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**Paper Accepted*** **ISSN Online 2406-0895**

**Original Article / Оригинални рад**

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# Immunohistochemical Evaluation of Insulin-like Growth Factor Receptor 1 in Breast Cancer

Процена имунохистохемијске експресије рецептора инсулину-сличног фактора раста 1 у карциному дојке

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**Received: July 7, 2017**
**Accepted: September 19, 2017**
**Online First: September 29, 2017**
**DOI: https://doi.org/10.2298/SARH170707177B**



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## Процена имунохистохемијске експресије рецептора инсулину-сличног фактора раста 1 у карциному дојке


**SUMMARY**

**Introduction/Objective** Activation of insulin-like growth factor receptor (IGF-1R) results in transition cell from growth phase (G1) to synthesis phase (S) of cells cycle. Breast cancer is categorised into prognostic and therapeutic subtypes based upon hormone receptor, estrogen receptor (ER) and progesterone receptor (PR) expression and human epidermal growth factor receptor (HER-2) expression. The objective of this study was to examine the expression of IGF-1R in specific subtype invasive breast cancer and its correlation with basic histopathological and immunohistochemical prognostic parameters.

**Methods** Formalin-fixed paraffin-embedded tumour samples were obtained from 129 female patients with invasive breast cancer (I- III stage disease) with follow-up ranging from 48 (36–108) months. For immunohistochemical staining, we used monoclonal antibodies for ER, PR, IGF-1R and polyclonal atibody HER-2.

**Results** IGF-1R inversely correlated with tumour stage (p=0.017), tumour grade (p=0.001), HER-2 (p=0.003), whereas significant positive correlation was found with multifocality/multicentricity of breast cancer (p=0.036), expression ER (p=0.001) and PR (p=0.0001). Cox-regression analysiss for relapse-free survival (RFS) showed that stage disease (p=0.039) and HER-2 (p=0.033) were independent prognostic factors. IGF-1R did not predict clinical outcome in patients with breast cancer (p= 0.488, Kaplan-Meier test for RFS).

**Conclusion** Patients with low stage and grade, hormone-dependent breast cancer had a significantly higher IGF-1R expression than patients with triple negative or HER-2 overexpressed cancer. The present findings also highlight expression IGF-1R in multicentric /multifocal breast cancer supports the key roles in tumor initiation.


**Keywords:** Insulin-like growth factor 1 receptor (IGF-1R); hormone-dependent breast cancer; HER-2


**САЖЕТАК**

**Увод/Циљ** Активација рецептора инсулину-сличног фактора раста 1 (*IGF-1R*) изазива покретање ћелијског циклуса из фазе раста (G1) у фазу синтезе (S). Оболели од карцинома дојке се деле на специфичне терапијске и прогностичке групе у зависности од експресије хормонских рецептора, естрогених (*ER*) и прогестеронских (*PR*), и експресије рецептора хуманог епидермалног фактора раста 2 (*HER-2*). Циљ рада је откривање степена експресије *IGF-1R* у туморском ткиву код одређених терапијских група оболелих од карцинома дојке и његова корелација са важећим патохистолошким и имунохистохемијским прогностичким параметрима.

**Методе** Истраживање је спроведено на 129 укалупљених узорака инвазивног карцинома дојке код жена (у стадијуму болести I–III) уз постоперативно праћење тока болести 48 (36–108) месеци. За имунохистохемијско бојење коришћена су моноклонска антитела за визализацију: ER, PR, IGF-1R) и поликлонско антитело за HER-2.

**Резултати** Експресија *IGF-1R* је била у негативној корелацији са стадијумом болести ($p$=0,017), степеном диферентованости тумора ($p$=0,001) и експресијом *HER-2* ($p$=0,003). Позитивна корелација овог рецептора налазила се између мултифокалног /мултицентричног макроскопског начина раста карцинома дојке ($p$=0,036) и експресије ER (p=0,001) и PR ($p$=0.0001). *Cox*-ова регресиона анализа времена без прогресије болести (*RFS*) показала је да стадијум болести (p=0,039) и *HER-2* ($p$=0,033) представљају независне прогностичке варијабле. Експресија *IGF-1R* није имала утицај на клинички ток болести код особа са раком дојке ($p$= 0,488, *Kaplan-Meier* test за *RFS*).

**Закључак** Пацијенти оперисани у почетном стадијуму са добро-диферентованим, хормон-зависним раком дојке имају већу *IGF-1R* експресију у односу на пацијенте са троструко негативним и *HER-2* амплификованим туморима дојке. Повећана *IGF-1R* експресија код карцинома са мултифокалним / мултицентричним макроскопским начином раста указује на значајну улогу овог рецептора у фази настанка тумора.


**Кључне речи:** рецептор инсулину-сличном фактору раста 1 (IGF-1R); хормон-зависни рак дојке; HER-2



 

## INTRODUCTION

Insulin receptor family represents an activator of tyrosine kinase second classes with three members**:** insulin receptor (IR), insulin-like growth factor receptor 1 (IGF-1R) and insulin-like growth factor receptor 2 (IGF-2R). IR activation in a vertebrate influence on the metabolic activity. IGF-1R activating results in proliferation and differentiation of the cells. IGF-2R is structurally and functionally different from the IR and IGF-1R, it is a monomer without tyrosine kinase activity. IGF-1R is a dimmer made of α and β subunits and has the same structure as the IR with which build hybrid receptors (IR/IGF-1R) [1-3]. Insulin receptors can be activated by insulin and two insulin like growth factors (IGFs): insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2). Many cells have been identified as producing as well as responding to the IGFs, including fibroblasts, chondrocytes, osteoblasts, granulosa cells, and epithelial breast cells. In circulation IGF1 and IGF2 are attached to 6 insulin-growth binding proteins (IGFBP 1-6) and protected from the action of proteases (Figure 1). IGF-1R together with the hormone receptors regulates the development of the epithelium of the normal glandular breast tissue [4,5]. Breast cancers are categorised into subtypes based on immunohisto-chemical hormone recep-tors expression (ER and PR) and human epidermal growth factor 2 (HER-2) expression. There are two major groups: *hormone-dependent/luminal breast* cancer involves luminal A (ER +, PR+, HER-2/-, Ki67low) and luminal B (ER+, PR+/-, HER-2 +/-, Ki67high), and *hormone-independent/basal-like* breast cancer involves triple negative (TNBC) breast cancer (ER−, PR−, HER2−) and HER-2 overexpressed (ER-, PR-, HER-2+). Among others, the TNBC subtype does not express therapeutically targetable ER, PR, or HER-2 receptors making aggressive subtype difficult to treat [6]. Nowadays, IGF-1R makes an attractive target for investigation for a different type of malignancy and anticancer therapy. The prognostic and predictive role of IGF-1R in breast cancer is still unknown. The optimal cut-point and standardised immunohistochemical expression of this receptor are subject of discussion [7]. The few studies have

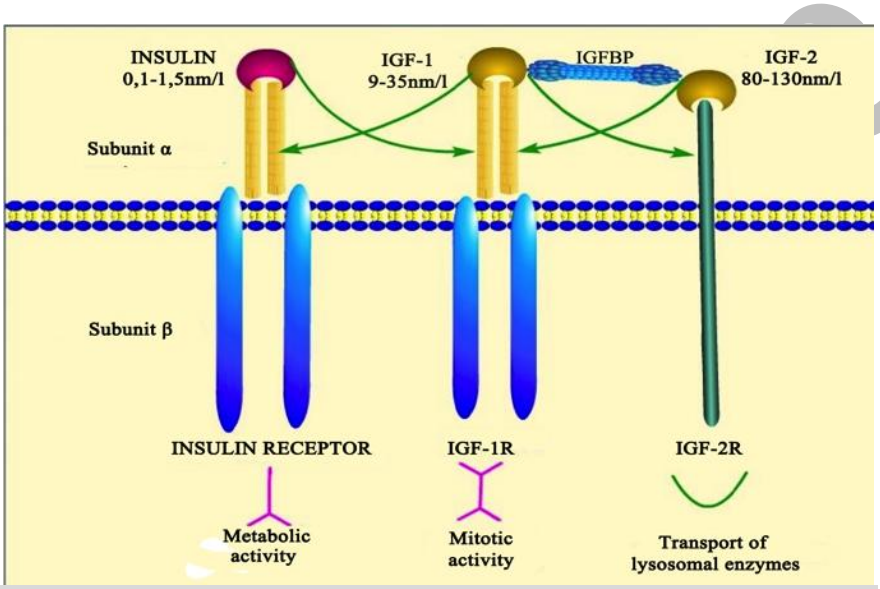


**Figure 1. The structure of the insulins receptors and concentrations IGFs in the blood.**
Insulins growth factors (IGFs): insulin, insulin-like growth factor 1 (IGF-1) and insulin-like growth factor 2 (IGF-2); Insulin-like growth factor-binding protein (IGPB); Insulin receptor (IR); Insulin-like growth factor 1 receptor (IGF-1R); Insulin-like growth factor 2 receptor (IGF-2R); extracellular subunit of IGF-1R and IR (subunit α); intracellular subunit of IGF-1R and IR (subunit β).



 

examined the relationship of the IGF-1R expression according to the hormone and epidermal growth factor receptors 2 (HER-2) and resistance to antiestrogen therapy [8,9]. Some in vitro studies have given promising results support the rationale for dual targeting HER-2 or/and IGF-1R as an improved treatment regimen for advanced therapy tailored to difference types of cancer [10].

## METHODS

### Patient selection

The biopsy specimens for 129 invasive breast cancer in stage I-III diagnose in Department of Pathology University hospital Foča (Republic of Srpska) from January 2008 to January 2013 were made from the study. We retrospectively analysed the Clinical Centre medical data collected from the Department of Surgery, Department of Oncology and records of family doctors. The prospective follow-up was 48 months (range 36-108) with last data obtained in November 2016. Subjects did not receive preoperative chemo-/radio- or hormone therapy. Minimum distance resection margin of invasive cancer or *in situ* component was 3 mm. Postoperative therapy for individual subtypes of breast cancer was determined following *St Gallen* consensus from 2008 [11]. The stage of breast cancer was determined following AJCC classification from 2010. Histologic grade of the tumour is determined by *Elston-Ellis* modification *Scarff-Bloom-Richardson* grading systems [12].

### Immunohistochemical Staining Methods

Formalin-fixed, paraffin-embedded tissue samples were cut at 3-5 μm. Following standard procedures; drying (30 minutes in the air), "baking" (60 minutes at 65°C) in a thermostat, dewaxing in xylene (two changes of 5 minutes), the drop-down rehydration concentrations of ethyl alcohol (100%, 96%, 70%, by 5 minutes for each change) and rinse in distilled water. Endogenous peroxidase activity was blocked by 3% $H_2O_2$ (10 min at ambient temperature), and the unmasking of antigens derived by heat treatment of tissue in the microwave oven. Sections were incubated with primary antibodies: *Mouse monoclonal anti -IGF 1R (clone 24-31 ab4065, Abcam, dilution 1:50); Mouse monoclonal anti-ERα clone 1D5 (M7047, DAKO Corporation, USA, dilution 1:60); Mouse monoclonal anti-PR clone 636 (M3569, DAKO Corporation, USA, dilution 1:100); and Polyclonal rabbit anti-HER-2 clone 340 (A0485, DAKO Corporation, USA, dilution 1:60).* After washing, primary antibodies were treated with streptavidin peroxidase for 15 minutes. Chromogen DAB was added in the final step procedures to visualise a positive. During a short incubation period (± 51 minutes), a pre-formed complex was able to develop a brown colour in the interaction with the DAB chromogen. Following immunohistochemical staining (IHC), of tissue sample, specimens were stained with Mayer□s hematoxylin, dehydrated through a series of ethyl alcohols up to absolute alcohol (70%, 90% and 100%), washed in xylene and mounted in Biomont. The IGF-1R protein was located to the plasma membrane (α subunit) and the cytoplasm (β subunit). Placental tissue was utilised as an adequate external control. Stainability was estimated semiquantitatively based on Allred scoring system.



 

Summarizing of the percentage positive tumour cells (<1% – 1; 1– 10% – 2; 11-33% – 3; 33-66% – 4; 67-100% – 5) and staining intensity (1 = weakly staining can easily be observed at high-power field; 2 = moderate staining can easily be seen under moderate power objective magnification; and 3 = strong staining can easily be observed under low power objective magnification), expression was scored: negative (0-2), low 1 + (3-4), moderate 2+ (5-6) and strong 3+ (7-8). Score 0 and 1 were considered as negative and score 2 and 3 as positive finding. The same method was applied to scoring ER and PR. Hormone receptor positivity is defined as Allred score >2 [13,14]. For the evaluation of HER-2, only staining of the tumour cell membranes was considered to be specific. Positive cases were defined as IHC 3+ and IHC 2+ FISH retested with amplification ratio C > 2.0 [15].

**Table 1. Clinical, histopathological and immunohistochemical data of 129 patients with breast cancer.**

| Variable | Number of patients (%) |
|---|---|
| median age (range) | 59 (33–84) |
| **Menopausal status** | |
| yes | 105 (81.4) |
| no | 24 (18.6) |
| **Tumor stage** | |
| I | 10 (7.8) |
| II | 56 (43.4) |
| III | 63 (48.8) |
| **Tumour type** | |
| ductal | 71 (55) |
| lobular | 32 (24.8) |
| other | 26 (20.2) |
| **Tumour size** | |
| <2cm | 16 (12.4) |
| 2–5cm | 75 (58.1) |
| >5cm and inflammatory carcinoma | 38 (29.5) |
| **Lymph node metastasis** | |
| node negative | 40 (31) |
| 1–3 node positive | 37 (28.7) |
| 4–9 | 32 (24.8) |
| >10 | 20 (15.5) |
| **Postoperative therapy:** | |
| Tamoxifen | 97 (75) |
| Chemotherapy | 89 (69) |
| Chemotherapy+Herceptin | 33 (25,6) |
| Radiotherapy | 99 (76,8) |
| **Estrogen receptor:** | |
| 0 | 32 (24.8) |
| 1 | 13 (10.1) |
| 2 | 16 (12.4) |
| 3 | 68 (52.7) |
| **Progesterone receptor:** | |
| 0 | 53 (41) |
| 1 | 13 (10) |
| 2 | 22 (17.2) |
| 3 | 41 (31.8) |
| **HER-2** | |
| Negative case* | 96 (74.4) |
| Positive case ** | 33 (25.6) |

* Immunohistochemical expression 0, 1 and 2 with FISH retested negative
** Immunohistochemical expression 3 and 2 with FISH retested positive (amplification ratio C>2.0).

### Statistical analysis

The association between the intensity of expression with tumour grade, lymph node status, and tumour size was studied with linear correlation method based on the Pearson correlation coefficient (r). For relapse-free survival (RFS) we used *Kaplan-Meier* test, for multivariate analysis Cox proportional hazard regression model. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was established at the $p<0.05$ level.

## RESULTS

Characteristics (clinical and histopathological data) of 129 patients with breast cancer are shown in table 1. 117 patients (90.7%) were alive without evidenced progression of the disease; 12 patients (9.3%) had a relapse of the disease. Bone metastases were registered in 5 (41.7%) patients, locoregional recurrence in 2 (16.7%) and one patient (8.3%) had a metastasis in lungs, liver, brain, remote lymph node and metastasis in two organic systems.



 

**IGF-1R Expression**

Forty-seven of the 129 samples (37.2%) of breast cancer showed no or weak staining (score 0 and 1+), 41 (31.8%) moderate (score 2+) and 42 (32.6%) strong immunohistochemical expression (score 3+) (Figure 2). Neither IGF-1R, ER nor PR were significant predictors of RFS (p=0.48, p=0.26,

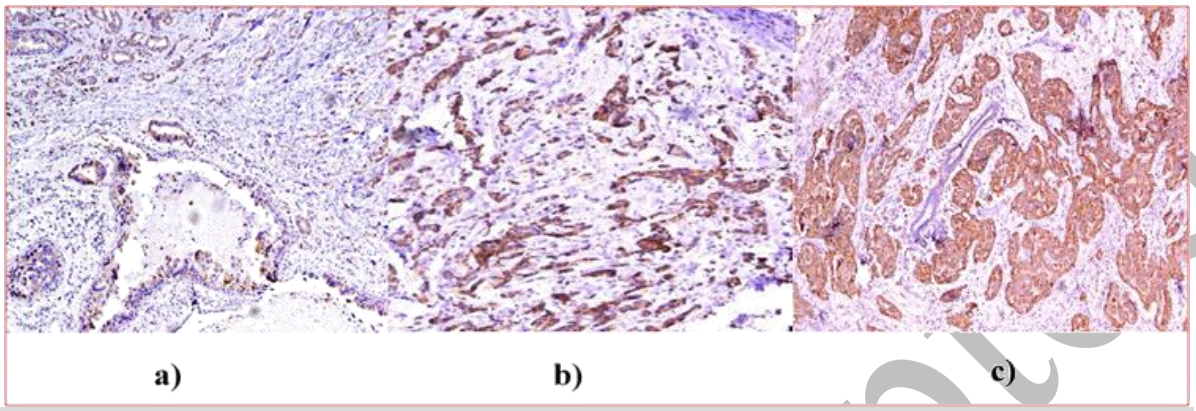


**Figure 2. Immunohistochemical expression of IGF-1R in breast cancer (formalin-fixed paraffin-emdedded sections, x40 magnification). Expression was scored according to area and intensity of membranous or citoplasmatic staining: a) score 1+ b) score 2+ c) score 3+).**

p=0.28, respectively; Kaplan-Meier test). We confirmed the prognostic value of tumour stage, lymph node metastasis, and HER-2 expression (Figure 3). Stage disease and HER-2 expression were

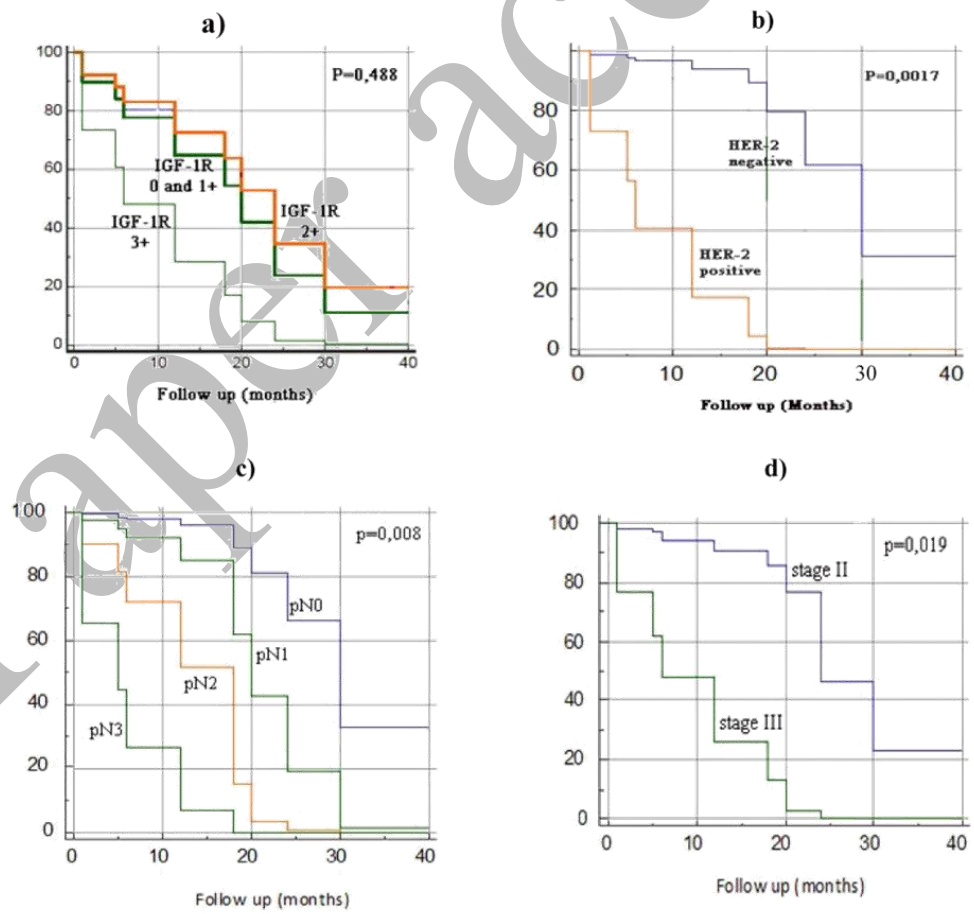


**Figure 3. Relapse free survival analysis *(Kaplan-Meier test*) progniostic value of a) IGF-1R, b) HER-2, c) lymph node metastases and d) stage disease.**

 

prognostic significance on relapse-free survival (RFS) in the final Cox proportional hazard multivariate analysis (Table 2).

**Table 2. Multivariate Cox proportional hazards regression analysis for RFS in breast cancer patients.**

| Variable | B | SE | HR | *p*-value | 95%CI |
|---|---|---|---|---|---|
| Stage disease | 4.8068 | 2.3302 | 122.344 | 0.0391 | 1.3008–11506.4 |
| Lymph node stage (pN) | -0.1966 | 0.3923 | 0.8216 | 0.16164 | 0.3823–1.765 |
| HER-2 | 1.3284 | 0.6259 | 3.7748 | 0.00338 | 1.1140–12.7915 |
| IGF-1R | 0.2511 | 0.2930 | 1.2854 | 0.3914 | 0.7260–2.2758 |

**B** – beta coefficient, **HR** – hazard ratio.

### Correlation among expression of IGF-1R and ER, PR and HER-2

IGF-1R was positively associated with ER (p=0.001), PR (p=0.001) and multifocality /multicentricity of breast cancer (p=0.039). Inverse correlation existed between IGF-1R and stage disease (p=0.017), tumor grade (p=0.0001) and HER-2 (p=0.003) expression. Other parameters did not show statistically significant correlation with IGF-1R (Table 3).

**Table 3. Correlation IGF-1R expression and prognostic parametars in breast cancer.**

| Number of cases (n=129) | 95% CI | r | *p*-value |
|---|---|---|---|
| Stage disease | -0.3671 – -0.0372 | -0,2081 | 0,0175 |
| Tumour size | 0,1782 – 0,1661 | -0,006221 | 0,9440 |
| Lymph node stage (pN) | -0.319 – 0.162 | -0,1564 | 0.075 |
| Tumor grade | -0.492 – -0.189 | -0,3501 | 0.0001 |
| Lymphatic invasion (L1) | -0.249 – 0.092 | -0,0812 | 0.3584 |
| Venous invasion (V1) | -0.127 – 0.216 | 0,04615 | 0.602 |
| Menopausal status | -0.211 – 0.132 | -0,04105 | 0.642 |
| Multifocal/multicentric cancer growth | 0.011 – 0.344 | 0,1832 | 0.036 |
| Age | -0.166 – 0.178 | 0,006337 | 0.943 |
| ER | 0.397 – 0.645 | 0,5328 | 0.0001 |
| PR | 0.331 – 0.598 | 0,4754 | 0.0001 |
| HER-2 | -0.410 – -0.088 | -0,2567 | 0.003 |

**r** – Pearson correlation coefficient.

## DISCUSSION

Up to now, the prognostic value of the IGF-1R expression on disease outcome has been controversial, with studies reporting both positive and negative findings [16,17,18]. In our study, IGF-1R expression did not independently predict on relapse-free survival and clinical outcome. Conflicting results may arise from discordant methodological approaches; distinct molecular subtypes studied, genetic differences between different populations and tumour heterogeneity. Our study demonstrated high expression (2+ or 3+ score) of IGF-1R in 64.4% of the samples. This is in line with some other studies [19]. Up to 50% of breast tumours express the activated form of type 1 Insulin-like growth factor receptor. In our study, IGF-1R was predominantly expressed in well-differentiated and hormone-dependent breast cancers. Insulin-like growth factor 1 receptor with estrogen receptor are critical for mammary gland development. Estrogen receptor and the IGF pathway show dynamic and



 

intricate interference, resulting in bidirectional regulation of expression and activity. ER transcriptionally upregulates IGF-1R expression. Positive correlation between cyclin D1 and ER expression which has already been explained in both experimental and clinical studies, because ER acts as the main mitogen stimulator in breast cancer [20]. The role of insulin-like growth factor receptor type 1 in mammary stem cell maintenance and a necessity for lineage differentiation suggest that aberrantly expressed IGF-1R may be capable of enhancing cell potential and changing cell fate in a tumour, perhaps even in tumours composed of fully differentiated cells. As discussed above, the IGF1R expression is essential for driving luminal alveolar differentiation, linking IGF-1R to the luminal lineage [21,22]. Furthermore, many studies indicate a down-regulation of IGF-1R upon cancer progression, whereas others report elevated levels in metastatic stages. Once cancer has been confirmed, the importance of IGF-1R for disease progression remains unclear. Whereas in our study IGF-1R was highly expressed in patients with early breast cancer and overall positively associated with good prognostic variables. We have indicated the decrease of IGF-1R expression with disease progression. High-level IGF-1R expression had low stage breast cancer with multiple/multicentric unilateraly or bilateraly growth. We emphasised that IGF-1R could have effects in early phases of development of luminal breast cancer. Numerous *in vitro* studies demonstrate IGF-1R as a driver of self-renewal, stem cell surface markers, migration, and invasion in both normal and cancerous tissues and tumour initiation in hepatic, lung, prostate and breast cancers [23]. Approximately 40 to 60% of ER-positive tumours express IGF-1R, while expression in ER-negative tumours is only 10 to 20%. Taken together with the correlation of IGF-1R with hormone-dependent tumor tipe and early stage, we assume that ER/ IGF-1R axis might represent a distinct proliferative pathway during breast cancer development. Other studies report that IGF-1R is receptor expressed in basaloid type breast cancer and has a role to anti-HER-2 resistance (Herceptin) [24]. We found the negative correlation between IGF-1R overexpressed and HER-2 positive breast cancer. In general, IGF-1R correlates with good prognostic markers, such as ER and PR- positivity and human epidermal growth factor receptor 2 (HER-2)-negativity. However, the IGF-1R expression has differential effects in different breast cancer subtypes. For example, its expression has been shown to be positively correlated with improved breast cancer-specific survival among patients with ER-positive tumours, while its expression was associated with an inferior prognosis in patients with HER2-overexpressing or triple negative tumours. In models of breast cancer cells that overexpress HER-2, anti-HER-2 activity is disrupted by increased expression of IGF-1R. Nowadays, antibody-based molecular therapies have been developed for HER-2. The IGF-1R can form heterodimers with the HER-2 tyrosine kinase and contribute to the development of resistance to HER-2 inhibition with the monoclonal antibody. An association between IGF-1R and HER-2 in IGF-1R-dependent tumour transformation has been reported in mammary luminal epithelial cells, indicating that the IGF-1/HER2 cross-talk may occur via autocrine and paracrine signalling. Recent study concluded that neoadjuvant therapy can induce changes in IGF-1R expression. Therefore, there are many studies with opposite results [25, 26]. It is possible that IGF-1R



 

expression is dependent not only on the specific cell type and stage disease, but also it is dependent on specific therapy and another factor. In some other tumors, like lung cancer, expression of IGF-1R correlated with a worse outcome [27]. This indicates that IGF-1R activities might be not only diverse but also tissue-specific. To test this hypothesis, we evaluated the protein expression of the most important components of the IGF-1R signalling pathway in hormone- dependent breast cancer and their significance according to the tumour subtypes. This clearly indicates other functions of IGF-1R which are not related to cell cycle progression and tumor aggressiveness, which may include cell differentiation and growth arrest.

## CONCLUSION

IGF-1R is particularly important for the establishment and maintenance of the transformed phenotype and for the survival of tumour cells with anchorage-independent growth in breast carcinoma with luminal differentiation.

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