ORIGINAL ARTICLE

Significant expression of IGFBP2 in breast cancer compared with benign lesions

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Background/Aim: Insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) play a role in the normal development of breast tissue, and possibly in breast cancer aetiology. IGFBP2, one of six members of the IGFBP superfamily, acts as regulator of the IGFs and has pleiotropic effects in normal and neoplastic tissues. Because IGFs have mitogenic effects on mammary epithelia, this study investigated IGFBP2 expression in mammary tissues of different benign and malignant entities.

Methods: Immunohistochemistry was used to study correlations between the presence and intensity of IGFBP2 staining and tumour type and grade, in addition to steroid hormone receptor status, in 120 breast specimens. Expression was measured by quantitative colour video image analysis and semiquantitative evaluation, and the measurements correlated well ($r=0.92$; $p<0.05$).

Results: Both methods found no significant expression of IGFBP2 in normal glandular cells and hyperplasia (group I). Atypical hyperplasia showed a slightly increased cytoplasmic expression of IGFBP2, and carcinoma in situ showed a distinctive, membrane associated and cytoplasmic expression (group II). Infiltrating carcinomas strongly expressed cytoplasmic IGFBP2 (group III). There were significant differences between group I and II, and between group II and III. There were no significant differences between invasive lobular and invasive ductal carcinoma, or between grades I, II, and III within these entities. There was no significant correlation between IGFBP2 immunostaining and oestrogen or progesterone receptor positivity within the malignant group.

Conclusions: IGFBP2 mitogenic signals of autocrine/paracrine regulatory mechanisms may be responsible for the growth of breast carcinomas and IGFBP2 may be an independent indicator of malignancy.

Insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs) play important roles in cellular growth and development in both normal and neoplastic tissues.[1 2] IGFs promote the growth of a range of tumour types, including mammary carcinoma, and enhance the proliferation of several mammary carcinoma cell lines. Because IGFs have mitogenic effects on mammary epithelia,[1 2] attention has been focused on the role and localisation of the IGFBPs in benign and malignant mammary tissues.

> "Insulin-like growth factors promote the growth of a range of tumour types, including mammary carcinoma"

IGFBP2 is not glycosylated and contains an integrin receptor recognition sequence, suggesting that it has cell association properties,[3] which may be necessary for its actions, because mutation of the membrane binding domain has been reported to block IGFBP2 potentiation of IGF2 stimulated mitogenesis.[4] The cellular actions of IGFBP2 on IGF mediated functions have been reported to be both inhibitory and stimulatory.[5] By using homologous recombination and embryonic stem cell technology to generate mice in which the IGFBP2 gene was mutated,[6] few developmental abnormalities were discovered. IGFBP2 expression was found to be reduced in lung carcinoma cell lines, and was inhibitory to IGF2 action when added exogenously.[7] We have previously shown the increased expression of IGFBP2 in prostatic intraepithelial neoplasia and invasive prostatic carcinoma, compared with normal prostatic glands.[8] We have also shown a significant correlation between malignant potential in adult soft tissue sarcomas and the expression of IGF mitogenic signals.[9]

Most of the scientific literature to date focuses on the expression of IGFBP2 in malignant, but not normal, breast epithelial cells. In our study, we have used an immunohistochemical approach to clarify the expression and localisation of IGFBP2 in malignant and benign breast tissues.

MATERIALS AND METHODS

Case selection

Our study material consisted of 120 archival surgical breast resections from the department of pathology, University Hospital of Northern Norway. Histological classification of breast diseases was made according to the criteria of the World Health Organisation.[10] Invasive ductal carcinomas were further divided into three subgroups according to the modified Bloom and Richardson classification.[11] The series included invasive lobular carcinomas, invasive ductal carcinomas of grade I, II, and III, ductal or lobular carcinoma in situ, atypical ductal or lobular hyperplasia, ductal or lobular hyperplasia, non-proliferative fibrocystic change, and breast specimens from women showing no histological abnormalities. All the normal breast specimens and benign breast lesions were from patients without cancer. The breast specimens were selected from the four most recent years of the archive. There were no major changes to the procedures in the laboratory during this period. No appreciable differences in staining intensity could be detected in the earliest diagnosed specimens compared with the latest one

Abbreviations: ER, oestrogen receptor; DAB, diaminobenzidine tetrahydrochloride; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IR, immunoreactivity score; PBS, phosphate buffered saline; PR, progesterone receptor; VIA, video image analysis

Table 1 Distribution and grouping of histological diagnosis, hormone receptor status of malignant cases, and patient age

Characteristics	N	%
Group I		
Normal	11	9
Non-proliferative fibrocystic change	12	10
Ductal or lobular hyperplasia	9	8
Group II		
Atypical ductal or lobular hyperplasia	11	9
Ductal/lobular carcinoma in situ	13	11
Group III		
Ductal carcinoma, grade I	17	14
Ductal carcinoma, grade II	19	16
Ductal carcinoma, grade III	15	12
Lobular carcinoma, grade I–III	13	11
Total specimens	120	100
Hormone receptor status (group III)		
ER+	34	53
ER−	30	47
PR+	33	52
PR−	31	48
Mean age (years)		
Group I	54 (range, 22–68)	
Group II	52 (range, 29–62)	
Group III	59 (range, 32–90)	
Menopausal/postmenopausal		
Group I	25	78%
Group II	20	83%
Group III	60	92%

ER, receptor; PR, progesterone receptor.

diagnosed. From 10 to 20 specimens were selected randomly from the different entities.

Haematoxylin and eosin stained sections from formaldehyde fixed and paraffin wax embedded breast tissues were reviewed independently by two pathologists (HS and LTB) to establish the diagnosis.

Immunohistochemistry

For each procedure, the order of samples was chosen randomly from each of the groups. A standard immunohistochemical method was performed. The 4 µm thick sections of phosphate buffered saline (PBS)/formalin fixed and paraffin wax embedded specimens were routinely dewaxed in xylene, rehydrated in a graded series of ethanol, and washed in distilled water. Antigen retrieval was achieved by placing the specimens in 0.01M citrate buffer at pH 6.0 and exposing them to repeated microwave heating for periods of 10 minutes at 450 W. The buffer was replenished after each interval because of evaporation. The specimens were cooled at room temperature for 15 minutes and washed in sterile water for five minutes and then in PBS at pH 7.6 for five minutes. Endogenous peroxidase or phosphatase activities were quenched in 0.3% H_2O_2/H_2O (Sigma, St Louis, Missouri, USA) for 30 minutes, followed by blocking of non-specific antibody binding in 10% goat serum for 30 minutes at room temperature. Tissue sections were incubated overnight at 4℃ in a humidifier with primary goat anti-IGFBP2 (Santa Cruz Biotechnology, Santa Cruz, California, USA; polyclonal antibody diluted 1/150 in 1% bovine serum albumin). As negative staining controls, the antibodies were replaced with either 1% bovine serum albumin in PBS to rule out the effect of endogenous peroxidase, or isotype matched irrelevant mouse immunoglobulin to test for specificity. The specificity of the antibody was also confirmed by western blotting. Biotinylated rabbit antigoat $F(ab')_2$ fragments (Dako, Carpinteria, California, USA; diluted 1/400) were used as the secondary antibody. Specific intracellular immunoreactivity was detected by incubation with avidin–biotin–horseradish peroxidase complex for 30 minutes at room temperature. After extensive washing in PBS, the bound antibody was detected with a three stage avidin–biotin–peroxidase complex technique using diaminobenzidine tetrahydrochloride (DAB) as chromogen (Dako). The specimens were counterstained in haematoxylin, dehydrated in a graded series of ethanol, cleared in three changes of xylene, and mounted in Eukitt. Appropriate positive and negative controls were included in each antibody run. No significant variations could be seen between different immunohistochemistry runs by repeating 10% of the batches.

The demonstration of oestrogen receptors (ERs), using antibody 1D15 (Dako), and progesterone receptors (PRs), using antibody NCL-PGR (Abbott Laboratories, Maidenhead, UK), was performed on paraffin wax embedded sections, according to a previously published protocol.[12]

Semiquantitative analysis

The degree of expression of IGFBP2 was then graded semiquantitatively and classified into one of four grades. The staining was scored as: 0, no staining; 1, weak staining; 2, strong staining of 25% tumour cells or moderate staining of $< 80\%$; 3, strong staining of 25–50% or moderate staining of $> 80\%$; 4, strong staining of $> 50\%$ tumour cells. In each case, 10 high power fields of representative areas were counted. The maximal staining intensity was typically higher in those cases where more cells were positive. Most positive cells showed cytoplasmic staining. In less than 1% of the cytoplasmically weak stained cells, an apparent coexistent membrane staining was seen. However, on closer inspection, this membrane staining was found to result from margins of epithelial cells pushing against necrotic lumina of carcinoma in situ. This pseudostaining of the membranes never exceeded the cytoplasmic level of staining, and was therefore recorded as cytoplasmic staining. Slides were examined and scored independently by two of the authors (TB and LTB), blinded to other pathological information. In 80% of cases, the data were similar, and the remaining cases were reviewed until final agreement was achieved.

The staining of ERs and PRs was estimated using the "quick score" technique,[13] as follows: slides were assessed for both the proportion of cells stained and staining intensity. Proportions were scored as 0, 0%; 1, 1–25%; 2, 25–50%; 3, 50–75%; or 4, 75–100% stained cells. The intensity was scored as 0, no staining; 1, weak; 2, moderate; or 3, strong staining. The two scores were added to give a final score of 0–7. A final score < 3 was regarded as negative.

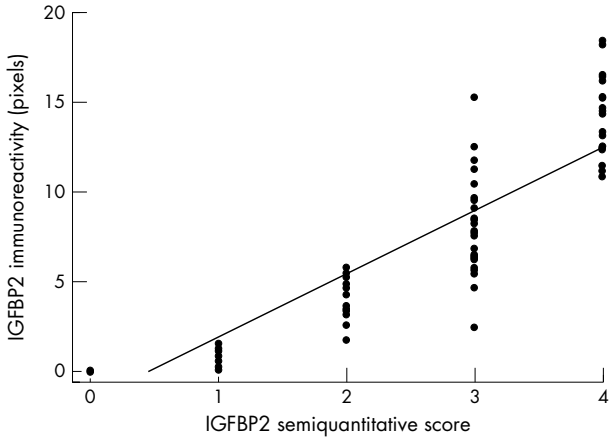

Figure 1 The two procedures, quantitative colour video image analysis and semiquantitative evaluation, correlated well for insulin-like growth factor binding protein 2 (IGFBP2) measurement (Spearman's correlation coefficient, $r = 0.92$; $p < 0.001$).

Table 2 Semiquantitative and quantitative scores of insulin-like growth factor binding protein 2 expression in benign and malignant human breast tissue

	Semiquantitative	Quantitative
Group I		
Normal breast tissue	0.18 (0.12)	0.06 (0.03)
Non-proliferative fibrocystic change	0.17 (0.11)	0.09 (0.05)
Hyperplasia	0.67 (0.17)	0.58 (0.15)
Group II		
Atypical hyperplasia	1.27 (0.19)	1.99 (0.45)
Carcinoma in situ	1.92 (0.18)	3.42 (0.49)
Group III		
Ductal carcinoma, grade I	2.94 (0.18)	7.33 (0.96)
Ductal carcinoma, grade II	3.58 (0.14)	11.59 (0.93)
Ductal carcinoma, grade III	3.33 (0.16)	11.52 (0.97)
Lobular carcinoma	3.15 (0.19)	9.56 (1.08)
Dunnett's test	p Value	
Group I *v* group II	<0.001	<0.001
Group II *v* group III	<0.001	<0.001
Group I *v* group III	<0.001	<0.001

Values are mean (SEM).

Colour video image analysis

The immunostained sections were examined using a Leica (Leitz Laborlux S; Leica, Wetzlar, Germany) microscope (×400) coupled to a video camera (Sony Hyper HAD; colour CCD-IRIS/RGB; model DXC-151AP), connected to a computer aided colour video image analysis (VIA) system (Multi-Sync 3V; model JC-1535VMR; NEC Corporation, California, USA).[14] After being captured and digitalised on to the video screen, microscopic images were analysed using an image analysis software program (Leica Q500MC; Microsoft Windows hosted image analysis system; Leica Australia P/L). The images stored in the software were composed of up to 512 × 512 pixels separated into 8 bit brightness. The transmitted light intensity was standardised by using a fixed rheostat setting at the microscope light source. The stability of the light output was frequently checked during all procedures.

Because of the high cellularity of some samples, sequential fields were systematically examined using a ×40 objective and a ×10 eyepiece within a fixed frame of 45 000 × 450 pixels (0.2025 mm^2). Colour values for each of the red, green, and blue components were calculated for each pixel from 1 to 255. After counterstaining with Harris's haematoxylin, which allowed excellent separation from DAB staining, a cutoff value of 170 was determined for the blue colour component to exclude false or weakly positive staining. This value was chosen after experience from 10 slides (two from each group), which were originally analysed for this purpose.

Because of the heterogeneity of the tumour samples, 10 fields were examined for each slide. This number was determined using a progressive mean graph to achieve a meaningful result in statistical terms. Each slide was initially examined at ×10 magnification for an overall view of the lesion and counterstain intensity. This practice allowed an area to be chosen that was the most representative, with no tissue folding or overlapping, and minimal background staining. The fields were then randomly selected within this area. The degree of staining intensity was estimated as the mean DAB area stained from a minimum of 900 cells in 10 microscopic fields at ×400 magnification. Evaluation of the staining reaction was performed using the immunoreactivity (IR) score, which is the staining intensity in pixels multiplied by the percentage area stained.

Data from the VIA were divided into five distinct IR groups similar to the semiquantitative evaluation of staining. Cutoff points defining these groups were determined before analysis to ensure roughly equal numbers in each group. The IR scale was as follows: IR 0, (0,1); IR 1, (1,5); IR 2, (5,80); IR 3, (80,110); IR 4, (110,∞).

Ethics clearance

Our study was approved by the research ethics committee of Northern Norway (REK, Ref. 200303108-3/IAY/400).

Statistical analysis

Data are presented as mean (SEM). Associations between the semiquantitative estimates of the expression of IGFBP2 and the quantitative measurements by colour VIA analysis were tested by Spearman's correlation coefficient. Individual groups were compared using the parametric Students's *t* test. The differences in expression of IGFBP2 between groups were determined by Dunnett's test. The relation between IGFBP2 expression and receptor status in malignant tissue was tested by the non-parametric Mann-Whitney U test. All reported p values are two sided. For all these statistical analyses a p value of < 0.05 was considered significant.

RESULTS

Characteristics of the 120 cases

Table 1 shows the distribution and grouping of the histological diagnoses. Thirty two specimens were in group I, 24 were in group II, and 64 were in group III. The four subgroups in group III each consisted of 13 to 19 cases. Thirty four of the 64 cases in group III were ER positive and 33 were PR positive. The mean age of the patients in group III was slightly higher than in groups I and II. This difference was not significant. The percentage of menopausal and postmenopausal patients was slightly higher in group III compared with group II, and in group II compared with group I. This difference was not significant.

Correlations between quantitative colour VIA and semiquantitative scores

The two procedures, quantitative colour VIA and semiquantitative evaluation, correlated well for IGFBP2 measurement (Spearman's correlation coefficient, $r = 0.92$; $p < 0.001$), as shown in fig 1.

Expression of IGFBP2 in groups I, II, and III

Table 2 shows the semiquantitative and quantitative scores of IGFBP2 expression in benign and malignant breast tissues.

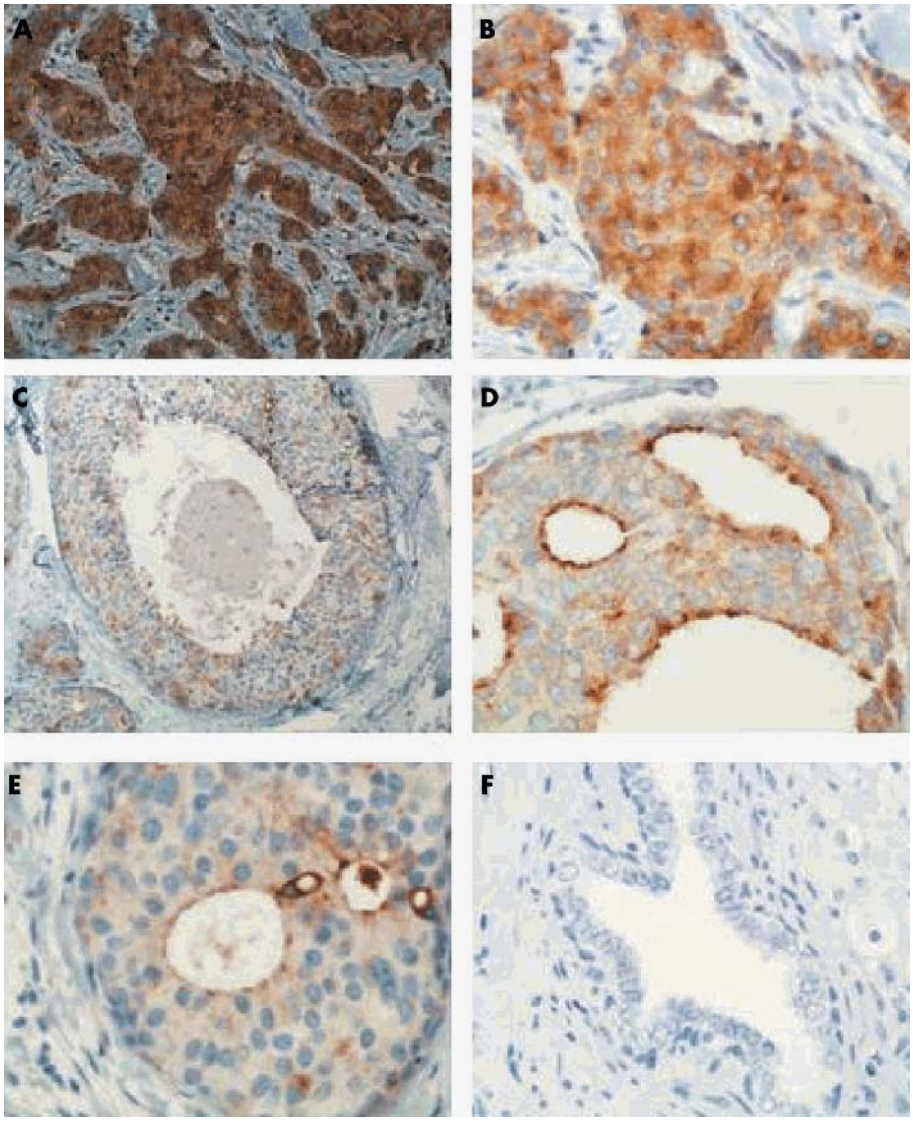

Figure 2 (A, B) Strong cytoplasmic staining for insulin-like growth factor binding protein 2 (IGFBP2) in the neoplastic cells of invasive ductal carcinoma. (C–E) Weak, dot-like positivity and abluminal staining of cribriform spaces for IGFBP2 in ductal carcinoma in situ. (F) Normal ducts show no staining.

The expression of IGFBP2 was mostly confined to the epithelial cells, with no appreciable stromal staining. Heterogeneity of staining, which was mostly intracellular, was noted within individual tumour samples, which showed either variations in intensity or patchiness of DAB staining. Significant differences in the immunohistochemical expression of IGFBP2 between the three histological groups were seen for both semiquantitative and quantitative scores ($p < 0.001$). In group I, the quantitative data generated by colour VIA were between 0.06 and 0.58 (mean semiquantitative scores were between 0.18 and 0.67), in group II the mean quantitative scores were between 1.99 and 3.42 (mean semiquantitative scores between 1.27 and 1.92), whereas in group III the quantitative scores were between 7.33 and 11.59 (mean semiquantitative scores between 2.94 and 3.58). There were no significant differences between invasive lobular carcinomas and invasive ductal carcinomas, or between grades I, II, and III within these entities (Dunnet's test). The highest scores were recorded in invasive ductal carcinomas of grades II–III.

Table 3 Expression of IGFBP2 in invasive breast carcinoma according to hormone receptor status

	Semiquantitative	Quantitative
ER+	3.18 (0.13)	9.74 (0.74)
ER−	3.37 (0.11)	10.35 (0.75)
PR+	3.36 (0.12)	10.37 (0.72)
PR−	3.16 (0.12)	9.66 (0.78)
Mann-Whitney test		
ER+ *v* ER−	NS	NS
PR+ *v* PR−	NS	NS

Values are mean (SEM).
ER, oestrogen receptor; IGFBP2, insulin-like growth factor binding protein 2; NS, not significant; PR, progesterone receptor.

Immunohistochemical images of malignant and benign entities

As shown in fig 2A and B strong cytoplasmic staining for IGFBP2 was seen in the neoplastic cells of invasive ductal carcinoma. Weak, dot-like positivity for IGFBP2 was seen in

ductal carcinoma in situ (fig 2C–E), whereas normal ducts showed no staining (fig 2F).

Correlation between receptor status and IGFBP2

No significant differences were found in the semiquantitative and quantitative scores of IGFBP2 expression in ER+ versus ER− tumours or PR+ versus PR− tumours of invasive carcinoma (Mann-Whitney test) (table 3). No correlation between ER or PR, and IGFBP2 could be seen when they were considered as continuous variables (data not shown).

DISCUSSION

The expression of growth factors and their receptors may be an important mechanism by which some cancers develop abnormal growth. Raised concentrations of IGFBP2 have been found in blood from patients with prostate carcinoma compared with those with benign prostatic hyperplasia, indicating a correlation between this protein and malignancy.[15]

In vitro studies using established breast tumour cell lines have suggested that tumours expressing IGF1 receptors may be responding, not only to circulating serum concentrations of IGFs produced by the liver, but also to IGFs produced by the tumour itself in an autocrine and/or paracrine manner.[2 16] Both IGF1 and IGF2 stimulate cell growth and are expressed in breast carcinomas.[17 18] The IGFBPs act by either enhancing or inhibiting the action of IGFs.[19] The abundant and variable presence of the different IGFBPs in human breast tumours suggests that these molecules play a physiological role as modulators of IGF action in breast cancer. This effect may be different in different tumours, not only depending on which IGFBPs are expressed, but also on individual factors, such as age, concentrations of endogenous hormones, and menopausal status.

> ''The epithelial cells of invasive carcinoma expressed significantly more cytoplasmic insulin-like growth factor binding protein 2, compared with those of carcinoma in situ, hyperplasia, or normal glandular cells''

In our study, we have determined the expression of IGFBP2 in 120 specimens from women undergoing evaluation of breast pathology. To our knowledge, the expression of IGFBP2 in breast carcinoma has not been evaluated by immunohistochemistry previously. The epithelial cells of invasive carcinoma expressed significantly more cytoplasmic IGFBP2, compared with those of carcinoma in situ, hyperplasia, or normal glandular cells. This finding is in accordance with previous reports where IGFBP2 has been shown to augment the IGF1 induced mitogenicity of cancer cells.[20] Both IGF1 and IGF2 are thought to be partly synthesised by the stromal cells of the tumour,[21] emphasising the importance of stromal–epithelial interactions in the control of breast cancer cell proliferation by IGFs.

Oestrogen positive breast cancer tumour cell lines have shown increased secretion of IGFBP2 compared with oestrogen negative cell lines.[22] In our study, we did not find such an association between the ER status of the specimens and IGFBP2 expression. A more heterogeneous population of cells in human tumours and the selective properties of continuous cultures of tumour cell lines may account for this difference.

By extracting IGFBP2 protein from breast cancer specimens a ligand blot analysis revealed a positive correlation between the amount of IGFBP2 and the receptor status of the specimens.[23] The difference between these studies can be explained by methodological variations. The immunohistochemical techniques used in our study measured the proteins expressed exclusively by the epithelial cells, ligand blot analysis measures the total amount of protein in the tumour tissue (stroma and neoplastic epithelial cells).

However, there were no significant differences in IGFBP2 expression between invasive lobular carcinomas and ductal carcinomas, or between grades I, II, and III within these entities. These findings are in agreement with the lack of a correlation between IGFBP2 and receptor status, because low grade of malignancy is positively correlated with ER positivity.

Both semiquantitative and a microcomputer based colour VIA technique revealed significant differences in the immunohistochemical expression of IGFBP2 between histological groups. The colour VIA technique for assessing immunohistochemistry staining provides an objective quantitative measurement of tissue features with high sensitivity and specificity.[14] There was a good correlation between the colour VIA and semiquantitative analysis, despite the subjectivity involved in the second method.

These data suggest that IGFBP2 mitogenic signals of autocrine/paracrine regulatory mechanisms may be responsible for the growth of breast carcinomas, and that IGFBP2 is an independent indicator of malignancy. Because IGF and IGFBP signals are directly involved in the perturbation of the proliferative and apoptotic pathways of malignant growth, further examination of the relation between the IGF axis and tumorigenic processes will not only provide new insights into the intracellular mechanisms of cellular growth control, but raise the possibility of targeting components of the IGF system in cancer treatment.

Take home messages

- Using immunohistochemistry (and quantitative and semiquantitative methods) no significant expression of insulin-like growth factor binding protein 2 (IGFBP2) was seen in normal breast glandular cells or breast hyperplasia
- IGFBP2 expression was gradually increased from hyperplasia through atypical hyperplasia and carcinoma in situ to invasive carcinoma
- No significant correlation was found between IGFBP2 immunostaining and oestrogen or progesterone receptor positivity within the malignant group
- IGFBP2 mitogenic signals of autocrine/paracrine regulatory mechanisms may be responsible for the growth of breast carcinomas, and IGFBP2 may be an independent indicator of malignancy

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