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DOI: https://doi.org/10.1007/s10549-011-1873-8

Breast Cancer Res Treat (2012) 133:929–935
DOI 10.1007/s10549-011-1873-8

PRECLINICAL STUDY

Topoisomerase 2A gene amplification in breast cancer. Critical evaluation of different FISH probes

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Received: 15 September 2011 / Accepted: 1 November 2011 / Published online: 15 November 2011

Abstract The *HER2* amplicon on chromosome 17q is variable in size and occasionally includes *Topoisomerase 2A* (*TOP2A*) at 17q21-22. It has been suggested that *TOP2* co-amplification, not *HER2* amplification on chromosome 17q11.2-12, is a useful predictive marker of response to anthracycline-based chemotherapy in breast cancer patients. Given the significant toxicities of anthracyclines, the detection methods of *TOP2A* gene amplifications have to be standardized. We determined *TOP2A* gene alterations using two different fluorescence in situ hybridization (FISH) DNA probes. HER2 amplifications were identified with the PathVysion™ probe. *TOP2A* status of 42 HER2 amplified breast cancers was tested by FISH with PathVysion™ covering 160 kb and DAKO pharm DX™ covering 228 kb of the TOP2A amplicon. TOP2A protein expression was tested by immunohistochemistry. Multiplex-ligation dependent probe amplification (MLPA) was performed retrospectively in cases showing discrepancies. *TOP2A* was amplified in 15 of 42 cases (35%) with DAKO pharm DX™ and in 11 of 42 cases (26%) with PathVysion™. In all four discrepant cases, MLPA showed no TOP2A amplification, but instead amplification of an upstream region including HER2. *TOP2A* was deleted in the same seven of 42 carcinomas (17%) with both probes. TOP2A protein expression was detected in all 42 tumours (100%) with high intratumoral heterogeneity. *TOP2A* amplification rate depends on the length of the hybridized probes for the *TOP2A* locus. Because *TOP2A*, not *HER2*, is a target of anthracyclines, non-overlapping DNA probes should be used to evaluate any associations between such alterations and response to anthracycline-based chemotherapy.

Keywords Topoisomerase 2A · Breast cancer · FISH · Different probes

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Introduction

Currently, anthracyclines represent the main adjuvant treatment of early stage breast cancer. Not all patients benefit from such treatment and these agents have significant toxicities. Evaluation of the *TOP2A* gene in *HER2* positive breast cancer has been recently addressed as a predictor of the response to anthracycline-based chemotherapy [9, 20, 21]. HER2 positive patients with simultaneously amplified *TOP2A* gene were associated with longer survival and increased response to anthracycline-based chemotherapy than HER2 positive patients without *TOP2A* amplification [9, 14, 18, 21, 23]. HER2 expression alone does not alter anthracycline sensitivity. Furthermore, *HER2* and *TOP2A* co-amplification predicts improved overall and disease free survival more reliably in breast cancer patients [1, 14]. Conversely, lack of *TOP2A* gene amplification (i.e. a normal gene status or a deletion of the *TOP2A*) has been associated with anthracycline resistance [1, 21, 23].

HER2 is located on chromosome 17q11.2-12. In vivo and in vitro studies have shown that HER2 overexpression alone does not alter anthracycline sensitivity [11]. *TOP2A* at 17q21-22 is in close proximity to *HER2*, resulting in a proportion of *HER2* amplified breast cancer cases also

containing co-amplification of *TOP2A* [12, 13, 16]. Because *TOP2A* is a target of anthracyclines, it is possible that this gene, not *HER2*, is responsible for anthracycline responsiveness [21].

Larger studies have shown that 33–60% *of HER2* positive cancers contain concurrent *TOP2A* amplifications, while 20–42% are *TOP2A* deleted. According to some studies, TOP2A alterations are extremely rare in the absence of *HER2* amplification, while others report a 10–20% *TOP2*A amplification or deletion rate in *HER2* negative breast cancer [6, 10, 20, 21].

This wide variability may have different causes and is likely related to the fluorescence in situ hybridization (FISH) technology. Fluorescence or chromogenic labelled in situ *hybridization* technology (FISH, CISH) or immunohistochemistry (IHC) have been the choice of methods in many previous studies [1, 4–6, 14, 18, 20–23]. Numerical analysis of *TOP2A* gene alterations has been conducted in many studies in an analogous way as it is usually done in *HER2* testing and a cut-off >2 *TOP2A/CEP17* ratio was used to determine an amplified *TOP2A* status [5, 6, 14, 18, 21, 23]. Deletion of the *TOP2A* gene conversely has been set to different *TOP2A/CEP17* ratios ranging from 0.5 to 1 [5–7, 14, 18, 20].

In the current study, we addressed the question whether the rate of *TOP2A* amplification and deletion depends on the length of the FISH detection probes using different FISH probes (PathVysion™ and DAKO pharm DX™).

Materials and methods

Patients

Forty-two consecutive *HER2* amplified breast cancer patients were selected from the diagnostic service of the Institute of Surgical Pathology, University Hospital Zurich. HER2 status was primarily determined with fluorescence in situ hybridisation (FISH) during routine diagnostics. All tumours had a *HER2/CEP17* ratio of more than 2.2. Mean age of patients was 57 years (range 29 to 95 years). Paraffin blocks with tumour tissue were available in all cases. All analyses were carried out on whole tissue sections.

Thirty-four of 42 cases (81%) were invasive ductal carcinomas, 8 cases (19%) corresponded to special types of breast cancer (5 micropapillary, 2 invasive pleomorphic lobular carcinomas and 1 case of clear cell mammary carcinoma).

Twenty of 42 patients had hormone receptor positive carcinomas (48%), 22 of 42 patients had hormone receptor negative carcinomas (52%). Tumours were graded according to the modified Bloom and Richardson grading system for invasive breast cancer [24]. Thirty-six carcinomas (86%) were poorly differentiated, and 6 cases (14%) were moderately differentiated.

TOP2A immunohistochemistry

Protein expression was determined immunohistochemically on 2-µm thick paraffin embedded sections by using a mouse anti TOP2A monoclonal primary antibody (DAKO, DakoCytomation, Denmark A/S, Glostrup, clone: M7186, dilution: 1:400). The entire procedure was carried out on the Ventana Benchmark automated staining system by applying Ventana reagents. Pretreatment was performed by cooking with CC1-Buffer (Ventana) for 36 min at 95°C.

TOP2A antibody was detected by the iVIEW DAB detection kit and the signal was enhanced using the amplification kit.

As to stain intensity and specificity of TOP2A immunohistochemistry, we analysed normal breast tissue on large sections in a similar fashion as seen with MIB-1 or ER immunohistochemistry. Vessels and interstitial spaces were not stained, only nuclei in the ductulo-lobular units was stained. TOP2A immunohistochemistry was scored as follows: clear nuclear staining regardless of stain intensity was considered as positive. The extent of positive cells was estimated semi-quantitatively over the tumour area on intermediate power view (10× magnification) and was scored in a percentage.

TOP2A fluorescence in situ hybridisation

TOP2A gene status on 17q21-22 was determined by using two dual colour DNA probe kits and a three colour DNA probe kit (Fig. 1).

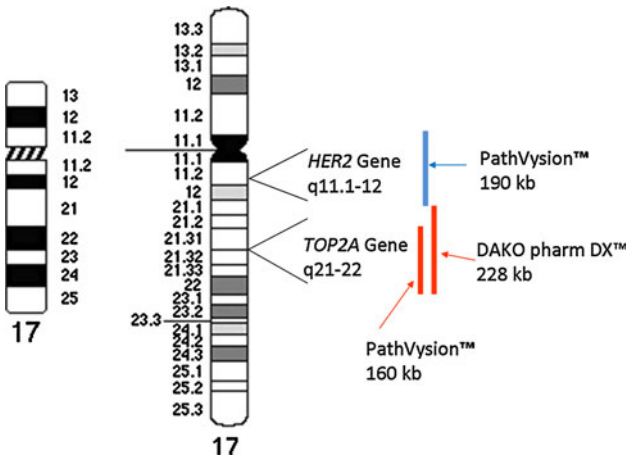

Fig. 1 Graphical illustration of the hybridized DNA length for *TOP2A* and *HER2*

Kit 1: TOP2A/CEP17 (PathVysion™, Code Nr: 32-190095, Vysis, Abbott AG, Diagnostic Division Baar, Switzerland).

This dual colour probe hybridizes the *TOP2A* gene on 17q21-q22 labelled with spectrum orange. The DNA clones cover a total of 160 kb of the *TOP2A* amplicon on 17q21-q22. The centromer (CEP17) is hybridized with spectrum green on 17p11.1-q11.1.

Kit 2: TOP2A/HER2/CEP17 (PathVysion™, Code Nr: 32-19095, Vysis, Abbott AG, Diagnostic Division Baar, Switzerland).

This three colour probe contains the *HER2* Gene (17q11.2-q12, labelled with spectrum green), the CEP17 region (17p11.1-q11.1, labelled with spectrum aqua) and the *TOP2A* gene (17q21-q22, labelled with spectrum orange). The DNA clones cover a total of 160 kb of the *TOP2A* amplicon on 17q21-q22 and a total of 190 kb of the *HER2* amplicon on 17q11.2-q12.

Kit 3: TOP2A/CEP17 (DAKO pharm DX™, Code Nr.: K5333, DakoCytomation, Denmark A/S, Glostrup).

This dual probe contains a mixture of *TOP2A* gene and *CEP17*. The *TOP2A* gene is labelled with Texas Red, which covers a total of 228 kb of the *TOP2A* amplicon on 17q21-q22. The *CEP17* is labelled with spectrum green and covers the region of 17p11.1-q11.1.

For all fluorescence in situ *hybridization* analyses, paraffin embedded sections of two micrometer thickness were used. All procedures for the FISH analyses were carried out by following the recommended protocol of the manufacturers. Probe mixes were hybridized at 37°C between 14 and 20 h, washed in Rapid-Wash-Solution I at 73°C for 5 min, Rapid-Wash-Solution II and H_2O for 7 min, air dried and counterstained with DAPI. The analysis was done by using an Olympus computer guided fluorescence microscope (BX61). For each tumour case, a new haematoxylin and eosin (H&E) stain was available in order to identify the invasive tumour component.

Scoring of all FISH kits was performed according to the recommendations in the literature [1, 6, 21]. The absolute copy number of both *CEP17* and *TOP2A* were counted and manually transferred into an excel data sheet. If gene copies were present in clusters, they were recorded as clusters. Following recommendation for clusters in the ASCO/CAP guidelines for HER2 testing, larger clusters were documented as equivalent of approximately 12–14 gene copies per cluster [26, 27]. Each FISH reaction was additionally documented on at least one digital photograph.

Deletion of *TOP2A* gene was defined as the presence of one gene copy in more than 90% of the examined area. Amplification of the *TOP2A* gene was defined as at least 6 gene copies in a minimum of 100 invasive tumour cells. As aneuploidy of higher grade was not a relevant feature in this cancer cohort, it was not necessary to measure *TOP2A/CEP17* ratio. *TOP2A* gene status was considered as normal if the gene copy number was at least 2 and did not exceed 5 gene copies.

Immunohistochemistry and FISH reactions were carried out by one technician (SB), the reactions were analysed and scored by one pathologist (ZV).

Multiplex-ligation dependent probe amplification (MLPA)

This assay was conducted retrospectively on those cases which revealed discrepant amplification status with the FISH probes. The test was performed as previously described using the P004-C1 chromosome 17 kit (MRC-Holland, Amsterdam, The Netherlands) containing 3 TOP2A probes [15, 16]. All tests were performed on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA) and fragment analyses were run on an ABI 3730 DNA Analyser (Applied Biosystems). Copy number ratios were analysed using Genemapper (Applied Biosystems) and Coffalyser software (MRC-Holland). For genes with more than one probe present in the kit, the mean of all the probe peaks of this gene in duplicate was calculated. A mean value below 0.7 was defined as loss, a value between 0.7–1.3 was defined as normal, 1.3–2.0 as gain and values >2.0 were defined as (high level) amplification, as established previously [3].

Results

TOP2A immunohistochemistry

There was heterogeneous TOP2A positivity in all 42 carcinomas. In 4 of 42 cases (9.5%) immunopositivity was found in 10% of the tumour cells. In 38 of 42 cases (90.5%), immunopositive nuclei were detected in more than 10% of the tumour cells (on average 50–80%). There was no correlation between TOP2A immunopositivity and *TOP2A* copy number by FISH.

TOP2A and CEP17 fluorescence in situ *hybridisation* (Fig. 2)

Kit 1 and 2 (PathVysion™):

The FISH analysis with the dual and the three colour probe kits resulted in completely identical results.

CEP17 was diploid in 31 of 42 cases (74%) and aneuploid (3–4 gene copies) in 11 cases (26%). There was no monosomy or aneuploidy of higher grade (> 4 copies) of CEP17 detected.

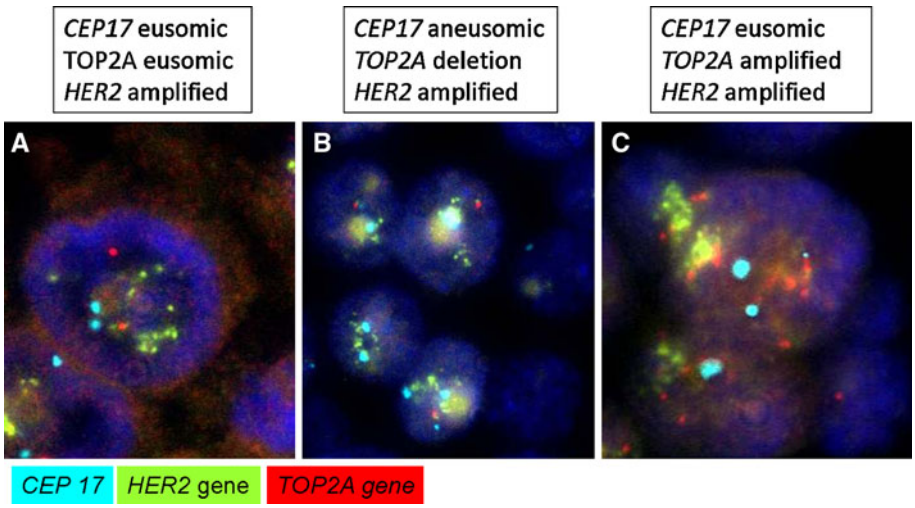

Fig. 2 Different patterns of fluorescent in situ hybridisation (FISH) for *TOP2A, HER2* and *CEP17* in *HER2* positive breast cancer samples. All reactions originate from the series with PathVysion™ kit. Coloration: *TOP2A*: *orange*, *HER2*: *green*, *CEP17*: *aqua*. **a** Normal *TOP2A* status (2 gene copies). CEP17 is present in 2 copies, the *HER2* gene in up to 10 gene copies. **b** Deletion of *TOP2A gene*: one copy of *TOP2a*, three copies of *CEP17* (aneusomic), small clusters and up to 8 copies of the *HER2* gene. **c** Amplification of TOP2A gene: *TOP2a*, three copies of *CEP17* (aneusomic), small clusters and up to 8 copies of the *HER2* gene

TOP2A was diploid in 9 cases (21%), deletion was found in 7 cases (17%). Low-aneuploidy (3–5 gene copies) was present in 15 cases (36%). Amplification of the *TOP2A* was present in 11 cases (26%). Among the amplified cases, *TOP2A* copy number reached 6–8 in 4 tumours. *TOP2a* was present in smaller clusters in 7 other amplified cases.

Kit 3 (DAKO pharm DX™):

CEP17 was diploid in 33 of 42 cases (79%) and aneuploid (3–4 gene copies) in 8 cases (19%). Monosomy was detected in one case (2%). Aneuploidy of higher grade (>4 copies) of CEP17 was not detected.

TOP2A was diploid in 8 of 42 cases (19%), deletion was found in 7 cases (17%). Low-aneuploidy (3–5 gene copies) was present in 12 cases (28%). Amplification of the *TOP2A* gene was present in 15 cases (36%). Among the amplified cases, *TOP2A* copy number was 6–8 in 6 cases, larger clusters or in more than 12 gene copies were detected in 9 cases.

Multiplex-ligation dependent probe amplification (MLPA)

The four cases with discrepant amplification status with the FISH probes, showed amplification of the *HER2* gene, *GRB7* and *IKZF3* (one of the cases also *RARA*) but no amplification of the *TOP2A* gene with the MLPA methodology. The TOP2A copy number ratios of the four discrepant cases were 0.86, 1.07, 1.21 and 0.99.

FISH and IHC results are summarised in Table 1.

Discussion

In our study, a discrepancy in the *HER2/TOP2A* co-amplification status was observed in 4 of 42 patients using different TOP2A probes. *TOP2A* was amplified in 26% by PathVysion™ and in 35% using DAKO pharm™ probe. There was no difference in the detection rate of *TOP2A* gene deletion. Both probes identified 7 identical cases with *TOP2A* gene deletion. Deletion or amplification of the *TOP2A* gene did not show any correlation to TOP2A protein expression. All 42 tumours showed at least 10% nuclear TOP2A positivity.

The results of our study suggest that the difference in detection rate of *TOP2A* amplification is due to the different length of the DNA probes hybridising to the *TOP2A* region (17q21-q22). The DAKO pharm™ probe covers a total of 228 kb of the *TOP2A* region, while the Pathvysion™ probe envelops a shorter DNA segment of 160 kb. The external MPLA control could not detect amplification of the *TOP2A* gene in any on the four discrepant cases; however, the HER2 gene was amplified with this methodology. These results strongly imply that the larger TOP2A DNA probe most likely overlaps with the *HER2* gene region and thus results in 'overdetection'.

Differential detection rate depending on the length of the mapping probe has also been identified by Jacobson et al., by using three different probes of Genome Systems covering three regions telomeric from the *HER2* gene [7]. Jacobson et al. [7] reported that the *TOP2A* amplification rate varies from 23–46% depending on the length of the

Table 1 Summary of *TOP2A* status via FISH testing and Immunohistochemistry (IHC) in 42 *HER2* amplified breast cancers

	TOP2A amplified PathVysion™	*TOP2A* amplified DAKO pharm™	*TOP2A* deletion PathVysion™	*TOP2A* deletion DAKO pharm™	Top2A IHC
ER positive $n = 20$	5 (25%)	7 (36%)	5 (25%)	5 (25%)	20 (100%)
ER negative $n = 22$	6 (22%)	8 (36%)	2 (9%)	2 (9%)	22 (100%)
Total $n = 42$	11 (26%)	15 (35%)	7 (17%)	7 (17%)	42 (100%)

ER estrogene receptors

hybridizing probe, which is very similar to our results. Interestingly, a differential *TOP2A* gene deletion was detected as well, ranging from 8 to 23% [7]. In our study, *TOP2A* gene deletion rate was found in 7 identical cases (17%), independently of the FISH probe. The length of the FISH probe apparently does not influence the detection of gene deletion.

Amplification rate of the *TOP2A* gene in *HER2* amplified breast cancer has been reportedly as high as 35% on average varying from 7 to 50% [4, 6–10, 14, 20, 21]. The variable incidence of *TOP2A* aberrations is most likely due to heterogenous study populations, divergent cut-offs/scoring systems as well as to the different specificity of the in situ hybridisation probes used for detection [2, 6, 20, 21]. Literature data are based on at least two different in situ hybridisation kits including fluorescent (FISH) and chromogenic (CISH) technology of three different companies (DAKO pharm™, Pathvysion™ and Zymed®) [1, 4–6, 14, 18, 20–23, 25].

As to scoring of *TOP2A* gene numeration, in most studies an analogous system to *HER2* scoring has been applied using a cut-off >2 *TOP2A/CEP17* to determine an amplified *TOP2*A status [5, 6, 14, 18, 21, 23]. However, in some earlier studies, *TOP2*A amplification was defined as a *TOP2A/CEP17* ratio >1.5 [4, 7, 20]. When using the absolute copy number for *TOP2*A amplification, most studies set the cut-off of at least 6 TOP2A gene copies [1, 23]. The same is true for the definition of *TOP2*A gene allelic deletion in the literature. Cut-offs for deletion include different *TOPAa/CEP17*ratios ranging from <0.50, <0.70, <0.75, <0.8 to <1.0 [5–7, 14, 18, 20].

In most studies, *TOP2A* gene amplification has been detected in a setting of co-amplification with the *HER2* gene, but rarely in *HER2* non-amplified patients [6, 20, 21]. The two genes are located on two distinct chromosomal regions and deletion of the *TOP2A* gene can commonly occur in the presence of *HER2* amplification [7, 8]. HER2 gene amplification is often accompanied by co-amplification of several adjacent genes comprising 17q12-q21, e.g. *TOP2A* or *RARA* [12, 13, 16]. It is important to note, that both commercially available *TOP2A* FISH probes hybridize a DNA region, which is positioned approximately 679 kb in distance from the *HER2* gene [17, 19].

Intratumoral heterogeneity of *TOP2A* gene signals can give an additional explanation on conflicting literature data regarding the frequency of *TOP2A* gene alterations [7, 10]. The number of cells showing *TOP2A* gene alterations can be largely influenced by the material used for the study: cell lines, imprint preparations, tissue microarrays or whole tissue sections can result in different percentages of *TOP2A* altered cells due to the heterogeneous distribution of the altered cells [7, 10]. As described by Jarvinen et al. [7, 10], *TOP2A* amplified and deleted cells can be found within the same tumour. Such heterogeneity was seen in up to 14% of breast cancer samples [7, 10]. Interestingly, the critical evaluation of our breast cancer samples revealed intratumoral heterogeneity of *TOP2A* alterations in most breast carcinomas. We did not see mixed patterns of deleted or amplified cells, but rather a mixture of amplified and normal cells or deleted and normal cells within the same tumour. This is in contrast to the *HER2* gene amplification in our cohort, which was in general a homogenous finding over the whole tissue sections.

Therapeutic benefit of adjuvant anthracycline containing regiments at HER2 and TOP2A positive breast cancer patients has been controversial. Depending on the study cohort and diagnostic criteria for *TOP2A* status determination, predictive and prognostic value of *HER2* and/or *TOP2A* amplification for treatment response could be confirmed or questioned in several previous mostly retrospective studies [1, 2, 14, 18, 21–23, 25]. Tubbs et al. [25] reported earlier in a prospective tissue microarray based study that *HER2* gene status and not *TOP2A* gene alterations are prognostic in anthracycline treated patients. Data from another prospective recent trial by Press et al. [21] demonstrate that *TOP2A* co-amplification but not *HER2* amplification is the clinically predictive marker for response to anthracycline-based chemotherapy.

This discrepancy cannot be explained by technical issues, because DNA probe kits from PathVysion™ and the same cut-offs for signal interpretation were used in both studies [21, 25]. It is more likely that the interpretation of a

heterogeneous distribution of *TOP2A* alterations has caused divergent results in both tissue microarray based studies [21, 25].

A recent meta-analysis from five randomised adjuvant trials suggests that not only patients with *HER2* gene amplified tumours, but also those with non-amplified *HER2* and normal *TOP2A* status can benefit from anthracycline-based adjuvant therapy [15]. In this meta-analysis, Leo et al. [15] propose the need for increased standardisation in *TOP2A* testing by FISH methodology due to suboptimal reproducibility.

The biological relevance of TOP2A protein overexpression is controversial. Arriola et al. [1, 14] reported that TOP2A protein overexpression correlates to high proliferation fraction, hormone receptor positivity and either to HER2 or to basal phenotype. Moelans et al. [12, 13, 16] found an association between TOP2A overexpression and *TOP2A* gene amplification but not with gene deletion. According to our data, TOP2A protein was expressed in all breast cancer cases independently of any other factors.

In summary, our data clearly underscore the relevance of appropriate standardisation of the FISH technology and probes for *TOP2A* testing with predictive purposes. Additional testing, for example MLPA technology to double-check amplification status, is a possibility to be addressed in future studies.

Conflict of interest None.

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