Effect of low doses of estradiol and tamoxifen on breast cancer cell karyotypes

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Abstract

Evidence supports a role of 17β-estradiol (E_2) in carcinogenesis and the large majority of breast carcinomas are dependent on estrogen. The anti-estrogen tamoxifen (TAM) is widely used for both treatment and prevention of breast cancer; however, it is also carcinogenic in human uterus and rat liver, highlighting the profound complexity of its actions. The nature of E_2- or TAM-induced chromosomal damage has been explored using relatively high concentrations of these agents, and only some numerical aberrations and chromosomal breaks have been analyzed. This study aimed to determine the effects of low doses of E_2 and TAM (10^{-8} mol L^{-1} and 10^{-6} mol L^{-1} respectively) on karyotypes of MCF7, T47D, BT474, and SKBR3 breast cancer cells by comparing the results of conventional karyotyping and multi-FISH painting with cell proliferation. Estrogen receptor (ER)-positive (+) cells showed an increase in cell proliferation after E_2 treatment (MCF7, T47D, and BT474) and a decrease after TAM treatment (MCF7 and T47D), whereas in ER− cells (SKBR3), no alterations in cell proliferation were observed, except for a small increase at 96 h. Karyotypes of both ER+ and ER− breast cancer cells increased in complexity after treatments with E_2 and TAM leading to specific chromosomal abnormalities, some of which were consistent throughout the treatment duration. This genotoxic effect was higher in HER2+ cells. The ER−/HER2+ SKBR3 cells were found to be sensitive to TAM, exhibiting an increase in chromosomal aberrations. These *in vitro* results provide insights into the potential role of low doses of E_2 and TAM in inducing chromosomal rearrangements in breast cancer cells.

Key Words
- breast cancer cells
- estradiol
- tamoxifen
- chromosomal abnormalities
- chromosomal instability

Endocrine-Related Cancer (2016) **23**, 635–650

Introduction

17β-estradiol (E_2) is the main estrogenic hormone that through the estrogen receptors (ER) acts on the mammary gland regulating a wide variety of biological processes including differentiation, cell proliferation, and development at puberty and during sexual maturity. E_2 may be procancerogenic by inducing (i) ER-mediated cell proliferation, (ii) gene mutation through a cytochrome P450-mediated metabolic activation, and (iii) aneuploidy

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-16-0078

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(Russo & Russo 2006), through overexpression of Aurora-A (Aur-A), a centrosome kinase, and centrosome amplification (Li *et al*. 2004). In addition, in both ER+ and ER− breast cancer cells, E_2 may induce chromatin structural changes through the estrogen-related receptors (ERR) (Hu *et al*. 2008). Although high levels of E_2 are implicated in breast cancer in postmenopausal women (Bernstein & Ross 1993), constant low E_2 concentrations, in the range of picograms, are sufficient to increase breast cancer risk in premenopausal women (Chetrite *et al*. 2000).

Tamoxifen (TAM) is a non-steroidal anti-estrogen with partial agonistic activity, extensively used in the treatment of ERα-positive breast cancer. Response to TAM is frequently of limited duration due to the development of resistance (Pearce & Jordan 2004, International Breast Cancer Study *et al*. 2006). Although ERα positivity is a well-established predictor of response to TAM and ERα-negative patients are considered nonresponders, it is known that 5–10% of ERα-negative tumors do benefit from adjuvant TAM treatment (McGuire 1975, Early Breast Cancer Trialists' Collaborative Group 1992, 1998, Early Breast Cancer Trialists' Collaborative Group *et al*. 2011, Gruvberger-Saal *et al*. 2007).

Paradoxically, it has been reported that TAM possesses a high mutagenic potential causing chromosome ruptures in animal models (Mizutani *et al*. 2004). However, data on type and frequency of chromosome abnormalities induced by TAM are scant (Mizutani *et al*. 2004). In particular, cytogenetic studies about the effects of low doses of TAM, as it is suggested for treatment of pre-invasive low-grade breast lesions (e.g., low-grade ductal carcinomas *in situ* or lobular intraepithelial neoplasia), are limited (Kedia-Mokashi *et al*. 2010). The nature of E_2- or TAM-induced chromosomal damage has been explored using relatively high concentrations of these agents, and only some numerical aberrations and chromosomal breaks have been analyzed (Tsutsui & Barrett 1997, Mizutani *et al*. 2004, Quick *et al*. 2008, Kedia-Mokashi *et al*. 2010).

The aim of this study was to determine the effects of low doses of E_2 and TAM on chromosomal rearrangements by comparing the results of conventional karyotyping and multicolor fluorescence *in situ* hybridization (M-FISH) painting with cell proliferation activity of human breast cancer cells with differential expression of ER and HER2.

Materials and methods

Cell lines

The human breast cancer cell lines MCF7 and T47D (ER+/progesterone receptor (PR)+/HER2−), BT474 (ER+/PR+/HER2+), and SKBR3 (ER−/PR−/HER2+) were obtained from the American Type Culture Collection (ATCC) in March 2010. Cell lines were expanded and stocked at −80°C and cells obtained from these stocks were thawed and used for the experiments. At the end of experiments, short tandem repeat (STR) profiles were performed to confirm the authentication of the cell lines used. All experiments were carried out in each cell line at passages (P) below 30.

MCF7 (P19), T47D (P20), and SKBR3 (P16) were cultured in RPMI-1640 medium (Sigma), whereas BT474 (P18) was cultured in DMEM medium (Sigma). All culture media were supplemented with 10% fetal bovine serum (FBS) (Sigma), antibiotic–antimycotic solution (1X) (Sigma), and L-glutamine (2 mM) (Invitrogen GmbH). Cells growing in 75 cm^2 flasks were maintained at 37°C and 5% CO_2. The absence of contamination with mycoplasma was demonstrated by PCR assay.

E_2 and TAM treatment

In order to remove endogenous serum steroids and exclude the weak estrogen agonistic activity of phenol red (Berthois *et al*. 1986), 48 h before the addition of E_2 (E2758; Sigma) and TAM (T5648; Sigma) cells were washed with 5 mL phosphate-buffered saline (PBS) and then switched to phenol red-free RPMI-1640 (Sigma) containing 10% charcoal-stripped FBS (Sigma). E_2 and TAM were dissolved in absolute ethanol and diluted in the media at 10^{-8} mol L^{-1} and 10^{-6} mol L^{-1}, respectively, and then added to the culture medium at 24, 48, and 96 h. These concentrations have been demonstrated to be the lowest to induce an effect on the architecture of the cytoskeleton in breast cancer cells *in vitro* (Sapino *et al*. 1986).

Cells without treatment at 24 h (T24 h) and at 96 h (T96 h) were used as controls.

Proliferation assay

Cells were seeded at a density of $2.5–5 \times 10^3$ cells per 100 µL of phenol red-free medium in a 96 multi-well plate and after 24 h were treated with E_2 and TAM for 24, 48, and 96 h. At the end of each treatment, cell proliferation was assessed using the cell proliferation ELISA kit, BrdU (Roche Diagnostics Deutschland GmbH). Measurement of absorbance was performed by using a MultiSkan Bichromatic reader (Labsystems, Midland, Canada) against a background control as blank. Each treatment was performed in 24 replicates and expressed as means ± standard deviation (S.D.).

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Metaphase spreads and G-banding

To determine whether E_2 and TAM treatment resulted in the induction of chromosomal abnormalities, we performed conventional and molecular cytogenetic analysis in parallel with the evaluation of cell proliferation. Metaphases were obtained by using standardized harvesting protocols in order to perform conventional and molecular cytogenetic analysis (multi-FISH and FISH). Briefly, colcemid solution (0.03 µg/mL) (Sigma) was added to cultures 2.5 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. For analysis of chromosomal alterations, the slides were banded with G-banding. Glass slides were baked at 70°C for 24 h, incubated in HCl, and placed in 2xSSC buffer before treatment with Wright's stain. Metaphase image acquisition and subsequent karyotyping were performed using a Nikon microscope with the cytogenetic software CytoVision System (Applied Imaging, Santa Clara, CA, USA). According to the International System of Cytogenetic Nomenclature (Shaffer *et al*. 2013) "The general rule in tumor cytogenetics is that only the clonal chromosomal abnormalities should be reported", whereas a minimal number of metaphases to be analyzed is not indicated. In this respect, we indicated only those alterations present in at least two metaphases, which is indicative of clonal chromosomal alterations (Shaffer *et al*. 2013). Based on these premises, we systematically analyzed 100 metaphases in order to establish the frequency of ploidy after treatments, by counting the number of chromosomes. As a second step, out of these metaphases, only those with good morphology and proper separation of chromosomes were analyzed by M-FISH and G-banding (between 11 and 26). Chromosome aberrations were described according to the International System of Human Cytogenetic Nomenclature (ISCN 2013) (Shaffer *et al*. 2013).

Multi-FISH (M-FISH)

M-FISH was performed with the aim of identifying complex chromosomal rearrangements. The probe cocktail containing 24 differentially labeled chromosome-specific painting probes (24xCyte kit MetaSystems, Altlussheim, Germany) was used according to the protocol recommended by Human Multicolor FISH kit (MetaSystems, Altlussheim, Germany). Briefly, the slides were incubated at 70°C in saline solution (2xSSC), denatured in NaOH, dehydrated in ethanol series, air-dried, covered with 10 µL of probe cocktail (denatured), and hybridized for 2 days at 37°C. Slides were then washed with post-hybridization buffers, dehydrated in ethanol series, and counterstained with 10 µL of DAPI/antifade. Signal detection and subsequent metaphase analysis were done using the Metafer system and Metasytems' ISIS software (software for spectral karyotypes) (Carl Zeiss, Metasystems, GmbH, Germany) (Rondon-Lagos *et al*. 2014*a*,*b*).

Immunohistochemistry (IHC)

Immunohistochemistry for ER and PR was carried out on MCF7, T47D, BT474, and SKBR3 cells at baseline and treated with E_2 (10^{-8} mol L^{-1}) and TAM (10^{-6} mol L^{-1}) for 24, 48, and 96 h. At each time point, cells were harvested, formalin-fixed, and paraffin-embedded according to standard procedures. Sections of the representative cell block were cut at 3 µm and mounted on electrostatically charged slides. Immunohistochemistry was performed using an automated immunostainer (Ventana BenchMark XT AutoStainer; Ventana Medical Systems, Tucson, AZ, USA) with antibodies against ER (Clone SP1, prediluted, Ventana) and PR (Clone 1A6, 1:50 diluted; Leica Biosystems). Positive and negative controls were included for each immunohistochemical run. IHC slides were scanned by using the Aperio system (ScanScope CS System, Vista, CA, USA) for automated counting. To ensure the reliability of the automatic assessment, stainings were reviewed by two pathologists (A S and C M).

Data analysis

The profile of numeric and structural chromosomal changes observed after treatments was determined in comparison with the control. Student's *t*-test was performed to compare cell proliferation of treated cell lines with untreated cell lines. Fisher's exact test was applied to compare conventional and molecular cytogenetic results from treated cell lines with the results from control cell lines (differences in single chromosomal alterations between control and treated cells). In addition, Pearson's χ^2 test was used to investigate a possible association between occurrence of specific chromosomal aberrations at each time point and effect on proliferation. The coefficient of variation, CV (=100 × standard deviation/mean), was used to calculate the variability in the frequency of new chromosomal alterations, observed after E_2 and TAM treatments (24, 48, and 96 h). P values <0.05

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were considered as statistically significant. All statistical analyses were performed using the SPSS v.20 program.

Results

General effects on chromosomes induced by low doses of E_2 and TAM

Control cells harbored the same alterations previously reported (Rondon-Lagos *et al.* 2014*a*,*b*). Both E_2 and TAM treatments rapidly induced *de novo* chromosomal alterations.

The frequency of new chromosomal alterations changed along E_2 and TAM treatments for all cell lines, and while the frequency of some chromosomal abnormalities remained constant along treatments, other increased or decreased (CV range: 3–96%) (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). This variability is not surprising, considering that genetic diversification, clonal expansion, and clonal selection are events widely reported in cancer and also associated with therapeutic interventions (Greaves & Maley 2012).

More in detail, compared with control cells (T24 h and T96 h without treatment), low doses of E_2 increased the chromosome ploidy in all cell lines (Table 1A), whereas TAM was effective on ploidy only in HER2+ cell lines (Table 1B). Some of the alterations were observed in more than one cell line and were induced by both E_2 and TAM (Fig. 2 and Supplementary Table 2). In Fig. 3, the chromosomal aberrations induced or increased after E_2 or TAM treatments as compared with control cells are represented. Low doses of E_2 produced

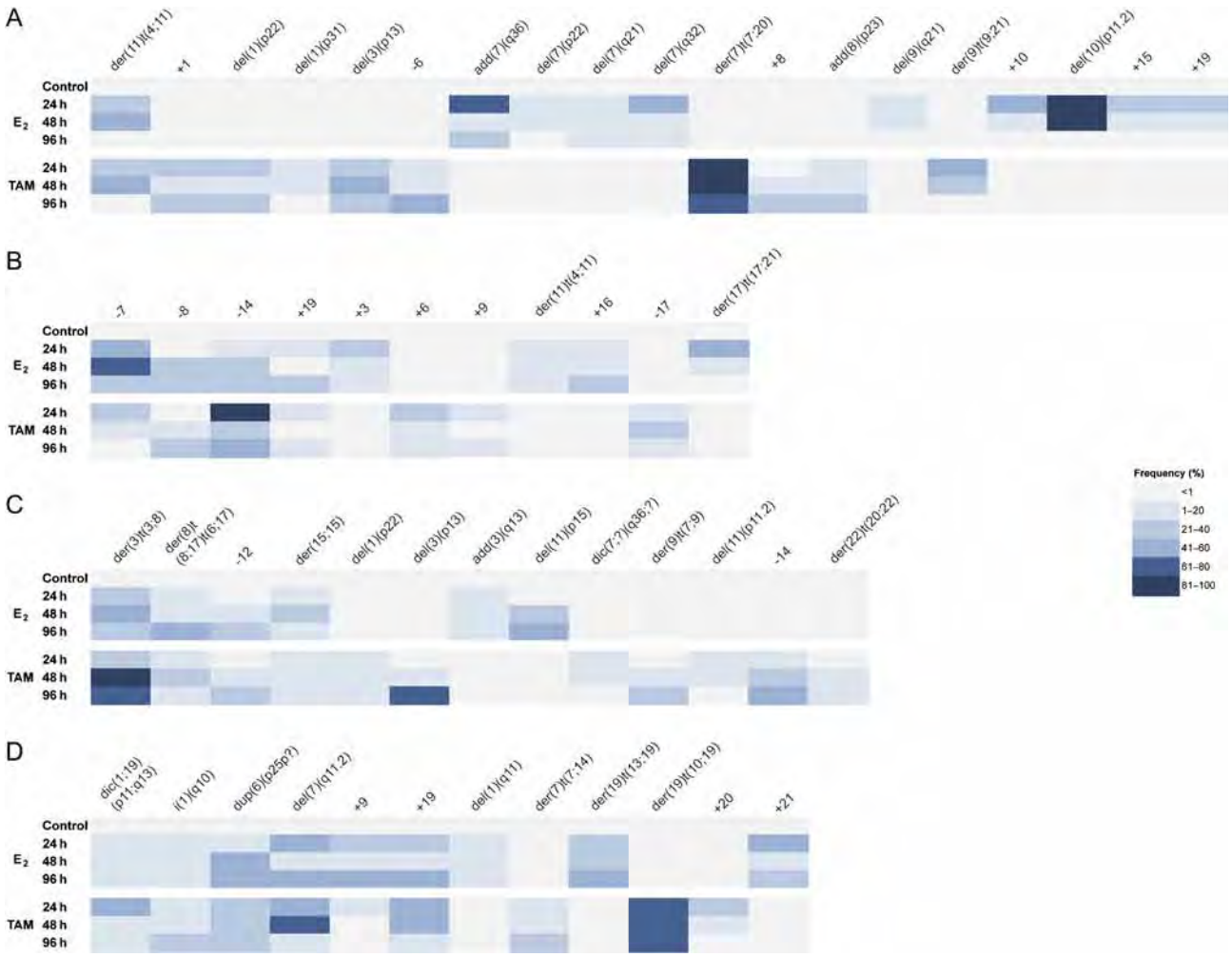

Figure 1
Frequency of chromosomal alterations observed *de novo* after E_2 and TAM treatments. The frequency of each chromosomal alteration is indicated along the treatments (24, 48, and 96 h) using a color code for each category. (A) MCF7 cells. (B) T47D cells. (C) BT474 cells. (D) SKBR3 cells. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

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Table 1 Percentage of cells with polyploidy in MCF7, T47D, BT474, and SKBR3 cell lines. (A) Control and E_2 treated. (B) Control and TAM treated. A hundred metaphases were analyzed for both control and for each of the treatments with E_2 and TAM.

	MCF7		T47D		BT474		SKBR3	
Treatments	4n	>4n	3n	>3n	4n	>4n	4n	>4n
A								
Control	98	2	96	4	100	0	81	19
E2. 24 h	85	15	87	13	88	12	63	37
E2. 48 h	80	20	78	22	77	23	52	48
E2. 96 h	61	39	67	33	70	30	50	50
B								
Control	98	2	96	4	100	0	81	19
TAM. 24 h	97	3	85	15	94	6	24	76
TAM. 48 h	99	1	98	2	98	2	24	76
TAM. 96 h	99	1	100	0	84	16	30	70

numerical alterations represented mainly by gain of whole chromosomes in all cell lines. Low doses of both E_2 and TAM induced *de novo* structural aberrations such as isochromosomes (i) in BT474 and SKBR3 cells and dicentric (dic) chromosomes in T47D and BT474 cells. Both treatments increased derivative (der) chromosomes in HER2+ cells only, whereas additional material of unknown origin (add) was a *de novo* observation only in T47D after E_2 treatment.

Many of the altered chromosomal regions in the cell lines analyzed contain important genes involved in breast cancerogenesis including *BCAR3* (1p22), *CENPF* (1q41), *ENAH* (1q42), and *AKT3* (1q44) associated with aneuploidy, chromosomal instability, and anti-estrogen resistance (Nakatani *et al*. 1999, Di Modugno *et al*. 2006, O'Brien *et al*. 2007); *FHIT*, *FOXP1*, and *LRIG1* on 3p14 correlated with chromosomal instability and anti-estrogen resistance (Campiglio *et al*. 1999, Banham *et al*. 2001,

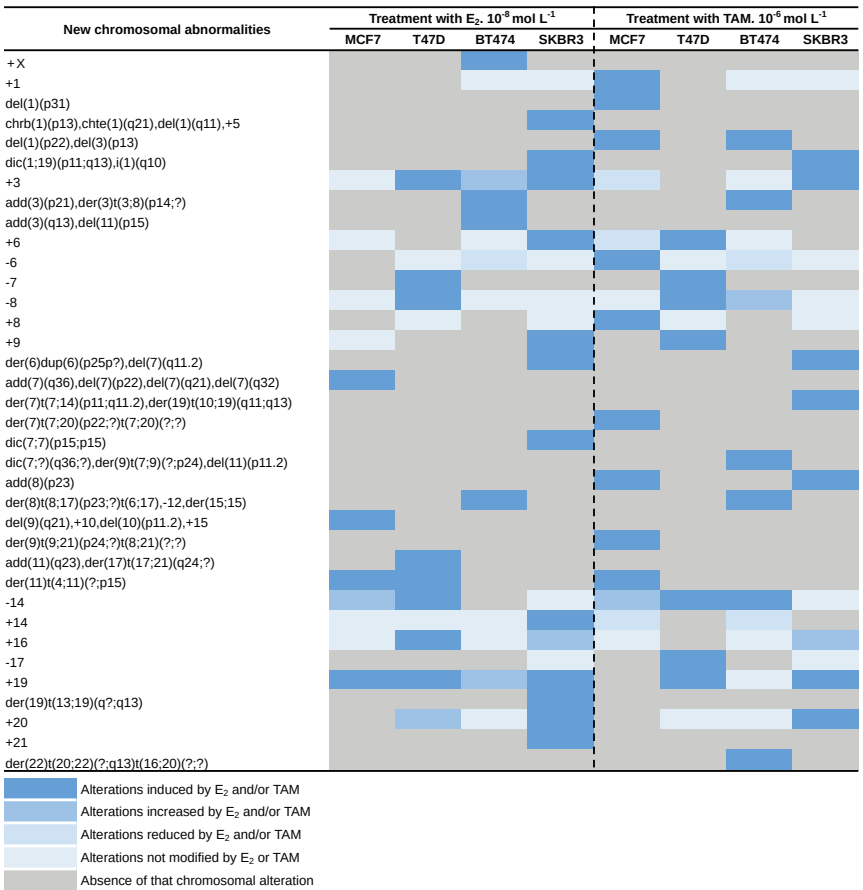

Figure 2
Clonal chromosomal abnormalities induced by E_2 and TAM in four breast cancer cell lines at each treatment time point. The presence of a given chromosomal alteration after E_2 and/or TAM treatment in one or more cell lines is color coded according to the legend at the bottom. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

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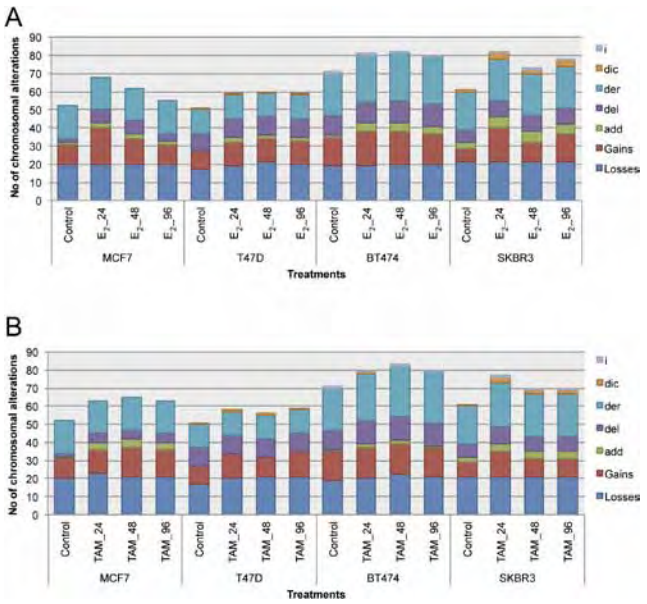

Figure 3
Total number of chromosomal aberrations induced after E_2 (A) and TAM (B) treatment at 24, 48, and 96 h in MCF7, T47D, BT474, and SKBR3 cell lines. Numerical chromosomal alterations: gains and losses. Structural chromosomal alterations: add, additional material of unknown origin; del, deletion; der, derivative chromosome; dic, dicentric chromosome; i, isochromosome. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

Ljuslinder *et al*. 2005); *AKAP9* (7q21), *DMTF1* (7q21), and *HIPK2* (7q32) involved in the assembly of protein kinases to the centrosome and in growth arrest (Edwards & Scott 2000, Sreeramaneni *et al*. 2005, Pierantoni *et al*. 2007); *E2F1* (20q11.22) and *MAPRE1* (20q11.1-11.23) involved in the regulation of the mitotic cell division process, regulation of microtubule dynamic instability, and in cell cycle control (Stender *et al*. 2007), among others (Table 2).

Combined effects on cell proliferation and chromosomal alterations

We then more specifically analyzed the chromosomal alterations in comparison with the effects on proliferation induced by E_2 and TAM in each cell line. Although we did not observe a specific pattern of chromosomal aberrations that significantly correlated with either increased or decreased proliferation rates across cell lines, single aberrations significantly correlated with increase or decrease of proliferation within each cell line, as detailed below.

In MCF7 cell line, as expected, E_2 treatment significantly stimulated cell proliferation ($P<0.0001$, Student's *t*-test; Fig. 4A) and induced more structural than numerical chromosomal alterations ($P\leq0.05$, Fisher's exact test; Fig. 2, Supplementary Tables 2, 3 and 4). However, only a statistically significant increase in nullisomy of chromosome 18 and 20 ($P<0.01$) together with del(7)(q21) and del(7)(q32) was constantly observed at all treatment time points (Figs 2 and 4A, Supplementary Tables 3 and 4).

TAM treatment inhibited significantly MCF7 cell proliferation ($P<0.01$) (Fig. 4B). Eleven chromosomes (1, 2, 6, 7, 8, 10, 11, 17, 15, 19, and 20) varied in their copy number, but most of these alterations, except for +1 and −6, were observed only in one of the treatment time points and were considered as sporadic (Supplementary Table 3). As compared with control cells, six additional complex chromosomal aberrations, del(1)(p22), del(3)(p13), der(7)t(7;20)(p22;?)t(7;20)(?;?), add(8)(p23), der(9)t(9;21)(p24;?)t(8;21)(?;?), and der(11)t(4;11)(?;p15) (Figs 2, 4B, 5A and Supplementary Table 2), were identified and constantly present at each time point. In addition, der(11)t(4;11)(?;p15) was observed in both E_2- and TAM-treated cells. An increase in the frequency of two pre-existing alterations del(7)(q11.2) and del(12)(p11.2) was also observed after both E_2 and TAM treatment (Supplementary Table 4).

T47D cells responded to E_2 treatment with the highest growth advantage at 96 h (Fig. 6A). This effect corresponded to a more complex karyotype of E_2-stimulated cells than control cells with the following additional alterations, +3, −7, −8, der(11)t(4;11)(?;p15), −14, +16, and der(17)t(17;21)(q24;?) ($P<0.01$), observed at least at two time points (Figs 2, 5B, 6A and Supplementary Table 2). In analogy to MCF7 cells, an increase in the frequency of some pre-existing numerical alterations was observed after both treatments in T47D cells (Supplementary Table 5).

The effect of TAM on cell growth inhibition was much lower than that observed in MCF7 cells and disappeared at 96 h (Fig. 6B). As compared with untreated controls, only three additional numerical alterations were constantly present (+6, −14, and −17) ($P<0.01$, Fisher's exact test) after TAM (Fig. 6B, Supplementary Tables 5 and 6). On the contrary, some chromosomal rearrangements present in the control cells could not be observed after E_2 and TAM treatment (Supplementary Table 6). In T47D, both E_2 and TAM induced loss of chromosomes 7, 8, and 14, whereas an additional chromosome 19 was induced by both treatments in T47D and SKBR3 cells.

In BT474 cells, both E_2 and TAM treatments induced two peaks of proliferation at 24 and 96 h. G-banding and M-FISH analyses of both E_2- and TAM-treated BT474 cells identified the same new chromosomal complex rearrangements der(3)t(3;8)(p14;?), der(8)

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Table 2 Selected breast cancer oncogenes and tumor suppressor genes present in the chromosomal regions affected by chromosomal abnormalities in MCF7, T47D, BT474, and SKBR3 cell lines following treatment with E_2 and TAM for 24, 48, and 96 h.

Chromosomal region	Genes	Cell line				Function	References
		MCF7	T47D	BT474	SKBR3		
1p13.3	*CSF1*				X	Cell proliferation	www.ncbi.nlm.nih.gov
1p22	*BCL10*	X		X		Oncogene, apoptosis	Lin (2009)
1p22	*BCAR3*	X		X		Cell proliferation, resistance in breast cancer cell lines	Nakatani *et al.* (1999), Di Modugno *et al.* (2006), O'Brien *et al.* (2007)
1p32p31	*JUN*				X	Oncogen	www.ncbi.nlm.nih.gov
1p36.21	*PRDM2*				X	Tumor suppressor gene, binds to ER. Transcriptional regulation, E_2 effector action	www.ncbi.nlm.nih.gov
1q11	*MUC1*				X	Cell physiology and pathology, up-regulated in breast cancer	Zaretsky *et al.* (2006)
1q21.1	*CA14*				X	Basic cellular metabolism; breast cancer	Orsetti *et al.* (2006), Beroukhim *et al.* (2010), Bignell *et al.* (2010)
1q21.3	*PIP5K1A*				X	Cell proliferation, breast cancer	Orsetti *et al.* (2006), Beroukhim *et al.* (2010), Bignell *et al.* (2010)
1q25.2-q25.3	*COX2*				X	Inflammation and mitogenesis	www.ncbi.nlm.nih.gov
1q32	*KISS*				X	Cell motility, oncogene	Orsetti *et al.* (2006), Bignell *et al.* (2010), Beroukhim *et al.* (2010)
1q31	*PTGS2*				X	Inflammation, tumorigenesis	Dossus *et al.* (2010)
1q41	*CENPF*				X	Kinetochore assembly	Nakatani *et al.* (1999), Di Modugno *et al.* (2006), O'Brien *et al.* (2007)
1q42.12	*ENAH*				X	Cell shape and movement	Nakatani *et al.* (1999), Di Modugno *et al.* (2006), O'Brien *et al.* (2007)
1q44	*AKT3*				X	Proliferation, cell survival, and tumorigenesis	Nakatani *et al.* (1999), Di Modugno *et al.* (2006), O'Brien *et al.* (2007)
3p14	*FHIT*			X		Tumor suppressor gene; resistance to tamoxifen in MCF7 cells	Campiglio *et al.* (1999)
3p14	*FOXP1*			X		Tumor suppressor gene, multiple types of cancers	Banham *et al.* (2001)
3p14	*LRIG1*			X		Suppressor of receptor tyrosine kinases, breast cancer	Ljuslinder *et al.* (2005)
6p25	*TFAP2A*				X	Tumor supressor gene, breast cancer	Scibetta *et al.* (2010)
6p25	*DUSP22*				X	Signaling pathway, breast cancer	Curtis *et al.* (2012)
7p22	*GPR30*	X				G protein-coupled receptor 30, drug resistance	Wang *et al.* (2010)
7p22	*SDK1*	X				Cell adhesion protein, breast cancer	Curtis *et al.* (2012)
7q11.2	*LIMK1*				X	Organization of actin cytoskeleton	Laskowska *et al.* (2010)
7q11.2	*HSPB1*				X	Oncogenesis and resistance to various anti-cancer therapies	Laskowska *et al.* (2010)
7q11.2	*AUTS2*				X	Breast cancer	www.ncbi.nlm.nih.gov
7q21	*AKAP9*	X				Protein that assembles protein kinases on the centrosome	Edwards & Scott (2000)
7q21	*DMTF1*	X				Transcriptional activator promoting p53/TP53-dependent growth arrest.	Sreeramaneni *et al.* (2005)
7q32	*HIPK2*	X				Tumor supressor gene, breast cancer	Pierantoni *et al.* (2007)
7q36	*MNX1*	X				Transcription factor, breast cancer	Nik-Zainal *et al.* (2012)
7q36	*MLL3*	X				Transcriptional coactivation, breast cancer	Nik-Zainal *et al.* (2012)
8p22	*MTUS1*	X		X		Tumor suppressor gene, breast cancer	Rodrigues-Ferreira *et al.* (2009)
8p23	*CTSB*	X		X		Metabolism, angiogenesis, invasion, and metastasis in breast cancer	Rafn *et al.* (2012)
8p23	*CSMD1*	X		X		Tumor supressor gene, poor survival in breast cancer	Kamal *et al.* (2010), Curtis *et al.* (2012)

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Table 2 *Continued.*

Chromosomal region	Genes	Cell line				Function	References
		MCF7	T47D	BT474	SKBR3		
8p23	*DLC1*	X		X		Tumor suppressor gene, breast cancer	Popescu & Zimonjic (2002)
9p24	*JAK2*	X		X		Protein tyrosine kinase of the non-receptor type, breast cancer	Curtis *et al*. (2012)
9p24	*RLN2*	X		X		Development of mammary gland. Invasion in breast cancer	Radestock *et al*. (2008)
9p24	*KANK1*	X		X		Tumor supressor gene, breast cancer	Curtis *et al*. (2012)
9p24	*JMJD2C*	X		X		Demethylase, breast cancer	Curtis *et al*. (2012)
10p11.2	*ABI1*	X				Cell growth inhibitor, cancer progression, and prognosis	Cui *et al*. (2010)
11p15	*HRAS*	X	X			Signal transduction, tumor aggressiveness in breast cancer	Hae-Young Yong *et al*. (2011)
11p15	*CTSD*	X	X			Invasion and metastasis	www.ncbi.nlm.nih.gov
11p15	*CD151*	X	X			Signal transduction, breast cancer	Ivyna Bong *et al*. (2011)
11p15	*RRM1*	X	X			Tumor supressor gene, DNA repair	Kim *et al*. (2011)
11p15	*MMP26*	X	X			Migration and angiogenesis, breast cancer	Curtis *et al*. (2012)
11p15	*CDKN1C*	X	X			Negative regulator of cell cycle	www.ncbi.nlm.nih.gov
11q23	*ATM*		X			Tumor supressor gene, DNA repair	Roy *et al*. (2006)
11q23	*CRYAB*		X			Molecular chaperone function, metastasis in breast cancer	Chelouche-Lev *et al*. (2004)
11q23	*ETS1*		X			Transcripction factor, breast cancer	Lincoln & Bove (2005)
11q23	*CCND1*		X			Cell cycle G1/S transition, tumorigenesis in various carcinomas	Lundgren *et al*. (2008)
11q23	*PGR*		X			Signal transduction, breast cancer	www.ncbi.nlm.nih.gov
15q10	*BUB1B*			X		Mitotic spindle checkpoint, chromosomal instability in breast cancer	Scintu *et al*. (2007)
15q15	*THBS1*			X		Invasion, metastasis, angiogenesis	www.ncbi.nlm.nih.gov
15q26.3	*IGF1R*			X		Cell growth and survival control, breast cancer	Kang *et al*. (2014)
17q24	*BIRC5*		X			Apoptosis inhibition	www.ncbi.nlm.nih.gov
18q21.1	*SMAD4*	X				Transcription factor, breast cancer	Curtis *et al*. (2012), Nik-Zainal *et al*. (2012)
18q21.1	*BCL2*	X				Cell death, breast cancer	Curtis *et al*. (2012)
18q21.2	*DCC*	X				Apoptosis, breast cancer	Koren *et al*. (2003)
19q13	*ATF5*				X	Cell cycle progression, breast cancer	Al Sarraj *et al*. (2005), Watatani *et al*. (2007)
19q13	*LILRA6*				X	Receptor for class I MHC antigens, breast cancer	Curtis *et al*. (2012)
19q13	*CYP2A6*				X	Metabolism of pharmaceutical drugs, directly induced by estradiol	Higashi *et al*. (2007)
19q13	*TGFB1*				X	Cell division and death, imply in tamoxifen resistance in breast cancer	Achuthan *et al*. (2001), Popescu & Zimonjic (2002), Jansen *et al*. (2005), Ivanovic *et al*. (2006)
19q13	*CEACAM1*				X	Cell survival, differentiation, and growth, breast cancer	Luo *et al*. (1997), Riethdorf *et al*. (1997)
20q11.22	*E2F1*	X				Tumor suppressor gene	Stender *et al*. (2007)
20q13.1	*CDH4*	X				Cell adhesion proteins, breast cancer	Curtis *et al*. (2012)
20q13.1	*MMP9*	X				Metastasis and cancer cell invasion, breast cancer	Kousidou *et al*. (2004)
20q13.31	*AURKA*	X				Cell proliferation, breast cancer	Cox *et al*. (2006)
22q13	*ATF4*			X		Adaptation of cells to stress factors, multidrug resistant gene	Igarashi *et al*. (2007)
22q13	*SERHL2*			X		Breast cancer	Curtis *et al*. (2012)
22q13	*LARGE*			X		Breast cancer	Curtis *et al*. (2012)
22q13	*XRCC6*			X		Apoptosis induction, breast cancer	Nik-Zainal *et al*. (2012)

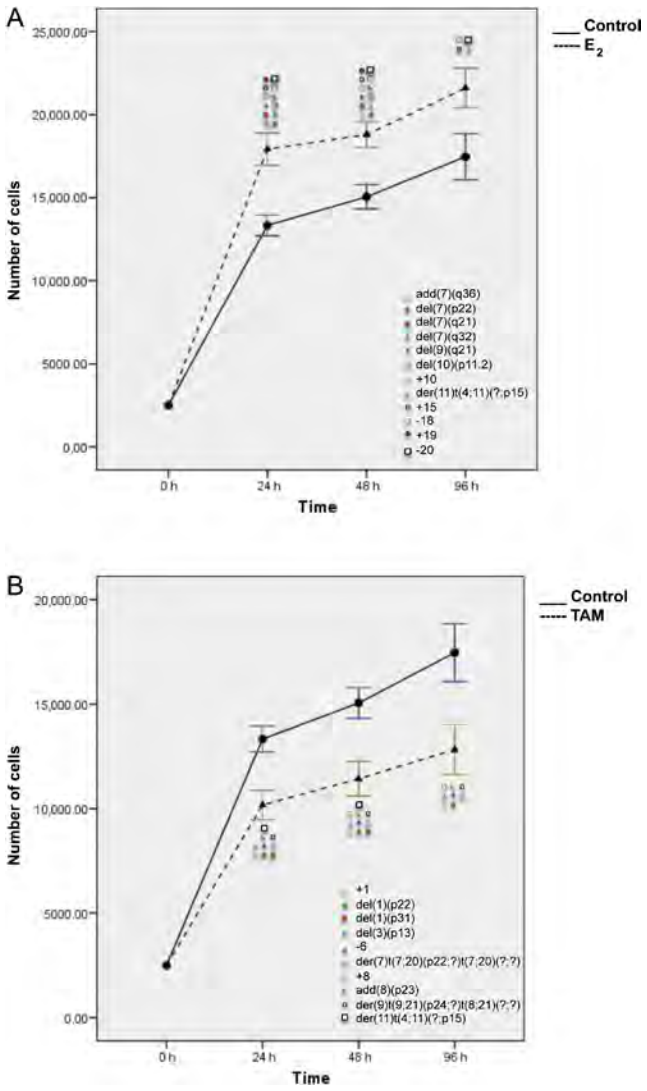

Figure 4
Effects of E_2 (A) and TAM (B) treatment for 24, 48, and 96 h on proliferation and corresponding chromosomal alterations in MCF7 cells. Error bars represent mean standard deviation of 24 separate experiments. Chromosomal abnormalities induced at each treatment time point are indicated. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

t(8;17)(p23;?)t(6;17)(?;?), and der(15;15)(q10;q10) at each time point (Figs 2, 5C, 7 and Supplementary Table 2). Additional new rearrangements were observed after E_2 (Fig. 7A, Supplementary Tables 7 and 8) or after TAM treatment (Fig. 7B) at least at two time points. An increase in the frequency of some preexisting chromosomal alterations ($P \leq 0.01$) was also observed (Supplementary Tables 7 and 8).

Finally, in SKBR3 (ER−/HER2+), only 96 h of E_2 and TAM treatment significantly increased cell proliferation ($P<0.006$ and $P<0.024$) (Fig. 8), as compared with controls. However, *de novo* chromosomal alterations were already observed after 24 h of treatment. SKBR3 control cells displayed a complex karyotype with a particularly high frequency of chromosome 1 aberrations. After 24 h of E_2 and TAM treatment, the karyotype became even more complex with the appearance of new chromosome 1 abnormalities, such as for instance dic(1;19)(p11;q13) and i(1)(q10) ($P<0.05$) (Figs 2, 5D, 8A, B and Supplementary Table 2). A statistically significant increase in the frequency of some pre-existing chromosomal abnormalities was observed in SKBR3 as well (Supplementary Tables 9 and 10).

Expression of hormone receptors following treatment

IHC analysis showed that ER levels remained unchanged after E_2 and TAM addition in MCF7, T47D, and SKBR3 cells, whereas in BT474 cells we observed an increase in both ER and PR expression after TAM treatment in parallel with an increase in proliferation (all time points; data not shown). These results support the hypothesis that TAM could play an estrogen agonist role in ER+/HER2+ cells (BT474), as it has been previously suggested (Pietras & Marquez-Garban 2007, Chang 2011, Kumar *et al*. 2011) and shown in other cell line models (Shou *et al*. 2004). In addition, increased PR expression in human breast cancers has been associated with TAM resistance (Cui *et al*. 2005).

E_2 addition increased PR expression also in the other ER+ cell lines (MCF7 and T47D). In contrast, after TAM treatment, a reduced PR expression was observed in MCF7 and T47D cells (data not shown). This is in line with previous observations showing that when estradiol is acting, TAM is not able to increase the level of occupied estrogen receptors and it acts as an anti-estrogen by decreasing the high level of progesterone receptors previously induced by estradiol (Castellano-Diaz *et al*. 1989).

Discussion

Short-term endocrine treatment has been proposed as an alternative to long-term neoadjuvant therapy to assess tumor response (Dowsett *et al*. 2007). In addition, low doses of TAM have been proposed for chemoprevention in women at high risk of developing breast cancer (Lazzeroni *et al*. 2012). Hypersensitivity to low levels of estrogen has been suggested as a potential mechanism of endocrine therapy resistance (Johnston & Dowsett 2003). In addition, residual amounts of estrogen may still be present after treatment with aromatase inhibitors, which function by reducing estrogen biosynthesis (Dowsett

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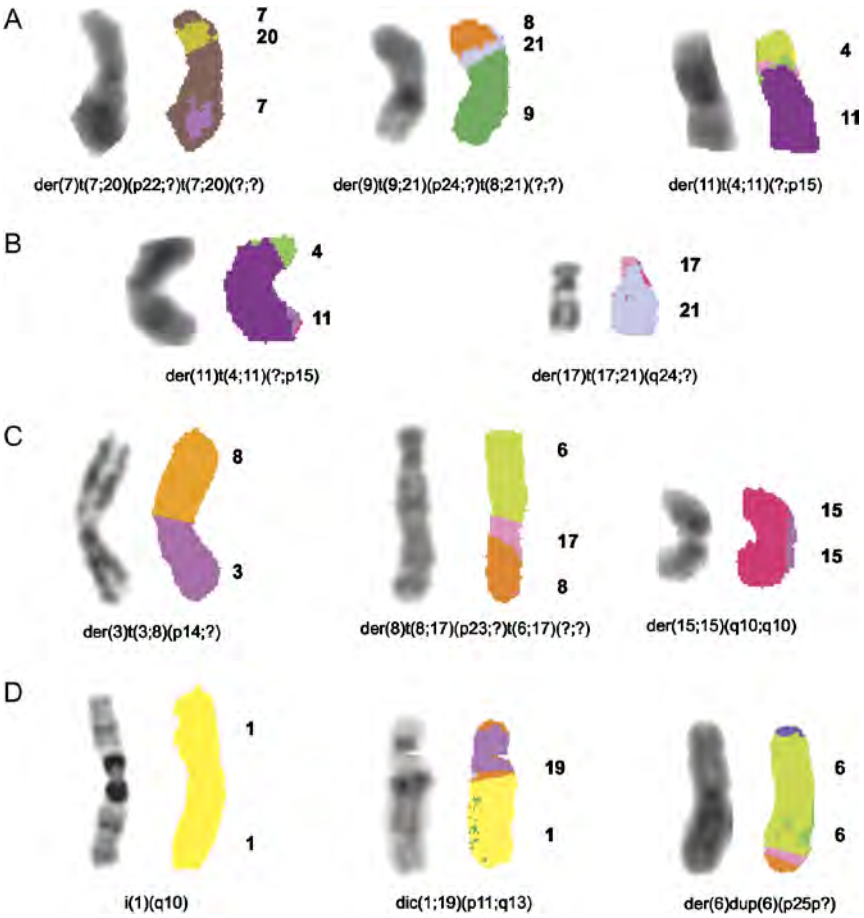

Figure 5
Representative images of chromosomal abnormalities observed throughout the treatment duration with either E_2 or TAM. (A) MCF7 cells, (B) T47D cells, (C) BT474 cells, and (D) SKBR3 cells. Rearranged chromosomes are visualized by G-banding technique on the left and by M-FISH on the right. The chromosomes involved in the rearrangement are numbered on the right hand side of the chromosomes. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

1999). E_2 binding to tubulin may induce a cell cycle arrest in G2/M and generate chromosomal instability (Sato *et al.* 1992, Sattler *et al.* 2003, Azuma *et al.* 2009, Lee *et al.* 2015).

In this study, we observed that low doses of both E_2 and TAM were able to induce structural chromosomal aberrations (deletions, isochromosomes, translocations, and dicentric chromosomes) in both ER+ and ER– breast cancer cells.

Dicentric chromosomes, which contain two functional centromeres, can lead to extensive chromosomal rearrangements, including translocations with other chromosomes (Gascoigne & Cheeseman 2013). Chromosomal translocations, a frequent event observed after E_2 and TAM treatment, may lead to the production of tumor-specific fusion proteins, which are often transcription factors (Rabbitts 1994). For example, der(11)t(4;11)(?;p15) was observed in both E_2- and TAM-treated MCF7 cells and in E_2-treated T47D. Several genes are located in the imprinted gene domain of 11p15.5, an important tumor-suppressor gene region (Hu *et al.* 1997).

While some complex chromosomal alterations were consistent throughout the treatments, other disappeared. The above could be related with the instability of such alterations. After treatment, unstable chromosomal alterations could be randomly fused to form more complex chromosomal rearrangements including translocations, dicentric chromosomes, and duplications (Shen 2013, Zhang *et al.* 2013). Another possible explanation, which can be strictly connected to the previous, is the possibility of clonal selection of the fittest clone (Heng *et al.* 2006, Liu *et al.* 2014, Dayal *et al.* 2015).

When chromosomal alterations were analyzed with respect to proliferation, some specific patterns within each cell line were observed. For instance, T47D cells showed a poorer response to TAM compared with MCF7 cells and mainly displayed numerical chromosomal alterations following treatment. The ER+/HER2+ BT474 cells showed the highest increase in cell proliferation after 24 h of treatment with both E_2 and TAM compared with control cells. Cell growth increase after TAM treatment may indicate an estrogen agonist activity, possibly enhanced by the co-expression of ER and HER2 (Pietras & Marquez-Garban 2007, Chang 2011, Kumar *et al.* 2011). Indeed, the cross talk between ER pathways and growth factor receptor

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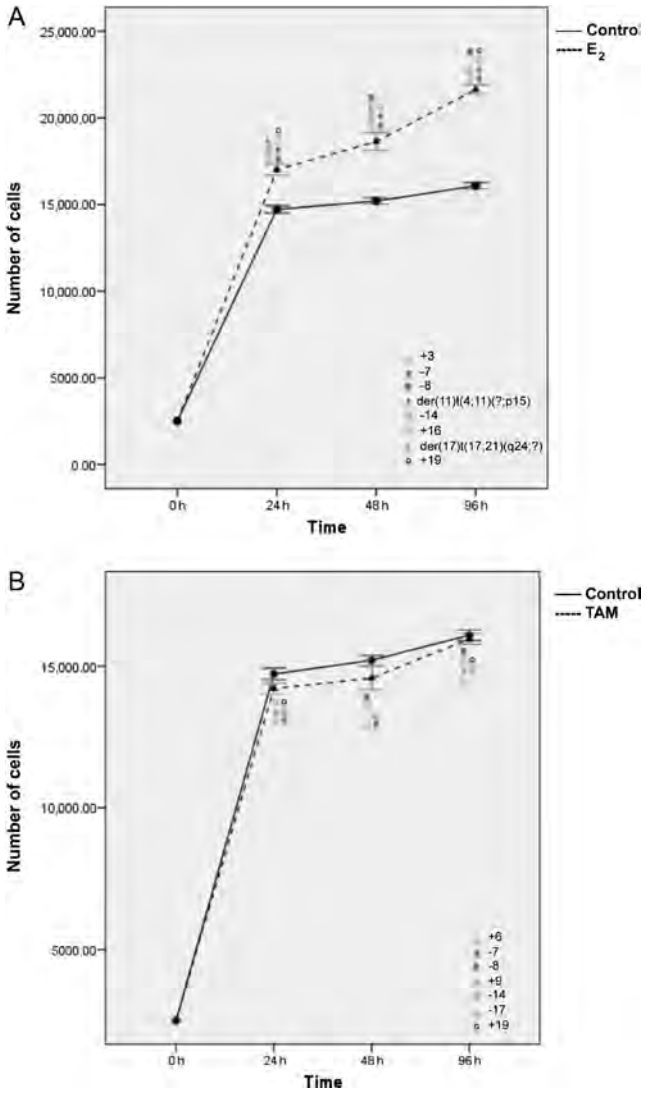

Figure 6
Effects of E_2 (A) and TAM (B) treatment for 24, 48, and 96 h on proliferation and corresponding chromosomal alterations in T47D cells. Error bars represent mean standard deviation of 24 separate experiments. Chromosomal abnormalities induced at each treatment time point are indicated. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

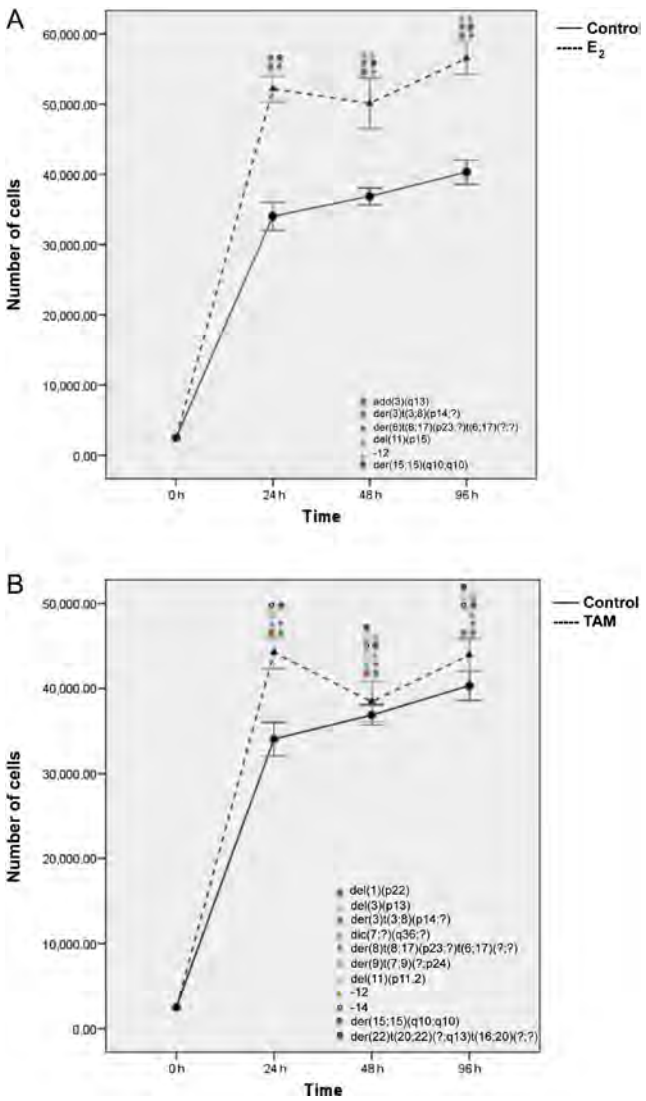

Figure 7
Effects of E_2 (A) and TAM (B) treatment for 24, 48, and 96 h on proliferation and corresponding chromosomal alterations in BT474 cells. Error bars represent mean standard deviation of 24 separate experiments. Chromosomal abnormalities induced at each treatment time point are indicated. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

pathways (EGFR, IGF-1, and HER2) has been involved in cell proliferation, survival, and resistance to endocrine therapy (TAM) in breast cancer (Yager & Davidson 2006, Pietras & Marquez-Garban 2007, Chang 2011). However, in our study, after 48 h of TAM treatment cell proliferation decreased and increased again at 96 h. This decrease/increase may be explained through a clonal selection, with survival of those cells that acquired chromosomal abnormalities fostering proliferative and survival advantages.

As expected, our results confirm that the induction and inhibition of cell proliferation by E_2 and TAM, respectively, is dependent on the presence of ER. However, in the ER−/HER2+ SKBR3 cells, these agents induced a high frequency of chromosomal abnormalities and a small increase in proliferative activity at 96 h of treatment. Both effects may be due to the presence of the G protein-coupled receptor 30 (GPCR30), an estrogen transmembrane receptor, which modulates both rapid non-genomic and genomic transcriptional events of estrogen (Thomas *et al.* 2005, Chen & Russo 2009, Li *et al.* 2010, Cheng *et al.* 2011). On the other hand, E_2 may induce chromatin structural

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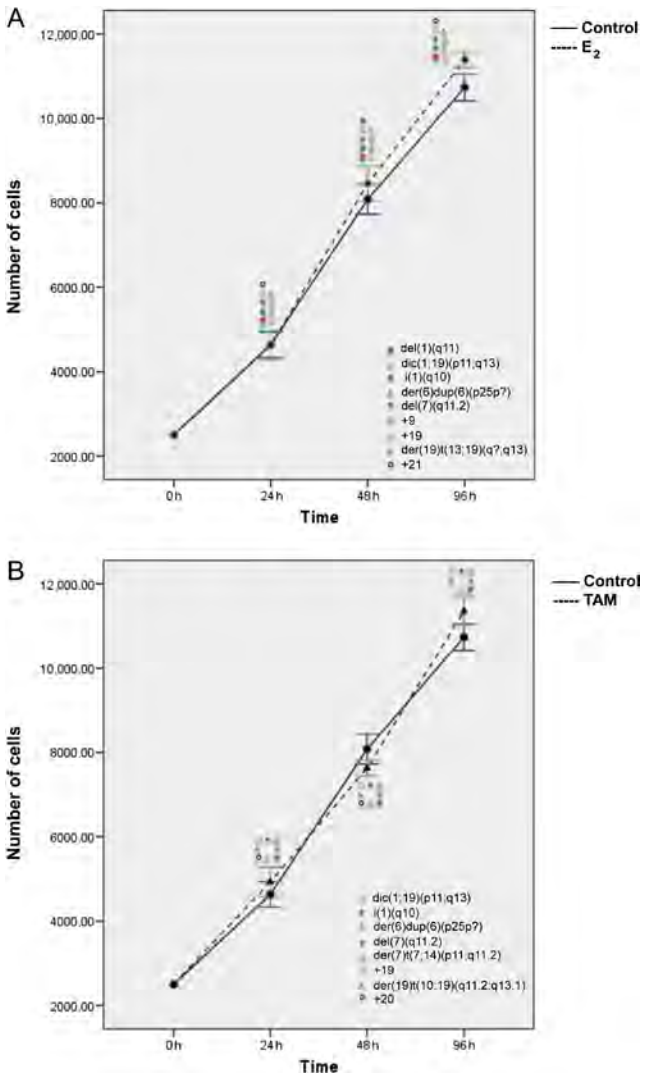

Figure 8
Effects of E_2 (A) and TAM (B) treatment for 24, 48, and 96 h on proliferation and corresponding chromosomal alterations in SKBR3 cells. Error bars represent mean standard deviation of 24 separate experiments. Chromosomal abnormalities induced at each treatment time point are indicated. A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-16-0078.

changes in both ER+ and ER− breast cancer cells through ERR (Hu *et al*. 2008). The ability of estrogens and its metabolites (catechol estrogens) to induce mutations in cancer cells has been demonstrated both *in vivo* and *in vitro* (Liehr 2000, Yager 2015), being observed that estrogens induce overexpression of the *Aurora A* and *B* genes (Li *et al*. 2004), cause genomic instability (Barrett *et al*. 1981, Tsutsui & Barrett 1997, Ahmad *et al*. 2000, Jeruss *et al*. 2003, Lam *et al*. 2011, Yager 2015), and induce chromosomal aberrations, thus confirming its properties as mutagenic and carcinogenic factor. Along the same lines, in luminal breast tumors, up-regulation of ER signal pathway has been associated with cell proliferation, cell survival, and therapy resistance (Yager & Davidson 2006, Pietras & Marquez-Garban 2007, Chang 2011). Although factors such as local synthesis of estrogen (Fabian *et al*. 2007), autocrine regulation of cell proliferation (Fabian *et al*. 2007, Tan *et al*. 2009), and cross talk with signaling from other growth factors have been associated with this up-regulation, the mechanisms underlying the action of ER are still not fully understood.

In summary, our results demonstrate that low doses of E_2 and TAM may favor the production of specific chromosomal abnormalities in both ER+ and ER- breast cancer cells. This genotoxic effect is higher in those cell lines with *HER2* gene amplification. The induction of chromosomal alterations by E_2 and TAM observed *in vitro* may support the contention that a careful assessment of the risk and the benefit of E_2 and TAM administration should be considered. Indeed, the novel chromosomal rearrangements originated following E_2 and TAM exposure may contribute to stimulate cell proliferation leading to survival advantages and allowing for selection of clones with new chromosomal abnormalities. *In vivo* studies that may help address the biological effect of such alterations and ascertain whether or not these may be responsible for treatment resistance are warranted.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0078.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was funded by the Italian Association of Cancer Research, AIRC (MFAG13310 to C M), by the Ministry of University (Ex 60% 2014 and 2015 to C M) and by Fondazione Piemontese per la Ricerca sul Cancro (ONLUS) 5 X 1000 Fondi Ministero della Salute 2013 (to A S).

Authors' contribution statement
M R L performed the experiments and analyzed and interpreted the data. L V d C acquired and analyzed G-banding and M-FISH karyotypes. R R and L A participated in cell culture experiments. T M performed IHC. N R performed statistical analyses and participated in data analysis. I C participated in data analysis. C M and A S conceived and supervised the study and analyzed and interpreted the data. M R L, C M, and A S wrote the manuscript.

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Acknowledgements

The authors would like to acknowledge technical support in immunohistochemical and M-FISH analysis by Mrs Maria Stella Scalzo, Mrs Stefania Bolla, and Mr Marco Cupo as well as assistance in manuscript preparation by Mrs. Paola Critelli, Mr Jacopo De Gregori, and Mr Lorenzo Di Filippo (Liceo Scientifico Scienze Applicate, Asti).

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Received in final form 8 June 2016
Accepted 29 June 2016
Accepted Preprint published online 29 June 2016

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