate for radiotracer binding and ER, PGR and HER2 protein expression, which may, despite our current and previously published (*22,23*) positive correlations turn out not to be entirely equivalent. Secondly, for the prognostic part only, even though our study is relatively large no independent validation was performed. In addition this is a retrospective study and might not completely represent the current situation in patients.

CONCLUSION

We successfully identified potential breast cancer patient groups for the application of radioligands targeting GRPR, SSTR2 or CXCR4 by analyzing associations between receptor expression and clinico-pathological, biological and prognostic factors. Our data show compelling evidence that sensitive and specific nuclear medicine-based imaging and therapy using radioligands might be of great benefit for selected breast cancer patients in a personalized setting. GRPR and SSTR2 radioligands in ER-positive, PR-positive tumors and CXCR4 radioligands in ER-negative patients might offer new, promising tools for imaging and therapy of breast cancer.

DISCLOSURE

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FIGURES

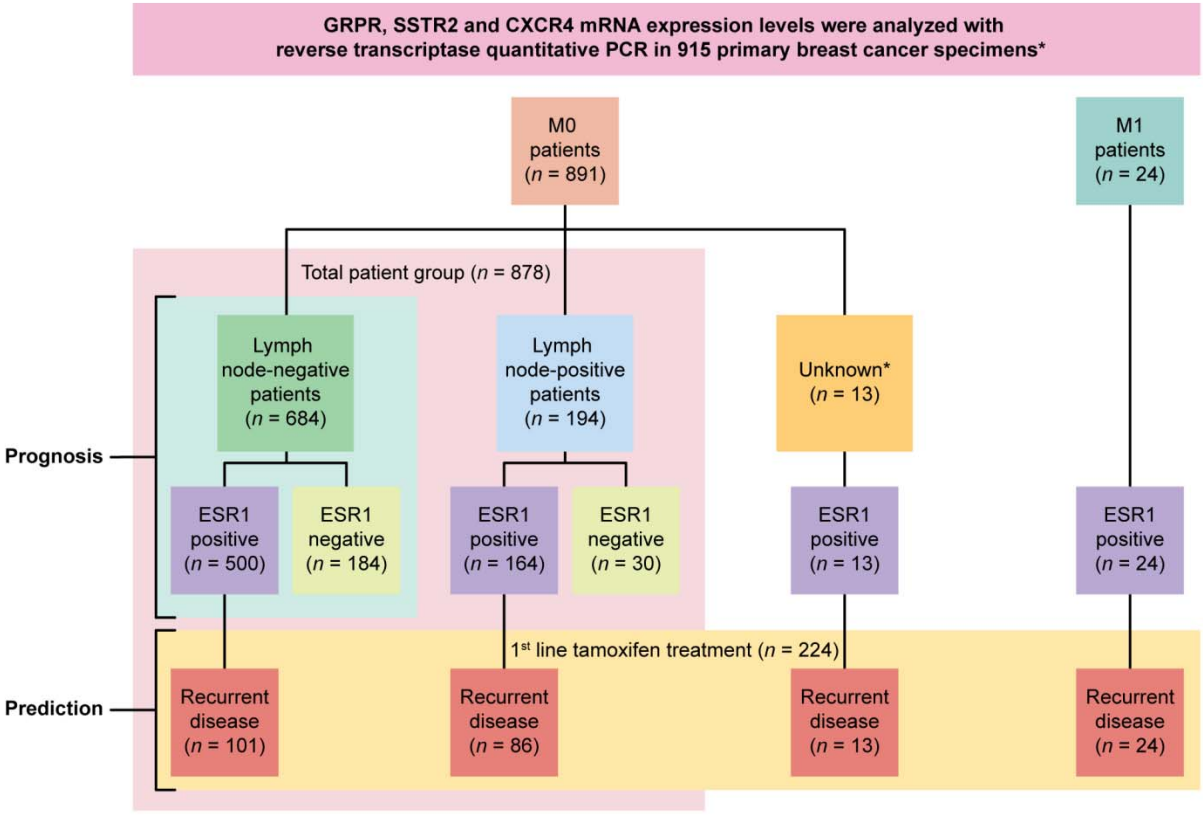
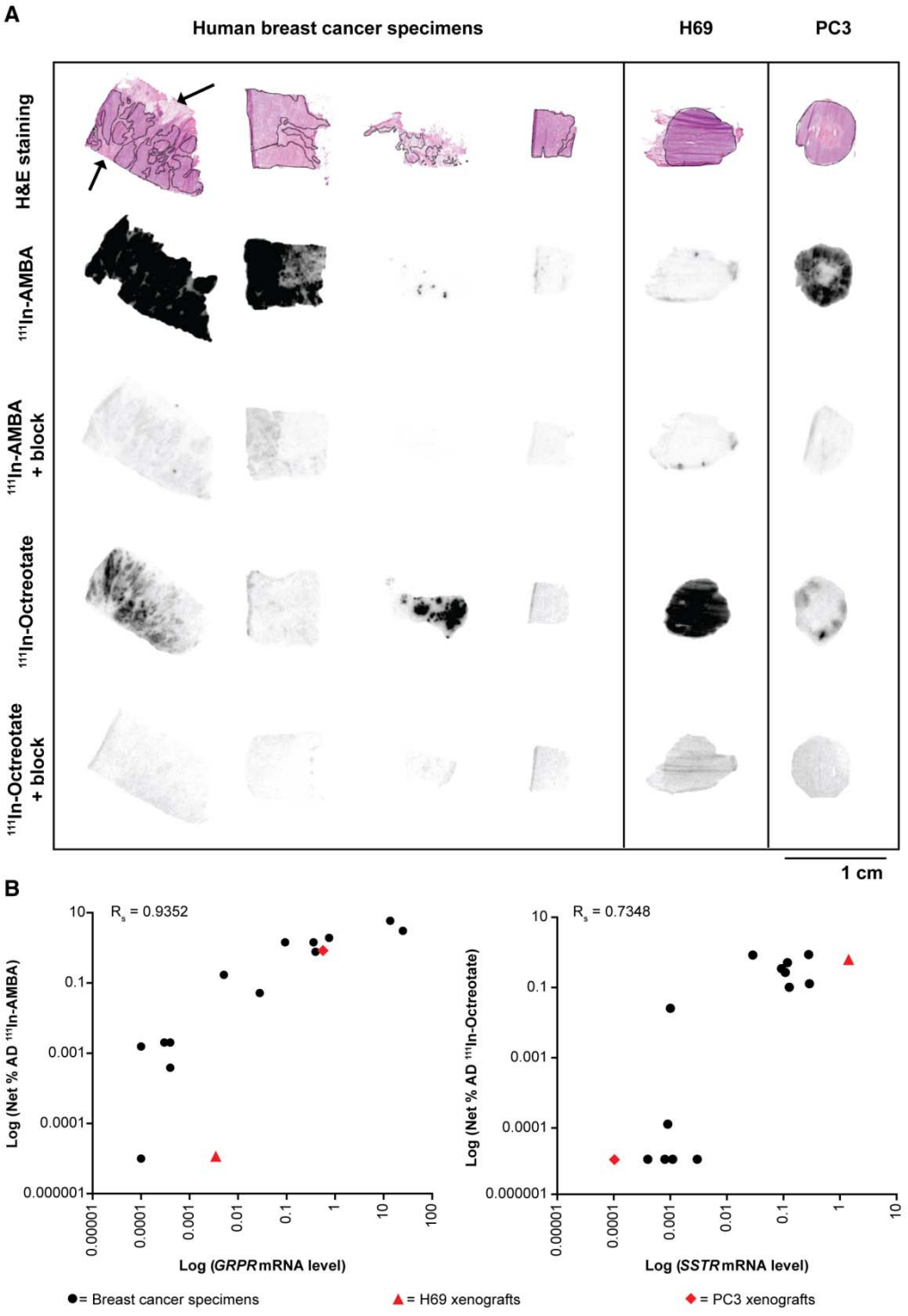


Figure 1. Study design. mRNA expression levels of GRPR, SSTR2 and CXCR4 of 915 primary breast cancer specimens (684 M0 LNN, 194 M0 LNP, 13 specimens with unknown nodal status and 24 M1 patients) were analyzed using RT-qPCR. The LNN and LNP M0 patient group were used to study the association of GRPR, SSTR2 and CXCR4 expression and clinico-pathological and biological factors, with a focus on the M0 LNN patient group. The association of GRPR, SSTR2 and CXCR4 with prognostic factors was studied in the M0 LNN patients. mRNA levels of ER-positive primary tumors of patients with recurrent breast cancer, who received first line tamoxifen treatment were used to study the association of GRPR, SSTR and CXCR4 mRNA expression and PFS.


A
Human breast cancer specimens
H69
PC3
H&E staining
111In-AMBA
111In-AMBA + block
111In-Octreotate
111In-Octreotate + block
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B
Rs = 0.9352
Log (Net % AD 111In-AMBA)
Log (GRPR mRNA level)
Rs = 0.7348
Log (Net % AD 111In-Octreotate)
Log (SSTR mRNA level)
= Breast cancer specimens
= H69 xenografts
= PC3 xenografts

Figure 2. A) In vitro autoradiography of human breast cancer specimens using ^{111}In-AMBA (GRP analog) and ^{111}In-DOTA-Tyr^{3}-octreotate (SST analog) with and without block, demonstrating specific binding of the radiotracers to receptor positive tumor tissue. H69 (SSTR+, GRPR-) and PC3 xenografts (SSTR-, GRPR+) were used as controls. Tumor containing area's are encircled in the hematoxilin-eosin (H&E) stainings. As an example, arrows indicate non-tumor containing tissue in the first H&E staining.

B) Significant correlation between GRPR and SSTR2 mRNA levels and quantification of in vitro autoradiography results analyzed in 13 breast cancer specimens with variable receptor expression, demonstrating that mRNA levels of the receptors can be used as a predictor for radiotracer binding.

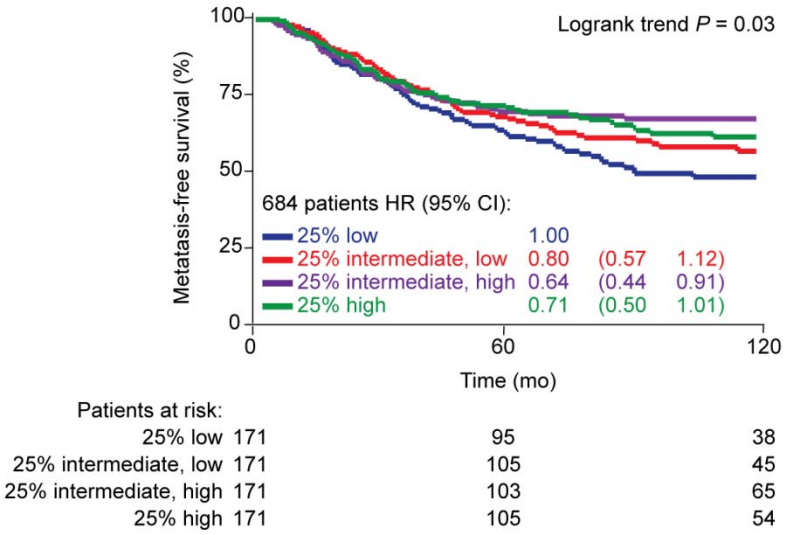

Figure 3. Distant metastasis-free survival in 684 LNN patients as a function of the levels of CXCR4.

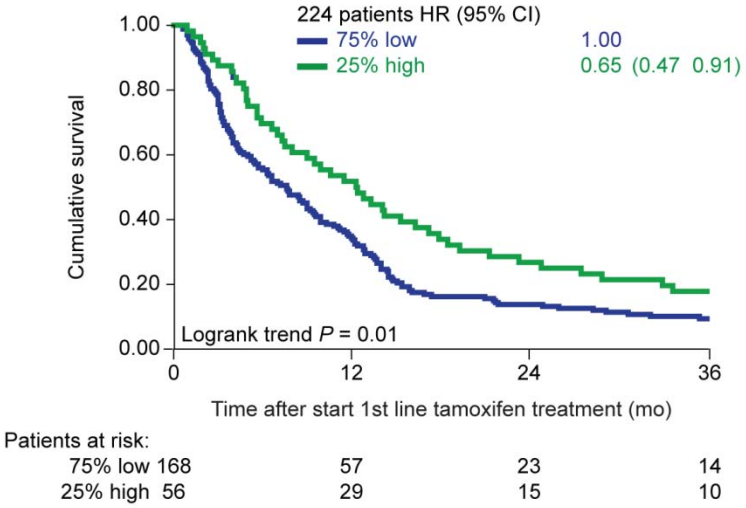

Figure 4. Association of GRPR expression with PFS on 1st line tamoxifen treatment.

Table 1. Associations of *GRPR*, *SSTR2* and *CXCR4* mRNA levels in LNN M0 patients

Characteristic	No of patients*		GRPR mRNA (x10^{-2}) Median	GRPR mRNA (x10^{-2}) Interquartile range	SSTR2 mRNA (x10^{-2}) Median	SSTR2 mRNA (x10^{-2}) Interquartile range	CXCR4 mRNA (x10^{-2}) Median	CXCR4 mRNA (x10^{-2}) Interquartile range
All patients in this cohort	**684**	**100%**	**0.72**	**7.07**	**0.58**	**1.75**	**11.78**	**13.13**
Age at surgery (years)								
≤40	60	9%	1.17	12.72	0.90	2.99	14.06	13.75
41-55	252	37%	0.97	9.20	0.61	1.64	11.58	13.18
56-70	218	32%	0.52	5.38	0.52	1.68	12.19	11.34
>70	154	23%	0.72	4.44	0.62	1.61	9.99	12.98
P†			*0.52*		*0.68*		***0.0403***	
Menopausal status								
Premenopausal	273	40%	1.26	10.95	0.62	1.82	11.81	13.73
Postmenopausal	411	60%	0.60	4.87	0.55	1.57	11.76	12.12
P†			*0.13*		*0.53*		*0.39*	
Surgery								
Lumpectomy	378	55%	0.61	7.69	0.57	1.82	11.67	13.15
Ablation	306	45%	0.90	6.79	0.60	1.56	11.90	13.00
P†			*0.69*		*0.59*		*0.65*	
Pathological tumor size								
pT1	307	45%	1.25	8.54	0.69	1.87	12.03	13.40
pT2+unknown	351	51%	0.41	5.25	0.51	1.65	11.53	12.84
pT3 + pT4	26	4%	0.58	3.05	0.50	1.38	12.19	13.53
P†			***0.0014***		*0.24*		*0.92*	
ESR1 mRNA status‡								
Negative < 0.2	184	27%	0.09	0.13	0.28	0.42	14.74	13.83
Positive ≥ 0.2	500	73%	2.46	10.98	0.81	2.59	10.98	12.16
P‡			***< 0.001***		***< 0.001***		***< 0.001***	
PGR mRNA status‡								
Negative < 0.1	285	42%	0.12	0.32	0.32	0.56	14.36	14.31
Positive ≥ 0.1	399	58%	3.67	12.68	1.02	2.98	10.45	11.09
P‡			***< 0.001***		***< 0.001***		***< 0.001***	
ERBB2 mRNA status‡								
Negative < 18	574	84%	0.99	8.28	0.61	1.92	11.64	12.96
Positive ≥ 18	107	16%	0.30	1.51	0.49	1.00	13.88	13.32
P§			***< 0.001***		***0.0344***		*0.22*	
Grade (GGI)								
1	227	33%	2.42	10.46	0.75	2.11	10.83	11.03
2	229	33%	0.89	6.92	0.63	2.41	11.44	14.59
3	224	33%	0.13	1.42	0.34	0.98	13.83	13.58
P§			***< 0.001***		***< 0.001***		***< 0.001***	
% Invasive tumor cells								
≤ 70%	470	69%	0.81	6.84	0.63	1.88	12.57	13.92
> 70%	214	31%	0.64	8.28	0.43	1.28	9.13	10.70
P†			*0.87*		***0.002***		***< 0.001***	

** Due to missing numbers, not all categories add up to 684.*

† P for Mann-Whitney U or Kruskal-Wallis test when appropriate.

‡ ESR1, PGR and ERBB2 were determined by real-time PCR, cut points were as follows ESR1=0.2, PGR=0.1 and ERBB2=18.0 (mRNA level relative to reference gene set).

§ P for Spearman rank correlation test.