Insulin-like growth factor-1 receptor gene expression is associated with survival in breast cancer: a comprehensive analysis of gene copy number, mRNA and protein expression

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Abstract

Insulin-like growth factor-1 receptor (IGF1R) plays a key role in the initiation and progression of breast cancer. However, its prognostic relevance to breast cancer patients has long been a matter of debate. In a series of 325 primary invasive breast cancer patients, we performed a comprehensive analysis of IGF1R at the levels of gene copy number, mRNA expression and protein expression. The relationship between the IGF1R status and the clinicopathological characteristics and prognosis was evaluated. IGF1R mRNA levels not only correlated with protein expression, but also significantly associated with several clinicopathological parameters and prognosis of the patients. Patients with low nuclear grade, negative axillary lymph node, positive hormone receptor, negative HER2, negative Ki67 and luminal subtype tumors showed higher level expression of IGF1R mRNA, which was shown to be a significant univariate parameter for both relapse free survival (RFS) and breast cancer specific survival (BCSS) as well as significant multivariate parameter for BCSS. IGF1R protein expression showed an association with a prolonged BCSS in univariate analysis. By contrast, IGF1R gene copy number did not correlated with mRNA and protein expression, and harbored no prognostic value. When studied in breast tumor subtype groups relatively, IGF1R mRNA level was still significantly associated with a better BCSS. Overall, our data indicated a correlation among IGF1R mRNA expression and protein expression in primary breast cancer. In particular, the IGF1R mRNA expression appeared to be a good prognostic marker either in the entire cohort or in the luminal subtype group. These data may serve as background information for IGF1R-targeted therapy.

Key words: Insulin-like growth factor-1 receptor, Breast cancer, Prognosis

Abbreviations:

IGF1R	insulin-like growth factor-1 receptor
RFS	relapse free survival
BCSS	breast cancer specific survival
MAPK	mitogen activated protein kinase
PI3K	phosphatidylinositol-3-kinase
ER	estrogen receptor
Her2	human epidermal growth factor receptor 2
PgR	progesterone receptor
BMI	body mass index

Introduction

Recent studies support a key role for insulin-like growth factor 1 receptor (IGF1R) in initiation and progression of many cancers, including breast cancer [1, 2]. In addition, targeting the IGF1R represents a promising strategy in the development of novel anti-cancer therapeutics [3-5].

IGF1R is a transmembrane tyrosine kinase, consisting of two extracellular α-subunits and two intracellular β-subunits. The α-subunits bind ligands IGF-1 and IGF-2, while β-subunits transmit ligand-induced signals through the RAS/RAF/mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K)-Akt pathway. Dysregulation of the IGF1R signaling is linked to diverse pathologies, ranging from growth deficits to cancer development [6]. The changed expression of IGF1R has been documented in breast cancer [7, 8]. However, the results of some of these studies are controversial. Except for the well established correlation with estrogen receptor (ER) status, its prognostic relevance to breast cancer patients has long been a matter of debate [7, 9, 10].

On the other hand, IGF1R has recently emerged as one of the most promising molecular targets in cancer treatment. Various technologies are being employed to downregulate IGF1R expression and signaling [5]. In breast cancer, with the evidence that IGF1R is involved in resistance to endocrine therapy, anti-human epidermal growth factor receptor 2 (Her2) therapy and chemotherapy, targeting the IGF1R has particular appeal [11, 12]. So far, phase Ⅱ studies with IGF1R inhibitors indicated favorable toxicity profiles and promising activity [13]. As no biomarkers are available yet to select patients who have the potential to benefit from IGF1R-targeted therapy, it is urgent that we develop a good understanding of the factors

contributing to it.

In the present study, we performed a comprehensive analysis of IGF1R at the levels of gene copy number, mRNA expression and protein expression in a cohort of patients with primary invasive breast cancer. The relationship between the IGF1R status and the clinicopathological characteristics and prognosis was evaluated. These data may serve as background information for selecting patients for IGF1R-targeted therapy.

Patients and Methods

Patients and breast cancer tissues

Breast tumor specimens from 325 female patients with invasive breast carcinoma, who were treated at Kumamoto University Hospital between 2001 and 2009, were included in this study. The patients were from a consecutive series, excluding those with other malignancies or bilateral breast cancer. Samples were snap frozen in liquid nitrogen at the time of the pretherapeutic biopsy or surgical treatment and stored at -80°C until simultaneous total RNA and genomic DNA extraction. Informed consent was obtained from all patients. The ethics committee of Kumamoto University Graduate School of Medical Sciences approved the study protocol. The median age of the patients was 59 years (range, 21-93 years). Adjuvant and neoadjuvant treatment was administered in accordance with the recommendations of the St. Gallen international expert consensus on the primary therapy of early breast cancer [14-16]. On recurrence, patients with hormone receptor negative tumors were treated with chemotherapy such as anthracycline containing regimens, taxanes, trastuzumab (for patients overexpressing Her2), capecitabine, and vinorelbine. Patients with hormone receptor-positive tumors and nonvisceral metastases were treated with endocrine therapy, such as antiestrogens, aromatase inhibitors, and medroxyprogesterone acetate. Patients were followed postoperatively every three months. The median follow-up period was 44 months (range, 5-112 months).

Immunohistochemistry and the scoring system

Histological sections (4 μm) were deparaffinized and incubated for 10 min in methanol containing 0.3 % hydrogen peroxide. We used rabbit polyclonal antibody against IGF1R (#3027, 1:600; Cellsignaling, Boston, USA) which was sensitive to detect the endogenous levels of β subunit of this transmembrane tyrosine kinase and did not cross-react with insulin receptor. Rabbit polyclonal antibody was also used for detection of Her2 (1:200; Dako Japan, Tokyo, Japan), and mouse monoclonal antibodies were used for estrogen receptor α (ERα) (1D5, 1:50; Dako Japan), progesterone receptor (PgR) (PgR636, 1:800; Dako Japan) and Ki67 (MIB-1, 1:50; Dako Japan). Expression using these antibodies was determined by the Histofine Simple Stain MAX-PO® (Nichirei, Tokyo, Japan) method as described previously [17]. IGF1R expression was scored according to the different staining patterns respectively. Membranous staining (range, from 0 to 3+), analogue to Her2 expression, was considered positive with strong staining pattern in >10% of tumor cells, while cytoplasmic staining was scored by H score, multiplying the products of the percentage of cells stained at a given staining intensity (0–100) by the staining intensity score (0, none; 1, weak; 2, moderate; and 3, intense): 0-10 points were considered to be negative, 11-100 points as weak, 101-200 points as moderate, and 201-300 points as strong positive. Her2 immunostaining was evaluated using the same method as the HercepTest (Dako), the membranous staining was scored on a scale of 0 to 3+. Tumors with scores of ≥3 or with a ≥2.2-fold increase in

HER2 gene amplification as determined by fluorescence *in situ* hybridization were considered to be positive for Her2 overexpression. ERα and PgR status was considered positive when there was ≥1 % of nuclear staining [18]. Ki67 was scored for the percentage of nuclear staining cells out of all cancer cells in the invasive front of the tumor at ×40 high-power field (Ki67 labeling index).

RNA extraction, primers, and real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from tissue specimens using the Allprep DNA/RNA Mini Kit (Qiagen, Germantown, Md, USA). Total RNA (0.5 μg) was reverse transcribed to cDNA using PrimeScript RT reagent Kit (Takara Bio Inc., Otsu, Japan), according to the manufacturer's protocol. Each PCR was performed with 2 μl of the cDNA and 0.2 μmol / l of each probe in a LightCycler System with SYBR Premix Dimer Eraser (Takara Bio Inc.). PCR primer sequences were as follows: for IGF1R, forward 5′-TGAAAGTGACGTCCTGCATTTC-3′ and reverse 5′-GGTACCGGTGCCAGGTTATG-3′; for β-actin, forward 5′-TGGCACCCAGCACAATGAA-3′ and reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. Each reaction (20 μl samples) was performed under the following conditions: initialization for 10 s at 95 °C, and then 45 cycles of amplification, with 5 s at 95 °C for denaturation and 20 s at 60 °C for annealing and elongation. For each PCR run, a standard curve was constructed from cDNA from the MCF7 cell line. The level of expression of IGF1R mRNA is given as relative copy numbers normalized against β-actin mRNA.

Gene copy number

Patient and control genomic DNA was extracted using the Allprep DNA/RNA Mini Kit (Qiagen) following the manufacturer's protocol. The concentration and purity of the genomic DNA preparations were measured. IGF1R gene amplification was analyzed with gene copy number assay by real time polymerase chain reaction on a 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). RNase P was chosen as a reference for gene dosage because of its single copy number. Reactions were performed in triplicate in a total volume of 20 μL, including 4 μL of gDNA, 1μL of IGF1R TaqMan Copy Number Assay (4400291, Applied Biosystems), 1 μL of RNase P TaqMan Copy Number Reference Assay (4316844, Applied Biosystems) and 10 μL of Master Mix. Thermal cycling conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Calculation of the gene copy number was carried out using the comparative Ct method (△△Ct) that requires a healthy control sample (diploid) as a calibrator and the RNase P gene as an internal standard. IGF1R gene status was defined by the ratio of IGF1R versus RNase P gene: >2.0 indicated amplification.

Statistical analysis

The Spearman rank correlation coefficients were used to assess the correlation between IGF1R gene copy number, mRNA level and cytoplasmic protein expression. The nonparametric Mann-Whitney U test and contingency analysis was adopted for statistical analysis of associations between different IGF1R status and the clinicopathologic characteristics of the patients. Disease-free survival and overall survival curves were generated using the Kaplan-Meier method and verified by the log-rank (Mantel-Cox) test. Cox's proportional hazards model was used for the univariate and multivariate analyses of prognostic values. Statistical significance was defined as P

< 0.05. JMP software version 8.0.1 for Windows (SAS institute Japan, Tokyo, Japan) was used for statistical analysis.

Results

Correlation between IGF1R gene copy number, mRNA expression and protein expression

We analyzed the IGF1R gene copy number in 319 primary invasive breast tumors. The median value of IGF1R versus RNase P gene was 1.7. 211 (66.1%) cases showed a ratio from 1.0 to 2.0, 64 (20.1%) cases showed a ratio from 2.0 to 4.0, and 20(6.3%) cases were >4.0. To identify a clinically meaningful cutoff for IGF1R gene copy number, various levels of IGF1R gene copy number between 2.0 and 4.0 were tested by the Kaplan-Meier method and verified by the log-rank (Matel-cox) test. However, no significant association with relapse-free survival (RFS) or breast cancer-specific survival (BCSS) could be found. Therefore, >2.0 were set for the cutoff as gene amplification. Results of IGF1R mRNA expression were obtained from 307 patients. Relative expression of IGF1R mRNA ranged from 0.006 to 52.6, which were not normally distributed and transformation of the data did not result in a sufficiently normalized distribution. A median of 0.557 was used as the cutoff point. Immunohistochemistry was used to evaluate 297 cases. As scored according to the different staining patterns (Fig. 1), negative, weak, moderate and strong membranous IGF1R expression was present in 192 (64.6%), 82 (27.6%), 14 (4.7%), 9 (3.0%) cases, respectively. Cytoplasmic staining for IGF1R was negative in 53 (17.8%) cases, weakly positive in 132 (44.4%) cases, moderately positive in 83 (27.9%) cases and strongly positive in 29 (9.8%) cases. Pure membranous IGF1R expression was present in 0.3%, pure cytoplasmic in 47.1%, and both membrane and cytoplasmic staining in 35.0%.

When we dichotomized IGF1R gene copy number and protein expression into positive and negative, IGF1R mRNA level was higher in the patients with gene amplification (n=299, P=0.026) and positive protein expression whether in membranous staining pattern (n=294, P=0.0002) or cytoplasmic staining pattern (n=294, P=0.003). Using continuous data, the results of IGF1R gene copy number (n=299, P=0.026), mRNA expression and cytoplastic protein expression were compared each other to study the correlation in our entire cohort and different tumor subtypes respectively (Table 1). A significant correlation was observed between IGF1R mRNA expression and IGF1R cytoplasmic expression whatever in entire cohort (γ= 0.20, P=0.0006) or in the luminal tumor subtype group (γ= 0.17, P=0.01), but not in Her2 subtype group and tripple negative subtype group. On the contrary, no significant correlation was indicated between IGF1R gene copy number and IGF1R mRNA expression or cytoplasmic expression.

Association of IGF1R gene copy number, mRNA expression and protein expression with clinicopathological parameters

The clinicopathological characteristics for the 325 cases analyzed in the present study are summarized in Table 2. The level of IGF1R mRNA expression was observed to be significantly associated with several clinicopathological parameters. Higher IGF1R mRNA levels were indicated in the groups of patients with a negative axillary lymph node (P=0.002), lower nuclear grade (P<0.0001), positive hormone receptor status (for ER, P<0.0001; for PR, P<0.0001), negative Her2 status (P=0.0003), negative Ki67(P=0.03) as well as luminal tumor subtype (P<0.0001). No relationship could be found between IGF1R mRNA level with body mass index (BMI; P=0.22), menopausal status (P=0.23) and tumor size (P=0.57) of the patients.

The IGF1R gene copy number had a less remarkable relationship with clinicopathological factors. Except for a relationship with ER status (P=0.03), higher IGF1R gene copy number was associated with smaller tumor size (P=0.02). Positive IGF1R protein expression was associated with positive ER status (P=0.0002, in membranous staining; P=0.0009, in cytoplasmic staining) positive PR status (P=0.004, in membranous staining; P=0.001, in cytoplasmic staining) and luminal subtype (P=0.007, in membranous staining; P=0.03, in cytoplasmic staining). Nevertheless, positive IGF1R cytoplasmic staining was also present in lower nuclear grade group (P=0.03).

In luminal tumor subtype group (n=237), higher IGF1R mRNA expression was associated with a negative lymph node status (P=0.001) as well as negative Her2 status (P=0.02). IGF1R cytoplasmic expression was frequently observed in the patients with negative Her2 status (P=0.04). However, no correlation was indicated between clinicopathological parameters and IGF1R gene copy number (Supplementary table 1). In Her-2 tumor subtype group (n=30) and triple negative tumor subtype group (n=39), neither IGF1R mRNA level, gene copy number nor protein expression showed any significant association with clinicopathological parameters.

Prognostic relevance of IGF1R gene copy number, mRNA expression and protein expression

In the analysis of RFS, local recurrences and distant metastases were considered as an event. Among 32 recurrent cases, there were 27 cases of distant metastases and 5 cases of local recurrences. 20 cases died as a result of breast cancer, which were regarded as events when analyzing BCSS.

Prognostic relevance of IGF1R gene copy number, mRNA expression and protein expression are summarized in Table 3 and Table 4. Our data indicate that IGF1R mRNA expression is a good prognostic factor for primary invasive breast cancer. Using the median as the cutoff point, patients showing higher IGF1R mRNA expression were associated with prolonged RFS (P=0.002) and BCSS (P=0.0002) when tested by Kaplan-Meier method and verified by the log-rank (Mantel-Cox) test (Fig. 2). In the Cox regression model, including menopausal status, BMI, tumor size, nodal status, nuclear grade, ER, PgR, Her2 and Ki67, IGF1R mRNA expression proved to be a significant prognostic univariate parameter for both RFS (P=0.002) and BCSS (P<0.0001) as well as significant multivariate parameter for BCSS (P=0.04).

When we defined the IGF1R protein expression in two groups (positive and negative), it also showed a significant correlation with BCSS. Regardless of their staining patterns, positive cases seemed to have a longer survival than negative ones in Kaplan-Meier method analysis (membranous staining, P=0.03; cytoplasmic staining, P=0.02; Fig. 3). As univariate factors, membranous IGF1R expression and cytoplasmic IGF1R expression were related to the BCSS (membranous staining, P=0.01; cytoplasmic staining, P=0.04), but when studied with other significant factors in the Cox regression analysis, they were no longer significant (membranous staining, P=0.29; cytoplasmic staining, P=0.25). No association could be found between IGF1R protein expression and RFS.

In contrast to IGF1R mRNA and protein expression, there was no prognostic difference between the IGF1R gene amplification group and non-amplification group.

Furthermore, we studied the prognostic value of IGF1R in different subtypes of our cohort. In luminal subtype group (Table 5), IGF1R gene expression was significantly associated with BCSS (univariate analysis, P=0.03), even after adjusting the Cox model for menopausal status, BMI,

tumor size, nodal status, nuclear grade, PgR, Her2 and Ki67 (multivariate analysis, P=0.04). Kaplan-Meier showed the association of higher IGF1R gene expression with prolonged survival time (Fig. 4, P=0.037). In the same group, IGF1R membranous expression indicated a marginal significance with BCSS (P=0.04) in univariate analysis. However, it lost the significance in the multivariate analysis of the Cox model and couldn't be verified by Kaplan-Meier curve.

Regarding to the Her-2 and triple negative tumor subtype group, no association was apparent between prognosis and IGF1R gene copy number, mRNA expression or protein expression.

Discussion

Gene amplification, as a kind of the gene copy number variation, might increase the expression levels of genes [19, 20]. In breast cancer, multiple different oncogenes have been described previously as being amplified, including HER2 gene [21, 22], c-Myc gene [23], cyclin D1 gene [24] and estrogen receptor α gene [25]. However, there have been few reports considering the frequency of IGF1R gene amplification. Using a quantitative real time PCR-based assay, we found that 26% of samples in our group showed IGF1R gene amplification, which was similar to the result of Dziadziuszko's research in a cohort of operable non-small-cell lung cancer [26] but not consisted with the previous reports of rare amplification rate in breast cancer [27, 28]. The different assays adopted might lead to the discrepancy of the results. While Adélaïde J found that the amplification of IGF1R was exclusively in basal-like breast cancer, our data showed no significance in distribution of IGF1R gene amplification in different subtype (p=0.56). However, the methodologies for accurate measurement of gene copy number remain challenging, and still limit the scope of present genetic studies associating CNV and disease. In our study, IGF1R gene amplification did not have a prognostic relevance. One explanation is that the transcriptional and translational control mechanisms have diminished the effect of gene amplification. A second copy of a gene might result in additional expression if the new copy is functional or no change in expression if the genomic location of the new copy is unfavorable. A partial second copy might even abolish expression of the first copy by inserting along side it [20]. This is reflected in a lower correlation of gene copy number vs. mRNA compared with mRNA vs. protein. Hence, efforts to find a simple correlation between gene copy number and the prognosis of the disease might occasionally meet with frustrations. Further studies, using accurate genotyping assays in large population cohorts are needed to define more precisely the overall mechanism involved.

Expression of IGF1R is a frequent finding in breast cancer. However, there is no universally valid immunohistochemical scoring system for IGF1R expression with respect to both intensity and pattern of the IGF1R staining. Since the clinical relevance of IGF1R localization is yet unknown and protein localization of tyrosine kinase receptors might be a highly dynamic process, we conducted a separate analysis for membranous and cytoplasmic staining. As IGF1R is a transmenbrane tyrosine kinase, it is reasonable to find the membranous staining. For the cytoplasmic staining, two factors should be taken into consideration. Firstly, the antibody used in present research detected β subunit of IGF1R which located at the internal side of the membrane. Secondly, IGF1R might translocated from the cell membrane to the cytosol with cytoplasmic IGF1R representing a bound internalized and thus a potential activated receptor. [29] Although the biologic significance of cytoplasmic localization of the IGF1R has received little attention in the literature up to now, cytoplasmic localization has been observed before [10, 30]. Some workers have reported a similar kind of IGF1R staining pattern, while others have chosen to merely focus

on the membranous staining [26, 31]. Different antibodies and diverse immunohistochemistry protocols and scoring systems might result in the disparity among the different studies. In our samples, since cytoplasmic staining patterns of tumor incorporated in the same slide show different staining intensity, we considered it was not an artifact but a relevant observation which should not be ignored. At first sight, there seems to be a discrepancy between the results of IGF1R membranous staining and cytoplasmic staining. As compared to 244/297 (82.2%) cases presenting cytoplasmic staining, only 105/297 (35.4%) cases presented membranous staining. However, with further analysis, we observed that 104/105 (99.0%) membranous staining positive cases also showed positive cytoplasmic staining. Moreover, these two different staining patterns were identically related to the same clinicopathological factors (ER status and PR status) and subsequently favorable BCSS. Taken together, our data suggest that IGF1R cytoplasmic staining, as well as membranous staining, was functional in evaluating protein expression and reflecting the prognosis, even though they were not independent biomarkers. The specific details concerning improving the accuracy of the IGF1R testing and the suitability of the scoring system need to be investigated in future studies.

In the present study, we observed that IGF1R mRNA expression was significantly higher in those patients who had favorable clinicopathological parameters such as lower nuclear grade, negative lymph node, positive hormone receptor status and negative HER2 status. Therefore, it was not surprising that, in our patients, IGF1R gene expression turned out to be significantly associated with RFS and BCSS. The favorable prognosis provided by higher IGF1R mRNA expression conflicts with some previous in vitro studies supporting a role for IGF1R in cell transformation and malignancy [32]. There are at least two possible explanations for this apparent discrepancy. The first one comes from the results of research using transgenic mice models [33]. Although IGF1R overexpression or constitutive activation has been shown to be sufficient to induce mammary tumor development in vivo, characterization of these in in vivo models has lead to a number of interesting observations regarding the role of IGF1R in breast cancer. First, the most critical stage for IGF1R activation, with the respect to mammary tumor development, is during the rapid proliferative stage of ductal morphogenesis. Second, tumors initiated by IGF1R overexpression have the ability to progress to an IGF1R independent state. Finally, IGF1R -induced tumors are weakly metastatic indicating that metastatic spread from the primary tumor probably requires additional genetic alterations. Our study provided powerful clinical evidence that coincides with these preclinical experiments. Regarding the second explanation, the prognostic significance of IGF1R may be similar to that of the ER [9]. Although ER is a receptor that mediates a mitogenic response to its ligand estradiol, its expression generally reflects well differentiated tumors with a favorable prognosis. Targeting of ER, by antiestrogens, is one of the most widely used and successful treatments of the breast cancer. IGF1R expression could also be used to identify a relatively well differentiated tumor that still requires IGF1R for proliferation. By analogy with ER, IGF1R could also be a useful therapeutic target for breast cancer.

As breast cancer isn't a homogenous disease, we focused further on the effect of IGF1R in the different tumor subtypes. We found in luminal subtype group, IGF1R mRNA expression was higher in the patients with negative lymph node status and negative HER2 status as well as acting as an independent prognostic biomarker for BCSS. These results might indicate that, given the predominant role of ER in this group, IGF1R could also serve as a supplementary target. In line with this, anti-IGF1R therapy is expected to find a place as a good supplementary approach in

combination with other therapeutic strategies. Recent research showed that IGF1R expression was strongly related to a shorter disease free survival in triple negative breast tumors [10]. However, in present study, no prognostic significance was indicated for the IGF1R in Her-2 group and triple negative group. Lower percentage of triple negative cases in our cohort may response to the disparity comparing with the previous research. To accumulate more cases and make further analysis is needed.

In conclusion, our study indicates a correlation among IGF1R gene expression and protein expression in primary breast cancer. IGF1R protein expression was associated with a prolonged BCSS whereas mRNA expression was not only associated with RFS but it also turned out to be an independent biomarker for BCSS. When studied in breast tumor subtype groups relatively, IGF1R mRNA level was still significantly associated with a better BCSS. It may not be possible to identify a single biomarker that correlates with response to IGF inhibitors. However, taking the status of the IGF1R mRNA expression into consideration may help us improve the ability to develop effective treatment modalities for those conditions in which IGF1R is involved.

Acknowledgments

We thank Y. Azakami and Y. Sonoda for excellent technical support, and A. Okabe, for clinical data management.

Disclosure statements

None declared

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Figure captions

Fig.1 a-d staining patterns of IGF1R immunohistochemistry: **a** membranous staining; **b** cytoplasmic staining; **c** mixed membranous and cytoplasmic staining; **d** negative staining (magnification x40)

Fig.2 Kaplan-Meier plots of association of IGF1R mRNA expression with RFS (**a**) and BCSS (**b**) in entire cohort

Fig.3 Kaplan-Meier plots of association of IGF1R protein expression with BCSS (**a** membranous expression; **b** cytoplasmic expression) in entire cohort

Fig.4 Kaplan-Meier plots of association of IGF1R mRNA expression with BCSS in luminal tumor subtype group

Supplementary material list:

1 table

Supplementary Table 1 Association of IGF1R gene copy number, mRNA expression and protein expression with clinicopathological parameters in luminal tumor subtype group

(when submitting the paper, the content below should be arranged in separate files)

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig.2 Kaplan-Meier plots of association of IGF1R mRNA expression with RFS (**a**) and BCSS (**b**) in entire cohort

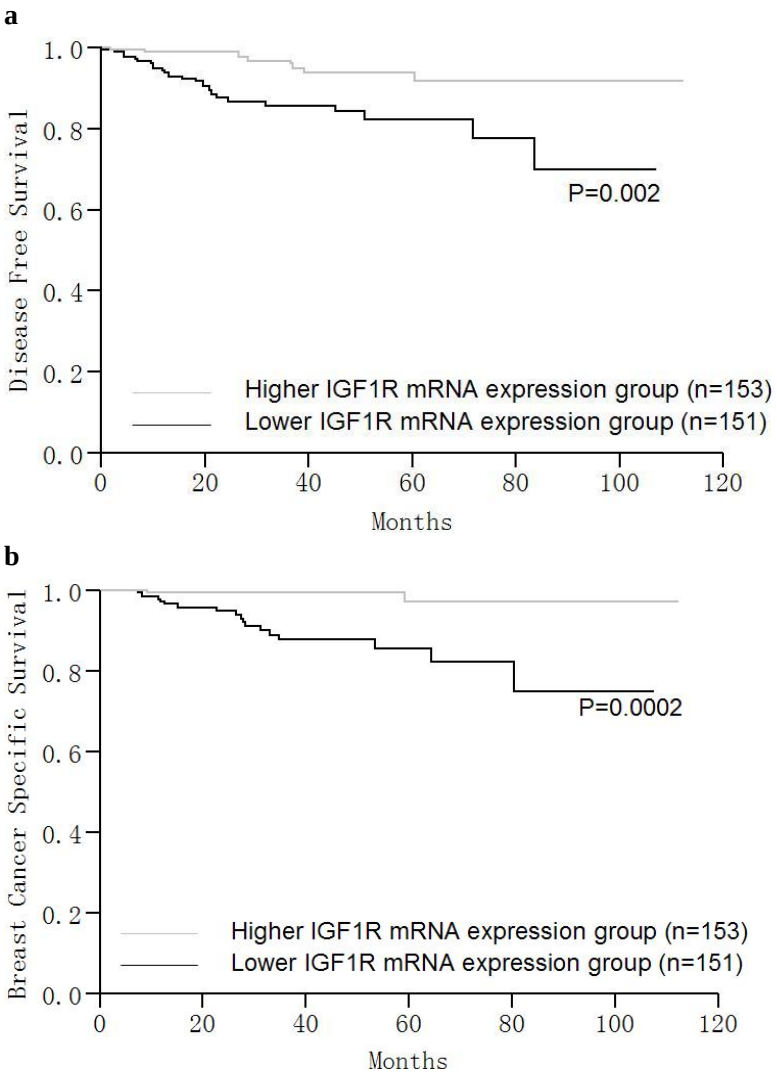
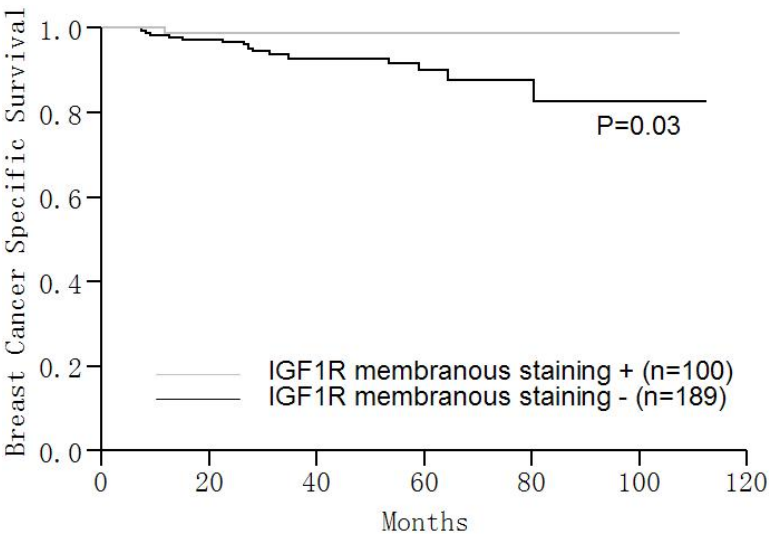


Fig.3 Kaplan-Meier plots of association of IGF1R protein expression with BCSS (**a** membranous expression; **b** cytoplasmic expression) in entire cohort

a



b

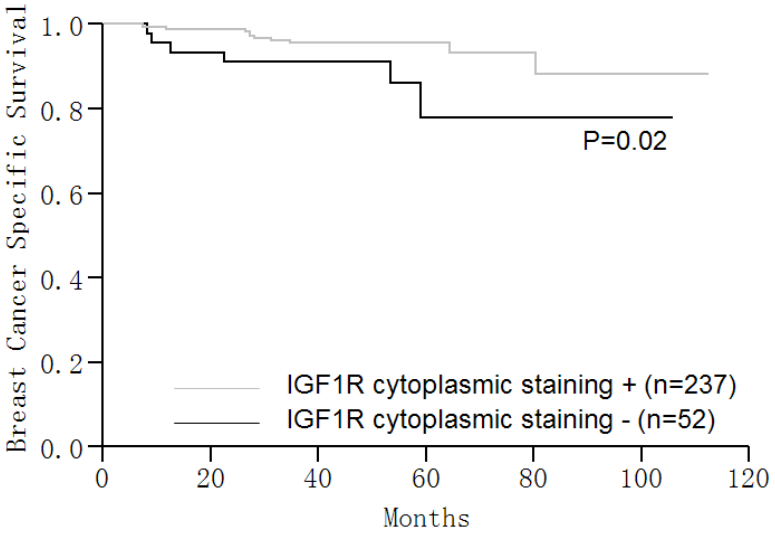


Fig.4 Kaplan-Meier plots of association of IGF1R mRNA expression with BCSS in luminal tumor subtype group

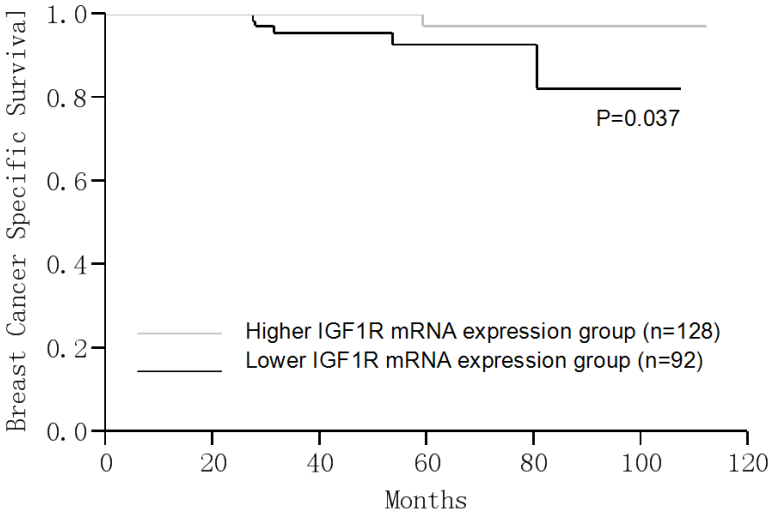


Table 1 Correlation between IGF1R mRNA expression and IGF1R gene copy number and cytoplasmic protein expression in entire cohort and luminal tumor subtype group

	Entire cohort		*Luminal subtype group*	
	r	*p*	*r*	*p*
IGF1R gene copy number vs. IGF1R mRNA expression	0.09	0.10	0.13	0.05
IGF1R gene copy number vs. IGF1R cytoplasmic expression	0.08	0.20	0.05	0.49
IGF1R mRNA expression vs. IGF1R cytoplasmic expression	0.20	0.0006	0.17	0.01

Table 2 Association of IGF1R gene copy number, mRNA expression and protein expression with clinicopathological parameters

Clinical parameters	IGF1R gene copy number			IGF1R mRNA expression			IGF1R protein expression				
	No	Median (25%,75%)	*P*	No.	Median (25%,75%)	*P*	No.	membranous -/+	*P*	cytoplasmic Median (25%,75%)	*P*
Menopause											
Pre-	83	1.71(1.43,1.93)	0.71	81	0.62 (0.33,1.22)	0.23	78	48/30	0.37	96.5(70,162.5)	0.09
Post-	229	1.70(1.41,2.05)		222	0.55 (0.28,1.02)		210	141/69		95(20,150)	
Body mass index											
<25	221	1.70(1.39,2.02)	0.22	213	0.59(0.32,1.06)	0.22	208	135/73	0.70	94(35,150)	0.35
≥25	96	1.72(1.53,2.02)		93	0.49(0.24,0.95)		88	56/32		98(35,167.5)	
Tumor size (mm)											
≤20	158	1.78(1.52,2.09)	0.02	151	0.59(0.34,1.06)	0.57	145	92/53	0.32	95(40,160)	0.07
>20	153	1.65(1.35,1.91)		151	0.55(0.25,0.97)		142	98/44		90(15,130)	
Nodal status											
-	194	1.72(1.47,2.01)	0.30	188	0.64(0.36,1.18)	0.002	176	122/54	0.15	90(25,130)	0.20
+	120	1.66(1.35,2.01)		116	0.47(0.23,0.73)		113	69/44		96(40,160)	
Nuclear grade											
1,2	234	1.72(1.49,2.01)	0.51	226	0.64(0.36,1.14)	<0.0001	217	140/77	0.29	95(40,150)	0.03
3	76	1.68(1.27,2.24)		75	0.35(0.17,0.55)		70	50/20		72(5,118.75)	
ER											
-	73	1.51(1.27,2.03)	0.03	69	0.19(0.12,0.42)	<0.0001	67	56/11	0.0002	71(4.25,100.5)	0.0009
+	244	1.72(1.52,2.02)		237	0.64(0.39,1.16)		229	135/94		98(42,165)	
PgR											
-	107	1.63(1.30,2.10)	0.12	102	0.33(0.14,0.68)	<0.0001	99	75/24	0.004	85.5(5,106.25)	0.001
+	210	1.72(1.52,2.01)		204	0.66(0.242,1.14)		197	116/81		98(44.5,170)	
Her2											
-	261	1.72(1.45,2.05)	0.18	254	0.61(0.34,1.14)	0.0003	241	153/88	0.07	95(35,155)	0.08
+	42	1.55(1.36,1.92)		39	0.25(0.16,0.52)		39	32/7		74(2,110)	
Ki67											
-	92	1.67(1.50,1.91)	0.95	84	0.67(0.37,1.17)	0.03	81	51/30	0.64	98(35,175)	0.14
+	218	1.72(1.36,2.08)		217	0.52(0.26,0.93)		211	139/72		93.5(35,132.5)	
Tumor Subtype†											
Luminal	237	1.73(1.52,2.03)	0.06	231	0.64(0.36,1.17)	<0.0001	223	135/88	0.007	95(40,155)	0.03
Her2	30	1.50(1.26,1.86)		27	0.19(0.13,0.49)		26	22/4		95(2,127.5)	
Triple Negative	35	1.74(1.26,2.30)		34	0.28(0.14,0.42)		32	6/33		72(2.75,100)	

† Tumor subtype was grouped according to combination of ER, PgR and Her2 status. Luminal: [ER(+), any PgR, any Her2], HER2: [ER(-)/PgR(-)/Her2(+)] and Triple Negative: [ER(-)/PgR(-)/Her2(-)].

Table 3 Univariate and multivariate analysis for relapse free survival (Cox proportional regression model)

	Univariate analysis			Multivariate analysis		
variable	HR	95%CI	*p*	HR	95%CI	*p*
Menopausal status	3.67	1.00-23.64	0.05			
BMI	1.09	0.49-2.24	0.83			
Tumor size	3.21	1.50-7.63	0.002*	2.20	0.93-5.86	0.07
Nodal status	3.04	1.49-6.45	0.002*	2.52	1.17-5.79	0.02
Nuclear grade	2.11	1.49-3.04	<0.0001*	1.95	0.85-4.58	0.12
ER	0.15	0.07-0.31	<0.0001*	0.16	0.04-0.58	0.004
PgR	0.29	0.14-0.59	0.0006*	2.13	0.60-7.45	0.25
Her2	1.83	0.72-4.01	0.18			
Ki67	2.27	0.95-6.71	0.07			
IGF1R gene amplification	0.50	0.17-1.20	0.13			
IGF1R gene expression	0.31	0.13-0.66	0.002*	0.54	0.22-1.21	0.14
IGF1R membranous expression	0.47	0.16-1.14	0.09			
IGF1R cytoplasmic expression	0.48	0.22-1.16	0.10			

Table 4 Univariate and multivariate analysis for breast cancer specific survival (Cox proportional regression model)

	Univariate analysis			Multivariate analysis		
variable	HR	95%CI	*p*	HR	95%CI	*p*
Menopausal status	1.49	0.34-10.30	0.61			
BMI	1.25	0.47-3.05	0.64			
Tumor size	4.15	1.52-14.49	0.004*	2.33	0.66-11.00	0.20†
Nodal status	4.03	1.59-11.49	0.003*	2.62	0.91-8.87	0.08†
Nuclear grade	2.46	1.56-4.03	0.0001*	1.40	0.49-4.39	0.54†
ER	0.05	0.01-0.15	<0.0001*	0.06	0.008-0.35	0.001
PgR	0.13	0.04-0.35	<0.0001*	2.38	0.41-10.09	0.32
Her2	1.54	0.44-4.23	0.45			
Ki67	7.71	1.60-138.50	0.006*	2.74	0.49-51.45	0.29
IGF1R gene amplification	0.30	0.05-1.06	0.06			
IGF1R gene expression	0.10	0.02-0.36	<0.0001*	0.26	0.04-0.96	0.04†
IGF1R membranous expression	0.14	0.01-0.71	0.01*	0.36	0.02-2.05	0.29§
IGF1R cytoplasmic expression	0.32	0.12-0.97	0.04*	0.49	0.15-1.74	0.25□

Considering the co-effect of IGF1R gene expression with IGF1R protein expression, each factor was used in the multivariate analysis respectively:

† Cox proportional regression model including IGF1R gene expression and tumor size, nodal status, nuclear grade, ER,PgR,Ki67.

§ Cox proportional regression model including IGF1R membranous expression and tumor size, nodal status, nuclear grade, ER,PgR,Ki67.

□Cox proportional regression model including IGF1R cytoplasmic expression and tumor size, nodal status, nuclear grade, ER,PgR,Ki67.

Table 5 Univariate and multivariate analysis for breast cancer specific survival in luminal subtype (Cox proportional regression model)

	Univariate analysis			Multivariate analysis		
variable	HR	95%CI	*p*	HR	95%CI	*p*
Menopausal status	0.84	0.16-6.06	0.84			
BMI	2.29	0.42-12.39	0.32			
Tumor size	3.22	1.23-8.47	0.001*	3.87	1.10-9.57	0.002†
Nodal status	3.39	0.66-24.48	0.14			
Nuclear grade	3.26	0.44-16.74	0.21			
PgR	0.57	0.10-4.18	0.53			
Her2	4.25	0.22-28.88	0.27			
Ki67	2.01	0.32-38.65	0.49			
IGF1R gene amplification	0.65	0.16-2.24	0.38			
IGF1R gene expression	0.14	0.01-0.86	0.03*	0.15	0.08-0.95	0.04†
IGF1R membranous expression	0.42	0.13-0.98	0.04*	0.26	0.11-1.35	0.06§
IGF1R cytoplasmic expression	0.21	0.03-1.60	0.12			

Considering the co-effect of IGF1R gene expression with IGF1R membranous expression, each factor was used in the multivariate analysis respectively:

† Cox proportional regression model including IGF1R gene expression and tumor size.

§ Cox proportional regression model including IGF1R membranous expression and tumor size.

Table S1 Association of IGF1R gene copy number, mRNA expression and protein expression with clinicopathological parameters in luminal tumor subtype group

Clinical parameters	IGF1R gene copy number			IGF1R mRNA expression			IGF1R protein expression				
	No	Median(25%,75%)	*P*	No.	Median(25%,75%)	*P*	No.	membranous		cytoplasmic	
								-/+	*P*	Median(25%,75%)	*P*
Menopause											
Pre-	63	1.72（1.45,2.01）	0.71	63	0.70(0.43,1.33)	0.35	61	36/25	0.77	98(70,150)	0.26
Post-	168	1.72（1.53，2.06）		164	0.63(0.37,1.16)		157	96/61		95(32.5,160)	
BMI											
<25	161	1.72(1.45,2.04)	0.13	157	0.67(0.43,1.22)	0.25	152	96/56	0.27	92(36.25,150)	0.25
≥25	71	1.76(1.59,2.05)		71	0.59(0.33,1.09)		67	37/30		99(44,180)	
Tumor size (mm)											
≤20	122	1.78（1.54,2.12）	0.09	117	0.65(0.41,1.20)	0.30	115	71/44	0.89	98(50,160)	0.16
>20	108	1.66（1.42,2.00）		109	0.64(0.35,1.07)		102	62/40		90(25,132.5)	
Nodal status											
-	149	1.72（1.53,2.07）	0.43	145	0.79(0.45,1.35)	0.001	138	90/48	0.08	95(35,140)	0.49
+	83	1.70（1.42,2.03）		83	0.54(0.30,0.83)		81	43/38		98(47.5,170)	
Nuclear grade											
1,2	198	1.72（1.50,2.02）	0.34	194	0.67(0.41,1.18)	0.12	188	119/69	0.09	95(40,150)	0.96
3	31	1.77（1.54,2.58）		32	0.44(0.24,1.24)		30	14/16		86(35,192.5)	
PgR											
-	38	1.73（1.50,2.21）	0.64	37	0.54(0.24,1.18)	0.20	36	22/14	0.96	85(20,110)	0.16
+	194	1.72(1.51,2.03)		191	0.64(0.42,1.17)		183	111/72		98(40,160)	
Her2											
-	218	1.73(1.50,2.05)	0.74	214	0.65(0.41,1.19)	0.02	204	122/82	0.22	96.5(33.75,160)	0.04
+	12	1.65(1.54,2.09)		12	0.33(0.22,0.80)		13	10/3		70(2,100)	
Ki67											
-	76	1.71(1.53,1.97)	0.68	72	0.70(0.40,1.20)	0.42	70	45/25	0.44	98(33.75,160)	0.82
+	155	1.76(1.48,2.12)		155	0.64(0.36,1.17)		148	87/61		95(41,150)	