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Short Communication

Growth Hormone Receptor Is Expressed in Human Breast Cancer

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Several clinical observations and experimental studies indicate that pituitary hormones, including growth hormone, play a role in the development of human breast cancer. We analyzed 48 human breast carcinomas using reverse transcription polymerase chain reaction, immunohistochemistry, and Western blotting techniques to assess growth hormone receptor expression. In 17 of these cases, adjacent normal breast tissue was similarly analyzed. These analyses revealed that growth hormone receptor (GHR) is expressed in human breast cancer and appears to be up-regulated compared to adjacent normal breast tissue. GHR expression correlated inversely with tumor grade and MIB-1 index. Progesterone receptor expression correlated positively with GHR expression. These findings, along with our observation of GHR expression in breast cancer stromal cells and previous reports of local production of growth hormone in breast carcinoma, suggest that GHR-mediated signaling pathways are involved in the development of human breast cancer, possibly via autocrine or paracrine mechanisms. *(Am J Pathol 2001, 158:1217–1222)*

Breast cancer is by far the most common malignancy in women, affecting one in eight in the United States and Western Europe. An increased risk of breast cancer is associated with early menarche, late menopause, and nulliparity. These observations suggest that ovarian hormones play an important role not only in normal breast development, but in the development of breast cancer and its progression. Pituitary hormones are also essential for normal breast development. Moreover, some observations suggest they are also involved in the development of breast cancer. Administration of growth hormone (GH) to aging primates induces a marked increase in mammary gland size and epithelial proliferation index.[1] Conditions with increased GH levels, such as acromegaly, are associated with an increased risk of malignancy, including breast cancer.[2–4] In the treatment of advanced breast cancer, hypophysectomy has beneficial, ovary-independent effects.[5] Breast cancer cell lines grow in response to both prolactin (PRL) and GH administration, and are inhibited by PRL and GH antagonists.[6–8] GH receptor (GHR) expression in human breast cancer and breast cancer cell lines has previously been detected using reverse transcription polymerase chain reaction (RT-PCR), *in situ* hybridization, and immunohistochemistry.[9–11] However, quantitative analyses for levels of GHR protein expression using Western blot techniques have not been performed. We studied 48 human breast carcinomas as well as adjacent normal mammary tissue using RT-PCR, Western blot, and immunohistochemistry. Our results indicate that GHR expression is up-regulated in breast cancer and suggest a role for GHR signaling in this disease.

Materials and Methods

The study included 48 breast carcinomas (47 primary tumors and one lymph node metastasis) from 47 patients who had surgery at the Sahlgrenska University Hospital in Göteborg, Sweden. Tumor size, histological type, and tumor grade (Bloom-Richardson-Elston score[12]), as well as axillary lymph node status (positive *versus* negative) were recorded in all cases. Material was snap-frozen in liquid nitrogen for RT-PCR (36 cases) and Western blotting (28 cases). Fresh frozen material of adjacent normal mammary tissue was obtained in 17 cases. Immunohistochemical analyses were performed in 47 cases; in all

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cases, the selected histological sections included carcinoma as well as adjacent normal breast tissue.

Immunohistochemistry

All immunostains were performed according to standardized protocols using the TechMate Horizon immunostainer (DAKO, Copenhagen, Denmark). For detection of GHR, the mouse mAb 263 (AGEN Biomedical, Brisbane, Australia) was used at a dilution of 1:500 with application of tyramide signal amplification system (NEN Life Science Products, Boston, MA). All primary tumors were also analyzed for estrogen receptor (ER; clone 105, DAKO), progesterone receptor (PR; clone PgR 636, DAKO), MIB-1 (Immunotech, Marseille, France), and p53 (D0–7, DAKO). The immunoreactions for mAb 263 were graded as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3). For ER, PR, MIB-1, and p53, the estimated percentage of positive tumor cell nuclei was recorded.

RT-PCR

Preparation of RNA was performed.[13] cDNA was synthesized from 0.5 μg RNA with 5 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and 0.5 μg oligo-(dt) primer (Promega). For the PCR reaction, the following primers were used: 5′-GCTGCTGTTGACCTTGGC-3′ (sense) and 5′-ACCTCATCTGTCCAGTGG-3′ (antisense) (Scandinavian Gene Synthesis, Köping, Sweden) located in exons 2 and 4, respectively. These primers will amplify a 201-bp fragment corresponding to nucleotides 58–258 of the human GHR cDNA. If the GHR lacks the nucleotides corresponding to exon 3, the amplified fragment will be 135 bp. PCR was performed following a standard protocol. Samples were amplified for 30 cycles at an annealing temperature of 55°C. Specificity of the PCR products was verified by specific cleavage with the restriction enzyme Bsp 1286 I (Promega), which cleaves the GHR in exon 3, rendering two fragments 119 and 82 bp in size when PCR products containing exon 3 are digested, whereas PCR products without exon 3 are left intact. Specificity of PCR products was also confirmed by Southern hybridization (not shown).

Western Blotting

The antibody used, GHR06, was a mouse monoclonal raised against amino acids 396–407 of the extracellular part of human GHR. It recognizes the human GHR protein by Western blotting, by immunoprecipitation, and by fluorescence-activated cell sorting (G Norstedt, unpublished data).

Soluble tissues were prepared by homogenization in PE buffer (10 mmol/L potassium phosphate buffer, pH 6.8 and 1 mmol/L EDTA) containing 6 mg/ml 3-(3-cholamidopropyl)dimethyl-ammonio 1-propane sulfate (CHAPS), aprotinin (200 kallikrein inhibitory units per milliliter), leupeptin (10 μg/ml), pepstatin (10 μg/ml), and Pefabloc (1 mg/ml; Boehringer Mannheim, Mannheim, Germany). After sonication and centrifugation, supernatants were collected and protein concentrations were determined by the Bio-Rad method. Supernatants were stored at −70°C until analysis. The samples were diluted in SDS sample buffer and denatured before loading on a SDS-polyacrylamide gel (8% Tris-glycine; NOVEX, San Diego, CA). Fifty micrograms of total protein were loaded into each lane. A prestained standard (SeeBlue, NOVEX) was used as weight marker. After electrophoresis, the proteins were transferred to a polyvinyldifluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) using a standard electroblotting system, followed by incubation with GHR06, diluted 1:1000. Immunoreactive protein was visualized by chemiluminescence using an ALP-conjugated secondary antibody (goat-anti-mouse, SIGMA), diluted 1:30000 and CDP Star (Tropix, Bedford, MA) as substrate. Membranes were exposed to ECL film (Amersham) for 10 seconds to 3 minutes and developed in a Curix 60 developing machine (AGFA). Autoradiograms were scanned and the bands corresponding to GHR protein were analyze by densitometry. Quantitative analyses were performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analyses

Correlation between GHR expression as determined both by mAb 263 immunostaining and by Western blot analysis, and patient age, tumor size, tumor grade, tumor type, axillary lymph node status, estrogen receptor expression, progesterone receptor expression, MIB-1 immunoreactivity, and p53 immunoreactivity was evaluated using Fisher's permutation test. Differences in GHR expression between tumors and adjacent normal mammary tissue as determined by Western blot analysis were evaluated using a paired *t*-test. P values <0.05 were considered significant.

Results

The patient and tumor characteristics are summarized in Table 1. The 47 patients ranged in age from 34 to 90 years (median, 59 years). Tumors ranged from 15 to 110 mm in greatest dimension. The vast majority of tumors were invasive ductal carcinomas ($n = 41$); the remainder were invasive lobular carcinomas ($n = 3$), atypical medullary carcinomas ($n = 2$), and invasive tubular carcinoma ($n = 1$). Axillary lymph nodes were removed in 42 patients; 22 patients had metastases and 20 did not.

Immunohistochemistry

The GHR (mAb 263) immunostaining results are summarized in Table 1. A variable degree of cytoplasmic staining of tumor cells was seen in all but two of the 47 analyzed tumors (Figure 1, a–c). Weak focal nuclear staining was observed in a few instances. Weak or moderate cytoplasmic staining also occurred in some stromal cells (Figure 1b). Adjacent normal breast tissue was neg-

Table 1. Summary of Clinical Data of 48 Carcinomas and RT-PCR, Immunohistochemical, and Western Blot Analyses

Case no.	Age (years)	Tumor size (mm)	Tumor type	BRE (3–9)	Axillary lymph node status	GHR detection			
						RT-PCR		Western blotting (arbitrary units)	IHC (0–3)
						GHR	Exon 3		
1	83	43	IDC	7	pos.	+	yes	1.05	3
2	50	26	IDC	ND	neg.	+	yes	0.47	1
3	51	25	IDC	6	neg.	+	yes	ND	3
4	62	34	IDC	7	pos.	+	yes	ND	1
5	79	90	IDC	7	pos.	+	yes	ND	3
6	59	40	IDC	8	pos.	+	yes	0.53	3
7	60	42	IDC	7	pos.	+	yes	1.12	3
8	68	29	IDC	6	neg.	+	yes	0.78	3
9	53	30	IDC	7	pos.	+	yes	0.56	3
10	39	25	IDC	8	neg.	+	yes	0.84	2
11	43	65	IDC	5	neg.	+	yes	0.91	3
12	62	60	IDC	7	pos.	+	yes	0.30	2
13	34	28	IDC	8	neg.	+	yes	0.63	3
14	88	30	IDC	7	ND	+	yes	0.58	1
15	71	25	IDC	6	neg.	+	yes	0.84	3
16	37	20	IDC	7	ND	+	yes	ND	1
17	37	110	IDC	6	pos.	+	yes	ND	3
18	56	40	IDC	8	neg.	+	yes	0.30	2
19	83	28	IDC	9	ND	+	yes	0.70	3
20	48	33	IDC	7	neg.	+	no	ND	3
21	34	40	IDC	9	neg.	+	no	0.47	2
22	42	30	IDC	6	neg.	+	no	0.77	3
23	39	30	IDC	7	pos.	+	no	1.31	3
24	49	25	IDC	7	pos.	+	both	0.80	2
25	46	55	IDC	9	pos.	+	both	1.00	1
26	33	40	IDC	9	neg.	+	both	0.97	2
27	44	40	IDC	9	pos.	+	both	0.52	ND
28	84	30	IDC	6	pos.	+	both	0.73	2
29	67	23	IDC	7	neg.	+	both	0.64	3
30	86	40	IDC	7	ND	+	both	1.27	3
31	71	30	IDC	6	ND	ND		ND	3
32	80	40	IDC	7	pos.	ND		ND	3
33	84	80	IDC	9	neg.	ND		ND	2
34	63	20	IDC	8	neg.	ND		ND	3
35	72	60	IDC	9	neg.	ND		ND	1
36	43	25	IDC	9	pos.	ND		ND	2
37	50	15	IDC	9	neg.	ND		ND	0
38	47	40	IDC	9	pos.	ND		ND	0
39	64	43	IDC	9	pos.	ND		ND	1
40	48	23	IDC	7	pos.	ND		ND	3
41	49	ND	IDC	9	pos.	ND		ND	3
42	90	110	ILC	5	pos.	+	no	0.52	3
43	73	22	ILC	8	pos.	+	both	0.28	1
44	73	35	ILC	5	neg.	ND		ND	3
45	90	ND	ILC, Ln M	ND	pos.	+	no	ND	1
46	66	60	AMC	9	neg.	+	no	0.22	2
47	48	45	AMC	8	neg.	+	yes	1.23	3
48	81	30	ITC	4	pos.	+	yes	ND	2

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; AMC, atypical medullary carcinoma; ITC, invasive tubular carcinoma; Ln M, lymph node metastasis; BRE, Bloom-Richardson-Elston score; IHC, immunohistochemistry; pos., positive; neg., negative; ND, not determined.

ative (Figure 1d), or weakly positive, sharply contrasting with mostly strongly positive tumors. A significant inverse correlation was found between GHR expression and tumor grade ($P < 0.05$).

Estrogen and progesterone receptor positivity, defined as >10% positive tumor cell nuclei, was observed in 26/44 and 18/46 tumors, respectively. MIB-1 positivity was ≤10% in 17/46 tumors, >10% and <50% in 18/46, and ≥50% in 11/46 tumors. p53 immunostaining >20% was seen in 14/47 tumors. There was a positive correlation between GHR expression and progesterone receptor expression ($P < 0.01$), and an inverse correlation between GHR expression and level of MIB-1 immunoreactivity ($P < 0.05$).

RT-PCR

Amplified fragments of the expected sizes (201 or 135 bp) were detected in all 36 tumor samples examined (Figure 2a and Table 1). To verify their identity, the PCR products were digested by the restriction enzyme Bsp 1286 I (Figure 2b). Twenty-one of 36 tumors expressed the GHR form containing exon 3, seven tumors expressed the GHR form without

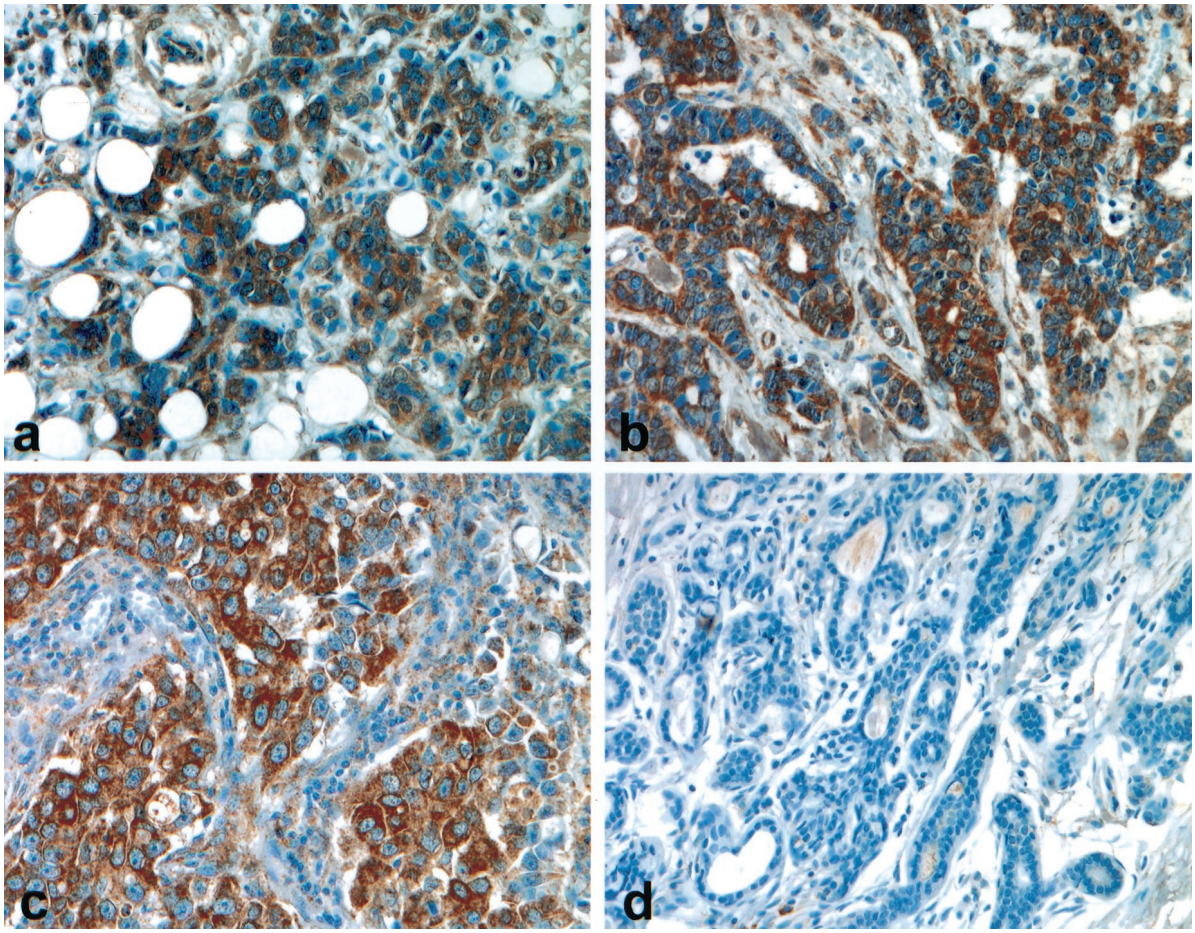

Figure 1. GHR (mAb 263) immunostaining of invasive ductal carcinomas of the breast (**a** and **b**) and an atypical medullary carcinoma (**c**). Strong cytoplasmic immunostaining of the epithelial component and weak staining of some stromal cells are seen (**b** and **c**). Adjacent normal breast tissue is negative (**d**) or weakly positive.

exon 3, and eight tumors expressed both forms. In 17 cases, GHR mRNA expression was also assessed in adjacent normal mammary tissue. GHR mRNA was detected in all normal mammary tissues and was always of the same form as the corresponding tumor.

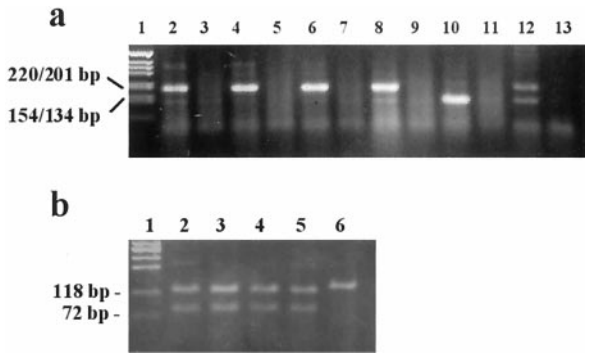

Figure 2. a: RT-PCR of GHR expression in six breast cancer cases. **Lanes 2**, **4**, **6**, **8**, **10**, and **12** represent RT-PCR of tumor tissues. **Lanes 3**, **5**, **7**, **9**, **11**, and **13** represent no template controls. **Lane 1** shows a DNA marker (1 kb, GIBCO BRL). The full-length GHR renders a fragment of 201 bp (**lanes 2**, **4**, **6**, and **8**). The GHR without exon 3 renders a fragment of 135 bp (**lane 10**). **Lane 12** represents a case in which both forms are expressed. **b:** Digestion of the PCR fragment with Bsp 1286 I. Cleavage occurs in the third exon, resulting in two fragments that are 119 and 82 bp in size (**lanes 2–5**) or, in the case of exon 3 deletion, leaving the fragment undigested (**lane 6**). **Lane 1** is the DNA marker X174 *Hae*III.

Western Blotting

GHR protein was detected in all 28 tumors analyzed. The amount of GHR varied between different tumors (Figure 3a and Table 1). The relative protein levels ranged between 0.22 and 1.31 arbitrary units (median, 0.71). In adjacent normal breast tissue ($n = 17$), the relative protein levels ranged between 0.09 and 0.75 arbitrary units (median, 0.26). Comparison of GHR expression levels in 16 tumors and adjacent normal mammary tissues (Figure 3b) revealed significantly higher levels of GHR in tumors ($P < 0.01$, paired *t*-test; Figure 3c). No significant correlation was found between GHR expression levels using Western blotting and any of the clinical, morphological, or immunophenotypic parameters recorded. However, there was a positive correlation between expression levels of GHR detected by Western blotting and the intensity of GHR immunoreactivity ($P < 0.05$).

Discussion

The RT-PCR, Western blotting, and immunohistochemistry results clearly demonstrate that GHR is expressed in human breast cancer. These findings agree with previous reports of GHR detected by RT-PCR in surgically re-

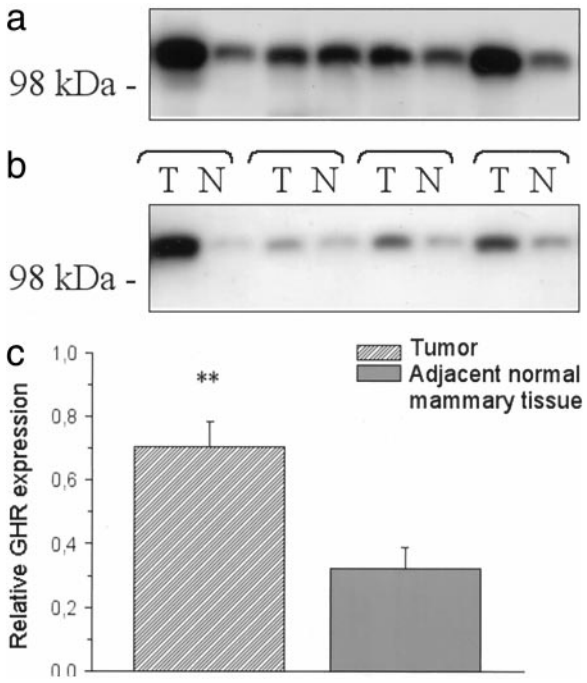

Figure 3. Western blots of GHR in human breast cancer tissues. **a:** Representative blot of GHR expression in eight breast cancer cases. **b:** Representative blot of GHR expression in tumor tissues and adjacent normal breast tissues. **c:** Relative GHR expression levels in breast cancer tissues are compared to relative GHR expression levels of adjacent normal mammary tissues ($n = 16$). Mean levels are lower in normal mammary tissues compared to cancer tissues ($P < 0.01$).

moved breast cancers and breast cancer cell lines,[9] or by using immunohistochemical[10,11] and *in situ* hybridization techniques.[11] Moreover, our immunohistochemical and Western blotting analyses demonstrated greater GHR expression in tumors than normal breast tissues. Thus, though adjacent normal breast tissues showed no or only weak immunoreactivity for GHR, all but a few carcinomas showed strong or moderate diffuse cytoplasmic staining. Also, the semiquantitative Western blotting results showed significantly increased GHR expression in tumors compared to adjacent normal breast tissues.

The predominantly cytoplasmic GHR immunoreactivity found in our study supports previous observations.[14–16] Weak nuclear staining was occasionally seen; this has also been previously reported[17] and is of interest since nuclear translocation of GHR may be induced by GH stimulation.[18] Another interesting observation was the detection of GHR in stromal cells of the breast carcinomas. The stromal compartment of the normal mammary gland is suggested to be the site of action for GH during normal mammary development in rodents.[19,20] GH, possibly via local insulin-like growth factor (IGF)-I production, acts synergistically with estradiol during normal mammary gland development in rodents[21,22] and primates.[23]

In this study of human breast cancer, there was an inverse correlation between GHR expression as determined by immunohistochemistry and proliferative activity (MIB-1 immunostaining) as well as tumor grade, whereas there was a positive correlation between GHR expression and progesterone receptor expression. These findings, as well as the previous detection of GHR in benign epithelial proliferations of the breast, all suggest that increased GHR expression does not correspond to aggressive biological behavior *per se*. Additional points of interest are the detection of GHR expression by RT-PCR and Western blotting, and strong immunoreactivity for GHR in both stromal and epithelial cells of a benign phyllodes tumor not included in this series.

Despite the significant correlation between GHR expression levels detected by Western blotting and immunostaining intensity, a significant correlation between progesterone receptor expression, tumor grade, and proliferative activity (MIB-1) was found only with immunostaining intensity. This apparent discrepancy could be explained by the relatively small number of cases analyzed using Western blotting.

The detection of an additional GHR cDNA lacking exon 3[24] (in this series, seen as the sole form in 7/36 tumors; 21/36 tumors expressed the full-length form; 8/36 expressed both forms) was originally believed to be due to an alternative splicing event. Subsequent studies suggested tissue-specific and individual-specific expression patterns.[25,26] The expression of GHR cDNA without exon 3, however, has recently been shown to be the result of a deletion of this part of the GHR gene, which, in turn, is due to a recombination of two retro-elements flanking exon 3.[27] Thus, our detection of the same GHR cDNA in tumors and normal breast tissues from the same individual supports these findings. Interestingly, the invasive ductal carcinomas of the breast with the exon 3-deleted GHR occurred in patients who were significantly younger than those who had full-length GHR (mean age 41 *vs.* 59 years). However, analysis of a larger series is required to draw any conclusions.

This study indicates that GH and GHR play a role in human breast cancer, but the exact mechanisms involved remain unclear. Autocrine/paracrine mechanisms have been suggested based on the detection of local GH production in normal breast tissue and carcinoma,[28] and transfection studies of GH expression plasmids in MCF-7 breast cancer cells in which increased growth response was recorded with autocrine/paracrine stimulation compared to exogenous GH administration.[8] Because GH-mediated stimulation of IGF-I production occurs in normal breast development, a similar phenomenon could occur in the development of breast cancer.

GH is closely related to PRL; although both hormones have specific receptors, GH also activates PRL receptors (PRLR) in primates.[29] Thus, in many studies of GH action, it is not possible to discriminate between GHR- and PRLR-mediated effects. Tumor-promoting effects have been shown to be mediated via PRLR and not GHR in mice.[30] The situation in human breast cancer, however, is probably more complex. Both GHR and PRLR are expressed in most human breast carcinomas[11,31] with higher expression levels of PRLR and GHR in breast cancers compared to normal breast tissues [32] (and this study). Local production of PRL, as well as GH, has also been demonstrated in breast carcinomas.[28,33] More potent mitogenic signals are suggested to be a result of PRLR activation rather than GHR activation.[7]

In conclusion, this study, which provides evidence of GHR expression and up-regulation in human breast cancer, indicates a role for GHR signaling in human breast cancer. To further understand the mechanisms involved, additional studies are necessary, including analyses of benign breast lesions and precancerous conditions.

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