Differential gene expression assessed by cDNA microarray analysis in breast cancer tissue under tamoxifen treatment

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Summary

Our purpose was to identify tamoxifen (TAM) responsive genes after 30 days of TAM treatment in tumor tissues obtained from women with breast cancer using microarray expression analysis. In our study, we identified 12 candidates to be considered as tamoxifen-modulated genes. Among them, we selected two candidates the TEGT BI-1 (testis enhanced gene transcript Bax Inhibitor-1) and the CD63 gene in order to further confirm their differential expression under tamoxifen effects. We observed that both were down-regulated in tumor tissues of patients during TAM treatment. TEGT is able to inhibit the expression of Bax, which is known to promote apoptosis. On the other hand, CD63 encodes a cell membrane protein and it seems to be involved in mechanisms of platelet activation, cell adhesion and cell motility. We therefore hypothesize that TAM would be able to modulate tumor growth by down-regulating genes involved in mechanisms such as cell cycle control, tumor invasion and metastasis.

Key words: cDNA microarray analysis; Breast cancer; Tamoxifen.

Introduction

It is well established that breast cancer cell proliferation is influenced by several peptide growth factors and hormones that appear to control cell growth by interacting with their specific cellular receptors including estrogen receptors (ER) and progesterone receptors (PR) [1, 2].

Estrogen primarily appears to stimulate normal ductal growth, whereas progesterone is responsible for lobule-alveolar development. These lipid-soluble molecules diffuse easily through cell membranes and bind to cytoplasm receptors, which are then transported to the nucleus where they interact with DNA [3, 4].

This interaction then stimulates appropriate genetic responses that dictate normal cellular growth and "housekeeping" functions [3, 4]. Recent advances in understanding the mechanisms by which nuclear hormone receptors exert their effects on gene transcription have greatly enhanced the prospects for tissue-selective regulation of these processes [5].

The recognition that the estrogen receptor (ER) may interact with more than one DNA response element, that various coactivators and corepressors may mediate these interactions and that the nature of these interactions may be dependent on the specific ligand that is bound to the receptor allows for the possibility of ligand-based transcriptional control [5, 6].

Many breast cancers express ER and PR, and cell growth appears to be hormone-dependent in these tissues [7]. It is believed that manipulation of the hormonal environment influences division and viability of steroid hormone-dependent breast cancer cells. Tamoxifen (TAM), a specific estrogen receptor modulator – SERM, has been used in the treatment of breast cancer for more than 20 years and more recently it has been administered for chemoprevention of breast cancer in high-risk women showing encouraging results [8].

Although many of the TAM actions are already established, very little is known about its influence on specific genes. Therefore, the main goal of our study was to identify new tamoxifen responsive genes in breast cancer tissue obtained from patients before and during tamoxifen therapy using microarray expression analysis.

Material and Methods

Specimens

Human breast cancer tissue was obtained at the Mastology Group, Gynecology Department, Federal University of São Paulo, Escola Paulista de Medicina, São Paulo, Brazil, according to a protocol approved by the Human Investigations Committee. The patients included 12 women who were diagnosed as having estrogen receptor-positive Stage II ($T_2N_1M_0$) infiltrating ductal carcinoma. The diagnosis was made by incisional biopsy; then, all women were treated with TAM (20 mg/day) for four weeks before the performance of a modified radical mastectomy. Neither patient had received radiotherapy, chemotherapy or any hormonal treatment during the six months before the diagnosis of breast cancer. A fragment of malignant tissue was obtained at the time of performing the incisional biopsies and during mastectomy. All of the tissue samples were immediately frozen in liquid nitrogen upon removal. The remaining tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin following standard histological procedures in order to include, in the present study, tumor fragments containing at least 70% of tumor cells.

RNA extraction and reverse transcription

Total RNA was extracted from tumor samples by using Trizol (Invitrogen) according to the manufacturer's instructions. RNA

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integrity was controlled on denaturing formaldehyde/agarose gel electrophoresis. Prior to reverse transcription, two pools of RNA were obtained by mixing 5 mg of total RNA from each individual case in two separated tubes representing tumors before (Group T) and during tamoxifen therapy (Group T+TAM). In order to obtain cDNA radiolabeled probes, the two RNA pools, containing 60 µg of RNA each, were reverse transcribed in the presence of oligodT primers and α-[^{33}P] (2000Ci/mmol) dATP by using the kit Superscript II (Invitrogen) following the manufacturer's instructions. Probes were purified by Probequant G50 microcolumns (Amersham Pharmacia Biotech).

cDNA arrays

The 2,000 cDNA array membranes were assembled at the Ludwig Institute for Cancer Research, São Paulo (Brazil branch). The spotted cDNAs were generated during the Human Cancer Genome Project [10] (Camargo *et al.,* 2001). This project consists entirely on the sequencing and analysis of short cDNA fragments generated preferentially from the central coding portions of expressed human genes obtained from tumors including breast, ovarian, prostate, stomach and colon. The cDNA fragments are termed ORESTES (Open Reading Frame EST's) and are generated using a strategy developed and patented by the Ludwig Institute for Cancer Research. We first selected known cancer-related genes to be spotted onto nylon membranes (Hybond N+, Amersham Pharmacia Biotech), followed by other genes including housekeeping genes from a list provided by the FAPESP-LICR Human Cancer Genome Project. cDNA segments ranging from 200 to 700 bp were PCR amplified using primers flanking the multiple cloning sites of the pUC vector. Clones containing repetitive elements or homopolymeric regions were not used.

Hybridization conditions

Hybridization took place at 42°C in an overnight reaction containing 20 x standard saline citrate (SSC), 50 x Denhardt's solution, 50% formamide, 10 x sodium dodecyl sulphate (SDS) and 100 µg/ml salmon-sperm DNA. The filters were washed at 50°C in 1 x SSC/0.1% SDS for 15 minutes. Images were obtained by scanning the membrane in a storage phosphor system (Cyclone™ - Packard BioScience Company, Meriden, CT, USA). DNA targets on the arrays were located using grid overlays and spot intensities were subsequently measured (ImageQuant, Molecular Dynamics). Spot intensities between arrays were normalized to the average intensities of a set of housekeeping genes present on each array. Relative levels of gene expression between arrays were calculated by the following formula [10]:

$$P\,(g_a.g_b) = \frac{(g_a+g_b)\,!}{g_a!g_b!\;2^{(ga+gb+1)}}$$

Small values for p correspond to large differences between ga (genes from group T) and gb (genes from group T+TAM), unlikely to arise by chance if the gene under scrutiny is expressed at the same level in conditions with or without TAM. Provided that all experimental factors are well replicated, statistically significant discrepancies (such as $p < 1\%$) between the values of ga and gb can thus be used to point out the gene most likely to be differentially expressed. A value of $p < 0.03$ was considered statistically significant (Figure 1).

RT-PCR

Validation of array data by semiquatitative RT-PCR was performed by pooling equal amounts of total RNA from each group, pools were then submitted to reverse transcription by using Superscript II RNAse H reverse transcriptase (Invitrogen) in a final volume of 20 µl according to the manufacturer's instructions. The PCR primers were as follows: glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used to normalize the reactions (sense GTGAAGGTCGGTGTGAACGGATTT) and (antisense CACAGTCTTCTGAGTGGCAGTGAT). TEGT Bax Inhibitor-1 (sense ATAGGGAAGGGGAATGGTTG) and (antisense CCTCGCTCTGTTGATGTGAA).

CD63 (sense CTACCCCTGGCTCTCTGTTG) and (antisense TCGGGTAATTCTCCATCTGC). Primers were designed by Prime3 program-http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi) and the expected size band 243 bp for CD63 and 200bp for TEGT Bax Inhibitor -1 (TEGT BI-1). Each semiquantitative analysis was repeated at least five times. Negative controls were also included in order to avoid false-positive results. Two microliters of the reverse transcribed cDNA was amplified in a final volume of 50 µl by 30 cycles of PCR under standard conditions: 1.5 mM MgCl2, 125 mM dNTP and 2.5 U Taq polymerase using specific primers at 10 µM concentration. The amplified PCR products were separated on a 2% agarose gel containing 0.1 µg/ml ethidium bromide. The visualized bands were analyzed semi-quantitatively using image-scanning densitometry (Kodak EDAS 120) (Figure 2).

Results

A total of 12 genes were shown to be up - or downregulated in tumors before (T) and during TAM therapy (T+TAM) (Table 1). Among them, we selected two candidates, the TEGT BI-1 GenBank Accession X75861) and the CD63 gene (GenBank Accession NM 00178) in order to further confirm their expression under tamoxifen effects. We could observe that both were downregulated in the tumor tissues of patients during TAM treatment.

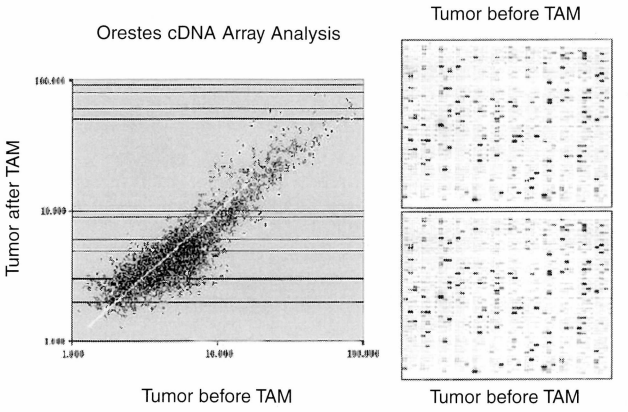
Discussion

TAM was proven, in the Breast Cancer Prevention Trial, to reduce breast cancer incidence by 49% in women at increased risk of the disease [8]. Based on these findings, the U.S. Food and Drug Administration (FDA) approved the use of tamoxifen to reduce the incidence of breast cancer in women at increased risk of the disease in October 1998.

In order to identify potential candidates to understand the in vivo TAM action, in the past five years we have been trying to isolate TAM responsive genes in human normal and breast cancer samples before and during TAM therapy. Indeed, we already showed that TAM down-regulates the expression of CD 36 in human normal and breast cancer samples [11].

This finding might represent an additional pathway of action of TAM affecting the function of genes involved in hematogenous tumor spread, invasion and angiogenesis.

In order to pursue our research further, in the present study we analyzed the differential expression of human



g. 1

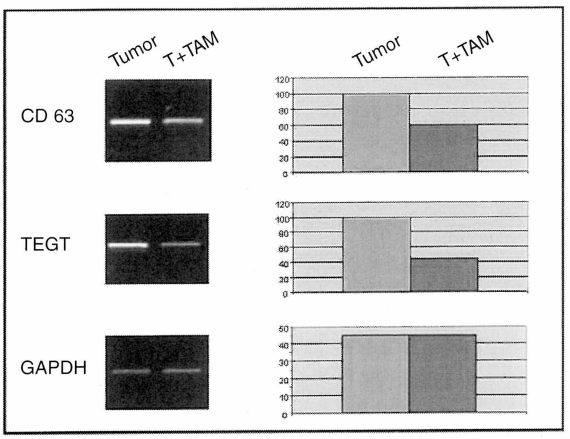
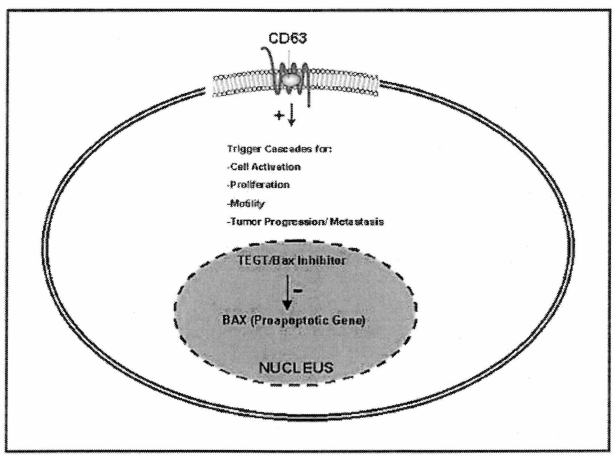


Fig. 2



g. 3

Figure 1. — cDNA array from tumor samples before and during TAM treatment.

Figure 2. — RT-PCR semi-quantitative analysis from CD63 and TEGT in breast malignant tumors before treatment and on the 30th day of tamoxifen treatment (T+TAM). The RNA pools were reverse transcribed and cDNAs were submitted to 30 cycles of PCR amplification. GAPDH expression levels were used to normalize reactions.

Figure 3. — Hypothetical model explaining how CD63 and TEGT proteins might be involved in cell activation, proliferation, motility, tumor progression/metastasis and apoptosis. The down-regulation of those genes exerted by tamoxifen might explain some of its actions.

genes in invasive breast cancer tissue samples, before and during tamoxifen treatment (20 mg/day) by using the cDNA array approach. Among the differentially expressed genes found here, we selected two genes, the TEGT BI-1 and the CD63, in order to further analyze their properties under tamoxifen effects.

Walter *et al.* [12] identified a single-copy gene in the rat for which two transcripts were found in each organ tested. The shorter transcript of about 1 kb was highly abundant in the postpubertal testis. The gene was therefore designated TEGT (for testis enhanced gene transcript).

Using a rat TEGT probe, Walter *et al.* [12] screened a human testis cDNA library and isolated the human homolog of the rat TEGT gene. The gene in the rat and humans does not belong to any known gene family of vertebrates.

The two different transcripts are due to alternative usage of two polyadenylation sites and the presence of a nuclear targeting motif indicates that the gene product must localize to the nucleus.

By Southern blot analysis of DNA from rat/human somatic cell hybrids, Walter *et al.* [13] mapped the TEGT gene to human chromosome 12 and fluorescence in situ hybridization refined the assignment to 12q12-q13.

Xu and Reed [14] transformed yeast cells containing a galactose-inducible Bax plasmid by using a human cDNA library (in which cDNAs were fused to a constitutively active yeast promoter) and isolated cDNAs that prevented Bax-induced lethality in response to galactose. This resulted in the identification of a gene, termed BI-1 *(Bax Inhibitor-1)*, which was identical to *TEGT*.

Therefore, regarding its function TEGT BI-1 is known to inhibit the Bax gene which is known to act as an anti-apoptotic element [15]. According to our results, TEGT BI-1 was downregulated in tumor tissues of patients during TAM treatment and therefore the TAM ability to downregulate this antiapoptotic gene seems to be another way to modulate apoptotic mechanisms in malignant breast tissue.

Virtual Northern blot analysis performed at the SAGE website (http://www.ncbi.nlm.nih.gov/SAGE) using the human TEGT BI-1 cDNA sequence (GenBank XM035490), indicate that TEGT can be found overexpressed in many studies using breast cancer samples.

In our study another down-regulated gene in breast cancer tissue, treated with tamoxifen, was the CD63. This protein was identified as a platelet activation marker and its gene is mapped to chromosome 12 p12-q33. This gene encodes a cell membrane protein and seems to be involved in mechanisms of platelet activation, cell adhesion and cell motility [16].

CD 63 belongs to a recently described gene family called TM4SF; members of this family are known to play

roles in signal transduction pathways and to regulate cell activation, development, proliferation, and motility [17]. Among the TM4SF family, CD9 [18] and CD63 [19] have been reported to modulate tumor progression or metastasis.

Virtual Northern blot analysis, at the SAGE website (http://www.ncbi.nlm.nih.gov/SAGE), using the CD63 cDNA sequence (GenBank NM001780) identified that breast cancer cells cultivated under tamoxifen treatment had their CD63 mRNA levels down-regulated during TAM treatment.

Studying breast cancer cells Stephen *et al.* [17] found that CD 63 expression is regulated by estrogen in human breast cancer cells MCF 7 and T47D. More recently, Sordat *et al.* [20], using cDNA array, found that changes in CD63 could affect migratory signals and progression of metastatic disease in colon cancer patients.

These findings are in agreement with our results and reinforce our hypothesis that TAM would be able to modulate breast carcinogenesis by down-regulating genes that might be involved in mechanisms of tumor invasion and metastasis.

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