Tamoxifen down-regulates CaMKII messenger RNA levels in normal human breast tissue

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Summary

Tamoxifen was proven to reduce the incidence of breast cancer by 49% in women at increased risk of the disease in the Breast Cancer Prevention Trial. In order to identify potential candidates to explain the preventive effect induced by tamoxifen on breast cancer, normal breast tissue obtained from 42 fibroadenoma patients, randomly assigned to receive placebo or tamoxifen, was analyzed by the reverse Northern blot and RT-PCR techniques.

The cDNA fragments used on Northern blot membranes were generated by the Human Cancer Genome Project funded by the Ludwig Institute for Cancer Research and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil).

Total RNA was obtained from normal breast tissue from patients with clinical, cytological and ultrasound diagnosis of fibroadenoma. After a 50-day treatment with tamoxifen (10 or 20 mg/day) or placebo, normal breast tissue adjacent to the tumor was collected during lumpectomy with local anesthesia.

One differentially expressed gene, Calcium/calmodulin-dependent protein kinase II (CaMKII), was found to be down-regulated during TAM treatment. CaMKII is an ubiquitous serine/threonine protein kinase that has been implicated in the diverse effects of hormones utilizing Ca2+ as a second messenger as well as in *c-fos* activation.

These results indicate that the down-regulation of CaMKII induced by TAM might represent alternative or additional mechanisms of the action of this drug on cell cycle control and response to hormones in normal human breast tissue.

Key words: Tamoxifen; Breast Tissue; CaMKII; Human Cancer Genome Project; Ca+2-signalling.

Introduction

Tamoxifen (TAM) exerts a long-term suppressive effect on breast cancer cell proliferation. It has been used in the treatment of breast cancer patients for more than 20 years [1]. TAM is a nonsteroidal triphenylethylene capable of adopting a structural conformation that resembles a steroid nucleus. It binds to an estrogen receptor in competition with this hormone, acting as both its antagonist and a partial agonist [2-4].

This orally effective, synthetic antiestrogen used first in women with metastatic disease has become an essential part of any therapeutic strategy for the control and prevention of breast cancer [5].

In addition, TAM was proven to reduce the incidence of breast cancer by 49% in women at increased risk of the disease in the Breast Cancer Prevention Trial [6]. 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 in October 1998.

In order to identify potential candidates to explain the preventive effect induced by TAM on breast cancer in the past four years, we have been trying to isolate TAM responsive genes in normal human breast tissue and breast cancer samples before and during TAM therapy. Indeed, we have already shown that TAM down-regulates the expression of CD 36 in normal human breast tissue

and breast cancer samples. This finding might represent an additional pathway of action of TAM affecting the function of genes involved in hematogenous tumor spread, invasion and angiogenesis [7].

To pursue our research further, we designed this study with the aim of isolating TAM responsive genes in normal human breast tissue. This time, we analyzed total RNA from 42 samples of normal breast tissue obtained from fibroadenoma patients receiving placebo or TAM (10 or 20 mg/day/50 days), in a prospective, double-blind, randomized, placebo-controled study using reverse Northern Blot and semi-quantitative RT-PCR.

The cDNA fragments used on Northern membranes were generated by the Human Cancer Genome Project funded by the Ludwig Institute for Cancer Research and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil). This is a program for human gene discovery and human coding region compilation based on a novel concept for the high throughput sequencing of human open reading frames [8].

The project consists entirely on the sequencing and analysis of short cDNA fragments generated preferentially from the central coding portions of expressed human genes [9] obtained from tumors including breast, ovarian, prostate, stomach and colon.

The cDNA fragments are termed ORESTES (Open Reading Frame ESTs) and are generated using a strategy developed and patented by the Ludwig Institute for Cancer Research.

Material and Methods

Normal breast tissue specimens

Human normal breast samples were obtained from the Mastology Group, Gynecology Department, Federal University of São Paulo according to a protocol approved by the Human Investigations Committee. Informed consent was obtained from subjects before enrollment.

This prospective, double-blind, randomized, placebo-controlled study included 42 women with a previous clinical, ultrasound, and cytological diagnosis (fine needle aspiration) of fibroadenoma.

Patients were divided into the following groups: group I (13 patients) received placebo/50 days; group II (14 patients) and group III (15 patients) received 10 and 20 mg/day/50 days, respectively. On the 50th day of TAM or placebo treatment, samples were collected from the normal breast tissue adjacent to the benign tumor during lumpectomy with local anesthesia.

None of the patients had received any hormonal treatment during the six months before the diagnosis of fibroadenoma. All the tissue samples were frozen in liquid nitrogen immediately upon removal. The remainder of the tissues was fixed in 10% neutral-buffered formalin and embedded in paraffin following standard histological procedures.

RNA preparation and reverse Northern blot hybridization

Total RNA was isolated from normal breast tissue using TRIZOL (Life Technologies, Gaithersburg, MD) and then, an aliquot of 1.5 μg of total RNA from each patient in the placebo group and from the 20-mg TAM group was pooled in different tubes. The pooled RNA from these two individual groups was then used as template for reverse transcriptase reaction (Superscript II, Life Technologies, Gaithersburg, MD) using oligo dT primers in the presence of [^{32}P] dCTP.

Two identical membranes each containing 96 different cDNA fragments amplified by PCR were assembled (Vacuum Blotter, Life Technologies, Gaithersburg, MD) using clones generated by the FAPESP-LICR Human Cancer Genome Project and hybridized overnight in separate tubes to the cDNA probes according to Zhang *et al.* [10]. 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 site of the pUC vector. Clones containing repetitive elements or homo-polymeric regions were not used.

After hybridization, membranes were washed and analyzed using a phosphoimager (STORM, Amersham Pharmacia Biotech). cDNAs that were shown to be differentially expressed after normalization with controls (ImageQuant, Amersham Pharmacia Biotech) between the two membranes were selected and specific PCR primers were designed to confirm the differences in expression by RT-PCR.

RT-PCR analysis of Ca+2/calmodulin-dependent protein kinase II (CaMKII) and signal recognition particle (SRP 72)

The differential expression of cDNAs between the placebo group and the 20-mg TAM group was confirmed by semi-quantitative RT-PCR. Briefly, an aliquot of 2 μg of total RNA from each pool (placebo, 10 and 20 mg) were reverse transcribed in a reaction volume of 20 μl (Superscript II, Life Technologies, Gaithersburg, MD). Two microliters of the reverse transcribed

cDNA were amplified in a final volume of 50 μl by PCR under standard conditions: 1.5 mM $MgCL_2$, 125 μM dNTP and 2.5 U *Taq* polymerase using specific primers for CaMK II (MIM 602123) (forward 5′- CATCGTCAGGAGACTGTGGA-3′ and reverse 5′-TTGGCAGCTGAGAAGTTCCT-3′); SRP 72 (MIM 602122) (forward 5′-GCCCTGCATTGTAAAGTGGT-3 and reverse 5′-TCATCTTGGGAGTTTCGGAC-3′) and finally for the truncated isoform of CaMKII (forward 5′-TCAGCATCCA-GAAAATGCAG-3′ and reverse 5′-CTTCTTCCGCC-CATATTTGA-3′) spotted on our membranes. This isoform encodes a truncated, trans-spliced gamma CaM kinase II in which the 3-prime end consists of 804 basepairs of the human SRP72 gene and therefore, specific primers from these two genes were independently designed in order to identify whether differences in expression were due to down-regulation in CaMKII and/or SRP72. The PCR conditions were 32 cycles at 94°C for 3 min; 94°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec. Glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) was used as an internal standard (sense GTGAAGGTCGGT-GTGAACGGATTT and antisense CACAGTCTTCTGAGTG-GCAGTGAT). For each experiment, a parallel control without RT was processed. 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).

Results

Effects of TAM on Normal Breast Tissue

Among the 96 cDNA fragments used in the reverse Northern blot, we were able to identify three differentially expressed genes between the two groups: Tissue inhibitor of metalloprotease-3 (TIMP-3), insulin-like growth factor binding protein-3 (IGFBP-3) and Ca2+/calmodulin-dependent protein kinase II (CaMKII). The Ca2+/calmodulin-dependent protein kinase II fragment (CaMKII) spotted onto our membranes, is a truncated isoform of CaMKII and it is composed of the 5′ sequence from the human CaMKII gamma gene joined to the 3′ end of the human signal recognition particle 72 (SRP72) gene (Figure 1).

```
10          20          30          40          50
TTTCGCGGCT  TGGGGGTATG  ATGTTACTTG  TAGAGGCAGA  TACTGTTGCA

60          70          80          90          100
GCACTGCCAG  GTCTTGGGGA  GGTGGGTGG   GCTGCTCACA  GTTTTACTGG

110         120         130         140         150
CATCCAGTTC  AGATGAAGCT  CCTGCAGTTG  CTCCCTGGGT  CCCTTTTCCA

160         170         180         190         200
ATCTGATCCT  TCTTTTTACC  CTTCTTTCTT  CCCCGGTAGT  AAGAACGTTCT

210         220         230         240         250
CGCATTGGC   AGCCATCTTT  CTGGATCTGG  GGTAACTTTT  GGGTCATAAT

260         270         280         290         300
TCTTAGGCAA  TTTTCCCTTC  TTTTTCTTTTT CTTCTTTTTC AAATCTCCCT

310         320         330         340         350
GTCCTTGTTC  CTTTGGTTGA  CTATCTCCAG  TAACTTTTCC  ACCCTTCTTC

360         370         380         390         400
CGAATGTATG  TAGCACCAGC  AGAATTTTCA  AGAGCCTCAA  CATCTACTTT

410         420         430
TAGAGACATA  CTATCTGAAG  ACGGCAAGCG  G
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Figure 1. — Nucleotide sequence of CaMKII truncated isoform.

This sequence was found to be expressed in the RNA pool from the placebo group but was down-regulated in the RNA pool obtained from patients receiving 20 mg/day of TAM (Figure 2A). In order to further confirm these results we used an RT-PCR based method, which confirmed the differential expression for the truncated isoform of the CaMK II in the group that received 20 mg TAM/day/50 days but not in the group that received 10 mg TAM/day/50 days which remained the same (Figure 2B).

The differentially expressed cDNA fragment is 431 bp in length and showed 100% similarity to the truncated isoform of Ca2+/calmodulin-dependent protein kinase II (251/251) (accession number ref NM 006947.1) mentioned above (Figure 1).
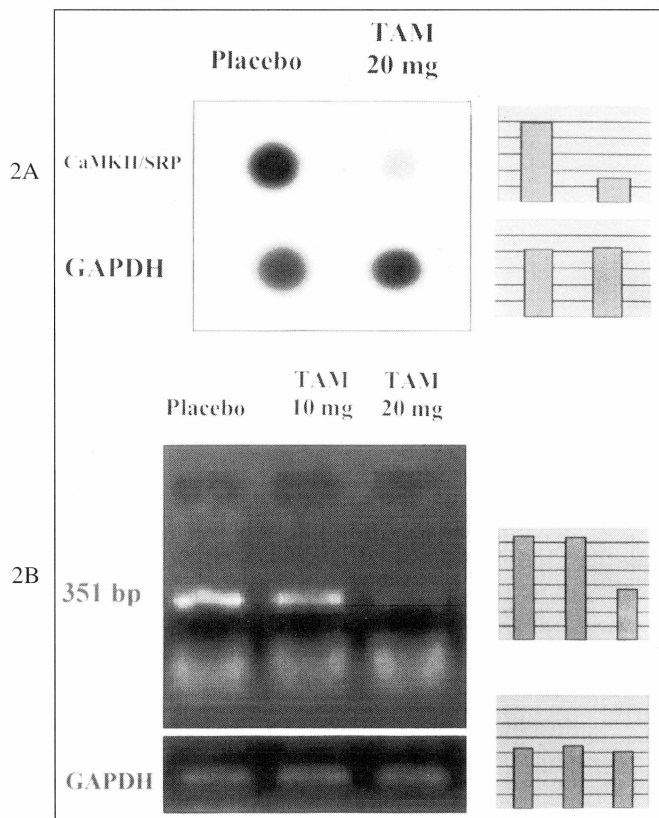
In order to identify whether the down-regulation induced by TAM was primarily due to its action on the CaMKII and/or SRP72 expression, specific primers were generated for CaMKII and SRP72. RT-PCR results showed that the down-regulation was exerted mainly on CaMKII expression (Figure 3).

The other two genes (IGFBP 3 and TIMP 3) were also down-regulated during the TAM treatment according to reverse Northern blot, however RT-PCR analysis did not confirm these results.
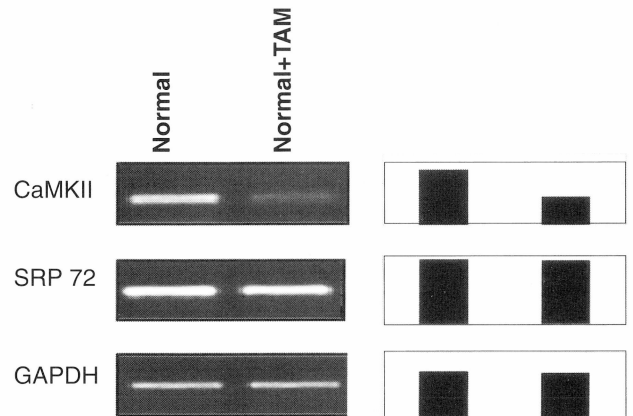

Figure 3. — Expression of the of CaMKII and SRP72 were investigated using RT-PCR during placebo and 20-mg TAM oral treatment in normal human breast tissue. RT-PCR using specific primers for CaMKII and SRP72 yielded single products of expected size 107 and 287 bp, respectively. RT-PCR using glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) specific primers yielded a single product of expected 555-bp. Histogram showing relative mRNA expression of CaMKII, SRP72 and GAPDH in the placebo and 20 mg/day TAM group.

Figure 2A. — Reverse Northern blot hybridization using [32]P labeled RT reaction from group 1 (placebo group) and group 3 (TAM 20 mg/day/50 days) as probes. The truncated isoform CaMKII is down-regulated during TAM treatment. GAPDH mRNA detection was used as a control for verifying the loading of RNA for RT reactions. Histogram showing relative mRNA expression of CaMKII and GAPDH in the placebo and TAM 20-mg group.
Figure 2B. — Expression of the truncated isoform of CaMKII was investigated using RT-PCR during placebo, 10-mg and 20-mg TAM oral treatment in normal human breast tissue. RT-PCR using specific primers yielded a single product of expected 351 bp size. RT-PCR using glyceraldehyde-3-phosphatase dehydrogenase (GAPDH)-specific primers yielded a single product of expected 555-bp. Histogram showing relative mRNA expression of CaMKII and GAPDH in the placebo and TAM groups (10 and 20 mg/day/50 days).

Discussion

In this prospective, randomized, placebo-controlled, double-blind study another gene was identified that seems to be down-regulated during TAM treatment in normal human breast tissue.

Indeed, a 50-day TAM treatment with 20 mg/day, but not with 10 mg/day, is able to significantly down-regulate mRNA expression of the calcium/calmodulin-dependent protein kinase II in normal human breast tissue.

Calcium has a specific role in mammary cell biology because normal differentiated breast epithelium has as a function, the concentration and transport of calcium for milk production. From the point of view of breast cancer, calcium metabolism is also very important and we recently demonstrated that the overexpression of calcium-binding protein S-100 placenta is associated with immortalization of human breast epithelial cells *in vitro* and human breast cancer progression *in vivo* [11].

In breast cancer cells TAM appears to play a very important role in calcium metabolism. In fact, TAM seems to induce variations in intracellular ionized

calcium leading to cell death of MCF-7 breast cancer cells [12]. In addition, studies have demonstrated the action of calcitonin in breast cancer cell lines [13]

Calcium/calmodulin-dependent protein kinase II (CaMKII) is an ubiquitous serine/threonine protein kinase that has been implicated in the diverse effects of hormones and neurotransmitters that utilize Ca2+ as a second messenger and cell growth.

The enzyme is an oligomeric protein composed of distinct but related subunits, alpha, beta, gamma, and delta, each encoded by a separate gene. Each subunit has alternatively spliced variants. Breen and Ashcroft [14] cloned and sequenced a novel CaM kinase II-gamma cDNA from human islet cell RNA. This isoform, designated gamma-SRP by the authors, encodes a truncated gamma CaM kinase II in which the 3-prime end consists of 804 basepairs of the human SRP72 gene.

In the present article we were able to demonstrate that the down-regulation of this TAM-induced isoform was exerted mainly on its CaMKII component rather than on its SRP72 counterpart (Figure 3). Using somatic cell hybrid analysis and fluorescence in situ hybridization, Li *et al.* mapped the CaMK γ gene to human chromosome 10q22 [15]. Since SRP72 and CaMK gamma map to different chromosomes, the authors suggest that this may represent the first example of trans-splicing producing a potentially functional protein in normal adult tissue.

Recent studies have shown very important links between Ca2+ signaling and cell cycle control. Indeed, CaMKII can activate the transcription of activating protein-1 (AP-1) member *c-fos* through a short promoter region (-99 to -53) containing the retinoblastoma control element (RCE) and cAMP response element (CRE) related sequences. It seems therefore, that CaMKII may represent a molecular link that controls cell cycle progression through the Rb/SP1 signaling pathway [16].

Another very interesting result suggesting that CaMKII is necessary for cell cycle progression through G1, is the fact that an inhibitor of CaMKII called KN-93, is able to induce cell cycle arrest in 95% of NIH 3T3 cells by the second day of treatment. In addition, KN-93 is able to block cell growth stimulated by basic fibroblast growth factor, platelet-derived growth factor, epidermal growth factor and insulin-like growth factor-1 [17].

The isolation and identification of tumor cell-specific CaMKII variants in breast cancer cell lines, and not in their normal counterpart, supports previous indications that CaMKII plays a role in cell cycle and growth control [18].

Indeed, the activity of another member of the calcium-calmodulin dependent protein kinase family, CaMKIII, is markedly increased in human breast cancer specimens compared with that of normal adjacent breast tissue [19].

Even though this is the first time that TAM has been shown to down-regulate CaMKII expression, this drug is also known to inhibit other members of the protein kinase family such as protein kinase C. In human astrocitoma cells this drug is able to selectively inhibit mitogen-activated protein kinase activation and c-fos phosphoprotein induction [20].

In breast cancer cells MCF-7, it seems that TAM selectively interacts with protein kinase C epsilon leading cells to apoptosis [21]. In addition, recent results have shown that CAMKII might be involved in the mechanisms of loss of cell adhesion in MCF 7 cells [22].

From the point of view of the signal recognition particle (SRP) this gene has at least three distinct functions: signal recognition, translational arrest and endoplasmatic reticulum membrane targeting. The signal recognition particle is a ribonucleoprotein complex that mediates the targeting of proteins to the endoplasmic reticulum. The complex consists of a 7S RNA and six different proteins SRP9, SRP 14, SRP19, SRP54, SRP68 and SRP72.

In conclusion, these results suggest that the down-regulation of CaMKII mRNA by TAM treatment might represent an alternative or another pathway of TAM action of this drug affecting the functions of genes involved in cell cycle control and response to growth factors in normal human breast tissue.

Acknowledgements

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