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## Low activation of Insulin-like Growth Factor 1-Receptor (IGF1R) is associated with local recurrence in early breast carcinoma

Gloria Peiró · Susana Benlloch · Laura Sánchez-Tejada · Encarna Adrover · Enrique Lerma · Francisca M. Peiró · José Sánchez-Payá · F. Ignacio Aranda

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**Abstract** *Background* The predictive value of IGF1R on local recurrence in invasive breast carcinoma (BC) is not well known. *Methods* In a series of 197 lymph-node negative BC patients treated with breast-conserving surgery and radiation therapy, we performed immunohistochemistry for  $\alpha$ -IGF1R,  $\beta$ -IGF1R (phosphorylated/active form) and Estrogen/Progesterone receptors. We further evaluated the IGF1R mRNA expression by quantitative RT-PCR and *IGF1R* mutations by direct DNA sequencing (exons 19 and 21) in 85 primary BC (42 control cases, 31 with local recurrence and 12 with distant metastasis) and in 31 local recurrences. Unconditional logistic regression analyses were performed to identify risk factors for recurrence. *Results* Local recurrences were associated with high-grade tumors, PR-negative and low active-IGF1R, which emerged as independent breast relapse predictors by multivariate analysis. *Conclusion* Patients with early BC

treated with lumpectomy and radiation who have low-grade tumors and favorable markers (increased content of active IGF1R and PR-positive) have a low risk of local recurrence. Therefore, do not benefit from a boost dose on the surgical scar.

**Keywords** Early breast carcinoma · IGF1R · mRNA · Mutation

### Introduction

Breast conservation has become standard treatment for patients with BC in early stage (I–II), although local recurrence (LR) occurs in 6–20% of patients, mainly in those without complementary radiation therapy (RT) [1–3]. Among the clinical-pathological factors determinants of risk for LR take into account the young age (<35 years) of the patients, pre-menopausal status, high histological grade, lymph-vascular invasion (LVI), multicentricity, presence of an extensive in situ component (EIC), margins status and the use of adjuvant therapy with hormone blockade and/or RT [4–6]. About 20–40% of LRs have been associated with poorer outcome [3]. Therefore, the early identification of those patients at a high risk is important for an adequate treatment.

Currently, in BC, there has been growing interest in identifying genetic factors that may influence the natural history and the response to treatment. Among them, the IGF1R pathway has been implicated in the initiation and progression of this neoplasia [7]. IGF1R is a glycosylated heterotetramer composed of two extracellular  $\alpha$ -subunits and two transmembrane  $\beta$ -subunits containing tyrosine kinase (TK) domains involved in signal transduction. The intracellular region of the  $\beta$ -subunit has a binding site for

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phosphorylation substrates at tyrosine residue 950, an ATP-binding at lysine 1003, a TK domain with three critical tyrosines (positions 1131, 1135 and 1136); and several tyrosines in the C-terminal domain (positions 1250, 1251 and 1316) [8]. Binding of the ligand to the  $\alpha$ -subunit leads to autophosphorylation/stimulation of the  $\beta$ -subunit intrinsic TK activity, which in turn induces activation of phosphoinositide 3'-kinase (PI3K)/Akt pathway, blocking apoptosis and activating cellular proliferation [9]. Experimental models have revealed several receptor-dependent functions: DNA synthesis (TK domain), apoptosis and metastasis (TK and C-terminal domains), migration/invasion (TK enhanced through Tyr1250/1251) [10–13] and radioresistance mediation (Tyr1251) [14].

Elevated IGF1R protein levels have been documented in various human malignancies [15, 16]. Overall, IGF1R overexpression occurs between 40% and 82% of BC [17–23]. Except for the well established correlation with ER status, its association with other prognostic parameters is still insufficiently documented [24–27]. On the other hand, the importance of IGF1R with respect to tumor control and response to therapy [14, 28] or survival [20, 21] is unclear. Currently, in lymph-node negative (LNN) BC patients, there are limited data evaluating the IGF1R protein receptor [17, 29, 30] and mutational status [14] and no previous studies have analyzed the relationship of IGF1R activation and mRNA levels with the risk of development of LR in patients with BC. So far, no data is available regarding a comprehensive analysis of IGF1R in primary breast tumors and their corresponding LR.

The present study was designed to examine the correlation of IGF1R protein expression and activation, IGF1R mRNA levels, and the presence of *IGF1R* mutations with the risk of LR in a series of primary LNNBC treated with breast-conserving surgery (CS) and RT. IGF1R results in primary tumors that locally recurred were compared with those in their corresponding LR specimen. All the data are presented following the recommendations for *tumor marker prognostic studies (REMARK)* [31].

## Materials and methods

Archival cases of LNNBC in stage I–II from 197 patients treated with CS and RT were retrieved from the archives of the Department of Pathology at the University General Hospital of Alicante, from the period of January 1990 to December 1999. All had undergone a complete gross excision of the primary tumor and axillary lymph nodes dissection, with a minimum of five nodes removed.

Post-lumpectomy RT was mandated and a total dose of 50 Gy to the tumor bed was given. Patients with ER and/or PR-positive tumors received tamoxifen for 5 years.

Furthermore, patients with high risk factors (young age, high grade or ER/PR-negative tumors) were treated with systemic chemotherapy (CT) with six cycles of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) or four cycles of doxorubicin plus cyclophosphamide (AC) after surgery. None received taxanes, trastuzumab (Herceptin<sup>TM</sup>) or aromatase inhibitors.

LR was defined as any recurrent infiltrating tumor at or near the primary site of the ipsilateral breast (parenchyma or skin).

Pathologic review was performed in all cases by two of the pathologists (G. Peiró, E. Lerma and F. I. Aranda) without knowledge of the outcome. Tumor size was determined by either gross or microscopic examination, histologic classification according to the World Health Organization (WHO) criteria, and histologic grade based on the Elston method [32]. Additional histologic features recorded were: presence of LVI, necrosis ( $\geq 5$  necrotic cells), and intraductal component (absent,  $< 25\%$  or  $\geq 25\%$ ). Pathological margins were classified as negative (inked margin  $> 5$  mm from the invasive component), close (0.1–5 mm), positive (at the inked margin or  $< 0.1$  mm) and indeterminate (biopsy not inked or non-valuable). The most representative area of the infiltrating tumor was selected for the tissue microarray (TMA) construction (Beecher Instruments; Silver Spring, MD) with 0.6 mm cores.

## Immunohistochemistry

TMA sections 2–4  $\mu$ m thick were mounted on poly-L-lysine-coated slides (DakoREAL<sup>TM</sup>, Glostrup, Denmark), deparaffinized and subjected to heat-induce epitope retrieval in 0.1 M sodium citrate buffer (pH 9).

IGF1R protein status was analyzed by using a mouse polyclonal IGF1R  $\alpha$ -subunit (1:200 for 1 h at RT; Neo-markers) and the phosphorylated (active) form of IGF1R  $\beta$ -subunit (p-IGF1R) (phospho-IGF1R (Tyr1131)/Insulin receptor (Tyr1146); rabbit polyclonal Ab; Cell Signaling; 1:80; overnight at 4°C). IHC assays were carried out in serial sections using the EnVision<sup>TM</sup> detection system peroxidase/DAB+, Rabbit/Mouse by using a TechMate 500-staining automat (Dako, Glostrup, Denmark). The staining (membranous and/or cytoplasmic) was semi-quantitatively scored according to the percentage of positive tumor cells and intensity (from 0 to 3+) (score 0–300). For ER/PR, antibody characteristics, methodology and scores have been previously described [33].

## Molecular analyses

We studied 116 tumor samples including 31 LR and 85 primaries BC (42 without recurrence—control cases matched for histologic grade—31 with LR and 12 with DM).

From 2 to 3 paraffin-embedded cylinders (0.6 mm-thick) from pre-selected tumor areas, we extracted genomic DNA with QIAmp DNA miniKit, and isolated total RNA by handling RNeasy FFPE Kit (QIAGEN, Hilden, Germany).

PCR amplification was performed in 25- $\mu$ l-reaction volume containing 1.25 mM  $MgCl_2$ , 0.2  $\mu$ M of each primer, 0.04 U/ $\mu$ l HotStar Taq polymerase (QIAGEN) and 3  $\mu$ l of extracted DNA. PCR reactions were amplified for 35 cycles with an annealing temperature of 58°C. The primer sets were obtained from RefSeq DNA NM000875.2, designed with Primer3 (TIB MOLBIOL Syntheselabor, Berlin). They were as follows: (a) TK domain IGF1R (exon 19): *IGF1R* Fw 5'-TGTAGGGTCCTCTGCTGTGA-3' *IGF1R* Rv 5'-ACCTTTA CTGCATTGCCAGC-3'. (b) C-terminal domain IGF1R (exon 21): *IGF1R* Fw 5'-GGCTGCAGGTTTGAAGTAT-3' *IGF1R* Rv 5'-CCAGAGAACATGGAGAGCGT-3'. The PCR products were purified by EXOSAP (Amersham Life Science Inc.). PCR amplicons were double strand sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and ABI Prism 310 automated sequencer. RT-PCR was performed in 20  $\mu$ l reaction volumes with random hexamer priming, 10  $\mu$ l of extracted RNA and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Santa Clara, CA). cDNA was diluted to 1:5 volume and 2.5  $\mu$ l of cDNA was amplified in a 12.5- $\mu$ l reaction volume for 40 cycles of RT-PCR using Taqman Universal PCR Master Mix and TaqMan® Gene Expression Assays of IGF1R hCG1640727,  $\beta$ -glucuronidase (GUSB) hCG18478 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hCG2005673 in the ABI Prism 7500 System (Applied Biosystems) in triplicate. RT minus controls and two commercial positive controls (total human and BC RNA) were included.

IGF1R quantification was normalized to GUSB and GAPDH genes, which are known to be relatively invariant. Since both were highly correlated (Spearman rank correlation coefficient:  $r = 0.82$ ;  $P < 0.000$ ), all the results were normalized to GUSB. Relative mRNA expression levels were carried out using the  $\Delta\Delta C_T$  method. The calibrator was a pool of 10 healthy breast tissue samples (mean  $\Delta\Delta C_T = 1.293$ ; SD  $\pm 0.298$ ).

### Statistical analyses

The chi-square or Fisher's tests were used, as appropriate, to determine the statistical significance of the qualitative results. Non-parametric Spearman's correlation test was used to determine the relationship between IGF1R protein and mRNA, and Mann-Whitney or Kruskal–Wallis test for the expressions' differences between pathologic features.

Multivariate analysis (unconditional logistic-regression model) was performed to determine the independent effect

of the prognostic variables by comparing the group of patient with no recurrence with the group with LR. A variable's effect on LR was estimated by the model's odds ratio (OR), and a 95% confidence interval (95%CI) was calculated for each OR.  $P$  values  $< 0.05$  were considered statistically significant.

### Results

Among our series of 197 LNNBC patients, 33 (16.8%) had LR and 16 (8.1%) DM. Patients' ages ranged from 23 to 88 years (average 52 years). Median follow-up was 100 months (range 16–234 months). The median time to LR was 50 months (range 7–201 months) and to metastasis 52 months (range 14–142 months).

Table 1 shows the distribution of the tumors in relation to clinical-pathologic features and development of LR/DM. Mean tumor size was 16 mm (SD  $\pm 6.90$  mm). They were predominantly of ductal type (98%), higher grade (148/197; 75%),  $<25\%$  of DCIS (165/197, 84%) and no necrosis (119/197; 60%) or LVI (140/197; 71%). Microscopic margins were negative in 148 (75%) tumors. Among the clinical-pathological features, histologic grade ( $P = 0.021$ ) and the presence of an EIC ( $P = 0.047$ ) correlated with the development of LR. ER and PR were positive in 75% and 65% of tumors, respectively. PR-negative tumors recurred locally more frequently than PR-positive (26% vs. 11%;  $P = 0.024$ ), but no differences were observed for ER (17% vs. 17%;  $P = ns$ ).

In addition to local RT, 45 (22.8%) patients received systemic CT, 54 (27.4%) patients tamoxifen and 98 (49.7%) both CT + tamoxifen. Analysis according to systemic therapy groups showed non statistical differences regarding the total recurrence: 29% (13/45) for those with CT, 25.5% (25/98) when tamoxifen was added and 20.4% (11/54) for those with only tamoxifen ( $P = ns$ ).

The median staining score for  $\alpha$ -IGF1R was 220 (range 90–300) and for p-IGF1R was 20 (1–60). For the purpose of the study, primary tumors were classified as having IGF1R low or high expression, using the median scores as cut-offs.[28]. We found not significant correlation between the  $\alpha$ -IGF1R and the risk of recurrence ( $P = ns$ ). However, lower p-IGF1R was associated with the presence of LR (31% vs. 9%;  $P = 0.004$ ).

$\alpha$ -IGF1R levels were no different depending on the patients' age, presence of LVI or EIC ( $P > 0.05$ ). Nevertheless, they were increased in low-intermediate grade tumors (58% vs. 41%;  $P = 0.039$ ), without necrosis (62%;  $P = 0.002$ ), ER-positive (62%;  $P = 0.001$ ) or PR-positive (61%;  $P = 0.018$ ) and in association with p-IGF1R (71.4%;  $P = 0.03$ ). Similarly, increased p-IGF1R correlated with PR-positive cases (44%;  $P = 0.02$ ), and with low-

**Table 1** Characteristics of the patients and tumors in relation to clinicopathologic features, ER/PR and IGF1R expression

	TOTAL cases (%)	no recurrence cases (%)	with LR cases (%)	with DM cases (%)	<i>P</i>
No. of cases	197	148 (75.1)	33 (16.8)	16 (8.1)	
<i>Age</i>					
<40	28 (14)	24 (86)	3 (11)	1 (3)	ns
≥40	169 (86)	124 (73)	30 (18)	15 (9)	
<i>Menopausal status</i>					
Postmenopausal	110 (68)	82 (75)	19 (17)	9 (8)	ns
Premenopausal	53 (32)	42 (79)	5 (10)	6 (11)	
<i>Tumor size</i>					
5–20 mm	149 (76)	111 (77)	24 (16)	11 (7)	ns
21–50 mm	48 (24)	34 (71)	9 (19)	5 (10)	
<i>Histologic type</i>					
Ductal	193 (98)	145 (75)	33 (17)	15 (8)	ns
Lobular	4 (2)	3 (75)	0 (0)	1 (25)	
<i>Histological grade</i>					
1	49 (25)	44 (90)	4 (8)	1 (2)	0.021
2 + 3	148 (75)	104 (70)	29 (20)	15 (10)	
<i>DCIS</i>					
<25%	165 (84)	125 (76)	24 (14)	16 (10)	0.047
≥25%	32 (16)	23 (72)	9 (28)	0 (0)	
<i>Necrosis</i>					
Absent	119 (60)	91 (76)	21 (17)	7 (6)	ns
Present	77 (40)	57 (74)	11 (14)	9 (12)	
<i>LVI</i>					
Absent/ND	140 (71)	109 (78)	22 (16)	9 (6)	ns
Present	56 (29)	39 (70)	10 (18)	7 (12)	
<i>Margins</i>					
Negative (>5 mm)	148 (75)	111 (75)	25 (17)	212 (8)	ns
Close (0.1–5 mm)	44 (22)	34 (77)	7 (16)	3 (7)	
Positive (<0.1 mm)	5 (3)	3 (60)	1 (20)	1 (20)	
<i>ER</i>					
Negative	47 (24)	34 (72)	8 (17)	5 (11)	ns
Positive	145 (76)	110 (76)	24 (17)	11 (7)	
<i>PR</i>					
Negative	68 (35)	45 (66)	18 (26)	5 (7)	0.024
Positive	125 (65)	100 (80)	14 (11)	11 (9)	
<i>α-IGF1R</i>					
≤220	80 (48)	56 (70)	14 (18)	10 (12)	ns
>220	85 (51)	64 (75)	17 (20)	4 (5)	
<i>p-IGF1R</i>					
≤20	71 (52)	42 (59)	22 (31)	7 (10)	0.004
>20	66 (48)	55 (83)	6 (9)	5 (8)	

\*  $\chi^2$  test; LVI, lymph-vascular invasion; ND, non definitive; ns, not significant; DCIS, ductal carcinoma in situ

intermediate grade (63%;  $P = 0.044$ ). Interestingly, further tumor stratification according to the IGF1R activity showed that those with ER/PR-negative recurred more frequently (38.5%) than ER-positive/PR-negative (25%) or ER-any/PR-positive (3.8%) ( $P = 0.004$ ).

Table 2 summarizes the IHC and molecular characteristics of the controls and those with recurrences by IGF1R expression, IGF1R mRNA and *IGF1R* mutations. We observed no differences regarding the  $\alpha$ -IGF1R median score for control cases and tumors with DM or LR (240 vs.

**Table 2** IHC and molecular characteristics of the tumors according to the development of recurrence by IGF1R protein and activity, mRNA and mutations

	$\alpha$ -IGF1R median score (P25–P75)	p-IGF1R median score (P25–P75)	IGF1R mRNA median levels (P25–P75)	IGF1R mutations no. (%)
<i>Primary tumors</i>				
Controls ( $n = 42$ )	240 (90–300)	30 (1–80)	2.55 (1.63–4.43)	3 (7)
With DM ( $n = 12$ )	120 (80–300)	11 (0.25–67)	3.65 (1.43–5.40)	0 (0)
With LR ( $n = 31$ )	240 (60–300)	4.5 (0–20)	2.54 (1.16–4.67)	3 (9.7)
	$P = \text{ns}^*$	$P = 0.008^*$	$P = \text{ns}^*$	$P = \text{ns}$
<i>Local recurrences</i>				
$n = 31$	240 (60–285)	2 (0–32.50)	1.22 (0.57–2.10)	1 (3.2)
	$r = 0.432$ ; $P = 0.019^{**}$	$r = 0.232$ ; $P = \text{ns}^{**}$	$r = 0.027$ ; $P = \text{ns}^{**}$	$P = \text{ns}$

IHC, immunohistochemistry; DM, distant metastases; LR, local recurrence; \* Kruskal–Wallis test; \*\* Spearman correlation test for the tumors with LR and their corresponding LR

120 vs. 240, respectively;  $P = \text{ns}$ ; Kruskal–Wallis test). However, we found significantly lower median levels of p-IGF1R (active form) in those with LR (median = 4.5; P25–P75 = 0–20) than with DM (median = 11; P25–P75 = 0.25–67) or controls (median = 30; P25–P75 = 1–80) ( $P = 0.008$ ; Kruskal–Wallis test). Further analysis of the primary tumors with LR with their corresponding recurrence samples showed only a positive correlation for  $\alpha$ -IGF1R (Spearman correlation coefficient:  $r = 0.432$ ;  $P = 0.019$ ).

The median relative IGF1R mRNA for primary tumors ( $n = 85$ ) was 2.57. Stratification of the data showed no differences in controls cases and those with DM or LR (median = 2.55 vs. 3.65 vs. 2.54, respectively;  $P = \text{ns}$ ; Kruskal–Wallis test). Moreover, no correlation was found between the mRNA levels in primary tumors with their corresponding LR (median = 1.22) (Spearman correlation coefficient:  $r = 0.027$ ;  $P = \text{ns}$ ) (Table 2). For the purpose of the study, we categorized the mRNA level of the tumors into two groups: low (median  $<2.57$ ) versus high (median  $\geq 2.57$ ) expression. We observed only a trend between the mRNA high expression and the presence of LVI (17/28, 61%;  $P = 0.16$ ). Moreover, IGF1R mRNA was higher in ER-positive than in ER-negative tumors (61% vs. 15%;  $P < 0.000$ ), which was similar for PR (61% vs. 33%;  $P = 0.014$ ). However, no association was found with  $\alpha$ -IGF1R or p-IGF1R ( $P = \text{ns}$ ).

Among the 116 tumors (42 controls, 31 with LR, 12 with DM, and 31 LR) analyzed for mutations in the  $\beta$ -subunit (TK-domain at positions Tyr1131, Tyr1135 and Tyr1136; and C-terminal domain at positions Tyr1250 and Tyr1251), we only detected IGF1R mutations at Tyr1131 (A3532G) in six primary tumors (three with LR and in three controls) and in one LR ( $P = \text{ns}$ ) (Table 3 and Fig. 1). Interestingly, five out of the six (83%) mutated tumors showed  $\alpha$ -IGF1R overexpression, but this was only marginally significant ( $P = 0.079$ ; Fisher test). No differences were observed regarding p-IGF1R (3/6; 50%;  $P = \text{ns}$ ) or IGF1R mRNA (3/6; 50%;  $P = \text{ns}$ ).

Only variables that were statistically significant in the univariate analysis (histologic grade, EIC, PR and p-IGF1R expression) were combined in the multivariate analysis. The results showed as independent breast relapse predictors the histologic grade (OR, 3.1;  $P = 0.04$ ), the levels of p-IGF1R (OR, 4.2;  $P = 0.004$ ) and PR status (OR, 2.8;  $P = 0.02$ ), and as a trend with the presence of an EIC (OR, 2.1;  $P = 0.10$ ) (see Table 4).

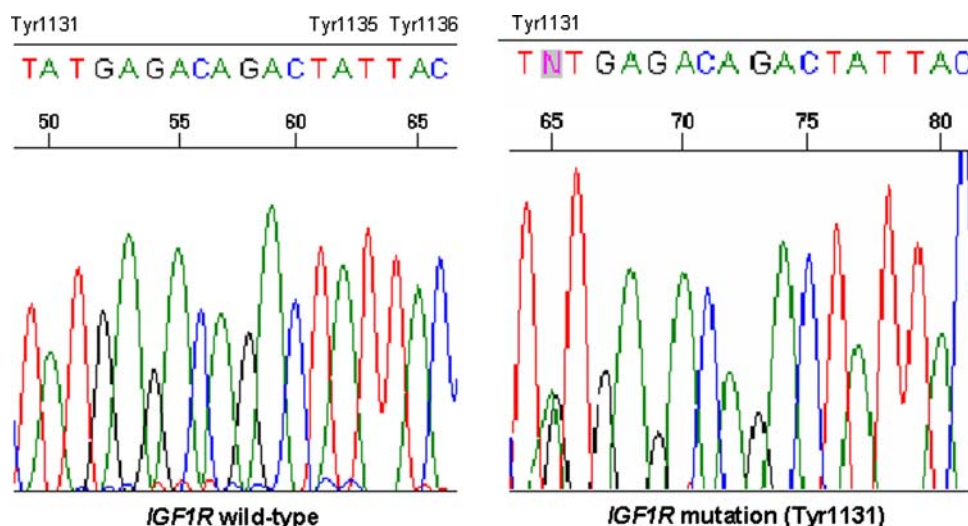
## Discussion

Over the past years there has been a large effort to identify predictive markers associated with LR [3–6]. 20–40% of LRs are associated with DM and a worse prognosis. LR in

**Table 3** Characteristics of primary tumors ( $n = 6$ ) and LR ( $n = 1$ ) with IGF1R mutation (Tyr1131)

Case no.	Grade	Size (Mm)	$\alpha$ -IGF1R score	p-IGF1R score	IGF1R mRNA levels	LR
6	2	32	300	160	1.730	No
15	3	20	300	60	0.417	No
16	3	18	300	90	12.933	No
74	2	22	285	0	15.889	Yes
109	3	18	300	2	4.329	Yes
164	3	15	60	5	2.553	Yes
LR	2	25	3	1	1.664	



**Fig. 1** *IGF1R* mutation at Tyr1131 (A3532G)**Table 4** Multivariate analysis (unconditional logistic-regression model) of histological and biological factors

Variables	$\beta$	Odds ratio	95% CI	P
Histol grade	1.32	3.1	1.0–9.1	0.04
EIC	0.75	2.1	0.8–5.2	0.10
PR	1.06	2.8	1.1–7.1	0.02
p-IGF1R	1.45	4.2	1.5–11.7	0.004

EIC, extensive intraductal component; PR, progesterone receptor; p-IGF1R, phosphorylated Insulin-like Growth Factor 1-Receptor

not uniformly defined in the articles reviewed; in some of them, all the lumpectomy area is considered as target volume for both boost dose or LR. Actual criteria are more restricted to 3 cm from the surgical margins. Also, true differences exist between early recurrence and resistance to RT, the last being defined as that which occurs in the first 12 months after finishing local treatment and is always associated with a worse prognosis. Later relapses on the other hand, occur beyond 1 year and are more dependent on medical treatments. In the present series of LNNBC treated with CS followed by RT and systemic treatment with tamoxifen and/or chemotherapy, histologic grade and the presence of an EIC correlated with LR risk. In addition, patients with PR-negative tumors showed the highest rate, supporting that ER-positive/PR-negative and ER/PR-positive BC are clinically and biologically distinct tumors [34]. However, no differences were observed for younger aged, positive/close margin of resection, LVI, or ER status. Further analysis according to systemic therapy groups showed no differences regarding the total recurrence. Randomized trials have shown not only decreased LR with RT but also an improvement in overall survival [1] being the magnitude greater with the combination of systemic therapy [2, 3, 35]. These analyses were out of the scope of the current study.

Similar to prior investigators in patients with lymph nodes-positive BC, we found increased IGF1R expression and activity more frequently in less aggressive tumors such as those with low-intermediate grade and no necrosis [18, 20, 27, 28]. Nevertheless, IGF1R may have still some relevance in aggressive triple-negative (ER/PR and HER2) tumors [36] and in ER-negative patients implying a shortened disease-free survival [30]. In our study, we found only a positive correlation of  $\alpha$ -IGF1R expression in primary tumors with their corresponding LR specimen, suggesting no changes of the receptor level when tumors recur.

Of note, IGF1R sends contradictory signals to cells depending on the availability of substrates. In the absence/low concentration of IRS-1, IGF1R induces cell differentiation, but when both are expressed, it has the ability to transform cells [9]. Schnarr et al. reported high levels of IRS-1 and IGF1R in breast tissues, in well-moderately differentiated carcinomas, but at low levels in poorly differentiated BC in agreement with our data [20]. Usually higher expression of IGF1R, autophosphorylation and kinase activity is detected in malignant than in normal breast tissue [19].

IGF1R and ER are frequently co-expressed and the two signalling systems are engaged in a cross-talk that regulates BC proliferation, protection from apoptosis and metastasis [9, 26]. We observed that tumors containing lower active IGF1R and PR-negative showed higher rates of LR, supporting their protective effect [20] and the interaction between both receptors [23, 25, 27, 37]. In agreement, Gee et al. [28] showed increased expression ( $\alpha$ -IGF1R) and activation (polyclonal antibody detecting Y1316 IGF-1R phosphorylation) in BC with good prognostic factors, and that was associated with the relevance to growth of tamoxifen-responsive disease, as seen by others [17, 29].

In contrast to our data, Turner et al. reported an association of IGF1R overexpression with higher LR which

was attributed to radiation resistance. Moreover, they found no association with ER/PR status, overall and disease-free survival, or axillary lymph-node involvement [14]. The discrepancies may be, on the one hand, due to the selection of patients. In fact, Turner presents an ancient series of early breast cancers with less than 30% of the patients treated with hormonotherapy according to old medical guidelines; we are led to suppose that at least 2/3 of the patients studied should be ER/PR positive and therefore were undertreated and did not benefit from chemoprevention with tamoxifen (radiation therapy cannot prevent progression from premalignant to malignant (in situ and infiltrating) lesions. On the other hand, the IHC assay results may not be comparable since a different antibody (against the IGF1R  $\beta$ -subunit) of ours (against the phosphorylated—active form—of IGF1R  $\beta$ -subunit and the IGF1R  $\alpha$ -subunit) and scoring system were applied.

In the current series, increased IGF1R mRNA expression was seen in neoplastic tissues, predominantly in ER/PR-positive tumors [23]. In contrast, Voskuil et al. reported higher levels in normal breast than in tumor tissue, and higher in tissue from women with a family history of BC [38]. In line with Al Sarakbi et al, we found no association with other clinical-pathological features [22]. In addition, we were not able to demonstrate in our cases any relationship between the mRNA levels in primary tumors with the corresponding LR as well as with the risk of LR/DM. To date, no other previous studies have quantitatively evaluated and compared the IGF1R mRNA in breast tumors tissues and recurrences for a comparison of our results.

Prior mutational analysis studies have suggested that signals derived from both regions of the IGF1-receptor cooperate to enhance metastasis. Experimental studies in M-27 cells (a clonal subline of the Lewis lung carcinoma) by Brodt using site-directed mutagenesis showed that mutation of any two of the tyrosines or a triple mutation in the TK domain abrogated the transformed phenotype and blocked metastasis [13]. However, a receptor mutated in the TK domain could still provide cell survival signals in a B cell lymphoma or a fibroblastic cell model as long as the tyrosines in positions 1250 and 1251 remained intact [39]. Consistently, among our cases with *IGF1R* mutations, all at Tyr1131 (A3532G), none developed DM, but three had LR and three did not recur. Only one LR specimen showed mutation that was not present in the corresponding primary tumor. Moreover, no mutations were detected in the C-terminal domain that could explain the relationship between LR and radioresistance reported in a previous study [14]. Turner's in vitro analyses based on the study of mouse fibroblasts cell lines (BALB/c3T3) to clarify radioresistance cannot parallel with human breast cancer models where a close cross-talk between IGF1R and ER

signalling, growth factors, tumor suppressor genes and apoptosis-involved proteins is present [14]. The IGF1R can probably utilize several distinct signalling pathways that may either involve, or to be independent of, the known receptor substrates to lead a determinate cellular response [39]. Therefore, the differences among the above reports may reflect unique properties of the different cell types used in these studies [40].

Of interest, recent studies suggest that gene-expression signature can be more powerful to predict LR after CS [41] metastasis and survival [42] and response to tamoxifen [43] than the standard clinical-pathological criteria. Nevertheless, IGF1R was not included in these studies.

In summary, our results indicate that in LNNBC patients treated with CS, local RT and adjuvant chemo- and/or hormone therapy, those with tumors showing low IGF1R activation, PR-negative and high grade have an increased risk of LR, which could be partially related with tamoxifen responsiveness. Apparently, IGF1R protein, mRNA or gene status do not seem major contributors to the radioresistance. Therefore, the subset of patients with low-grade tumours and favourable markers (increased content of active IGF1R and PR-positive) does not benefit from additional boost dose over the scar to prevent LRs. Nonetheless, more clinical research should be done to confirm our results and to elucidate unresolved questions regarding the complex mechanisms involved in LR in which IGF1R is involved as well as its prognostic value in BC patients in early stage.

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