Oncogene (2001) 20, 3497–3505

www.nature.com/onc

Downregulation of the potential suppressor gene IGFBP-rP1 in human breast cancer is associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation in estrogen receptor negative tumors

Göran Landberg[1], Hanna Östlund[1], Niels Hilmer Nielsen[2], Göran Roos[2], Stefan Emdin[3], Angelika M Burger[4] and Arun Seth*[,5]

[1]Division of Pathology, Department of Laboratory Medicine, Lund University, Malmö University Hospital, Malmö, Sweden; [2]Department of Medical Biosciences, Pathology, Umeå University, Sweden; [3]Department of Surgical and Perioperative Sciences, Surgery, Umeå University, Sweden; [4]Tumor Biology Center at the University of Freiburg, Germany; [5]Department of Laboratory Medicine and Pathobiology, University of Toronto and Laboratory of Molecular Pathology, Sunnybrook and Women's College Health Sciences Centre, 76 Greenville Street, Toronto, Ontario, Canada

The complex insulin-like growth factor network of ligands, receptors and binding proteins has been shown to be disturbed in breast cancer. In addition to defects in proteins controling cell cycle checkpoints, this type of aberrations could affect tumor growth and survival thereby influencing both tumor aggressiveness and potential response to treatments. We have previously identified the T1A12/mac25 protein, which is identical to the IGFBP-rP1, as a differentially expressed gene product in breast cancer cells compared with normal cells. Here we compare the expression of IGFBP-rP1 in 106 tumor samples with known status of cell cycle aberrations and other clinicopathological data. This was done using a tumor tissue section array system that allows for simultaneous immunohistochemical staining of all samples in parallel. Cytoplasmic staining of variable intensity was observed in most tumors, 15% lacked IGFBP-rP1 staining completely, 20% had weak staining, 32% intermediate and 33% showed strong staining. Low IGFBP-rP1 was associated with high cyclin E protein content, retinoblastoma protein (pRb) inactivation, low bcl-2 protein, poorly differentiated tumors and higher stage. There was a significantly impaired prognosis for patients with low IGFBP-rP1 protein tumors. Interestingly, IGFBP-rP1 showed an inverse association with proliferation (Ki-67%) in estrogen receptor negative tumors as well as in cyclin E high tumors suggesting a separate cell cycle regulatory function for IGFBP-rP1 independent of interaction with the estrogen receptor or the pRb pathway. *Oncogene* (2001) **20,** 3497–3505.

Keywords: IGFBP; breast cancer; proliferation; cell cycle; estrogen receptor

*Correspondence: A Seth
Received 15 January 2001; revised 1 March 2001; accepted 15 March 2001

Introduction

IGFBP-rP1 is a recently described member of the low-affinity IGFBPs that have the capacity to bind IGF. Identified independently by several groups the protein is also known as IGFBP-7 or mac25 (Burger *et al*., 1998; Swisshelm *et al*., 1995; Kato *et al*., 1996; Wilson *et al*., 1997). We isolated the IGFBP-rP1 cDNA from a subtractive cDNA cloning library derived by subtraction of normal breast cell line RNA (Hs578Bst), from RNA of the corresponding tumor cell line (Hs578T). IGFBP-rP1 has subsequently been reported as downregulated at the transcriptional level in other human breast cancer cell lines, possibly by hypermethylation, and to be upregulated in senescent cells and in mammary epithelial cells treated with all-trans-retinoic acid (Swisshelm *et al*., 1995; Komatsu *et al*., 2000). We reported that a fraction of invasive breast carcinomas showed LOH at the IGFBP-rP1 gene locus and this together with earlier publications implicate that IGFBP-rP1 is a potential tumor suppressor gene (Burger *et al*., 1998; Swisshelm *et al*., 1995; Sprenger *et al*., 1999).

The IGFs and IGFBPs form a network of ligands and binding proteins that co-operate in regulating signals from the receptors for insulin, IGF I, and IGF II, and are therefore central players in many important pathways regulating cell growth and apoptosis. This intricate network has been shown to be altered in many cancers potentially leading to disturbed tyrosine kinase signaling and growth advantages (Ellis *et al*., 1998). Besides direct disturbances through the IGF pathway some IGFBPs, such as IGFBP-3, can inhibit proliferation in an IGF independent manner (Martin *et al*., 1999). This proliferative inhibition can be abolished by MAP kinase activation through Ha-*ras*, pinpointing downstream pathways for IGFBPs that are separate from IGFs (Martin and Baxter, 1999).

We and others have shown that G1-S transition defects are found in up to 88% of primary breast

cancer samples and based on such figures it can be hypothesized that G1-S defects might be obligatory in tumor development (Nielsen *et al*., 1997, 1999; Landberg and Roos, 1997). Aberrations in G1-S regulatory proteins are common in other tumor types and overexpression of cyclin E and D1, downregulation of p16 and p27 as well as inactivation of pRb has frequently been observed in breast cancer as well as in other tumors (Lukas *et al*., 1995; Nielsen *et al*., 1996; Sherr, 1996).

In this study we wanted to examine the protein content of IGFBP-rP1 in invasive breast cancer and characterize potential associations with biological and clinical properties. We have earlier identified various types of aberrations in the expression of G1-S regulatory proteins as well as other proteins or systems involved in tumorogenesis in a set of 106 invasive breast cancer samples. These tumor samples have now been arranged in a tumor tissue section array and analysed by immunohistochemistry for IGFBP-rP1 protein content. We observed low IGFBP-rP1 protein content in a major fraction of the tumors and notable associations between low IGFBP-rP1, inactivation of pRb, as well as with proliferation in estrogen receptor negative/high cyclin E tumors.

Results

IGFBP-rP1 expression

The IGFBP-rP1 protein content was monitored by immunohistochemistry in 106 human breast cancers arranged in a tissue array system according to Material and methods. All 106 tumors were possible to evaluate and the T1A12 polyclonal antibody reacting with the IGFBP-rP1 gene product produced a strong cytoplasmic staining pattern in a fraction of the tumors with a generally low background staining in negative cells (Figure 1). The IGFBP-rP1 staining produced a rather homogenous staining pattern in all tumor cells if present and there was no need to count the fraction of positive cells but there were large variations in staining intensity of tumor cells. A classification into four grades (0–3) was therefore applied representing lack of staining, weak staining, intermediate staining and strong staining, respectively. Fifteen per cent of the tumors were negative for IGFBP-rP1 whereas 20% had weak staining, 32% intermediate and 33% strong IGFBP-rP1 staining. For some comparison later in the article, negative and weak staining was denoted low IGFBP-rP1 whereas intermediate and strong staining was called high IGFBP-rP1. The occasional normal breast epithelium in the array was strongly IGFBP-rP1 positive.

IGFBP-rP1 expression and clinico-pathologic parameters

The associations between IGFBP-rP1 and clinico-pathologic parameters are summarized in Table 1. There was a significant association between IGFBP-rP1 and tumor grade determined by Elston–Ellis. Within different sub variables of the grading system (I: presence of tubular formations, II: nuclear grade, III: mitotic index) low IGFBP-rP1 was associated with lack of tubular formations ($P=0.022$) as well as higher mitotic grading ($P=0.023$). There was also an association between higher stage and lower IGFBP-rP1 ($P=0.053$ and $P=0.019$, IGFBP-rP1 in four

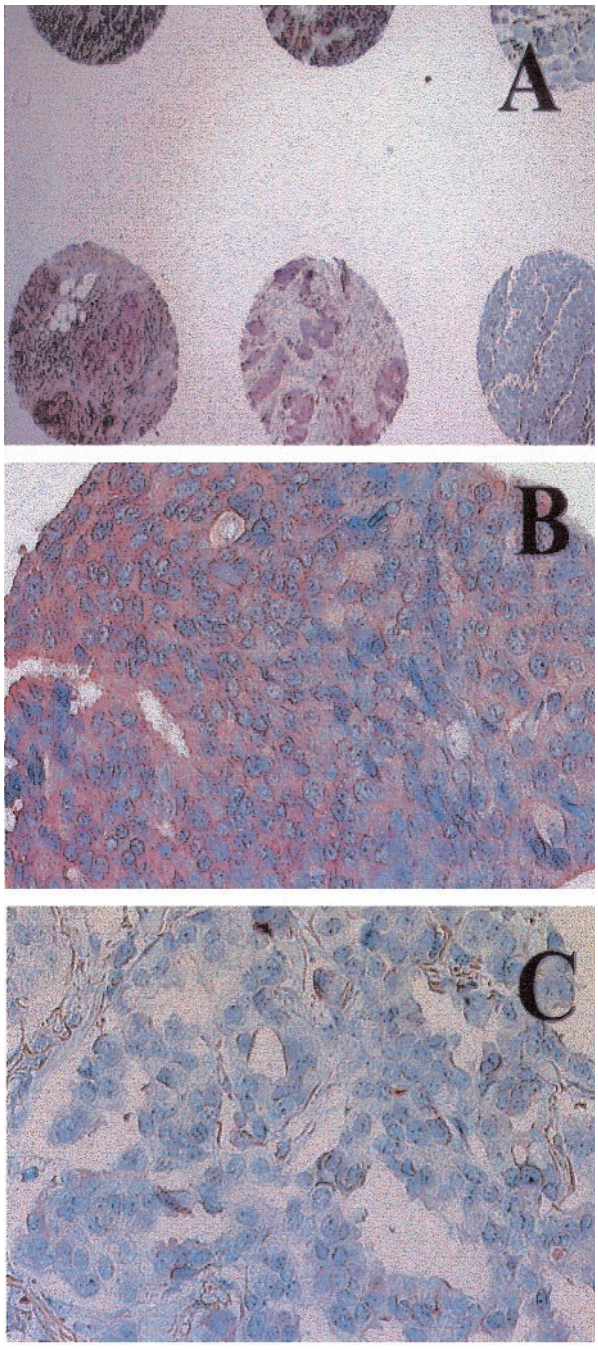

Figure 1 (**a**) A low magnification photo of the tumor array stained with IGFBP-rP1 antibodies showing duplicate biopsies from each tumor. (**b**) An example of a tumor with strong IGFBP-rP1 staining in the cytoplasm and (**c**) lack of staining

Table 1 Associations between IGFBP-rP1 and clinico-pathological data in 106 patients with invasive breast cancer

	IGFBP-rP1			
	Low	*High*	*Numbers*	*Significance*
Tumor type				
Ductal	28	63	105	0.050*
Lobular	4	3		
Mucinous	–	2		
Medullar	4	1		
Grade				
1	1	11	85	0.031*
2	10	21		
3	18	24		
Axillar lymph node				
Negative	16	34	96	0.466
Positive	34	28		
Stage				
1	8	19	98	0.053
2	20	41		
3	2	–		
4	5	3		
Menopausal status				
Pre	12	20	106	0.613
Post	24	50		
DNA-content				
Diploid	16	29	98	0.716
Aneuploid	17	36		
Estrogen receptor				
Positive	21	55	106	0.013*
Negative	16	14		
Progesteron receptor				
Positive	17	49	105	0.017*
Negative	19	20		
Tumor size* (mm)	25.99	22.00	102	0.157
Proliferation* (Ki-67%)	27.75	16.00	104	0.035*

*Mean rank used and Mann-Whitney *U*

respectively two groups). Estrogen receptor positive tumors were strongly associated with high IGFBP-rP1 ($P=0.013$) as illustrated in Figure 2. A similar association as for estrogen receptor was observed for the progesterone receptor (data not shown).

IGFBP-rP1 expression and survival

Ninety-six of the patients with unilateral and non-metastatic disease were included in the survival analysis with a median follow up time of 53 months. There was a non statistical significant difference in survival for patients divided according to IGFBP-rP1 protein content ($P=0.065$) even though patients with intermediate or high IGFBP-rP1 tumors seemed to have a better survival compared with patients with lower IGFBP-rP1 tumors (shown in Figure 3a). However, there was a significant difference in survival ($P=0.015$) when divided into low IGFBP-rP1 (including negative or low staining intensity), and high IGFBP-rP1, (including intermediate and high staining intensity (Figure 3b). This indicates that loss of IGFBP-rP1 was associated with unfavorable prognosis during the first years after surgery. However, in a multivariate analysis including Ki-67, estrogen receptor status, node status and IGFBP-rP1 the only independent significant prognostic factor was estrogen receptor status (data not shown).

IGFBP-rP1 expression and proliferation

The insulin-like growth factors and related binding proteins have profound effects on the growth and differentiation of normal and malignant cells. We compared IGFBP-rP1 staining pattern with the pro-

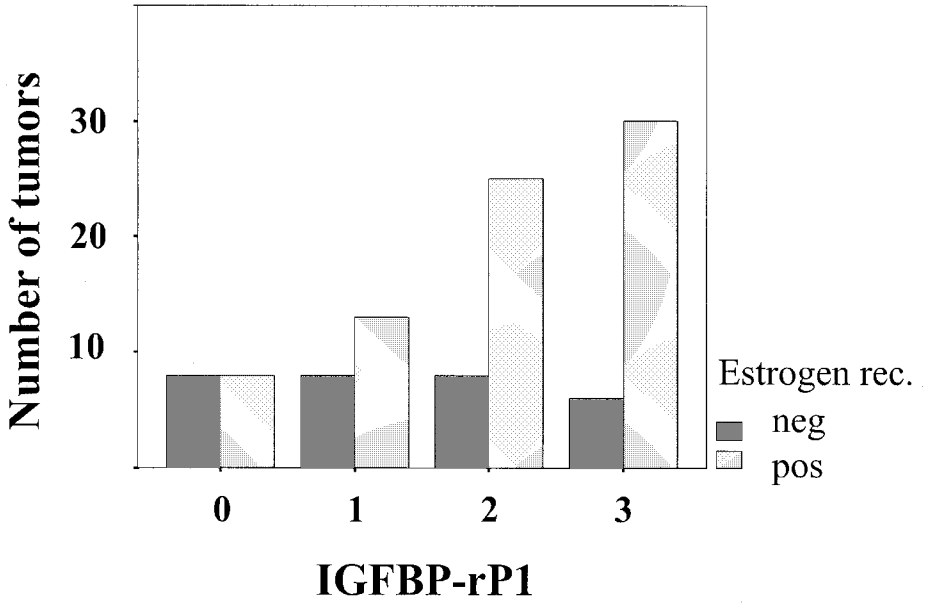

Figure 2 Histogram illustrating the fraction of estrogen receptor positive and negative tumors in the different IGFBP-rP1 categories

liferation marker Ki-67. The fractions of actively proliferating tumor cells in this set of breast tumors were previously characterized by using the well established Ki-67 antibody MIB-1 (Nielsen *et al*., 1996). We found an inverse significant association between the fraction of Ki-67 positive cells and IGFBP-rP1 ($P=0.01$). This association was nevertheless only observed in estrogen receptor negative tumors ($P=0.009$) in contrast to estrogen receptor positive tumors ($P=0.816$) as illustrated in Figure 4.

IGFBP-rP1 expression and cell cycle alterations

The G1/S checkpoint is frequently deregulated in breast tumors and the expression levels of various cell cycle regulatory proteins have been found to be altered (Nielsen *et al*., 1999; Sherr, 1996). Due to the observed association between IGFBP-rP1 and proliferation and the potential function for IGFBP-rP1 as a tumor suppressor gene, we wanted to examine possible associations between IGFBP-rP1 and specific alterations in G1/S regulatory proteins. In this group of breast tumors, the levels of retinoblastoma protein, cyclin E, cyclin D1, p16, p21, p27 and p53 have been characterized by us previously (Nielsen *et al*., 1996, 1997, 1999; Lodén *et al*., 1999). In addition, we examined expression of three other proteins involved in oncogenesis (c-erbB2, bcl-2, telomerase) as summarized in Table 2. We found a strong association between IGFBP-rP1 and cyclin E protein content. Tumors with low IGFBP-rP1 had in general higher and more dispersed cyclin E protein content in comparison to IGFBP-rP1 high tumors having lower and less variable cyclin E as illustrated in Figure 5. From this figure it can also be concluded that the relation between cyclin E and proliferation was different in tumors with high or low IGFBP-rP1. IGFBP-rP1 high tumors had only a moderate cyclin E increase with increasing proliferation (Figure 5a), a behavior that can be expected from a protein that is expressed in a fraction of the cell cycle and therefore should increase proportionally to the percentage of Ki-67 positive cells. In contrast, tumors with low IGFBP-rP1 showed a more scattered association between proliferation increase and cyclin E content (Figure 5b), a property that we earlier defined as a non-proliferation associated cyclin E increase, potentially indicating overexpression of the protein (Loden *et al*., 1999).

Besides the observed relationship between IGFBP-rP1 and cyclin E, we found an inverse association to cyclin D1 and therefore wanted to explore the correlation between cyclin D1, E and IGFBP-rP1 in detail. We have earlier shown that the protein content of cyclin E and D1 in breast cancer have a specific pattern with either high cyclin E/low cyclin D1, high cyclin D1/low E or low of both cyclins (Nielsen *et al*., 1997). As illustrated in Figure 6 all tumors with high cyclin E/low D1 were IGFBP-rP1 low. As shown earlier, tumors with pRb inactivation defined by several methods, were also found in the high cyclin E/low D1 arm (Nielsen *et al*., 1997). Low IGFBP-rP1 and pRb inactivation was therefore localized to the same cyclin E/D1 arm, and pRb inactivation was further strongly associated with low IGFBP-rP1 (Table 2). This links inactivation of Rb, one of the archetypal suppressor genes, with inactivation of the potential suppressor gene IGFBP-rP1 and both these events are associated with cyclin E high/D1 low tumors (Nielsen *et al*., 1997 and data in this article).

The relationship between proliferation and IGFBP-rP1 was also explored in tumors with different patterns of expression of cyclin E and cyclin D1. Interestingly, IGFBP-rP1 was only associated with proliferation in tumors with cyclin E high/D1 low ($P=0.015$) whereas tumors with either low cyclin D1/E or cyclin D1 high/E low, did not show any significant proliferation difference with varying IGFBP-rP1 ($P=0.929$, $P=0.757$, respectively, Figure 7). If tumors with high cyclin E were further subdivided according to function of pRb (determined by immunohistochemistry) there were indications of proliferative differences regarding IGFBP-rP1 for both pRb inactivated and pRb normal

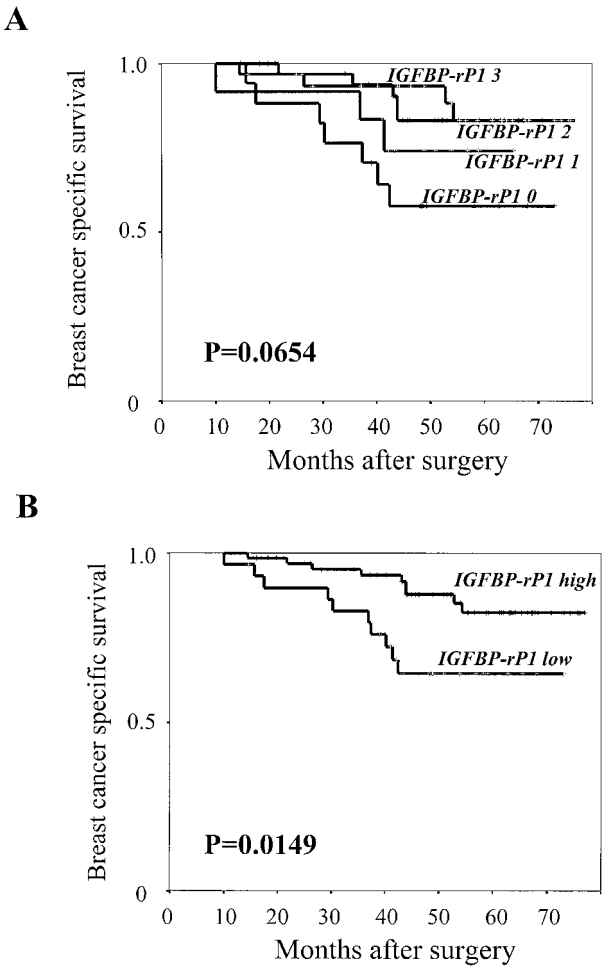

Figure 3 Breast cancer specific survival in 96 patients with non-metastatic disease at the time of diagnosis divided in (**a**) four different categories according to IGFBP-rP1 staining or (**b**) two categories

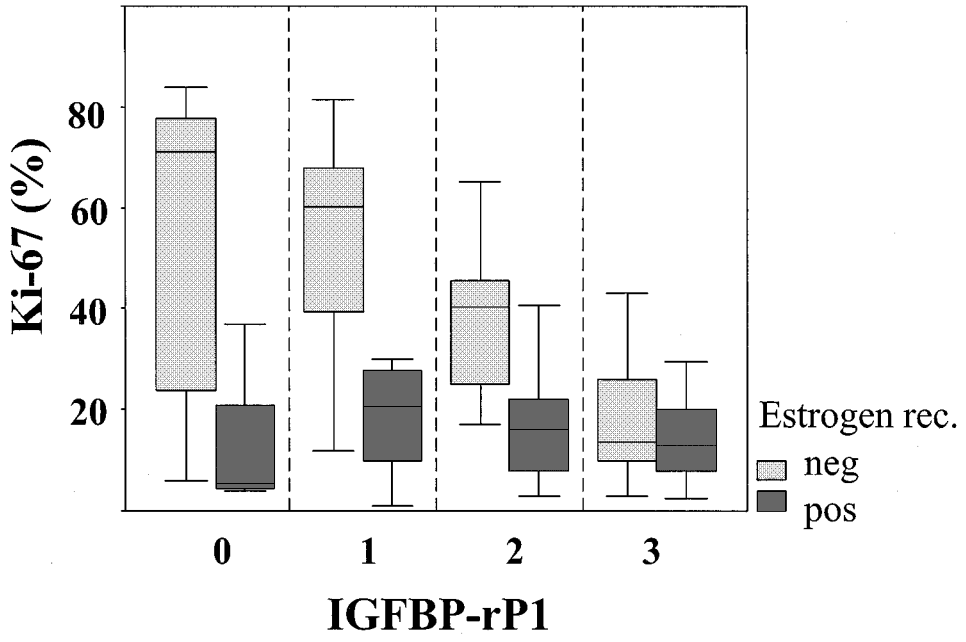

Figure 4 Box plots illustrating the proliferation (Ki-67) in the four IGFBP-rP1 categories separate for estrogen receptor positive and negative tumors

Table 2 Associations between IGFBP-rP1 and aberrations in selected cell cycle regulatory proteins and other proteins involved in oncogenesis

	IGFBP-rP1			
	Low	*High*	*Numbers*	*Significance*
Cyclin E	0.48	0.24	106	0.001*
Cyclin D1	0.28	0.65	106	0.008*
Teleomerase	2.14	1.50	100	0.546
p16				
0	20	52	106	0.001*
1	7	15		
2	10	2		
p21				
0	15	16	100	0.027*
1	12	25		
2	7	25		
p27				
0	–	5	102	0.836
1	9	5		
2	27	56		
pRB (immuno)				
Normal	24	63	94	0.002*
Altered	6	1		
p53 (immuno)				
Low	23	52	104	0.176
High	13	16		
p53 (SSCP)				
Normal	30	58	105	0.576
Mutations	7	10		
bcl-2				
0	7	7	105	0.014*
1	7	7		
2	11	16		
3	11	39		
c-erbB2				
Normal	30	54	103	0.926
High	7	12		

*Mean rank used and Mann-Whitney *U*

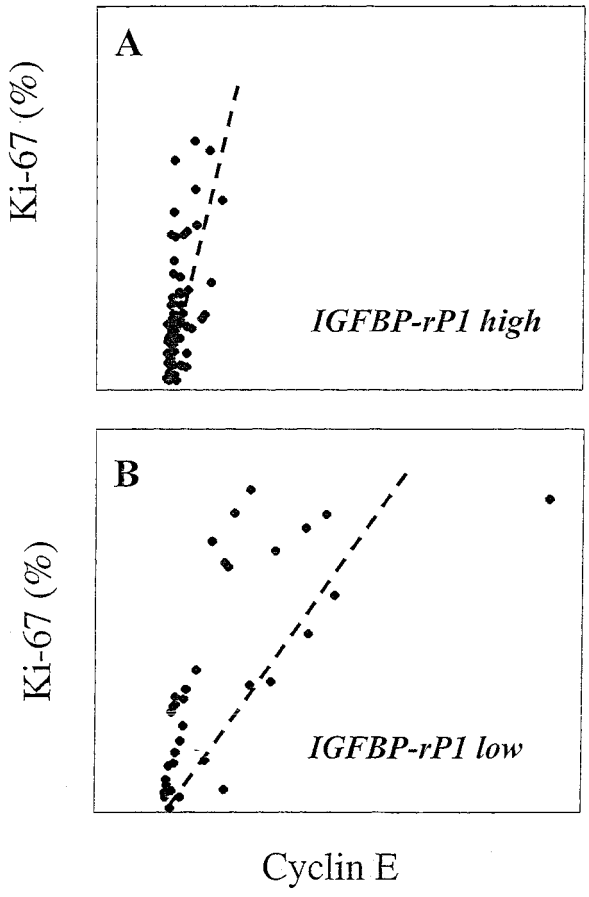

Figure 5 Scatter plots of relative cyclin E protein contents determined by Western blotting and percentage Ki-67 positive cells in (**a**) IGFBP-rP1 high tumors and (**b**) IGFBP-rP1 low tumors. Regression lines for the subgroups are indicated in the figure

tumors ($P=0.062$ and $P=0.169$ respectively). This suggests that IGFBP-rP1 could affect proliferation in pRb inactivated breast cancers, independent of a functional pRB pathway.

IGFBP-rP1 was not associated with c-erbB2 protein or telomerase but strongly and inversely associated with Bcl-2 ($P=0.014$) as presented in Table 2. This association was only detected in estrogen receptor positive tumors ($P=0.016$) and not in the estrogen receptor negative tumors ($P=0.688$).

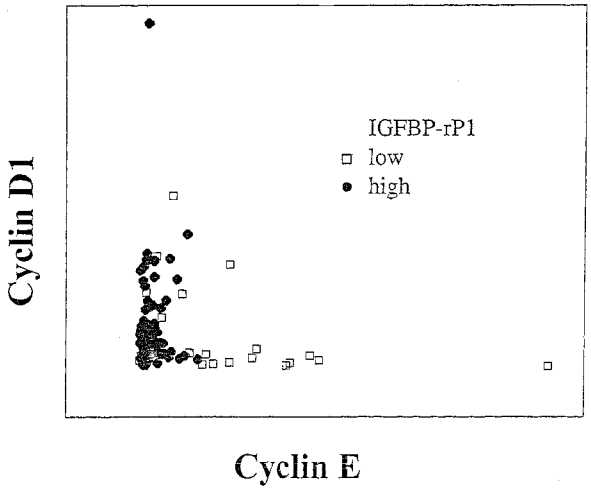

Figure 6 The relation between high (2–3) and low (0–1) IGFBP-rP1, cyclin E and cyclin D1 protein content in human breast cancer. Relative cyclin E and cyclin D1 protein contents were determined using densitometry and Western blot analysis

Discussion

In this study we have analysed the IGFBP-rP1 protein expression in a thoroughly characterized group of 106 invasive breast samples using the novel tumor tissue array technique and immunohistochemistry. The tumor tissue section array allows representative 'donor' biopsies from formalin fixed archival material to be arranged into a recipient paraffin block. Thus multiple tumors are included in each tissue section that are cut from the donor block. This enables parallel analysis by immunohistochemistry and FISH, saving time and better utilizing valuable collection of tumor samples (Kononen *et al*., 1998). In this study we have used a semi-quantitative evaluation of the IGFBP-rP1 immunostaining because of a clear difference between strongly positive cytoplasmic staining, moderate staining or lack of staining in the tissue samples, an approach that has been used in other studies using similar tumor tissue arrays (Richter *et al*., 2000). The only disadvantage with the method is that small areas of the tumors are analysed and if the tumor is heterogeneous regarding various factors, a non-representative area might be characterized. To circumvent

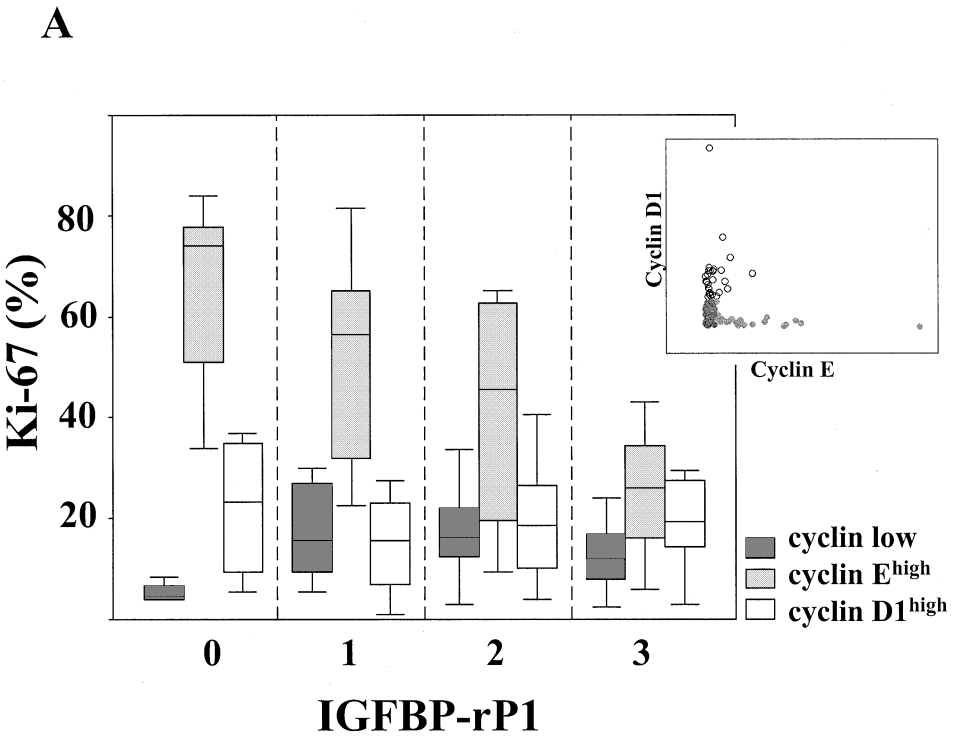

Figure 7 Box plots illustrating the proliferation (Ki-67) in the four IGFBP-rP1 categories separately for (1) cyclin E high/D1 low tumors, (2) cyclin D1 high/E low and (3) cyclins low tumors. A scatter plot of cyclin E and cyclin D1 protein content and the definition of the categories are projected in the plot

this problem we clearly marked the area of interest on each slide of the whole section thereby partly controling the examined tumor area. In the majority of the samples we did not notice any substantial variation between the two biopsies obtained in the tumor array. Other studies have shown some but minor variations between analysis of tumor arrays and regular slides (Moch *et al*., 1999; Schraml *et al*., 1999) supporting the reliability of tumor array analysis. In our hands the tumor array produced excellent morphology and immunohistochemistry and we believe that the method will be useful for characterization and perhaps screening of multiple gene products in tumors.

As suggested previously and also observed in this study IGFBP-rP1 has several characteristics of a potential suppressor gene. TGF b has been shown to up-regulate IGFBP-rP1 expression in various cancer cell lines. Swisshelm *et al*. (1995) have shown that variety of breast cancer cell lines lacked IGFBP-rP1 expression and Burger *et al*. (1998) have reported LOH at the gene locus for IGFBP-rP1 in breast tumors. In accordance with earlier data we observed that approximately 40% of the tumors had low or no IGFBP-rP1 staining suggesting that the gene or the gene product was inactivated in a fraction of transformed invasive breast cancer samples. As shown by us earlier (Burger *et al*., 1998), 50% of the informative samples (5/10) in a collection of 30 matched pairs of breast normal and invasive cancer tissues showed allele specific LOH suggesting that the IGFBP-rP1 gene was inactivated by mutation/deletions in at least a portion of the tumors. Further studies analysing potential mutations in the IGFBP-rP1 gene in human breast cancer will be needed to clarify the role for IGFBP-rP1 as a suppressor gene in breast cancer. There are also alternative mechanisms for inactivation of IGFBP-rP1 such as potential increased protein degradation or hypermethylation of CpG islands in the promoter region that have to be explored to fully understand the low IGFBP-rP1 protein content observed in a fraction of the tumors. In line with a suppressor function for IGFBP-rP1, tumors with low protein content were associated with higher stage and grade as well as lower differentiation. These are properties that are associated with aggressive tumor behavior and patients with low IGFBP-rP1 also had a significantly impaired prognosis in univariate survival analysis (Figure 3).

The relation between proliferation and IGFBP-rP1 was complex but interesting. When all tumors were analysed there was a significant difference in proliferation between the four IGFBP-rP1 groups but this was due to the strong association between IGFBP-rP1 and proliferation in the subgroup of estrogen receptor negative tumors. This suggests that downregulation of IGFBP-rP1 either is a secondary phenomenon to an increased proliferation in this sub group of tumors or that the actual loss of IGFBP-rP1 could increase proliferation. In favor for a direct growth regulatory role for IGFBP-rP1 is the observation that there are genetic events associated with the IGFBP-rP1 gene (LOH) supporting that the downregulation of IGFBP-rP1 is not a secondary event. The important observation is nevertheless that the association between IGFBP-rP1 and proliferation is observed only in estrogen receptor negative tumors, a difference from the majority of the IGF related functions that are closely associated with the insulin/IGF/estrogen receptor pathways. Another exception is the overexpression of IGFBP-3 that is mainly observed in estrogen receptor negative breast cancers with an inhibitory effect on proliferation in this subgroup (Martin *et al*., 1999). Thus, a similar effect on the sub-group of estrogen receptor negative tumors but with differences regarding gain and loss of function. The over representation of low IGFBP-rP1 in estrogen receptor negative tumors suggests a selective advantage for low IGFBP-rP1 in this tumor group. Low IGFBP-rP1 was further observed in estrogen receptor positive tumors but at a lower frequency, suggesting that loss of IGFBP-rP1 produces a selective advantage also in these tumors but probably not through a proliferative benefit. In general, our results support an estrogen independent proliferative effect for IGFBP-rP1 that could be IGFR independent. Our results further highlight the importance of analysing several variables in tumor materials to identify different behaviors in subgroups of tumors.

Tumors with high cyclin E/low cyclin D1 are strongly associated with lack of estrogen receptor (Nielsen *et al*., 1997) and it was therefore anticipated that this group of tumors would be similar to estrogen receptor negative tumors regarding association between IGFBP-rP1 and proliferation. Interestingly, tumors with high cyclin E/low cyclin D1 are either pRb inactivated (Nielsen *et al*., 1997) or seem to bypass the pRb pathway in growth control. This hypothesis is based on experiments showing lack of association between pRb phosphorylation and proliferation in cyclin E high tumors with apparently functional pRB (determined by immunohistochemistry) but strong association between cyclin E associated kinase activity and proliferation (Lodén *et al*., 1999 and unpublished data). This suggests that there are alternative substrates for cyclin E besides pRB that are the key regulators of proliferation in these tumors. Cell lines experiments have also shown that cyclin E can induce proliferation in serum starved $Rb^{-/-}$ (Ohtsubo *et al*., 1995) supporting the existence of important alternative cyclin E targets. Due to the strong association between IGFBP-rP1 and proliferation in cyclin E high tumors, IGFBP-rP1 could be linked with proliferation in a pRb independent manner.

Recently Spruck *et al*. (1999) showed that genetic instability could be induced in fibroblast and human breast cancer cell lines by overexpression of cyclin E. The mechanism for this chromosome instability could be that aberrant cyclin E expression delays the S phase transition time thereby affecting the accuracy of DNA synthesis with an impaired DNA replication. In agreement with these data we have earlier shown that there is an association between overexpression of cyclin E and presence of aneuploidy in breast cancer (Nielsen

et al., 1996 and unpublished data). LOH of the retinoblastoma gene is exclusively observed in cyclin E high tumors and we have also recently observed that p53 inactivation by insertions and deletions in contrast to p53 inactivation by missense mutations are over represented in cyclin E high tumors (unpublished data). This means that besides low IGFBP-rP1 protein content, both inactivation of pRb and p53 by gross DNA aberrations are associated with high cyclin E expression suggesting that overexpression of cyclin E could be an important primary event in the transformation process leading to secondary inactivation of multiple suppressor genes by the induced genetic instability.

IGFBP-rP1 has previously also been reported to have tumor suppressor function in prostate cancer by some (Sprenger *et al*., 1999), but to be highly expressed in prostate cancers by others (Degeorges *et al*., 1999), the latter being in contrast to the results presented in this article indicating loss of IGFBP-rP1 protein in a fraction of the breast cancer samples. Nevertheless, our results clearly suggest that IGFBP-rP1 might have important suppressor gene like functions for breast cancer. By using tumor arrays and a well characterized set of breast cancers we have been able to observe associations between IGFBP-rP1 and aberrations in important growth regulators such as cyclin E. This exemplifies the need to analyse several variables in tumors to better illustrate the complex pattern of multiple genetic events involved in tumorigenesis. Tumor arrays are valuable for associating candidate proteins with specific tumor properties, clinical behaviors or treatment responses and we found that the IGFBP-rP1 as well as cyclin E are key proteins that should be analysed in breast cancer samples in order to better evaluate and determine treatment strategies.

Materials and methods

Tumor samples and tissue array construction

Diagnostic tumor samples were collected from 106 patients with breast carcinoma. Twenty-seven patients had stage I, 61 patients stage II, two patients stage III and eight patients stage IV, and eight patients had unknown stage. No patient had been treated with radiation or chemotherapy before sampling. All tumors were attended immediately after excision with at least two pieces snap frozen in liquid nitrogen and stored in -80°C. Protein extracts were prepared as described earlier (Nielsen *et al*., 1996). Adjacent tissue samples were formalin fixed, paraffin embedded and used for routine morphological examination and tumor grading according to (Page *et al*., 1995), immunohistochemical staining and tumor tissue array construction.

In order to ensure that clearly defined areas of tumor samples were used for tissue section array construction, a slide bearing a fresh tissue section from the paraffin block of each tumor sample was prepared and stained with hematoxylin. Areas including representative tumor cells were indicated on the slide. At least two, 0.6 mm large biopsies corresponding to the marked area at the slide were then taken from each donor tumor paraffin block. Each section was mounted in a recipient paraffin block using a tissue array machine according to the manufacturer's instructions (Beecher Instruments, MD, USA). In order to minimize handling problems with the array, only 60–80 biopsies were arranged in each recipient paraffin block.

The order of the tumors was documented in a spreadsheet in order to link IGFBP-rP1 staining results for each unique tissue with the original donor tumor samples. The quality of the tissue array was monitored after completion by a hematoxylin stain and the few cases with lack of visible tumor cells in the duplicate biopsies were processed in a second round and mounted in a separate paraffin block. The quality of the tissues in the arrays was in general excellent with preserved morphology.

Immunohistochemistry

Six μm sections of the paraffin embedded tissue arrays were dried, deparaffinised, rehydrated and microwave treated for 10 min in a citrate buffer (pH of 7.0) before being processed in an automatic immunohistochemistry staining machine according to standard procedures (Ventana 320–202, Ventana inc., AZ, USA) using a polyclonal T1A12 antibody (Burger *et al*., 1998) diluted 1 : 300, recognizing IGFBP-rP1. Two biopsies from each tumor were examined to ensure reproducibility in the analysis. If neither of the examined biopsies were satisfactory regarding quality of the cells or staining, a second set of biopsies was examined in a separate tumor array. The tumor array was examined by two separate investigators and cases with divergent results between the examiners were mutually re-examined followed by a conclusive decision. The IGFBP-rP1 staining produced a rather homogenous staining pattern in all tumor cells if present and there was no need to count the fraction of positive cells but there were large variations in staining intensity of tumor cells. A classification into four grades (0–3) was therefore applied representing lack of staining, weak staining, intermediate staining and strong staining, respectively (Richter *et al*., 2000).

Determination of cell cycle and other aberrations in the tumor material

The material of breast tumors arranged in the tissue array has earlier been extensively characterized regarding protein contents of several important regulators of the G1/S transition in the cell cycle and the details of the separate characterizations have been published (Landberg and Roos, 1997; Nielsen *et al*., 1996; Lodén *et al*., 1999). In brief, cyclin E, cyclin D1, p16 and pRB were characterized using Western blotting and densitometry and denoted a relative value to a cell line standard included on each filter. The fraction of p21, p27, Ki-67, p53 positive cells and staining intensities of bcl-2 (clone 124, Dakopatts AB, Copenhagen, Denmark) and c-erbB2 (Dakopatts AB, Copenhagen, Denmark) have also been determined earlier using immunohistochemistry (Lodén *et al*., 1999; Roos *et al*., 1998 and unpublished data). The presence of nuclear pRb staining as an indicator of a functional gene product (detailed in Landberg and Roos, 1997) was monitored with the C15 antibody (Santa Cruz, CA, USA) and immunohistochemistry. SSCP analysis of exons 5–8 in the p53 gene was performed to screen for p53 mutations as reported elsewhere (Roos *et al*., 1998). Telomerase activity was determined by the TRAP assay (Roos *et al*., 1998).

Statistical methods

Associations between linear parameters were calculated using Spearman's two-tail significance test. The Kruskal-Wallis non-parametric significance test was applied when comparing a linear and a grouped parameter except for comparison between the four IGFBP-rP1 groups and Ki-67 where Spearman's significance test was used. Comparisons between groups were performed with the log-rank test and cross table analysis with the chi-square test. All calculations were performed using SPSS version 9.0 (SPSS inc., IL, USA).

Acknowledgments
We would like to thank Bodil Bäcklund and Britta Lindgren for skilful technical assistance and Martin Lodén for fruitful discussions. This work was supported by grants from the NCIC, Canadian Breast Cancer Research Initiative to A Seth and Swedish Cancer Society and Lion's Cancer research foundation, Umeå University to G Landberg.

References

Burger AM, Zhang X, Li H, Ostrowski JL, Beatty B, Venanzoni M, Papas T and Seth A. (1998). *Oncogene,* **16,** 2459 – 2467.

Degeorges A, Wang F, Frierson HF, Seth A, Chung LKW and Sikes RA. (1999). *Cancer Res.,* **50,** 2787 – 2790.

Ellis MJ, Jenkins S, Hanfelt J, Redington ME, Taylor M, Leek R, Siddle K and Harris A. (1998). *Breast Cancer Res. Treat.,* **52,** 175 – 184.

Kato MV, Sato H, Tsukada T, Ikawa Y, Aizawa S and Nagayoshi MA. (1996). *Oncogene,* **12,** 1361 – 1364.

Komatsu S, Okazaki Y, Tateno M, Kawai J, Konno H, Kusakabe M, Yoshiki A, Muramatsu M, Held WA and Hayashizaki Y. (2000). *Biochem. Biophys. Res. Commun.,* **267,** 109 – 117.

Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G and Kallioniemi OP. (1998). *Nat. Med.,* **4,** 844 – 847.

Landberg G and Roos G. (1997). *APMIS,* **105,** 575 – 589.

Lodén M, Nielsen NH, Roos G, Emdin SO and Landberg G. (1999). *Oncogene,* **22,** 2557 – 2566.

Lukas J, Aagaard L, Strauss M and Bartek K. (1995). *Cancer Res.,* **55,** 4818 – 4823.

Martin JL and Baxter RCJ. (1999). *Biol. Chem.,* **4,** 16407 – 16411.

Moch H, Schraml P, Bubendorf L, Mirlacher M, Kononen J, Gasser T, Mihatsch MJ, Kallioniemi OP and Sauter G. (1999). *Am. J. Pathol.,* **154,** 981 – 986.

Nielsen NH, Arnerlov C, Emdin SO and Landberg G. (1996). *Br. J. Cancer.,* **74,** 874 – 880.

Nielsen NH, Emdin SO, Cajander J and Landberg G. (1997). *Oncogene,* **14,** 295 – 304.

Nielsen NH, Arnerlov C, Cajander S and Landberg G. (1998). *Anal. Cell. Pathol.,* **17,** 177 – 188.

Nielsen NH, Lodén M, Cajander J, Emdin SO and Landberg G. (1999). *Breast Cancer Res. Treat.,* **56,** 105 – 112.

Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM and Pagano M. (1995). *Mol. Cell Biol.,* **15,** 2612 – 2624.

Page DL, Ellis IO, Elson CW. (1995). *Am. J. Clin. Pathol.,* **103,** 123 – 124.

Richter J, Wagner U, Kononen J, Fijan A, Bruderer J, Schmid U, Ackermann D, Maurer R, Alund G, Knonagel H, Rist M, Wilber K, Anabitarte M, Hering F, Hardmeier T, Schonenberger A, Flury R, Jager P, Fehr JL, Schraml P, Moch H, Mihatsch MJ, Gasser T, Kallioniemi OP, Sauter G. (2000). *Am. J. Pathol.,* **157,** 787 – 794.

Roos G, Nilsson P, Cajander S, Nielsen NH, Arnerlov C and Landberg G. (1998). *Int. J. Cancer,* **21,** 343 – 348.

Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, Mihatsch MJ, Kallioniemi OP and Sauter G. (1999). *Clin. Cancer Res.,* **5,** 1966 – 1975.

Sherr CJ. (1996). *Science,* **6,** 1672 – 1677.

Sprenger CC, Damon SE, Hwa V, Rosenfeld RG and Plymate SR. (1999). *Cancer Res.,* **59,** 2370 – 2375.

Spruck CH, Won KA and Reed SI. (1999). *Nature,* **16,** 297 – 300.

Swisshelm K, Ryan K, Tsughya K and Sager R. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4472 – 4476.

Wilson EM, Oh Y and Rosenfeld RGJ. (1997). *Clin. Endocrinol. Metab.,* **82,** 1301 – 1303.