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Original Paper

Expression of Insulin-like Growth Factor I (IGF-I) in Female Breast Cancer as Related to Established Prognostic Factors and Long-term Prognosis

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The expression of insulin-like growth factor I (IGF-I) was analysed immunohistochemically in a series of 211 breast cancers with special emphasis on its relationship to conventional prognostic factors and long-term prognosis. Altogether, IGF-I was expressed by the tumour cells in 91% of the breast carcinomas, and by stromal cells in 29%. The expression of IGF-I in cancer cells was weakly related to a high proportion of intraductal growth ($P = 0.032$), distinct tumour margins ($P = 0.048$) and high S-phase fraction ($P = 0.074$). In a univariate analysis, IGF-I expression in cancer cells was significantly related to a high survival probability in the entire cohort ($P = 0.0144$) as well as in the axillary lymph node positive tumours ($P = 0.0286$). Alternatively, expression of IGF-I in the stromal cells was related to metastasis at the time of diagnosis ($P = 0.05$), tumour diameter ($P = 0.04$), DNA ploidy ($P = 0.07$) and nuclear pleomorphism ($P = 0.025$), but it was without prognostic significance in a univariate analysis ($P > 0.1$). In a multivariate analysis, the conventional prognostic factors were superior to IGF-I expression in predicting the disease outcome, albeit expression of IGF-I in tumour stroma showed some independent prognostic significance in axillary lymph node negative tumours. The results suggest that IGF-I expression is related to malignant histopathological features in breast cancer, and expression of IGF-I has independent prognostic significance in the early phases of the disease.

Key words: IGF-I, breast cancer cells, stromal cells, prognosis, immunohistochemistry
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INTRODUCTION

INSULIN-LIKE growth factors (IGFs; IGF-I and IGF-II) consist of a family of hormones with structural homology to insulin. They regulate proliferation and differentiation of various types of cells, and are capable of exerting insulin-like metabolic effects. However, unlike insulin, they are produced by most tissues of the body and accordingly are abundant in the circulation. Thus, IGFs have the potential to act via endocrine as well as autocrine and/or paracrine mechanisms.

IGF-I (also known as somatomedin C) mediates many of the trophic effects of growth hormone, and it is synthesised predominantly in the liver [1]. The mitogenic effect of IGF-I is attributed to its ability to transfer the cells from the G_1-phase to the S-phase [2]. Distinct receptor for IGF-I has been described and cloned. The type I IGF receptor is homologous to the insulin receptor, contains a cytoplasmic tyrosine kinase domain and binds both IGF-I and IGF-II [3, 4]. Studies with blocking antibodies to the receptor suggest that it mediates many, if not all, of the known effects of IGF-I and IGF-II [5].

Binding data and growth studies also indicate that breast cancer cells express receptors for IGFs [6–8]. There are also extracellular IGF-binding proteins (IGFBPs) in the circulation, and to date, six different IGFBPs have been cloned [9]. Human breast cancer cell lines also express and secrete large quantities of IGFBPs (at least five distinct IGFBPs have been found), and the secretion seems to be related to the oestrogen receptor (ER) content [10]. Some species may act to inhibit the mitogenic effects on the IGFs [9].

Several lines of evidence suggest that the IGFs may be important regulators of breast cancer proliferation. Insulin and IGF-I have been reported to stimulate the growth of several human breast cancer cell lines [5, 11–14], and several human cancers have been reported to express IGF-I. Accordingly, IGF-I has been postulated to function as an important autocrine growth factor in transformed cells [13, 15–20]. Additionally, IGF-I has been shown to be synthesised by normal fibroblasts in culture [21]. In the human fetus, *in situ* hybridisation studies have localised IGF-I mRNA expression primarily in stromal cells [22]. Expression of IGF-I receptor has been related to good

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prognosis in hormone-dependent breast cancer [8], but IGF-I overexpression alone showed no effect on the prognosis [23].

In the present study, we examined the expression of IGF-I in tumour cells and in stromal connective tissue in a series of 211 female breast carcinomas using immunohistochemical staining. Special emphasis was placed on the associations of IGF-I expression to the known indicators of cell proliferation and cancer differentiation as well as on its prognostic significance during long-term follow-up.

MATERIALS AND METHODS

The present series consists of 211 women with breast cancer treated and followed-up at the Kuopio University Hospital during 1968–1990. Of all the patients, complete follow-up histories and histopathological specimens from the primary tumours were available for the current analysis. Axillary lymph nodes were examined histologically in 183/211 (87%) of cases, a clinical judgement being available in the remaining cases. Tumour size was recorded as the largest tumour diameter in fresh mastectomy specimens based on the estimation by the operating surgeon. The follow-up of the patients was conducted at 3-month intervals during the first year, at 6-month intervals during the next 2 years and annually thereafter. The pertinent data of the patients are summarised in Table 1.

Histological methods

The tissue specimens from the primary tumours were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin. Histological grading [24] and typing [25] of all tumours was completed, as detailed previously. Mitotic figures were counted using a dual-headed microscope by two observers with an objective magnification of ×40 (field diameter 490 μm) [26]. The volume-corrected index method was used which expresses the number of mitotic figures/mm^2 of the neoplastic epithelium in the section [26].

Flow cytometry, nuclear morphometry and steroid receptor assay

Flow cytometry and nuclear morphometry were completed as previously described [27, 28]. DNA index was available in 190/211 (90%) of cases and S-phase fraction (SPF) could be analysed in 129/211 (61%) of cases. Tumours with a DNA-index value ≤1.05 were considered diploid, and those with a DNA index >1.05 were aneuploid. Of the nuclear morphometric features, the mean nuclear area and its SD was used in this study. The sex steroid receptor content (ER, PR) was assayed biochemically using a charcoal-dextran method detailed previously [29], and the cut-off level of receptor positivity was 10 fmol/g cytosol protein.

Table 1. Patients' characteristics

Characteristic	
Number of patients	211
Age (years), Mean (SD)	57.1 (12.4)
Range	27–86
Follow-up time (years), Mean (SD)	14.1 (3.3)
Range	10.0–22.0
Diameter (mm), Mean (SE)	36.8
Range	0–150.0
Metastasis at diagnosis	
No	198
Yes	13
Axillary lymph node stage*	
Negative	85
Positive	98

*Histological examination.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 5-micron sections were deparaffinised in xylene, rehydrated in graded alcohol and treated with 0.5% pepsin in 0.01 N HCl for 45 min at 37°C. Slides were washed twice with 0.1% bovine serum albumin (BSA) in PBS for 5 min, treated with 5% hydrogen peroxide for 5 min. Slides were incubated for 15 min with 1.5% normal horse serum in PBS (pH 7.2), containing 1% BSA and 0.01% natrium azide followed by an overnight incubation with monoclonal mouse antibody to human IGF-I (SEROTEC, Oxford, U.K.) diluted in 1:100 PBS/1% BSA/0.01% natrium azide at 4°C. Slides were washed twice with PBS/0.1% BSA for 5 min and incubated for 30 min with horse antimouse biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, California, U.S.A.) diluted 1:200 in PBS/1% BSA/0.01% natrium azide. Slides were then washed with two changes of PBS/0.01% BSA for 10 min and incubated for 40 min in preformed avidin-biotinylated peroxidase complex (ABC, Vestastain Elite kit, Vector). Slides were washed twice with PBS/0.1% BSA for 5 min, developed with diaminobenzidine tetrahydrochloride substrate (Sigma Chemical Company, St Louis, Missouri, U.S.A.), counterstained with Mayer's haematoxyline, dehydrated, cleared and mounted with DePex (BDH Ltd, Poole, U.K.). Both positive and negative controls were processed in each staining, and they were shown to be positive or negative, respectively.

Scoring of immunoreactivity

Slides were examined on light microscopy. If at least one focus of positively stained malignant cells was observed, the staining was considered positive. The positive reactions were scored into three grades (1, weak; 2, moderate; 3, intense) according to the intensity of the staining. Those with no positivity for IGF-I were scored as negative. Tumours with weak just identifiable positivity of cancer cells were scored as weak (+) (Figure 1a) and tumours with a strong distinct staining of the cancer cells were scored as strong (+++) (Figure 1b). Tumours with intermediate staining intensity were scored as moderate (++) expressors. While grading the stroma, the same principles were applied.

Statistical analysis

In basic statistical calculations, the SPSS/PC+ program was used in an IBM computer and the statistical tests used are indicated in the results when appropriate. Frequency distributions were tested by the chi-square test and Yate's correction was applied when appropriate. The differences between the means of continuous variables were tested by analysis of variance. The univariate survival analysis (logrank analysis, SPSS-X) was based on life-table method with the statistics of Lee and Desu [30]. Several group limits were tested for the expression of IGF-I in univariate survival analysis. Because of this statistical approach, the results of univariate survival analysis have only a guiding character and not a confirming character. However, currently there are no generally accepted cut-off points for the analysis of the expression of IGF-I in breast cancer. Multivariate survival analysis [31] was carried out with the BMDP (2L)

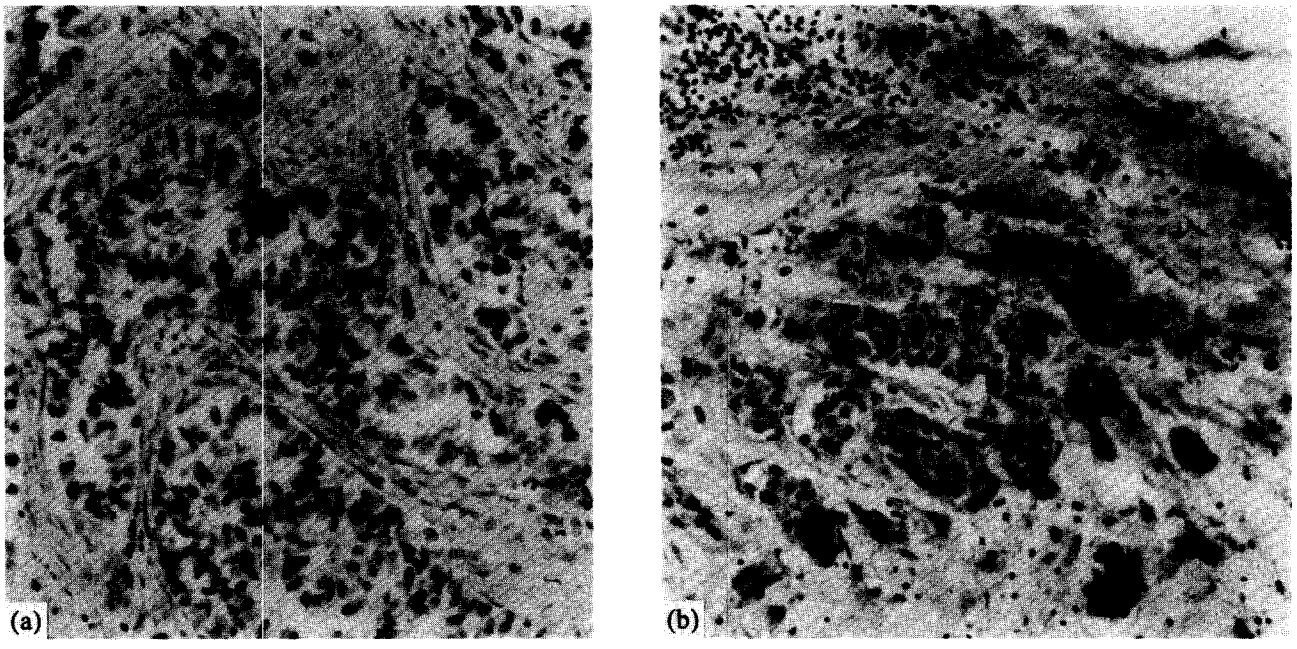

Figure 1. (a) The expression of IGF-I was weak (+) in 72 of cases in breast cancer. (b) The expression of IGF-I was strong (+++) in 42 of cases in breast cancer.

program package in a stepwise manner and continuous variables were used as absolute numbers in this analysis. Enter limit was $P < 0.1$ and removal limit was $P > 0.15$. Multivariate analyses included only cases with histologically confirmed axillary lymph node status. When the recurrence-free survival was analysed, the cases ($n = 13$) with distant metastasis at diagnosis were not included in the analysis (univariate/multivariate analysis).

RESULTS

Breast cancer cells

Altogether, 191/211 (91%) of the tumours were positive for IGF-I. In 72/211 (34%) of cases, the expression was weak (+) (Figure 1a), in 77/211 (36%) expression was moderate (++), and in 42/211 (20%) expression was strong (+++) (Figure 1b). IGF-I expression was independent of the histological type (chi-square $P = 0.7$), histological grade ($P = 0.8$), nuclear pleomorphism ($P = 0.16$), degree of tubule formation ($P = 0.4$), ER content ($P = 0.7$), PR content ($P = 0.8$), tumour diameter ($P = 0.7$), DNA ploidy ($P = 0.5$), mitotic index ($P = 0.2$) and proportion of intraductal growth ($P = 0.2$). Metastasis at the time of diagnosis was independent of IGF-I whereas axillary lymph node status, margin formation and SPF were weakly related to expression of IGF-I (Table 2). IGF-I expression was also more common in tumours with a marked intraductal component (71/76) (93%) (IGF-I expression 1, 2, 3) than in tumours without intraductal growth (112/127) (88%) (chi-square $P = 0.032$) (IGF-I expression 0). The expression of IGF-I was independent of the morphometrically analysed nuclear factors. Lack of IGF-I expression I in the cancer cells was associated with a low survival probability in the entire cohort (Figure 2) as well as in axillary lymph node positive tumours (ANP) (Figure 3), in contrast to axillary lymph node negative tumours (ANN), where the expression of IGF-I was not a significant prognostic factor ($P = 0.32$). Recurrence-free survival was not significantly related to expression of IGF-I in any of the subcategories ($P > 0.2$).

Table 2. Expression of IGF-I in breast cancer cells as related to other prognostic factors

Variable	Number of cases	Intensity of staining				P*
		Negative	Weak	Moderate	Intense	
Margin formation						
No	25	5	8	6	6	0.048
Questionable	174	13	59	67	35	
Definite	4	2	1	1	0	
Metastasis at diagnosis						
No	198	18	70	73	37	0.216
Yes	13	2	2	4	5	
Axillary lymph node						
Negative	85	4	28	32	21	0.046
Positive	98	13	38	35	12	
SPF ≤ 7%	82	4	32	26	20	0.074
SPF > 7%	47	5	17	21	4	

*Chi-square test; SPF, S-phase fraction.

Stromal connective tissue

IGF-I expression was found in the stromal connective tissue of 62/211 (29%) tumours. In 59/211 (28%) cases, the expression was weak, in 2/211 (0.9%) it was moderate and in 1/211 (0.5%) of cases the expression was strong. Stromal expression of IGF-I was independent of the histological type (chi-square $P = 0.5$), tumour grade ($P = 0.1$), degree of tubule formation ($P = 0.9$), proportion of intraductal growth ($P = 0.6$), margin formation ($P = 0.9$), ER content ($P = 0.5$), PR content ($P = 0.4$), SPF ($P = 0.3$), mitotic index ($P = 0.3$) and axillary lymph node

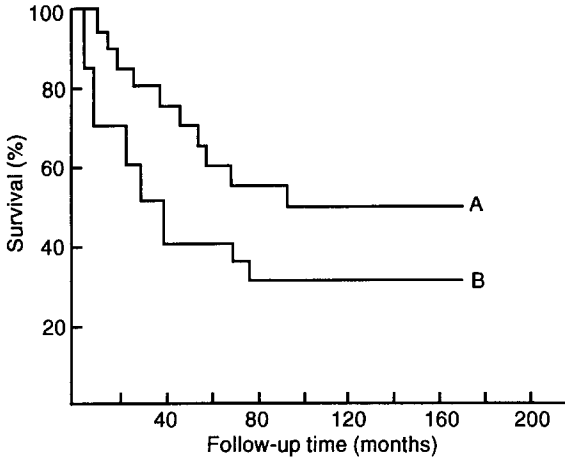

Figure 2. Survival of the patients categorised according to the expression of IGF-I. The difference in survival between the curves was significant ($\chi^2 = 5.9$, $P = 0.0144$). Curve A: Expression of IGF-I (weak, moderate or strong), $n = 190$. Curve B: IGF-I negative, $n = 20$.

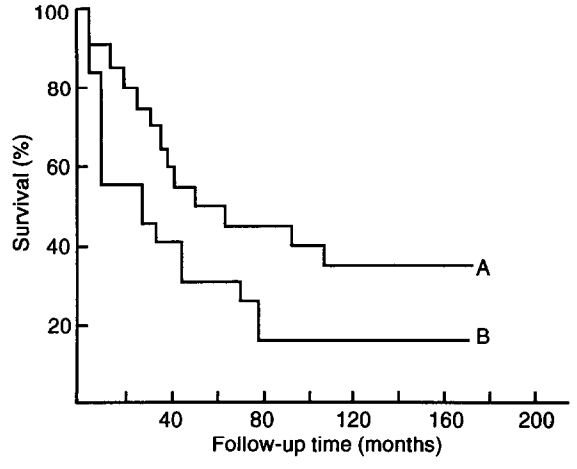

Figure 3. Survival of the patients with ANP tumours categorised according to the expression of IGF-I. The difference in survival between the curves was significant ($\chi^2 = 4.8$, $P = 0.0286$). Curve A: Expression of IGF-I (weak, moderate or strong), $n = 84$. Curve B: IGF-I negative, $n = 13$.

status ($P = 0.1$). Moderate and intense expression of IGF-I was confined to tumour diameter <2.0 cm ($P = 0.04$) and to DNA diploidy ($P = 0.07$). There was a significant relationship between stromal expression of IGF-I, metastasis at diagnosis and nuclear pleomorphism, as shown in Table 3. Nuclear morphology showed no correlation to expression of IGF-I in the tumour stroma. Similarly, expression of IGF-I was not significantly related to survival or recurrence-free survival ($P > 0.2$).

Multivariate analysis of prognostic factors

Independent predictors of recurrence-free survival in the entire cohort were: axillary lymph node status [β(s.e.) = 0.542(0.225), RR = 1.72, $P = 0.003$] and tumour diameter [β(s.e.) = 0.009(0.005), RR = 1.01, $P = 0.071$]. In ANN tumours, diameter [β(s.e.) = 0.022(0.009), RR = 1.02, $P = 0.082$] and expression of IGF-I in the tumour stroma [β(s.e.) = 0.574(0.286), RR = 1.77, $P = 0.056$] were signs of a short recurrence-free survival. In ANP tumours, the M/V index [β(s.e.) = 0.017(0.007), RR = 1.02, $P = 0.036$] predicted recurrence-free survival. Cancer-related survival was related to axillary lymph node status [β(s.e.) = 0.899(0.234), RR = 2.45, $P < 0.001$], tumour diameter [β(s.e.) = 0.016(0.004), RR = 1.02, $P < 0.001$] and M/V index [β(s.e.) = 0.013(0.005), RR = 1.01, $P = 0.028$]. In ANN tumours, tumour diameter [β(s.e.) = 0.031 (0.009), RR = 1.03, $P = 0.005$] and stromal expression of IGF-I [β(s.e.) = 0.608(0.320), RR = 1.83, $P = 0.07$] predicted a poor disease outcome. In ANP tumours, independent predictors were the M/V index [β(s.e.) = 0.022(0.007), RR = 1.02, $P = 0.003$] and tumour diameter [β(s.e.) = 0.013(0.005), RR = 1.01, $P = 0.011$].

Table 3. Expression of IGF-I in stromal cells as related to other prognostic factors

Variable	Number of cases	Intensity of staining: Negative	Weak	Moderate	Intense	P*
Metastasis at diagnosis						
No	198	142	54	1	1	0.050
Yes	13	7	5	1	0	
Nuclear pleomorphism						
Some	8	6	1	1	0	0.025
Moderate	120	81	38	0	1	
Severe	75	55	19	1	0	

*Chi-square test.

DISCUSSION

The present study analysed the expression of IGF-I in the cancer cells and in the stromal connective tissue of 211 female breast carcinomas with special emphasis on its role in tumour proliferation and disease progression. Previous reports on this subject are highly controversial. In one study, IGF-I was shown to be expressed by stromal cells only, but not by breast cancer cells or cell lines [7]. In our study, however, IGF-I expression in breast cancer cells was found in 91% of tumours, and in 29% of cases, IGF-I was also expressed by the stromal cells. This is partly contradictory to the findings of Mizukami and associates [23] who reported that IGF-I was absent in stromal fibroblasts of benign or malignant breast tissues, and exclusively confined to the epithelial cells (both benign and malignant).

In the present study, IGF-I expression was independent of the histological type or differentiation of breast tumours. However, tumours without axillary lymph node metastasis showed more intense expression of IGF-I than axillary node positive tumours. Similarly, tumours with a low SPF, significant proportion of intraductal growth and margin formation showed more often strong or moderate expression of IGF-I than those highly invasive tumours with a high SPF and without clear tumour demarcation from the surrounding tissues. This suggests that expression of IGF-I in breast epithelial cells indicates a lower malignant potential than the lack of such an expression. This is concordant with the results of survival analysis, where the lack of IGF-I expression was associated with an unfavourable prognosis. This is substantiated by some previous observations

on the presence of IGF-I receptor in breast cancer epithelium being associated with a favourable disease outcome [7]. However, in another previous immunohistochemical study, no correlation could be established between the histopathological features, prognosis and expression of IGF-I [23]. These variable results most probably reflect some differences in the intrinsic malignancy of breast carcinomas in different series studied.

Several groups have attempted to analyse the importance of IGF-I in the regulation of breast cancer growth by blocking its binding to the IGF-I receptor with the monoclonal antireceptor antibody (α-IR-3) [32]. Sex steroid receptor content of the cancer cells was not related to expression of IGF-I, although some studies have shown that IGF-I is the most potent growth factor for E2-dependent cell lines [12], and 17-β-oestradiol can influence on the effects of IGF. Expression of IGF-I receptor in breast tumours has also been associated with good prognosis in ER-positive cancer [8]. Moreover, Mizukami and colleagues [23] reported a positive correlation between ER content and IGF-I expression which could not be confirmed by the present results.

IGF-I expression in the stromal cells was significantly related to early metastasis (at diagnosis) in that the tumours with distant metastasis were IGF-I negative or expressed IGF-I only weakly. Similarly, nuclear pleomorphism, small tumour diameter and DNA aneuploidy were related to IGF-I negativity in the tumour stroma. These observations suggest that IGF-I expression might have its greatest significance in the early phases of breast cancer development, as is known to be the case with other growth regulating stimuli in breast cancer [33]. Previous analyses have already shown that the tumour–stroma interactions [34] may influence the growth rate of breast neoplasms, and stroma may modulate the growth rate of cancer cells by secreting paracrine and autocrine growth factors. It seems likely that IGF-I is one of these mediators [32].

Although the expression of IGF-I in tumour stroma was not a significant prognostic factor in univariate analysis, expression of IGF-I was related to poor outcome in ANN-tumours in a multivariate analysis. These contradictory prognostic results (ANN versus ANP) are not entirely unexpected, however. In small local tumours, the significance of various growth factors and factors related to the growth environment of tumours are probably more important than in disseminated tumours. As the tumours become invasive and disseminate, their genetic instability increases and their growth is also more independent of growth factors. A multivariate analysis of the prognostic factors clearly showed that the standard prognostic factors were superior to the IGF-I expression in predicting the recurrence-free survival and overall survival. The value of the standard prognostic factors in this same cohort of patients has been recently confirmed and discussed in detail elsewhere [27, 28, 34, 35].

In conclusion, expression of IGF-I by the cancer cells and stromal connective tissue is weakly related to several histopathological features in female breast cancer. Expression of IGF-I by the stromal cells in ANN tumours has also some prognostic value in that IGF-I negative tumours have a more favourable outcome. This might indicate that IGF-I in the tumour stroma acts as a paracrine growth promoter for breast cancer cells thus increasing the tumour malignancy.

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