EUROPEAN JOURNAL OF CANCER 42 (2006) 636–645

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.ejconline.com

Phosphorylation of tyrosine 1248-ERBB2 measured by chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients

Jonas Cicenas[a,c], *Patrick Urban*[a,c], *Willy Küng*[c], *Vincent Vuaroqueaux*[b], *Martin Labuhn*[b], *Edward Wight*[d], *Urs Eppenberger*[b,c], *Serenella Eppenberger-Castori*[a,*]

[a]*Stiftung Tumorbank Basel, Lörracherstrasse 50, CH-4125 Riehen, Switzerland*
[b]*OncoScore AG, Riehen, Switzerland*
[c]*Molecular Tumor Biology, Department of Research, University Basel, Basel, Switzerland*
[d]*University Clinics, Kantonsspital Basel, Switzerland*

ARTICLE INFO

Article history:
Received 29 April 2005
Received in revised form
31 October 2005
Accepted 28 November 2005
Available online 18 January 2006

Keywords:
Phosphorylation
ERBB2 (HER2/Neu)
Immunoassay
Biomarker
Prognosis
Breast cancer

ABSTRACT

ERBB2 (HER2/Neu) gene amplification and overexpression is associated with increased risk of metastases and shorter survival in breast cancer. Tyrosine 1248 is a major phosphorylation site of ERBB2 and reflects the activation status of the receptor. The aim of this study was to investigate the relationships between quantitative levels of pY1248-ERBB2 (p-ERBB2) and the expression of epidermal growth factor receptor (EGFR)-family members, and whether p-ERBB2 could provide additional prognostic value compared with established prognostic markers. For this purpose we developed a highly sensitive chemiluminescence-linked immunoassay (CLISA) and detected p-ERBB2 levels in 70 primary breast cancer biopsies. Phosphorylated ERBB2 correlated with EGFR and ERBB2, and inversely with oestrogen receptor (ER), progesterone receptor (PgR) and ERBB4 expression levels. Additionally, p-ERBB2 was associated with poor clinical outcome in univariate and multivariate Cox regression analysis. Further studies are needed to evaluate the predictive value of p-ERBB2.

1. Introduction

ERBB2 (HER2/Neu) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which comprises EGFR (HER1, ERBB1), ERBB3 (HER3) and ERBB4 (HER4).[1–3] Upon ligand binding, homo- and heterodimeric complexes are formed, with ERBB2 as the preferred dimerisation partner.[4] This leads to autophosphorylation of specific tyrosine residues, activation of downstream signalling cascades and, finally, initiation of biological processes such as proliferation.[5] ERBB2 contains five major tyrosine autophosphorylation sites, including Y-1248.[6]

In primary breast cancer, ERBB2 is amplified and overexpressed in 15–30% of patients and has been associated with poor prognosis.[7–9] Trastuzumab (Herceptin™), a humanised monoclonal anti-ERBB2 antibody is the first clinically available oncogene-targeted therapeutic agent for treatment of solid tumours, and is approved for use in metastatic breast cancer patients.[10] First-line trastuzumab in combination with chemotherapy resulted in a 25% improvement in overall

* *Corresponding author:* Tel.: +41 61 645 9304; fax: +41 61 645 9308.
E-mail address: s.eppenberger@tumorbank.org (S. Eppenberger-Castori).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2005.11.012

survival compared with chemotherapy alone. However, only up to 40% of patients respond to the therapy, suggesting that more accurate biomarkers are required to identify patients who are likely to respond to treatment such as trastuzumab. It was also reported that an inverse relationship exists between oestrogen receptor (ER) and ERBB2 expression, where ERBB2 overexpression is associated with decreased ER/progesterone receptor (PgR) levels and reduced sensitivity, possibly even resistance to endocrine therapy.[9,11,12]

ERBB2 gene amplification or overexpression per se may not reflect adequately the activated status of the ERBB2 receptor. It was hypothesised that the percentage of phosphorylated ERBB2, and thus activated receptor, could be different between tumours expressing similar amounts of ERBB2.[13]

The aim of the present study was to investigate the prognostic value of pY1248-ERBB2 detected with a newly developed chemiluminescence-linked immunoassay (CLISA), its association with protein and mRNA expression levels of the EGFR-family members including established prognostic markers in a set of 70 primary breast cancer patients.

2. Patients and methods

2.1. Patients and tumour characteristics

For all tumour samples the Stiftung Tumorbank Basel (STB) received a representative piece of fresh frozen tissue containing more than 65% tumour cells after surgery and pathological examination. Specimens were immediately processed or cryopreserved (−80 °C). For this study, 70 primary breast tumour samples were selected according to ERBB2 protein expression levels detected by enzyme immunoassay (EIA) at time of surgery. Tumours with ERBB2 protein levels >260 ng/mg total protein were considered positive, which corresponds to a previously published cut-off value of 500 U/mg total protein and correlates with the immunohistochemistry (IHC) DAKO 3+[8] as well as ERBB2 amplification detected by fluorescence in situ hybridisation (FISH) (Urban P, et al., submitted). ERBB2-negative tumours showed protein expression levels between 100 and 260 ng/mg. All patients underwent primary surgery before January 1996. Twenty-four patients (34%) relapsed within the median follow-up time of 55 months (range 30–89 months). Thirty-seven (53%) were nodal-positive, 50 (71%) were ER-positive and 40 (57%) patients were ERBB2-positive. None of the patients received neoadjuvant therapy.

STB is a non-profit organisation with an official Swiss permit that guarantees ethical issues and patient confidentiality. Patients and tumour characteristics are summarised in Table 1.

Table 1 – Tumour and patients characteristics

Feature	Number of patients (%)
Patients	70 (100)
Histology type	
Ductal	48 (69)
Lobular	11 (16)
Other	11 (16)
Tumour size	
T1	18 (26)
T2	42 (60)
T3–4	10 (14)
Lymph node status	
Node-negative	33 (47)
Node-positive	37 (53)
Histopathological grade	
I + II	27 (39)
III	34 (48)
Not analysed	9 (13)
Oestrogen receptor	
Positive (>20 fmol/mg)	50 (71)
Negative (⩽20 fmol/mg)	20 (29)
Median/mean (fmol/mg)	72/139
Progesterone receptor	
Positive (>20 fmol/mg)	38 (54)
Negative (⩽20 fmol/mg)	32 (46)
Median/mean (fmol/mg)	28/128
ERBB2	
Positive (>260 ng/mg)	40 (57)
Negative (<260 ng/mg)	30 (43)
Median/mean (ng/mg)	307/298

2.2. Cell lines and tissue culture

SKBr3 breast cancer cells were cultured in improved minimal essential medium with zinc option (IMEM-ZO) supplemented with 5% foetal bovine serum (FBS) and L-glutamine at 37 °C in a 5% CO_2 incubator. For the phospho-standard preparation sub-confluent SKBr3 cells were serum-starved for 48 h in serum-free medium, treated with NaF and Na_3VO_4 for 1 h, then with 10% FBS for 10 min. Cells were lysed in EB lysis buffer (0.5 M NaCl, 10 mM EDTA, pH 8, 1% Triton × 100, 20 mM Tris–Cl, pH 7.0, 20 mM NaF, 20 mM glycerophosphate, 2 mM Na_3VO_4, proteinase inhibitor cocktail, Roche) for 5 min on ice, centrifuged at 20,000*g* for 5 min and the supernatant stored at −80 °C.

2.3. Measurement of ER, PgR, ERBB2 and EGFR protein levels

Tissue homogenates were prepared in accordance with standard procedures for tumour marker EIA measurement, as described previously.[8] In brief, frozen tissues were powderised in liquid nitrogen (Micro-Dismembrator U, B. Braun AG, Melsungen, Germany) and homogenised (tissue homogeniser, Ultra-Turrax; Janke and Kunkel, IKA-Werke, Staufen, Germany) for 20 s in three volumes of ice-cold extraction buffer. The homogenate was centrifuged at 800*g* for 30 min at 2 °C, and the resulting supernatant recentrifuged in an ultracentrifuge (Beckman Instruments, Fullerton, CA, United States of America (USA)) at 100,000*g*. The resulting supernatants (cytosols) were used for measurement of the hormone receptors (ER, PgR by Abbott Laboratories, Abbott Park, IL, USA), while the membrane fractions were used for EIA measurement of ERBB2 (Oncogene Science Human HER-2/neu Quantitative ELISA Kit, Bayer, Leverkusen, Germany). Quantification of EGFR was done by radioligand binding assay (LBA) as described previously.[14] Quality control of ER and PgR measurements were carried out in collaboration with the Receptor