3. UNRAVELING THE CHROMOSOME 17 PATTERNS OF FISH IN INTERPHASE NUCLEI: AN IN-DEPTH ANALYSIS OF THE *HER2* AMPLICON AND CHROMOSOME 17 CENTROMERE BY KARYOTYPING, FISH AND M-FISH IN BREAST CANCER CELLS

3.1 INTRODUCTION

The Chr17 is the second most gene-dense chromosome in the human genome (141), containing many genes central to breast cancer development and progression, including oncogenes (*HER2, TOP2A, STARD3, TAU*), tumor suppressor genes *(TP53, BRCA1, HIC-1)* and DNA double-strand break repair genes (*RDM1*) (142-147). In particular, the *HER2* gene mapping to 17q11-q12 is amplified in 15-20% of all human breast cancers (130), is a prognostic marker for aggressiveness (130) and predicts the response to anti-HER2 agents (130). An accurate and definitive reporting of *HER2* status is thus essential for appropriate treatment determination. Fluorescence *in situ* hybridization (FISH) with dual probes for *HER2* and for the Chr17 centromere (CEP 17) is the most frequently used technique to determine the *HER2* gene status in interphase nuclei. The correction of *HER2* gene copy number using CEP17 signals is required to account for Chr17 polysomy. However, we have recently provided by array CGH-analysis the first direct evidence that true Chr17 polysomy is a rare event in breast cancer (102). Indeed, a number of CEP17 copies greater than 3.0 detected by FISH analysis is frequently related to either a gain or amplification of the centromere region, providing another line of evidence that Chr17 usually displays very complex rearrangements.

CGH, loss of heterozygosity (LOH), and molecular genetics studies have shown that chromosome 17 (Chr17) is rearranged in at least 30% of breast tumors (141, 148, 149) and presents a number of rearrangement breakpoints mapping to either its short or long arm. In particular, Chr17p is principally involved in losses, whereas CGH on 17q shows complex combinations of overlapping gains and losses (141, 150). In addition, CGH and spectral karyotyping (SKY) studies have shown that Chr17 is one of the most frequent chromosomes involved in translocations (12). However the frequency and type of specific structural Chr17 alterations in breast cancer have not been fully detailed.

The complexity of these Chr17 rearrangements detected by both array CGH and FISH analysis has called into question the accuracy of reported *HER2*/CEP17 ratios. Indeed, unsuspected Chr17 rearrangements may be contributing to the high percentage of inaccurate and equivocal results for *HER2* status in breast cancer. Although the numbers of these equivocal and discordant cases vary widely between laboratories, it is estimated in some studies to be as high as 20% of cases (151). This inaccuracy has been most often attributed to multiple pre-analytic,

analytic, and post-analytic variables inherent to the mechanics of performing the test in a clinical laboratory (130).

The aim of this study was to assess numerical alterations and structural rearrangements of Chr17 in breast cancer cells and to elucidate how these alterations may impact the *HER2*/CEP17 FISH evaluation of interphase nuclei.

3.2 METHODS

3.2.1 Cell Lines

Nine established breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). They have different characteristics related to the presence of estrogen receptors (ER+ or ER-) and HER2 genes status, HER2+ means amplified and HER2- no amplified. Then, MCF7, T47D and ZR-75-1 are ER+/*HER2*-; BT474 and, MDA-MB361 are ER+/*HER2*+; SKBR3, JIMT-1 and KPL4 are ER-/*HER2*+ and MDA-MB231 is ER-/*HER2*-]. The MCF7, T47D, ZR-75-1, SKBR3, JIMT-1 and KPL4 cell lines were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA), while the BT474, MDA-MB231 and MDA-MB361 lines were cultured in DMEM medium (Sigma). All culture media were supplemented with 10% fetal bovine serum (FBS) (Sigma), an antibiotic-antimycotic solution (1X) (Sigma) and L-glutamine (2 mM) (Invitrogen GmbH, Karslruhe, Germany). The cultures were maintained in an incubator at 37ºC and 5% CO2.

3.2.2 Tumor samples for primary culture

The study on primary culture was approved by the ethics institutional review board for "Biobanking and use of human tissue for experimental studies" of the Pathology Services of the Azienda Ospedaliera Città della Salute e della Scienza di Torino. Written informed consents are obtained from patients for the use in research studies of residual tissues from the diagnostic procedures.

We analyzed the cells of a triple negative breast carcinoma that metastasized to the peritoneum, giving rise to a peritoneal effusion. We received and managed the fresh effusion as follows: a quick smear was performed to confirm the presence of tumor cells, after which one part of the fluid was used to obtain a cell block, and the remaining part was used to set up a primary culture according to a protocol recently described (152). The cells grew adhering to the dish. Their epithelial origin was confirmed by the positive expression of cytokeratin (clones AE1/AE3 & PCK26, Ventana-Diapath, Tucson, AZ, USA) and by the absence of the mesothelial marker calretinin (polyclonal; 1:100 diluted, Invitrogen) using an immunohistochemical procedure on cells grown directly on sterilized slides. The triple negative phenotype was confirmed by immunohistochemistry (IHC) for the

estrogen receptor (ER) (Clone SP1, dilution 1/50. Cell Marque, Rocklin, California), progesterone receptor (PgR) (Clone 1A6, dilution 1/50. Leica Biosystems, Newcastle Upon Tyne, United Kingdom) and HER2 receptor (Dilution 1/700. Dako, Glostrup, Denmark) on the cell block obtained after centrifugation of the effusion.

3.2.3 G-Banding and karyotyping

Metaphases for performing conventional and molecular cytogenetic analysis (M-FISH and FISH) were obtained by using standardized harvesting protocols. Briefly, colcemid solution (0.03 µg/ml) (Sigma) was added to cultures 2.5 hours (h) before cell harvesting; cells were then treated with hypotonic solution, fixed three times with Carnoy's fixative (3:1 methanol to acetic acid) and spread on glass. Glass slides were baked at 70°C for 24 h, incubated in HCl and placed in 2xSSC buffer before treatment with Wright's stain.

Metaphases image acquisition and subsequent karyotyping were performed using a Nikon microscope with the cytogenetic software CytoVision System (Applied Imaging, Santa Clara, CA). Between 10 and 26 metaphase cells with good dispersion and morphology were analyzed for each cell line. Chromosome aberrations were described according to the International System for Human Cytogenetic Nomenclature 2013 (ISCN) (122).

3.2.4 Multi-color fluorescence in *situ* hybridization (M-FISH)

M-FISH was performed with the aim of identifying complex chromosomal rearrangements unidentified by conventional cytogenetic. A probe cocktail containing 24 differentially labeled chromosome-specific painting probes (24xCyte kit MetaSystems, Altlussheim, Germany) was denatured and hybridized to denatured tumor metaphase chromosomes according to the protocol recommended by the Human Multicolor FISH kit (MetaSystems). Briefly, the slides were incubated at 70ºC in saline solution (2xSSC), denatured in NaOH, dehydrated in an ethanol series, air-dried, covered with 10 µl of the probe cocktail (denatured) and hybridized for two days at 37ºC. The slides were then washed with post-hybridization buffers, dehydrated in an ethanol series and counter-stained with 10µl of DAPI/antifade. Signal detection and subsequent metaphases analysis were accomplished using the Metafer system and the Metasytems ISIS software (software for spectral karyotypes). At least 10 metaphases exhibiting the same derivative chromosomes were studied for each cell line.

3.2.5 FISH for the *HER2, STARD3* and *TOP2A* genes

FISH experiments were performed to define the *HER2, STARD3* (17q12) and *TOP2A* (17q21-q22) gene status and mapping. Two commercial dual-color probes for *HER2* (SpectrumOrange)/CEP17 (SpectrumGreen) and *TOP2A*

(SpectrumOrange)/CEP17 (SpectrumGreen), all from Abbott Molecular, Downers Grove, IL, USA, were used separately on each cell line.

For the *STARD3* gene, FISH studies were performed using both an alpha satellite probe specific for Chr17 (CEP17) that was directly labeled with a green fluorochrome (Abbott molecular) and a *STARD3* specific locus probe fosmid WI2-2398I17 (17q12) that was made in-house. The clone was obtained from BACPAC Resources Center (Children's Hospital Oakland Research Institute, CA, USA). The UCSC database (http://genome.ucsc.edu, February 2009 release) was queried to localize the probe. The fosmid was expanded, extracted using the QIAGEN Plasmid Purification Kit (Qiagen GmbH, Hilden, Germany) and then directly labeled with SpectrumOrange-dUTP (Abbott Molecular), using the Nick Translation Kit (Abbott Molecular) according to the manufacturer's instructions. The fosmid clone was tested on metaphase and interphase cells of healthy donors, obtained using conventional cytogenetic methods, to analyze the position and strength of the signal, the presence/absence of background and cross-hybridization and the hybridization efficiency.

FISH with the *HER2*/CEP17, *STARD3*/CEP17 *and TOP2A*/CEP17 probes was performed separately on each cell line on fresh slides from methanol acetic acid fixed cells according to the manufacturers' instructions. Briefly, the slides were washed at 37ºC in 2x saline-sodium citrate buffer (SSC), dehydrated in an ethanol series, air-dried, covered with 10 µl of probe, co-denatured in HYBrite System at 70ºC for 5 min and hybridized overnight at 37ºC. Slides were then washed with a post-hybridization buffer (2xSSC/0.3% Nonidet P-40), dehydrated in an ethanol series and counter-stained with 10 µl DAPI/antifade. Metaphases and nuclei were selected with an AxioImager Z1 epifluorescence microscope (Carl Zeiss, Germany). Analysis of the signal pattern on the nuclei and metaphases was performed on a screen with ISIS software. The number of FISH signals and the localization of the signals were analyzed in at less 10 metaphases and interphase nuclei.

3.3 RESULTS

3.3.1 Structural alterations of Chr17

The cytogenetic profiles of Chr17 in breast cancer cells are illustrated in Figures 18 - 22, and their specific chromosomal alterations are indicated in Table 10. We identified 44 different alterations of Chr17, involving mainly its long arm, in 8 out of the 9 cell lines analyzed. The triple negative MDA-MB231 cells showed no Chr17 alterations, while the *HER2* amplified MDA-MB361, SKBR3 and JIMT-1 cell lines carried no normal copies of Chr17. In particular, the SKBR3 cells harbored 10 different types of structural abnormalities on Chr17, making it the cell line with the highest frequency of structural abnormalities. The lowest frequency for Chr17

abnormalities was observed in HER2- cells, which carried between 2 and 3 different types of alterations (Table 10).

Table 10. Aberrations of Chr17 identified in nine breast cancer cell lines and one triple negative breast carcinoma by G-Banding, M-FISH and FISH.

CELL LINES	Type of Rearrangement
MCF7 (ER+/HER2-)	der(6)t(6;17;16)(q25;q21;?)[100],der(17)t(8;17)t(1;8)[100],der(17)t(17;19)(p11.1;p12)[65]
T47D (ER+/HER2-)	dic(9;17)t(9;17)(p12;p13)[100],der(11)t(11;17)(q23;q?)t(9;17)(q?12;?)[33]
ZR-75-1 (ER+/HER2-)	der(11)t(11;17)(p15;q?21)[100],der(11)t(11;17)(p15;q?21)t(11;17)(?;q25)[88],der(17)t(6;17)(p12;p11.2)[100]
BT474 (ER+/HER2+)	der(X)t(X;17)(q13;q11q12)del(X)(p21)hsr(17)(q11q12)x2[39],der(11)t(8;17)(q21.1;q11q12)t(11;17)(p15;q11q12)x2[100],der(11)t(11;17)(q?14;q?11.2)hsr(17)(q11q12)[39],der(11)t(11;17)(q?14;?)t(8;17)(?;q?11.2)hsr(17)(q11q12)x2[57],der(13)t(13;17)(q10;q11q12)t(13;17)(q10;q11q12)hsr(17)(q11q12)x2[87],der(17)t(6;17)(?;p13)t(15;17)(q11.2;q25)hsr(17)(q11q12)x2[96]
MDA-MB361 (ER+/HER2+)	der(8)t(8;17)(p21;q11q12)t(5;17)(?;q11q12)hsr(17)(q11q12)[100],der(8)t(8;17)(p21;q25)t(8;17)(q13;q11.2)[100],der(16)t(16;17)(p11.2;q21)[100],der(17)t(6;17)(?;q21)[100],der(17)t(7;17)(?;p13)[100],der(17)t(17;20)(p11.1;?)t(9;20)(?;q13.1)t(5;9)(q14;?)[100],der(17)t(17;21)(q21;q22)[100]
SKBR3 (ER-/HER2+)	der(X)t(X;8;17)(q13;q?21;q?)[33],der(X)t(X;17)(q21;q?21)hsr(17)(q11q12)x2[79],der(6)t(6;14;17)(q21;?;q11q12)del(6)(p23)[42],der(8)dup(8)(?)t(8;8)(?;p23)t(8;17)(q24;?)t(11;17)(?;?)[21],del(17)(p11)[37],der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12)[100],der(17)t(8;17)(?;q25)dup(17)(q22q25)[37],der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)hsr(17)(q11q12) [42],der(17)t(3;8;13;17;17;20)(?;?;q12;q12;?p;?)[74],der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?)[100]
JIMT-1 (ER-/HER2+)	der(3)t(3;12)(p21;?)t(2;3)(?;q12)t(2;17)(?;q11q12)hsr(17)(q11q12)[100],der(7)t(7;17)t(17;21)t(17;21)[56],der(8)t(8;17)(p23;q21)[100],der(8)t(8;17)(q13;q11q12)t(8;17)(q11.1;q12)hsr(17)(q11q12)[100],der(9)t(9;17)(q12;?)t(17;21)[33],der(17)t(8;17)(?;p13)[67],der(17)t(17;22)(p13;?)t(17;22)(q11.1;?)[100]
KPL4 (ER-/HER2+)	der(1)t(1;17)(p36.3;q11q12)hsr(17)(q11q12)[100],der(6)t(6;17)(p12;q11.2)t(8;17)(q25;?)[93],der(9;13)t(9;17)(p24;q11q12)t(13;17)(p11.2;q11.2)hsr(17)(q11q12)[100],der(11)t(11;17)(q13;?)t(8;17)(q21;?)[93],der(17)t(3;17)(q13;q11)t(6;17)(?;q11)[67]
MDA-MB231 (ER-/HER2-)	0
TNBC CASE (ER-/HER2-)	der(17)t(8;17)(q21;p12)[100],der(17)t(16;17)(q11.2;q11.1)[15],der(17)del(17)(p11.2)del(17)(q11.2)[69],der(17)t(17;19)(p11.1;?)[15],der(17)t(17;22)(p11.1;q11.2)[62]

The % of cells for which each abnormality was observed is indicated at the end of each abnormality within square brackets. The number of cells examined for chromosome count was 26 for MCF7 cells; 24 for T47D cells; 10 for ZR-75-1 cells for BT474 cells; 10 for MDA-MB361; 19 for SKBR3 cell; 18 for JIMT-1 cells; 15 for KPL4 cells; 14 for MDA-MB231 and 13 for the triple negative breast cancer case (TNBC).

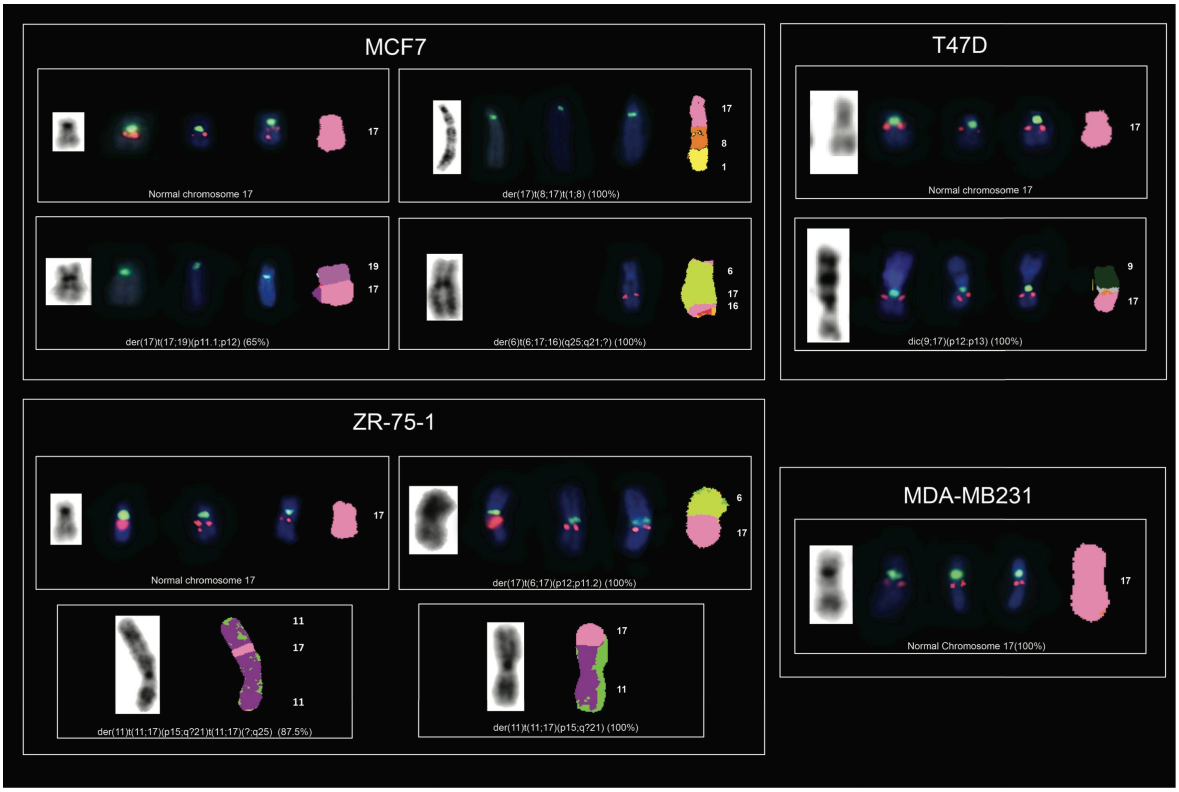

Figure 18. Analysis of Chr17 using G-Banding, dual-color FISH (*HER2*/CEP17*, STARD3*/CEP17 *and TOP2A*/CEP17) and M-FISH in the MCF7, T47D, ZR-75-1 and MDA-MB231 not *HER2* amplified breast cancer cell lines, showing one or three translocated chromosomes in addition to the normal-appearing copies of Chr17. The G-Banding technique was used to visualize both normal and derivative Chr17 copies, shown on the left. The Chr17 copies visualized by dual-color FISH for *HER2, STARD3* and *TOP2A* are shown in the middle. The *HER2, STARD3* and *TOP2A* genes are labeled with red, and the centromere of Chr17 is labeled with green. Chr17 visualized by M-FISH is shown on the right. By M-FISH, the classified color of Chr17 is shown in pink and the translocation partners are numbered beside the chromosomes. The frequency at which each abnormality was observed is indicated in brackets at the end of each abnormality. The derivative chromosome der(6)t(6;17;16)(q25;q21;?) observed in MCF7 cells, have deletion of *HER2* and *STARD3* genes, for this reason are only shown the images visualized by FISH (using dual probe *TOP2A*/CEP17) and M-FISH. In ZR-75-1 cells, for derivative chromosomes der(11)t(11;17)(p15;q?21)t(11;17)(?;q25) and der(11)t(11;17)(p15;q?21) only are shown the images visualized by M-FISH, because these derivatives have deletion of the *HER2, STARD3* and *TOP2A* genes.

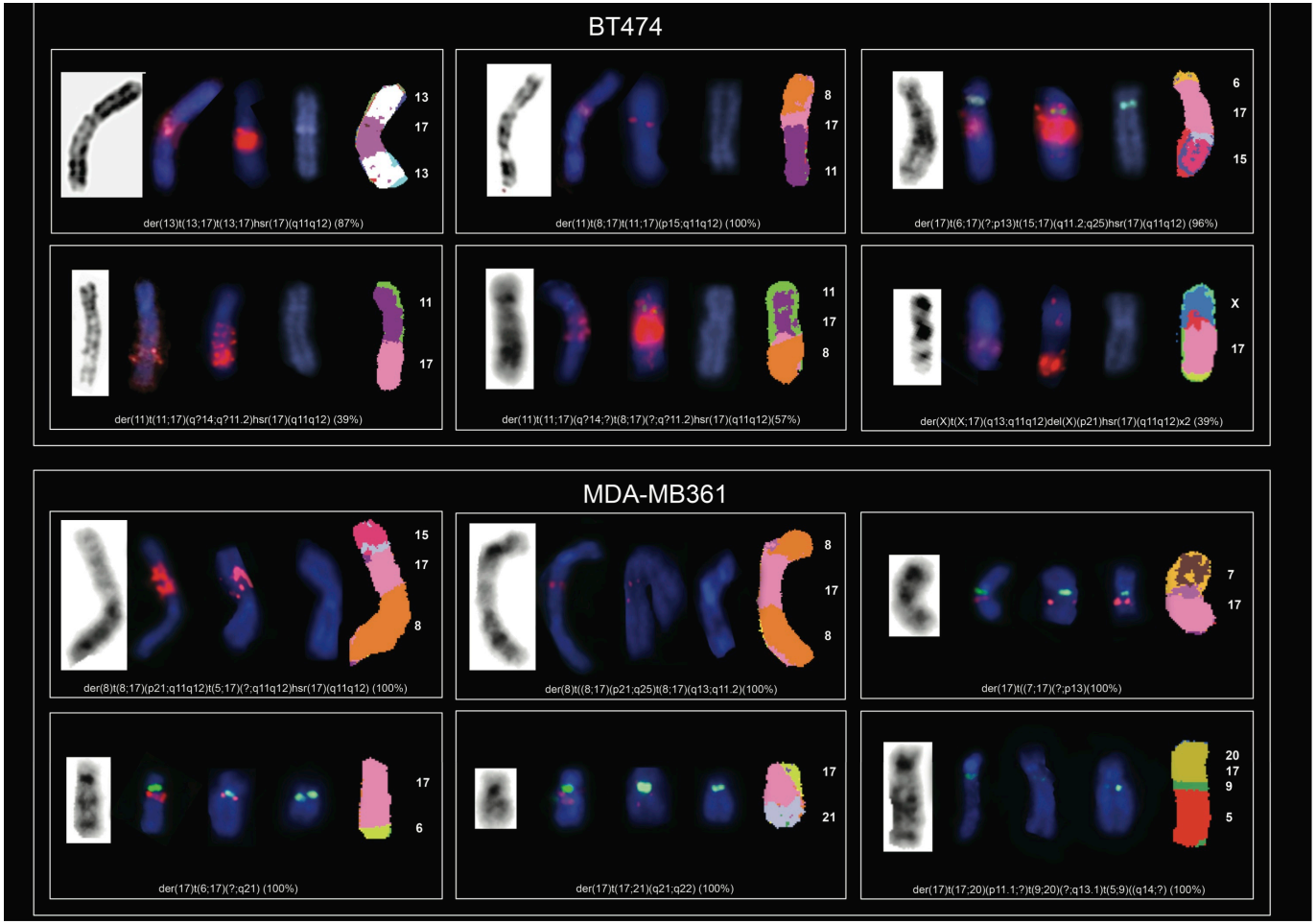

Figure 19. Analysis of Chr17 using G-Banding, dual-color FISH (*HER2*/CEP17*, STARD3*/CEP17 *and TOP2A*/CEP17) and M-FISH in BT474 and MDA-MB361 *HER2* amplified breast cancer cell lines showing six translocated copies of Chr17. By M-FISH, the classified color of Chr17 is shown in pink, and the translocation partners are numbered beside the chromosomes. The frequency at which the abnormality was observed is indicated in brackets at the end of each abnormality. Otherwise, the results are shown as in Figure 1.

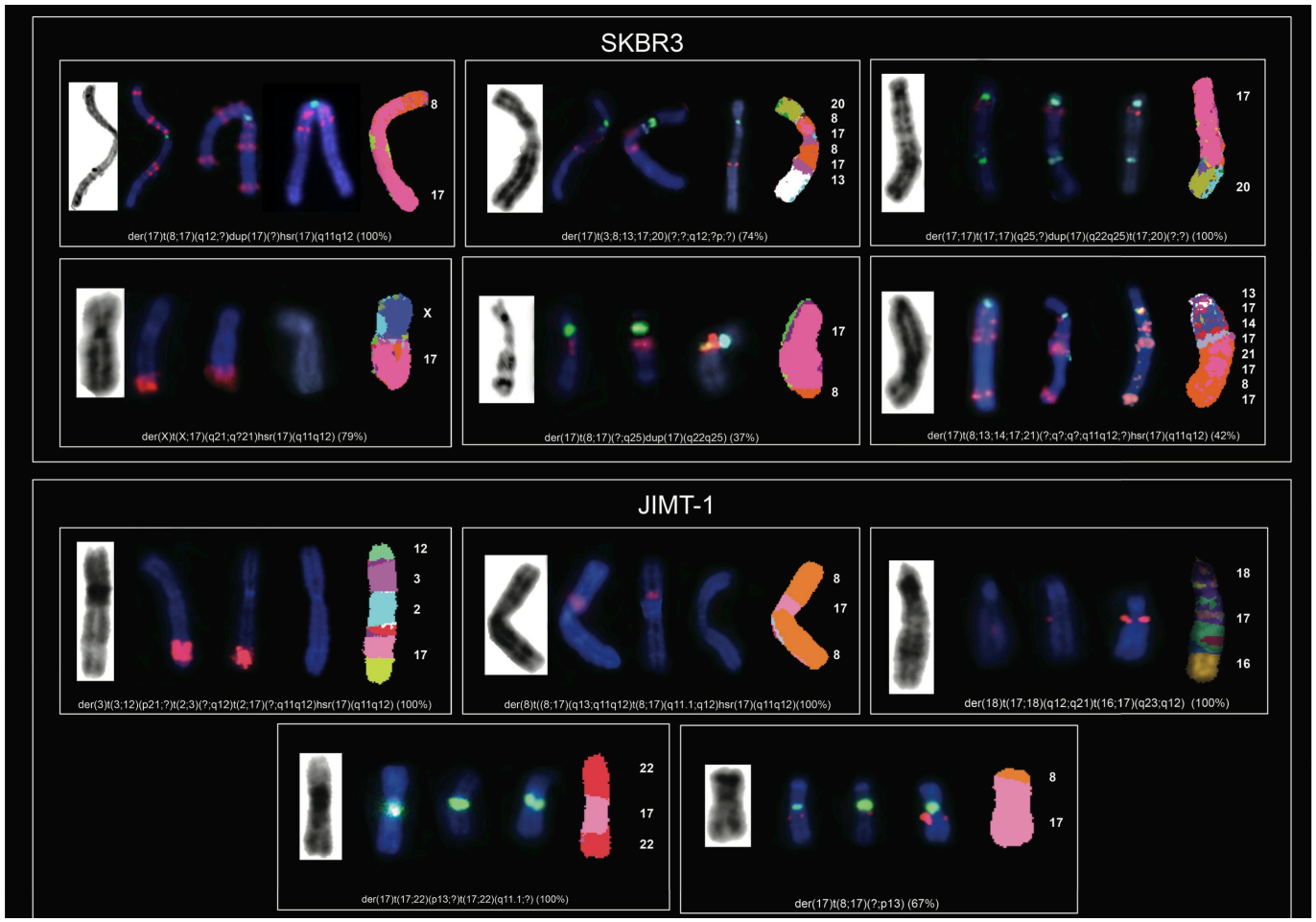

Figure 20. Analysis of Chr17 using G-Banding, dual-color FISH (*HER2*/CEP17*, STARD3*/CEP17 *and TOP2A*/CEP17) and M-FISH in SKBR3 and JIMT-1 *HER2* amplified breast cancer cell lines showing four or six translocated copies of Chr17. By M-FISH, the classified color of Chr17 is shown in pink, and the translocation partners are numbered beside the chromosomes. The frequency at which the abnormality was observed is indicated in brackets at the end of each abnormality. Otherwise, the results are shown as in Figure 1.

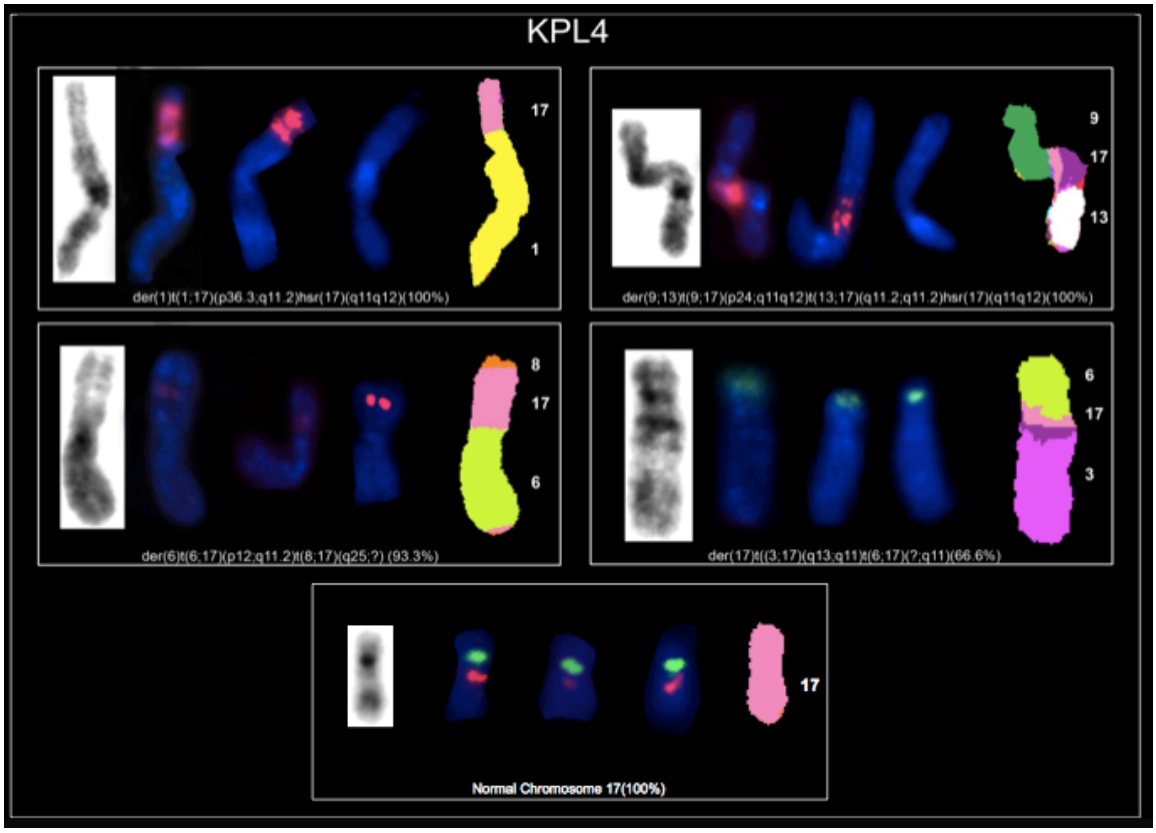

Figure 21. Analysis of Chr17 using G-Banding, dual-color FISH (*HER2*/CEP17*, STARD3*/CEP17 *and TOP2A*/CEP17) and M-FISH in KPL4 *HER2* amplified breast cancer cell line showing four translocated Chr17 in addition to the normal-appearing copies of Chr17. By M-FISH, the classified color of Chr17 is shown in pink, and the translocation partners are numbered beside the chromosomes. The frequency at which the abnormality was observed is indicated in brackets at the end of each abnormality. Otherwise, the results are shown as in Figure 1.

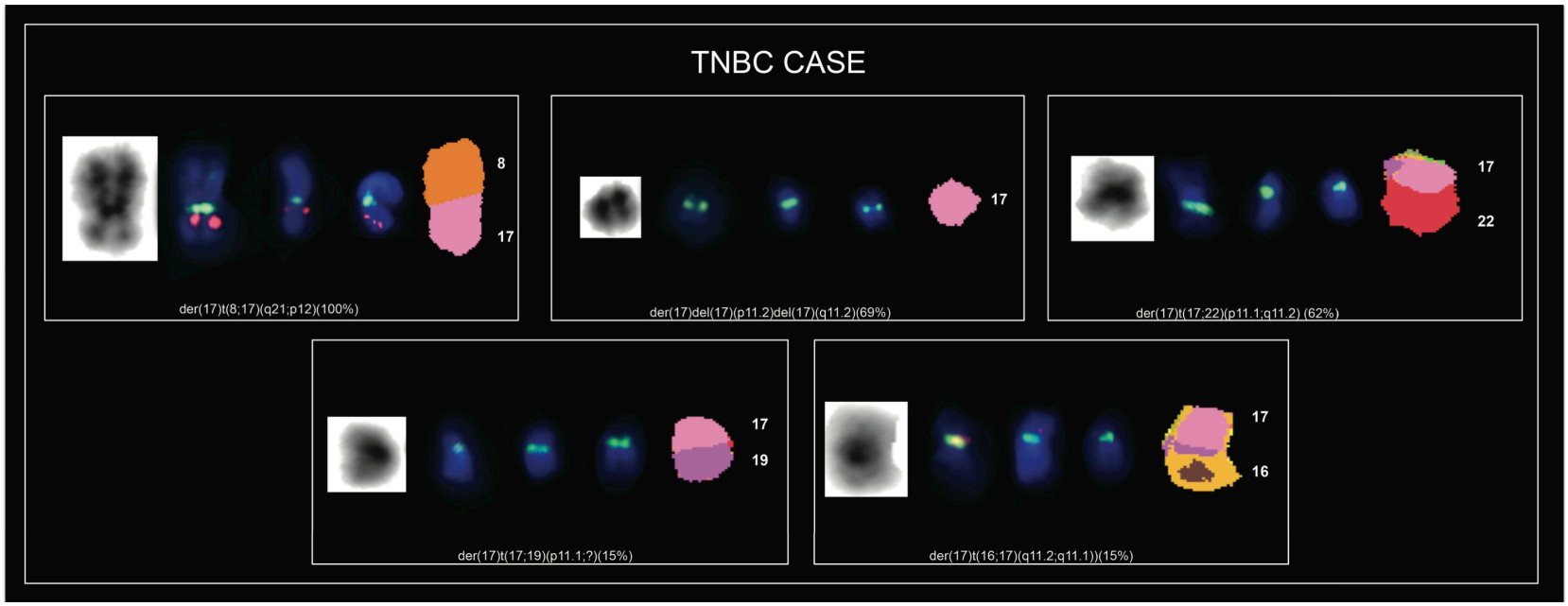

Figure 22. Analysis of Chr17 using G-Banding, dual-color FISH (*HER2*/CEP17, *STARD3*/CEP17 *and TOP2A*/CEP17) and M-FISH in one triple negative breast cancer case (TNBC) showing five translocated copies of Chr17. By M-FISH, the classified color of Chr17 is shown in pink, and the translocation partners are numbered beside the chromosomes. The frequency at which the abnormality was observed is indicated in brackets at the end of each abnormality. Otherwise, the results are shown as in Figure 1.

We defined nine regions of Chr17 frequently involved in the observed structural alterations: 17p11, 17p13, 17q11.2, 17q11-12, 17q12, 17q21, 17q22, 17q23 and 17q25. The 17q11-12 region was the most frequent long arm structural alteration. This region was affected in the BT474, MDA-MB361, SKBR3, JIMT-1 and KPL4 *HER2* amplified cell lines, while 17p11 and 17p13 were commonly affected in the MCF7, ZR-75-1, MDA-MB361 and SKBR3 cells and in the T47D, MDA-MB361 and JIMT-1 cells, respectively (Table 10).

Using G-Banding, numerous complex derivative chromosomes containing material from Chr17 were observed in all cell lines except for MDA-MB231. Some of the derivative chromosomes were present in duplicate (Table 10). Chr17 deletions and dicentric chromosomes were observed only in the T47D and SKBR3 cells.

Through M-FISH we found that Chr8 and Chr11 were the most frequent translocation partners with Chr17. Fifteen different rearrangements between Chr17 and Chr8, involving mainly their long arms (8q11.1, 8q12, 8q13, 8q21 and 8q24), were identified in MCF7, MDA-MB361, BT474, SKBR3, KPL4 and JIMT-1. Similarly, seven translocations between Chr17 (long arm) and Chr11 (11p15, 11q13 and 11q23) were identified in ZR-75-1, BT474 and KPL4. Translocations with Chr6 were observed in six cell lines, and translocations between Chr17 and chromosomes X, 1, 3, 7 and 16 were observed only in HER2+ cells (Table 11).

We identified five different alterations of Chr17 in the primary TNBC culture, involving both the short (17p11.1, 17p11.2, 17p12) and the long (17q11.1 and 17q11.2) arms. Numerous complex Chr17 derivatives containing material from chromosomes 8, 16, 19 and 22 were observed. One of these derivative chromosomes, the der(17)t(8;17)(q21;p12) was present in duplicate (Table 10, Figure 22).

Table 11. Frequency of translocation partners of Chr17 in nine breast cancer cell lines.

Translocation partner	Chromosomal abnormality	Number of abnormalities	No of cell lines	Cell lines
Chromosome 8	der	15	4	MCF7, MDA-MB361, SKBR3, JIMT-1
Chromosome 11	der	7	3	ZR-75-1, BT474, KPL4
Chromosome 6	der	6	6	MCF7, ZR-75-1, MDA-MB361, BT474, SKBR3, KPL4
Chromosome X	der	3	2	BT474, SKBR3
Chromosome 9	der dic	3 1	3	JIMT-1, KPL4, T47D T47D
Chromosome 3	der	2	2	JIMT-1, KPL4
Chromosome 7	der	2	2	MDA-MB361, JIMT-1
Chromosome 16	der	1	1	MDA-MB361
Chromosome 1	der	1	1	KPL4
Chromosome 13	der	1	1	BT474
Chromosome 17	der	1	1	SKBR3
Chromosome 19	der	1	1	MCF7
Chromosome 20	der	1	1	MDA-MB361
Chromosome 21	der	1	1	MDA-MB361
Chromosome 22	der	1	1	JIMT-1

der= derivative chromosome; dic= dicentric chromosome

3.3.2 Mapping the CEP17 and the 17q12–q21 amplicon

We considered the spatial chromosomal correlation of 3 genes mapping to 17q12–q21, (*HER2, STARD3* and *TOP2A*) as single genes with CEP17.

3.3.2.1 Triple negative cell lines

Three copies of the normal Chr17 were present in the MDA-MB231 triple negative cells, each with one CEP17 green signal and one red signal corresponding to either *HER2,* or *STARD3* or *TOP2A* (Table 12, Figures 18 and 23).

Table 12. *HER2* and *STARD3* FISH pattern and complex Chr17 rearrangements in nine-breast cancer cell lines and one primary culture raised from a triple negative breast carcinoma.

Cell line	CEP 17 signals (green)	*HER2* signals (red)		Chr17		Complex abnormalities imply in HER2 Amplification
		Cluster	Individual	Normal	Derivatives	
MCF7	4	0	2	2*	der(17)t(8;17)t(1;8)	
					der(17)t(17;19)(p11.1;p12)	
T47D	4	0	4	2*	dic(9;17)(p12;p13)*x2	
ZR-75-1	3	0	3	2*	der(17)t(6;17)(p12;p11.2)*	
BT474	6	9	6	4*	der(17)t(6;17)(?;p13)t(15;17)(q11.2;q25)hsr(17)(q11q12**)x2	der(X)t(X;17)(q13;q11q12)del(X)(p21)hsr(17)(q11q12**)x2 der(11)t(8;17)(q21.1;q11q12*)t(11;17)(p15;q11q12)x2 der(11)t(11;17)(q?14;q?11.2)hsr(17)(q11q12**) der(11)t(11;17)(q?14;?)t(8;17)(?;q?11.2)hsr(17)(q11q12**)x2 der(13)t(13;17)(q10;q11q12)t(13;17)(q10;q11q12)hsr(17)(q11q12**)x2 der(17)t(6;17)(?;p13)t(15;17)(q11.2;q25)hsr(17)(q11q12**)x2
MDA-MB361	4	1	4	0	der(17)t(6;17)(?;q21)*	der(8)t(8;17)(p21;q11q12)t(5;17)(?;q11q12)hsr(17)(q11q12**)
					der(17)t(7;17)(?;p13)*	der(8)t(8;17)(p21;q25)t(8;17)(q13;q11.2*)
					der(17)t(17;20)(p11.1;?)t(9;20)(?;q13.1)t(5;9)(q14;?)	
					der(17)t(17;21)(q21;q22)*	

SKBR3	7	16	4	0	der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12**/**/**/**/**/**)x2 der(17)t(8;17)(?;q25)dup(17)(q22q25)* der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)hsr(17)(q11q12**/**) der(17)t(3;8;13;17;17;20)(?;?;q12*;q12*;?p;?) der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?)*	der(X)t(X;17)(q21;q?21)hsr(17)(q11q12**)x2 der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12**/**/**/**/**/**)x2 der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)hsr(17)(q11q12**/**)
JIMT-1	2	2	2	0	der(17)t(8;17)(?;p13)* der(17)t(17;22)(p13;?)t(17;22)(q11.1;?)	der(3)t(3;12)(p21;?)t(2;3)(?;q12)t(2;17)(?;q11q12)hsr(17)(q11q12**) der(8)t(8;17)(q13;q11q12)t(8;17)(q11.1;q12)hsr(17)(q11q12**)
KPL4	3	2	3	2*	der(6)t(6;17)(p12;q11.2*)t(8;17)(q25;?) der(17)t(3;17)(q13;q11)t(6;17)(?;q11)	der(1)t(1;17)(p36.3;q11q12)hsr(17)(q11q12**) der(9;13)t(9;17)(p24;q11q12)t(13;17)(p11.2;q11.2)hsr(17)(q11q12**)
MDA-MB231	3	0	3	3*	0	0
TNBC CASE	4	0	2	0	der(17)t(8;17)(q21;p12)*x2 der(17)del(17)(p11.2)del(17)(q11.2) der(17)t(17;22)(p11.1;q11.2)	

* Indicates the presence of one red signal (*HER2*) on a normal Chr17 or on a derivative(s) Chr17
** Indicates the presence of one red cluster (*HER2*) on a derivative(s) Chr17
/ Indicates the presence of two red clusters (*HER2*) on a derivative(s) Chr17
//**/**/**/** Indicates the presence of six red clusters (*HER2*) on a derivative(s) Chr1

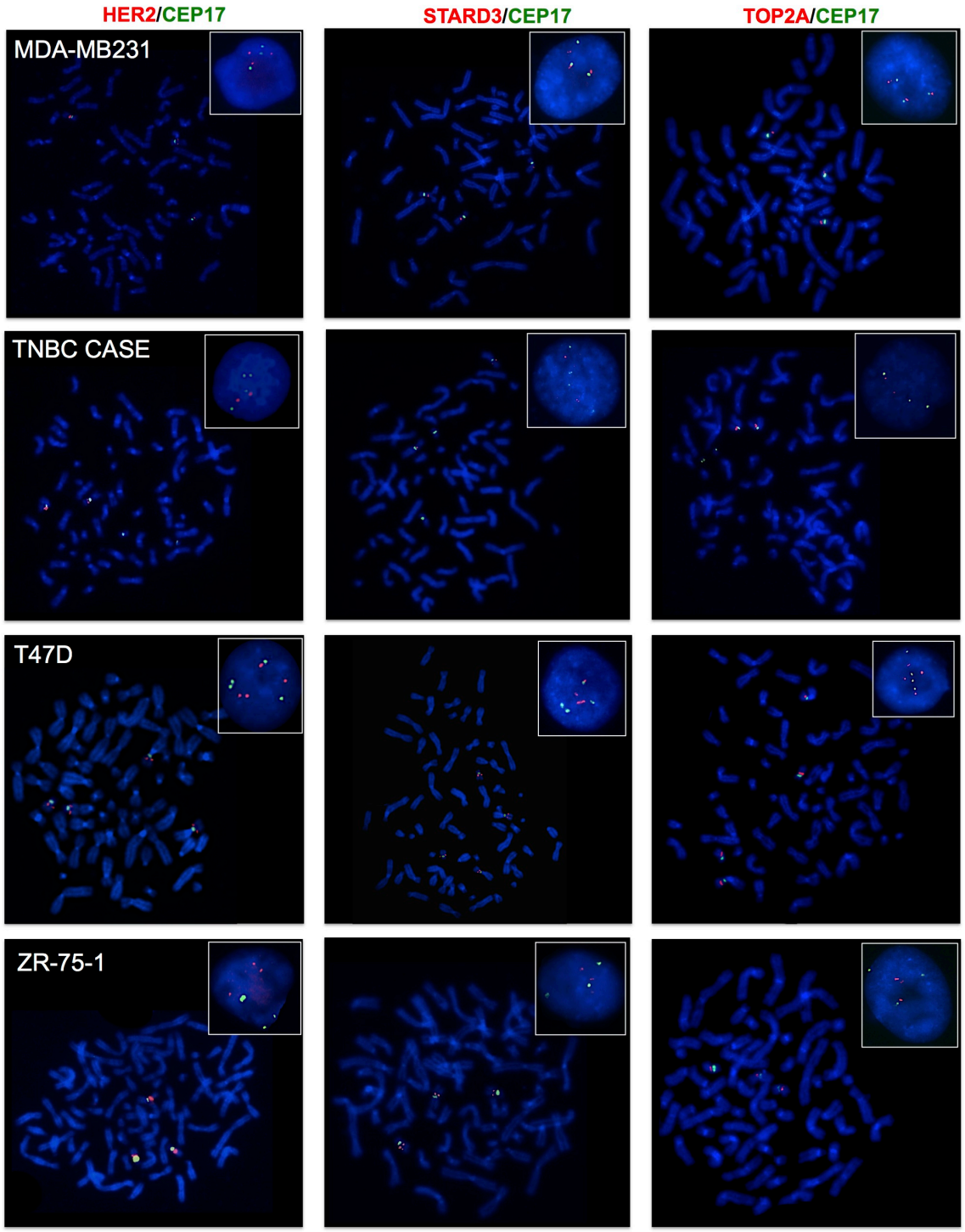

Figure 23. Representative FISH images of the MDA-MB231, T47D and ZR-75-1 breast cancer cells and one TNBC case using *HER2*/CEP17, *STARD3*/CEP17 and *TOP2A*/CEP17 dual-color probes. None of these cell lines showed

amplification of the *HER2, STARD3* or *TOP2A* genes. The MDA-MB231 cells had three normal copies of Chr17, each with a CEP17 green signal and one red signal corresponding to either *HER2,* or *STARD3* or *TOP2A*. The TNBC primary culture cells displayed the same FISH pattern for the *HER2, STARD3*, *TOP2A* genes and CEP17; four CEP17 green signals and two red signals were observed. The T47D and ZR-75-1 cells showed the same copy number for the *HER2, STARD3* and *TOP2A* genes (four and three red signals, respectively) and for CEP17 (four and three green signals, respectively). Boxes indicate representative nuclei in each case. All pictures were taken at 100x magnification.

The TNBC primary culture cells displayed the same FISH pattern for the *HER2, STARD3*, *TOP2A* genes and CEP17. Four green CEP17 signals and two red signals were observed (Figure 23). Two red and two green signals corresponded to two Chr17 derivatives, namely der(17)t(8;17)(q21;p12)x2 (100%), while the other two green signals (without the *HER2, STARD3* and *TOP2A* genes) mapped to der(17)del(17)(p11.2)del(17)(q11.2) (69%) and der(17)t(17;22)(p11.1;q11.2) (62%). Two additional complex alterations of Chr17 were also observed but at a lower frequency, including der(17)t(16;17)(q11.2;q11.1) (15%) and der(17)t(17;19)(p11.1;?) (15%) (Table 12, Figure 22 and 23).

3.3.2.2 ER± and *HER2 gene* not amplified cell lines

In T47D and ZR-75-1 interphase nuclei, the same copy numbers of *HER2, STARD3* and *TOP2A* genes and of CEP17 were observed (Table 12, Figure 23). Four copies were observed in the T47D nuclei and three in the ZR-75-1 nuclei (Table 12, Figure 23).

In the T47D interphase nuclei, FISH showed two normal copies of Chr17 and two Chr17 derivatives that also involved Chr9, which carried both CEP17 and the three genes. M-FISH showed that the derivative chromosome previously reported as der(9)t(9;17)(p13;q11) (120) was a dic(9;17)t(9;17)(p12;p13) (Figures 18 and 23).

In ZR-75-1 (Table 12, Figure 18 and Figure 23), M-FISH showed two normal Chr17 and one derivative Chr17: der(17)t(6;17)(p12;p11.2), both carrying the *HER2, STARD3* and *TOP2A* genes.

MCF7 interphase nuclei displayed four CEP17 green signals and two red signals for the *HER2* and *STARD3* genes (Table 12, Figure 24). This pattern corresponded to one CEP17 signal and one copy of the *HER2* and *STARD3* genes located on two normal Chr17 and two CEP17 signals on two Chr17 derivatives: der(17)t(8;17)t(1;8) (100%) and der(17)t(17;19)(p11.1;p12) (65%), as confirmed by M-FISH (Figure 18). The FISH pattern for *TOP2A* was similar to that observed for the *HER2* and *STARD3* genes, with the only exception of having an additional

TOP2A copy mapping on a derivative Chr6, namely der(6)t(6;17;16)(q25;q21;?) (Figure 18) without CEP17 signals.

3.3.2.3 HER2 gene amplified cell lines

HER2, TOP2A and STARD3 gene amplifications were found within chromosomes as homogeneously staining regions (HSRs) but not in extra-chromosomal, double-minute chromosomes (DMs). All of these cell lines showed *HER2* and *STARD3* co-amplification.

In BT474 interphase nuclei, six CEP17 signals and nine clusters and six individual red signals (*HER2* and *STARD3*) were observed by FISH (Figure 24), which corresponded on the FISH karyotype to four CEP17 and four red signals on four normal copies of Chr17, and two CEP17 signals and two clusters of red signals located on two Chr17 derivatives: der(17)t(6;17)(?;p13)t(15;17)(q11.2;q25)hsr(17)(q11q12)x2 (96%). The remaining seven clusters of red signals mapped to five previously unreported highly rearranged chromosomes: der(11)t(8;17)(q21.1;q11q12)t(11;17)(p15;q11q12) (100%), der(13)t(13;17)(q10;q11q12)t(13;17)(q10;q11q12)hsr(17)(q11q12) (87%), der(11)t(11;17)(q?14;?)t(8;17)(?;q?11.2)hsr(17)(q11q12)x2 (57%), der(X)t(X;17)(q13;q11q12)del(X)(p21)hsr(17)(q11q12)x2 (39%) and der(11)t(11;17)(q?14;q?11.2)hsr(17)(q11q12) (39%) (Table 12, Figures 19 and 24). BT474 cells showed normal *TOP2A* gene copy number, and four red signals were observed on four normal copies of Chr17 (Figures 19 and 24).

In the MDA-MB361 nuclei, four CEP17 signals and one red cluster and four individual red signals (*HER2* and *STARD3*) were observed. None of these green and red signals were located on normal copies of Chr17 but were instead located on highly rearranged chromosomes (Table 12, Figures 19 and 24). Three individual red signals were correlated with the centromeric locus and located on three Chr17 derivatives: der(17)t(6;17)(?;q21), der(17)t(7;17)(?;p13) and der(17)t(17;21)(q21;q22). The other individual red signal mapped to a Chr8 derivative, der(8)t(8;17)(p21;q25)t(8;17)(q13;q11.2), and the only red cluster, indicative of *HER2* and *STARD3* amplification, was located on the Chr8 derivative, der(8)t(8;17)(p21;q11q12)t(5;17)(?;q11q12)hsr(17)(q11q12). The remaining CEP17 signal, without red signal (*HER2* and *STARD3* deletion), mapped to a complex translocation of Chr17 involving chromosomes 5, 9 and 20, i.e., der(17)t(17;20)(p11.1;?)t(9;20)(?;q13.1)t(5;9)(q14;?). These cells harbored a *TOP2A* deletion, as four chromosomes with CEP17 were identified, but only one of them had a *TOP2A* signal, appearing on der(17)t(7;17)(?;p13) (Figures 19 and 24).

In the SKBR3 cells, *HER2* and *STARD3* co-amplification was observed in 100% of metaphases and interphase nuclei analyzed. Seven CEP17 signals and sixteen clusters and four individual red signals (*HER2* and *STARD3*) were observed on numerous chromosomes (Figure 24). Because in these cells no

normal copies of Chr17 were observed, these signals were located on complex rearranged chromosomes as follows: one CEP17 signal and six red clusters (indicative of *HER2* and *STARD3* gene amplification) mapped to two Chr17 derivatives, der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12)x2 (100%); one CEP17 and two red cluster signals were located on another Chr17 derivative, der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)hsr(17)(q11q12) (42%); and two red clusters (without CEP17 signals) were located on two derivatives of ChrX, der(X)t(X;17)(q21;q?21)hsr(17)(q11q12)x2 (79%). The remaining four CEP17 and four individual red signals mapped as follows: two CEP17 and one red signal mapped to the dicentric Chr17, der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?) (100%), which had not been previously reported. One CEP17 and two red signals mapped to der(17)t(3;8;13;17;17;20)(?;?;q12;q12;?p;?) (74%), and one CEP17 and one red signal mapped to der(17)t(8;17)(?;q25)dup(17)(q22q25) (37%) (Table 12, Figures 20 and 24). In addition, SKBR3 cells showed *TOP2A* gene amplification with eleven predominantly red signals (Figures 20 and 24); however, the pattern of this amplification was different from that observed for the *HER2* and *STARD3* genes. *TOP2A* was co-amplified with *HER2* in the same region of *HER2* locus on der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)hsr(17)(q11q12) as well as in a different region from *HER2* in der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12). In addition, *TOP2A* deletion was detected on der(X)t(X;17)(q21;q?21)hsr(17)(q11q12). In the remaining derivative chromosomes without gene amplification, *TOP2A* showed the same FISH pattern observed for *HER2*, in which all solitary *HER2* genes were accompanied by solitary *TOP2A* genes (Figures 20 and 24).

In the JIMT-1 cells, two CEP 17 signals and two clusters and two individual red signals were observed (Figure 25). The two clusters of red signals (indicative of *HER2* and *STARD3* gene amplification) mapped to two chromosomes lacking CEP17, including der(3)t(3;12)(p21;?)t(2;3)(?;q12)t(2;17)(?;q11q12)hsr(17)(q11q12) (100%) and der(8)t(8;17)(q13;q11q12)t(8;17)(q11.1;q12)hsr(17)(q11q12) (100%). One of the two CEP17 signals and one of the two individual red signals were observed on a Chr17 derivative, i.e., der(17)t(8;17)(?;p13) (67%), while the remaining red signal was present on a Chr18 derivative, der(18)t(17;18)(q12;q21)t(16;17)(q23;q12) (100%). We also observed *HER2* and *STARD3* deletion (one CEP17 signal without red signal) on der(17)t(17;22)(p13;?)t(17;22)(q11.1;?) (100%). *The TOP2A* gen was not amplified, and the FISH pattern showed two red and two CEP17 signals: one CEP17 and one red signal mapped to the Chr17 derivative der(17)(8;17)(?;p13), while one red signal mapped to der(18)t(17;18)(q12;q21)t(16;17)(q23;q12). In addition, a deletion of the *TOP2A* gene was observed on der(17)t(17;22)(p13;?)t(17;22)(q11.1;?), similar to that observed for the *HER2* and *STARD3* genes. *TOP2A* signals were not observed on derivative chromosomes with *HER2* gene amplifications (Figures 20 and 25).

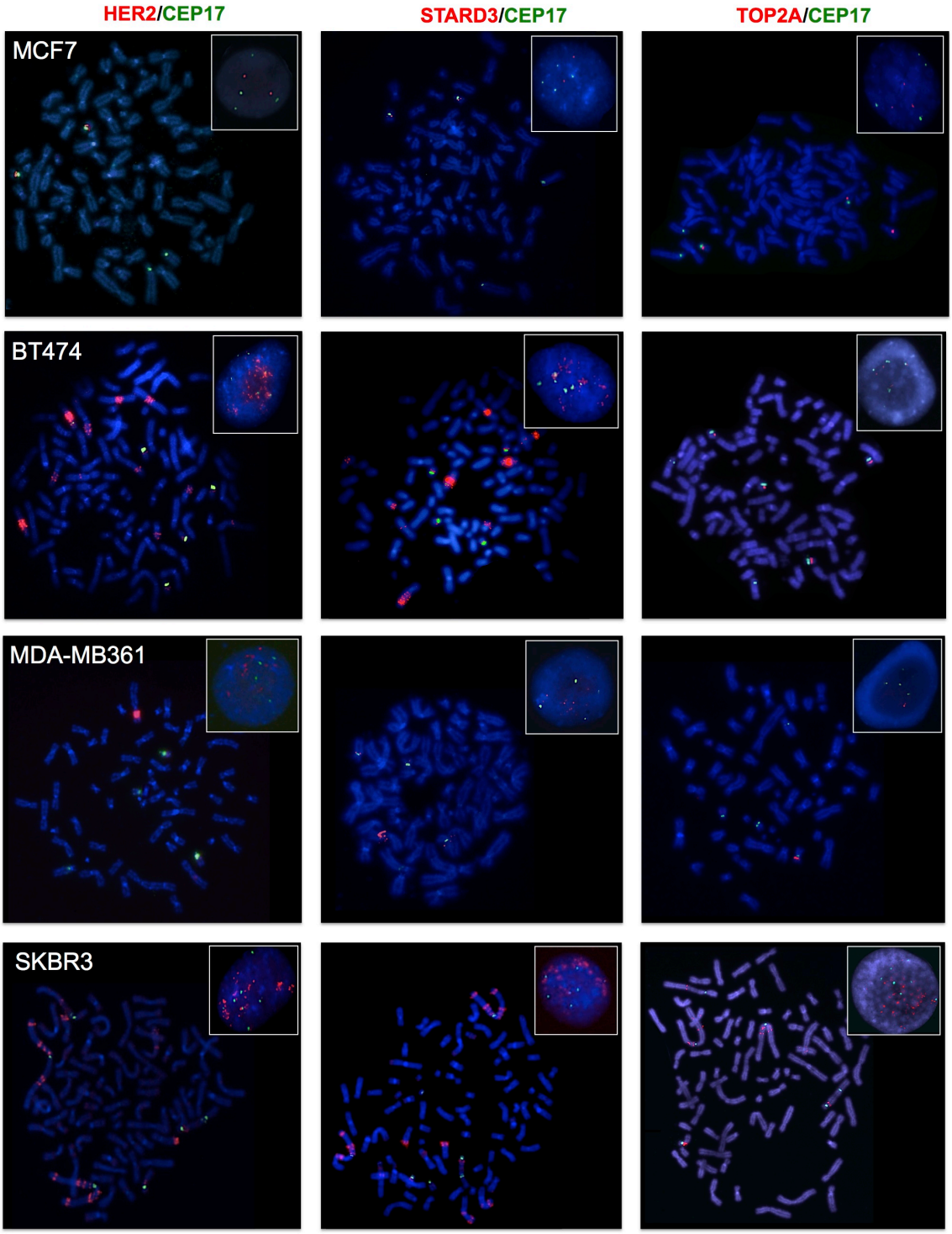

Figure 24. Representative FISH images of the MCF7, BT474, MDA-MB361 and SKBR3 breast cancer cell lines using *HER2*/CEP17, *STARD3*/CEP17 and *TOP2A*/CEP17 dual-color probes. FISH images for the BT474, MDA-MB361 and SKBR3 cells demonstrated that *HER2* and *STARD3* genes were amplified (red

signals), while *TOP2A* gene amplification was only observed in the SKBR3 cells. The MCF7 cells displayed four CEP17 green signals, two red signals for *HER2* and *STARD3* and three red signals for *TOP2A*. The BT474 cells showed six CEP17 green signals, multiple red signals for the *HER2* and *STARD3* genes and four red signals for the *TOP2A* gene. The MDA-MB361 cells displayed four CEP17 green signals, several red signals for the *HER2* and *STARD3* genes and one red signal for the *TOP2A* gene. SKBR3 showed seven CEP17 green signals and multiple red signals for the *HER2, STARD3* and *TOP2A* genes. Boxes indicate representative nuclei in each case. All pictures were taken at 100x magnification

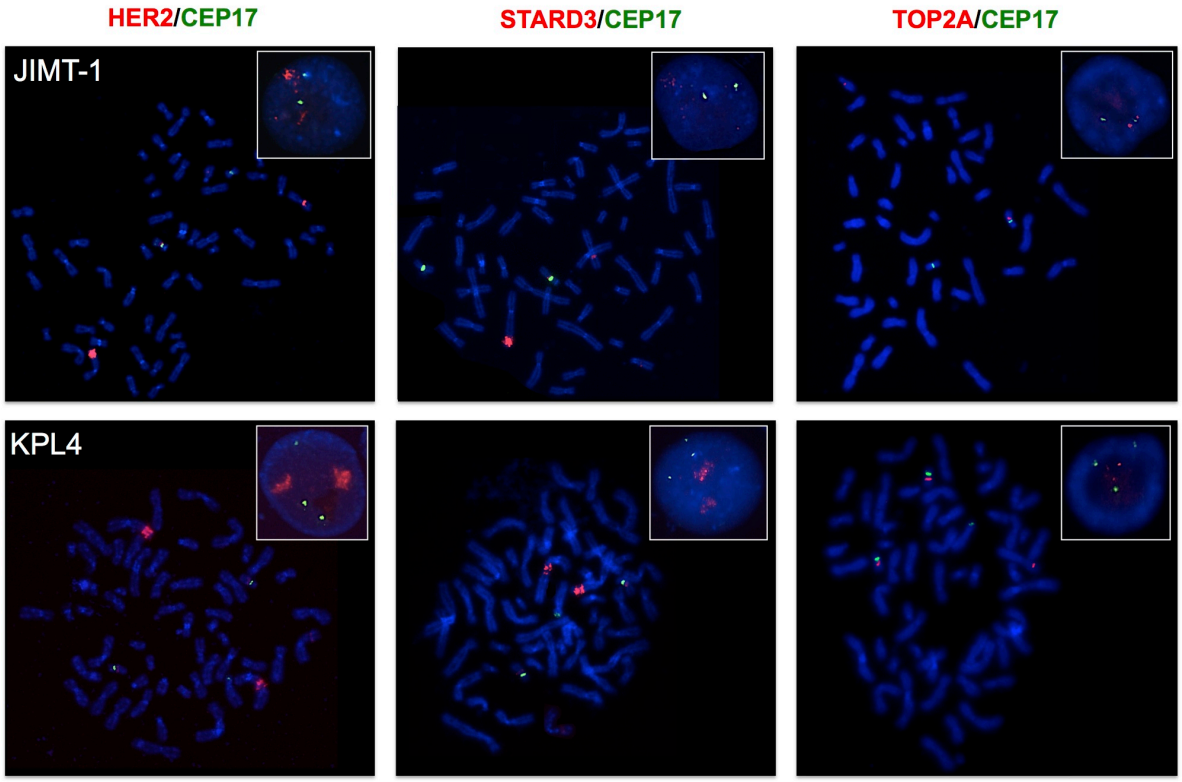

Figure 25. Representative FISH images of the JIMT-1 and KPL4 breast cancer cell lines using *HER2*/CEP17, *STARD3*/CEP17 and *TOP2A*/CEP17 dual-color probes. FISH images for the JIMT-1 and KPL4 cells demonstrate *HER2* and *STARD3* gene amplification (red signals), while *TOP2A* gene amplification was not observed in these cells. JIMT-1 cells displayed two CEP17 green signals, several red signals for the *HER2* and *STARD3* genes and two red signals for the *TOP2A* gene. The KPL4 cells showed three CEP17 green signals, many red signals for the *HER2* and *STARD3* genes and three red signals for the *TOP2A* gene. Boxes indicate a representative nuclei image for each case. All pictures were taken at 100x magnification.

The KPL4 cells showed three CEP17 signals and two clusters and three individual red signals (Figure 25). Two CEP17 and two red signals were located on two normal copies of Chr17. The other green and red individual signals corresponded to complex rearrangements involving Chr17 der(17)t(3;17)(q13;q11)t(6;17)(?;q11) (67%) and der(6)t(6;17)(p12;q11.2)t(8;17)(q25;?) (93%) (Table 12, Figures 21 and 25). Similar to the JIMT-1 cells, the *HER2* and *STARD3* gene clusters were not present on normal Chr17 copies but were instead located on highly rearranged chromosomes, with one cluster found on der(1)t(1;17)(p36.3;q11q12)hsr(17)(q11q12) (100%) and the other one on the der(9;13)t(9;17)(p24;q11q12)t(13;17)(p11.2;q11.2)hsr(17)(q11q12) (100%).
These cells did not show *TOP2A* gene amplification. Instead, two CEP17 and two red signals were observed on two normal Chr17 copies, and one red signal mapped to one der(6)t(6;17)(p12;q11.2)t(8;17)(q25;?) (Figure 21 and Figure 25).

3.4 DISCUSSION

In this study, we reported the type and frequency of complex Chr17 alterations in nine breast cancer cell lines and in primary cells obtained from a TNBC culture. This extensive cytogenetic analysis of Chr17 demonstrated that only the MDA-MB231 triple negative cell line and the BT474 HER2+ cell line showed true Chr17 polysomy (normal chromosome acquisition). In ER+/*HER2*- cell lines, normal copies of Chr17 coexist with rearranged Chr17 copies that either harbor or do not harbor the *HER2* genes. On the other hand, some of the *HER2*+ cell lines did not show any normal copies of Chr17; the *HER2*-*STARD3* gene clusters were observed as HSR on complex rearranged chromosomes, and the Chr17 derivatives carrying CEP17 did not always carry *HER2* gene clusters, and these latter were not only observed in Chr17 derivatives. In particular, in the BT474 cells, 7 of 9 *HER2* gene clusters were found on derivatives lacking CEP17.

In breast cancer specimens, the analysis of the *HER2* gene in interphase nuclei is requested after an equivocal immunohistochemical result (score 2+) of HER2 expression (130, 153). Either double-signal (*HER2* and CEP17) or single-signal (*HER2*) assays may be performed assessing the *HER2*/CEP17 ratio or the absolute *HER2* copy number, respectively. In the case of the double-signal assay, the recent ASCO/CAP guidelines (153) recommend using the *HER2*/CEP17 ratio to screen for amplified or not amplified breast cancer. However, our data provide another line of evidence that the interpretation of the results of the ISH analyses on interphase nuclei using a dual-signal assay should be performed “with caution”, given the high frequency of complex Chr17 abnormalities observed here. By comparing the *HER2*/CEP17 FISH pattern in metaphases *versus* interphase nuclei, the present study demonstrated that a CEP17 signal rarely corresponds to a single intact Chr17, in both HER2+ and HER2- cell lines as well as in cells cultured from a metastatic effusion of a TNBC. Thus, the evaluation of the *HER2*/CEP17 ratio as

the only parameter to define *HER2* amplification for double-signal assays does not reflect the real status of the gene. In this respect, the introduction by ASCO/CAP 2013 of an algorithm that takes into account the ratio first and then the *HER2* gene copy numbers represents an improvement in the identification of HER2 positive tumors by dual-signal assays. However, we should point out that the sole *HER2* gene copy number methods, as used in single-signal assay, best identifies *HER2* gene amplification in interphase nuclei, as CEP17 copy numbers does not reflect Chr17 copy numbers.

Analysis of the five *HER2* amplified cell lines revealed *HER2* and *STARD3* co-amplification. The simultaneous amplification of *HER2* and *TOP2A* was only observed in SKBR3 cells, in which these genes mapped to distinct chromosomes. This observation provides another line of evidence that, despite the genomic proximity of *HER2* and *TOP2A* and the observation that *TOP2A* amplification seems to be restricted to tumors harboring *HER2* amplifications, these two genes are likely to pertain to separate amplicons, as suggested previously (154, 155). Then one may speculate that secondary rearrangements may intervene to separate the two genes from the primary amplicons.

The Chr17 was frequently translocated with Chr8 and Chr11. These chromosomes have been observed in translocations in many breast cancer cell lines (12, 156) and have also been shown to participate in translocation events in cases of primary breast carcinoma (157). Cytogenetic analysis of primary cultures would be of invaluable help in understanding whether such alterations recapitulate those of primary tumors. In the primary culture analyzed here, Chr8 was involved in one of the translocations in the primary culture from a TNBC.

3.5 CONCLUSIONS

In conclusion, the results of the traditional karyotyping and of FISH and M-FISH assays on metaphase and nuclei reported in this study, highlight that complex structural alterations of Chr17 encompassing *HER2* genes and CEP17 are common in breast cancer cell lines. This may reflect the scenario found in breast carcinomas, as this finding was also observed in the primary cell culture raised from a TNBC. The *HER2*/CEP17 ratio of interphase nuclei is routinely used to select patients for eligibility for anti-HER2 treatment. However, this assessment should be considered with caution and always coupled with the *HER2* gene copy number values in order to not misinterpret *HER2* gene amplification, as recently updated in the ASCO/CAP 2013.