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Detection of the HER-2/*neu* Proto-oncogene Protein p185^{erbB2} by a Novel Monoclonal Antibody (MAB-145ww) in Breast Cancer Membranes from Oestrogen and Progesterone Receptor Assays

Matthias W. Beckmann, Leighton J. Toney, Anton Scharl, Robin Fuchs-Young, Geoffrey L. Greene and John A. Holt

Amplification of the proto-oncogene HER-2/*neu* and/or overexpression of the transmembrane protein p185^{erbB2} that it encodes occur in approximately 30% of human breast and gynaecological cancers seen clinically and are strongly associated with an unfavourable outcome. We report on the use of a new monoclonal antibody (Mab-145ww) together with immunoblotting for detection of p185^{erbB2} in membranes that remain after routine processing of breast cancer tissue for steroid receptor assays. Human breast cancer cell lines SKBR3 and MCF-7 were used as high and low controls, respectively, for p185^{erbB2} expression. Mab-145ww was detected p185^{erbB2} in more than half of the breast cancer specimens; the expression was intense in SKBR3 cells, but only faint in MCF-7 cells. These results demonstrate that routine processing of cancer tissue for steroid receptor status can include providing a preparation with which to assess p185^{erbB2} expression and, thus, can provide information potentially useful for the clinical management of individual cancer patients.
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INTRODUCTION

NEW IMMUNOMOLECULAR biological techniques can be employed for probing of breast and gynaecological cancers which provides clinically useful information on individual patients. In the present study, we used a specific marker protein, p185^{erbB2}, to illustrate this capability. The proto-oncogene HER-2/*neu*, with its protein product p185^{erbB2}, is representative of a family of normal cellular genes and proteins that are involved in cell growth and differentiation. Alterations in the gene structures for these growth regulators, in the number of gene copies (amplification), or in overexpression of their mRNA in and/or of their corresponding proteins, are believed to be important in the pathogenesis of many human breast, and genital cancers [1–4]. Amplification of the proto-oncogene HER-2/*neu* occurs in approximately 30% of these cancers and is strongly associated with a poor prognosis [2–5]. HER-2/*neu* gene amplification is commonly accompanied by overexpression of its protein, p185^{erbB2}, which itself is a strong index of tumour aggressiveness [5–7]. Overexpression of HER-2/*neu* in transgenic mice resulted in malignant transformation of all breast epithelial cells in both males and females [8]. The p185^{erbB2} protein shows the typical functional domains seen in growth factor receptors, such as the epidermal growth factor receptor (EGF-R), which include extracellular, transmembrane, and intracellular regions, and it exhibits tyrosine kinase activity. The extracellular domain spans 653 aminoacids with two cysteine-rich regions, which are thought to participate in binding of the specific ligand gp30 [9]. A monoclonal antibody (Mab-145ww) against a partially purified synthetic peptide corresponding to an epitope of the internal domain of p185^{erbB2} was recently developed and was characterised with respect to its high specificity [10].

Surgical specimens of breast cancers and selected gynaecological malignancies that are seen clinically are commonly processed in the laboratory for measurement of their oestrogen and progesterone receptor content (ER and PR, respectively) [11, 12]. The biochemical extraction for the ER and PR measurements usually results in residual pellets that contain cell membranes. With the hope of obtaining additional information concerning the prognosis for individual cancer patients, we tested whether p185^{erbB2} could be detected in these unused pellets. We used immunoblot analysis with a new monoclonal antibody, Mab-145ww, which is highly specific for p185^{erbB2} [10]. As an approach to partial clinical validation of the p185^{erbB2} expression results, we compared p185^{erbB2} overexpression to the presence of steroid receptors, whose prognostic value is well established [12].

MATERIALS AND METHODS

Materials

All media and cell culture reagents were purchased from Gibco. Insulin and calf serum were obtained from the Sigma Chemical Co. Culture medium CR-ITS+ was purchased from Collaborative Research Incorporated (Bedford, Massachusetts). Culture dishes and plates were obtained from Falcon or Nunc.

Electrophoresis chemicals were purchased from Bio-Rad (Rockville Center, New York). The production of the mouse monoclonal antibody Mab-145ww has been described previously [10]. The rabbit antimouse antibody (IgG) used was a product of Zymed Laboratories (San Francisco). The [^{125}I]-labeled pro-

Correspondence to M.W. Beckmann. M.W. Beckmann is at Universitätsfrauenklinik Johann Wolfgang Goethe-Universität, Theoder-Stern-Kai 7, 6000 Frankfurt am Main 71; A. Scharl and J.A. Holt are at the Department of Obstetrics and Gynecology; R. Fuchs-Young and G.L. Greene are at the Ben May Institute; and L.J. Toney is at the University of Chicago Medical Laboratories, The University of Chicago, Chicago, Illinois, U.S.A.
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Table 1. Selected characteristics of the breast cancer donors and their tumours, together with frequency and levels of occurrence of HER-2/neu* protein p185^{erbB2}. *Measured with Mab-145ww on western blots of the residual pellets after processing for ER and PR assay†, expression as a function of tumour grade§ and histological type§*

	Distribution* no. (%)	HER-2/*neu* protein expression‡ no. (%)					
		<1+	1+	2+	3+	4+	≥1+
Age (years)							
≤50	28 (37%)						
≤50	48 (63%)						
Menopause							
pre-	23 (30%)						
post-	53 (70%)						
Grade§							
I	18 (24%)	12(27%)	6	—	—	—	6(19%)
II	35 (46%)	21(48%)	1	7	3	3	14(43%)
III	23 (30%)	11(25%)	—	1	4	7	12(38%)
Total no. (%)	76(100%)	44(58%)	7	8	7	10	32(42%)
Histol. type§							
A	67 (88%)	39(58%)	4	7	7	10	28(42%)
B	9 (12%)	5(55%)	3	1	—	—	4(45%)
Total no. (%)	76(100%)	44(58%)	7	8	7	10	32(42%)

*Data are for 76 donors; all specimens were from sites of primary cancer.
†DCC-type assay.
‡For ranking of p185^{erbB2} overexpression (≥1+ through 4+) see legend to Fig. 1 and *Materials and Methods*.
§*Manual for Staging of Cancer*, by Beahrs *et al.* [13]. Type A: all common invasive breast carcinomas except invasive lobular forms; type B: only invasive lobular breast cancers.

Table 2. HER-2/neu protein p185^{erbB2} *expression detected by Mab-145ww in pellets from primary* (n = 76) *and metastases* (n = 14) *of breast cancers (total* n = 90) *as a function of ER and PR**

Steroid receptor status*	Primary tumours No. in cat./total (%)	Specimens ≥1+ HER-2/*neu* protein p185^{erbB2} expression †	Metastases No. in cat./total (%)	Specimens ≥1+ HER-2/*neu* protein p185^{erbB2} expression †
ER-positive PR-positive	38/76 (50%)	5/38 (8%)	7/14 (50%)	1/7 (14%)
ER-positive PR-negative	4/76 (5%)	1/4 (25%)		
ER-negative PR-positive	2/76 (2%)	0/2 (0%)		
ER-negative PR-negative	32/76 (42%)	25/32(78%)	7/14 (50%)	6/7 (86%)
Total	76(100%)	31/76(41%)	14(100%)	7/14(50%)

*Less than 3 fmol/mg soluble cytosolic protein was interpreted as ER-negative, and ≥3 fmol/mg soluble cytosolic protein as ER-positive. The values for PR status were considered negative when <5 and positive when ≥5 fmol/mg soluble cytosolic protein. ER = estrogen receptor; PR = progesterone receptor; cat. = category.
†See legend to Fig. 1 and *Materials and Methods*.

tein A used for immunoblot signal detection and quantification was purchased from Amersham.

Human breast cancer tissue processing and receptor analyses

Clinical staging, grading and histological typing of the cancer specimens was according to Beahrs *et al.* [13] (see legend to Table 1). Cell membrane samples from 76 primary human breast cancers and from 14 breast cancer metastases, representing a total of 90 patients, were pulverised, homogenised, and centrifuged (1 h, 100000 *g*, 0°C) for a saturation and dextran-coated charcoal (DCC) steroid receptor assay [14]. Receptor content was expressed as fmol per mg soluble cytosolic protein, with bovine serum albumin (BSA) used as a standard [15] (see legend to Table 2).

Cell cultures

Human breast cancer cell lines MCF-7, characterised as having low p185^{erbB2} expression, and SKBR3, characterised as having significant p185^{erbB2} overexpression [7, 10], were cultured at 37°C under 95% O_2, 5% CO_2 in Dulbeccos modified Eagles medium (DMEM) containing L-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l), glucose (3.5 mg/ml), MEM-vitamin solution (1 mmol/l), insulin (5 μg/ml), $NaHCO_3$ (3.7 mg/ml), penicillin (10 U/ml), streptomycin (10 μg/ml), and calf serum (10% v/v). The medium was changed every third day. Upon confluency, the cells were split 1:3 with trypsin–EDTA (0.05% trypsin, 0.53 mmol/l EDTA). Three days before the final harvest, the MCF-7 cell medium was replaced by DMEM without phenol red, supplemented with CR-ITS+ instead of calf serum to promote ER expression [16]. The status of the cells with respect to ER and PR was confirmed in two ways: by immunohistochemical staining with mouse monoclonal anti-ER, H-222 and H-226 (1/40 dilutions of each, combined in equal volumes), and immune complexes of alkaline phosphatase and monoclonal antialkaline phosphatase for amplification of recognition (APAAP complexes) [17], and by biochemical receptor analysis as described for human breast cancer tissues. MCF-7 cells contained 190 fmol ER/mg soluble cytosolic protein and 22 fmol PR/mg soluble cytosolic protein; the SKBR3 cells were negative for both ER and PR. The immunocytochemical assay gave corroborative results.

Tissue pellets and membrane processing

The pelleted cells from the breast cancer cell lines and the residual pellets from tumour tissue processing were prepared with a slight modification of the procedure described by McCaffrey *et al.* [18]. In brief, the samples were washed in PBS-EDTA-phenylmethylsulfonyl fluoride (PMSF) and buffer A without salt (20 mmol/l Tris, pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l PMSF, 0.025 U/ml aprotinin, 5 μmol/l leupeptin, 25 mmol/l benzamidine), disrupted in a cooled dounce homogeniser (25 strokes), centrifuged (1 h, 30000 *g*, 0°C), resuspended in buffer A plus salt (salt: 150 mM NaCl), and rehomogenised. After centrifugation (1 h, 35000 *g*, 0°C) and resuspension in buffer A without salt, the resuspended membranes were flushed through 25-gauge needles (1.5 inches long). The centrifugation was repeated, and, after a final resuspension, the total protein in an aliquot was assayed [15]. Portions of the remaining protein were analysed by polyacrylamide gel electrophoresis (PAGE) and immunoanalysis (western blotting) as described below.

Gel electrophoresis and western blot analysis

In preliminary experiments, the yield of the membrane preparation and the protein detection limits were assessed by loading different concentrations of cell lysates from the controls and from processed membranes. The yield was >93%, protein concentrations of less than 0.75 μg soluble protein showed no detectable signal. Equal amounts of protein (10 μg) from each surgical specimen, and various amounts from the cell line preparations (20 μg for MCF-7 cells and 20 or 30 μg for SKBR3 cells) were loaded on separate lanes of a 7% sodium dodecyl sulphate (SDS)–PAGE gel. Electrophoresis was performed for 18 h at 4°C. The proteins were then transferred from the gels to nitrocellulose. The nitrocellulose strips were incubated with PBS containing 3% dry milk (w/v), which reduced low-affinity binding. Strips were then incubated for 1 h with the monoclonal antibody Mab-145ww diluted in PBS, 0.05% Tween 20, and 1% dry milk. After rinsing in the vehicle buffer, the blots were incubated for 1 h in rabbit anti-mouse antibody, rinsed again, and incubated with [^{125}I]-labeled protein A at 1 to 2 × 10^6 cpm/ml incubation solution for 1 h. After rinsing and drying, the radiolabeled blots were exposed to Kodak XAR-5 films. The optical density (O.D.) of spots that appeared in selected regions on the developed autoradiograms was quantitated by laser-densitometric scanning (LKB 2202 Ultrascan Laser Densitometer), and the O.D. signals were integrated (IBM LC/9540 Chromatography Data Integrator) and ranked as 0 through 4+ (see legend to Fig. 1). Different blots were exposed for the same length of time, each blot included positive (SKBR3) and negative (MCF-7) controls. All signals were normalised to the appropriate controls from that same blot.

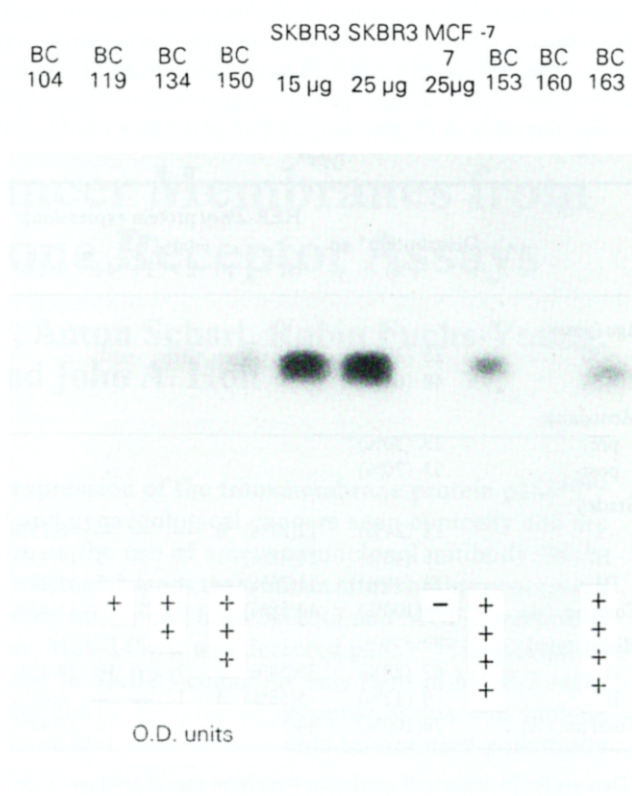

Fig. 1. Immunoblotting after gel electrophoresis (western blot) with use of the monoclonal antibody Mab-145ww for detection of p185^{erbB2} protein in cell membranes from human breast cancers, which were residues after routine tissue processing for steroid receptor (ER and PR) assays, and in membranes from cancer cell lines MCF-7 and SKBR3. Tumours were grouped into p185^{erbB2} expression categories as follows: A value below 0.3 O.D. units was considered negative; this category encompassed the assay results for p185^{erbB2} abundance in MCF-7 breast cancer cells (low-overexpression control). The range 0.3 to 0.8 O.D. units was designated 1+; >0.8 to 1.5 O.D. units, 2+; >1.5 to 2.5 O.D. units, 3+, and values >2.5 O.D. units, 4+. The overexpression of p185^{erbB2} in the SKBR3 cell line (high-overexpression control) had an O.D. unit value of >2.5 O.D., 4+.

RESULTS

Table 1 lists characteristics of our principal study group of 76 donors of tumour specimens from the site of primary disease, including their ages and menopausal status, as well as some details on their cancers, including the histopathology grade (I to III) and histologic classification [13]. In addition, in the section in which ER and PR data are presented (Table 2), we provide data on 14 donors whose specimens were taken from metastases of breast cancers. The primary tumours, from which the metastases derived, were positive for ER and PR in 7 cases and negative for ER and PR in 7 cases.

Consistent with previous reports [10, 19], in the present study the recently raised monoclonal antibody Mab-145ww detected a highly abundant 185 kD protein in SKBR3 cell membranes, but only very low levels of this protein were detected in MCF-7 cells. The detection of p185^{erbB2} in pellets left over from routine preparation of the cytosol for ER and PR assay was achieved by western blotting and compared with the western blotting results for the breast cancer cell lines. The 185 kD protein, which, we assume, was also p185^{erbB2} because of the monospecific binding of the antibody [10], was detected in some, but not all of our breast cancer samples. A representative western blot is illustrated in Fig. 1. The relative O.D. of the bands on the different autoradiographs in the western blotting analyses ranged from 0.1 to 16 O.D. units (see Fig. 1 legend).

Specimens of histopathology grade II (Table 1) had the highest incidence of p185^{erbB2} overexpression (tumour ranked ≥1+); the level of overexpression with the highest abundance was 2+ in this histopathological grade II (Table 1). Grade I specimens had overexpression only at level 1+, grade III specimens had overexpression at levels of 3+ and 4+ (Table 1). The distribution of protein overexpression as a function of histologic type revealed a 42% incidence among the cases of histologic type A; 4 of the 9 cases of type B showed overexpression, measuring at most 2+ O.D. (Table 1). Statistical analyses were not warranted because of the small number of specimens in the different sub-groups.

When grouped according to ER and PR status (Table 2), the patients' tumours included nearly equal numbers that were positive for both of the steroid receptors and those that were negative for both of the steroid receptors. Qualitatively, the strength of association of p185^{erbB2} expression was inversely related to the occurrence of steroid receptors. In three specimens, in which a combination of ER positivity and p185^{erbB2} protein overexpression was seen, the amount of ER was less than 50 fmol/mg soluble cytosolic protein, and the overexpression was moderate, measuring at most 2+ O.D.

The metastases of breast cancers were from 14 donors. The metastases tested positive for ER and PR in 7 cases, an equal number of specimens tested negative for ER and PR. The cases positive for ER and PR had an overexpression of p185^{erbB2} (2+ in O.D.) in one case. In the group of metastases which tested negative for ER and PR, 6 cases had an overexpression of p185^{erbB2} (3+ and 4+ rankings). (Table 2).

DISCUSSION

We used the monoclonal antibody Mab-145ww to evaluate the potential for improved care of individual cancer patients. Our choice of reagents was guided in part by the interest in the HER-2/*neu* oncogene product as a target for immuno-based therapy [20–22].

After preparation of cell membranes and immunoblot analyses, the antibody Mab-145ww detected high levels of a single protein with a molecular weight of 185 kD in ER/PR negative SKBR3 cell membranes and very low levels of the same protein in ER/PR-positive MCF-7 cells. Previous characterisation and validation of the specificity of this monoclonal antibody [10] had established that the reactive protein was p185^{erbB2}, the gene product of the HER-2/*neu* oncogene. Cell membrane pellets remaining after routine processing of human breast cancer tissues for determination of steroid receptor status were prepared in fashion similar to that used for the cultured cells and were also subjected to immunoblotting. p185^{erbB2} could be detected in 31 of the clinical primary breast tumour specimens and in seven specimens of metastases from human breast tumours, for a total of 38 positives amongst 90 specimens.

The level of p185^{erbB2} expression in these cancers was evaluated with an approach modified slightly from that used by Slamon *et al.* [4]. Levels for patient specimens were compared with values obtained from simultaneous blotting of SKBR3 and of MCF-7 cells [10, 19]. In this way, we found that the percentage of cancers in our study group that exhibited p185^{erbB2} overexpression was moderately higher than the numbers reported by other authors [4, 6]. To place our data in proper context, it must be taken into account that our study group was not chosen randomly—it was simply taken from available specimens—and that the number of specimens examined was modest.

Slamon *et al.* [4] had shown that 6% of their results from western blot analysis of human breast cancer samples were inconsistent with data obtained by Southern blot, northern blot, and/or immunohistochemistry. The presence of excessive stromal elements with increased susceptibility of this analytic method to dilutional effects have to be kept in mind for the evaluation of the results by western blotting, as well as the presence of p185^{erbB2} levels in human breast cancer in the absence of detectable gene amplification [23, 25].

Our approach to the use of Mab-145ww for p185^{erbB2} detection proved feasible in clinical material. A partial clinical validation in the absence of 5-year survival data was made by comparisons showing expression of p185^{erbB2} relative to steroid receptor status. It has been established that there is an inverse relationship between expressions of the sex steroid receptor proteins and the cell membrane receptor for this specific growth factor [19, 23, 24]. Patients with endocrine-sensitive tumours for which the steroid receptor proteins are usually considered to be expressed and functional have a somewhat improved prognosis, whereas the overexpression of p185^{erbB2} is associated with a poor prognosis [4, 12].

We were able to confirm the relationship of absence of p185^{erbB2} overexpression and positive ER in the ER-positive MCF-7 cell line analysed. We therefore included some clinical samples with absent or low levels of ER and PR in the study group in order to test this relationship. Our results for association between overexpression of p185^{erbB2} and a negative ER and PR status confirmed the previous reports by Berger *et al.* [23], Roux-Dosseto *et al.* [24], and Dati *et al* [19]. For tumours with ER and PR content below the chosen cut-off value, the incidence of specimens with p185^{erbB2} overexpression (78%) was much higher than that in the group of ER-positive and PR-positive tumours (8%). Furthermore, the measured levels of p185^{erbB2} expression in the steroid receptor-negative group were higher than those in the steroid receptor-positive group, indicating inhibition of HER-2/*neu* oncogene mRNA and of protein expression, by oestrogens [19]. Thus, with respect to the prospect of determining the clinical outcome for individual patients, our data provide support for continued studies on residual membranes with measurements of HER-2/*neu* expression.

Based on our findings, we think the processing of human breast cancer tissue that is commonly performed for the determination of steroid receptor status allows concurrent evaluation of p185^{erbB2} protein expression. The analysis of the otherwise unused sample material therewith can provide information that is potentially useful for the clinical management of individual patients with breast cancer, and potentially also for patients with ovarian and endometrial cancer.

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Definition of Immunogenic Determinants of the Human Papillomavirus Type 16 Nucleoprotein E7

Annette Altmann, Ingrid Jochmus-Kudielka, Rainer Frank, Heinrich Gausepohl, Ulrich Moebius, Lutz Gissmann and Stefan C. Meuer

Specific T lymphocyte lines and T cell clones were established from peripheral blood mononuclear cells of asymptomatic seropositive individuals employing synthetic peptides which correspond to the sequence of the human papillomavirus (HPV) type 16 transforming protein E7. Specificity analysis of T cells as determined by means of [^{3}H] thymidine incorporation after stimulation with individual peptides revealed three immunogenic determinants of E7 that are recognised in association with at least two different HLA haplotypes. One N-terminal region (aminoacids 5–18) was recognised by one T cell line. T cell clones and the corresponding T cell line established from another donor responded to a different N-terminal (17–38) and to a C-terminal region (69–86). The N-terminal sequence 5–18 and the C-terminal determinant contain a periodicity of hydrophilic and hydrophobic residues that have been found in many T cell epitopes. Phenotypic characterisation of T cell clones by indirect immunofluorescence revealed that the T cell clones expressed the CD4 surface glycoprotein suggesting that the specific E7 determinants were recognised in association with major histocompatibility complex (MHC) class II molecules. With regard to functional properties, at least three T cell clones exhibited specific cytotoxic activity towards autologous B lymphocytes transformed by Epstein–Barr virus in the presence of the relevant HPV16 E7 peptides. The implications of these results regarding the development of vaccination strategies and host–virus interaction are discussed.
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INTRODUCTION

THE GENOME of human papillomavirus (HPV) has been detected in the majority of cervical carcinomas. Of more than 60 characterised HPV types HPV16 is most commonly associated with severe dysplasias and malignant tumours of the uterine cervix [1].

The nuclear protein E7 of HPV16 has been demonstrated to play a key role in both the transformation and maintenance of the malignant phenotype in cell culture [2–4]. Most recently, it has been demonstrated that the E7 oncoprotein can form complexes with p105-RB (the gene product of the RB1 retinoblastoma gene) similar to the adenovirus E1A protein and the simian virus 40 (SV40) large T antigen [5, 6]. These findings suggest that these three DNA viruses may employ similar mechanisms in transforming host cells and implicate RB-binding as a possible step in human papilloma virus-associated carcinogenesis.

Immunisation of mice with a syngenic non-tumorigenic fibroblast-cell line that contains the transfected HPV16 E7 gene confers protection against transplanted cells from an HPV16 E7-positive syngeneic tumour suggesting that the human papilloma virus type 16 nuclear protein E7 represents a 'tumour specific' antigen which can serve as a target for cellular immune responses [7]. These findings could provide a basis for the development of highly specific means of immunotherapy against HPV16 associated tumours and, in addition, for their prevention by means of vaccination.