Research Article

Estrogen-Responsive Genes Overlap with Triiodothyronine-Responsive Genes in a Breast Carcinoma Cell Line

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It has been well established that estrogen plays an important role in the progression and treatment of breast cancer. However, the role of triiodothyronine (T<sub>3</sub>) remains controversial. We have previously shown its capacity to stimulate the development of positive estrogen receptor breast carcinoma, induce the expression of genes (PR, TGF-alpha) normally stimulated by estradiol (E<sub>2</sub>), and suppress genes (TGF-beta) normally inhibited by E<sub>2</sub>. Since T<sub>3</sub> regulates growth hormones, metabolism, and differentiation, it is important to verify its action on other genes normally induced by E<sub>2</sub>. Therefore, we used DNA microarrays to compare gene expression patterns in MCF-7 breast adenocarcinoma cells treated with E<sub>2</sub> and T<sub>3</sub>. Several genes were modulated by both E<sub>2</sub> and T<sub>3</sub> in MCF-7 cells (Student’s t-test, P < 0.05). Specifically, we found eight genes that were differentially expressed after treatment with both E<sub>2</sub> and T<sub>3</sub>, including amphiregulin, fibulin 1, claudin 6, pericentriolar material 1, premature ovarian failure 1B, factor for adipocyte differentiation-104, sterile alpha motif domain containing 9, and likely ortholog of rat vacuole membrane protein 1 (fold change > 2.0, pFDR < 0.05). We confirmed our microarray results by real-time PCR. Our findings reveal that certain genes in MCF-7 cells can be regulated by both E<sub>2</sub> and T<sub>3</sub>.

1. Introduction

Most breast cancer risk factors are associated with prolonged exposure of the mammary gland to high levels of estrogen. The biological effects of estrogen are predominantly mediated by two estrogen receptors (ER) that bind to estrogen response elements (EREs) in the promoter region of target genes [1]. Although the involvement of thyroid hormones (TH) in the development and differentiation of normal breast tissue has been established [2–4] and epidemiological and experimental studies have associated TH pathologies with an increased risk of breast cancer, the role of TH remains controversial [5–14]. Vorherr [15] described an increase in the survival of hyperthyroid patients with breast cancer, whereas we have identified a biological link between breast cancer in postmenopausal women and subclinical hyperthyroidism [16]. Most, if not all, major triiodothyronine (T<sub>3</sub>) actions are mediated by specific high affinity nuclear receptors (thyroid receptor, TR), which are encoded by the two genes THRA and THRβ that are also ligand-regulated transcription factors that act via DNA response elements [17]. Recent results have revealed substantial changes in the expression profile of thyroid hormone receptors, suggesting that their deregulation may be involved in breast cancer development [18].
Thyroid receptor is present in both MCF-7 and MDA-MB-231 breast cancer cell lines [19]. We previously demonstrated that T₃ mimics the effects of estradiol (E₂) in the ER-positive breast cancer cell line MCF-7, stimulating growth, modulating mRNA transcription of growth factors, and inducing the expression and activity of E₂-inducible proteins. In addition, these T₃ effects were antagonized by the simultaneous addition of tamoxifen (TAM), which is a competitive inhibitor of E₂ that binds to ER. However, we did not observe similar effects in the ER-negative MDA-MB-231 breast cancer cell line, in spite of high amounts of TR. This suggests that in MCF-7 cells both ligands share a common signaling pathway via ER, since the sequence similarity of these hormone receptors allows interactions of TR with ERs or ER with TREs [19]. These results are consistent with those of Zhou-Li et al. (1992), but contradictory to Dinda et al. [20, 21], who found no evidence that T₃ competitively displaces E₂ from ER. Recently, Hall et al. [22] showed that both E₂ and T₃ promoted proliferation in MCF-7 and T47D cell lines, which was suppressed by coadministration of the ER antagonist fulvestrant (ICI 182780), and T₃ induction of activated ER-mediated gene expression (ERα, ERβ, and PR) in MCF-7 cells. They also demonstrated that T₃ enhanced the effect of E₂ on cell proliferation in a dose-dependent manner.

We have demonstrated that tamoxifen inhibits transforming growth factor alpha (TGFα) gene expression in human breast carcinoma samples treated in vitro with T₃ [23]. These results suggest that T₃ may play a role in breast cancer development and progression by regulating proliferation, gene expression, and activity of E₂-inducible proteins such as progesterone receptor (PR) and TGFα and indicate an interaction between E₂ and T₃ signaling systems. Here we systematically examined the transcriptional effects of E₂ and T₃ in the MCF-7 breast adenocarcinoma cell line using DNA microarrays in order to better understand the actions of these two hormones. We identified their effects on the expression of a large number of genes by using a microarray platform containing 4,608 open reading frames (Orestes) [24]. We demonstrated that the expression of eight genes was significantly altered by both E₂ and T₃ in MCF-7 cells (fold change > 2.0, positive false discovery rate (pFDR) < 0.05). Out of these eight genes, amphiregulin (AREG), fibulin 1 (FBLN1), and claudin 6 (CLDN6) were the most differentially expressed.

2. Materials and Methods

2.1. Cell Line. MCF-7 cells were grown for 14 days before harvesting in RPMI 1640 supplemented with L-glutamine (2.8 mM), insulin (8 mM/mL), penicillin-streptomycin (100 U/mL), and 5% charcoal-stripped calf serum (FCS) and kept at 37°C in humidified 5% CO₂ and air. The medium was changed every two days.

2.2. Cell Treatment Conditions. MCF-7 cells were propagated in 150 cm² culture flasks until they reached 40% confluence. Before starting hormone treatments, the medium was replaced with phenol red free RPMI 1640 to eliminate all known sources of estrogen [25]. After 24 h (day 0), the medium was changed and cells were treated in triplicate with 10⁻⁸ M E₂ (Sigma-Aldrich, St. Louis, MO, USA, E8874), 10⁻⁸ M T₃ (Sigma-Aldrich, T2752), and absolute ethanol (vehicle control) for 72 h. Medium was changed every 24 h. Cells were harvested at the times indicated and cell numbers were counted.

2.3. RNA Isolation and Reverse Transcription. Total RNA was extracted from cultured MCF-7 cells by the guanidinium thiocyanate method and analyzed by electrophoresis using 1% agarose gels. One microgram of total RNA was reverse-transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA, 18080-051).

2.4. DNA Microarrays. The microarray glass slide was generated in conjunction with the Laboratório de Biologia Computacional (LBC—Computational Biology Laboratory) at the Hospital do Câncer, São Paulo, Brazil, together with the Laboratório de Análise de Expressão Gênica (LGEA—Gene Expression Analysis Laboratory) at the Instituto Ludwig de Pesquisa sobre o Câncer, São Paulo. The slide contained 4,608 genes from the Human Cancer Genome Project Bank, Instituto Ludwig para a Pesquisa do Câncer, São Paulo. Microarray hybridization and analysis were performed as previously described [26].

2.5. Real-Time RT-PCR. Assay-on-Demand Gene Expression Product (Applied Biosystems, Foster City, CA, USA, 4331882), consisting of unlabeled PCR primers and a TaqMan MGB probe (FAM dye-labeled) optimized to work with the TaqMan Universal PCR Master Mix (P/N 4304437) in an ABI Prism 7700 system (Perkin Elmer Life Sciences, Boston, MA, USA), was employed to quantitatively measure AREG, FBLN1, CLDN6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. All assays were performed in triplicate, mRNA content was normalized to the GAPDH mRNA level and differences in expression were determined by the Ct method described in the ABI user’s manual (Applied Biosystems).

2.6. Statistical Analysis. “Permutation” Student’s t-test (10,000 permutations) was performed on microarray results without bootstrapping, with a positive false discovery rate (pFDR) less than 0.05, and fold change greater than 2.0.

3. Results

The influence of T₃ on the gene expression profile of MCF-7 cells was examined and compared to the effects of treatment with E₂. RNA samples extracted from triplicate samples of MCF-7 cells after 24 h of E₂ or T₃ treatment were analyzed for E₂- or T₃-regulated gene expression by comparing to cells treated with vehicle control. Genes with P < 0.05 (Student’s t-test) in paired group comparisons were considered as differentially expressed. We verified that 393 genes were differentially expressed after both treatments (up- or downregulated) (Figure 1). After applying a 2-fold change
Table 1: Genes significantly modulated by E<sub>2</sub> and T<sub>3</sub> in MCF-7 cells.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene</th>
<th>Biological process</th>
<th>Fold change (adj. P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG</td>
<td>Amphiregulin, colorectum cell-derived, growth factor</td>
<td>Epidermal growth factor</td>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>FADI04</td>
<td>Fibronectin type III domain containing 3B</td>
<td>Positive regulation of fat cell differentiation</td>
<td>18.13</td>
</tr>
<tr>
<td>FBLN1</td>
<td>Fibulin 1</td>
<td>Interspecies interaction between organisms</td>
<td>3.89</td>
</tr>
<tr>
<td>POIFB</td>
<td>Premature ovarian failure, IB</td>
<td>Protein encoded by this gene binds nonmuscle actin filaments</td>
<td>2.60</td>
</tr>
<tr>
<td>CLDN6</td>
<td>Claudin 6</td>
<td>Calcium-independent cell-cell adhesion</td>
<td>2.58</td>
</tr>
<tr>
<td>PCMI</td>
<td>Pericentriolar material 1</td>
<td>G2/M transition of mitotic cell cycle/microtubule anchoring</td>
<td>2.53</td>
</tr>
<tr>
<td>FLJ20073</td>
<td>Sterile alpha motif domain containing 9</td>
<td>Regulation of cell proliferation and apoptosis</td>
<td>2.38</td>
</tr>
<tr>
<td>VMP1</td>
<td>Likely ortholog of rat vacuole membrane protein 1</td>
<td>Cell-cell adhesion</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Fold: expression difference; P value: significance value of Student’s t-test; FDR: false discovery rate.

**FIGURE 1: Dispersion of fold changes for the 393 genes modulated by E<sub>2</sub> and T<sub>3</sub> in MCF-7 cells.** Each point represents the expression of one gene. The dispersion is similar in both treatments for all except two genes.

As a cut-off point and a pFDR < 0.05, MCF-7 cells treated with E<sub>2</sub> displayed 39 genes that were differentially expressed compared to the control, whereas after T<sub>3</sub> treatment only 25 genes were differentially expressed relative to control cells. Eight genes were commonly modulated after treatment with both E<sub>2</sub> and T<sub>3</sub> (Table 1), although the response to T<sub>3</sub> was less pronounced than to E<sub>2</sub>. Out of these eight genes, AREG, FBLN1, and CLDN6 were strongly expressed after treatment with both T<sub>3</sub> and E<sub>2</sub>. In order to confirm our microarray data, we quantified AREG, FBLN1, and CLDN6 mRNAs using quantitative RT-PCR. Treatment with both E<sub>2</sub> and T<sub>3</sub> at 10<sup>-8</sup> M resulted in overexpression of AREG (26.3- and 13.8-fold more, resp.), FBLN1 (5.3- and 1.9-fold more, resp.), and CLDN6 (4.4- and 2.2-fold more, resp.) (Figure 2).

4. Discussion

In breast cancer, several clinicopathological markers are frequently used alone or in combination to assess patient risk. For example, lymph-node stage, tumor size, and histologic grade are important elements of the major prognostic indices, whereas ER status is widely regarded as the primary predictor of response to hormonal (antiestrogen) therapy. Microarray data sets from large studies of breast carcinomas have revealed several underlying signatures associated with the primary physiology of the tumor that have important prognostic and predictive implications.

In previous studies, we have demonstrated that T<sub>3</sub>, in supraphysiological doses, is able to increase the progression of ER-positive breast cancer, enhancing the expression of genes normally stimulated by E<sub>2</sub> and suppressing genes normally inhibited by E<sub>2</sub> [19]. Based on those results, we sought to identify genes that are influenced by both hormones in order to identify additional markers of progression.

Many genes were equally modulated by E<sub>2</sub> and T<sub>3</sub> in MCF-7 breast carcinoma cells (Student’s t-test, P < 0.05) (Figure 1). Using more stringent criteria (2-fold cutoff and pFDR < 0.05), the number of genes modified by E<sub>2</sub> and T<sub>3</sub> decreased to eight. Out of these eight genes, AREG, FBLN1, and CLDN6 were strongly expressed by both T<sub>3</sub> and E<sub>2</sub> treatment. We validated our microarray results using real-time PCR on AREG, FBLN1, and CLDN6 expression (Figure 2).

Amphiregulin (AREG), the most differentially expressed gene, codes for a glycoprotein, that is, a member of the epidermal growth factor family (EGF), the members of which are ligands that can bind EGFR. AREG was discovered in concentrated conditioned medium from MCF-7 cells [27]. Numerous studies have sought to characterize the transcriptional network associated with estrogen response using cell culture experiments. Kenney et al. [28] inserted pads with recombinant AREG in the breast of oophorectomized rats, which reestablished the premature development of the ductal mammary epithelium and prompted hyperplasia. Therefore, AREG seems to play an intermediary role in maturing the mammary gland and in stimulating the initiation of mammary oncogenesis. Martinez et al. [29] treated MCF-7 cells with 10<sup>-9</sup> M E<sub>2</sub>, 10<sup>-9</sup> M E<sub>2</sub> with 10<sup>-6</sup> M TAM (E<sub>2</sub> + TAM), TAM alone, and vehicle control for 24 hours.
They observed that adding TAM to the treatment with E2 decreased AREG mRNA expression by 38%, suggesting that E2 stimulates AREG expression via ER. Using microarrays, Frasor et al. [30] observed an upregulation of AREG in MCF-7 cells treated with E2, which was reversed by tamoxifen. When Vendrell et al. [31] treated MVLN cells, a breast carcinoma cell line derived from MCF-7 cells, for 4 days with E2, they observed that AREG was one of the differentially expressed genes using cDNA miniarrays. A recent microarray analysis identified E2-regulated genes in a model in which human breast tissue was transplanted into mice, which were then treated with estradiol [32]. Interestingly, AREG was the most upregulated gene. Several studies have examined AREG expression in breast carcinomas by immunohistochemistry and found that AREG expression is higher in infiltrative breast carcinoma than in normal epithelium and is associated with regional lymph-node metastasis [33–35]. In addition, AREG upregulates the expression of genes associated with invasion [27]. Modulation of the AREG gene by T3 has not been previously reported. While the increase in AREG expression in MCF-7 cells after T3 treatment that we found was lower than the increase after E2 treatment, it was highly significant. Thus, AREG appears to be an important target gene for E2 and T3 in MCF-7 breast cancer cells.

**Fibulin 1 (FBLN1)** is the prototype member of the fibulin family of ECM proteins and binds to many ECM proteins, including fibronectin, laminin-1, fibrinogen, aggrecan, and versican. Fibulin 1 may be involved in cell motility and anchorage-independent growth of tumor cells [36] and is overexpressed in breast cancer specimens and breast cancer cell lines [37–39]. FBLN1 was previously reported to be induced by E2 in MCF-7 cells [40] and in ER-positive ovarian cancer cell lines [41]. We have shown for the first time that FBLN1 is upregulated in breast cancer cell lines treated with T3.

**Claudin 6 (CLDN6),** a member of the Claudin family, is involved in the formation of the GAP junction [42]. While Offner et al. [43] reported the expression of CLDN6 in breast cancer cell lines, its role in carcinogenesis remains controversial [44–46]. Wu et al. [47] noted that cells with a high level of expression of claudin 6 grew slowly and had a higher rate of death than control cells, suggesting that claudin 6 may function as a cancer suppressor. Its downregulation may contribute to the malignant progression of certain types of breast cancers [48]. To our knowledge, this is the first study showing that E2 and T3 modulate CLDN6 expression in a breast cancer cell line.

Some of the other genes that we identified as being regulated by E2 and T3 are directly involved in cell proliferation,
such as EMP1, IFNAR2, VMP, FLJ20073, MYC, and AREG, and some are involved in nucleotide binding and/or protein binding, such as PFKFB3, APPBP2, SSFA2, and NALP7. Other genes, such as CSTA, show altered expression in the tissue invasion process during breast carcinogenesis [49].

Expression of BRAP, responsible for ubiquitination of the product of the tumor suppressor gene BRCAl, was increased in our study and in other studies [50] and FBLN1 and ADAM9 are associated with migration and tissue invasion [51, 52]. We also identified genes that had not been previously correlated with breast cancer, including TERF2IP, which is involved in telomere regulation [53], IGSF1, which is involved in intracellular adhesion and transcription and is a signal transduction regulator [54], NMT2, which acts in the protein myristoylation process [55], FADI104, which positively regulates fat cell differentiation [56], PCM1, involved in centromere amplification and genomic instability [57], VWF, involved in cellular adhesion [58], CIR, which is involved in immune response and complement system activation [59], and POIF1, which binds nonmuscle actin filaments [60].

This is the first report of T3 modulation of FBLN1, CLDN6, and AREG. The magnitude of the increase in expression of AREG, FBLN1, and CLDN6 in MCF-7 cells treated with T3 was less than when treated with E2 but highly significant. This may be because these genes have more binding consensus sequences for E2 than T3 receptors. Specifically, within 5,000 bp upstream of the transcriptional initiation site, AREG has 10 EREs and 8 TREs, FBLN1 has 6 TREs and 6 EREs, and CLDN1 has 12 EREs and 10 TREs.

5. Conclusion

We have shown that T3 treatment results in a gene expression pattern similar to E2 treatment, up- or downregulating a group of the same genes, suggesting that these two hormones can cause similar phenotypes. Our in vitro observation suggests a molecular mechanism by which thyroid hormone can be a relevant factor for breast cancer progression through the induction of genes involved in growth and invasion.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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