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Expression of c-*erb*B2, TGF-β1 and pS2 Genes in Primary Human Breast Cancers

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The presence of c-*erb*B2, TGF-β1 and pS2 mRNAs was examined in primary breast tumours. The c-*erb*B2 mRNA was overexpressed in 34% of the tumours. There was a positive, statistically significant correlation between c-*erb*B2 gene overexpression and nodal status. TGF-β1 mRNA was detected in 84% of the tumours, regardless of their clinical status. When possible, the c-*erb*B2 and TGF-β1 proteins were identified immunohistochemically on frozen sections from the same tumours. For TGF-β1, the mRNA and immunohistochemical results were divergent in 6 cases, 5 of which did contain clearly detectable mRNA but did not stain with the antibody. The pS2 mRNA was detected in 22% of the tumours and in the BT474 cell line. There was a significant correlation between the presence of pS2 mRNA and of oestrogen receptors. No statistically significant correlation was observed between pS2 and TGF-β1 genes expression and the clinical parameters of the tumours.
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INTRODUCTION

THE c-*erb*B2 oncogene is coding for an EGF-receptor-related transmembrane growth factor receptor with tyrosine kinase activity [1]. This gene is amplified and overexpressed in a number of adenocarcinomas, such as mammary adenocarcinomas [2]. It appears that in human tumours the amplified gene is not mutated [3]. The overexpression of the normal c-*erb*B2 protein is highly oncogenic in the NIH3T3 assay [4]. Slamon and his coworkers [5] first showed that c-*erb*B2 gene overexpression has a prognostic value. Since than, numerous studies have addressed this question and the use of c-*erb*B2 in predicting relapse-free or overall- survival is controversial. The data have been recently reviewed and discussed by Perren [6].

TGF-β1 is a growth factor with growth inhibitory properties for breast cancer cells [7, 8]. The presence of TGF-β1 mRNA and protein in the tumour could thus be a positive factor as far as the evolution of the disease is concerned.

The pS2 cDNA was cloned as an oestrogen-induced mRNA in the MCF7 mammary adenocarcinoma cell line. Later it was found that the gene is also expressed in the normal gastric

mucosa. The sequence of the pS2 gene shows close similarities to a family of peptides including the porcine spasmolytic polypeptide. The effect of pS2 on normal neoplastic breast cells is unknown, since the mammary glands of transgenic mice overproducing the pS2 protein do not present morphological or functional alterations.

The pS2 mRNA was found in a fraction of the oestrogen receptor-positive breast tumours [9]. The absence of pS2 in breast tumours was associated with shorter disease-free survival and defines a subset of tumours corresponding to a high risk for recurrence and death, among the oestrogen and progesterone receptor-positive tumours [10]. The presence of pS2 mRNA is predictive of response to hormonal therapy [11].

Here we present preliminary results of a prospective study aimed at identifying a group of factors which, used in combination, might improve the prediction of outcome mainly in the node-negative breast cancer subgroup. A combination of factors positively (TGF-β1 and pS2) and negatively (c-*erb*B2) correlated with prognosis was analysed in 47 primary breast cancers. Although this is a very small group, a statistically significant relationship was detected between c-*erb*B2 mRNA overexpression and some clinical features of the tumours.

A larger number of tumours will have to be analysed and the patients followed over a longer period of time in order to establish statistically significant correlations between these results and the other parameters which characterise the biology of the disease.

MATERIALS AND METHODS

Tissue collection

The tumours were surgically removed at the Institut Bordet. Fragments were taken for hormone receptor dosage and histology. The tumour fragments for nucleic acid analysis were quickly frozen in liquid nitrogen and kept at −80°C until used. All the relevant data are stored in a data base at the Institut Bordet.

Probes

The 400bp *NcoI-Eco*RI fragment of pMAC117 [1] was used as the c-*erb*B2 probe. The TGF-β1 probe was the 513bp *Sac*I-*Bam*HI fragment of the cDNA. The pS2 mRNA was detected with the 400bp *Pst*1 fragment of the pS2 cDNA [12]. The 36B4 specific probe was the 700 bp *Pst*I fragment of the 36B4 cDNA [12]. These fragments were subcloned in the pSPT18 (Pharmacia) or pGEM3Z (Promega) plasmids for the synthesis of antisens riboprobes with the Promega SP6/T7 transcription kit, using P32CTP (Dupont). 18S rRNA was hybridised with a 5.8 kb genomic rDNA fragment randomly labelled with the PrimeIt Random kit (Stratagen) using P32dCTP (Dupont).

Antibodies

The Ne-1-488 anti-neu specific antibody (Dupont) was used at a 1/25 dilution to detect the c-*erb*B2 protein. The antihuman TGF-β1 specific antiserum was raised by immunising turkeys against purified human TGF-β1. In radioimmunoassay it does not cross-react with TGF-β2 (J.C. Hendrick *et al.*, in preparation).

Nucleic acids extraction and analysis

DNA and RNA were extracted from separate tumour fragments.

The RNAs were extracted by RNAzol according to the instructions of the manufacturer (Cinna/Biotecx). The RNAs, dissolved in RNase free water were quantitated by spectrophotometry at 260 and 280 nm and their concentration and quality were checked by electrophoresis through a 1% formaldehyde-agarose gel. Aliquotes of 10 or 20 μg were ethanol precipitated and kept at −80°C.

The DNAs, extracted according to Maniatis *et al.* [13] were dissolved in Tris 10 mmol/l, EDTA 1 mmol/l, pH8 (TE), quantitated by spectrophotometry at 260 and 280 nm and their concentration and quality checked by electrophoresis through a 0.4% agarose gel.

Total RNA and 5 μl of the Gibco-BRL 9–0.24 kb molecular size markers were submitted to electrophoresis through a 1% agarose–formaldehyde gel [14] and transferred with 10 × SSC to GeneScreen Plus membrane (NEN-Dupont) for 2 h with a Vacugene 2016 apparatus (LKB-Pharmacia). The membranes were treated according to the instructions of the manufacturer. The blots were hybridised with 7.5×10^6 cpm of the appropriate riboprobe according to the conditions described by Melton *et al.* [15], except that 1% sodium dodecylsulphate (SDS) was included in the prehybridisation and hybridisation buffers. Hybridisations were carried out at 55°C in a shaking water bath. The blots were washed five times in 0.1 × SSC, 0.1% SDS at 65°C under shaking. Since the molecular weight of the c-*erb*B2 mRNA is similar to that of the 28S rRNA (4.8 kb), and the probe cross-hybridises with this rRNA species, the blots were washed in addition with 2 μg/ml of RNase A (Boehringer Mannheim) in 2 × SSC for 30 min at room temperature. The blots were autoradiographed with regular intensifying screens at −70°C. They were rehybridised with 18S rRNA or 36B4 specific probes to correct for the differences in the actual amounts of RNA loaded on the gel.

RNase protection experiments were performed as described in Lambert *et al.* [16].

DNAs digested with *Eco*RI (Boehringer Mannheim) were elctrophoresed through a 1% agarose gel, denatured, transferred and cross-linked by UV light to a Hybond-N membrane (Amersham). The blots were hybridised and washed according to the instructions of the membrane manufacturer.

The autoradiographic signals were quantitated by Ultroscan (LKB).

Immunohistochemistry

Frozen sections, 7 μm thick were cut with a cryostat. The sections, unfixed or quicly fixed in ice-cold acetone were pretreated with 1% bovine serum albumin. The incubation with the primary antibody (dilutions: Ne-1-488: 1/25; TGF-β1: 1/10) was for 16 h at 4°C. The revelation was carried out with the second antibody coupled to the peroxydase-antiperoxidase system, and revealed with diaminobenzidine. The cells were counter-stained with Harris's haematoxylin or methyl green.

Histology and hormone receptor dosage

The grading was independently assessed by two pathologists according to the classification of Scarff and Bloom [17]. Oestrogen and progesterone receptors were measured according to the EORTC recommendations [18].

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Table 1. Number of tumours analysed for c-erbB2, TGF-β1 and pS2 genes expression by different methods

	c-*erb*B2			TGF-β1		pS2
Method	Northern	Southern	IHC	Northern	IHC	Northern
Tumour no.	47	11	24	39	25	43

Statistical analysis of the results

Univariate analysis used χ^2 and non-parametric tests (Wilcoxon–Mann-Whitney and Kruskal–Wallis). Stratified analysis was done using the Mantel–Haenszel method [19]. Trend assessment was done using the Mantel–Haenszel χ^2 for trend. All significance levels are two-sided.

RESULTS

Identification of the messenger RNAs and proteins

Undegraded RNA was extracted from 47 primary breast tumours. For the northern blotting experiments, special care was taken to load equal amounts of RNA in each well. The c-*erb*B2 mRNA was quantitated in 47 tumours, pS2 in 43 tumours and TGF-β1 in 39 tumours. Positive and negative controls were included on each blot. RNA from BT474 cells, a cell line overexpressing the c-*erb*B2 mRNA 80 times, represented the respective positive control. RNA from healthy human kidney gave the level of c-*erb*B2 gene expression in healthy tissues [20]. Placental and liver RNAs were the positive and negative control, respectively for TGF-β1 mRNA quantification. Measurable amounts of TGF-β1 mRNA were detected in normal liver, confirmed by RNAse protection (results not shown). The c-*erb*B2 gene copy number was measured in eight tumours over-expressing the mRNA and three tumours expressing basal levels of the message. Immunohistochemical detection of c-*erb*B2 and TGF-β1 proteins was performed on 24 and 25 tumours, respectively. These data are summarised in Table 1.

*Expression and amplification of the c-*erbB2 *gene*

The c-*erb*B2 mRNA levels in 47 breast tumours and normal kidney were measured by northern blotting. A representative result is presented in Fig. 1. The BT474 cells (lane 1) and tumours from lanes 5 and 8 overexpress the c-*erb*B2 mRNA, while RNAs from lanes 6, 7, 11 and 12 contain moderate amounts of the message. The kidney c-*erb*B2 mRNA signal intensity was arbitrarily taken as 1. Sixteen tumours overexpress 3–77 times the message, in agreement with the data from the literature [6]. No aberrantly sized mRNAs were detected.

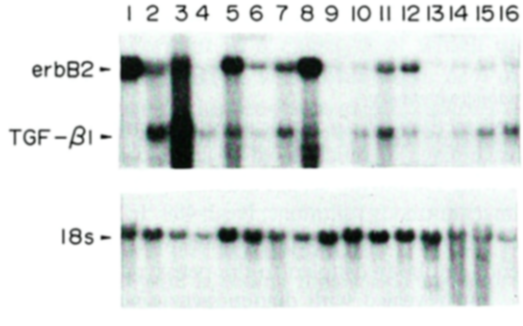
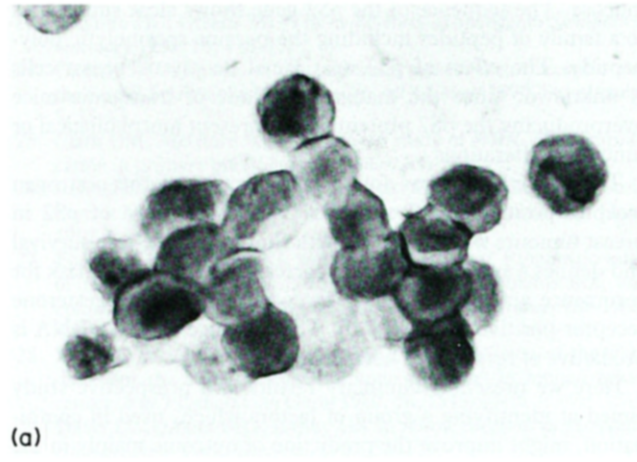


Fig. 1. Simultaneous detection of c-*erb*B2 and TGF-β1 mRNAs in breast tumors. 10 μg of total cellular RNA were separated on a 1% formaldehyde-agarose gel, transferred and hybridised with 7.5 × 10^6 cpm c-*erb*B2 and TGF-β1 specific riboprobes as described in Materials and Methods. The lower panel shows the result of the rehybridisation of the blot with the 18S rDNA probe. Lane 1: BT474 cell line; 2 normal kidney; 3 term placenta; 4 normal liver; 5–16: different tumour samples.



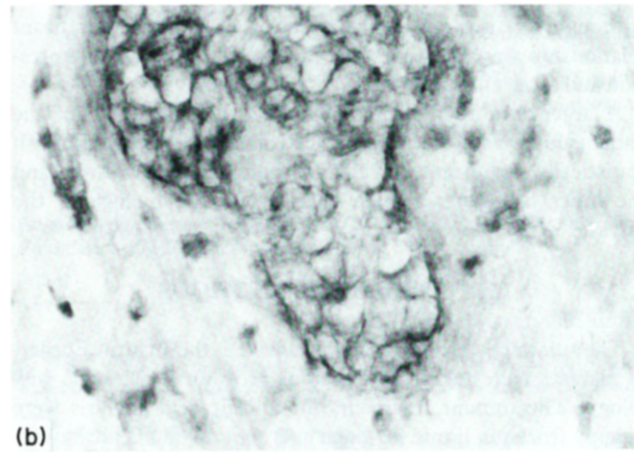


Fig. 2. Immunodetection of c-*erb*B2 protein in BT474 (a) and a tumour section (b). The frozen tumour sections were treated as described in Materials and Methods. The cells, cultured on coverslips, were fixed with cold acetone and treated as described in Materials and Methods.

Gene amplification was detected in seven out of eight tumours overexpressing the mRNA, but in none of the tumours expressing normal levels of c-*erb*B2 message (data not shown). The overexpression of the c-*erb*B2 mRNA without the amplification of the gene has already been signalled [21–24].

The presence of the c-*erb*B2 protein was investigated by immunohistochemistry (IHC) on frozen sections from 24 tumours using the Ne-1-488 anti-neu antibody (Dupont). Fig. 2(a) presents the immunoreactivity observed on BT474 cells. The positivity is located at the cell membrane. The immunoreactivity detected on one tumour is presented in Fig. 2b. There was an excellent correlation between the RNA and IHC data. All tumours overexpressing the mRNA were positive by IHC. Four out of five tumours where no mRNA was detected were also negative by IHC. Among 10 tumours

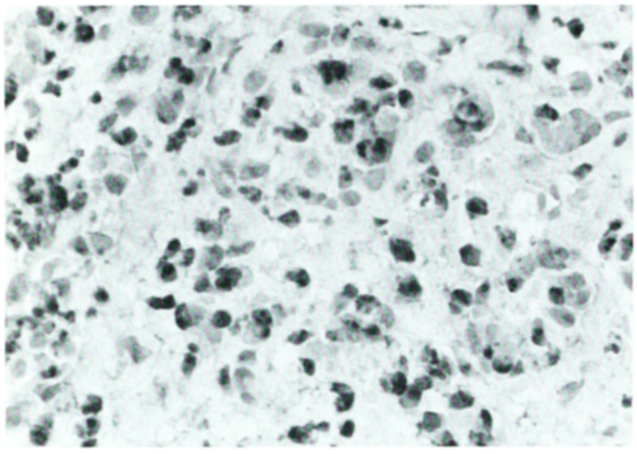


Fig. 3. Immunodetection of TGF-β1 in a frozen tumour sample. The immunoreactivity is essentially nuclear.

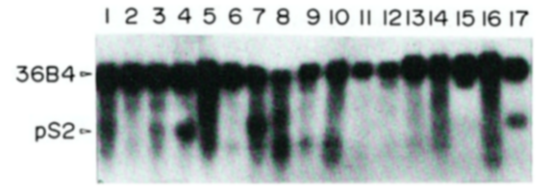


Fig. 4. pS2 and 36B4 mRNA detection. 10 μg of total cellular RNA were submitted to electrophoresis and northern blotting and hybridised simultaneously with 7.5 × 10^6 cpm of pS2 and 36B4 riboprobes as described in Materials and Methods. Lanes 1 to 13 contain RNAs extracted from different breast tumours. Lane 14: liver; 15: HBL100; 16: normal kidney; 17: BT474.

expressing normal levels of c-*erb*B2 mRNA, six stained positively with the antibody.

Expression of the TGF-β1 gene

The RNAs extracted from 37 tumours were analysed by northern blotting for the presence of TGF-β1 mRNA. A representative blot is shown in Fig. 1. Thirty one tumours expressed moderate to high levels of the mRNA (lanes 7 and 11), comparable with the positive controls (lanes 3 and 4). Six tumours showed little or no expression of the mRNA (lane 9). The expression levels in the tumours never exceeded significantly those measured in the positive controls. No aberrant mRNA was observed.

The presence of the protein was investigated by immunohistochemistry on frozen section from 25 tumours. A representative result is shown in Fig. 3. When present, the immunoreactivity was important, diffuse and mainly nuclear. The results obtained with the two techniques are compared in Table 2. RNA and IHC data were available for 21 tumours. They can be divided in four groups according to the correlation between RNA and IHC results: (1) RNA- and IHC-positive: 12 tumours; (2) RNA-positive IHC-negative: five tumours; (3) RNA-negative, IHC-positive: three tumours; (4) RNA- and IHC-negative: one tumour.

Expression of the pS2 gene

The presence of pS2 mRNA was investigated by northern blotting in 41 tumours. Representative results are presented in Fig. 4. The expected 600bp mRNA was clearly present in nine tumours (22%). In eight other tumours one or two different pS2 hybridising bands were observed. The pS2 mRNA was detected in the oestrogen receptor-positive BT474 cell line (lane 17).

All tumours where the 600bp pS2 signal was detected were considered as positive. There was a statistically significant correlation between the presence of pS2 mRNA and of oestrogen receptors. ($0.02 < P < 0.05$, Table 3).

Statistical analysis of the correlation between of c-erbB2 gene expression and clinical parameters

The results are presented in Table 4. 47 patients, 41–83 years old, were included. The cut-off point for c-*erb*B2 overexpression was established at 3. 31 women were negative (66%) and 16 positive (34%). Interestingly, in spite of the small sample size, the relationship between c-*erb*B2 gene overexpression and nodal status is statistically significant. For tumour grade and Scarff and Bloom classification, a positive and significant relationship with *erb*B2 positivity may exist.

In this series it has been noted that when age decreases, more patients overexpress c-*erb*B2, more node status are positive (χ^2 for trend: $P = 0.12$), and tumour size tends to be greater ($P = 0.08$). A stratified analysis of node status according to c-*erb*B2 positivity adjusted for tumour size (greater dimension < 20 mm versus > or equal to 20 mm) was carried out and yielded more significant results (Mantel–Haenszel χ^2: $P < 0.01$). When adjusting for age, the relationship between nodal status and c-*erb*B2 overexpression is also more significant (Mantel–Haenszel χ^2: $P < 0.01$).

There is at present no statistically significant correlation between TGF-β1 and pS2 genes expression and the variables mentioned in Table 4.

DISCUSSION

These are the first results of a prospective study which combines factors positively and negatively related to prognosis

Table 2. Comparison between northern blotting (RNA) and IHC results for TGF-β1

	RNA +	RNA −	Total
IHC+	12 (70)	3 (75)	15
IHC−	5 (30)	1 (25)	6
Total	17	4	21

(% of column)

Table 3. Correlation between the pS2 mRNA and oestrogen receptor status

	pS2 +	pS2 −	Total
ER+	9 (90)	15 (52)	24
ER−	1 (10)	14 (48)	15
Total	10	29	39

χ^2: 4.61; $0.02 < P < 0.05$.
(% of column)

Table 4. c-erbB2 overexpression according to tumour and malignancy characteristics

	*erb*B2- (n = 31)	*erb*B2+ (n = 16)	Statistical significance (P)
Tumour size (mm)			
Mean	20.2	24.0	0.41
S.D.	9.5	13.8	
Median	19.0	17.5	
Node status			
Negative	17 (55)	3 (19)	0.02
Positive	14 (45)	13 (81)	
Grade (data for 41 patients)			
3–5	6 (23)	2 (13)	0.01
6–7	18 (69)	5 (33)	(trend)
8–9	2 (8)	8 (54)	
Scarff and Bloom (data for 41 patients)			
3–5	10 (38)	2 (13)	0.01
6–7	14 (54)	7 (47)	(trend)
8–9	2 (8)	6 (40)	
Oestrogen receptors			
= 0	8 (26)	6 (38)	0.41
> 0	23 (74)	10 (62)	
< 100	18 (58)	13 (81)	0.11
≥ 100	13 (42)	3 (19)	

(% of column)

in breast cancer. Different techniques were used: northern and Southern blotting and immunohistochemistry.

The prognostic value of c-*erb*B2 overexpression has been studied by more than 20 research teams. The state of the art concerning the correlation between c-*erb*B2 expression and increasing tumour aggressiveness was discussed by Perren [6]. Although our sample is small, a statistically significant positive correlation between c-*erb*B2 overexpression and nodal status was observed. A similar trend exists for tumour grade and Scarff and Bloom classification. Our data are too preliminary to allow a valid comparison with the data from the literature.

The effect of TGF-β1 on tumours is not well understood. Identified in the conditioned medium from transformed tumour cell lines [25], its secretion is increased at least 40 times upon viral transformation of the cells [26]. The levels of TGF-β1 mRNA are usually higher in tumours than in the adjacent normal tissue [27]. TGF-β1 inhibits the growth of epithelial tumour cell lines *in vitro* [7]. An anti-TGF-β1 antibody suppressed the TGF-β1-induced growth inhibition of breast cancer cells in culture [28]. Oestrogen receptor-negative breast cancer cells are specially responsive to the growth inhibitory properties of TGF-β1 [29].

The possible prognostic significance of TGF-β1 in breast cancers was investigated by Coombes *et al.* [30]. The disease-free survival was significantly longer in patients bearing TGF-β1 mRNA-rich tumours. Most of our tumours (31 out of 39) express the TGF-β1 mRNA. We do not think that this parameter alone can give an indication for the outcome of the disease. However, a proportion of the TGF-β1 mRNA positive tumours were negative when analysed immunohistochemically. The RNA hybridisation specificity was tested by the RNase protection assay, and the antiserum was shown to be specific by RIA. Multiple mechansims could explain the discrepencies observed between RNA and protein results in tumours from groups 2 and 3. In group 2 tumours, TGF-β1 mRNA might not be translated. Alternatively, the immunohistochemistry might identify the molecules which are fixed on the receptor and not those actually produced by the cells. In these tumours, the proteins might not be retained, due for instance to the absence of the receptor. Indeed, cells might loose the TGF-β receptor, thus becoming resistant to the action of the factor [31]. The TGF-β1 detected by the antibody in group 3 tumours might have been produced elsewhere and captured by the tumour cells.

The expression of the pS2 gene in gastric and breast tumours has been investigated. In primary human stomach tumours, pS2 gene expression is down-regulated when compared with the normal tissue [32]. Others have identified the pS2 mRNA and protein in undifferentiated human gastric tumour cell lines [33]. In breast cancers, pS2 mRNA or protein were detected mainly in the oestrogen receptor-positive tumours. The correlation between pS2 and oestrogen receptor varied according to pS2 detection methods and the cut-off point for pS2 positivity. Very significant correlations ($P < 0.001$) were observed when pS2 protein and oestrogen receptor data were compared [11, 34, 35]. Our results are more comparable with those of Skilton *et al.* [36] who correlated the oestrogen receptor-status to the presence of pS2 mRNA. The lower level of significance could be explained by the heterogeneous expression of pS2 in the tumours, apparent in immunohistochemical studies [34]. We detected the pS2 mRNA in only 37.5% of the oestrogen receptor-positive tumours, less than the values reported by Rio *et al.* [9] and Skilton *et al.* [35]. This might be due to the fact that a smaller amount of RNA was used in our northern blotting experiments. We shall re-evaluate the pS2 status of the tumours by using more sensitive detection methods.

The functional significance of oestrogen and progesterone receptor-positive, pS2-negative tumours is unknown. Some authors [9, 11, 34] hypothesised that these might correspond with the receptor positive tumours which do not respond to hormonal treatment.

At present we are analysing more tumours in order to improve the statistical correlation between the parameters that we are investigating. Moreover, new parameters are included, such as the identification of p53 gene mutations.

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