Original Article

Study of the Effect of Neoadjuvant Chemotherapy on the Status of Her2/neu

(breast cancer / neoadjuvant chemotherapy / Her2/neu / IHC / FISH / real-time PCR)

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Abstract. *Her2/neu* proto-oncogene amplification and protein over-expression is observed in 20–40 % of patients with breast cancer and plays a crucial role in invasive breast cancer and its treatment. A number of studies postulated the stability of *Her2/neu* gene expression, showing that in most patients the status of expression had not significantly changed after the neoadjuvant treatment. In the present study, we investigated samples from 20 patients with invasive breast carcinoma who had undergone neoadjuvant chemotherapy and subsequent surgery. In all cases, the expression level of *Her2/neu* was evaluated in both pre-therapeutically obtained tumour tissue by core needle biopsy and from specimens obtained during final surgery using immunohistochemistry. Fluorescence *in situ* hybridization and quantitative reverse transcription polymerase chain reaction methods were used for verifying the results obtained by immunohistochemistry. *Her2/neu* status determined by immunohistochemistry remained unchanged in 12 of 20 (60 %) patients after neoadjuvant treatment. In six cases (30 %) minor changes were observed after the treatment. However, in two cases (10 %) we found altered *Her2/neu* expression from strongly positive in the pre-treatment biopsy to negative in the post-treatment surgery specimen. Moreover, this is the first report describing the changes in *Her2/neu* status at all protein, RNA and DNA levels by using immunohistochemistry, quantitative reverse transcription polymerase chain reaction and fluorescence *in situ* hybridization, respectively. By using variable methods we demonstrated possible new ways for *Her2/neu* detection and their dependability. Improvement in specific molecule detection can prevent the use of tailored targeted therapy in an untargeted manner.

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Abbreviations: DAPI – 4,6-diamidino-2-phenylindol, DCIS – ductal carcinoma *in situ*, ER – oestrogen, FISH – fluorescence *in situ* hybridization, HE – haematoxylin and eosin, IHC – immunohistochemistry, MMLV – Moloney-Murine leukaemia virus, PR – progesterone receptors, qRT-PCR – quantitative reverse transcription polymerase chain reaction, RPL23 – ribosomal protein L23.

Introduction

The *Her2/neu* gene (also known as *ERBB2* or *EGFR2*) encodes a 185 kDa transmembrane glycoprotein with tyrosine kinase activity. *Her2/neu* has a high sequence homology with other members of the epidermal growth factor receptor family (Duffy, 2005). The function of these receptors is in the regulation of cell growth, differentiation and survival. Receptor activation requires three components: a ligand, a receptor, and a dimerization partner. When a specific ligand binds to a Her2 receptor, it must combine with another receptor of similar structure and undergo dimerization. This initiates a cascade of phosphorylation and signal transduction events that affect the transcription of specific genes involved in cell proliferation and survival (Davoli et al., 2010).

Her2/neu proto-oncogene amplification and protein over-expression is observed in 20–40 % of patients with breast cancer (Slamon et al., 1989) and it plays a crucial role in the biological behaviour and pathogenesis of invasive breast cancer and its treatment. Both node-positive and node-negative breast cancer patients whose tumours exhibit *Her2/neu* amplification have a poor prognosis, an increased risk of recurrence and a high risk of disease-related death, showing an overall shorter survival time (Slamon et al., 1987; Ro et al., 1989; Paterson et al., 1991; Press et al., 1997; Andrulis et al., 1998).

However, it does predict a favourable response to neoadjuvant chemotherapy and anti-Her2 antibody treatment (Quddus et al., 2005) because the humanized anti-Her2 antibody binds to the Her2 receptor, thereby

preventing heterodimerization and interrupting the downstream signalling pathway. Moreover, the bound antibody induces FcR-mediated cytotoxicity (Davoli et al., 2010). Therefore, it is very important to know the status of *Her2/neu* when thinking about biological therapy.

A number of studies have postulated the stability of the *Her2/neu* gene, showing that in most patients the status of expression had not significantly changed after they were given neoadjuvant therapy (Taucher et al., 2003; Tinari et al., 2006; Lee et al., 2007; D'Alfonso et al., 2010). On the other hand, other studies have presented patients with a higher rate of altered status. In 2005, Varga and collaborators (Varga et al., 2005) reported a decreased expression of *Her2/neu* in six patients (26 %) and an increased status in two cases (9 %). In a later similar study, another group of authors (Adams et al., 2008) demonstrated a change from an initial Her2--negative tumour to a positive one in two patients (9 %) and from a previously Her2-positive tumour to a negative one in six patients (26 %) after the treatment.

In the present study, we investigated samples from 20 patients with invasive breast carcinoma who had undergone neoadjuvant chemotherapy and subsequent surgery (lupmectomy or mastectomy) at the General University Hospital in Prague (Prague, Czech Republic). In all cases, the expression level of *Her2/neu* was evaluated both in pre-therapeutically obtained tumour tissue by core needle biopsy and from specimens removed during final surgery using immunohistochemistry (IHC). Although in 12 cases the expression of *Her2/neu* remained unchanged, there were minor changes observed in six cases, but we also revealed a remarkable alteration in two patients. One possible explanation for this phenomenon is the co-existence of at least two clones of tumour cells with different levels of *Her2/neu* expression and thus different sensitivities to neoadjuvant therapy. In contrast to other reports (Taucher et al., 2003; Varga et al., 2005; Tinari et al., 2006; Lee et al., 2007; Adams et al., 2008; D'Alfonso et al., 2010), both fluorescence *in situ* hybridization (FISH) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) methods were used for verifying the results obtained by IHC.

Material and Methods

Patients and treatment

Our study included 20 patients, all females, aged 54 ± 13 (mean ± standard deviation) years, neoadjuvantly treated for breast carcinoma at the General University Hospital in Prague between 2005 and 2010. Twelve patients (60 %) had tumour stage II, four patients (20 %) stage III, three patients (15 %) stage IV and one patient (5 %) stage 0 (ductal carcinoma *in situ*, DCIS). The scheme of neoadjuvant chemotherapy is summarized in Table 1. In all cases, tumour tissue was obtained from both diagnostic core needle biopsy and from a specimen removed during final surgery (lupmectomy or mastectomy).

Histological evaluation

The specimens from the core needle biopsy and surgery were fixed in 10 % formalin and embedded in paraffin wax. Histological evaluation was done on slides routinely stained with haematoxylin and eosin (HE). Tumour stage was determined according to WHO guidelines (Tavassoli and Devilee, 2004) regarding the use of clinical data.

Immunohistochemistry

Immunohistochemistry included the assessment of Her2, oestrogen (ER) and progesterone receptors (PR). Evaluation of Her2 was done using HerceptTest (Dako Denmark A/S, Glostrup, Denmark) according to the manufacturer's instructions. The slides were immersed in a preheated Epitope retrieval solution (95–99 °C), in a water bath for 40 min, cooled-down for 20 min at room temperature and then rinsed by a wash buffer. A peroxidase-blocking reagent, a primary antibody, a visualization reagent and a substrate-chromogen solution were applied; the slides were incubated and after each step they were rinsed in a wash buffer. The evaluation of Her2 over-expression was performed as defined by the HercepTest scoring guidelines: 0: no staining or membrane staining in less than 10 % of the tumour cells. 1+: partial faint membrane staining in more than 10 % of the tumour cells. 2+: weak to moderate complete membrane staining in more than 10 % of the tumour cells. 3+: strong complete membrane staining in more than 10 % of the tumour cells. HercepTest was interpreted as negative (score 0 and 1+), weakly positive (2+), and strongly positive (3+) for Her2 protein over-expression.

Antibody clone ER-6F11 for the ER receptor and PGR-312 for the PR receptor were applied according to manufacturer's instructions (both Novocastra, Leica Microsystems, IL). A positive score for ER and PR was defined as higher than or equal to 10 % of tumour cells showing nuclear staining, with intensity graded from 1+ (weak) to 3+ (strong).

Fluorescence in situ hybridization

Five μm thick sections from paraffin-embedded tissue were processed for FISH using a PathVysion HER-2 DNA Probe Kit from Abbott Vysis (Downers Grove, IL, USA). The assay procedure was carried out according to the manufacturer's recommendations. First, the slides were deparaffinized in xylene, then pre-treated in 0.2 N HCl and subsequently in an NaSCN solution at 80 °C; the next step was proteolytic treatment. The protease digestion plays a crucial role in terms of obtaining readable and conclusive FISH results. We used Protease II from Abbott Vysis, 25 mg in a 50 ml saline solution at pH 2, digestion time 45 min for the pre-treatment core needle biopsy and 60 min for the post-treatment mastectomy samples. Afterwards, the sections were fixed in buffered formalin. Then we applied the FISH probe, sealed it with liquid rubber cement and co-denatured the specimen and the DNA probe for 1 min at 85 °C and

Table 1. Clinical, histological and immunohistochemical characteristics of patients before and after neoadjuvant therapy

No.		Her2 (0-3+)	ER (%)	PR (%)	Clinical stage	Diagnosis	Neoadjuvant treatment
Her2 positive							
1	Before	3	>90	<1	IIA	ILC	4× doxorubicin and cyclophosphamide + 4× docetaxel and trastuzumab
	After	1	30-40	<1	I		
2	Before	3	80	80-90	IIIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel + 6× paclitaxel and trastuzumab
	After	2	50-60	20-30	I		
3	Before	3	20-25	<1	IIA	IDC	4× doxorubicin and cyclophosphamide
	After	3	20	0	IIA		
4	Before	3	0	0	IIB	IDC	5× fluorouracil, epirubicin and cyclophosphamide + 12× paclitaxel and trastuzumab
	After	0	20	10	I		
5	Before	3	0	0	IV	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	3	10-15; 70-80*	<5; 5-10*	IV		
6	Before	3	0	0	IIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	3	10	10	IIIA		
7	Before	3	0	0	IIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel and trastuzumab
	After	3	40	5-10	0 (DCIS)		
8	Before	3	<5	0	IIA	IDC	4× doxorubicin and cyclophosphamide +12× paclitaxel and trastuzumab
	After	2	<1	0	0 (DCIS)		
Her2 negative							
9	Before	0	40-50	10	IIIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	5-10	10	IIIC		
10	Before	1	60-70	70-80	IIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	>90	100	I		
11	Before	0	>90	100	IIA	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	70-80	10-15	IIA		
12	Before	1	80-90	<1	IV	ILC	tamoxifen + letrozole + 4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	2	40	<1	IIA		
13	Before	1	80-90	20	0 (DCIS)	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel + 6× goserelin
	After	0	80	20	I		
14	Before	1	20	>90	IV	ILC	6× fluorouracil, epirubicin and cyclophosphamide + 4× docetaxel
	After	0	30	0	IV		
15	Before	0	50-60	100	IIIB	ILC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	70	80	IIB		
16	Before	1	90	20	IIA	IDC	4× doxorubicin and cyclophosphamide + 9× paclitaxel
	After	1	80-90	20-30	IIIC		
17	Before	0	65-75	35-45	IIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	40	<5	IIA		
18	Before	0	80	100	IIA	IDC	3× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	70-80	80-90	N/A		
19	Before	0	80	20	IIB	IDC	4× doxorubicin and cyclophosphamide + 1× docetaxel + 9× paclitaxel
	After	0	80	30	I		
Triple negative							
20	Before	0	0	0	IIB	IDC	4× doxorubicin and cyclophosphamide + 4× docetaxel
	After	0	0	0	IIA		

* inv; DCIS
ILC - Invasive Lobular Carcinoma
IDC - Invasive Ductal Carcinoma
N/A - not available

then hybridized overnight in ThermoBrite (Vysis, Downers Grove, IL) at 37 °C. After hybridization, the unbound probe was removed in 0.4x SSC/0.3% NP-40 wash solution at 74 °C, the slides were dehydrated and counterstained with 4,6-diamidino-2-phenylindol (DAPI).

For each sample, a minimum of 20 cells were evaluated in an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan) for the presence of amplification signals. A positive result was defined as the ratio Her2 : CEP17 > 2.

RNA isolation, cDNA preparation and qRT-PCR analysis

Deparaffinizing slides of formalin-fixed, paraffin-embedded tissue, isolation of total RNA and synthesis of cDNA were performed by standard procedures described in our previous work (Tvrdík et al., 2005). Briefly: total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hamburg, Germany), reverse transcription was performed by a RevertAid – H Minus First Strand cDNA

Synthesis Kit (Fermentas, St. Leon-Rot, Germany), which employs a random hexamer primer and Moloney-Murine leukemia virus (MMLV) reverse transcriptase.

Her2/neu mRNA expression levels were quantified by means of a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using the LightMix *Her2/neu* kit (Tib MolBiol, Berlin, Germany) according to manufacturer's protocol. This kit contained a calibrator DNA provided to generate a calibration curve.

The PCR conditions were: initial denaturation at 95 °C for 10 min, followed by 50 cycles with the denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s.

The level of *Her2/neu* mRNA was normalized to the level of ribosomal protein L23 (RPL23) as a housekeeping gene. PCR amplification of each transcript was performed twice.

The normalized ratio Her2 : RPL23 > 2 was considered positive for *Her2/neu* gene over-expression.

Statistical analysis

Q-PCR data is presented as the arithmetic mean of an absolute copy number ± standard deviation of the mean. Two-sided Student's *t*-test was used to evaluate the statistical significance of the results. Differences with P values < 0.05 were considered significant.

Results

Histologic findings, tumour staging

Out of 20 patients, 16 (80 %) had infiltrating ductal carcinoma, four (20 %) had infiltrating lobular carcinoma. Tumour stage was determined both before therapy and after therapy (Table 1). In pre-therapeutical investigation 12 (60 %) patients had tumour stage II, four (20 %) patients stage III, three (15 %) patients stage IV and one (5 %) patient stage 0 (DCIS). In post-therapeutial investigation six (30 %) patients had tumour stage I, six (30 %) patients stage II, three (15 %) patients stage III, two (10 %) patients stage IV and one (5 %) patient stage 0 (DCIS). These results indicate tumour reduction and a decrease in tumour stage after therapy in eight (40 %) patients. No significant change was observed in seven (35 %) patients. Progression of disease was found in three (15 %) patients. In two patients (10 %) the tumour stage after therapy was not known, but one of them died with generalized tumour spread.

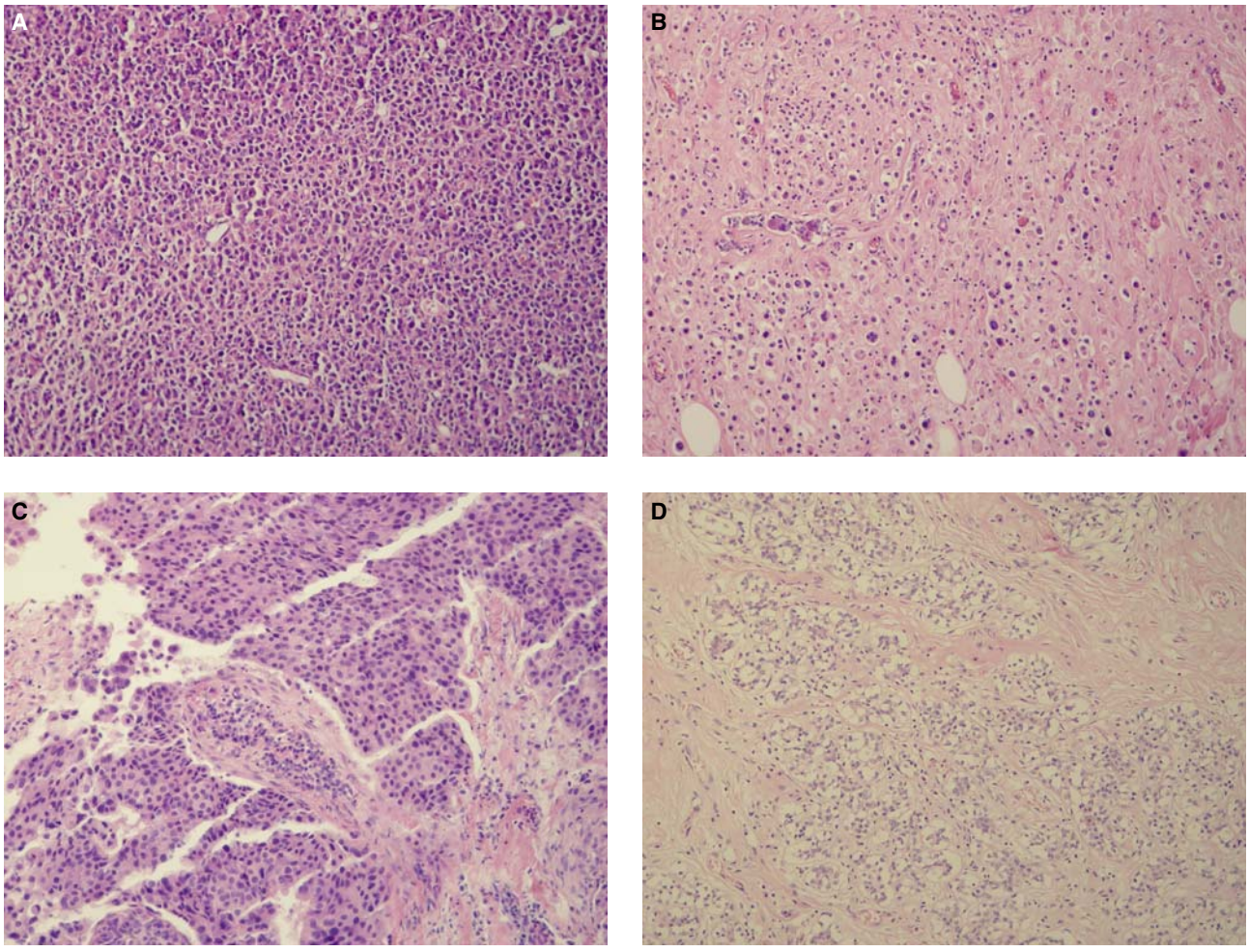

Fig. 1. Histomorphology. Case No. 1: Infiltrative lobular carcinoma before treatment – core needle biopsy (a) and with regressive changes after treatment (b). Case No. 4: Infiltrative ductal carcinoma before treatment – core needle biopsy (c) and after treatment (d). HE, magnification ×100.

We focused on two patients with significant *Her2/neu* alteration. Patient No. 1 had infiltrating lobular carcinoma, in post-treatment surgery specimen with regressive changes (Fig. 1a,b) and tumour stage decreased from IIA to I. Patient No. 4 had multiloculated infiltrating ductal carcinoma, and in post-treatment surgery, the specimen was partially necrotic and with regressive changes (Fig. 1c,d). The tumour stage decreased from IIB to I.

Immunohistochemistry

Initially, we determined the Her2 status of 20 neoadjuvantly treated patients. We found that in 12 of the 20 (60 %) patients the Her2 status remained unchanged after neoadjuvant treatment. In six cases (30 %) minor changes were observed after the treatment. However, in two cases (10 % of the whole set of patients and 25 % of Her2-positive cases; cases Nos. 1 and 4) we found Her2 expression alterations from strongly positive to negative after the treatment (Table 1).

In both pre-treatment biopsies there was a strong membrane staining in more than 10 % of tumour cells, and the finding was scored as 3+ (strongly positive for Her2/neu over-expression) (Fig. 2a,c). In the post-treatment specimen only a barely perceptible partial membrane staining in less than 10 % of tumour cells was found in case No. 1 (Fig. 2b), and no staining in case No. 4 (Fig. 2d). The finding was scored as 1+ and 0, respectively (negative for Her2 over-expression).

We also determined the ER and PR receptor status on the treatment samples both before and after the treatment (see Table 1). In four patients (20 %), the originally negative tumour cells were found to express ER and PR weakly. In other two patients (10 %), a change from strongly positive to negative was observed. We also found minor changes in the expression of ER and/or PR in five (25 %) other cases. When we compared the two patients with significant Her2 expression alteration (Nos. 1 and 4) regarding the receptor status, we found decreased expression of ER and unchanging very low expression of PR in patient No. 1 and contrariwise slight increase of both in patient No. 4 (see Table 1).

However, a change in the expression of hormone receptors is a more common event and therefore we then concentrated on the change of *Her2/neu* amplification/over-expression by using FISH and quantitative reverse qRT-PCR, respectively.

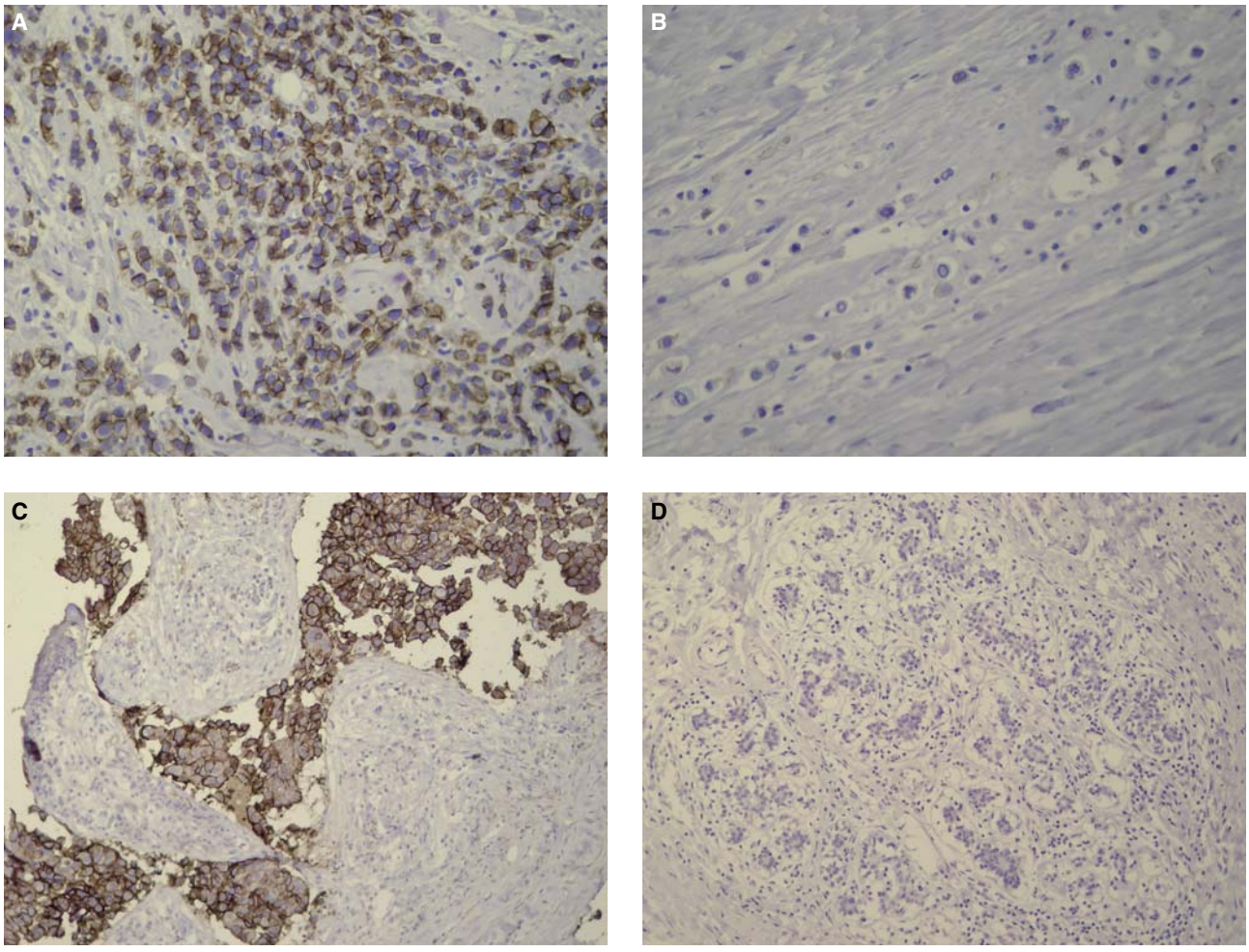

Fig. 2. Immunohistochemical staining for Her2. Strong membrane positivity before treatment in both patients (a – case No. 1, c – case No. 4). Faint partial membrane positivity after treatment (b – case No. 1). Absolute negative staining after treatment (d – case No. 4). Magnification ×200.

Fluorescence in situ hybridization

In order to verify correct results of immunohistochemistry in cases No. 1 and No. 4, we analysed the amplification of *Her2/neu* at the DNA level.

The *Her2/neu* probe kit consists of a mixture of two DNA probes: the first labelled in SpectrumOrange spans the whole *Her2* gene, and the second one labelled in SpectrumGreen hybridizes to the α satellite DNA located at the centromere of chromosome 17 (17p11.1-q11.1), which allows determination of the relative copy number of the *Her2/neu* gene.

Concerning the results of hybridization in the pre-treatment sample of case No. 1, the average number of copies of CEP17 was 2.15 and the average number of copies of the *Her2/neu* gene was 7.75. The ratio of Her2/CEP17 was 3.6, which we interpreted as strong amplification of the *Her2/neu* gene (Fig. 3a). By contrast, in the same case after the treatment (Fig. 3b), the average number of copies of CEP17 was 2.25 and the average number of copies of the *Her2/neu* gene was 3.3. The ratio of Her2/CEP17 was 1.5, which meant that amplification was not observed.

In case No. 4 before the treatment, the average number of copies of CEP17 was 2.3 and the average number of copies of the *Her2/neu* gene was 14.6. The ratio of Her2/CEP17 was 6.4, which means that strong amplification of the *Her2/neu* gene was observed (Fig. 3c). In the post-treatment sample (Fig. 3d), the average number of copies of CEP17 was 2.2 and the average number of copies of the *Her2/neu* gene was 2.9. The ratio of Her2/CEP17 was 1.3, after which we concluded that amplification was not found.

Quantitative RT-PCR

Finally, we analysed the over-expression of Her2/neu at the RNA level by real-time PCR. In case No. 1 (Fig. 4a), we found $(6.04 \pm 0.34) \times 10^5$ copies of Her2, $(3.09 \pm 0.23) \times 10^4$ copies of RPL23 and the ratio of Her2/RPL23 was 19.6 ± 2.5 in the sample before the treatment. However, we found $(1.88 \pm 0.26) \times 10^4$ copies of Her2, $(8.66 \pm 0.82) \times 10^4$ copies of RPL23 and the ratio

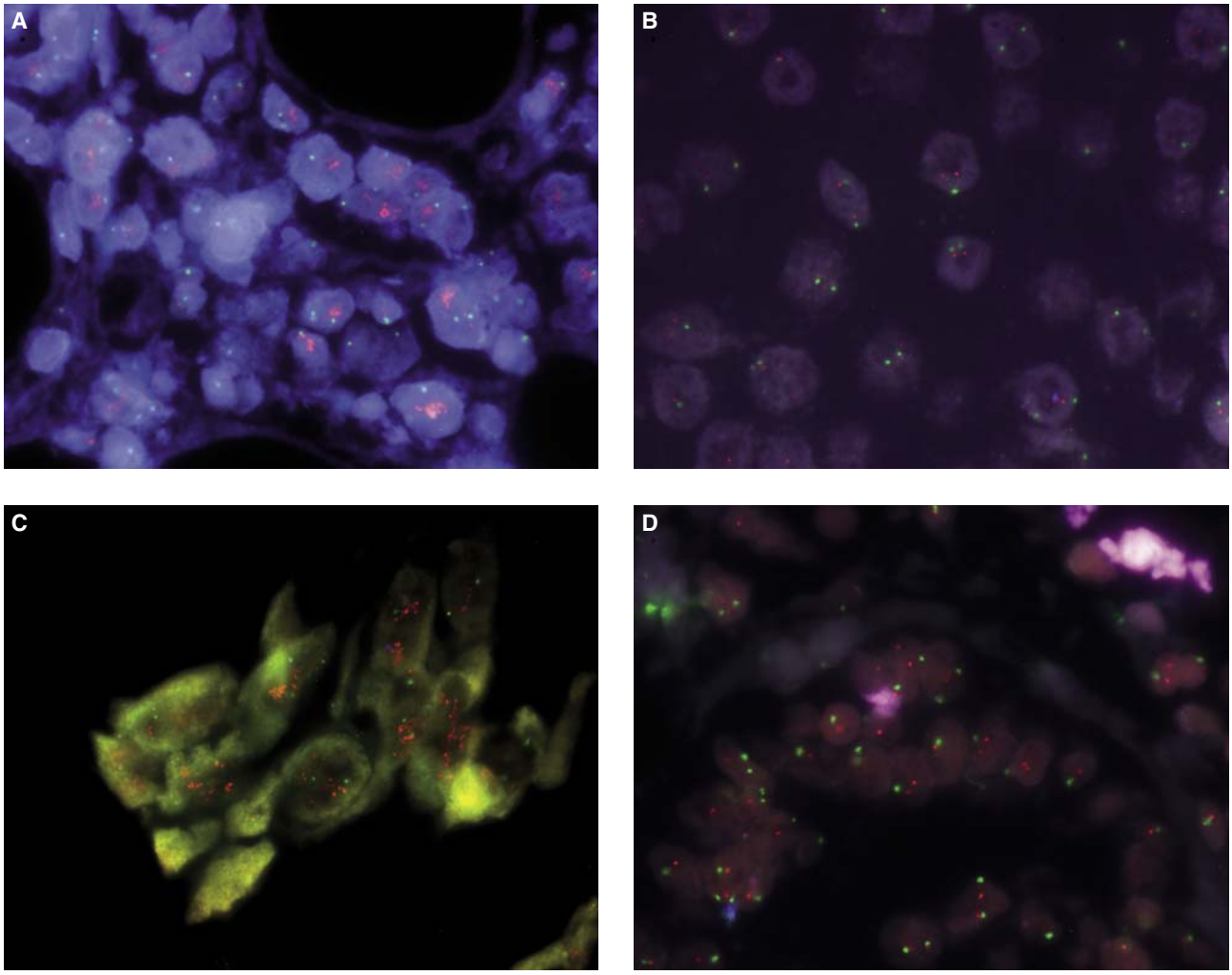

Fig. 3. Fluorescent *in situ* hybridization. Two-colour fluorescent *in situ* hybridization analysis using probe specific for the *Her2/neu* gene (red signals) and for the centromere of chromosome 17 (green signals). The analysis was performed in all samples before (a – case No. 1, c – case No. 4) and after (b – case No. 1, d – case No. 4) the treatment. The hybridization signals were visualized in a fluorescence microscope using appropriate filters. Magnification ×1000.

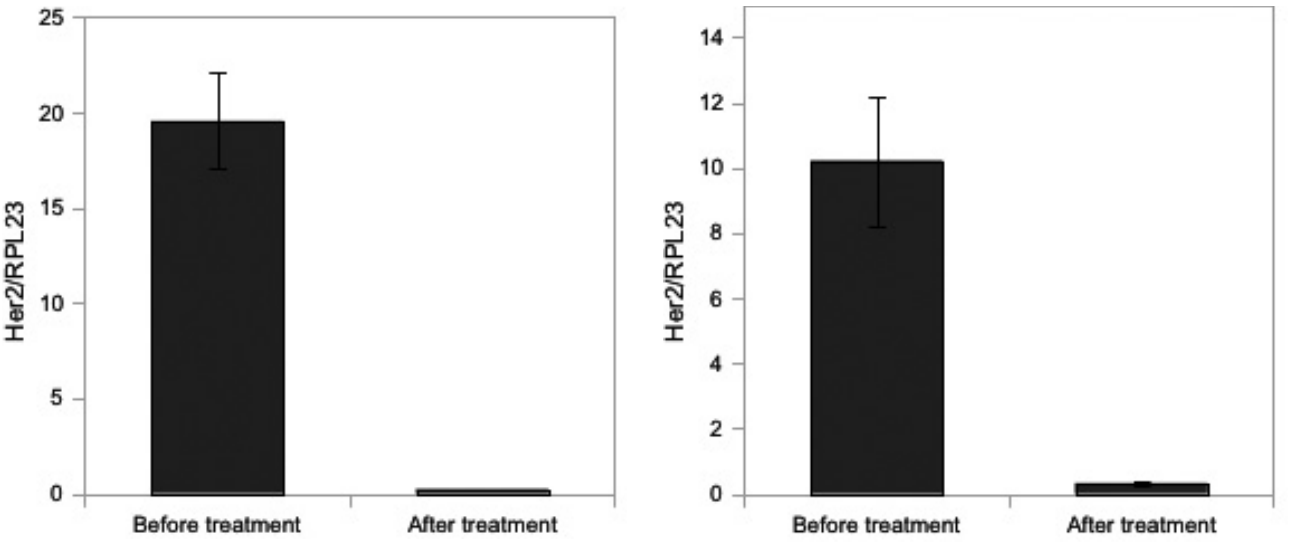

Fig. 4. The expression of Her2/neu mRNA. The evaluation of the *Her2/neu* transcript expression was performed by quantitative RT-PCR analysis. The analysis was performed in both cases (a – case No. 1, b – case No. 4). The level of *Her2/neu* expression was given as normalized ratio Her2/RPL23. ($P < 0.05$, two-sided Student's *t*-test).

of Her2/RPL23 was 0.22 ± 0.01 in the sample after the treatment ($P < 0.05$ for before vs. after the treatment, 2-sided Student's *t*-test).

In case No. 4 (Fig. 4b), we found $(6.49 \pm 0.99) \times 10^1$ copies of Her2, 6.35 ± 0.26 copies of RPL23 and the ratio of Her2/RPL23 was 10.2 ± 1.9 in the pre-treatment biopsy. However, we found 2.57 ± 0.55 copies of Her2, 8.03 ± 0.17 copies of RPL23 and the ratio of Her2/RPL23 was 0.32 ± 0.06 in the post-treatment surgery specimen ($P < 0.05$ for before vs. after the treatment, 2-sided Student's *t*-test).

In other words, we found over-expression of the *Her2/neu* gene at the RNA level in both pre-treatment biopsies, while in both post-treatment samples we found down-regulation of *Her2/neu* transcripts.

Discussion

With the development of tailored therapies targeting specific molecules, Her2, ER, and some other molecular markers have become important predictive factors. For instance, Her2 positivity predicts a response to trastuzumab, the oestrogen receptor positivity predicts a response to hormonal therapy, the presence of c-kit mutation predicts a response to imatinib, and the presence of the epidermal growth factor receptor mutation predicts a response to gefitinib (Van de Vijver, 2005).

Her2 protein over-expression is also indicative of a more aggressive tumour phenotype, increased number of lymph node metastases, shorter time to treatment failure, and a shorter overall survival time (Ro et al., 1989; Slamon et al., 1989; Paterson et al., 1991; Press et al., 1997; Andrulis et al., 1998). Moreover, through unknown mechanisms, it is correlated with the grade and the type of breast cancer and is associated with a poor prognosis (Hoff et al., 2002).

Neoadjuvant chemotherapy substantially reduces the size of the primary tumour and lymph node metastasis in more than 80 % of cases, and increases the probability that breast-conserving surgery can be performed instead of mastectomy. However, surgery is the main treatment method for patients with early breast cancer, and surgery alone or together with radiotherapy may control local disease in most patients. In spite of these treatments, subclinical tumour cells that remain following surgery are responsible for the progression of the disease at a later date. Adjuvant hormonal or cytotoxic treatment is widely used with the goal of eradicating these remaining tumour cells, in order to reduce the subsequent risk of relapse and death (Early Breast Cancer Trialists' Collaborative Group, 1998).

As we have shown in cases No. 1 and No. 4, the expression of Her2 was markedly changed after neoadjuvant chemotherapy from strongly positive to negative. This situation is infrequent but not unknown as most of the published studies that involved stability of Her2/neu described some cases of alteration as well. Overall these changes were usually not statistically significant (Adams et al., 2008), despite that they definitely occur.

The causes for these discrepancies are still unclear. We used three methods (IHC, FISH, qRT-PCR) to eliminate possible sampling error or insufficient fixation of post-treatment specimens, which could lead to the consecutive loss of epitopes and decreased sensitivity for IHC.

The very presumable explanation lies in mono- versus oligoclonality of the tumours. We, like other authors of similar study (Adams et al., 2008), assume a co-existence of at least two clones of tumour cells. One that over-expresses *Her2/neu* and hence is more sensitive to neoadjuvant therapy, including anti-Her2 antibody, and another without over-expression and thus with lower sensitivity. The result of this theory is that the number of

cells with *Her2/neu* over-expression decreases more rapidly than the clone without over-expression, which afterwards becomes more prevalent in the surgery specimen through clonal selective advantage.

It has already been described that many breast carcinomas are karyotypically complex, often with massive structural chromosomal rearrangements (Saint-Ruf et al., 1990, 1991; Pandis et al., 1995a, b). Interestingly, some groups of authors have demonstrated that cytogenetic polyclonality can be found in nearly half of all breast carcinomas that are short-term *in vitro*-cultured and analysed by G-banding and comparative genomic hybridization (CGH) (Teixeira et al., 1994, 2001; Pandis et al., 1995b). Moreover, comparisons between breast carcinomas and their lymph node metastasis have shown that polyclonality may exist in both the primary and secondary tumours (Pandis et al., 1994, 1998). All of these findings suggest that the oligoclonality of breast cancer is not an artefact.

However, in routine practice, it is not common to determine the *Her2/neu* status in breast cancer in samples both before and after the treatment, and in addition, by using two independent methods to minimize possible sampling errors and to distinguish over-expression/amplification. In the light of our results, we concluded that the patients neoadjuvantly treated with anti-Her2 antibody should be retested for the *Her2/neu* status if adjuvant, and that anti-Her2 treatment be considered because the prerequisite for efficiency of targeted therapy is the presence of a target structure at the molecular level. This dual testing would avoid the use of targeted biological therapy in an untargeted manner.

The most common methods for testing the *Her2/neu* status are immunohistochemistry for the detection of gene expression at the protein level, and FISH for the detection of gene amplification at the DNA level. Immunohistochemical evaluation can be affected by variations among antibodies, fixatives, and subjective interpretation. The FISH technique allows the analysis of individual cells, and it can detect whether amplification is the result of chromosome duplication or of gene amplification. However, FISH is expensive, time-consuming and requires several hours for hybridization (often overnight) and considerable time to count amplification in individual cells. Moreover, FISH cannot identify cases in which the gene product is over-expressed in the absence of gene amplification.

Quantitative PCR techniques allow the detection of *Her2/neu* at both the DNA and RNA levels, and may provide alternatives to these methods (Biéche et al., 1999; Lyon et al., 2001). In the future, PCR methods are likely to become more widely used for the same purpose because they are more sensitive, faster, easy to perform, and allow screening multiple samples at the same time. On the other hand, the use of formalin-fixed paraffin-embedded tissue for this purpose at the RNA level may be problematic because the RNA often becomes degraded in this material (Riehle et al., 2010).

In conclusion, in the recent wide discussion and published articles about specific molecules as potential markers of breast cancer behaviour and targets for specific therapy that we know of, this is the first report describing the changes in the *Her2/neu* status at all protein, RNA and DNA levels by using IHC, qRT-PCR and FISH, respectively. As we have shown, quantitative PCR is a valuable tool for the evaluation of *Her2/neu* gene over-expression and correlates well with the results of IHC and FISH, respectively. Moreover, the results obtained by quantitative PCR are not encumbered with any subjective error on the part of the evaluator.

There needs to be more studies encompassing more patients with breast carcinoma to reveal the causes of these differences in expression of the *Her2/neu* gene after the treatment, and to facilitate their possible application in advancement of neoadjuvant therapy. However, repeated testing of the *Her2/neu* status can prevent clinicians from using tailored targeted therapy in an untargeted manner.

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