

# Sporadic Amplification of the Insulin-like Growth Factor 1 Receptor Gene in Human Breast Tumors<sup>1</sup>

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## Abstract

A principal difference between malignant and normal cells is the aberrant expression of oncogenes. Previously, we have reported on the expression of the insulin-like growth factor 1 receptor (IGF-1-R) in 93% of the human primary breast cancers studied. In the present study, we observed an increased gene copy number of the IGF-1-R in only 19 (2%) of 975 cases studied. The gene copy number of tumors with an amplified *IGF-1-R* gene varies between 3 and 56 (median, 24 copies). In 11 breast tumor samples with high ( $\geq 20$  copies) *IGF-1-R* gene copy numbers, an additional amplification of either the *c-myc* gene ( $n = 3$ ) or *int-2/bcl-1* genes ( $n = 5$ ) was observed, whereas no amplification of the *HER2/neu* gene was detected. The *c-fes* gene (like the *IGF-1-R* gene located on chromosome 15q25-qter), was found coamplified with the IGF-1-R in two cases, in one case to the same high extent (38 gene copies, each) and in the other case to only a moderate extent (4 copies of the *c-fes* gene and 21 copies of the *IGF-1-R* gene). Tumors with an amplified *IGF-1-R* gene showed a noticeable increased expression of the IGF-1-R as measured by ligand binding assays on membrane preparations. The median amount of the IGF-1-R protein of the amplified tumors was observed to be 35 times higher when compared to nonamplified tumors ( $P < 0.001$ ). Patients with tumors containing a high ( $\geq 20$  copies) *IGF-1-R* gene copy number tend to have a shorter median overall survival (42 months; range, 14–120+;  $n = 8$ ) than patients with tumors having a low amplified (3–10 copies) *IGF-1-R* gene copy number (median, 77 months; range, 19.5–98+;  $n = 4$ ).

## Introduction

IGF-1<sup>3</sup> (somatomedin C) is one of the most potent mitogens for (IGF-1 receptor positive) human breast cancer cells *in vitro* (1). IGF-1-like activities have been detected in human breast tumor biopsies (2) and plasma IGF-1 concentrations are higher in primary breast cancer patients, when compared to age-matched control women (3). IGF-1 acts through the IGF-1-R, a tetrameric glycoprotein composed of 2 extracellular  $\alpha$ -subunits (135 kDa) which bind the ligand and 2 intracellular  $\beta$ -subunits (90 kDa) which cross the membrane and have intrinsic, intracellular tyrosine kinase activity, with both  $\alpha$ - and  $\beta$ -units joined by disulfide bridges (4–6). IGF-1 receptors have been demonstrated in 50–93% of the human primary breast tumors studied and were found to be positively related to the amount of ER and PgR (2, 7–10). Compared to tumor tissues, receptors for IGF-1 were less frequently present or undetectable in benign breast disease and normal tissue (11).

Enhanced expression is often consequent to a process of DNA amplification and is a mechanism by which a number of growth controlling genes can be converted to oncogenic forms.

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<sup>3</sup> The abbreviations used are: IGF-1, insulin-like growth factor 1; IGF-1-R, insulin-like growth factor 1 receptor; ER, estrogen receptor; PgR, progesterone receptor.

In human breast cancer, gene amplification has been reported most frequently for oncogenes such as *HER2/neu*, *c-myc*, and *int-2* in up to 40% of the cases, average of 15–20% [for a review, see the report of Callahan (12)]. In contrast to these oncogenes, the epidermal growth factor receptor gene is highly expressed, at average in 45% of the tumors, whereas this gene is amplified in only a few cases (2–13%) of human primary breast cancers (12, 13). The gene of another growth factor receptor, the *IGF-1-R* gene, is also highly expressed but there are no reports on *IGF-1-R* gene amplification in human (primary) breast cancer. We have therefore studied *IGF-1-R* gene copy number and IGF-1-R expression, in human breast cancer tissues. Moreover, in tumors with an amplified *IGF-1-R* gene, expression of the IGF-1-R protein was studied together with steroid receptors and other (onco)genes which may be relevant in breast cancer development and progression.

## Materials and Methods

**Patients and Tissues.** Tumor specimens were drawn from a pool of frozen specimens (liquid nitrogen) originally submitted to our laboratory for steroid hormone receptor analysis. This study includes a series of 1052 breast tumors collected from 1978 to 1991.

**Receptor Assays.** Tumor tissue (0.4–0.8 g) was pulverized and homogenized as recommended by the European Organization for Research and Treatment of Cancer for processing of breast tumor tissue for cytosolic ER and PgR determinations (14). The homogenate was centrifuged for 30 min at  $100,000 \times g$  at 4°C, and the supernatant fraction (cytosolic extract) was used for ER and PgR determinations, either with enzyme immunoassays (ER enzyme immunoassay and PgR enzyme immunoassay kits; Abbott Laboratories, Abbott Park, IL) or with radioligand binding assays as described before (15) and as recommended by the European Organization for Research and Treatment of Cancer (14). The membrane fraction for IGF-1-R determinations was obtained in the supernatant fraction after homogenization of the  $100,000 \times g$  pellet in 2.5 ml 10 mM phosphate buffer (pH 7.4), containing 0.15 M NaCl and 40  $\mu\text{g/ml}$  bacitracin with three 5-s bursts of an Ultraturrax tissue homogenizer and subsequent centrifugation for 10 min at  $1000 \times g$ . Following aspiration of the supernatant, an aliquot was taken for protein estimation and bovine serum albumin (final concentration, 0.5%) was added to the remainder of the membrane fraction prior to IGF-1-R analysis. Scatchard analysis of specific IGF-1 binding was performed after separation of bound and free ligand by high-speed centrifugation as described previously (9). In a number of preparations, specific IGF-1 binding was that extremely high that Scatchard analysis was pointless. In those cases the membrane preparations were diluted 10 times with assay buffer and analyzed again for IGF-1-R content.

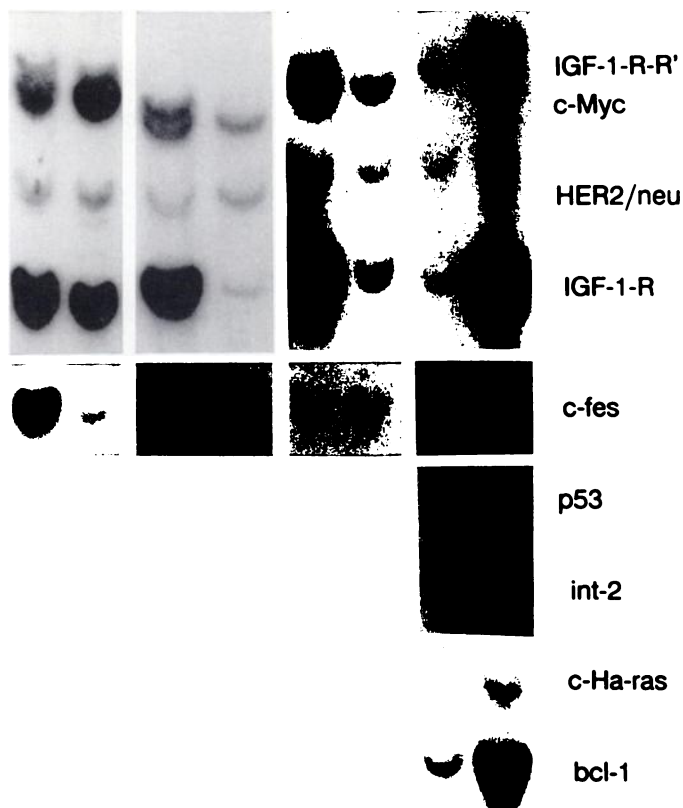
**DNA Analysis.** DNA was isolated from an aliquot of the total tissue homogenate. Southern blotting of *EcoRI*-digested DNA was performed by standard techniques (16). In brief, digested DNA was size fractionated on a 0.6% agarose gel and transferred to a nylon membrane (Hybond N<sup>+</sup>; Amersham, Buckinghamshire, United Kingdom), and hybridized overnight at 65°C with randomly primed [<sup>32</sup>P]dATP-labeled probes (specific activity,  $1-2 \times 10^9$  cpm/ $\mu\text{g}$  DNA). *HER2/neu* (pHER2-436-1, ATCC 59296); *c-myc*, an *EcoRI*-*Clal* human exon 3-specific c-

*myc*; and IGF-1-R (pIGF-9-R.8, ATCC 59295); p53 (php53B, ATCC 57254); c-Ha-ras, a 6.6-kilobase fragment obtained from the American Type Culture Collection (Rockville, MD), *bcl-1* (a 2.1-kilobase *SstI* fragment), *c-fes* (a 0.95-kilobase fragment) and *int-2*; SS6 (a 0.91-kilobase *SacI-SacI* fragment), a gift from Drs. Gordon Peters and Clive Dickson, ICRF, London, United Kingdom. After washing at high stringency ( $0.3 \times$  standard saline-citrate), autoradiography with intensifying screens was performed for 1–2 days at  $-70^\circ\text{C}$  using Kodak XAR-5 films, and autoradiograms were scanned with a Bio-Rad Video densitometer 620. In our recent experience concerning measurement of *c-myc* and *HER2/neu* amplification in approximately 1000 breast tumors, the incidence for *c-myc* amplification was 17% and that of *HER2/neu* amplification was 19% (17).

## Results and Discussion

**Frequency of IGF-1-R Gene Amplification.** IGF-1-R gene copy numbers were studied in DNA isolated from homogenates of 1052 human breast cancer samples. Seventy-seven samples (7% of the cases) were not evaluable due to very low recovery of DNA or because the DNA could not be digested with *EcoRI*. The IGF-1-R gene copy number was increased ( $>2$  copies) in 19 of the remaining 975 evaluable samples (median, 24 gene copies; range, 3–56 copies). An example of a Southern blot is shown in Fig. 1. The filters were dehybridized and then re-probed with a human *c-fes* probe (both the IGF-1-R and *c-fes* gene are located on chromosome 15q25-qter); in two samples with an increased IGF-1-R gene copy number, an increased copy number of *c-fes* gene was seen as well (Fig. 2; Table 1). In one case the gene copy number of *c-fes* was moderately (4 copies) increased when compared to the IGF-1-R gene (21 copies), shown in Fig. 2, and in the other case identical gene copy numbers (38 copies) for both genes were observed (Fig. 2; Table 1). In the latter case an increased number of chromosome 15 is very likely. Interestingly, Saint-Ruf *et al.* (18) also described an amplification of the *c-fes* gene in one mammary tumor, and in this tumor the homogeneously stained region was composed of the coamplification of *fes* and adjacent sequences included in band 15q26. Two cases showing IGF-1-R amplification were examined in duplicate, respectively in triplicate. Homogenates separately prepared from different parts of the same tumor specimen were analyzed. All these DNA samples showed IGF-1-R amplification, indicating that these tumors were not heterogeneous with respect to amplification of the IGF-1-R gene. In all cases with high ( $\geq 20$  gene copies) amplification, an additional hybridizing band of 14 kilobase pairs (IGF-1-R-R'; Fig. 1 and 2; Table 1) was present. In only two of these cases was this band also amplified. The nature of this extra band is, as yet, unknown. Interestingly, both cases with this additional amplified band also showed an *int-2/bcl-1* (both located on chromosome 11q13) amplification (see Fig. 2 and Table 1).

**Fig. 1.** Southern blot of DNA from breast tumor tissue samples. Total DNA was digested with *EcoRI*, subjected to electrophoresis on a 0.6% agarose gel, transferred to a nylon membrane, and hybridized simultaneously with *c-myc*, *HER2/neu*, and IGF-1-R probes (left, showing different magnitudes of amplification) or hybridized to the IGF-1-R probe (right) with either *c-myc* (middle) or *c-myc* and *HER2/neu*. The filters were washed at high stringency and exposed. "Normal" refers to two copy numbers of the respective protooncogene. "Amplified" refers to more than two copies of the gene (for the IGF-1-R: low, 3–10 copies; high,  $\geq 20$  copies).



**Fig. 2.** Southern blot of DNA from breast tumor tissue samples. Same blotting and hybridization-procedure as described under Fig. 1. Membranes were dehybridized and re-probed with *c-fes*, *bcl-1*, *int-2*, p53, or c-Ha-ras. Filters were washed at high stringency and exposed. "Normal" refers to two copy numbers of the respective protooncogene. "Amplified" refers to more than two copies of the gene (low, 3–10 copies; high,  $\geq 20$  copies).

With respect to other tumor types, in none of 40 ovarian cancer biopsies investigated did we observe, an amplification of the IGF-1-R gene. In view of the low frequency of IGF-1-R gene amplification, no conclusions with respect to possible specificity of IGF-1-R gene amplification in breast cancer can be drawn.

**IGF-1-R Gene Amplification and Expression.** The relationship between IGF-1-R gene amplification and protein expression was examined in breast tumor tissues without and in available membrane preparations from tumor biopsies with gene amplification. Scatchard analysis revealed a single class of specific  $^{125}\text{I}$ -IGF-1 high affinity binding sites (median Kd, 0.11 nM; range, 0.03–0.59). The median IGF-1-R content calculated from Scatchard analysis in tumor tissue with gene amplification available from our tumor bank (median, 4,850; range, 1,350–18,850 fmol/mg protein;  $n = 5$ ) was 35-fold higher ( $P < 0.001$ )



Table 1 Frequency of IGF-1-R gene amplification

Patient		Tumor T/N/M	Receptors (fmol/mg protein)		Gene copy numbers							Survival (mo)
Age (yr)	Meno <sup>a</sup>		ER	PgR	IGF-1-R	FES	HER2	MYC	INT-2	BCL-1	IGF-1-R-R'	
63	Post	3/0/0	245	0	56	n	n	15	n	n	n	69
58	Post	X/X/1	349	27	32	n	n	14	n	n	n	31
40	Pre	4/9/0	253	101	26	n	n	n	4	4	n	14
67	Post	2/x/1	65	23	25	n	n	n	4	4	n	16
33	Pre	2/3/0	278	431	48	n	n	n	10	10	A	37
74	Post	2/0/0	949	40	38	n	n	n	n	n	n	82 <sup>b</sup>
76	Post	2/1/0	80	55	38	38	n	n	n	n	n	120 <sup>b</sup>
51	Post	2/0/0	718	0	30	n	n	n	5	7	A	46
44	Pre	x	13	195	20	n	n	n	4	4	n	x
68	Post	x	47	3	24	n	n	16	n	n	n	x
72	Post	x	110	2	21	4	n	n	n	n	n	x
48	X	2/0/0	113	463	9	n	n	n	n	n	n	x
57	Post	x	290	649	5	n	n	n	n	n	-	x
74	Post	2/2/0	0	0	6	n	n	n	n	n	-	19
61	Post	3/0/0	0	7	4	n	n	n	n	n	-	98 <sup>b</sup>
78	Post	2/2/0	1187	778	3	n	n	n	n	n	-	80.5 <sup>b</sup>
49	X	1/1/0	34	255	3	n	n	n	n	n	-	74 <sup>b</sup>

<sup>a</sup> Meno, menopausal status; Post, postmenopausal; Pre, premenopausal; X, not known; T, tumor size (T1,  $\leq 2$  cm; T2, 2–5 cm; T3/4,  $\geq 5$  cm). N, amount of positive nodes; M, distant metastasis (0, absent; 1, present at time of diagnosis). Gene copy numbers; number of gene copies present for each (onco)-gene (n, normal = 2 copies, -, absent).

<sup>b</sup> Patient still alive after months indicated.

when compared to tumors without *IGF-1-R* gene amplification (median, 140; range, 90–320 fmol/mg protein;  $n = 12$ ). Scatter plots of two additional tumors with gene amplification were not evaluable due to a too high amount of IGF-1-R present, even after a 10-fold dilution. Although the number of tumor biopsies studied is small, these data suggest that there is a strong correlation between IGF-1-R overexpression and gene amplification.

**Relationship of *IGF-1-R* Gene with Other Variables and Prognosis.** The relationships between *IGF-1-R* gene amplification and patient and tumor characteristics and prognosis were determined. Updated clinical information including age of the patient and steroid receptor status was available for 17 patients (as shown in Table 1); additional data on menopausal status, nodal status, and size of the tumor were available for 13 patients, and data on survival were available for 12 patients (in our computerized data base). Two patients had metastasis at time of diagnosis of the primary tumor (Table 1). The median age of the patients with tumors containing an increased *IGF-1-R* gene copy number was 61 years (range, 33–78 years;  $n = 17$ ). Twelve patients were postmenopausal and five were pre- or perimenopausal. No clear relation between *IGF-1-R* gene amplification and age, menopausal status, or nodal status was observed. Amplification was found more frequently in larger ( $>2$  cm) tumors, since only one tumor that demonstrated an increased number of the *IGF-1-R* gene, was T1, eight tumors were T2, and three were T3/4. Of the 17 tumors with IGF-1-R amplification 15 (88%) were highly positive for the ER (ER-positive values; median, 179 fmol/mg protein; range, 13–1187), whereas 11 (65%) of 17 tumors were PgR positive (PgR-positive values; median, 148 fmol/mg protein; range, 23–778). It is of interest to note that a positive relationship was described for the expression levels of IGF-1-R with steroid receptors (2, 7, 8). No association of IGF-1-R with epidermal growth factor receptor, lymph node status (7, 15), tumor size (9), differentiation grade (7, 9), or menopausal status (9) were reported.

In 10 of 11 breast tumor samples with high gene copy number ( $\geq 20$  copies), an amplification of *IGF-1-R* gene with either *c-myc* ( $n = 3$ ) or *int-2/bcl-1* ( $n = 5$ ) or *c-fes* ( $n = 2$ ) was detected (Table 1). Interestingly, in none of these cases a simultaneous amplification of both the *IGF-1-R* and the *HER2/neu* genes

was observed. One should keep in mind that *HER2/neu* amplification is associated with ER-negative tumors (17), while IGF-1-R amplification was found predominantly in ER-positive tumors.

The median overall survival of patients with tumors having a low (3–10 copies) IGF-1-R gene copy number was 77+ months (range, 19–98+ months) and the median duration of overall survival in patients with tumors having high *IGF-1-R* gene copy numbers ( $\geq 20$  copies) was 42 months (range, 14–120+ months). Whether this shorter survival in patients with high number of copies is caused by just one or the combination of oncogene amplifications is, at present, unclear. For instance amplification of *int-2/hst* has been associated with ER-positive tumors and worse prognosis (19) and *c-myc* amplification has been associated with a shorter overall survival (12) as well. However, this series of patients, having tumors with IGF-1-R gene amplification, is too small for definite conclusions in this respect. The two studies on the prognostic value of IGF-1-R overexpression showed different results. Bonnetterre *et al.* (20) showed a longer (disease-free) survival of patients with very high levels of IGF-1-R protein, when compared to those with lower levels of IGF-1-R, whereas we found no relationship between IGF-1-R levels and (disease free) survival (9).

The role of *IGF-1-R* gene amplification in human breast cancer is unclear. It has been suggested that the expression of the IGF-1-R is associated with the malignant transformation of breast epithelial cells (8). We showed a strong association of amplification with overexpression of the IGF-1-R. *IGF-1-R* gene amplification (which mechanism is unclear) may be related to estrogen receptor positivity and to *c-myc* (regulated by estrogens) or *int-2/bcl-1* (correlated with ER positivity) amplified tumors, and may point to an estrogen-related/regulating process.

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