Evidence for a Tumour Suppressive Function of IGF1-binding Proteins in Human Breast Cancer

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Abstract. *The role of the insulin like growth factor (IGF) system in various human malignancies has been well established. The aim of this study was to determine the levels of mRNA expression of insulin-like growth factor-binding protein (IGFBP)-1, -3 and -7 genes in benign and malignant breast tissue and explore their relationship with various prognostic parameters. Materials and Methods: Breast cancer tissue (n=127) and normal background tissue (n-33) were prospectively collected and analysed for levels of IGFBP-1, -3 and -7 mRNA using real-time Q-PCR. mRNA levels were then analysed against tumour grade, nodal status, Nottingham prognostic index (NPI)/TNM stage and tumour type. Results: For IGFBP-1 and -3, mRNA expression was higher in normal tissue. This was significant for IGFBP-1 when comparing NPI 3 with NPI 1 (p=0.050) and the normal group (p=0.040). With respect to TNM analysis, there was less IGFBP-1 mRNA when comparing TNM 3 with normal (p=0.017), TNM 1 (p=0.047) and TNM 2 (p=0.019) tumours. This was also found when comparing TNM 4 samples with normal tissue (p=0.017), TNM 1(p=0.046) and TNM 2 (p=0.019). For IGFBP-3 mRNA, there was less mRNA when comparing TNM3 with TNM 1 (p=0.017) and TNM 2 (p=0.050), and also less mRNA expression when comparing TNM 4 with TNM 1 (p=0.030). For IGFBP-7 mRNA, both TNM 1 (p=0.0077) and TNM 2 (p=0.015) had significantly more expression than TNM 3 samples. Conclusion: This study supports the role of IGFBP-1, -3 and -7 as potential tumour suppressor genes in human breast cancer.*

The insulin-like growth factor (IGF) axis plays a key role in the growth, differentiation and proliferation of mammalian cells and, although vital in many organ systems, has been shown to play a particular role in the normal development of the human mammary gland and has also been heavily implicated in mammary carcinogenesis (4, 7, 25).

The IGF axis comprises two growth factors (IGF-1 and IGF-2), their receptors (IGFR-1 and IGFR-2) together with a group of IGF-binding proteins. Both IGF-1 and 2 bind to IGFR-1, although IGF-2 also binds to IGFR-2 with high affinity. Together, these factors interact to influence cell signalling pathways which control the eventual growth, proliferation, differentiation and eventual survival of their target cells.

The eventual action of the IGF ligand is modulated by interaction with a family of seven IGF-binding proteins (IGFBP-1 to -7) which share 40-60% amino acid homology. The majority (97%) of serum IGF-1 is bound to these binding proteins (particularly to IGFBP-3) which binds the ligand with an affinity equal to or greater than the IGFR-I (9). The action of the IGF-binding proteins is primarily to regulate the bioavailability of the IGF-1 ligand in the circulation and thereby its eventual action at the receptor, but they also appear to have other IGF-dependent and -independent actions at the cell surface which are equally important.

Recent evidence however suggests that the binding proteins have a ligand-independent action at the cellular level acting *via* independent receptors both at the cell surface and possibly also at the nucleus and that the cumulative action of these effects is a tumour suppressive action to counter the pro-neoplastic effect of the main growth factor.

The aim of our study was to determine the level of mRNA expression of IGFBP-1, -3 and -7 genes in benign and malignant breast tissue and examine their association with clinical outcome and several other tumour prognosticators including nodal status, stage, grade, Nottingham prognostic index (NPI) score and ER status.

We used quantitative PCR (Q-PCR), which has been shown to be a sensitive and specific method of measuring copy number changes of a given gene transcript. This

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Table I. *Clinical data.*

Parameter	Category	Sample number
Node status	Node positive	54
	Node negative	73
Tumour grade	1	24
	2	43
	3	58
Tumour type	Ductal	98
	Lobular	14
	Medullary	2
	Tubular	2
	Mucinous	4
	Other	7
TNM staging	1	70
	2	40
	3	7
	4	4
Clinical outcome	Disease-free	90
	Alive with: metastasis	7
	local recurrence	5
	Died of: breast cancer	16
	unrelated disease	9

method has the advantage of a high throughput capacity and no post-PCR manipulations. The study reports the clinical and prognostic value of selective IGFBPs in human breast cancer.

Materials and Methods

Materials. RNA extraction kits and reverse transcription kits were obtained from AbGene and SIGMA Ltd. (Epson, Surrey, England, UK and Dorset, England, UK, respectively). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by SigmaGENESIS (Gillingham, Dorset, England, UK). Custom made hot-start Master mix for quantitative PCR was from Abgene (AbGene, Epsom, UK) (16, 17).

Sample collection. Breast cancer tissues (n=127) and normal background tissues (n=33) were collected immediately after surgery and stored at –80˚C until use. Patients were routinely followed up after surgery. The median follow-up period was 120 months. A Consultant Pathologist (A.D-J.) who examined H&E-stained frozen sections verified the presence of tumour cells in the collected tissues.

Details of histology were obtained from pathology reports. Follow-up data was recorded in a custom database. Table I shows the tumour characteristics and clinical outcome.

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5 to 10 μm and were kept for routine histology. An additional 15-20 sections were mixed and homogenised, using a hand-held homogeniser, in ice-cold RNA extraction solution. The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried out using a reverse transcription kit with an anchored olig(dT) primer supplied by Abgene, using 1 μg of total RNA in a 96-well plate. The quality of cDNA was verified using ß-actin primers (primers 5'-ATGATATCGCCGCGCTCGTC-3' and 5'-CGCTCGGTGAGGATCTTCA-3')

Table II. *IGFBP primer sequences.*

Binding protein	Primer sequence
BP-1	GGAGGTCCTCCTCAGTTATC, IGFBP1zR, CTGCGTGCAGGAGTCTGA, IGFBPF1
BP-3	GCGCCTACCTGCTGCCAG, igfbp3F1, GACGGGCTCTCCACACTGC, igfbp3Zr1, AGTCTCAGAGCACAGATACC, igfbp3f2, GAGGAACTTCAGGTGATTCAG, igfbp3Zr2,
BP-7	ACATCTGGAATGTCACTGGT, IGFBP7F2, AACTCCCATAGTGACCCCTTT, igfbp7zr2

Quantitative analysis of IGFBP-1, -3 and -7. The level of IGFBP-1, -3 and -7 transcripts from the DNA prepared above was determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported elsewhere (17).

PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK), was added. The product expands one intron. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer with the Z sequence, 10 pmol of FAM (6-carboxyfluorescein) tagged probe (Intergen Inc., Oxford, England, UK) and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (BioRad, Hercules, CA, USA) equipped with an optic unit that allowing real-time detection of 96 reactions under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s and 72˚C for 20 s. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. Levels of binding protein transcript were then normalized against CK19 expression already measured in these specimens, to correct for varying amounts of epithelial tissue between samples. Primer sequences are shown in Table II.

Results

Data were analysed using both parametric (Student's *t*-test) and non-parametric tests (Mann-Whitney). Raw values of IBFBP-1, -3 and -7 transcripts are given in Table III.

Tumour vs. normal tissue. For IGFBP-1 and -3 the mean mRNA expression was higher in normal *vs.* tumour tissue but this did not reach statistical significance. For IGFBP-7, this relationship was reversed and reached statistical significance ($p=0.045$). It can be seen however that when the tumour group was subdivided further into grade and NPI score, statistically significant differences emerged.

Table III. *Levels of IGFBP -1, -3 and -7 mRNA and tumour stage and type (raw data).*

Parameter	Subgroups	IGFBP-1 mRNA			IGFBP-3 mRNA			IGFBP-7 mRNA		
		N	Mean	SD	N	Mean	SD	N	Mean	SD
All samples	Normal tissue	24	311.0	574.0	20	588.0	2063.0	23	9.6	19.5
	Tumour tissue	102	166.1	608.1	91	555.0	1644.0	100	37.3	130.4
NPI	1	52	178.9	444.2	44	712.0	2121.0	48	13.8	22.3
	2	33	209.0	917.0	30	226.3	405.8	35	62.9	202.9
	3	13	52.7	70.8	13	935.0	1775.0	13	41.0	107.7
Grade	1	16	89.7	262.0	11	385.0	695.0	16	33.6	65.1
	2	37	94.3	295.3	31	217.0	769.0	34	13.4	22.1
	3	48	250.0	832.0	48	819.0	2131.0	49	55.6	180.6
TNM staging	1	55	114.0	378.4	47	686.0	1895.0	52	14.1	36.7
	2	34	131.3	286.6	31	576.0	1567.0	35	52.4	121.0
	3	5	8.9	19.3	5	0.5	0.7	5	0.01	0.02
	4	4	9.5	7.5	4	55.9	108.9	4	16.6	27.0
Tumour type	Ductal	78	197.5	685.6	71	589.0	1779.0	77	45.3	147.6
	Tubular	12	47.6	114.3	11	408.0	1277.0	11	11.2	13.9
	Mucinous	2	0.0	0.0	2	886.0	1252.0	2	0.4	0.6
	Medullary	2	0.1	0.141	2	23.0	30.5	2	19.7	27.4
	Tubular	2	27.2	38.3	1	1985.4	0.0	2	24.1	34.1
	Other	6	9.11	22.2	5	761.0	1023.0	6	14.7	22.6

mRNA expression with respect to NPI.
For IGFBP-1: There was significantly less mRNA expression in the NPI3 group when compared with the 'normal' group ($p=0.040$) and the NPI1 group ($p=0.050$).
For IGFBP-3: There were no significant differences in mRNA expression with NPI.
For IGFBP7: There were no significant differences in mRNA expression with NPI.

mRNA expression with regard to grade. There was no significant association between mRNA expression and grade of tumour for any of the IGFBPs studied.

mRNA expression with regard to TNM status.
For IGFBP-1: There was significantly less mRNA expression when comparing TNM3 samples with 'normal' tissue ($p=0.017$), with TNM1 samples ($p=0.047$) and with TNM 2 samples ($p=0.019$).

There was also significantly less mRNA expression when comparing TNM4 samples with 'normal' tissue ($p=0.017$), with TNM1 samples ($p=0.046$) and TNM2 samples ($p=0.019$).
For IGFBP-3: There was significantly less mRNA expression when comparing TNM3 samples with TNM1 ($p=0.017$) and with TNM2 samples ($p=0.50$). There was also significantly less mRNA expression when comparing TNM4 samples with TNM1 samples (0.030).
For IGFBP-7: There was significantly more mRNA expression when comparing TNM2 samples with the 'normal' samples ($p=0.048$), but significantly less expression when comparing TNM3 samples ($p=0.028$). Both TNM1 ($p=0.0077$) and TNM2 samples ($p=0.015$) had significantly more expression than TNM3 samples.

mRNA expression and tumour type.
For IGFBP-1: There was significantly less mRNA expression for the medullary group ($p=0.013$) and the tubular group ($p=0.044$) when compared with the ductal group.
For IGFBP-3: There was significantly less mRNA expression for the medullary group when compared to the ductal group ($p=0.0095$).
For IGFBP-7: There was significantly less mRNA expression in the mucinous group when compared with the ductal group ($p=0.0094$).

mRNA expression and ER positivity. There was significantly higher IGFBP-1 mRNA expression in ER+ tumour samples when compared with ER– tumour samples ($p=0.0362$). This association was not found for IGFBP-3 or -7.

Correlation between binding proteins. Using Pearson's correlation coefficient neither IGFBP-1 *vs.* -3 (0.068, ns), nor IGFBP-3 *vs.* -7 (0.056, ns) was significant, but there was a significant association between IGFBP-1 and -7 ($r=0.696$, $p<0.001$).

Discussion

The IGFBP 'superfamily'. As already mentioned, the biological activity of the IGF ligand is modulated by the presence of at least 6 binding proteins which circulate in the serum together with ten cysteine-rich related proteins (IGFBPrP) with

structural and functional similarities to IGFBPs but with a 100x lower affinity for IGF-1. The first protein characterized in this related group was named IGFBP-rP1/Mac 25 and is now synonomous with IGFBP-7. Together these proteins constitute the IGFBP 'superfamily' (14).

Differential secretion of IGFBPs. All human breast cancers secrete IGFBPs to some extent but expression varies according to cell type and receptor status. In one study, mRNA of IGFBPs 2 to 6 was expressed in all of 40 primary breast tumours, with IGFBP-4 and -5 being expressed to a greater extent in ER+ tumours compared with IGFBP-3 which was expressed more in ER– tumours. IGFBP-1 mRNA was absent (10). Several other studies confirm these findings to a lesser or greater degree with one consistent finding of the production of IGFBP-3 being significantly higher in ER- tumours both *in vivo* and in breast cancer cell lines *in vitro*. *In vitro* studies appear to suggest ER– cell lines produce mainly IGFBP-1, -3 and -4, whereas ER+ lines produce GFBP -2 and -4. Interestingly, in one study, the IGFBP content was significantly lower in adjacent normal tissue than the tumour sample although the sample set was small (n=5) (8, 24, 29). In another cell culture study using MCF-7/hs578T cells (4) *vs.* MCF-10A cells (benign), it was shown that IGFBP-3 had a growth promoting effect on normal cells compared with an anti-growth effect on the tumour cells which was reversed by fibronectin. The function of tissue IGFBP-3 and its microenvironment appears therefore to be at least as important as the concentration of local tissue levels in breast cancer.

The role of the cell matrix in determining IGFBP effects. The role of the cell matrix has been further investigated by recent studies which show that IGFBP-1 and -6 do not affect cell adhesion to a general cell matrix in an IGFR-I null breast cancer cell line, whilst IGFBP-4 and -5 increase it and IGFBP -2 and -3 reduce it. When this was repeated on a fibronectin-rich matrix, attachment was increased by IGFBP-3 and reduceded by IGFBP-5 whilst also rendering the effect of IGFBP-3 anti- rather than pro-apoptotic (22). This was also the case in MCF-7 ER+ cell lines. Interestingly, on the MCF-10A normal breast epithelial cell line, IGFBP-3 appears to be anti-apoptotic when cultured on a general matrix which then changes to pro-apoptotic when cultured on fibronectin (4). These findings are of particular clinical significance as breast cancers with a particularly aggressive phenotype often express high concentrations of stromal fibronectin (15).

IGFBP-dependent signalling pathways. The mechanisms of action of IGFBP-3 are complex and at present poorly understood (23). It appears that IGFBP-3 inhibits oestrogen-stimulated cell proliferation in breast cancer cell (28) lines with the ability to potentiate paclitaxel- and ceramide-induced apoptosis (11-13), whilst other *in vivo* studies suggest mitogenicity *via* the EGFR and ras/MAPK signalling pathways (6, 21). It has also been shown that transfection of breast cancer cell lines with IGFBP-3 induces growth inhibition and apoptosis in a p53-independent manner which also renders them more susceptible to radiation-induced apoptosis (5). Therefore, at the cellular level, whilst IGF-1 is anti-apoptotic, IGFBP-3 can be either tumour suppressive or pro-malignant which may be a function of IGF sequestration, IGF-independent actions, the action of its degradation fragments (1) or the nature of the tissue matrix which it is associated with.

IGFBP-7 as a tumour suppressor gene. There is accumulating evidence that IGFBP-7 acts predominantly as a tumour suppressor in breast cancer with reduced levels found in invasive *vs.* ductal carcinoma *in situ* (DCIS) tumours *vs.* normal breast tissue and a worse prognosis seen in those tumours with reduced expression of BP7 (2). Methylation of IGFBP-7 at CpG islands in a 1 kb region containing the promoter and exon 1 in liver carcinoma lines has been shown to lead to down-regulation of protein expression. This evidence, and the knowledge that regional DNA hypermethylation of CpG islands acts to silence tumour suppressor genes such as E-cadherin and VHL, has led to the hypothesis that IGFBP-7 may act as a tumour suppressor gene (18, 19).

In breast cancer lines, allelic loss of heterozygosity has been shown to occur on chromosome 4q in 50% of invasive cancers, which is not only the location of IGFBP-7 but also a suspected tumour suppressor locus in hepatocellular, bladder, cervical, lung, oesophageal, papillary thyroid cancers and leukaemia. In the same study, of 60 primary breast carcinomas, all the invasive tumours were negative for IGFBP-7, 16 cases of DCIS showed weak expression and 12 normal/benign samples showed strong expression using immunohistochemistry (3), which also adds weight to the theory of this protein having a tumour suppressor function. Correlations have also been established between low IGFBP-7 levels and poor prognostic factors particularly in ER-7 invasive breast cancers (20, 26). This data together with the evidence that IGFBP-7 accumulates in senescent mammary epithelium also supports its tumour suppressive function (27).

In our study, it appeared that mRNA expression for IGFBP-1 and -3 was reduced in tumour tissue *versus* normal tissue which although not statistically significant points towards these binding proteins having a putative tumour suppressive function. This theory is supported when analysing mRNA expression of IGFBP-1 with relation to the Nottingham prognostic index (NPI), as the poor prognostic group (NPI3) had significantly reduced expression when compared with both the benign and the

NPI1 group. This relationship is also found when analysing both IGFBP-1 and IGFBP-7 for the TNM classification as the poor prognosis groups, TNM3/4, were associated with significantly lower mRNA expression. For IGFBP-7, although there was significantly higher expression in the tumour group when compared to the 'normal' group as a whole, the putative tumour suppressor function was again highlighted on further subgroup analysis, with 'normal', TNM1 and TNM2 groups having significantly greater expression than the poorer prognosis TNM3 group.

In general these binding proteins have a tumour suppressive function and as such are associated with high expression in favourable prognostic tumours but this does not hold true when performing subgroup analysis for tumour type. Although classically associated with favourable prognoses, the groups medullary (for IGFBP-1 and -3), tubular (for IGFBP-1) and mucinous (for IGFBP-7) were associated with significantly lower mRNA expression when compared with the ductal group. This may be due to different interactions between the binding proteins and the interstitial matrix in these unusual cancer types and may be of particular interest in those with a family history of breast cancer where medullary carcinomas are more common.

Conclusion

The role of IGF and its receptors in the pathogenesis of breast cancer has been extensively investigated and has been shown to be strongly implicated in tumour growth and malignant progression. The eventual physiological effect of IGF however is not only dependent upon receptor ligand binding, but also on IGF bioavailability and metabolism, which in turn relies on the IGF-binding proteins and digestive proteases.

IGFBP-1, -3 and -7 stand out as important members of the IGF superfamily which appear to modulate the anti-apoptotic effects of IGF-1 on human breast cancer, both through IGF-dependent and -independent mechanisms. Increasing evidence is emerging highlighting their roles as potential tumour suppressor genes.

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