TOPOISOMERASE II-ALPHA GENE COPY NUMBER ALTERATIONS IN *ERBB2*-POSITIVE PRIMARY BREAST CARCINOMAS: A FLUORESCENCE *IN SITU* HYBRIDIZATION STUDY.

Colomer A[1], Erill N[1], Górriz M[1,2], Verdú M[2], Roman R[1], Ibáñez R[1], Cordon-Cardo C[1], Puig X[1,2].
[1]BIO**PAT**, Biopatologia Molecular, Grup Assistència & [2]HISTOPAT Laboratoris, Barcelona, Spain.

INTRODUCTION

- Chemotherapy containing anthracyclines, which target the enzyme topoisomerase II-alpha, is commonly employed in the treatment of invasive breast carcinoma.
- A number of retrospective studies had attributed responsiveness to anthracycline-based regimens to *ERBB2* amplification, but recent data has raised the hypothesis that *ERBB2* is rather to be viewed as a surrogate marker for amplification of the *TOP2A* gene, located next to the *ERBB2* locus on chromosome 17q21-q22.
- *TOP2A* amplification occurs almost exclusively in patients with *ERBB2* amplification (50% *ERBB2*-positive *vs.* 5% *ERBB2*-negative breast tumors), and has been associated with increased sensitivity to anthracyclines.
- *TOP2A* deletion, as frequent as *TOP2A* amplification in a number of series of invasive breast carcinoma, seems to predict response to anthracycline-based treatment too.
- Patients with primary breast cancer that exhibit *ERBB2* and *TOP2A* coamplification have significantly increased recurrence-free and overall survival when treated with anthracycline-based regimens; nevertheless, a nearly identical hazard ratio is found in patients with *TOP2A* deletion.
- All these data support the evidence that *ERBB2* amplification along with *TOP2A* changes (either amplification or deletion) may define a subgroup of high-risk breast cancer patients who would benefit from individually tailored and dose-escalated anthracycline-based adjuvant chemotherapy.

AIM

The present study was undertaken to examine the presence of *TOP2A* gene copy number alterations -either amplifications or deletions- in a series of primary breast invasive carcinomas obtained from patients in the area of Barcelona, Catalonia, Spain. Selected tumors had been previously characterized as *ERBB2*-positive by dual-color fluorescence *in situ* hybridization (FISH).

PATIENTS AND TISSUES

Patients. A cohort of 32 women surgically treated for invasive breast carcinoma in the area of Barcelona, Catalonia, Spain, was included in this retrospective study according to *ERBB2*-positive status. Median age of patients was 59.7±15.0 years (range 37-87).

Tissues. Surgical specimens were routinely fixed in 10% buffered formalin and embedded in paraffin. Representative hematoxylin and eosin-stained sections of each case were examined microscopically by a pathologist, who selected viable representative areas for FISH assessment.

Concerning histopathology, breast carcinomas were classified as 29 ductal (7 with histological grade 2 and 22 with grade 3), 2 lobular and 1 medullary. **Table 1** summarizes clinicopathologic and molecular features of the cases studied.

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METHODS

Dual-Color FISH. *ERBB2* status had been previously studied using PathVysion (Vysis Inc.) that includes a centromeric probe (CEP17 SpectrumGreen) and a locus specific probe (LSI HER SpectrumOrange). Relative gene copy numbers were calculated as ratios between mean number of LSI signals and mean number of CEP17 signals. Tumors were considered *ERBB2*-positive when showing evidence of amplification (LSI HER/CEP17 ≥ 2.0).

TOP2A status was studied using the same centromeric probe and a locus specific probe (LSI TOP2A SpectrumOrange), also from Vysis. FISH results were classified in 3 categories according to Hicks *et al.*, 2005: amplification (LSI TOP2A/CEP17 ≥ 2.0), deletion (LSI TOP2A/CEP17≤ 0.7), and non-alteration (0.8 ≤ LSI TOP2A/CEP17 ≤ 1.9).

Multi-Color FISH. A more recently developed multi-color probe kit was used in selected cases, which were restudied just with iconographic purposes. The probe mixture includes LSI TOP2A SpectrumOrange, LSI HER SpectrumGreen and CEP17 SpectrumAqua. Examples of *TOP2A* amplification and deletion are respectively shown in **Figures 1** and **2**.

Statistical Analysis. Amplification levels of the tumors exhibiting coamplification of *ERBB2* and *TOP2A* after FISH assessment were investigated using the Wilcoxon exact test for paired data. The *p*-value was considered statistically significant when less than 0.05. Data were analyzed using the exactRankTest of the R package v 2.4.1 (R Development Core Team).

RESULTS

Copy Number Studies. Cases included in the series were selected since they had been previously classified as *ERBB2*-positive by dual-color FISH. Changes in the *TOP2A* gene copy number were identified in 25 out of 32 *ERBB2*-positive tumors (78%). *TOP2A* was coamplified with *ERBB2* in 15 cases (47%), deleted in 10 cases (31%), while non-alteration was found in 7 cases (22%). Seven out of 15 *ERBB2* and *TOP2A*-coamplified tumors exhibited monosomy, whereas 6 were polysomic, and 2 were eusomic. No tumors exhibited monosomy amongst those with *TOP2A* deletion, 6 out of 10 were polysomic, and the 4 left were eusomic. **Table 1** summarizes the relative *TOP2A* gene copy numbers of all the cases studied.

TOP2A Status vs. Clinicopathological Features. **Table 1** reviews relationship of tumor data in detail. Of interest, all the tumors with *TOP2A* deletion were histological Grade 3, exhibiting high percentages of c-erbB2/neu immunostaining (scored 3+) with the CB11 monoclonal antibody. We also found that patients whose tumors lacked *TOP2A* copy number changes hardly ever had nodal involvement (only 1 case), these data not reaching statistically significance though.

ERBB2 vs. TOP2A amplification. **Table 2** summarizes the results obtained after FISH assessment of the 15 cases that exhibited *ERBB2* and *TOP2A* coamplification. The mean ratio of LSI HER/CEP17 was 4.6 (range 2.0-10), while the mean ratio of LSI TOP2A/CEP17 was 2.8 (range 2.0-5.4), thus suggesting that the level of amplification for *ERBB2* was higher than the level of *TOP2A*. Significant statistical analysis corroborated this observation (Wilcoxon exact test; V = 66, $p < 0.0005$).

CONCLUSIONS

- Our results are in agreement with those previously published reporting high incidence of alterations in the *TOP2A* gene copy number, either amplification (47%) or deletion (31%), within the group of *ERBB2*-positive invasive breast carcinomas.
- FISH seems to play a role as a predictor of breast tumor sensitivity to anthracycline-based chemotherapies; actually, in our series, it allowed identification of 78% *ERBB2*-positive patients that could have got benefit from the inclusion of an anthracycline in their regimens.
- As a variety of adjuvant treatments is now available, more studies should be addressed to identify predictors of response for effectively tailoring therapy in order to maximize cost/benefit ratios while minimizing toxicity.

FIGURES

Figure 1. Example of *ERBB2* and *TOP2A* coamplification. Breast invasive tumor cells were hybridized with a multi-color probe kit. (a) Nuclei exhibit coamplification of *ERBB2* (SpectrumGreen) and *TOP2A* (SpectrumOrange) as indicated by multiple signals of each color; (b) Alternative vision of *TOP2A* amplification using a yellow filter; (c) The CEP17 probe (SpectrumAqua) shows evidence of eusomy (disomy); and, (d) DAPI nuclei counterstain. (magnification, x1000)

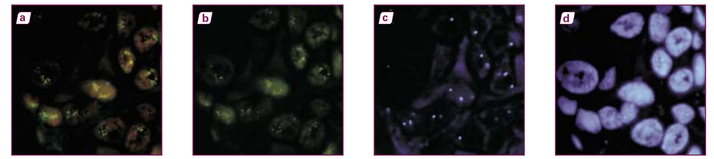
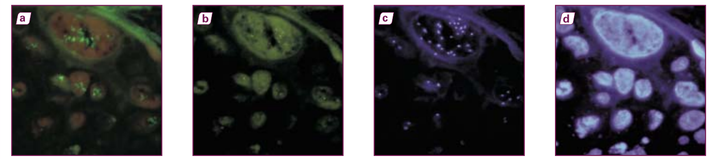


Figure 2. Example of *TOP2A* deletion. (a) *ERBB2*-amplificated nuclei (SpectrumGreen) show *TOP2A* deletion as indicated by a reduced number of signals (SpectrumOrange) with respect to CEP17. (b) Alternative vision of *TOP2A* deletion using a yellow filter; (c) The presence of three or more CEP17 signals per nucleus (SpectrumAqua) reflects polysomy; and, (d) DAPI nuclei counterstain. (magnification, x1000)



TABLES

Table 1. Relationship between *TOP2A* status and clinicopathologic features of study cases (n= 32). Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HG, histologic grade; A, amplification; D, deletion; and N, non-alteration.

Case No.	*TOP2A*	CEP17	c-erbB2*	ER	PR	Ki67	p53	Type	Age	HG	pT	Nodes
M037T	A	monosomy	<10 (1+)	95	0	10	0	ductal	68	3	1	yes
M150T	A	polysomy	60 (3+)	90	<10	30	10	ductal	58	2	1	no
M170T	A	polysomy	90 (3+)	90	0	25	<10	ductal	81	3	2	no
M172T	A	monosomy	85 (2+)	40	95	50	0	ductal	40	2	1	no
M196T	A	monosomy	80 (2+)	20	0	15	0	ductal	77	3	4	yes
M291T	A	eusomy	95 (3+)	80	<10	15	0	ductal	70	2	2	yes
M490T	A	eusomy	90 (3+)	80	60	60	0	ductal	72	3	1	yes
M514T	A	polysomy	95 (3+)	95	95	50	45	lobular	45	-	1	no
M532T	A	polysomy	<10 (1+)	95	60	0	20	ductal	74	3	1	yes
M551T	A	monosomy	0	0	<10	70	90	ductal	41	3	1	no
M733T	A	polysomy	15 (1+)	90	90	<10	0	ductal	73	2	1	no
M756T	A	monosomy	0	95	80	15	10	ductal	75	2	1	no
M764T	A	monosomy	<10 (1+)	0	0	50	0	medullary	81	-	2	no
M770T	A	polysomy	90 (3+)	<10	0	25	25	ductal	67	3	2	yes
M825T	A	monosomy	10 (1+)	40	<10	<10	0	lobular	57	-	3	yes
M021T	D	polysomy	**95 (3+)**	<10	0	30	-	ductal	53	3	-	yes
M056T	D	eusomy	**95 (3+)**	0	0	70	0	ductal	50	3	2	yes
M060T	D	eusomy	**95 (3+)**	0	0	30	<10	ductal	48	3	4	yes
M180T	D	polysomy	**80 (3+)**	95	10	25	<10	ductal	40	3	1	no
M325T	D	eusomy	**80 (3+)**	90	90	70	0	ductal	45	3	2	yes
M356T	D	polysomy	**95 (3+)**	<10	0	30	0	ductal	87	3	4	no
M515T	D	eusomy	**95 (3+)**	0	0	90	95	ductal	37	3	1	yes
M713T	D	polysomy	**80 (3+)**	0	0	30	0	ductal	47	3	2	yes
M773T	D	polysomy	**95 (3+)**	0	<10	60	0	ductal	65	3	2	yes
M818T	D	polysomy	**80 (3+)**	95	95	40	60	ductal	43	3	1	no
M033T	N	polysomy	25 (2+)	95	35	50	25	ductal	54	3	2	yes
M103T	N	polysomy	90 (3+)	0	0	40	0	ductal	72	2	2	**no**
M634T	N	eusomy	15 (3+)	85	<10	15	0	ductal	70	2	1	**no**
M643T	N	eusomy	0	40	0	15	0	ductal	41	3	2	**no**
M707T	N	polysomy	<10 (1+)	95	90	15	0	ductal	70	3	1	**no**
M754T	N	polysomy	50 (2+)	95	10	50	50	ductal	68	3	2	**no**
M771T	N	polysomy	<10 (1+)	90	85	10	<10	ductal	41	3	1	**no**

*c-erbB2 was scored by intensity (in brackets) in addition to the percentage of immunoreactive cells used to score the other immunohistochemical features.

Table 2. Comparative FISH results of the 15 cases exhibiting coamplification. Notice that *ERBB2* relative copy numbers were always higher or equal than *TOP2A* ones, which reached statistical significance (Wilcoxon exact test; V = 66, $p < 0.0005$).

Case No.	LSI HER/CEP17	LSI TOP2A/CEP17	CEP17
M291T	>10.0	5.4	eusomy
M170T	>10.0	2.3	polysomy
M196T	>10.0	2.3	monosomy
M514T	6.0	4.8	polysomy
M490T	5.3	3.6	eusomy
M770T	4.8	2.0	polysomy
M172T	3.3	3.0	monosomy
M037T	3.0	2.0	monosomy
M756T	2.8	2.1	monosomy
M150T	2.5	2.5	polysomy
M733T	2.5	2.2	polysomy
M764T	2.1	2.0	monosomy
M532T	2.0	2.0	polysomy
M551T	2.0	2.0	monosomy
M825T	2.0	2.0	monosomy