Insulin-like Growth Factor 1 (IGF-1) and its Receptor mRNA Levels in Breast Cancer and Adjacent Non-neoplastic Tissue

Y.M. CHONG, S.L.J. WILLIAMS, A. ELKAK, A.K. SHARMA and K. MOKBEL

Department of Breast Surgery, St. George's Hospital and Medical School, University of London, London SW17 0RE, U.K.

Abstract. *Previous studies investigating the insulin-like growth factor 1 receptor (IGF-1R) expression in breast cancer tissue and adjacent non-neoplastic breast tissue (ANCT) have produced conflicting results. The IGF-1 and IGF-1R expression in pairs of breast cancer tissue and ANCT were investigated using RT-PCR and immunohistochemistry. The results of both methods were compared. Materials and Methods: IGF-1 and IGF-1R mRNA from 31 specimen pairs were estimated using RT-PCR. Immunohistochemistry for IGF-1R was carried out on 20 specimen pairs and the strength of staining was scored. Results: The mean relative IGF-1 mRNA level was lower in the cancerous tissue (mean 0.450±0.206) than in the ANCT (mean 0.632±0.384) (paired t-test, p=0.001). There was no measurable difference in relative IGF-1R mRNA levels in the cancerous tissue (mean 0.146±0.08) and the ANCT (mean 0.14608±0.108) (paired t-test, p=0.807). Using immunohistochemistry, there was no statistical difference (paired t-test, p=0.910) in IGF-1R staining scores between cancer (mean 1.93) and ANCT (mean 1.90). The comparison between the two methodologies showed no correlation (Pearson's Correlation Coefficient=–0.393). Discussion: It can be concluded that IGF-1 expression is lower in cancerous tissue, thus supporting a paracrine relationship between cancerous tissue and ANCT, which may be useful in the prevention, diagnosis and treatment of breast cancer. There was no difference in the expression of the IGF-1 receptor in both types of tissue, as proven by RT-PCR and immunohistochemistry. Conflicting results in previous studies may be due to the different methods used to measure IGF-1R expression.*

Insulin-like growth factor-1 (IGF-1) has been established as a growth factor with pro-mitogenic and anti-apoptotic effects on a variety of human cancers (1, 2). Although, it was originally discovered as part of the growth hormone axis, it is currently implicated in the malignant transformation of certain human cells, the promotion of tumour cell proliferation, the salvaging of cancer cells from natural or induced apoptosis (3) and the increase in metastatic potential of malignant tumours (4). These effects of IGF-1 are mediated through the IGF-type-1 receptor (IGF-1R), a tyrosine kinase receptor, and a series of intracellular pathway cascades. Increased circulating serum levels of IGF-1 are associated with an increased risk of breast cancer, especially in pre-menopausal women (5). The effects of IGF-1 on cancer cells are complex and are determined by an interplay involving the serum IGF-1 ligand, IGF-binding proteins (IGFBP), IGFBP proteases and the local paracrine or autocrine production of IGF-1 in the breast tissue (6). The relationship between IGF-1 and IGF-1 receptor expression in cancer breast tissue and adjacent non-cancer breast tissue (ANCT) has been previously investigated, with conflicting results (7 - 18).

In this study, the relationship between the expression of IGF-1 and IGF-1 receptor (IGF-1R) mRNA levels in breast cancer specimens and ANCT was investigated using TaqMan RT-PCR. Immunohistochemistry analyses, using IGF-1 R monoclonal antibody alpha-IR3, were carried out on some breast cancer samples, and the strengths of membrane immunostaining were compared with IGF-1 R mRNA levels.

Materials and Methods

Specimens. Institutional guidelines, including ethical approval and informed consent, were followed. Small pieces from the tumour and the ANCT were obtained after surgical excision and were Spap-frozen in liquid nitrogen and then stored at –80˚C. Paired specimens, consisting of tumour tissue (an invasive ductal carcinoma) and ANCT, were selected.

RNA extraction. The total cellular RNA was extracted from cancer tissue and ANCT using the Total RNA Isolation Reagent (Advanced Biotechnologies, Maryland, USA), according to the manufacturer's

Correspondence to: Kefah Mokbel, Department of Breast Surgery, St. George's Hospital, Blackshow Rd., London SW17 OQT, U.K.

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Table I. *Sequence of Taqman primers and probes for RT-PCR.*

Name of gene	Forward Primer	Reverse Primer	Probe
IGF-1	5-CTT CAG TTC GTG TGT GGA GAC AG-3	5-CGC CCT CCG ACT GCT G-3	5-TTT TAT TTC AAC AAG CCC ACA GGT ATG GC-3
IGF-1 Receptor	5-CTC CTG TTT CTC TCC GCC G-3	5-ATA GTC GTT GCG GAT GTC GAT-3	5-TGG CCC GCA GAT TTC TCC ACT CGT-3

protocol. Approximately 10 mg of cancer tissue was homogenised. A larger amount of ANCT (20 – 50 mg) was used as the high fat content made it difficult to obtain a sufficient RNA concentration for analysis. The quantification of RNA following treatment with DNase (Promega, Madison, USA) was carried out in triplicate using Ribogreen reagent (Molecular Probes Europe BV, Leiden, The Netherlands), according to the manufacturer's protocol.

RT-PCR. Taqman RT-PCR was carried out on each sample using the SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 7700 Sequence Detector (Perkin Elmer Applied Biosystems), according to the manufacturer's protocol. The RNA extraction and the preparation of the RT-PCR mix were carried out in different rooms. The preparation of the RT-PCR mix was carried out in an extraction hood.

The sequences for the forward and reverse oligonucleotide primers and probes were designed using Primer Express Software (PE-Applied Biosystems, Warrington, UK) and were intron spanning to prevent amplification of genomic DNA. The oligonucleotide sequences of these primers and probes are given in Table I.

The conditions for the reverse transcription were 50˚C for 2 min, 60˚C for 10 min and 92˚C for 5 min. The polymerase chain reaction step was carried out for 50 cycles for 20 sec at 92˚C and 25 sec at 62˚C. For negative controls, RNase-free water was used in the RT-PCR instead of RNA. For positive controls, 18S ribosomal mRNA was used. The relative levels of IGF-1 and IGF-1 R mRNA expression were calculated by comparing their readings (arbitrary units) to the readings of a housekeeping gene (18S ribosomal mRNA). The association between the relative levels of IGF-1 and IGF-1 R mRNA in cancer and ANCT was calculated using the paired *t*-test.

Immunohistochemistry. Immunohistochemistry (IHC) was carried out on 20 specimen pairs. Sections of the block were made and mounted on microscope slides. After peroxidase blocking, the slides were incubated with 20 μg/ml of IGF-1R monoclonal antibody, α IR 3 (purchased from Sigma Aldrich, Dorset, UK), which is specific to the alpha subunit of human IGF-1R. Following standard incubation with anti-mouse IgG antibody and peroxidase-conjugated streptovidin, the immune complex was visualised using DAB under the microscope. All steps were carried out according to the manufacturer's protocol. The strength of staining of IGF-1R was scored by an experienced histopathologist: score 1 (no staining), 2 (weak staining), 3 (moderate staining) and 4 (strongest staining) based on the subjective degree of membrane and cytoplasmic staining as well as the percentage of cells positively stained (19). In slides where the strength of staining was between 2 scores, a mid-score was given, *i.e.,* if the staining score was between 3 and 4, a score of 3.5 was given. For each specimen, the strength of IGF-1R was compared with the relative value of IGF-1 mRNA obtained through RT-PCR using Pearson's Correlation test.

Results

Demographics. All 31 patients underwent either a mastectomy or a wide local excision together with lymph node clearance or sampling during the period between January 1997 and December 1998. The median age was 69 and 7 patients were below the age of 50. Six patients were pre-menopausal. All tumour specimens contained invasive ductal carcinoma and 14 of these also contained ductal carcinoma *in situ* (DCIS). Eighteen cases were positive for estrogen receptor (ER) immunostaining. Histological grading of cancer tissue revealed 3 cases of grade 1, 10 cases of grade 2 and 17 cases of grade 3. The histology grade of one case could not be determined.

RT-PCR. All 31 specimen pairs had detectable levels of mRNA for both IGF-1 and IGF-1R. In the tumour specimens, the mean IGF-1 mRNA was 0.450±0.206. For the ANCT, the mean level was 0.632±0.384. Twenty-seven (87%) out of the 31 specimen pairs had higher levels of IGF-1 mRNA in the ANCT than in the cancer tissue (Figure 2A). In 15 cases, the relative value of IGF-1mRNA was more than 1.2 times and of these, 6 cases were more than 1.5 times. The results of the paired *t*-test showed that the higher levels of IGF-1 mRNA in normal adjacent tissue was statistically significant ($p=0.001$).

For IGF-1R mRNA, a mean of 0.146±0.083 was found for cancer tissue, while a mean mRNA level of 0.14608±0.108 was found for the ANCT (Figure 2B). Eleven specimen pairs had IGF1-R mRNA relative levels higher in the tumour than that of the corresponding normal adjacent tissue and in 6 cases the relative mRNA levels were higher by more than 1.5 times, however, this difference was not statistically significant ($p=0.807$). In addition, another 11 pairs showed the opposite relationship, thus suggesting a universal expression of IGF-1R in cancer and normal tissue.

Immunohistochemistry. Due to limited tissue specimens, IHC was carried out successfully in only 23 tumour

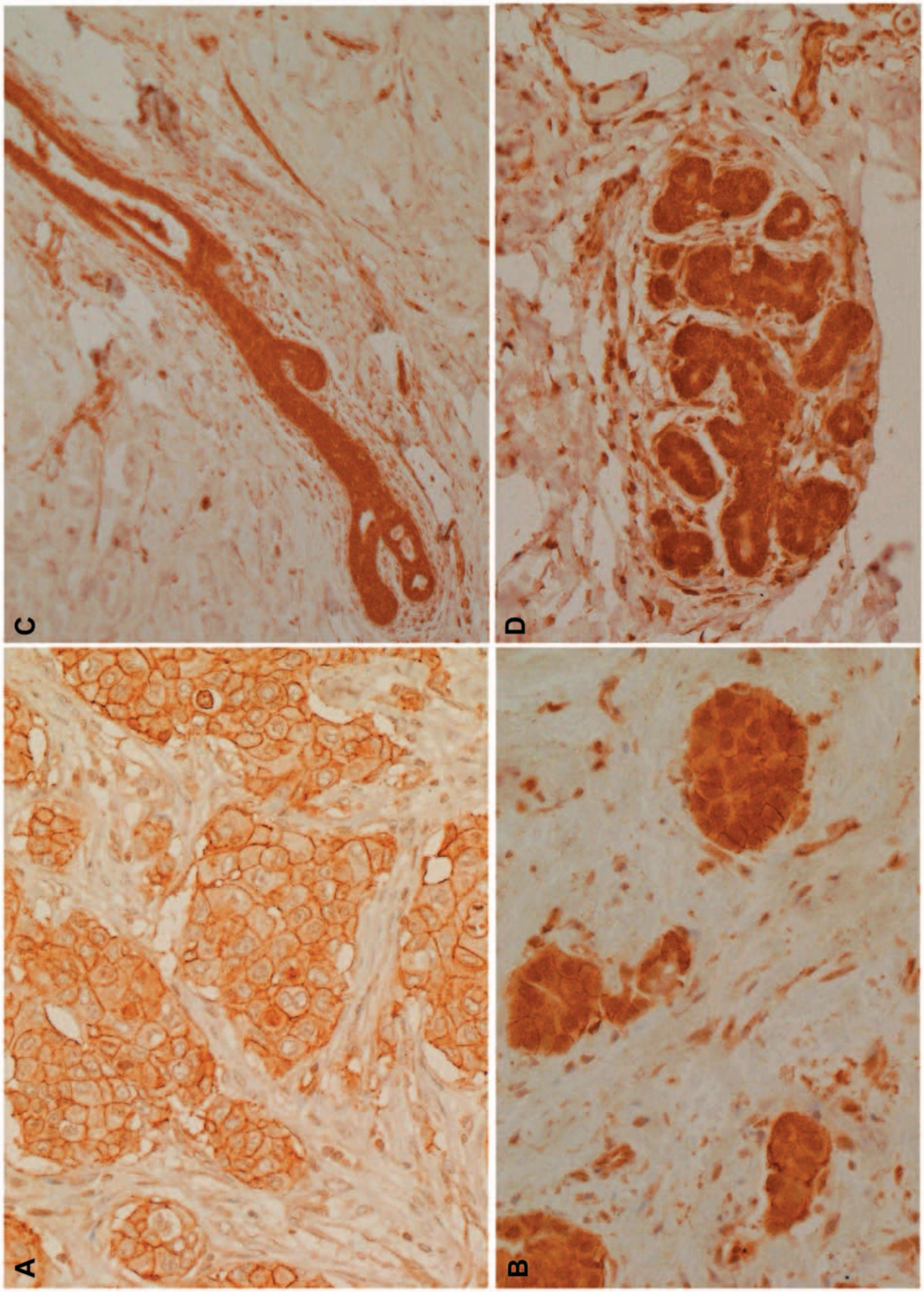

Figure 1. *Immunostaining of IGF-1 Receptor with α IR 3 monoclonal antibody. In invasive ductal carcinoma (A) shows strong membrane staining with a staining grade of 3/4, while (B) shows both strong membrane and cytoplasmic staining with a staining score of 3/4. Adjacent normal breast tissue (C) shows a normal duct with strong staining of 3/4 and (D) shows a normal lobule with similar staining.*

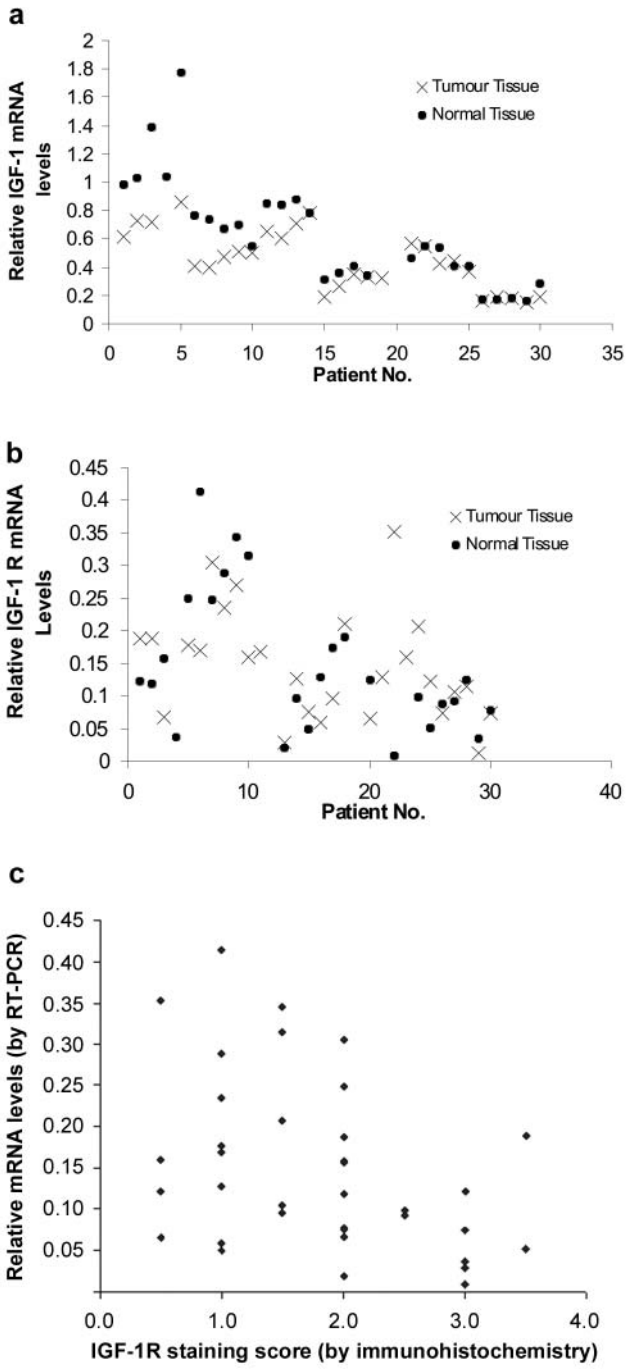

Figure 2. *a) Scatterplot of patient code no. vs. relative IGF-1 mRNA levels. b) Scatterplot of patient code no. vs. relative IGF-1 R mRNA levels. c) IGF-1R protein against IGF-1 R mRNA in normal and cancer tissue.*

specimens and 22 ANCT. When paired, there were only 20 complete pairs of specimens available for comparison. Higher IGF-1-R staining in the cancer tissue was evident in 6 pairs, while another 6 pairs showed the opposite relationship. Eight pairs showed similar IGF-1R staining in both specimens (Figure 1). The mean of the IHC scores was 1.93 and 1.90 for cancer tissue and ANCT, respectively, and a paired *t*-test showed that this small difference was not statistically significant ($p=0.910$).

Correlation between IHC and RT-PCR in measuring IGF-1R expression. It was presumed that higher levels of IGF-1R mRNA would translate into a higher density of IGF-1R protein expression and, hence, a higher strength of IGF-1 R immunostaining. However, of the 9 specimen pairs which had higher IGF-1R mRNA in the cancer tissue according to RT-PCR, only 4 pairs were in agreement with the IHC results. In the 5 specimen pairs where IGF-1R mRNA levels were higher in the normal tissue, only one pair showed a similar pattern. When comparing results of both methodologies, by Pearson's Correlation Coefficient (Figure 2C), there was no positive correlation between the results for IGF-1R mRNA and protein expression (correlation coefficient=–0.393).

Discussion

The GH/IGF-1 axis has been implicated in breast cancer for the past 30 years since hypophysectomy was used in the treatment of metastatic breast cancer (20). It has become evident that the breast cancer cell lines can be stimulated by IGF-1 and that alterations of the components of the IGF-1 system, including the IGF-1 hormone itself, IGF-1R, its binding protein (IGF BP 1 to 5) and binding protein proteases, could alter the behaviour of the breast cancer cell. IGF-1 ligand binding and overexpression of its receptor have been reported to stimulate mitosis (2, 21), induce malignant transformation of normal cells to cancer cells *in vitro* and *in vivo* (2, 21, 22), oppose apoptosis (1, 23, 24), promote metastasis (4) and contribute to the development of resistance to radiation treatments (3).

IGF-1 can affect breast cells through 3 mechanisms. Firstly, endocrine mechanisms, in which serum IGF-1 (predominantly produced by the liver and other tissues) can stimulate breast cancer cells, have been implicated in leading to a higher incidence of premenopausal breast cancer (5, 25). The second, or paracrine mechanism refers to adjacent normal breast and stromal cells including fibroblasts (26), which can produce IGF-1 to stimulate neighbouring breast cancer cells (8). The third is an autocrine mechanism in which the tumour cell produces its own IGF-1.

This is the third reported study to use real-time RT-PCR to directly measure IGF-1 and IGF-1 R mRNA levels in normal and canceous human breast tissue. Our results indicate that higher levels of IGF-1 mRNA are produced by ANCT than by tumour tissue. This finding favours a paracrine relationship between the surrounding normal

breast tissue and breast cancer cells and this information may be useful in the prevention, diagnosis and treatment of breast cancer. Other experimental evidence now supports this hypothesis (7-9, 12, 13). Voskuil *et al.* also used RT-PCR to show that higher levels of IGF-1 are expressed in normal tissue than in tumour tissue. Furthermore, the authors showed a positive correlation between IGF-1 and -2 in breast tissue from patients with a family history of breast cancer (7). Gebauer *et al.* used RT-PCR to deduce that IGF-1 was produced predominantly in the stromal parts of cancer tissues when they found IGF-1 mRNA present in cancer breast tissue specimens but not in cancer cell cultures (12). Similar results were reported by Yee *et al.* (8) and Cullen *et al.* (13) using *in situ* hybridization.

ANCT may be composed of non-cancer breast cells and stromal cells, including fibroblasts and vascular elements. It is still unclear as to which cell types mainly contribute to the higher levels of IGF-1 mRNA. Most studies were unable to determine the percentage of stromal tissue present in tumours and normal specimens and their contribution to the expression of IGF-1(7). Some studies have used *in situ* hybridisation techniques to localise mRNA expression and found it to be limited to stromal cells (8, 27). Other investigators have demonstrated that it is the stromal-epithelial interaction that plays a central role in malignant progression and metastasis in breast cancer (8, 28). Stromal cells can regulate breast cancer cell proliferation, migration and invasion by cell-cell contact involving the extracellular matrix (ECM) (29, 30), by producing cytokines such as IL-6, IL-11 and LIF (31, 32) or by producing other growth factors such as EGF, PDGF, HGF, bFGF and TGF-alpha and beta (33, 36). Particular attention has been given to the role of fibroblasts in this paracrine mechanism. Kees *et al.* showed that conditioned serum-free media, derived from primary breast fibroblasts, stimulated the proliferation of all breast cancer cell lines. They also showed that conditioned media derived from fibroblasts taken from malignant breast tissue significantly stimulated cell line proliferation to a greater extent than fibroblasts obtained from normal breast tissue taken from the same specimens (reduction mammoplasties and benign fibroadenomas). Some attempts to identify the fibroblast-derived growth factor in this paracrine relationship have concluded that this is the IGF-1 and/or IGF-II system (10, 26). However, other studies involving co-culturing of cancer-associated and normal fibroblast cells with breast cancer cells lines revealed conflicting results, with some showed a growth promotion while others showed an inhibitory effect (26). It is still unclear what role tumour-associated fibroblasts play in breast cancer progression.

In this study, there was no measurable difference in levels of IGF-1R mRNA in cancer tissue and adjacent non-cancer tissue according to both RT-PCR and immunochemistry. This is in not in agreement with some previous studies which showed that breast cancer tissues generally express the IGF-1 receptor at higher levels (10, 11, 37) and are more activated with respect to normal or benign breast tissue (18). However, these studies used a radio-immunoassay (16-18) and IHC (15) to measure IGF-1R levels and, thus, making direct comparisons between results which utilise wholly different methods of detection difficult. Happerfield *et al.* (14) reported that radio-immunoassay experiments involving radiolabelled IGF-1 ligands show cross-reactivity with other receptors and produced falsely high rates in tumours. There may also be a difference between the expression of IGF-1 R mRNA and the actual protein expression due to post-translational modification. In Voskuil's *et al's* study (7), the levels of IGF-1 R mRNA were also higher in normal tissue than in cancer tissue and some IHC studies (14, 38, 39) showed no difference in the IGF-1 R levels in either type of tissue. Happerfield *et al.* (14) also reported a strong IGF-1 R immunostaining in normal epithelial cells adjacent to tumours as well as in normal and fibroadenoma samples not related to breast cancer. Schnarr *et al.* (38) reported a marked expression of IGF-1 R in ducts and lobules of normal breast tissue as well as in ductal and lobular tumours.

The difference in results obtained by different scientific methods of measuring IGF-1 R expression has often led to inconsistent results regarding IGF-1 receptor expression in cancer and its surrounding tissue. The direct comparison of each specimen in this experiment failed to show a positive correlation between the IHC scores of IGF-1 R relative to its mRNA levels and this further emphasizes that such findings should be compared using the same modalities. We used the mouse receptor monoclonal antibody α IR 3, which has been used in several similar studies (14, 39). In Happerfield's *et al's* study (14), the authors mentioned that the IGF-1 receptor molecule may be modulated between the cytosol and the cell membrane with the cytoplasmic IGF-1 R representing bound, internalised receptors. It is possible that, depending on the grade and pathological features of the invasive tumour, there may be a failure to transport IGF-1 R to the surface and, hence, it cannot be concluded that the overall production of IGF-1R within the cell always correlates with the IGF-1 R mRNA. Many other intracellular signalling pathways may modify the final protein product (14). More studies are needed to clarify whether the IGF-1 receptor is deemed as a potential prognostic marker.

In conclusion, we have demonstrated, by RT-PCR, that the IGF-1 is produced in higher amounts in tissue adjacent to breast cancers and this reinforces the theory that a paracrine relationship exists within the local environment of cancerous breast tissue. The same method used to measure the IGF-1 R levels did not support the general belief that breast cancer cells express higher levels of IGF-1 receptor

than their adjacent normal tissue, but this may be due to different methods of measuring IGF-1 R expression.

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