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Mannose-6-phosphate/insulin-like growth factor-II receptor expression levels during the progression from normal human mammary tissue to invasive breast carcinomas☆

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Abstract

The putative role of mannose-6-phosphate/insulin-like growth factor-II receptor (M6P/IGFII-R) as a tumour suppressor and its value as a prognostic marker of breast cancer was studied in 42 benign breast diseases (BBD), 61 *in situ* carcinomas (CIS) and 133 invasive carcinomas. The receptor was quantified by immunohistochemistry with a computerised image analyser, using specific polyclonal IGY antibodies. The M6P/IGFII-R level varied markedly according to the different patient samples, but median values and distributions were similar in lesions and normal adjacent glands. However, the receptor level was significantly increased in high-grade ductal carcinomas *in situ* (DCIS) and decreased in invasive carcinomas relative to adjacent normal tissue. The M6P/IGFII-R protein concentration in invasive breast carcinomas was mostly independent of prognostic parameters: tumour size, histological grade, lymph node (N) invasiveness and oestrogen receptor α (ERα) status. The only positive correlation was with cathepsin D, the progesterone receptor (PgR) and with patients aged >60 years. These results do not support the hypothesis of a frequent and early inactivation of the M6P/IGFII-R gene in breast cancer. Clinical follow-up of patients might reveal a prognostic value for one of the cathepsin receptors.

Keywords: M6P/IGFII receptor; Cathepsin D; Tumour suppressor; Benign breast disease; *In situ* mammary carcinoma; Invasive breast carcinoma; Carcinogenesis; Immunohistochemistry

1. Introduction

The multifunctional mannose-6-phosphate/insulin-like growth factor-II receptor (M6P/IGFII-R) has been proposed to play a role in carcinogenesis as a tumour suppressor activating the growth inhibitor transforming growth factor β1 (TGFβ1) [1], neutralising the mitogen insulin-like growth-factor-II [2] in lysosomes, and preventing cathepsin secretion [3–5]. In hepatocarcinomas, there is fairly substantial evidence linking the receptor with a role as a tumour suppressor and for an alteration in its function due to loss of heterozygosity (LOH) and mutations in the remaining allele [6,7]. In breast cancer, its functional role is more debated, since the argument is only supported by evidence of LOH in the 6q25-27 region coding for the receptor in two high-grade ductal carcinomas *in situ* (DCIS) out of five studied [8,9].

We have considered that M6P/IGFII-R could be a regulator of pro-cathepsin D trafficking in cancer cells based on the assumption that a defect in the receptor might explain the increased secretion of pro-cathepsin D observed in several types of cancer cells and particularly in breast and ovarian cancers [3–5] as well as due to M6P-independent targeting into lysosomes in these cells [10,11]. However, no point mutations in the receptor were observed in three breast cancer cell lines hypersecreting pro-cathepsin D [12]. Moreover by *in situ* hybridisation, we found a positive

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correlation with cathepsin D mRNA level measured in serial sections of invasive breast cancers [13].

We recently obtained high affinity polyclonal antibodies raised against purified human M6P/IGFII-R prepared from hen's egg yolks [14]. These antibodies allow M6P/IGFII-R quantification in frozen tissue sections, as previously described [14], and in formalin-fixed paraffin sections as shown in this study. A pilot study on frozen sections of 21 breast cancers suggested a decrease in the receptor protein in the tumour tissue compared with adjacent normal ducts, and this decrease seemed to be correlated with lymph node (N) invasiveness, in agreement with a tumour suppressor function [14]. In an attempt to determine whether this receptor could be useful as a prognostic marker to complement assays of hormone receptors and proteases such as cathepsin D [15,16], we studied the M6P/IGFII-R level in 133 invasive breast cancer tumours in frozen sections and in 95 patients, including 42 benign breast diseases (BBD) and 61 *in situ* carcinomas (CIS), using formalin-fixed paraffin-embedded samples.

2. Patients and methods

2.1. Patients and tissue sampling

Two populations were studied separately using different staining protocols.

a—95 patients with BBD or CIS were included in a multicentre prospective study from 1994 to 1998 at the University Hospitals of Montpellier and Nîmes, France. Their age ranged from 30 to 79 years, with a median of 51 years. Forty-six patients were premenopausal and 49 menopausal. Written consent was obtained for all patients. Investigations were approved by the local ethical committee. Oestrogen receptor α (ERα) and β (ERβ), cathepsin D and Ki67 levels had been assayed previously [17,18].

All tissues were collected during breast surgery for diagnostic or therapeutic purposes. BBD were separated into three groups (non-proliferative, proliferative without atypia and proliferative with atypia) of at least 10 patients each and classified according to their relative risk of invasive breast cancer using the Dupont and Page histological criteria [19]. DCIS were classified according to their nuclear grade [20,21]. For each patient, formalin-fixed and paraffin-embedded tissue blocks containing the highest risk lesion and histologically-normal glandular structure at the periphery of lesions were selected. Different histological lesions with the same risk were included, explaining why 103 lesions were analysed from only 95 patients. Low-grade and intermediate-grade DCIS and lobular carcinomas *in situ* (LCIS) were pooled for the statistical analysis. Two high-grade DCIS were associated with a LCIS. Three atypical hyperplasias and 37 CIS were located at the periphery of invasive carcinomas. There were no significant differences in M6P/IGFII-R staining in these lesions compared with isolated CIS and atypical hyperplasias without invasive carcinoma, so these lesions were included in the study.

b—133 invasive breast cancer tissues, collected in liquid nitrogen from 1997 to 2000, were assayed for ERα, progesterone receptor (PgR) and cathepsin D, mostly using an immunohistochemical technique on serial frozen sections (106 patients) or biochemical assays for 27 larger (>0.1 g) tumour samples. Among them, 41 contained normal mammary duct structures. Only breast tumours without clinical distant metastasis were included. The patient age ranged from 29 to 88 years, with a median of 56 years. 37 patients were <50 years and $90 \geqslant 50$ years.

2.2. Immunohistochemistry

M6P/IGFII-R immunohistochemical analysis was performed using purified chicken polyclonal anti-human M6P/IGFII-R IGY antibodies (IGY 415) previously produced from egg's yolks and characterised in Ref. [14]. Their specificity had been shown by Western blotting and the validity of immunohistochemistry by incubation with the purified receptor [14].

a—For the paraffin study, 5 μm thick tissue sections were dewaxed, rehydrated, and treated with 0.1% pronase in phosphate buffered solution (PBS) prewarmed at 37 °C for 10 min. After endogenous peroxidase blocking (H_2O_2 1%), slides were incubated with normal goat serum (1:40 dilution) in PBS + 0.5% bovine gamma globulin (BGG) for 30 min at room temperature. Then the sections were incubated overnight at 4 °C with IGY 415 (1:1800 dilution) in PBS + 0.5% BGG. The polyclonal antibody was revealed with anti-chicken biotinylated antibody (1:300 dilution) (Vector, Compiègne, France) for 30 min at room temperature and streptavidin-peroxidase complex (1:1000 dilution) (Dako, Trappes, France) for 20 min at room temperature, using 3,3′ diaminobenzidine tetrachloride (Sigma, Saint Quentin Fallavier, France) as a substrate. The samples were counterstained with haematoxylin (Dako), dehydrated and mounted. Tissue sections were washed with 0.1% Tween 20 in PBS between each immunostaining step.

b—In the frozen sections, M6P/IGFII-R immunohistochemical assays were performed in the 133 breast cancers as described in Ref. [14]. Tissue sections were incubated for 30 min at room temperature with IGY 415 (1:300 dilution) in PBS. The antibodies used for

immunohistochemical staining of ERα and PgR were the 1D5 [22] and the 1A6 clones from Dako, respectively. They were applied on serial frozen sections according to the manufacturer's instructions. Immunohistochemical staining of cathepsin D was performed as described in Ref. [23].

c—In both cases, the specificity of the IgY 415 staining was tested using IgY from a non-immunised hen (Nordic, Tilburg, The Netherlands and Sigma for the paraffin and frozen tissues, respectively). Pellets of MDA-MB-231 cells expressing a constant level of M6P/IGFII receptor were either embedded in paraffin or frozen at −80 °C and stored. Sections of these pellets were used in each experiment as a positive external control for the reproducibility of the M6P/IGFII-R staining.

2.3. Immunostaining quantification

A computerised image analyser (SAMBA 2005 TITN Alcatel Grenoble, France) was used as described in Refs. [17,23]. For M6P/IGFII-R and cathepsin D, the results were expressed by a quantitative immunochemical (QIC) score = [(percentage of surface stained in epithelial cells)×(mean staining intensity)×10], expressed in arbitrary units (AU). The non-specific staining due to control IgY was zero or weak and subtracted. ER and PgR and Ki67 were quantified as a percentage of the stained nuclear surface. M6P/IGFII-R staining was quantified in the lesions and adjacent histologically normal ducts and lobules. Reproducibility of M6P/IGFII-R staining between experiments was evaluated on MDA-MB-231 cell pellets and found to be slightly variable within a QIC score range of 100–150 AU for the frozen sections and 80–100 AU for the paraffin-embedded sections.

2.4. Statistical analysis

The Kruskal–Wallis test was used to compare the different marker values between all groups. When the test was significant, we used the Bonferroni correction to determine the significance between the subgroups. The non-parametric Wilcoxon test was used for the paired samples. Correlations between the former variables were estimated using the Spearman test. The overall significance threshold was 0.05.

3. Results

In the first study on paraffin-embedded sections, the population of BBD and *in situ* carcinomas, previously studied for ER and cathepsin D content [17,18], was used to stain for M6P/IGFII-R. The optimal conditions for sample fixation and staining were first established on pelleted MDA-MB-231 breast cancer cells embedded in paraffin. The staining, mostly in the perinuclear region, corresponded to the Golgi network and confirmed the staining pattern seen using frozen sections [14]. The same pattern was also observed using paraffin sections of mammary lesions, with cellular cytoplasmic staining being observed in both epithelial and stromal cells (Fig. 1b–e). An absence of the brown staining was observed using the negative control antibodies (Fig. 1a). In normal ducts (Fig. 1e) and lobules (Fig. 1d), staining was cytoplasmic and mostly apical in epithelial polarised cells and also present in some of the stromal cells. The extent and staining intensity in epithelial cells were quantified using a QIC score and compared with QIC scores in adjacent normal tissues. The overall distribution of M6P/IGFII-R expression and median values were similar in the pooled population of 95 normal tissues and 103 non-invasive lesions, but the level was very heterogeneous according to samples studied (from 0 to 320 AU) (Fig. 2). This heterogeneity was not due to a lack of reproducibility from one experiment to another, but was real, and due to a variable level of the M6P/IGFII receptor according to the patient samples. In general, 6–10 tumour samples were analysed in the same experiment and compared with the same MDA-MB-231 cell pellet. In Fig 2a, the QIC score varied between 38 to 124 AU, in Fig 2b between 28 to 320 AU and in Fig. 2c between 50 and 190 AU, while the MDA-MB-231 pellet value only varied between 80 and 100 AU. Thus, this clearly showed that the heterogenous staining was not due to inter-experiment variations, but to large variations in the expression of this receptor in different patients. When all patients were considered, the median values were similar in normal tissues (Fig. 2a) and in pooled non-invasive lesions (Fig. 2b) except for high-grade DCIS which showed a significant increase compared with the normal adjacent tissues (Fig. 3), as illustrated in Fig. 1b for 1 patient. Since there was a large variation in the M6P/IGFII receptor level according to patient sample used, we also compared in the same experiment and in the same patient, the receptor level in the lesion and in the normal adjacent glands in an attempt to detect variations due to the tumourigenic process. As shown in Fig. 3, the M6P/IGFII-R levels in benign lesions and adjacent normal glands varied according to different patient samples and were highly correlated using the Spearman test ($r = 0.675$, $P > 0.0001$). There was no influence of the type of lesion on the value in adjacent normal tissues. In a limited number of patients, however, as shown in Fig. 3, there was a strong variation ($\geqslant 2$ fold) in expression in the lesion compared with the normal glands. Interestingly, this variation summarised in Table 1 was not homogeneous. Staining in the lesion was substantially increased for a few patients in both benign and malignant lesions and mostly in high-grade DCIS. By contrast, a large decreased level was only observed in the

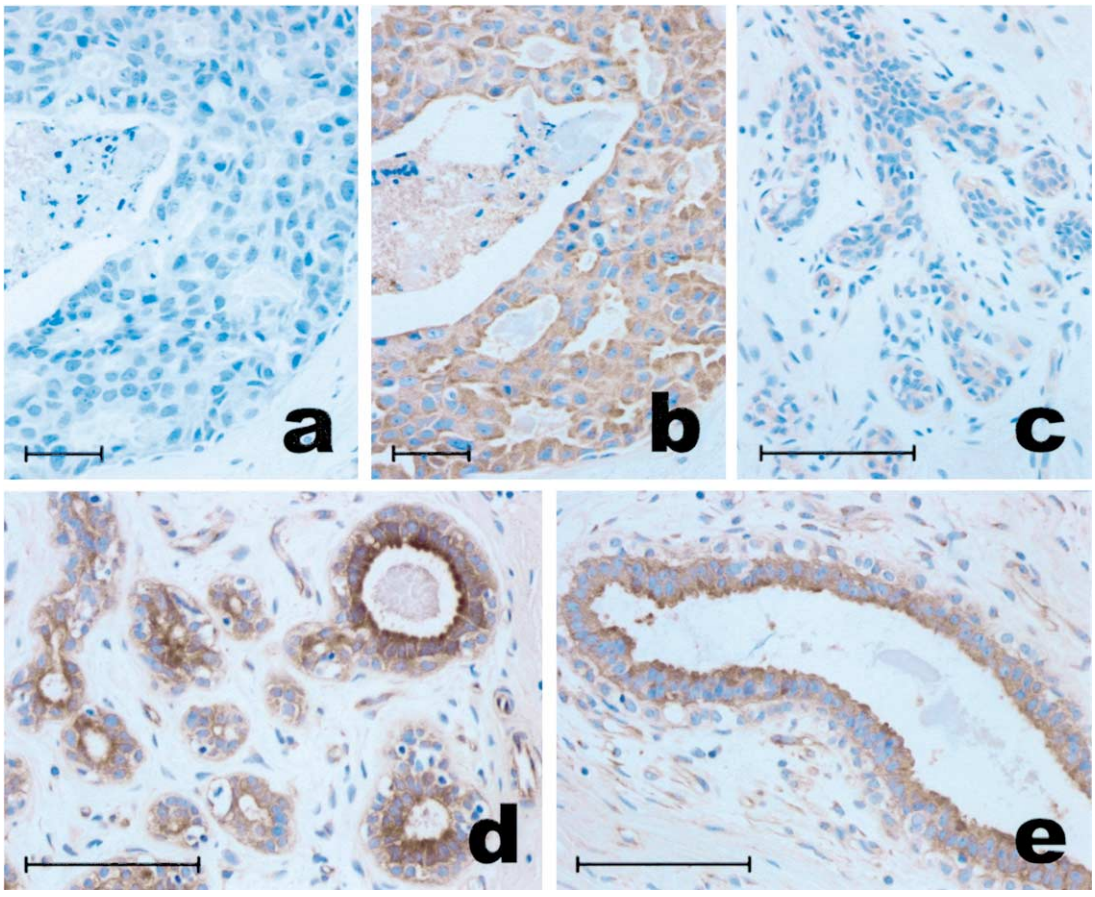

Fig. 1. Immunostaining of M6P/IGFII-R in paraffin sections. Specificity of immunohistochemical staining of the M6P/IGFII-R. Adjacent sections from a high-grade ductal carcinoma *in situ* (DCIS) were treated with hen polyclonal anti-human M6P/IGFII-R 415 IgY (b), or with non-specific IgY (a). Strong M6P/IGFII-R staining in the cytoplasm of a high-grade DCIS (b) contrasts with weak staining in adjacent normal glands of the same tissue section (c). Normal lobules (d), and normal ducts (e) at the periphery of lesions from different patients show strong staining of the apical pole of epithelial cells and staining in stromal cells. Bar = 50 μm.

cancerous tissues, in 10% of cases with CIS and 24% of invasive carcinomas. This decrease suggested a late alteration of this receptor in cancer cells and was compatible with the tumour suppressor gene hypothesis.

The M6P/IGFII-R level was also compared with other biological parameters evaluated in adjacent sections. Table 2 shows the absence of correlation of the M6P/IGFII-R level with ERβ and Ki67. There was a slight, but significant, correlation with ERα in normal mammary glands, but not in the lesions. There was a significant positive correlation with the cathepsin D level in proliferative lesions with atypia, as was also observed in the invasive breast carcinomas (Table 3). Although both cathepsin D and M6P/IGFII-R were significantly increased in high-grade DCIS compared with normal tissue, there was no correlation between the two markers in this lesion group, since many tumours had independently increased cathepsin D or M6P/IGFII receptor levels.

The second study on invasive breast carcinomas was performed on frozen sections, in parallel to routine assays for hormone receptor and cathepsin D levels. The relationship between the two immunohistochemical techniques was determined by comparing results obtained with the two techniques on pellets of the same MDA-MB-231 breast cancer cells and on serial sections of some breast cancer tissues. In general, the QIC scores were slightly higher (~25%) in the frozen sections than in formalin-fixed sections, justifying the reporting of the results separately. Fig. 2c shows the overall distribution of M6P/IGFII-R in 133 invasive breast carcinomas, with heterogeneity ranging from 5 to 230 AU and a median value of 95, which is higher than the results on paraffin sections, because of the higher sensitivity of this technique. In 41 samples, the M6P/IGFII-R level could be compared in both invasive breast cancers and adjacent non-tumoral tissues using the same technique (Fig. 3, second panel). The expression of M6P/IGFII-R was significantly decreased in the invasive cancers according to a paired non-parametric Wilcoxon test. Moreover, 24% showed a large decrease in the M6P/IGFII-R level relative to normal glands (Table 1). Finally, in 29 tumour samples analysed on paraffin sections, the M6P/IGFII-R QIC scores were compared in invasive cancers and adjacent CIS. In 10 of these patients, the level was significantly higher in the CIS

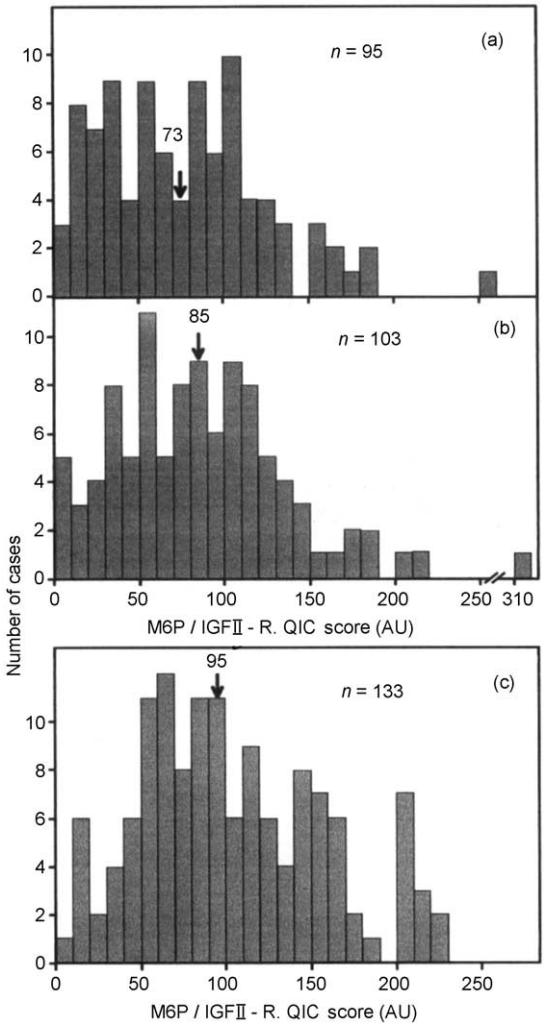

Fig. 2. Overall distribution of M6P/IGFII-R level (expressed as a quantitative immunochemical (QIC) score in arbitrary units) in normal glands (a), in adjacent non-invasive mammary lesions (b), and invasive breast carcinomas (c). a and b were measured in formalin-fixed sections and c in frozen sections. Arrows indicate median value. n: number of cases. AU, arbitrary units.

samples relative to the invasive carcinomas, in 15 samples it was constant and it was lower in only four cases.

These results suggest that the overall expression of this receptor is often lower in invasive breast carcinomas compared with CIS, consistent with a late alteration of the putative tumour suppressor M6P/IGFII-R. Interestingly, M6P/IGFII-R staining in stromal cells was correlated to that of the adjacent invasive breast cancers (data not shown).

As shown in Table 3, this marker was independent of the classical prognostic parameters, including tumour size, histological grade, regional lymph node invasiveness and ERα level. A slight, but significant, positive correlation was found with cathepsin D status ($P=0.04$), that became less significant when continuous concentrations were considered ($r=0.17$, $P=0.07$). A strong positive correlation was noted with the PgR status, which remained significant in a continuous analysis ($r=0.26$, $P=0.006$). Finally, the M6P/IGFII-R level progressively increased with age. It was not significant using a cut-off level of 50 years, but became significant with a cut-off level of 60 years ($P=0.01$) or 65 years ($P<0.003$). The clinical follow-up of these patients will be required to determine whether or not the M6P/IGFII-R level could improve the prognostic significance of cathepsin D levels.

4. Discussion

The M6P/IGFII-R protein level determined by computer-aided immunohistochemistry was found to be heterogeneous according to different patient samples in normal mammary glands adjacent to lesions, in non-invasive lesions and in breast cancers. However, there were marked significant differences for some patients when the lesions were compared with their adjacent normal tissue. There was an overall trend towards an increase in the receptor level in the non-invasive lesions, with a significant increase in high-grade DCIS compared with normal adjacent tissues. A similar increased level was observed in primary thyroid cancers [24]. This overall increase contrasted with a significant decrease in the invasive breast carcinomas. This strongly suggests that the mechanisms involved in these variations are different.

Interestingly, the proportion of lesions showing a significantly (at least 2-fold) decreased level of M6P/IGFII-R compared with normal tissue appeared to increase as the tumour progressed (Table 1). This decrease is compatible with the receptor acting as a tumour suppressor. Point mutations of the M6P/IGFII-R appear to be rare in breast cancer [12] and the decrease of the M6P/IGFII-R protein concentration observed in 24% of the breast cancers could also be explained by a somatic gene deletion on one allele or by an increased secretion of the receptor protein stimulated by mitogens such as oestradiol or IGFs [25]. A 50% decrease in expression might be sufficient to increase the aggressiveness of the tumour as shown by transfection of the normal receptor in a colon cancer cell line with a single allele mutation [26].

The proposal that LOH of the M6P/IGFII-R are early events in breast [9] and liver [7] carcinomas is not supported by our results since an overall and significantly increased expression of this receptor was noted in the high-grade DCIS.

Among the different biological parameters that were quantified in the serial sections, ERα was found to be correlated with M6P/IGFII-R in normal glands. There was also a significant increase of the M6P/IGFII-R level in invasive breast cancers in patients over 60 years old and a positive correlation with the PgR level. The M6P/IGFII-R may be regulated *in vivo* in mammary glands

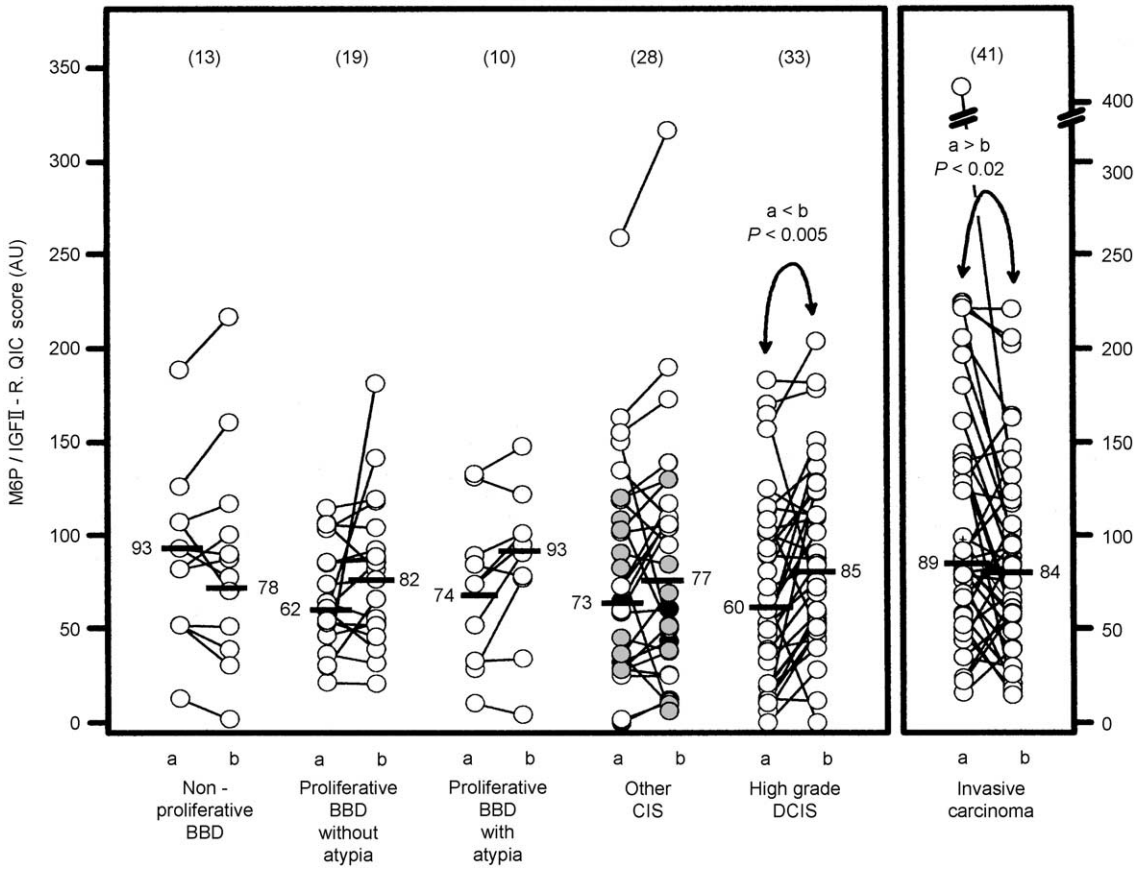

Fig. 3. Comparison of M6P/IGFII-R level between adjacent normal breast tissue (a) and lesions (b). *First panel*: comparison of M6P/IGFII-R level between normal adjacent glands and the indicated non-invasive lesions in paraffin sections. BBD: benign breast disease. High-grade DCIS: high-grade ductal carcinomas *in situ*. Other CIS include lobular CIS (●), low-grade (○) and intermediate (◍) DCIS. *Second panel*: comparison of M6P/IGFII-R level between normal adjacent glands and invasive carcinomas in the frozen sections. The number of different samples is in brackets. Bar: median value. *P* value between a and b according to the paired non-parametric Wilcoxon test is indicated when significantly increased (high-grade DCIS) or decreased (invasive carcinomas).

Table 1
Per cent of significant variations in the M6P/IGFII receptor level in lesions versus normal adjacent glands

	Proliferative BBD	*In situ* low-grade	*In situ* high-grade	Invasive
Increase *n*	3/29	1/28	5/33	3/41
%	10%	4%	15%	7%
Decrease *n*	0/29	3/28	3/33	10/41
%	0%	11%	9%	24%

From Fig. 3 the high variations (arbitrarily defined as $\geqslant 2$ fold) of the M6P/IGFII-R level in the indicated lesions relative to normal adjacent glands have been selected and represented in this table. Even though the small number of cases with this large variation is small, it is remarkable that the cases with a marked decreased staining in the lesions are only observed in the carcinomas, particularly the invasive carcinomas. The number of cases with marked increased staining is also low, but not restricted to the carcinomas. In most other cases, there is a correlation between the receptor level in the lesion and the adjacent normal glands.

as it has been shown to be regulated in cell lines [25] and in regenerating liver [27]. A significant correlation with cathepsin D level was observed in both proliferative BBD with atypia and invasive breast carcinomas, confirming our previous study of RNA levels [13]. Cathepsin D and one of its receptors could complement each other in terms of their prognostic significance and a cathepsin D/M6P/IGFII receptor ratio may have a better prognostic significance than the two markers separately. While the M6P/IGFII-R has been proposed to behave as a tumour suppressor [6,8], cathepsin D is associated with the aggressiveness of the cancer [15,16]. The interaction of Cathepsin D with an alternative receptor [11] facilitated by a decrease expression of M6P/IGFII-R [5] may be important for mediating mitogenic activity [15,28,29]. The decrease of this receptor might also modulate the effect of other cathepsins involved in cancer [30].

This is the first study using immunohistochemistry to assess M6P/IGFII-R levels in a large number of breast cancers and non-malignant lesions. Measuring expression

Table 2
Correlation of M6P/IGFII-R with other biological parameters in normal tisues and non-invasive structures

	Total ($n=198$)	All lesions ($n=103$)	Normal tissues ($n=95$)	Non-proliferarive BBD ($n=13$)	Proliferarive BBD ($n=19$)	BBD with atypia ($n=10$)	Other CIS ($n=28$)	High-grade DCIS ($n=33$)
Cathepsin D	NS	NS ($r=0.12$, $P=0.09$)	NS	NS	NS	$r=0.73$, $P<0.03$	NS	NS
ERα	$r=0.19$, $P<0.007$	NS	$r=0.27$, $P<0.01$	NS	NS	NS	NS	NS
ERβ	NS	NS	NS	–	–	–	–	NS
Ki67	NS	NS	NS	–	–	–	–	NS

All lesions ($n=103$) were quantified for cathepsin D and ERα, only 91 lesions for ERβ and 52 lesions for Ki67. The correlation between M6P/IGFII-R and the other biological markers was estimated in a continuous analysis using the Spearman test. The overall significance threshold was 0.05. –: not determined. NS: non-significant. *n*: number of samples.

Table 3
Correlation of M6P/IGFII-R level with prognostic parameters

Parameters	M6P/IGFII-R Mean value	M6P/IGFII-R Median value	*P* value
A Histological grade			
I (24)	96.4	95	
II (69)	110	102	NS
III (27)	107.1	101	
Lymph-node involvement			
Negative (97)	105.7	98	NS
Positive (36)	97.3	88	
Tumour size			
1 (92)	105.4	99	NS
2 (38) + 3–4 (3)	99.1	86	
Age (years)			
⩽60 (75)	94.8	79	0.01
>60 (52)	115.5	101.5	
B Oestrogen receptor α			
Negative (30)	99.5	94.5	NS
Positive (76)	107.4	101	
Progesterone receptor			
Negative (19)	64.8	65	0.003
Positive (87)	111.3	103	
Cathepsin D			
⩽211 AU (53)	95.5	96	0.04
>211 AU (53)	115.7	107	

A—In a total of 133 invasive breast cancer tumours, 106 were ductal, 20 lobular, 5 medullary, 1 tubular and 1 mucinous. All carcinomas were included to determine correlations with classical parameters. 13 breast cancers were not graded and 6 were not aged. B—Only the 106 carcinomas for which biological parameters were determined by immunohistochemistry (IHC) were included, the 27 carcinomas in which biochemical assays were used were excluded. Oestrogen receptor α and progesterone receptor positivity was set at 20% and 10% of stained nuclei, respectively. The cathepsin D median value of the 106 samples analysed by IHC was used as the cut-off value. Populations were analysed by the Wilcoxon non-parametric test. *P*-value significance was set at 0.05. NS: non-significant. Number of tumours analysed in brackets.

at the protein level could be more useful than analysing the RNA level, since the protein is directly responsible for the receptors' function. However, one disadvantage is that our polyclonal antibodies may interact with both functional and non-functional receptors including spliced variants, mutated receptors or defective receptors that have been altered by phosphorylation or glycosylation.

We conclude that levels vary in different patient samples, but can also be markedly increased or decreased relative to adjacent normal tissues in some lesions, with a significant proportion of invasive breast cancers showing a decrease of expression. This first large scale quantification of the M6P/IGFII-R level in human mammary tumours provides a basis for further genetic studies, aimed at determining the mechanism of the variation and for prospective clinical studies to define the prognostic significance of this receptor in both mammary carcinogenesis and tumour progression.

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