

ORIGINAL ARTICLE

TOP2A and HER2 gene amplification as predictors of response to anthracycline treatment in breast cancer

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Abstract

The purpose of this study was to evaluate amplification of topoisomerase II α (TOP2A) and HER2 genes as predictors of response to chemotherapy in advanced breast cancer. Gene copy number of TOP2A and HER2 were analysed with chromogenic in situ hybridization (CISH) on paraffin-embedded tissue sections from the primary tumour of 85 patients treated with anthracycline containing chemotherapy. TOP2A gene amplification was present in 14 (16%) and HER2 gene amplification in 38 (45%) of the primary tumours. Two of the 14 cases with TOP2A amplification were amplified without concurrent HER2 amplification. Neither TOP2A nor HER2 gene amplification were significantly associated with response to chemotherapy ($p=0.35$ and $p=0.49$, respectively).

The objective response rate achievable with chemotherapy in advanced breast cancer is about 40–60% [1]. The most active chemotherapeutic agents are anthracyclines and taxanes [1,2]. Very few patients, probably less than 5%, are long-time survivors even after achieving complete remission [3]. The limited efficacy of chemotherapy is due to intrinsic or acquired resistance to the drugs in use. Despite decades of research on tumour biology there is today no single factor explaining the enormous difference in chemotherapy sensitivity between different tumours. The value of a factor that could reliably predict efficacy of chemotherapy in both the metastatic and adjuvant setting is obvious.

The nuclear enzyme topoisomerase II α (TOP2A) and the proto-oncogene c-erbB-2 (HER2) are two factors that have shown predictive potential with regard to anthracycline-based chemotherapy in breast cancer.

Anthracyclines belong to the anti-cancer agents called topoisomerase II inhibitors. Topoisomerases

are enzymes regulating topological changes in DNA that are vital for many cellular processes such as replication and transcription. They perform their function by introducing transient protein-bridged DNA breaks on one (topoisomerase I) or both DNA strands (topoisomerase II) [4]. There are two isoenzymes of topoisomerase II, with genetically and biochemical distinct features. TOP2A with a molecular weight of 170 kd is located on chromosome 17, and topoisomerase II- β (TOP2B) with a molecular weight of 180 kd is located on chromosome 3. TOP2A is the primary drug target for anthracyclines [4].

Topoisomerase II inhibitors inhibit the rejoining step in the breakage-rejoining cycle of TOP2A and thereby shift the equilibrium toward a key covalent reaction intermediate termed the cleavable complex [5]. This leads to double-stranded DNA breaks, which can by a partly unknown mechanism lead to cell death. In vitro studies on cell lines from different human malignancies has shown that sensitivity to

topoisomerase II inhibitors is dependent on the cellular level of TOP2A [6].

HER2 is located closely to TOP2A on chromosome 17 and encodes a transmembrane tyrosine kinase growth factor receptor [7]. HER2 gene amplification or protein over-expression is present in 10–34% of breast cancers and is associated with poor clinical outcome [8]. While HER2 is an established prognostic factor its role as predictive factor for response to chemotherapy is still a controversial issue.

Fluorescence in situ hybridization (FISH) is currently considered the most accurate method for detection of oncogene amplification in human tumours [9]. Because FISH has its technical disadvantages, a new modification of FISH, chromogenic in situ hybridization (CISH), has been developed [10]. In CISH the probe detection is based on the use of a peroxidase reaction, the end product of which is visible in conventional light microscopy. A good concordance between CISH and FISH in the detection of oncogene amplification in archival breast cancer tissue has been shown [10–12].

The aim of the present study was to evaluate TOP2A and HER2 gene amplification analysed with CISH as predictive markers of response to anthracycline-based chemotherapy in advanced breast cancer.

Material and methods

Patients and tumour material

The patient population of the present study consists of a subgroup of 283 patients who took part in a randomised multicenter trial comparing docetaxel (T) to sequential methotrexate and 5-fluorouracil (MF) in advanced breast cancer [2]. To enter the randomised trial, patients were required to have histologically proved primary breast cancer that had progressed during or after first line anthracycline treatment for advanced disease or relapsed within 12 months after discontinuation of adjuvant anthracycline therapy. Only patients receiving anthracycline as first line treatment for advanced disease having measurable or evaluable metastatic lesions were included in the present study. Response evaluation was performed according to WHO recommendations. Seven different centres participated in the actual study and paraffin-embedded blocks for 85 patients were available. As this study is not directly connected to the randomised study comparing T to MF the patient characteristics has not been compared with the patients in the latter study. Of the 85 patients 17 were treated with anthracycline monotherapy and 68 with anthracycline containing

polychemotherapy, 13 according to a weekly schedule and 72 every three weeks. The main tumour and chemotherapy characteristics of the 85 analysed patients are shown in Table I.

Preparation of Tissue arrays

Tumour tissue array blocks were made as follows: three tissue cylinders with a diameter of 0.6 mm was punched through selected tumour areas from each “donor” tissue block and inserted into “recipient” tissue array paraffin blocks using a specific custom-made instrument as described by Kononen et al. [13]. Sections of the recipient block were cut at 5 µm, collected on SuperFrost Plus glass slides, and

Table I. Primary tumour and chemotherapy characteristics of the 85 investigated patients.

Factor	No	%
Age		
Median (range)	50 (29–69) years	
Histologic type		
Ductal	79	93
Lobular	5	6
Medullary	1	1
Estrogen receptor status		
Positive	43	51
Negative	36	42
Unknown	6	7
MIB-1		
Low (<25%)	38	45
High (≥25%)	37	43
Unknown	10	12
HER2 CISH		
No amplification	47	55
Amplification	38	45
TOP2A CISH		
No amplification	71	84
Amplification	14	16
Adjuvant chemotherapy ^a		
FEC	1	1
CMF	31	37
None	53	62
Weekly anthracycline treatment		
Yes	13	15
No	72	85
Anthracycline treatments ^a		
FEC	52	61
FAC	8	9
EV	5	6
Dosescaled FEC	3	4
E	4	5
E weekly	1	1
A weekly	12	14
Response to treatment ^b		
CR+PR	39	46
NC+PD	46	54

^aF (5-fluorouracil), E (epirubicin), C (cyclophosphamide), A (adriamycin), V (vinorelbine), M (methotrexate).

^bCR, complete response; PR, partial response; NC, no change; PD, progressive disease.

baked in a 60°C oven for 2 to 4 h before starting the CISH procedures.

CISH

The CISH was carried out according to Rummukainen et al. [14]. Before hybridization, the tissue sections were deparaffinized in xylene, washed in 100% ethanol, and air-dried. Slides were then incubated in 0.1 M Tris-saline (pH 7.3) at 121°C in an autoclave for 2 min. After cooling at room temperature for 15 min and rinsing twice in PBS for 3 min, the tissue sections were covered with 100 µl of pepsin solution (Digest-All 3; Zymed, South San Francisco, California) at 37°C for 6 to 8 min. The slides were then washed in PBS three times at room temperature for 2 min, dehydrated in graded ethanols, and air-dried. The ready-to-use digoxigenin-labeled HER2 probe (Zymed) was applied to the tissue sections. Slides were coverslipped and sealed with rubber cement to prevent evaporation of the probe solution. Slides and the probe mixture were codenatured at 94°C for 3 min on a thermal plate, and hybridization was carried out in a humid chamber at 37°C for 36 to 40 h (over two nights). After hybridization, the slides were washed with $0.5 \times$ SSC for 5 min in 75°C, followed by three washes in PBS for 2 min at room temperature. The HER2 probe was detected with sequential incubations with mouse anti-digoxigenin (Roche Biochemicals; diluted 1:300 in PowerVision Blocking solution), goat anti-mouse HRP polymer (PowerVision; ImmunoVision Technologies, Daly City, California), and DAB (ImmunoVision Technologies) as chromogens. Incubation in DAB Enhancer (Zymed) for 3 min was used to further enhance the signal intensity. After light counterstaining with hematoxylin, the slides were dehydrated and embedded. CISH with TOP2A probe (Zymed) was performed using the same method. CISH hybridizations were analysed using a Nikon Labophot transmitted light microscope with a 40 $\times$ objective. At least 50 non-overlapping nuclei in every tumour sample were scored to determine the number of HER2 and TOP2A signals. The sample, of the three adjacent samples from the same tumour, with the most representative histology and technically most successful hybridization was analysed. The results were expressed as the actual copy numbers per cell in each sample. Amplification was defined to be present when six or more gene copies were detected in at least 20% of the screened malignant cells or the presence of a gene copy cluster. In a previous study analysing HER2 amplification in 197 breast cancer patients this cutpoint was used to compare CISH with the Food and Drug Administration

(FDA)-approved FISH test (Vysis PathVysion) and showed very good agreement (kappa coefficient 0.875) [12].

Statistical Methods

The association between response rate and gene amplification for HER2 and TOP2A was tested by χ^2 test with clinical response divided into two categories: response (complete response (CR)+partial response (PR)), and non-response (no change (NC)+progressive disease (PD)). TOP2A gene amplification and variables that were statistically significant associated with response in the univariate analysis were further evaluated in a multivariate analysis using a logistic regression model.

Time to progression (TTP) was measured from the date of the first course of chemotherapy until disease progression and overall survival (OS) from start of chemotherapy till death. The univariate analysis of TTP and OS were done with the Cox proportional hazards model.

Results

The subset of patients investigated in the present study consisted of 85 patients with a median age of 50 years (range 29–69 years) and a median disease free interval of 26 months (range 0–286 months). With 51% of patients having estrogen receptor positive tumours and with 37% treated with adjuvant cyclophosphamide methotrexate and 5-fluorouracil (CMF) we regard the patient population representative for patients treated with first line anthracycline based chemotherapy for advanced breast cancer in the 1990s.

Among the 85 patients, all having measurable or evaluable metastatic lesions and a successful CISH, there were 8 CRs (9%), 31 PRs (36%), 18 patients with NC (21%) and 28 patients with PD (33%). The number of excluded patients and the success rate of CISH are illustrated with the patients from Helsinki, the centre with the majority of the patients. Of 77 patients included in the randomised phase III trial paraffin embedded blocks with tumour tissue left were available for 68 patients and CISH was successful in 66 cases (97%), which is in accordance with a larger study with identical method for CISH analysis [12]. TOP2A gene amplification was present in 14 (16%) and HER2 gene amplification in 38 (45%) of the primary tumours. Two of the 14 cases with TOP2A amplification were amplified without concurrent HER2 amplification. Neither TOP2A nor HER2 gene amplification was significantly associated with response to chemotherapy ($p=0.35$ and $p=0.49$, respectively). Only one of

eight patients showing CR had TOP2A gene amplification. The response rate was significantly higher in patients treated every three weeks than those treated weekly (51% vs. 15%, $p=0.02$). In the multivariate analysis weekly treatment remained significant, risk ratio (RR) 7.29 (95% CI 1.39–38.17), while TOP2A amplification was associated with a non-significant increase in response rate, RR 2.52 (95% CI 0.68–9.31).

TTP was similar irrespective of TOP2A status ($p=0.73$) while tumours with TOP2A amplification showed a trend towards worse OS ($p=0.09$). TTP and OS were similar irrespective of HER2 gene amplification ($p=0.25$ and $p=0.11$). TTP was significantly worse for patients treated weekly than for those treated every three weeks ($p=0.01$), and also for patients treated with anthracycline monotherapy compared to those treated with an anthracycline containing combination regimen ($p=0.03$).

Among patients with HER2 gene amplification response rate, TTP and OS did not differ between those with simultaneous amplification of TOP2A and those with normal TOP2A status ($p=0.49$, $p=0.13$ and $p=0.66$, respectively). Treatment response according to different combinations of TOP2A and HER2 gene amplification are illustrated in Table II.

Discussion

Today there is no established factor for clinical use in prediction of chemotherapy efficacy in breast cancer. HER2 and TOP2A are two factors that have shown predictive potential in some previous studies [15–18].

HER2 has been extensively studied for the last years and in the vast majority of these studies overexpression of HER2 has been determined by immunohistochemistry. In the adjuvant setting patients with tumours over expressing HER2 have been shown to benefit from addition of doxorubicin to L-phenylalanine mustard and 5-FU [15], and from dose-escalation of anthracycline based chemotherapy [16]. No association between treatment re-

sponse and HER2 expression was seen either in patients treated with neoadjuvant 5-FU/doxorubicin/cyclophosphamide [19] or in patients with metastatic breast cancer treated with 5-FU/epirubicin/cyclophosphamid [20]. The conflicting results observed with regard to the role of HER2 as predictive marker seen in these studies using immunohistochemistry may be due to differences in antibodies used and scoring systems resulting in very different proportions of tumours classified as HER2 over expressive. In the present study HER2 and TOP2A status were analysed with CISH, a recently introduced technique to evaluate gene amplification that has shown high concordance with FISH [10–12], currently considered as the gold standard, but with several practical and economical disadvantages. We found HER2 gene amplification in 45% of tumours but no significant association between gene amplification and response to chemotherapy, TTP or OS. We regard this as an expected finding as there is no obvious molecular rationale for a relationship between HER2 status and anthracycline efficacy. In a study by Pegram et al. it has been shown that there is no difference in chemosensitivity in vitro or in vivo between HER2-negative and transfected breast cancer cells and breast cancer xenografts [21]. The patients in the present study were treated with either docetaxel (T) or sequential methotrexate and 5-FU (MF) after progressing during or after first-line anthracycline treatment for advanced disease. Immunohistochemical determination of HER2 expression could not predict response to either T or MF [22].

As the primary drug target for anthracyclines TOP2A is a potential predictive factor for anthracycline efficacy in breast cancer. In vitro studies on breast cancer cell lines have shown that TOP2A gene amplification is associated with increased TOP2A protein expression and increased sensitivity to the TOP2A inhibitor doxorubicin [23]. Whilst immunohistochemical analysis of TOP2A has failed to predict response to epirubicin in metastatic breast cancer [24], a pilot study of 59 patients with locally advanced or metastatic breast cancer indicate that concomitant use of TOP2A gene amplification and protein over expression seem to correlate with response to anthracycline [25]. Two studies on locally advanced breast cancer has shown coamplification of HER2 and TOP2A to be associated with increased response to neoadjuvant anthracycline containing chemotherapy [17,26]. In the first study of 35 patients gene amplification was evaluated with FISH [26], and in the second study of 67 patients with CISH [17]. In 119 patients with operable breast cancer treated with neoadjuvant anthracycline chemotherapy HER2 and TOP2A gene amplification

Table II. Treatment response according to different combinations of TOP2A and HER2 gene amplification.

Gene amplification	Number	CR+PR (%)	NC+PD (%)
All patients	85	39 (46)	46 (54)
HER2 Am/TOP2A Am	12	7 (58)	5 (41)
HER2 Am/TOP2A U	26	12 (46)	14 (54)
HER2 U/TOP2A Am	2	1 (50)	1 (50)
HER2 U/TOP2A U	45	19 (42)	26 (58)

CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

Am, amplified; U, unamplified.

evaluated by real-time polymerase chain reaction (PCR) were not predictive of response [27]. The experience from gene amplification of TOP2A as predictor or response to anthracycline in advanced breast cancer is thus still limited and the results contradictory. This might partly be explained by different methods for evaluation or by different cut-off points for amplification. In the study by Park *et al.* [17] amplification was determined as more than four gene copies or the presence of a gene copy cluster. In this study we used six or more gene copies or the presence of a gene copy cluster based on experience from previous studies [10,12,28]. In the adjuvant setting information is available from one large study comparing anthracycline-based therapy with cyclophosphamide, methotrexate and 5-fluorouracil (CMF) [18]. In this study only those patients with HER2 amplification and simultaneous TOP2A amplification seemed to benefit from the anthracycline therapy, however the number of patients with TOP2A amplification was only 23 and the estimated prevalence of TOP2A amplification in node-positive breast cancer patients was only 6%. A meta-analysis of adjuvant studies comparing anthracycline-based chemotherapy with CMF is ongoing and will hopefully define the predictive value of HER2 and TOP2A in this clinical situation.

The major advantage to study predictive factors in patients with metastatic or ideally locally advanced disease over adjuvant treatment is the possibility to objectively and reliably monitor the clinical response rather than to measure prolongation of disease-free or overall survival. In the present study TOP2A gene amplification was present in 16% of the primary tumours. This frequency is higher than the 6% found in patients with stage II disease [18], but is comparable with the proportions of 17% [26] and 28% [17] found in patients with locally advanced disease. A higher frequency of TOP2A gene amplification in patients with more advanced disease is expected as TOP2A expression is related to HER2-positivity and worse prognosis in primary breast cancer [29]. No association between TOP2A gene amplification and response to chemotherapy, TTP or OS was found. The lack of correlation may partly be due to the limited number of patients treated, and the fact that 13 patients (15%) were treated according to a weekly schedule with low response rate.

In the present study HER2 and TOP2A were evaluated on primary tumour samples. Regarding HER2 a newly published review concluded that HER2 is generally over-expressed to the same extent in both lymph node and distant metastasis as in the corresponding primary tumour [30]. The data regarding TOP2A is more limited and based on few studies, but the level of concordance between

the primary tumour and the corresponding metastasis has been good [28,31]. In the study by Tanner *et al.* [28], TOP2A amplification and deletion were identical in ten of 13 paired tumours studied, however in the remaining three cases the predominant cell population in metastatic tissue was present only as a subpopulation in the primary tumour. The same degree of discordance in the present study could have influenced the results as the number of patients evaluated is limited.

HER2 and TOP2A genes are located close to each other in chromosome 17q12-21, and previous reports have indicated that TOP2A gene aberrations, deletion or amplification, is only seen in connection with HER2 amplification [26,32]. However, in the present study we had two tumours with TOP2A gene amplification without HER2 amplification, a finding supported in a study by Olsen *et al.* [33] in which they found TOP2A gene alterations, amplification in one and deletion in three, in 90 tumours with normal HER2 status.

Whereas *in vitro* studies has shown TOP2A amplification to be associated with increased sensitivity to anthracycline, TOP2A deletion, in turn, induces resistance [23]. These observations are supported by a previous study in patients with metastatic breast cancer treated with anthracycline, where the response rate in patients with simultaneous amplification of HER2 and TOP2A was 79%, whereas in patients with HER2 amplification and TOP2A deletion it was only 17% [34]. Another factor that may have implications for the predictive value of TOP2A is the intratumoural heterogeneity at the TOP2A locus with both TOP2A amplified and deleted breast cancer cells adjacent to each other in the same tumour [32,35]. The presence of different cell populations with different chemosensitivity may explain both the variable clinical responses seen in otherwise similar patient populations, and the gradual loss of efficacy and development of chemoresistance. The significance of TOP2A gene deletion could not be analysed in the present study as simultaneous analyses of TOP2A and chromosome 17 centromere copy numbers is not possible with CISH. This is a disadvantage of CISH as compared to FISH.

Conclusion

In conclusion, HER2 or TOP2A gene amplifications were not predictive markers of response to first-line anthracycline treatment in advanced breast cancer in the present study. Still there was a trend for increased response rate in TOP2A amplified tumours and a predictive value can not be ruled out, as the number of patients are relatively few and the anthracycline treatment rather heterogeneous. Due

to tumour heterogeneity and the fact that most patients are treated with a combination of several drugs it is not likely that a single factor can be used to predict treatment efficacy in a group of patients. Probably a combination of several markers will be necessary and much of the current interest in this field is now focused on gene microarrays and proteomics [36].

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