

ORIGINAL ARTICLE

Insulin-like growth factor system gene expression in women with type 2 diabetes and breast cancer

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Background/Aims: A twofold increased risk for breast cancer has been reported recently for women with late onset diabetes. Most studies showed that there were differences in serum concentrations of insulin-like growth factors and related proteins between women with and without diabetes who have breast cancer. This study investigated the expression of these markers at the cellular level in a cohort of women with and without type 2 diabetes who underwent biopsy because of a breast lump.

Methods: Relative quantitative analysis of specific mRNA sequences was performed after extraction and reverse transcription polymerase chain reaction amplification from formalin fixed and paraffin wax embedded tissues. Sixty seven breast surgical specimens from women with and without diabetes who did not have cancer and from women with and without diabetes who did have cancer were studied for insulin-like growth factor I (IGF-I), the IGF-I receptor (IGF-IR), insulin-like growth factor binding protein 3 (IGFBP-3), and oestrogen receptor 1 gene expression.

Results: The expression of IGF-I and IGF-IR was significantly lower in the cancer groups, whereas there was no significant difference for IGFBP-3 between women with and without cancer. Moreover, there was a good correlation between the expression of IGF-I and IGF-IR in women without cancer: this link was still present in breast tissue from patients with diabetes and cancer, whereas it was lost in patients without diabetes but with cancer.

Conclusions: These differences in IGF-I/IGF-IR expression could contribute to the increased risk for breast cancer in women with type 2 diabetes.

Recent epidemiological studies have shown an association between diabetes mellitus and breast cancer: there is a twofold increased risk for breast cancer in women with late onset diabetes.^{1–3} The hyperinsulinaemia seen in late onset diabetes may promote breast cancer because insulin is a growth factor for human breast cancer cells.^{4,5} Some of the mitogenic effects of insulin might result from the structural similarities between insulin and insulin-like growth factor I (IGF-I).⁶ The structurally and functionally insulin related IGF-I polypeptide is a systemic hormone with mitogenic and anti-apoptotic potential, which can powerfully affect the growth of breast cancer cells, and has been proposed to be involved in breast tumorigenesis.^{7–9} IGF-I is synthesised mainly in the liver, but also in the mammary gland, and acts as an autocrine/paracrine growth factor; the activity of IGF-I involves its cell surface receptor (IGF-IR) and IGF-II.^{10–13} Most of the circulating IGF-I is bound with high affinity to IGF binding protein 3 (IGFBP-3), and only free IGF-I is able to bind to IGF-IR and thus exert its effects.¹⁴ Therefore, IGFBP-3 seems to play a regulatory role in the proliferation of mammary cells because of its inhibitory effect on IGF-I.^{15–18} Amplification of IGF-I activity may take place at the tissue level in women with chronic hyperinsulinaemia, as a result of altered plasma concentrations of IGFBPs.⁴ Many studies have shown that both the insulin receptor and IGF-IR are overexpressed in most human breast cancer cells and in normal tissues from patients with type 2 diabetes.^{19–22} Furthermore, evidence exists of a complex crosstalk between the IGF system and the oestrogen receptor (ESR)—ligand bound ESR enhances both IGF-I and IGF-IR transcription, and IGF-I, in turn, causes transcriptional activation of ESR.^{23–27} Little is known about how these events are affected by the diabetic condition, in which an alteration of the normal ESR status seems to occur.²⁸

“Insulin-like growth factor I is a systemic hormone with mitogenic and anti-apoptotic potential, which can powerfully affect the growth of breast cancer cells, and has been proposed to be involved in breast tumorigenesis”

With this background in mind, we studied the expression of IGF-I, IGF-IR, IGFBP-3, and ESR1 mRNA in a cohort of women with and without diabetes, who underwent biopsy because of a breast lump. Moreover, we compared IGF-I, IGF-IR, and IGFBP-3 expression with ESR1 expression in normal and cancer tissues of women with and without diabetes. Our hypothesis was that altered expression of IGF-I, IGF-IR, or IGFBP-3 might be connected to the increased susceptibility to breast cancer seen in patients with diabetes.

MATERIALS AND METHODS

Patients and samples

Our patients were selected from 1905 consecutive women submitted for breast biopsy from January 1994 to December 1998, in the province of Trieste, Italy. These women were subdivided according to the presence or absence of a positive breast cancer biopsy: 938 women had breast cancer and 967 had a negative biopsy. The individual histological diagnoses were re-examined, both by going back to the clinical reports and through the revision of the single slides. According to the new guidelines of the American Diabetic Association,²⁹ the study cohort was then subdivided into four groups: women

Abbreviations: BMI, body mass index; ESR, oestrogen receptor; IGFBP-3; insulin-like growth factor binding protein 3; IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; T_a, annealing temperature

Table 1 Clinical and histological findings of subjects

	No cancer		Cancer	
	No diabetes	Diabetes	No diabetes	Diabetes
Number	15	13	19	20
Mean age in years (SD, range)	39* (15, 17–62)	62 (14, 43–80)	65 (12, 41–88)	69 (10, 38–84)
Mean (SD) BMI	28 (5)	30 (6)	26 (4)	29 (5)
Histology				
Ductal			14	13
Lobular			4	2
Other			1	2
Unknown			0	3
Bilateral			1	2
Multicentric			4	6
Grading			0 G1, 11 G2, 4 G3, 4 unknown	1 G1, 13 G2, 3 G3, 3 unknown
Staging				
T			12 T1, 4T2, 0 T3, 2 T4, 1 TX	11 T1, 5 T2, 2 T3, 2 T4
N			12 N0, 3 N1, 3 N2, 1 NX	11 N0, 4 N1, 3 N2, 2 NX
M			18 M0, 1 M1	17 M0, 2 M1, 1 MX

BMI, body mass index (weight in kg divided by height squared in metres).

* $p < 0.001$; denotes the statistical difference between normal women and the other groups.

with diabetes and a positive breast cancer biopsy (D+/C+), women without diabetes but with cancer (D-/C+), women with diabetes but without cancer (D+/C-), and women without diabetes and without cancer (D-/C-). The molecular study was carried out on random samples of formalin fixed and paraffin wax embedded biopsies, of which 19 were D-/C+, 20 D+/C+, 13 D+/C-, and 15 D-/C-. For those patients with cancer, the analysis was performed both on tumorous and normal peritumorous tissue. In four D-/C+ and five D+/C+ cases, there was insufficient peritumorous material to perform the analysis. All the specimens were obtained before chemotherapy.

Table 1 summarises the clinical and histological findings of the patients with and without cancer selected for molecular study. Specimens from women without cancer included samples of fibroadenosis, intraductal papillomatosis, fibroadenoma, acute mastitis, and chronic mastitis.

RNA extraction, reverse transcription polymerase chain reaction, and relative quantitation

Total RNA was extracted from formalin fixed, paraffin wax embedded tissue sections from the 67 patients by means of a proteinase K and phenol/chloroform method, as described previously.^{30–31} In breast cancer specimens, a careful microdissection was performed to avoid contamination with normal tissue before extraction. All the specimens were analysed for the expression of β actin, IGF-I, IGF-IR, IGFBP-3, and ESR1 mRNA. For each gene, three oligonucleotides were synthesised, two of which were mRNA sense and one of which was antisense. For all the sequences studied, the first sense oligonucleotide and the antisense oligonucleotide were in two successive exons of the gene, and they were used for amplification of the sequence. The second sense oligonucleotide spanned the included intron and was used as a probe for the amplified product. Sequence GenBank accession numbers were as follows: β actin, M10277.1; IGF-I, M37484; IGFBP-3, M35878; IGF-IR, M24599; and ESR1, U47678. Oligosequences are available from the authors upon request. For every sequence studied a fixed amount of RNA was reverse transcribed as described previously.^{32–33} Relative quantification of each mRNA was possible, with the initial amount of RNA and amplification conditions being consistent with a linear relation between the log of the target RNA and the log of the amplified product.^{32–33} These conditions were satisfied when using 10 ng of total RNA for β actin and 50 ng for IGF-I with 40 standard polymerase chain reaction cycles, annealing temperature (T_a)

55°C; 150 ng for IGFBP-3, 45 cycles, T_a 60°C; 250 ng for IGF-IR, 40 cycles, T_a 57°C; and 30 ng for ESR1, 33 cycles, T_a 54°C.

Amplicons were tested by dot blot hybridisation using the second sense oligonucleotide as the probe labelled with [γ^{32} -P]ATP (Amersham, Little Chalfont, Buckinghamshire, UK), as described previously.^{32–33} The amount of radioactivity was measured by means of a computerised phosphor imaging system. The results were standardised for each sample against the amount of β actin.

Immunohistochemistry

The primary transcript of the ESR1 gene undergoes several alternative splicing events that yield a range of mRNA isoforms.³⁴ Immunohistochemical evaluation of ESR1 expression was performed to confirm data obtained at the mRNA level. Immunohistochemistry was carried out on tissue

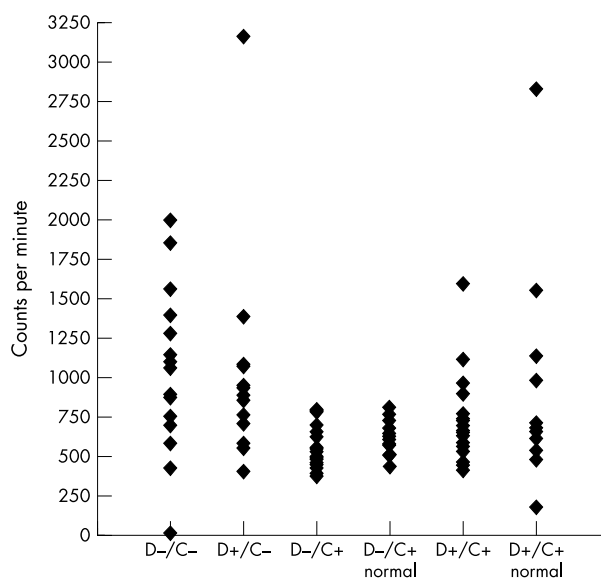


Figure 1 Relative standardised quantitation of insulin-like growth factor I mRNA in patients without diabetes who do not have cancer (D-/C-), patients with diabetes who do not have cancer (D+/C-), patients without diabetes who have cancer (D-/C+), normal breast tissue from patients without diabetes who have cancer (D-/C+ normal), patients with diabetes who have cancer (D+/C+), and normal breast tissue from patients with diabetes who have cancer (D+/C+ normal).

Table 2 Relative standardised mRNA expression quantitation in the four groups

	No cancer		Cancer			
	No diabetes (15)	Diabetes (13)	No diabetes		Diabetes	
			Tumorous (19)	Peritumorous (15)	Tumorous (20)	Peritumorous (15)
IGF-I	1045 (535)	1031 (696)	568 (135)	615 (111)	714 (274)	903 (708)
IGF-IR	1360 (1131)	1092 (632)	682 (258)	692 (168)	852 (271)	1106 (1015)
IGFBP-3	572 (575)	334 (235)	522 (283)	506 (524)	389 (522)	327 (271)
ESR1	132 (68)	182 (131)	485 (305)		460 (333)	

Results are given as mean of counts/minute (SD). The number of samples analysed is given in parenthesis in the headings.

sections from the same blocks used for RNA extraction. Antigen retrieval was achieved by treatment in a microwave oven. The primary antibody was mouse monoclonal anti-ER α protein ID5 (Dako A/S, Glostrup, Denmark). The secondary biotin conjugated polyclonal antimouse antibody was detected with peroxidase-streptavidin (VectaStain kit; Vector Laboratories, Burlingame, California, USA). Staining was performed with diaminobenzidine as chromogen and H₂O₂ (DAB kit; Dako A/S). Sections of normal breast epithelium were used as positive controls for immunostaining. Two operators assessed the immunoreactivity in three fields for each slide: the ESR status was scored as the percentage of positively stained cells. This figure was the average number of stained cells counted as a percentage of the total number of epithelial cells counted in three high magnification fields. Readings from the two operators were averaged to account for minor discrepancies arising from individual variability (< 5%). Variability between readings for the three sections for each sample ranged from 2% to 5%.

Statistical analyses

The mean and SD were calculated for each parameter. The relation between variables was tested by the least squares method. The bivariate correlation was tested by the Pearson product moment correlation coefficient. To adjust for the influence of covariates on the outcome variable, ANCOVA was

also used. Because most of the data obtained from the relative quantitation of gene expression were not normally distributed, they were logarithmically transformed in the statistical analysis. A value of $p < 0.05$ (two sided) was chosen as the limit of significance.

RESULTS

Gene expression: relative quantitation

IGF-I expression was significantly lower in the cancer groups (D-/C+ and D+/C+) than in the control groups (D-/C- and D+/C-) (mean, 769; SD, 235; mean, 1235; SD, 927, respectively; $p = 0.001$; age and body mass index (BMI) normalised) (fig 1). Moreover, the cancer groups showed a narrower range of expression for IGF-I than did the control groups. IGF-I expression was higher in patients with diabetes and cancer than in those without diabetes but with cancer; however, when the results were adjusted for age and BMI no significant difference was found (mean, 714; SD, 274; mean, 568; SD, 135, respectively; $p = 0.73$). In normal tissue adjacent to carcinoma, IGF-I expression was not significantly different between patients with and without diabetes (mean, 903; SD, 708; mean, 615; SD, 111, respectively; $p = 0.43$) (table 2).

The pattern of IGF-IR expression was similar to its ligand: there was a significant difference between the groups with and without cancer after adjusting for age and BMI (mean, 643; SD, 227; mean, 1038; SD, 603, respectively; $p = 0.01$) (fig

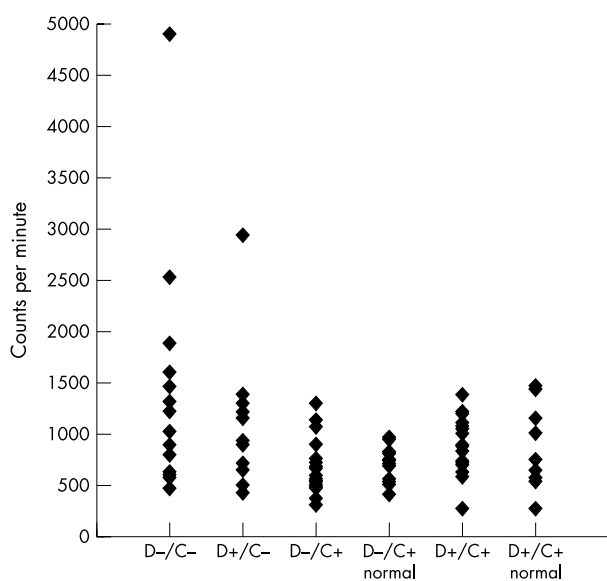


Figure 2 Relative standardised quantitation of insulin-like growth factor receptor mRNA in patients without diabetes who do not have cancer (D-/C-), patients with diabetes who do not have cancer (D+/C-), patients without diabetes who have cancer (D-/C+), normal breast tissue from patients without diabetes who have cancer (D-/C+ normal), patients with diabetes who have cancer (D+/C+), and normal breast tissue from patients with diabetes who have cancer (D+/C+ normal).

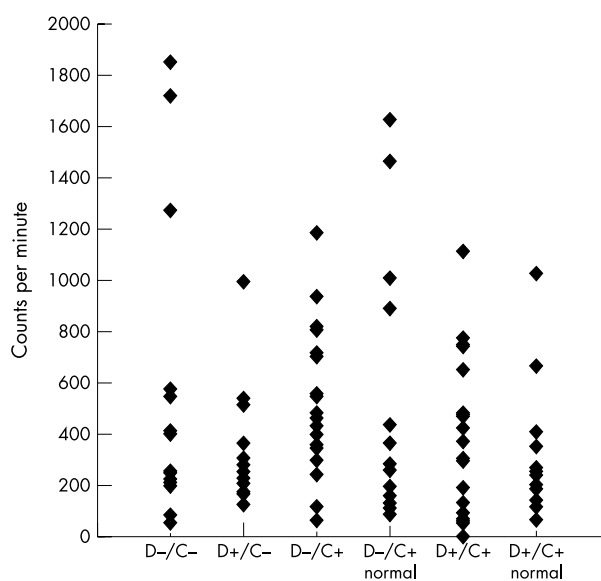


Figure 3 Relative standardised quantitation of insulin-like growth factor binding protein 3 mRNA in patients without diabetes who do not have cancer (D-/C-), patients with diabetes who do not have cancer (D+/C-), patients without diabetes who have cancer (D-/C+), normal breast tissue from patients without diabetes who have cancer (D-/C+ normal), patients with diabetes who have cancer (D-/C+ normal), patients with diabetes who have cancer (D+/C+), and normal breast tissue from patients with diabetes who have cancer (D+/C+ normal).

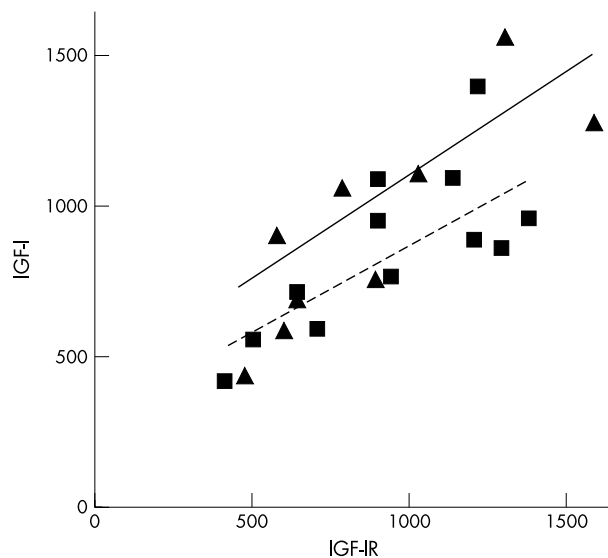


Figure 4 Correlation between relative standardised quantitation of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR) mRNA expression in breast tissue from patients without diabetes (triangles, solid trendline; $p = 0.006$) and patients with diabetes (squares, dotted trendline; $p = 0.005$) who do not have cancer.

2). Women with cancer and diabetes expressed higher amounts of IGF-IR than did those with cancer but without diabetes, although this difference was not significant after adjusting for age and BMI (mean, 852; SD, 271; mean, 682; SD, 258, respectively; $p = 0.26$). In normal tissue adjacent to carcinoma, IGF-IR expression was not significantly different

between those with and without diabetes (mean, 1106; SD, 1015; mean, 692; SD, 168, respectively; $p = 0.39$) (table 2).

No significant differences in IGFBP-3 expression were seen when comparing groups with and without carcinoma (mean, 454; SD, 296; mean, 462; SD, 458, respectively; $p = 0.5$) or when comparing women with and without diabetes who had cancer (mean, 389; SD, 299; mean, 522; SD, 283, respectively; $p = 0.67$) (fig 3). In addition, normal tissue from the cancer groups showed no significant difference in IGFBP-3 expression between women with and without diabetes (mean, 327; SD, 271; mean, 506; SD, 524, respectively; $p = 0.42$) (table 2).

The expression of ESR1 was not significantly different between women with and without diabetes who did not have cancer (mean, 182; SD, 131; mean, 132; SD, 68, respectively). Similarly, there was no significant difference between patients with and without diabetes who had cancer (mean, 460; SD, 334; mean, 485; SD, 305, respectively) (table 2). Analysis of variance of ESR1 expression between groups with and without cancer showed a highly significant difference ($p = 0.0002$). ESR1 expression at the mRNA level was consistent with data obtained by means of immunohistochemistry, with a good correlation between the two methods ($r = 0.774$; $p < 0.0001$).

Correlation between gene expression

We found a positive correlation between IGF-I and IGF-IR expression in patients with and without diabetes who did not have cancer ($r = 0.765$; $p = 0.006$; $r = 0.740$; $p = 0.005$, respectively) (fig 4). In patients with diabetes and cancer, there was also a significant correlation between IGF-I and IGF-IR expression ($r = 0.579$) (fig 5B), whereas there was no correlation in the tumorous and peritumorous tissues of women without diabetes who had cancer (fig 5A,C). However, we found a strong positive correlation between IGF-I and

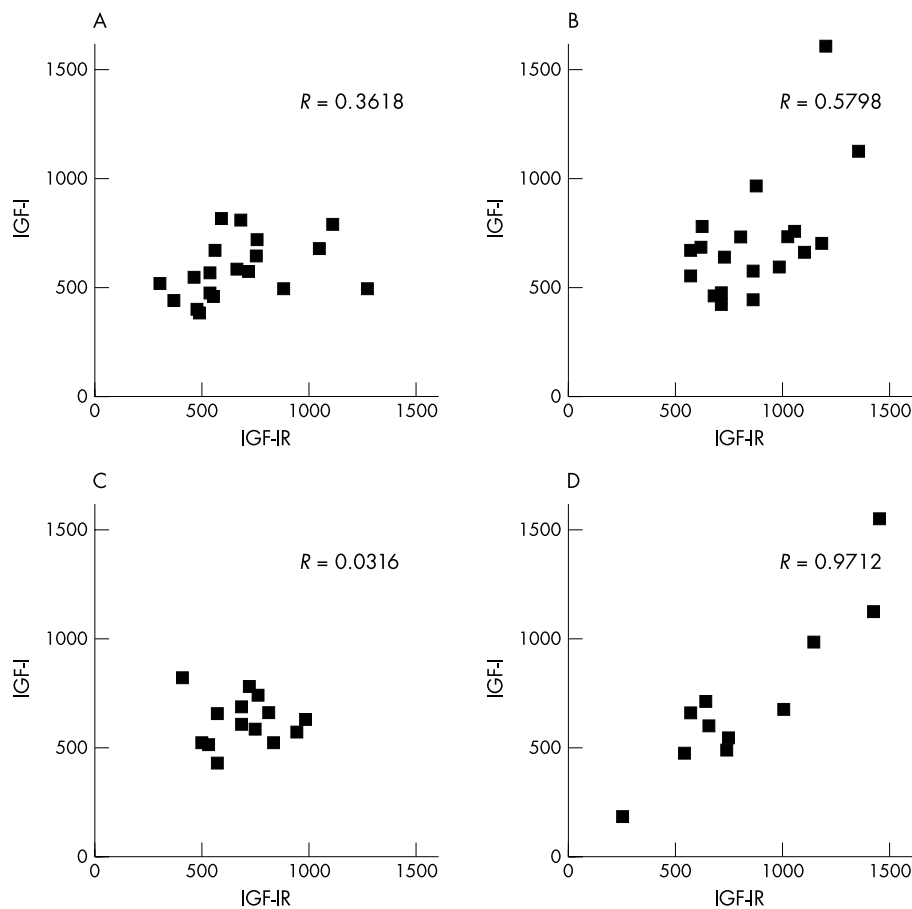


Figure 5 Correlation between relative standardised quantitation of insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR) mRNA expression in neoplastic tissue from (A) patients without diabetes and (B) patients with diabetes, and in normal breast tissue from (C) patients without diabetes who have cancer, and (D) patients with diabetes who have cancer. The p values were: (A) 0.1279, (B) 0.0093, (C) 0.9148, and (D) < 0.001 . R, correlation coefficient.

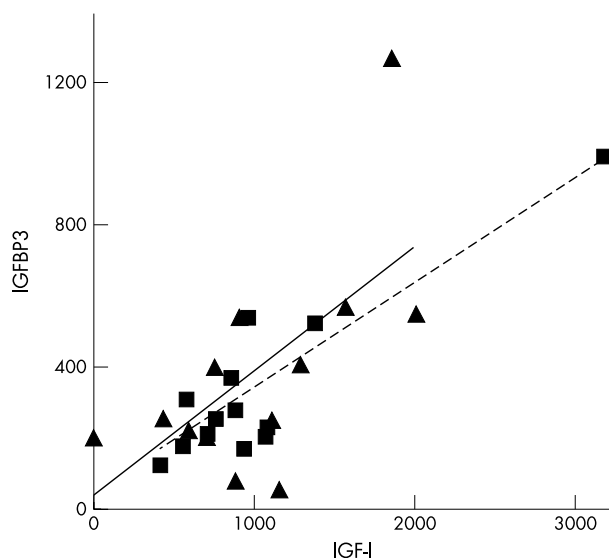


Figure 6 Correlation between relative standardised quantitation of insulin-like growth factor binding protein (IGFBP-3) and insulin-like growth factor I (IGF-I) mRNA expression in breast tissue from patients without diabetes (triangles, solid trendline; $p = 0.018$) and those with diabetes (squares, dotted trendline; $p < 0.001$) who do not have cancer.

IGF-IR expression in peritumorous tissue from women with diabetes cancer ($r = 0.971$) (fig 5D). There was a positive correlation between IGF-I and IGFBP-3 expression only in the women with and without diabetes who did not have cancer ($r = 0.640$; $p = 0.018$; $r = 0.893$; $p < 0.001$, respectively) (fig 6). There was no correlation between IGF-I and IGFBP-3 in the cancer groups either in the tumorous or the peritumorous tissues (not shown). The ESR1 status did not correlate with the expression of IGF-I or IGF-IR, either at the mRNA or at the protein level, in women with or without cancer.

DISCUSSION

We studied the expression of IGF-I, IGF-IR, IGFBP-3, and ESR1 mRNA in a cohort of women with and without diabetes who underwent biopsy because of a breast lump. Surprisingly, IGF-I and IGF-IR expression at the mRNA level was higher in the mammary tissue of patients without cancer than in those with cancer. These data are inconsistent with the higher concentrations of circulating IGF-I and IGF-IR previously reported in women with breast cancer.^{9 19–22 35 36} This discrepancy could be explained by our methodological approach, which analysed the autocrine production of IGF-I and IGF-IR at the mRNA level in mammary tissues. The expression of IGF-I in the mammary tissue surrounding cancer tissue was comparable to that found in the tumorous tissue. This result is consistent with a paracrine induction of the mitogen,^{10–13} and it raises the possibility that locally produced IGF-I influences the growth of breast cancer. In fact, paracrine sources of IGF-I in tissues surrounding malignant breast epithelium are thought to increase the rate of cellular proliferation of malignant breast epithelium expressing the IGF-IR.³⁶

In our present study, there was a good correlation between the expression of IGF-I and IGF-IR mRNA in the normal mammary tissue of women with and without diabetes. This correlation was maintained in tumorous and particularly in peritumorous tissue only in women with diabetes, and was lost in those without diabetes. In breast cancer tissue from women without diabetes, the growth factor IGF-I and its receptor were deregulated and the correlation present in normal mammary tissue was completely lost. In contrast, in patients with diabetes who also had cancer the positive correlation between IGF-I and IGF-IR expression was still

Take home messages

- Insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR) expression was significantly lower in patients with and without diabetes who had cancer, whereas there was no significant difference for IGF binding protein 3 between women with and without cancer
- There was a good correlation between the expression of IGF-I and IGF-IR in women without cancer and in patients with diabetes and cancer, but not in those patients without diabetes who had cancer
- These differences in IGF-I/IGF-IR expression might contribute to the increased risk for breast cancer seen in women with type 2 diabetes

apparent. It is possible that this difference in the expression of IGF-I and its receptor could be a factor that contributes to the increased risk of breast cancer in women with type 2 diabetes. Further tests are needed to demonstrate a causal connection between the signalling pathways mediated by the IGF system and carcinoma growth in women with diabetes.

“It is possible that this difference in the expression of insulin-like growth factor I and its receptor could be a factor that contributes to the increased risk of breast cancer in women with type 2 diabetes”

The correlation between IGF-I and IGFBP-3 expression was present only in patients without cancer, whereas it was lost in women with cancer. This suggests that a deregulation in the expression of the mitogen and its inhibitor at the autocrine/paracrine level may be associated with an increased risk of breast cancer. In our present study, we found no significant correlation between IGF system gene expression and ESR status: this is not surprising because previous studies detected overexpression of the IGF-IR in neoplastic tissues,^{24 36} whereas we found decreased IGF-I/IGF-IR expression in our patients with cancer. As a whole, our results do not agree with most of the experimental evidence collected to date. Nevertheless, recent studies support the methodological approach that we used as a useful means of pointing out autocrine/paracrine mechanisms in normal and neoplastic tissues. In fact, the correlated expression of two genes at the mRNA level suggests a common underlying mechanism of regulation: disruption of this signalling loop can contribute to the cancer phenotype.³⁷

In conclusion, our study suggests that the IGF-I system plays a role in the malignant transformation of breast epithelium in women with diabetes. Such findings provide further support for the need for anti-IGF targeted treatment of breast cancer in women with diabetes.

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Insulin-like growth factor system gene expression in women with type 2 diabetes and breast cancer

E Nardon, I Buda, G Stanta, et al.

J Clin Pathol 2003 56: 599-604

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