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The effects of lycopene on the proliferation of human breast cells and *BRCA1* and *BRCA2* gene expression

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Abstract

The purpose of this study was to demonstrate the effects of lycopene, the major tomato carotenoid, on the expression of the *BRCA1* and *BRCA2* genes in three breast tumour cell lines, MCF-7, HBL-100, MDA-MB-231 and the fibrocystic breast cell line MCF-10a. Flow cytometry analysis showed a G_1/S phase cell cycle-arrest after treatment of the cells with 10 μM lycopene for 48 h. mRNA expression was studied by quantitative reverse transcription-polymerase chain reaction using the Taqman® method. We observed an increase of *BRCA1* and *BRCA2* mRNA in the oestrogen receptor (ER)-positive cell lines (MCF-7 and HBL-100), and a decrease (MDA-MB-231) or no change (MCF-10a) in the ER-negative cell lines. BRCA1 and BRCA2 proteins were quantified by perfusion affinity chromatography. No variation in their expression was observed. These preliminary results on the effects of lycopene on the expression of *BRCA1* and *BRCA2* oncosuppressor genes in breast cancer may reflect cross-talk between the oestrogen and retinoic acid receptor (RAR) pathways.

Keywords: Lycopene; Breast cancer; *BRCA1*; *BRCA2*; Quantitative RT-PCR; Affinity chromatography; Protein quantification

1. Introduction

Germline mutations are responsible for 5–10% of female breast cancer [1–3]. Half of these are mutations in the *BRCA1* and *BRCA2* genes. In sporadic cancer, no mutation of *BRCA1* has been found, although variation in its expression has been observed [4] and was associated with the inactivation of the promoter by methylation [5]. For *BRCA2*, Bieche and colleagues demonstrated overexpression in 20% of breast carcinomas [6]. BRCA1 and BRCA2 interact with other proteins to maintain genomic integrity and repress tumour formation [7]. Alternately spliced forms of *BRCA1* have been characterised by Thakur and colleagues [8] who described the isolation and expression of two *BRCA1* cDNAs, one of them generated by exclusion of exon 11 and producing a 4.6-kb mRNA. They observed a complex tissue-specific pattern of multiple spliced forms of *BRCA1* and suggested that splicing may play a role in the regulation of BRCA1 function [8]. Bieche and Lidereau [9] identified an alternatively spliced *BRCA2* variant that was widely expressed in all normal tissues examined. This Δ12-*BRCA2* transcript was found to be overexpressed in steroid receptor-negative breast tumour tissues, suggesting that dysregulation of the Δ12-*BRCA2* isoform may contribute to progression in human breast cancer.

Numerous epidemiological studies point to the likelihood that nutritional pre-emption may be a useful strategy to influence cancer risk at multiple sites [10–12]. The past decade has witnessed the publication of several articles that question the role of diet in the cancer process [13,14] and thus have raised concerns about what is physiologically important and the circumstances that may dictate the overall response [10,15].

Tomatoes are the main foodstuffs contributing to the dietary intake of the red pigment lycopene which has

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shown promise for the prevention of breast cancer. Additionally, apricots, guavas, watermelons, papayas, pink grapefruits [16] and rosehip [17] contain lycopene. Lycopene is known to have antioxidant properties.

Lycopene is a good example of the controversy that exists within the literature. Considerable variability exists in the literature about the potential protective effects of lycopene against breast cancer. Ten case-control studies [18–27] support a protective effect against breast cancer resulting from a high tomato or lycopene consumption. However, another three case-control studies [28–30] and one cohort study [31] did not support a pre-emptive relationship. The reasons for these inconsistencies are not clear, but may relate to the quantity or duration of exposure to lycopene or to genetic differences among subjects in the studies. Increasingly, genetics is thought to have an intimate involvement in directing the cancer process and is also a likely modifier of the response to diet [32]. A host of studies are appearing in the literature that demonstrate the nutrigenomic effects of bioactive food components that result in marked increases or suppression in the expression of multiple genes [33–35]. Recently, our group reported changes in mRNA expression of the *BRCA1* and *BRCA2* genes in breast cell lines after treatment with $n-3$ and $n-6$ polyunsaturated fatty acids [36], phyto-oestrogens such as genistein and daidzein [37] and resveratrol [38].

Here, we studied the effects of lycopene on the expression of *BRCA1* and *BRCA2* in human breast cancer cell lines at the transcriptional level using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and at the translation level by perfusion chromatography.

2. Materials and methods

2.1. Cell lines

A fibrocystic breast cell line MCF-10a and breast cancer cells MCF-7, HBL-100 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA) [39–42]. Roswell Park Memorial Institute (RPMI) 1640, L-15, Dulbecco's modified Eagle's medium (DMEM)/HAM's F-12 and McCoy's media, fetal bovine serum and horse serum were obtained from Life Technologies (Rockville, MD).

2.2. Lycopene treatment of cells and flow cytometry analysis

MCF-7, HBL-100, MDA-MB-231 and MCF-10a cells were maintained in medium supplemented with 2, 5, 10 or 20 μM lycopene (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France) dissolved in Tetra Hyro Furane (THF) (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France) and 0.25 g/l of Butylated Hydroxy Toluene (BHT) (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France). A control was performed with THF and BHT (0.25 g/l). Each medium was changed every 12 h to compensate for lycopene's instability. Cells were collected after 12, 24 or 48 h by trypsinisation and the DNA content was assessed by flow cytometry according to Krishan's method [43]. Each experiment was performed in triplicate.

2.3. RNA extraction

Cells were plated at a density of 3×10^6 cells per T75 cm^2 flasks. At 80% confluence, cells were washed twice with phosphate-buffered saline (PBS). Total RNA was isolated using 1 ml of RNA-B™ (Qbiogene, Illkirch, France) according to the manufacturer's protocol. Total RNA samples were dissolved in diethyl-pyrocarbonate-treated water and their concentrations determined by A_{260} measurements using a Hitachi spectrophotometer U-2000.

2.4. cDNA synthesis

One microgram of total RNA was used for the synthesis of first-strand cDNA using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions.

2.5. BRCA1 and BRCA2 mRNA quantification

For *BRCA1* and *BRCA2* expression analysis, probes and primers used for the quantification were chosen with the help of Primer Express (Applied Biosystems, ZA Courtabœuf, France) and were designed so that they overlapped splice junctions, thereby avoiding the potential amplification of genomic DNA. The sequence of forward primers, Taqman probes and reverse primers were, respectively, for *BRCA1* 5′-^{5566}CAGAGGACAA TGGCTTCCATG^{5586}-3′, 5′-^{5588}AATTGGGCAGATG TGTGAGGCACCTG^{5613}-3′, 5′-^{5646}CTACACTGTCC AACACCCACTCTC^{5623}-3′ ; for *BRCA2* 5′-^{9794}CCAA GTGGTCCACCCCAAC^{9812}-3′, 5′-^{9818}ACTGTACTTC AGGGCCGTACACTGCTCAAA^{9847}-3′, 5′-^{9895}CACA ATTAGGAGAAGACATCAGAAGC^{9870}-3′; for Δ11-*BRCA1* 5′-^{4157}AAGAGGAACGGGCTTGGAA^{4175}-3′, 5′-^{4177}AAAATAATCAAGAAGAGCAAAGCATGGA TTCAAACTTA^{4214}-3′, 5′-^{4236}CACACCCAGATGCTG CTTCA^{4217}-3′; for Δ12-*BRCA2* 5′-^{7120}GAAAATCAAG AAAAATCCTTAAAGGCT^{7147}-3′, 5′-^{7153}AGCACTC CAGATGGCACAATAAAAGATCGAAG^{7184}-3′, 5′-^{7220}GTAATCGGCTCTAAAGAAACATGATG^{7195}-3′. All probes were labelled with 6-carboxy-fluoresceine (FAM) as a fluorescent reporter. The sequence of probes for 18S rRNA are 5′-CGGCTACCACATCCAA-GGAA-3′, 5′-TGCTGGCACCAGAC-TTGCCCTC-3′

and 5′-GCTGGAATTACCGCGGCT-3′, and were labelled with VIC [38].

Multiplex PCRs were carried out in 96-well plates on cDNA equivalent to 10 ng of total RNA using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) in a 25-μl reaction mixture containing 12.5 μl TaqMan® Universal PCR Master Mix (2×). The mix is optimised for the 5′ nuclease assay using TaqMan® probes and contains AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, desoxynucleotide triphosphate (dNTPs) with desoxyuridine triphosphate (dUTP). We added 200 nM of *BRCA1*, Δ11-*BRCA1*, *BRCA2* or Δ12-*BRCA2* primers and TaqMan® probes, and 50 nM of 18S rRNA primers and TaqMan® probe. Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. Real-time PCR assays were conducted in triplicate. Relative quantification of mRNA *BRCA1*, Δ11-*BRCA1*, *BRCA2* and Δ12-*BRCA2* level was done by the comparative C_T method [44], which consists of the quantitative normalisation of cDNA in each sample to an internal control (i.e., 18S rRNA), designated as the calibrator, in order to normalise the quantity and quality of the cDNA samples. To guarantee the reproducibility of the mRNA determination, two independent total RNA extractions were performed. Two independent RTs were carried out for one RNA extraction, while only one was performed for the second extraction. Each RT was analysed in triplicate and expressed as a mean ± standard deviation (SD) [45].

2.6. BRCA1 and BRCA2 protein quantification

Cell lines were incubated in 75-cm^2 flasks with 10 μM lycopene during 24 h at 37 °C with or without CO_2 (MDA-MB-231). At 80% confluence, the medium was supplemented with 100 μCi [^{35}S]methionine (1000 Ci/mM; Amersham International plc, England) for 24 h. Metabolic radiolabelling was stopped with 10 ml cold PBS and cells were washed twice with cold PBS. Labelled cells were solubilised in 5 ml 0.1 M Tris–HCl, pH 7.1, containing 0.5% Nonidet-P40 at 4 °C for 30 min. The lysates were ultracentrifugated at 40 000*g* for 30 min.

Labelled DNA-binding proteins were detected using a BioCAD Sprint high-pressure chromatography system (PerSeptive Biosystems Inc., Framingham, MA) equipped with a fraction collector (Gilson Inc., Middelton, WI). The flow rate was 5 ml/min. Labelled DNA-binding proteins specifically bound to a POROS®20 HE Media (heparin) column (PerSeptive Biosystems Inc, Framingham, MA) were isolated with a NaCl gradient from 0.15 to 1 M in 20 mM MES (2-[*N*-Morpholino] ethanesulphonic acid), pH 5.5. Detection of proteins was performed at 280 nm. Fractions (0.5 ml) containing DNA-binding proteins were collected. Radioactivity was measured on 10 μl of each fraction in 5 ml of scintillation cocktail (Packard Ready Safe).

Radiolabelled BRCA1 or BRCA2 proteins were then immunoprecipitated by the addition for 30 min at 37 °C of 20 μl of anti-BRCA1 antibody (556441, BD PharMingen, San Diego, CA) specifically recognising the zinc finger domain (amino acids 2–20 in *BRCA1*) or anti-BRCA2 antibody (556448, BD PharMingen, San Diego, CA), which recognises the epitopes of amino acids 2587–2601 of *BRCA2*.

The BRCA1 or BRCA2 antigen–antibody complexes were isolated by fixation on a POROS A (protein A) column (PerSeptive Biosystems, Inc. Framingham, MA). Elution was performed by a fall in pH to 2 with 0.1% (v/v) HCl/15 mM NaCl (flow rate: 5 ml/min). Detection of the immune complex was performed at 280 nm. Radioactivity of each 1 ml fraction was measured as described below. The protein A affinity chromatography by means of the immune complex gave the amount of DNA-binding proteins that bind specifically to anti-BRCA1 antibody or anti-BRCA2 antibody, and a ratio was calculated as follows: [Activity (dpm) of proteins eluted from protein A column]/[activity (dpm) of proteins eluted from heparin column] × 100. All data are means ± SD [38,46,47].

3. Results

3.1. Effect of lycopene on the proliferation of breast cell lines

MCF-7, HBL-100, MDA-MB-231 and MCF-10a were exposed to 2, 5, 10 or 20 μM lycopene for 12, 24 or 48 h. A control without lycopene and an assay with the lycopene solubilisation buffer, THF-BHT, were also done for each exposure time (Fig. 1).

For each cell line, an effect was observed on the cell cycle after 48 h of treatment with 10 μM of lycopene. The results showed a late G_1-phase cell cycle arrest corresponding to an increase in the G_1 phase cell number.

3.2. Effect of lycopene on BRCA1, Δ11-BRCA1, BRCA2 and Δ12-BRCA2 mRNA expression

To evaluate the effect of lycopene on *BRCA1* and *BRCA2* mRNA and their Δ11-*BRCA1* and Δ12-*BRCA2* splice variants, a study by quantitative RT-PCR was performed on the breast cell lines MCF-7, HBL-100, MDA-MB-231 and the fibrocystic breast cell line MCF-10a (Fig. 2). Expression of each mRNA species in lycopene-treated cells was normalised to their expression levels in untreated cells, normalised to an arbitrary value of 1.

Lycopene significantly increased *BRCA1*, Δ11-*BRCA1*, *BRCA2* and Δ12-*BRCA2* RNA expression in MCF-7 ($P < 0.05$) and HBL-100 ($P < 0.05$) ER-posi-

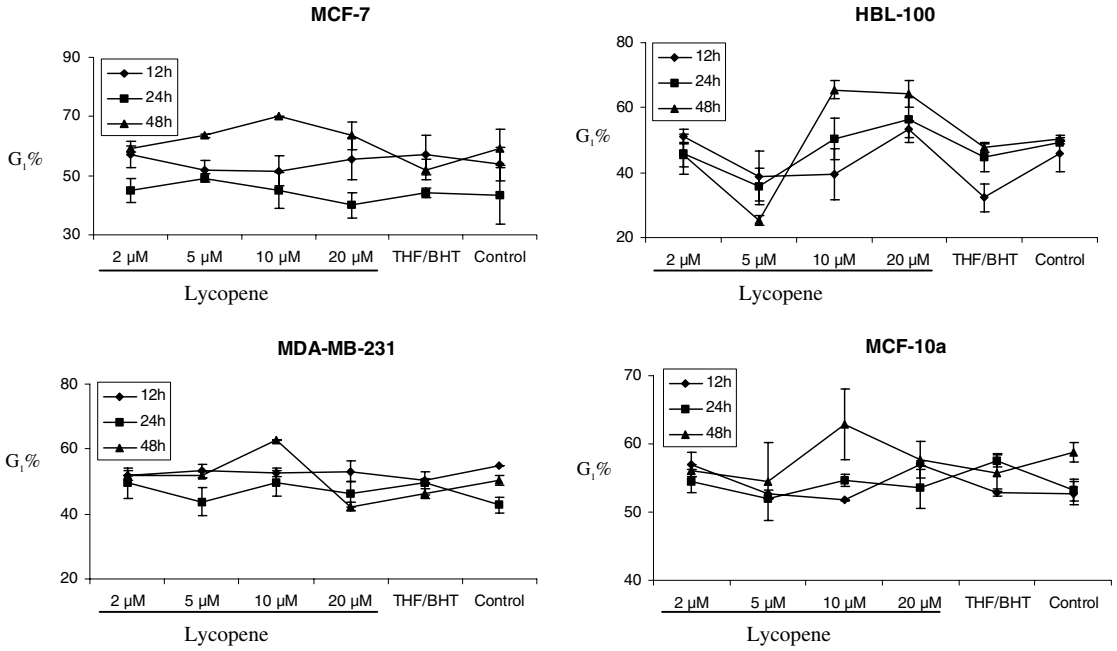

Fig. 1. Effect of different concentrations of lycopene on the cell cycle of MCF-7, HBL-100, MDA-MB-231 and MCF-10a breast cell lines by flow cytometry analysis. Cells were cultured with 2, 5, 10 or 20 µM lycopene in normal medium supplemented with THF-BHT or in normal medium (control) and harvested after 12, 24 and 48 h (each value of G_1 is the % of cells in G_1 phase and corresponds to mean ± standard deviation (SD) of three assays for each group).

tive cells. Conversely, in ER-negative MDA-MB-231 cells, lycopene significantly decreased mRNA gene expression ($P < 0.01$). No effect of lycopene was obtained in the fibrocystic breast cell line MCF-10a, except for Δ11-*BRCA1* ($P < 0.01$).

3.3. Effects of lycopene on BRCA1 and BRCA2 protein expression

BRCA1 and BRCA2 proteins were quantified by affinity chromatography after 48 h treatment with 10µM lycopene. No variation in the protein level was found (Fig. 3).

4. Discussion

Breast cancer is the most common cancer and cause of death in women. It is commonly known that diets high in fruits and vegetables probably reduce the risk of developing cancer. Recent research [48,49] on the functional effects of carotenoids, and particularly lycopene, showed a correlation between nutrition and degenerative disease prevention.

Herein, we studied the transcription and translation of the *BRCA1* and *BRCA2* genes and their different splicing variants in breast cell lines with different characteristics (MCF-7, HBL-100 and MDA-MB-231) and a fibrocystic breast cell line (MCF-10a) after lycopene treatment.

First, we determined that exposure to 10 µM lycopene for 48 h induced a G_1/S cell cycle arrest. Lycopene has also been observed to inhibit cell cycle progression [50]. This is consistent with another study in which 10 or 20 µM resulting in maximum growth cessation in MCF-7 and MDA-MB-231 [51]. We did not observe growth inhibition at 20 µM due to the toxicity of this product.

It is well known that the expression of *BRCA1* and *BRCA2* mRNA are regulated with the cell cycle and associated with proliferation in normal and tumour-derived breast epithelial cells. Cells arrested in G_0 or early in G_1 contain low levels of *BRCA1* and *BRCA2* mRNA. After release into a proliferative state, cells produce maximum levels of *BRCA1* and *BRCA2* mRNA in late G_1 and the S-phase [52,53], implying that these two tumour suppressor genes are utilised during growth and may have a protective role in cellular proliferation. Thus, we chose this checkpoint to perform the quantification of these two genes after treatment with lycopene.

We observed differential expression between the ER-positive cells (MCF-7 and HBL-100) which had increased expression of *BRCA1*, *BRCA2*, Δ11-*BRCA1* and Δ12-*BRCA2* mRNA, and the ER-negative cells (MDA-MB-231) which exhibited decreased *BRCA1*, *BRCA2*, Δ11-*BRCA1* and Δ12-*BRCA2* mRNA. No effect was observed in MCF-10a fibrocystic breast cells.

The biochemical mechanisms underlying the inhibitory effects of lycopene on the growth of cancer cells are largely unknown. It has been hypothesised that lycopene derivatives may act as ligands for a nuclear receptor

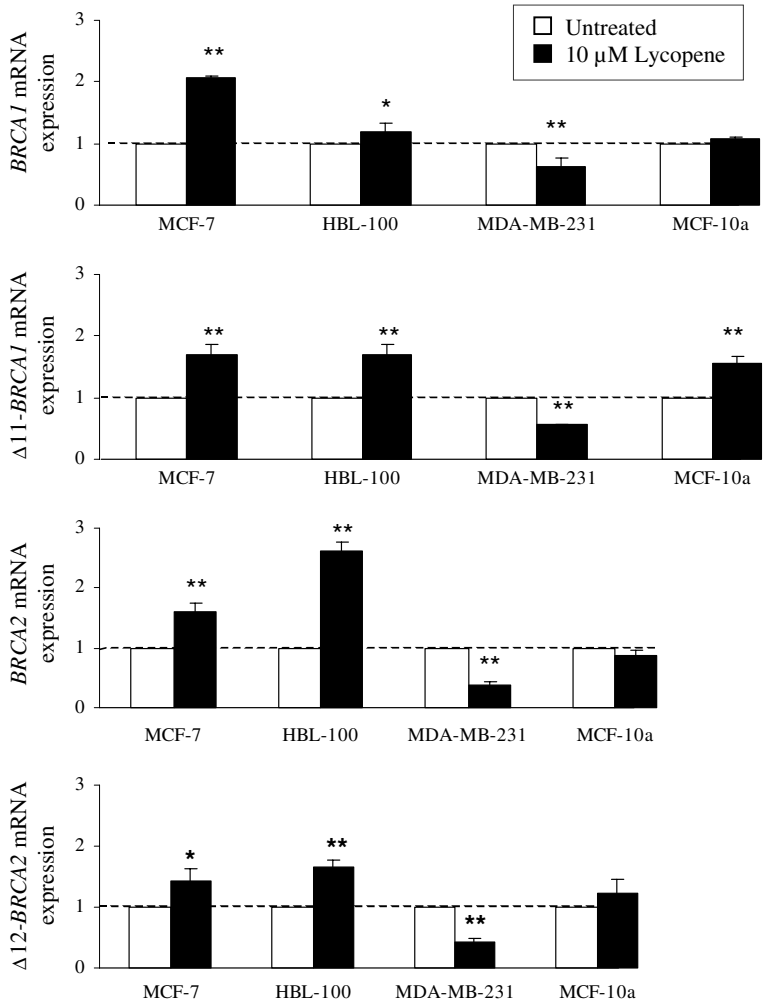

Fig. 2. Difference in the expression of *BRCA1*, Δ11-*BRCA1*, *BRCA2* and Δ12-*BRCA2* mRNA in MCF-7, HBL-100, MDA-MB-231 and MCF-10a breast cell lines after treatment with 10 μM lycopene for 48 h. Expression in the treated cells was normalised to untreated controls (corresponding to an arbitrary value of 1). Each measure was performed on two extractions and three reverse transcriptions (RT) and is expressed as mean ± SD. Statistical analysis was performed using the Student's *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$).

(RAR), in analogy to retinoic acid, the hormone derived from β-carotene [54]. Three subtypes of RAR are known: α, β and γ. MCF-7 cells express RARα, RARγ and retinoic X receptor (RXR), but not RARβ [55]. RARα has been shown to play a role in retinoic-induced growth inhibition of human breast cancer cell lines that express the ER. The dogma in the field has been that ER-positive breast cancer cell lines respond to retinoid treatment because they express RARα, whereas ER-negative breast cancer cell lines, such as MDA-MB-231, are refractory to retinoid treatment and have been thought to express little or no RARα [56,57]. In contrast, it has been shown that RARγ mRNA is expressed at relatively high levels in most tumour samples independent of the ER status, while RARβ mRNA is expressed at low levels in ER-positive cell lines [58]. Moreover, the loss of functional RARs may be a frequent event, leading to retinoic acid unresponsiveness of ER-negative breast cancer cells. This implies that both the steroid and retinoic receptor status of breast tumours may be important following treatment with retinoids [59].

We suggest that lycopene may interact with RARα in ER-positive cells and with RARβ in ER-negative cells, resulting in an increase in *BRCA1*, Δ11-*BRCA1*, *BRCA2* and Δ12-*BRCA2* mRNA expression in ER-positive cells and a decrease in *BRCA1*, Δ11-*BRCA1*, *BRCA2* and Δ12-*BRCA2* mRNA expression in ER-negative cells due to the underexpression of RARs and the loss of functional RARs.

It is well established that selected biomarkers play a key role in breast carcinoma, including certain steroid receptors (ER, progesterone receptor and RARβ), members of the HER/*erb*B family and selected tumour

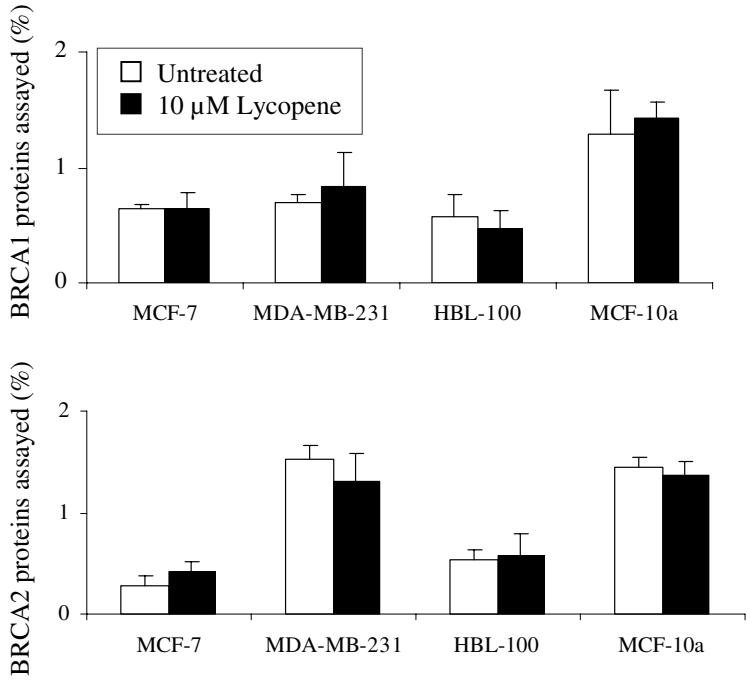

Fig. 3. Amount of BRCA1 and BRCA2 proteins expressed by MCF-7, MDA-MB 231, HBL-100 and MCF-10a breast cells lines after treatment with 10 μM lycopene for 48 h. BRCA1 and BRCA2 proteins were obtained after DNA-binding protein purification on heparin column, specific immunoprecipitation with anti-BRCA1 (556441) or anti-BRCA2 (556448) antibodies and protein A affinity chromatography, and expressed in percentages calculated as follows: $100 \times$ dpm DNA-binding proteins bound specifically to antibodies raised against BRCA1 or BRCA2/dpm of total DNA-binding proteins purified on heparin affinity chromatography. All data are expressed as means $\pm$ SD of three assays.

suppressor/susceptibility genes (e.g., *p53*, *BRCA1* and *BRCA2*) [60].

The protein quantification of BRCA1 and BRCA2 did not show any differences in any of the cell lines that we studied, confirming our previous results using affinity chromatography after treatments with different micronutrients (resveratrol [38], genistein and daidzein [37,61] and $n-3$ and $n-6$ polyunsaturated fatty acids [36]).

A post-transcriptional regulation might maintain the steady-state level of BRCA1 and BRCA2 proteins in cancer cells by modifying the stability of their mRNA [62]. Alternatively, the level of *BRCA1* mRNA in cells may be translationally regulated by antisense RNA transcripts.

Post-translational regulation is also possible and it would be interesting to check the phosphorylation status of BRCA1 and BRCA2, as well as their acetylation or ubiquitination level. Lycopene treatment has been shown to be responsible for a decrease in pRb phosphorylation and inhibition of G_1/S transition in MCF-7 and T-47D cells. Nahum and colleagues [50] suggested that the inhibitory effect of lycopene on cell cycle progression is mediated primarily through the downregulation of cyclin D, this action leads directly to a reduction in cdk4 kinase activity. The decrease in cyclin D levels is probably related to retention of p27 in the cyclin E–cdk2 complex resulting in the inhibition of cdk-2 kinase activity.

In conclusion, lycopene seems to have an effect on the mRNA levels of *BRCA1*, *BRCA2* and their splice variants, although there was no variation in the protein levels. To better understand the relationship between these genes and the impact of lycopene on other genes involved in mammary tumorigenesis, it will be necessary to use newly developed technologies such as microarrays.

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