IN VITRO THREE-DIMENSIONAL MICROENVIRONMENT INDUCES DIFFERENTIAL GENE EXPRESSION OF BREAST CANCER BIOMARKERS

MICROAMBIENTE TRIDIMENSIONAL IN VITRO *INDUZ A EXPRESSÃO GÊNICA DIFERENCIAL DE BIOMARCADORES DO CÂNCER DE MAMA*

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Abstract

In vitro three-dimensional culture has an invaluable role in the study of cell biology because it can provide a more physiologic microenvironment than the conventional two-dimensional culture, much more similar to in vivo features. The present study aimed to assess the expression of breast cancer biomarkers (*ERBB2*, *KRT5*, *MKI67*, *CDH3* and *TP63*) in breast cancer cell lines (BT-549 and Hs 578T) in these different in vitro culturing (2D x 3D) by quantitative PCR. The results showed a differential expression of those genes when the 2D cell lines were cultured in Matrigel™. This analysis provides indications of their expression in Matrigel™, highlighting ERBB2 and TP63 that emerge like promising markers of cancer stem cell. These findings offer a better understanding of the biology of the breast cancer cell lines analyzed for further use of these models in drug cancer cytotoxicity assays.

Keywords: three-dimensional culture; breast cancer; cell lines; gene expression; Matrigel™.

Resumo

O cultivo tridimensional in vitro *tem um papel inestimável no estudo da biologia das células, pois proporciona um microambiente mais fisiológico do que a cultura bidimensional, muito mais semelhante a características* in vivo. *O presente estudo teve como objetivo avaliar a expressão de biomarcadores do câncer de mama (*ERBB2, KRT5, MKI67, CDH3 and TP63*) em linhagens celulares de câncer de mama (BT-549 e Hs 578T) em sistemas de cultura* in vitro *2D x 3D por PCR quantitativo. Os resultados mostraram uma expressão diferencial dos genes quando as linhagens celulares cultivadas em 2D foram cultivadas em Matrigel™. Esta análise fornece indícios da expressão desses genes em Matrigel™ com destaque para ERBB2 e TP63, que surgem como promissores marcadores de células-tronco do câncer. Estes resultados oferecem uma melhor compreensão da biologia das linhagens celulares de câncer de mama estudadas para futuramente utilizar esse modelo em ensaios de citotoxicidade contra o câncer.*

Palavras-chave: *cultura tridimensional; câncer de mama; linhagem celular; expressão gênica; Matrigel™.*

1. Introduction

Breast cancer is the most frequent cancer in women worldwide with an estimated 1.67 million new cancer cases diagnosed in 2012, representing 25% of all cancers (GLOBOCAN, 2012). It is a heterogeneous disease with distinct subtypes in terms of clinical implications, evolution, therapeutic response and molecular profile (PEROU *et al*., 2000; Sorlie *et al*., 2001). The hormone receptors (estrogen and progesterone) and the human epidermal growth factor receptor 2 (ERBB2) are used in routine clinical management of patients with breast cancer (ALLRED *et al.,* 2010). They are very important to determine patients' treatment and outcome (Slamon *et al*., 2001). The markers Ki-67, p53, vascular markers, p63, cytokeratin 5 and P-cadherin have also been used (FASCHING *et al*., 2011; FITZGIBONS *et al*., 2000; MATOS e*t al*., 2005; MILLAR *et al*., 2011; PAREDES *et al*., 2005.

In vivo models offer a relevant physiological environment to study carcinogenesis and drug screening, but they are limited in yields and are difficult to dissect biochemically (JACKS e WEINBERG, 2002), besides the ethical issue involved. The two-dimensional (2D) in vitro models create an environment that differs markedly from the breast microenvironment (VARGO-GOGOLA; ROSEN, 2007). Then they do not provide the optimal system for fully understanding cell proliferation, death and differentiation (BISSEL e RADISKY, 2001; O'BRIEN *et al*., 2002; WELGELT e BISSEL, 2008). Therefore three-dimensional (3D) models have the advantage of simulating the in vivo interactions that regulate morphology and tissue-specific function providing the appropriate structural and functional context for the study of cancer genes (DEBNATH *et al*., 2003), being a powerful alternative way to perform genetic and biochemical analysis (JACKS e WEINBERG, 2002).

Nowadays, 3D in vitro models have become very important in the studies of normal and malignant development and in molecular studies in the search of new therapeutic targets and in the screening of new therapeutic drugs in cancer (FOURNIER *et al*., 2006; KENNY *et al*., 2007; MARTIN *et al*.,2008; WEAVER *et al*., 2002; WELGELTe BISSEL, 2008). Taking into account the great value of 3D models in cellular signaling and morphology the present work aims to evaluate gene expression profile of important molecular markers in breast cancer in two breast cancer cell lines cultured in 3D model using Matrigel™, a basement membrane matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse tumor rich in extracellular matrix proteins, which offers a biologically active matrix that is effective for the attachment and differentiation of cells (LACROIX e LECLERQ, 2004).

2. Materials and methods

2.1. Cell culture

BT-549 (Cat. # HTB-122™) and Hs 578T (Cat. # HTB-126™) cells were obtained from American Type Culture Collection (ATCC) and propagated in monolayer, respectively, in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gibco), 0.10 µg/mL of bovine insulin (Sigma) and DMEM (Sigma) with 10% fetal bovine serum (Gibco), 10 µg/mL of bovine insulin (Sigma).

For three-dimensional (3D) cultures, the parental cell lines were trypsinizated and single cells (4 x 10^4 cells/well) were seedied on top of Matrigel™ (BD Bioscience) in 24-well plates using the culture mediums described above without supplementation (LEE *et al*., 2007). These cultures were maintained for three days. All cultures were incubated in a 5% CO_2 incubator at 37 °C.

2.2. Fluorescence optical microscopy

Cells were stained with Image-iT™ LIVE Plasma Membrane and Nuclear Labeling kit (Invitrogen), Phalloidin Alexa Fluor® 488 (Molecular Probes) and the nuclei were counterstained with 4,6-diaminidino-2-phenylindole (DAPI; Molecular Probes) according to the manufacturer's instructions. 3D cells culture were stained directly in the Matrigel™

but the incubation time was doubled. Image acquisition was performed using AxioVert 200 microscope (Zeiss).

2.3. RNA isolation and Quantitative PCR analysis

Total RNA was isolated from parental cell lines using TRIzol® (Life Technologies) according to the manufacturer's instructions. 3D cells cultures were isolated by enzymatic digestion with 0.46 mL/well of Dispase 10mg/mL (Gibco) for 1h/37 °C followed by TRIzol® homogenization. Total RNA concentration and 260/280 nm absorbance was measured using NanoVue spectrophotometer (GE Healthcare). Total RNA was treated with RNase-free DNase Set (Qiagen). cDNA was synthesized using 1µg of total RNA with M-MLV Reverse transcriptase (Promega). Quantitative PCR (qPCR) was performed with Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions and followed with the Stratagene Mx3005P detection system (Agilent Technologies). Two negative controls (no cDNA and with no transcriptase reverse) were prepared for every set of reactions. The genes analyzed in this study were human epidermal growth factor receptor 2 (*ERBB2*), keratin 5 (*KRT5*), marker of proliferation Ki-67 (*MKI67*), P-cadherin (*CDH3)* and tumor protein p63 (*TP63*). Primers are listed in Table 1. The qPCR cycling conditions were performed as follows: 95 °C for 10 min and 40 cycles of 95 °C for 30 sec, annealing at 60 °C for 60 sec and extension at 72 °C for 30 sec. The values obtained were normalized using the housekeeping gene TATA box binding protein (*TBP*) and relative expression level were calculated with the $2^{-\Delta\Delta CT}$ method (LIVAK e SCHMITTGEN, 2001) using parental cell lines as calibrator.

Table 1: Primers used for quantitative PCR

GENE	GENEBANK ACCESSION Nº	LOCATION	AMPLICON	REFERENCE	CONCENTRATION
ERBB2	NM_004448	17q21.1	182pb	PrimerBank ID 4758298a3	200nM FW/150nM RV
KRT5	NM_000424	12q13.13	152pb	PrimerBank ID 119395753b3	250nM FW/150nM RV
MKI67	X65551	10q26.2	250pb	PrimerBank ID 415821a3	250nM FW/250nM RV
TP63	AJ315499	3q28	182pb	PrimerBank ID 169234656c1	250nM FW/150nM RV
CDH3	NM_001793	16q22.1	204pb	PrimerBank ID 14589891a1	250nM FW/250nM RV
TBP	NM_003194	6q27	132pb	Li et al., 2009	300nM FW/300nM RV

2.4. Statistical analysis

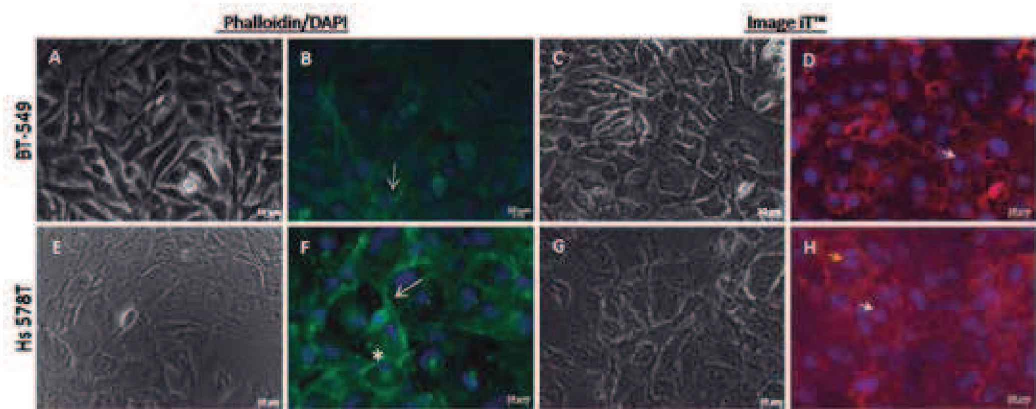
Statistical analysis was performed using REST 2009 (Relative Expression Software Tool) from Qiagen. A p value less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1 Cell line morphology

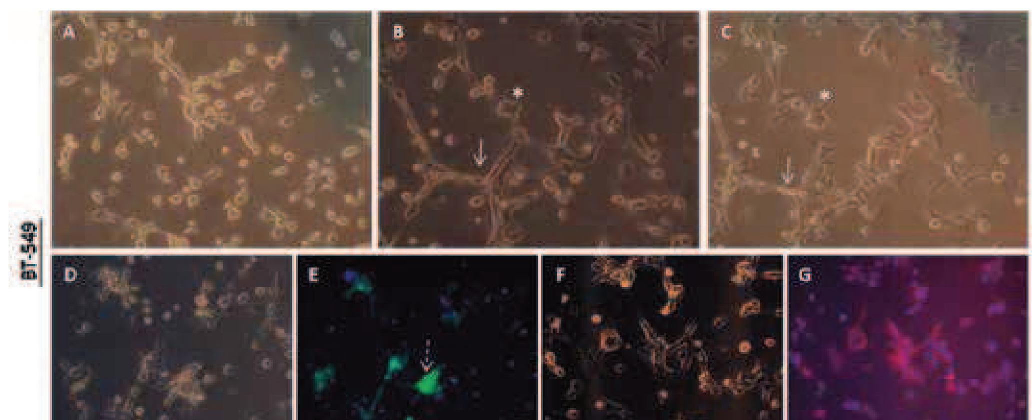
We cultured the parental cell lines, BT-549 and Hs 578T, in 2D and 3D systems and analyzed them through optical microscopy. The BT-549 cell line in 2D culture presents a polymorphic population with epithelial-like components and multinucleated giant cells (ATCC, data sheet). The Hs 578T cell line in monolayer presents a mixed polygonal morphology and a stellate cell type with aggregates of casein protein granules (ATCC, data sheet) and a nucleus-cytoplasm ratio greater than the BT-549 (Figure 1: B and F). Both cell lines grew in a disorganized manner, displaying overlapping layers (Figure 1).

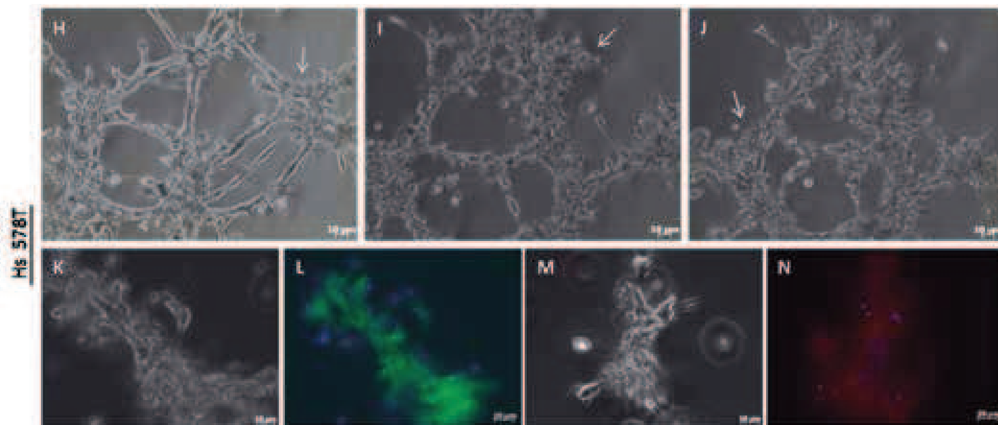
Figure 1: BT-549 and Hs 578T cell lines growing in 2D culture: BT-549 (A-D) and Hs 578T (E-H) cells stained with Phalloidin/DAPI (green/blue) and Image iT™ (red/blue). Differential interference contrast images are shown in A and C for BT-549 and in E and G for Hs 578T. Actin staining cytoplasmic projections (arrow) and peripherically to the plasma membrane (asterisk). Small spherical nuclei of BT-549 cell (arrow) and polymorphic nuclei of Hs 578T cell (spherical – white arrow; reniform – orange arrow).



In Matrigel™ culture, the BT-549 cell line, after three days, showed an invasive phenotype, with stellate projections that often creates bridges between multiple cell colonies (KENNY *et al.*, 2007) (Figure 2: A-G). The Hs 578T cell line presented a structure tubular-like with multiple ramifications and multiple cell colonies (Figure 2: H-N). Both cells were able of differentiation in complex three-dimensional structures. The cellular architecture of the structures formed on Matrigel™ is visualized in Figure 2 through Phalloidin/DAPI and Image iT™ staining. The differences in the actin filaments distribution observed between the cell lines (Figure 1: B and F) were probably responsible for the organization and architecture of each cell exhibited in 3D culture.

Figure 2: BT-549 and Hs 578T cell lines growing in 3D culture: BT-549(A-G): at day 2 cells were organized, juxtaposed and aligned. Stellate projections (asterisk) and bridges between multiple cell colonies (arrow). Very organized cellular architecture is revealed with Phallodin/DAPI (green/ blue) and Image iT™ (red/blue) and strong staining of actin filaments in the multiple cell colonies (dotted arrow) with Phallodin/DAPI. Hs 578T cell (H-N): multiple cell colonies and branching from day 1 (arrow). Phallodin/DAPI and Image iT™ shows homogeneous distribution of actin filaments and cellular architecture.



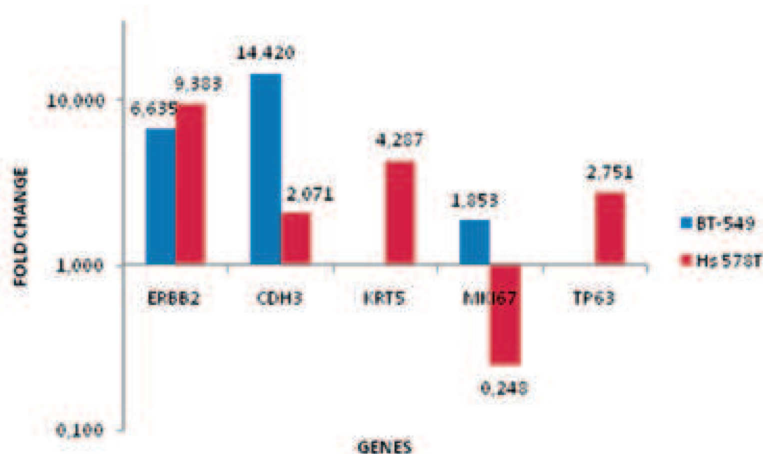


3.2 Gene expression analysis

Next we performed qPCR assays from samples obtained from Hs 578T and BT-549 cells cultured in 2D and 3D systems (Figure 3). *ERBB2, KRT5, MKI67, CDH3* and *TP63* presented a differential expression when the cell lines were cultured in Matrigel™ compared with their expression in cells cultured in the 2D system. We found that *ERBB2* and *CDH3* were up-regulated (p = 0.000) in BT-549 and in Hs 578T. In Hs 578T cells cultivated in the 3D system, the *KRT5* and *TP63* were up-regulated (p = 0.000) in relation to these cells in the 2D system. Both these genes were not amplified in BT-549 cells cultivated in the 3D system, which may indicate a negative regulation of these genes since they were expressed in 2D system. *MKI67* expression did not change in BT-549 (p = 0.496) and was down-regulated in Hs 578T (p = 0.000) in the 3D in comparison to the 2D culture systems.

Taken together the genes *ERBB2*, and *CDH3* for BT-549 showed statistically significant differences between the two types of cultures (2D x 3D). For Hs 578T cells, all the genes analyzed (*ERBB2, KRT5, MKI67, CDH3* and *TP63*) showed statistically significant differences between the two types of cultures (2D x 3D) with *ERBB2* presenting the highest up-regulation and *MK167* presenting the highest down-regulation.

Figure 3: *Relative gene expression of BT-549 and Hs 578T cells in 3D culture. Relative gene expression levels ERBB2, KRT5, MKI67, CDH3 and TP63 genes in both cells showed a differential expression when these cells were cultured in Matrigel™ compared with the 2D culture. The genes KRT5 and TP63 showed no amplification in the 3D cultures for BT-549 cells. The values were obtained by REST 2009 software.*



The Matrigel™ culture system seems to be effective for cell growth and differentiation. Thus, the pronounced overexpression of *ERBB2* suggests that BT-549 and Hs 578T cells cultured in this system may include a population of undifferentiated cells involved in the formation of 3D structures observed in Figure 2. This suggestion is based on a correlation made by Korkaya *et al.* (2008) between *ERBB2* overexpression and the increase in stem/progenitor cell populations.

The *CDH3* gene codes for P-cadherin, a protein known for its role in cellular differentiation and growth (GAMALLO *et al.*, 2001). Its up-regulation in BT-549 and Hs 578T 3D cultures is compatible with the formation of 3D structures on Matrigel™ (Figure 2).

TP63 and *KRT5* are highly expressed in basal and myoepithelial cells (KARANTZA, 2011 ; YANG *et al.*, 1998, 1999). The lack of amplification of these genes in BT-549 3D cultures suggest they may have originated from an invasive tumor, characterized as one that lost the basal layer of myoepithelial cells (STEFANOU *et al.*, 2004). *TP63* also regulates the expression of basal cytokeratins such as KRT5 and KRT14 (ROMANO *et al.*, 2009, YANG *et al.*, 1999). Indeed, Yang *et al.* (1999) showed that $p63^{-/-}$ mice do not express KRT5, which could be another explanation for the absence of *KRT5* in the BT-549 3D cultures. In Hs 578T 3D cultures, *KRT5* and *TP63* were up-regulated.

MKI67 was down-regulated in Hs 578T 3D cultures but did not significantly change in BT-549 3D compared to 2D cultures. Debnath *et al.* (2002) and Coppock *et al.* (2007) showed a balance between proliferation and apoptosis in the formation and maintenance of acinus in mammary epithelial cells in 3D culture. The result obtained by these authors suggests that the cell 3D structures studied herein may be in different stages of cell differentiation.

3. Conclusion

Individualized analysis of the genes *ERBB2*, *KRT5*, *MKI67*, *CDH3* and *TP63* provides indications of their expression in relevant physiological environment (Matrigel™) for the study of cell and molecular biology. We highlight the genes *ERBB2* and *TP63* due to their great importance on cell and tumor biology. These genes were linked with the stem cell population (KORKAYA *et al.*, 2008; YANG *et al.*, 1998, 1999) and so they may also be involved in the persistence of cancer stem cells. There are increasing evidence showing that these cells are responsible for sustaining tumorigenesis, tumor resistance to conventional cancer therapies and relapse (DEAN *et al.*, 2005; LEE *et al.*, 2011; LI *et al.*, 2008; PHILIPS *et al.*, 2006; REYA *et al.*, 2001; VISVADER e LINDERMAN, 2008; ZHOU *et al.*, 2009). A better knowledge of these genes in a matrix, like the Matrigel™, capable of recapitulating in culture the growth behavior as well as structural and functional differentiation characteristics of the cells in vivo (PETERSEN *et al.*, 1992) will be a breakthrough in cancer research field. This paper made it possible to understand the biology of the breast cancer cell lines BT-549 and Hs 578T in 3D culture for further use of these models in cytotoxicity assay in the search for new cancer drugs and biomarkers that could be utilized in the diagnosis, prevention and treatment of breast cancer patients. ■

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