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Expression of HER2 and its association with AP-2 in breast cancer

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Abstract

The aim of the study was to investigate the relationship between the expression of human epidermal growth factor receptor 2 (HER2) and activator protein 2 (AP-2), as well as the prognostic significance of HER2 in breast cancer. HER2 and AP-2 expressions were immunohistochemically (IHC) analysed in a large prospective, consecutive series of 425 breast cancer patients diagnosed and treated between 1990 and 1995 at the Kuopio University Hospital, Kuopio, Finland. In a subset of patients ($n = 71$), the gene amplification status was examined by using a chromogenic *in situ* hybridisation (CISH) analysis. Expression of HER2 was studied in relation to AP-2, clinicopathological parameters and patients' survival. Pathological membranous overexpression of the HER2 receptor was seen in 13% of the carcinomas, which was related both to gene amplification (78% of the cases) and high nuclear expression of AP-2 (67%, $P = 0.007$). HER2-positivity was most often seen in carcinomas having both high nuclear and high cytoplasmic AP-2 expression ($P < 0.001$). In the univariate survival analyses HER2-positivity predicted a shorter recurrence-free survival (RFS) ($P < 0.0001$) and a shorter breast cancer-related survival (BCRS) ($P = 0.0063$) in the whole patient group, as well as in the subgroup of node-negative patients. In the node-positive patients, HER2-positivity predicted only a shorter RFS. Combined expression of HER2 and nuclear AP-2 resulted in the separation of four groups with different prognoses, the best prognosis being for patients in the HER2-/AP-2+ group and the worse prognosis for those in the HER2+/AP-2- group. In the multivariate survival analyses, HER2-positivity independently predicted a shorter RFS in the whole patient group ($P = 0.0067$), as well as in the subgroup of node-positive patients ($P = 0.0209$). In conclusion, pathological membranous overexpression of the HER2 receptor in breast cancer is related both to gene amplification and a high AP-2 expression. Combining HER2 and AP-2 expressions may provide valuable information on the prognosis of breast cancer patients.

Keywords: HER2; AP-2; CISH; Breast cancer; Prognosis; Prospective

1. Introduction

Human epidermal growth factor receptor 2 (HER2) (ErbB-2) is a member of the subclass I receptor tyrosine kinase family including the epidermal growth factor receptor (EGFR) (ErbB-1), HER3 (ErbB-3) and HER4 (ErbB-4) [1]. These transmembrane receptors homo- and heterodimerise upon ligand stimulation resulting in active molecular complexes that are involved in various cell regulation processes [2,3]. HER2 has no known ligand, but it is a preferred partner in dimerisation [4] and, thus, can actively modulate signalling [3]. Pathological membranous overexpression of the HER2 receptor, which is related to gene amplification and increased transcriptional activity [5–7], occurs in approximately 10% to 34% of breast cancers [8], as well as

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in other malignancies [9,10]. Pathological overexpression of the HER2 receptor is associated with an adverse prognosis in breast cancer [11–13]. Recently, HER2 has become a target for monoclonal antibody therapy [14].

The transcription factor AP-2 is required for *HER2* transcription and has been related to HER2 overexpression in breast cancer cells [7,15–17]. However, experiments performed using clinical tumour material have yielded contradictory results [18,19]. AP-2 forms a protein family (AP-2α, -β, -γ; [16] and -δ; [20]), which is essentially involved in development, apoptosis and cell cycle regulation [21]. An aberrant function of AP-2 has recently been associated with malignant transformation [22–24]. In breast tissues, AP-2 proteins are a regular component of the expressed gene network [18,19,25]. Altered expression of AP-2 has been associated with the poor clinical outcome of breast cancer patients [26].

The relationship between pathological membranous overexpression of the HER2 receptor and AP-2 has not been previously studied in a large group of clinical breast cancer material. In the present study, we analysed the expression of HER2 and AP-2 using immunohistochemistry (IHC) in a large, prospective and unselected series of 425 breast cancer patients with a long follow-up (median 57.2 months, range 1.2–115.1 months). In a subset of patients, *HER2* amplification was examined by chromogenic *in situ* hybridisation analysis [27]. Interrelationships between HER2 and AP-2, clinicopathological data and patients' survival were also investigated.

2. Patients and methods

2.1. Patients

The present study is part of the Kuopio Breast Cancer Project, a prospective, long-term clinical study involving 520 breast cancer patients [28–31] diagnosed among the 2500 women who were referred to the Kuopio University Hospital (between April 1990 and December 1995) because of a clinical breast lump, suspicious mammographic findings or breast symptoms (e.g. pain, nipple discharge). Women willing to participate in the project were interviewed and examined by a trained study nurse before any diagnostic procedures were undertaken. The participation rate of the patients who were diagnosed with breast cancer was 98%. Thus, the patient series represents unselected typical breast cancer cases from the University Hospital catchment area. Altogether, 479 invasive and 41 non-invasive carcinomas were diagnosed. Following surgical treatment, the patients were offered adjuvant chemo- and/or hormonal therapy and radiotherapy depending upon the mode of the surgery, the patient's menopausal status and the stage of the disease, according to the national guidelines [32]. In brief, postoperative radiotherapy was given to all patients treated with breast-conserving surgery and to all patients with axillary node-positive status (pN+), irrespective of the mode of surgery. All premenopausal patients with pN+ and some with axillary node-negative status (pN−) presenting with other adverse prognostic factors, such as oestrogen/progesterone receptor (ER/PR)-negative or poorly differentiated tumours, were given adjuvant chemotherapy (intravenous, i.v., cyclophosphamide-methotrexate-5-fluorouracil) for six cycles, which was the national standard therapy at the time. All postmenopausal women with ER- and/or PR-positive tumours were adjuvantly treated with either tamoxifen or toremifene for 3 years within another study protocol. Thus, within a stage the postoperative treatment was rather uniform, with only a few exceptions due to, e.g. concurrent conditions. The stage was assessed by using the International Union Against Cancer (UICC) classification [33]. Patients with non-invasive carcinomas, earlier history of breast cancer, metastatic disease (Stage IV) or insufficient tumour material were excluded from the present study. Thus, 425 patients with sufficient primary tumours and complete clinical histories were available for the HER2 analyses, 417 for AP-2 and 420 for Ki-67. The mean age of the 425 patients was 59.1 years and the median 56.7 years (range 23.3–91.6 years). The mean follow-up time was 54.8 months and the median 57.2 months (range 1.2–115.1 months). During the first five years of follow-up, 76 patients of the 425 (18%) had a recurrence, 50 patients (12%) died from breast cancer and 37 patients (9%) died from other causes. The overall 5-year survival rate was 77% (95% CI 72–81%). The 5-year recurrence-free survival rate of the patients was 79% (95% CI 74–83%) and the breast cancer-related survival rate was 85% (95% CI 81–89%). The 5-year survival rate of the excluded stage IV patients ($n = 19$) was 21% (95% CI 7–41%). No patient was lost to follow-up. The clinicopathological data of the patients are summarised in Table 1.

2.2. Histology

The tumour samples were fixed in 10% neutral-buffered formalin, routinely processed and embedded in paraffin. The histological diagnosis was confirmed by reviewing 1–4 original sections of the primary tumour. All tumours were simultaneously re-evaluated for histological type and graded by two senior pathologists, who were unaware of the clinical data. The most representative blocks were selected to be cut into new 5-μm thick sections for the immunohistochemical and *in situ* hybridisation analyses.

2.3. HER2 immunohistochemistry

The HER2 IHC was performed according to a routine staining method by using a TechMate 500-staining automat (DAKO, Golstrup, Denmark) at the Kuopio University Hospital. The sections were deparaffinised in

Table 1
Clinicopathological data of the patients

Characteristic	*n*	(%)
Number of patients	425	(100)
Age (years)		
Mean (range)	59.1	(23.3–91.6)
Tumour size		
T1	224	(53)
T2	167	(39)
T3	24	(6)
T4	10	(2)
Lymph node status		
Negative	248	(58)
Positive	169	(40)
Unknown	8	(2)
Stage		
I	165	(39)
II	213	(50)
III	39	(9)
Unknown	8	(2)
Histological type		
Ductal	273	(64)
Lobular	69	(16)
Other	83	(20)
Histological grade		
I	114	(27)
II	191	(45)
III	120	(28)
ER-status		
Positive	328	(77)
Negative	95	(22)
Data not available	2	(1)
PR-status		
Positive	264	(62)
Negative	158	(37)
Data not available	3	(1)
Menopausal status		
Premenopausal	132	(31)
Postmenopausal	293	(69)
Recurrence at 5 years		
No	349	(82)
Yes	76	(18)
Cause of death at 5 years		
Alive	338	(79)
Breast cancer	50	(12)
Other	37	(9)

ER, oestrogen receptor; PR, progesterone receptor.

xylene, rehydrated in ethanol and washed twice with distilled water. For better antigen retrieval, the samples were boiled in a microwave oven for 3×5 min in a citrate buffer solution (ChemMate^{TM} Buffer for Antigen Retrieval, pH 6.0, 10× concentrated, DAKO). Endogenous peroxidases were blocked by 5% hydrogen peroxidase treatment and the samples were washed with phosphate-buffered saline (PBS, pH 7.2). The primary antibody used was NCL-CB11 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK), a mouse monoclonal antibody directed towards HER2. The samples were incubated in the primary antibody, diluted 1:100 at room temperature, whereafter the samples were washed twice with PBS. HER2 expression was visualised by the labelled streptavidin–biotin system (LSAB, ChemMate^{TM} Detection Kit, DAKO), which included a biotinylated secondary antibody, streptavidin peroxidase (HRP), diaminobenzidine solution (DAB) and HRP substrate buffer. LSAB detection kit was used according to the instructions of the manufacturer. The slides were counterstained with Mayer's haematoxylin, dehydrated and mounted with Pertex (Histolab Products AB, Göteborg, Sweden). Each staining series had positive and negative control slides, which stained as expected.

2.4. AP-2 and Ki-67 immunohistochemistry

The IHC procedures for AP-2 [26] and Ki-67 [34] have been reported previously. In brief, a rabbit polyclonal antibody for human AP-2 (C-18, specific for AP-2α, AP-2β and AP-2γ, Santa Cruz Biotechnology 2002, Santa Cruz, CA, USA) was used as a primary antibody at a working dilution of 1:2000. An AP-2-positive melanoma was used as a positive control. The Ki-67 staining was demonstrated using the Sequenza^{TM} Immunostaining Center (Shandon Scientific Limited, Astmoor, UK). The primary antibody in the Ki-67 staining (MIB-1, Immunotech, Marseille, France) was used at a working dilution of 1:600. The positive control for Ki-67 was a lung carcinoma sample.

2.5. Scoring of immunoreactivity

The specimens were analysed according to a scoring system proposed by the HercepTest (DAKO) by two observers, who were unaware of the patients' clinical outcome. Discrepancies between the observers were found in less than 10% of the slides examined and a consensus was reached on a further review. Samples having a weak, moderate or intense staining of the entire membrane in more than 10% of the tumour cells (2+ and 3+) were considered to be HER2-positive. Other staining patterns were considered to be negative (0 and 1+). For Ki-67 and nuclear AP-2 expressions the median value (20% for Ki-67 and 80% for AP-2) was used as a cut-off value [26,34]. Cytoplasmic AP-2 expression was considered positive if more than 10% of the cells in the tumour area were stained [26].

2.6. Chromogenic in situ hybridisation (CISH)

For CISH, the paraffin sections were first heated at 37 °C for 1 h and at 60 °C for $2\frac{1}{2}$ h followed by deparaffinisation in xylene and rehydration in 100%

ethanol and air-drying. The samples were heated in CISH pretreatment buffer (Spot-Light™ Tissue Pretreatment Kit, Zymed Laboratories Inc, San Francisco, CA, USA) at 93 °C for 10 min in a microwave oven and cooled for 15 min at room temperature. Then the samples were incubated with 100 μl of the pretreatment enzyme (Spot-Light™ Tissue Pretreatment Kit, Zymed) in a humid chamber for 5 min at 38–40 °C or alternatively for 7 min at room temperature, dehydrated with graded ethanol and air-dried. The digoxigenin-labelled (DIG) HER2 probe (Spot-Light™ HER2 probe, Zymed) was applied on the slides (25–30 μl) and a coverslip was placed onto the sections. The edges of the coverslip were sealed with rubber cement (Sanford Corporation, Bellwood, USA). The specimens were denatured for 3 min at 94 °C on a heat block (GENOtronics, Biozym, Holland) and incubated in a humid chamber for 17h at 38–40 °C. The samples were washed with 7.5 mM tri-Na-citrate buffer for 5 min on a heat block at 80 °C and with 0.025% Tween–PBS. Endogenous peroxidase activity was blocked in 3% H_2O_2 diluted with methanol for 10min. The visualisation of HER2 CISH was performed by using a commercial Spot-Light™ CISH™ Detection Kit (Zymed). The non-specific staining was blocked by CAS-block (nonspecific blocking solution, CAS-block™) for 10 min following incubation with fluorescein isothio-cyanate (FITC)-conjugated sheep anti-DIG antibody for 40 min. After a wash in Tween–PBS, the HRP-conjugated goat anti-FITC antibody was applied onto the slides for 40 min. The DAB chromogen was applied for 24 min to visualise *HER2* expression. The slides were counterstained with hematoxylin, dehydrated with graded ethanol and cleared in xylene and mounted with Histomount (Zymed). Specimens both negative and weakly as well as strongly positive in HER2 immunohistochemical stain were included in the series.

2.7. Evaluation of CISH

Three observers evaluated the samples. The *HER2* gene was considered amplified if >2 brown-coloured dots were present at least in 10% of the tumour cell nuclei (Fig. 1(b)). A negative control slide was used in each staining batch (Fig. 1(c)).

2.8. Statistical analyses

The statistical analyses were carried out by using the SPSS for Windows 9.0 programme (SPSS Inc., Chicago, IL, USA). The associations between HER2 IHC, AP-2 expression and clinicopathological parameters were tested with contingency tables and a χ^2 test. Univariate survival analyses were performed using the Kaplan–Meier's log-rank analysis and the independent prognostic value of variables was further examined with Cox's regression analysis. Probability values $\leqslant 0.05$ were considered as significant in the analyses. In the

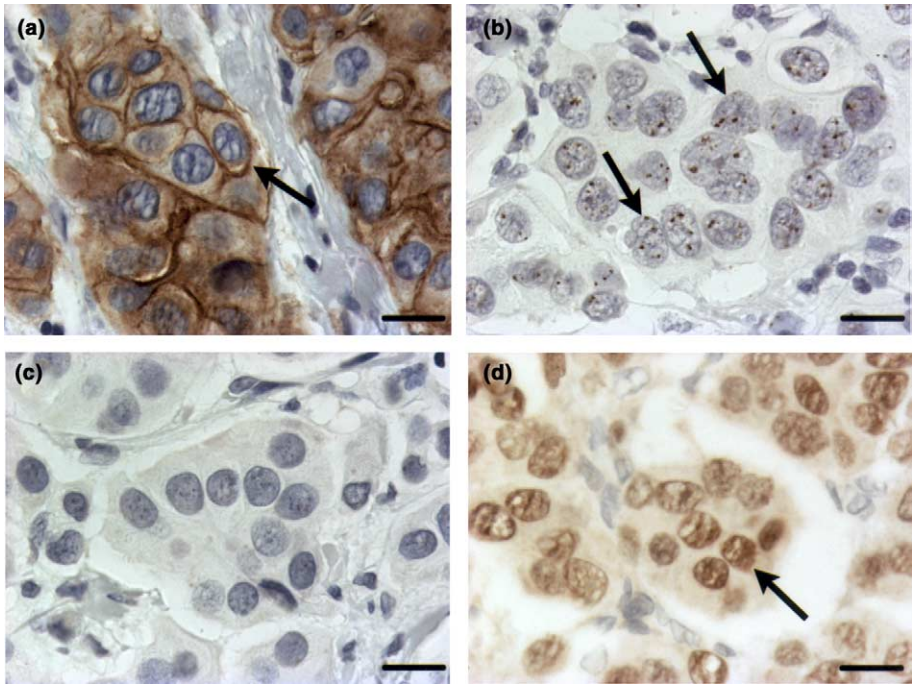

Fig. 1. Ductal carcinoma, grade III, with a pathological membranous overexpression (arrow) of HER2 receptor in IHC staining (a), *HER2* gene amplification (arrows) demonstrated by chromogenic *in situ* hybridisation (b), a negative control slide of chromogenic *in situ* hybridisation (c), and high nuclear (arrow) and cytoplasmic expression of AP-2 (d). Bar = 20 μm.

Cox's multivariate analysis, the Enter-method was used with an additional removal limit of $P < 0.10$. Both breast cancer-related survival (BCRS) and recurrence-free survival (RFS) were examined. The recurrence-free time was defined as a time between the diagnosis and the date of the first local recurrence or a distant metastasis, which ever one appeared first. Patients who remained healthy or died without breast cancer during the follow-up were censored at the time of the last follow-up visit or death.

3. Results

3.1. HER2 expression

Only membranous HER2 IHC staining was recorded as positive (Fig. 1(a)). The proportion of HER2-positive tumours (i.e. 2–3+) was 13%. Expression was lowest in lobular carcinomas of which only 4% were considered positive compared with 15% ductal and 12% for the other carcinoma subtypes (Table 2).

Table 2
Relationships between HER2 expression and AP-2, cell proliferation (Ki-67) and clinicopathological variables

Variable	HER2 expression (%)			χ^2	df	*P* value
	Sample	Negative	Positive			
	n	0–1+	2–3+			
Stage				8.1	2	0.017
I	165	(92)	(8)			
II	213	(85)	(15)			
III	39	(77)	(23)			
Lymph node status				3.3	1	0.069
Negative	248	(90)	(10)			
Positive	169	(83)	(17)			
Histological type				5.7	2	0.058
Ductal	273	(85)	(15)			
Lobular	69	(96)	(4)			
Other	83	(88)	(12)			
Histological grade				22.7	2	<0.001
I	114	(97)	(3)			
II	191	(88)	(12)			
III	120	(77)	(23)			
ER-status				53.1	1	<0.001
Positive	328	(94)	(6)			
Negative	95	(65)	(35)			
PR-status				25.5	1	<0.001
Positive	264	(94)	(6)			
Negative	158	(77)	(23)			
Menopausal status				8.4	1	0.004
Premenopausal	132	(80)	(20)			
Postmenopausal	293	(90)	(10)			
Recurrence at 5 years				22.0	1	<0.001
No	349	(91)	(9)			
Yes	76	(71)	(29)			
Nuclear AP-2				7.4	1	0.007
Low <80%	211	(91)	(9)			
High $\geqslant$ 80%	206	(83)	(17)			
Combined AP-2 (nuclear/cytoplasmic)				19.1	3	<0.001
AP-2 −/−	118	(90)	(10)			
AP-2 −/+	93	(94)	(6)			
AP-2 +/−	103	(90)	(10)			
AP-2 +/+	103	(75)	(25)			
Cell proliferation (Ki-67)				25.6	1	<0.001
Low <20%	235	(94)	(6)			
High $\geqslant$ 20 %	185	(78)	(22)			

df, degrees of freedom.

3.2. Chromogenic in situ hybridisation (CISH)

HER2-positive cases with sufficient tumour material ($n = 51$), as well as 20 negatively stained samples, were included in the analysis. Gene amplification was observed in 78% of the HER2 IHC-positive tumours (Fig. 1(b)). The amplification rate was 100% in the 3+ stained tumours and 61% in the 2+ stained tumours. Two of the HER2- IHC negative samples also showed a positive signal.

3.3. Expression of AP-2 and Ki-67

The expression of nuclear AP-2 (range 0–100%) was high ($\geqslant 80\%$) in 49% of the carcinomas (Fig. 1(d)), whereas cytoplasmic AP-2 expression was detectable in 47% of the cases. Ki-67 expression was nuclear and the proportion of cases considered as high expressors ($\geqslant 20\%$) was 44%.

3.4. Relationship of HER2 expression to AP-2

HER2-positivity was associated with a high nuclear AP-2 expression ($\chi^2 = 7.4$, $P = 0.007$, Table 2) in the whole patient group, as well as in the node −/+ subgroups ($\chi^2 = 4.1$, $P = 0.042$ for N− and $\chi^2 = 4.7$, $P = 0.030$ for N+). In addition, an association was seen in ER- ($\chi^2 = 8.9$, $P = 0.003$) and PR- ($\chi^2 = 8.2$, $P = 0.004$) negative tumours. The association between HER2 and cytoplasmic AP-2 expression did not reach statistical significance ($\chi^2 = 3.7$, $P = 0.056$).

When nuclear and cytoplasmic AP-2 expressions were combined, HER2-positivity was most often seen in carcinomas having both high nuclear and high cytoplasmic AP-2 expressions ($\chi^2 = 19.1$, $P < 0.001$, Table 2). Indeed, 48% of HER2+ cases fell within this category. This association was also seen in the subgroup of node-positive patients ($\chi^2 = 22.7$, $P < 0.001$).

When nuclear AP-2 expression was investigated in relation to CISH in the HER2-positive cases, 70% of amplified cases expressed AP-2 highly compared with 55% in non-amplified cases. However, this did not reach statistical significance. AP-2 and *HER2* amplification showed coexpression in 55% of the cases, 23% were amplified only, 12% were AP-2-positive only and 10% were negative for both.

3.5. Relationship of HER2 expression to the clinicopathological data and cell proliferation

HER2-positivity was associated with an advanced stage ($P = 0.017$), poor differentiation ($P < 0.001$), ER- and PR-negativity ($P < 0.001$ for ER and $P < 0.001$ for PR), premenopausal status ($P = 0.004$), high fraction of proliferating cells measured by Ki-67 expression ($P < 0.001$) and recurrent disease ($P < 0.001$). Expression was not associated with the lymph node status. The results are summarised in Table 2.

3.6. Univariate survival analysis

In the univariate survival analyses, HER2-positivity predicted a shorter RFS ($P < 0.0001$, Table 3, Fig. 2) and a shorter BCRS ($P = 0.0063$, Table 3) in the whole patient group, as well as in the subgroup of node-negative patients ($P = 0.0456$ for RFS and $P = 0.0342$ for BCRS). In the node-positive patients, HER2-positivity predicted a shorter RFS ($P = 0.0002$).

In addition, we investigated the prognostic value of HER2 and AP-2 coexpression with regard to RFS and BCRS. Interestingly, nuclear AP-2 expression separated the patients into four groups with different prognoses. The 5-year RFS in patients having HER2-/AP-2+ tumours was 89% compared with 76% in the HER2-/AP-2- group, 65% in the HER2+/AP-2+ group and 39% in the HER2+/AP-2- group ($P < 0.0001$) (Fig. 3). Similar observations were made in the BCRS analysis ($P = 0.0103$) and for the RFS analysis of the N+ patients ($P < 0.0001$). The same trends, although non-significant, were also seen in the survival analysis of the N- patients, as well as in the BCRS analysis for the N+ patients.

The other statistically significant predictors of BCRS and RFS were stage ($P < 0.0001$ for both), lymph node status ($P < 0.0001$ for both), histological grade ($P = 0.0306$ for BCRS and $P = 0.0441$ for RFS), ER expression ($P < 0.0001$, BCRS only), PR expression ($P = 0.0011$, BCRS only) and Ki-67 expression ($P = 0.0063$ for BCRS and $P < 0.0001$ for RFS).

3.7. Multivariate survival analysis

There were 409 patients with a complete set of data available for RFS and 402 for the BCRS analyses. Cox's regression model included all statistically significant variables derived from the univariate survival analyses (stage, histological grade, HER2 and Ki-67 expressions in the RFS analyses, and stage, histological grade, ER- and PR-status, HER2 and Ki-67 expressions in the BCRS analyses). The independent predictors of a shorter RFS (Table 4) were HER2-positivity ($P = 0.0067$), advanced stage ($P = 0.0004$) and a high proliferation activity ($P < 0.0001$). Shorter BCRS (Table 4) was independently predicted by an advanced stage ($P < 0.0001$), poor differentiation ($P = 0.0439$) and a high proliferation activity ($P = 0.0007$).

In addition, we performed multivariate analyses using combined HER2/AP-2 expression instead of HER2 expression alone. In the RFS analyses ($n = 401$), HER2/AP-2 expression ($P = 0.0042$), stage ($P = 0.0013$), histological grade ($P = 0.0408$) and proliferation activity ($P < 0.0001$) independently predicted outcome (Table 4). In

Table 3
Immunohistochemical and statistically significant clinicopathological variables of breast cancer-related and recurrence-free survival in univariate survival analysis

Variable	Surviving breast cancer at 5 years (BCRS)			Recurrence-free at 5 years (RFS)		
	n	(%)	*P* value	*n*	(%)	*P* value
Stage			<0.0001			<0.0001
I	165	(92)		165	(87)	
II	213	(86)		213	(77)	
III	39	(55)		39	(55)	
Lymph node status			<0.0001			<0.001
Negative	248	(92)		248	(86)	
Positive	169	(77)		169	(68)	
Histological grade			0.0306			0.0441
I	114	(92)		114	(86)	
II	191	(85)		191	(78)	
III	120	(81)		120	(74)	
ER-status			<0.0001			0.1004
Positive	328	(89)		328	(80)	
Negative	95	(72)		95	(74)	
PR-status			0.0011			0.1748
Positive	264	(90)		264	(80)	
Negative	158	(79)		158	(76)	
HER2 expression			0.0063			<0.0001
Negative 0–1+	371	(88)		371	(82)	
Positive 2–3+	54	(71)		54	(56)	
Cell Proliferation (Ki-67)			<0.0001			<0.0001
Low <20 %	235	(93)		235	(90)	
High $\geqslant$ 20 %	185	(75)		185	(64)	

BCRS, breast cancer related survival; RFS, recurrence-free survival.

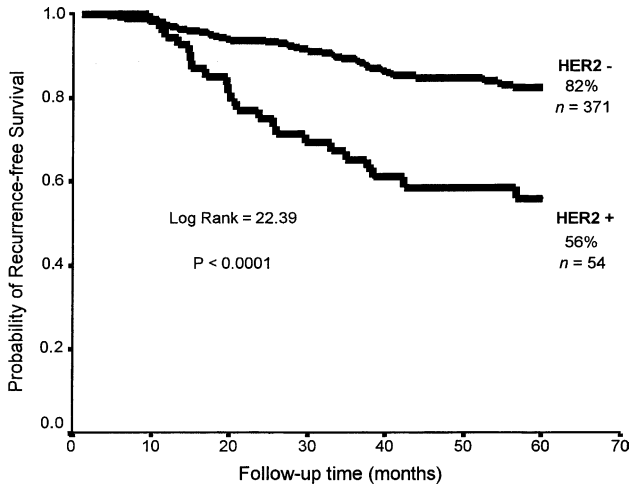

Fig. 2. Recurrence-free survival (RFS) in the whole patient group according to IHC HER2 expression. The HER2− group contains the patients who have HER2 (0–1+) tumours, whereas the HER2+ group consists of HER2 (2–3+) stained cases. The percentages given represent the 5 year RFS.

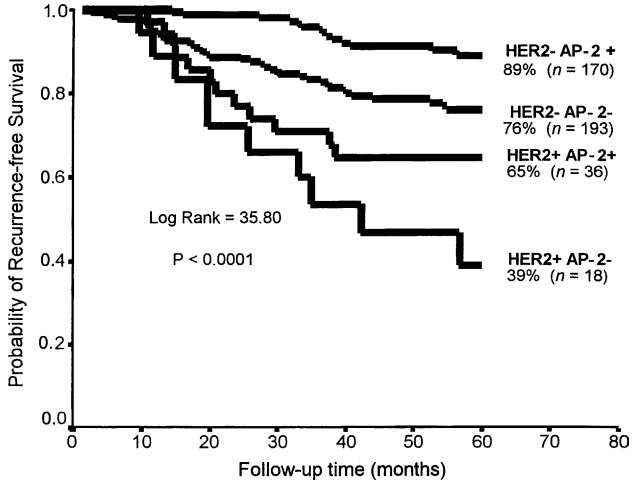

Fig. 3. Recurrence-free survival (RFS) in the whole patients group according to the combined IHC HER2 and nuclear AP-2 expressions.

the BCRS ($n = 395$) analyses, HER2/AP-2 expression did not have an independent prognostic value.

In the subgroup of node-positive patients, a shorter RFS ($n = 166$) was independently predicted by HER-positivity ($P = 0.0209$) and high proliferation activity measured by Ki-67 expression ($P = 0.0130$). High proliferation activity ($P = 0.0213$) and an advanced stage ($P = 0.0014$) predicted a shorter BCRS ($n = 165$). In the node-negative patient group, the only independent predictor of RFS and BCRS was proliferation activity

Table 4
Results of Cox's multivariate analysis for breast cancer-related and recurrence-free survival

Category (variable)	Beta (SE)	Relative risk (95% CI)	*P* value
Breast cancer-related survival			
Stage			<0.0001
I	Reference		
II	0.81 (0.41)	2.24 (0.99–5.04)	0.0523
III	2.04 (0.45)	7.65 (3.17–18.49)	<0.0001
Cell Proliferation (Ki-67)			
Low <20%	Reference		
High ⩾ 20%	1.29 (0.38)	3.65 (1.72–7.71)	0.0007
Histological grade			0.0439
I	Reference		
II	0.46 (0.47)	1.58 (0.63–4.00)	0.3335
III	−0.48 (0.56)	0.62 (0.21–1.84)	0.3857
Recurrence-free survival			
Stage			0.0004
I	Reference		
II	0.53 (0.29)	1.70 (0.96–3.00)	0.0697
III	1.41 (0.36)	4.08 (2.01–8.29)	0.0001
Cell proliferation (Ki-67)			
Low <20%	Reference		
High ⩾ 20 %	1.36 (0.29)	3.90 (2.20–6.93)	<0.0001
HER2 expression			
Negative 0–1+	Reference		
Positive 2–3+	0.73 (0.27)	2.07 (1.22–3.50)	0.0067
Recurrence-free survival using combined HER2/AP-2 expression			
Stage			0.0013
I	Reference		
II	0.39 (0.30)	1.47 (0.82–2.64)	0.1941
III	1.27 (0.36)	3.54 (1.74–7.20)	0.0005
Cell Proliferation (Ki-67)			
Low < 20%	Reference		
High ⩾ 20%	1.29 (0.29)	3.62 (2.05–6.41)	<0.0001
Histological grade			0.0408
I	Reference		
II	0.09 (0.35)	1.10 (0.55–2.18)	0.7898
III	−0.58 (0.40)	0.56 (0.26–1.21)	0.1406
HER2/AP-2 expression			0.0042
HER2-AP-2+	Reference		
HER2-AP-2−	0.72 (0.32)	2.05 (1.10–3.83)	0.0243
HER2+AP-2+	1.04 (0.41)	2.83 (1.25–6.37)	0.0121
HER2+AP-2−	1.56 (0.44)	4.75 (2.00–11.34)	0.0004

SE, standard error; CI, confidence interval.

($P = 0.0002$ for RFS, $n = 242$ and $P = 0.0257$ for BCRS, $n = 222$).

4. Discussion

In the present study, we analysed the expression of HER2 using IHC in a large prospective series of breast cancer patients. *HER2* amplification was investigated using a CISH method in a subset of the patients. Furthermore, we compared the expression of HER2 to that of the transcription factor AP-2 to investigate whether AP-2 may partly be responsible for HER2 overexpression *in vivo* in breast cancer, as has been suggested in [15,16,19]. According to our results, HER2-positivity seems to be related to the parameters that reflect the aggressive behaviour of breast cancer and that independently predict a shorter RFS. Pathological membranous overexpression of HER2 in breast cancer was related both to gene amplification (78% of the cases) and a high expression of AP-2 (67%), of which one or both were present in 90% of the HER2-positive cases. High nuclear AP-2 expression offered a prognostic advantage,

as the survival rate in HER2-/AP-2+ group was 89% compared with 39% in the HER2+/AP-2- group.

The HER2 overexpression has been reported in 10–34% of breast cancers [11]. In the present study, 13% of carcinomas stained positively, which falls within the category mentioned above. In addition, most of the tumours were small (53% T1, $n = 224$), a category in which 13% positivity appears correct [35]. However, the overexpression of HER2 that we observed is less than that previously described in breast cancer (23%, 19.7%) when the same antibody NCL-CB11 had been used [27,36]. Recently, CISH has been demonstrated to be a useful alternative to fluorescence *in situ* hybridisation (FISH) [27,37,38]. In this study, we established the CISH procedure to analyse *HER2* amplification in a subset of cases. The proportion of amplification among cases overexpressing HER2 was 78%, which is close to that described in literature (83%, 81%) [27,38]. Additionally, the amplification rate in the 3+ tumours (100%) is in agreement with previous reports (96%, 92%) [27,38]. Accordingly, the results of CISH are comparable and add valuable information to the present study.

In our study, HER2-positivity was associated with aggressive clinicopathological parameters as documented previously in [39–41]. In our series, the association with nodal status and histological type was of borderline significance. Indeed, HER2 overexpression has not always been associated with nodal status, but rather with the number of infiltrated nodes [39,41]. HER2 overexpression has rarely been observed in lobular carcinomas [42,43], which was a trend in this study too. In addition, an increased proliferation rate in HER2-overexpressing tumours has been well documented [39,44]. HER2 overexpression was a prognostic indicator in agreement with data from the literature [11]. HER2 overexpression also independently predicted a shorter RFS in the node-positive patient group but this correlation did not reach statistical significance in the BCRS analysis as demonstrated previously in [12,40]. This may be due to the small low number of patients as well as the number of cancer deaths that occurred in this subgroup. However, the traditional prognostic factors remained significant, thus verifying the value of our current material.

We demonstrated that HER2 overexpression is often related to the high expression of the AP-2 transcription factor. Indeed, 67% of HER2-positive cases highly expressed nuclear AP-2 and nearly half (48%) of the HER2-positive tumours belonged to a group of combined high nuclear and cytoplasmic AP-2 expression. Previously, the results concerning the relationship between HER2 and AP-2 expressions in breast cancer have been contradictory. Turner and colleagues [19] reported a positive association between HER2 and AP-2α/γ double positivity, whereas Gee and colleagues [18] in a small number of patients showed an inverse relationship using an AP-2α/β antibody. In childhood medulloblastoma, AP-2 expression has been positively associated with expression of HER2, HER3 and HER4, when the same AP-2 antibody was used as in our study [45]. As far as we are aware, this is the first study to report a relationship between HER2 and AP-2 in a large prospective breast cancer series.

Interestingly, by combining HER2 and nuclear AP-2 expressions four separate patient groups with different survival rates were observed, with respect to both RFS ($P < 0.0001$) and BCRS ($P = 0.0103$). Patients having a high nuclear AP-2 expression did better than patients with a low nuclear AP-2 expression, independent of their HER2-status. However, of these two factors, HER2 expression seemed to be the stronger predictor of clinical outcome. Furthermore, we investigated whether the expression of AP-2 in cases having *HER2* amplification/*HER2* overexpression was an attempt to halt cell proliferation via regulation of the cell cycle inhibitor $p21^{WAF1}$ [34,46], but the expressions of AP-2 and $p21^{WAF1}$ were not associated with each other (data not shown). However, the number of patients in the analysis was relatively small ($n = 40/54$).

The role of a high AP-2 expression in HER2-positive breast cancer remains unclear, as there are contradicting results concerning the function of AP-2. Recently, it is increasingly believed that AP-2 functions as a growth limiting tumour suppressor in breast tissue [18,25,26]. On the other hand, in breast cancer, AP-2 is related to overexpression of oncogenic HER2 [16] and is required for maximal *HER2* transcription [17]. High nuclear AP-2 expression in HER2-positive cases and the prognostic advantage that AP-2 offers may reflect an attempt to suppress malignant transformation. Cytoplasmic AP-2 expression was also common in HER2-positive cases, which may indicate an inappropriate function for AP-2 and a step to uncontrolled cell growth regulation. On the other hand, it is possible that on some occasions high AP-2 expression may be oncogenic [5,16,47], or the function of AP-2 may be modified by the expression of other growth regulating factors, such as Sp1 [48]. Thus, the role of the separate AP-2 proteins in HER2 regulation requires further investigations.

We conclude that immunohistochemically detected pathological membranous overexpression of HER2 in breast cancer is very often related to gene amplification as well as to a high AP-2 expression. However, the role of high AP-2 expression in these cases remains unclear. In addition, evaluating AP-2 expression and combining it with HER2 may provide important additional information on the clinical outcome of breast cancer patients.

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