Identification of Modulated Genes by Three Classes of Chemopreventive Agents at Preneoplastic Stages in a p53-Null Mouse Mammary Tumor Model

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Abstract

Genetically engineered mouse cancer models are among the most useful tools for testing the in vivo effectiveness of the various chemopreventive approaches. The p53-null mouse model of mammary carcinogenesis was previously characterized by us at the cellular, molecular, and pathologic levels. In a companion article, Medina et al. analyzed the efficacy of bexarotene, gefitinib, and celecoxib as chemopreventive agents in the same model. Here we report the global gene expression effects on mammary epithelium of such compounds, analyzing the data in light of their effectiveness as chemopreventive agents. SAGE was used to profile the transcriptome of p53-null mammary epithelium obtained from mice treated with each compound versus controls. This information was also compared with SAGE data from p53-null mouse mammary tumors. Gene expression changes induced by the chemopreventive treatments revealed a common core of 87 affected genes across treatments ($P < 0.05$). The effective compounds, bexarotene and gefitinib, may exert their chemopreventive activity, at least in part, by affecting a set of 34 genes related to specific cellular pathways. The gene expression signature revealed various genes previously described to be associated with breast cancer, such as the activator protein-1 complex member Fos-like antigen 2 ($Fosl2$), early growth response 1 ($Egr1$), gelsolin ($Gsn$), and tumor protein translationally controlled 1 ($Tpt1$), among others. The concerted modulation of many of these transcripts before malignant transformation seems to be conducive to predominantly decrease cell proliferation. This study has revealed candidate key pathways that can be experimentally tested in the same model system and may constitute novel targets for future translational research.

Human breast cancer therapeutic and preventive agents are primarily grouped based on their mechanism of action about the estrogen receptor-α status of the tumor. Human clinical trials have shown that selective estrogen receptor modulators such as tamoxifen and raloxifene and aromatase inhibitors are ineffective for the most part in the treatment of estrogen receptor-α–negative breast cancers (1, 2). Agents such as retinoids, cyclooxygenase-2 (Cox-2) inhibitors, and epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitors are being tested for the prevention and treatment of hormonally unresponsive estrogen receptor-α–negative breast cancers. Thus, there is much interest in testing these chemopreventive agents in preclinical models of breast cancer. Transgenic and other genetically engineered mouse cancer models are among the most useful tools for testing the in vivo effectiveness of the various chemopreventive approaches (3).

The p53-null mouse model of mammary carcinogenesis is a unique in vivo model of preneoplastic and neoplastic progression that reproduces many of the critical features of human breast cancer (4, 5). In this model, BALB/c p53-null mammary epithelial cells are transplanted into cleared mammary fat pads of p53 wild-type syngeneic hosts. More than 60% of these isogenic orthotopic transplants develop invasive mammary adenocarcinomas without hormonal stimulation (4). Most of these tumors are intraductal in origin and premalignant lesions can be observed closely mimicking human breast cancer (5). Importantly, the deregulations of transcripts related to the control of cell proliferation, differentiation, and apoptosis in tumors arising from p53-null mice and human mammary gland have been reported to be strikingly similar (6).
The effects of chemopreventive agents at the gene transcriptional level are poorly understood (7). To identify biomarkers of effectiveness and to elucidate molecular mechanisms of action, we performed a comparative transcriptome profiling from p53-null mammary epithelium obtained from mice treated with three chemopreventive agents: a retinoid X receptor agonist (bexarotene, LGD10169), an EGFR-TK inhibitor (gefitinib, ZD1839), and a Cox-2 inhibitor (celecoxib, SC58635). In a companion article, we assessed the antitumorigenic effectiveness of the same compounds in the same p53-null mammary epithelial cancer model (8). That study showed a significant decrease in mammary tumorigenicity when p53-null mammary epithelium recipient virgin mice were treated with bexarotene (75% reduction) or gefitinib (50% reduction; P < 0.05); however, no effect was observed when animals were treated with celecoxib.

In this article, we report gene expression changes detected in p53-null mammary epithelium as a result of treating mice with the aforementioned chemopreventive agents. The results are presented and analyzed in light of the antitumorigenic effectiveness of two of the three compounds studied.

Materials and Methods

Chemopreventive agents

The retinoid X receptor–selective retinoid used in this study LGD10169 (bexarotene, Targetin) was obtained from Ligand Pharmaceutical, Inc.; ZD1839 (gefitinib, Iressa) was obtained from AstraZeneca; and SC58635 (celecoxib, Celebrex) was purchased from Sigma.

p53-null mouse mammary model and treatments

Housing of mice and all experiments done with mice were done in accordance with NIH guidelines and regulations in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. BALB/c p53-null mammary epithelium was transplanted into the cleared mammary fat pads of 3-wk-old wild-type BALB/c mice (4). Transplanted mice were separated at random in two groups for each reagent (experimental versus control). Thus, each group included age-matched vehicle-treated controls and bexarotene-treated, gefitinib-treated, or celecoxib-treated mice, respectively. All mice were treated 6 d/wk for 2 mo starting at 11 wk of age. The retinoid bexarotene (100 mg/kg) was administered by gastric gavage using a 20-gauge gavage needle in a 0.1-mL volume of sesame seed oil. Mice were treated with gefitinib (100 mg/kg) suspended in distilled water containing 1% Tween 80. ZD1839 was administered in 0.1 mL by gastric gavage with a 20-gauge gavage needle. Celecoxib treatment was provided with the diet of mice supplemented with 500 ppm SC58635.

SAGE method

To decrease the chances of potential artifacts due to sample heterogeneity, RNA for SAGE was extracted from a pool of mammary epithelial samples (8-10 fat pads per pool, three separate pools from each treatment group) collected at 2 mo after initiation of treatment with the chemopreventive agent. Mammary epithelial enriched samples (>90% epithelial cells) were used for the analyses (9). All SAGE libraries were generated following standard procedures as described previously (10). Briefly, total RNA was extracted from frozen samples using TRIzol (Invitrogen). SAGE library construction was done with the I-SAGE kit (Invitrogen) according to the manufacturer’s protocol and introducing only minor modifications. The anchoring enzyme was NlaIII and the tagging enzyme used was BsmFI. Concatemerized di-tags were cloned into pZERO-1 and sequenced with an ABI 3700 DNA Analyzer (Applied Biosystems). SAGE libraries were generated at an approximate resolution of 60,000 tags per library (6, 9).

SAGE data processing and statistical analyses

SAGE tag extraction from sequencing files was done by using the SAGE2000 software version 4.0 (a kind gift of Dr. Kenneth Kinzler, John Hopkins University, Baltimore, MD). SAGE data management, tag-to-gene matching, as well as additional gene annotations and links to publicly available resources, such as Gene Ontology (GO), UniGene, and Entrez gene ID, were done using a suite of web-based SAGE library tools developed by us. In our analyses, we only considered tags with single tag-to-gene reliable matches.

To obtain a more complete picture to identify transcripts of potential relevance as biomarkers and to identify transcripts of relevance in chemoprevention, we performed two types of analyses: (a) The gene expression signature for each chemopreventive agent in normal p53-null mammary epithelium was obtained. To this end, SAGE profile of each chemopreventive agent was compared with its corresponding control. (b) To identify transcripts whose modulation could be of relevance in prevention of carcinogenesis, SAGE profiles obtained from each chemopreventive agent were also compared with transcripts deregulated in p53-null mammary tumors.

The mouse mammary tumors used developed spontaneously from intra-mammary fat pad transplanted p53-null mammary epithelium (4). As normal control for the SAGE analysis of tumors, p53-null enriched mammary epithelium derived from BALB/c female mice unexposed to hormonal stimulation was used as described previously (9). To decrease the chances of potential artifacts due to sample heterogeneity, the normal sample (MN2) represents a pool of mammary epithelial samples from five age-matched separate mice. In addition, two p53-null mammary tumor SAGE libraries (T2532 and T2539) derived from p53-null BALB/c female mice unexposed to hormonal stimulation were selected for the comparative analysis (6). These SAGE mammary tumor libraries were pooled, averaged, and normalized to 60,000 tags.

To compare the control (vehicle) versus treatment SAGE libraries for each chemopreventive agent (e.g., untreated p53-null mammary epithelium versus celecoxib treatment SAGE libraries) and the p53-null normal mammary epithelium (MN2 SAGE library) versus p53-null mammary tumors (T2532 and T2539 pooled SAGE libraries), we used the Audic and Claverie’s significance test (11). Tags with total counts of <4 in compared libraries were filtered out before the analysis. First, we compared the differences in gene expression profiles between p53-null normal mammary epithelium (SAGE library, MN2) and two pooled p53-null mammary tumors (SAGE libraries T2532 and T2539) previously generated by us (6, 9). Second, we compared the differentially expressed transcripts from each chemopreventive treatment (treated versus untreated epithelium) with the transcripts detected as differentially expressed between normal and tumor.

Statistical analysis and scatter-plot visualization of SAGE libraries were done with the Discovery Space 4 software (Genome Science Centre, BC Cancer Agency, Canada, Vancouver). For automated functional annotation and classification of genes of interest based on GO terms, we used the EASE (12) available at the Database for Annotation, Visualization and Integrated Discovery (DAVID; ref. 13). All of the raw SAGE data reported as supplementary files in this article are publicly available.

Identification of commonly deregulated genes among chemopreventive agents

Differentially expressed genes were compiled into an Excel spreadsheet pivot table for comparison of overlapping data between retinoid LGD10169, gefitinib, and celecoxib chemopreventive agents. Any combination of two lists was compared for matching gene identity. The number and identity of genes commonly affected in two chemopreventive agents (e.g., LGD10169 versus celecoxib) were determined.

4 http://www.bcgsc.ca/platform/bioinfo/software/ds
We used the normal approximation to the binomial distribution as previously described (14) to calculate whether the number of matching genes derived from each comparison was of statistical significance \((P < 0.05)\). To enable illustration of the co-occurring deregulated genes between transgenic mouse models, we used the TIGR MultiExperiment Viewer (MeV 3.0) software. This tool was used for average clustering of SAGE based on the fold change of tag counts for each transcript comparing treatment to control (vehicle) in each chemopreventive agent.

**Results and Discussion**

**p53-null mouse SAGE libraries**

We generated six mouse SAGE libraries from mammary epithelium obtained from the described p53-null mouse model from virgin mice treated with bexarotene, gefitinib, or celecoxib and their corresponding controls. In addition, we compared these data with SAGE profiles obtained from p53-null normal mammary epithelium (MN2) and from two p53-null mammary tumors (T2532 and T2539; refs. 6, 9). This resulted in a data set of almost 540,000 tags representing more than 25,000 transcripts from a total of nine SAGE libraries. The study approach underwent three phases: (a) identification of differentially expressed genes in mammary epithelium as a result of each chemopreventive agent treatment, (b) identification of commonly deregulated transcripts among treatments, followed by (c) assessment of modulation of the identified transcripts in p53-null mammary tumors.

**Bexarotene (rexinoid agonist) treatment**

Retinoids are biologically active derivatives of vitamin A that play essential roles modulating cell proliferation, differentiation, and apoptosis. Signal transduction is mediated by two classes of nuclear retinoid-dependent transcriptional activators: the retinoic acid receptors (RARα, RARβ, and RARγ) and the retinoid X receptors (RXRα, RXRβ, and RXRγ). A highly selective retinoid X receptor agonist, the retinoid bexarotene (Targetretin), can inhibit the growth of normal and malignant breast cells and was shown to suppress the development of breast cancer transgenic mouse models without apparent side effects (15,16).

The chemopreventive effects of bexarotene have been attributed to transcriptional modulation of genes related to cell proliferation, cell death/apoptosis, and cell differentiation (17). Our statistical analysis revealed 236 genes differentially expressed \((P < 0.05)\) between vehicle-treated p53-null mammary epithelium and bexarotene treatment (Fig. 1A). Among these transcripts, 120 were up-modulated and 116 were down-modulated by bexarotene treatment (see Supplementary Table S1). GO annotation of the 236 differentially expressed genes showed that \(\sim16\%\) of the transcripts are involved in signal transduction/transcriptional regulation, and \(12\%\) are related to cell adhesion/migration and cytoskeleton organization (Fig. 1B). Table 1 shows the most highly deregulated transcripts by bexarotene treatment in p53-null mammary epithelium (fold change \(\geq7\); \(P < 0.01\)).

**Gefitinib (EGFR-TK inhibitor) treatment**

The EGFR family members (HER1-HER4) are commonly overexpressed in estrogen receptor-α-negative human breast carcinomas, providing a new target for anticancer drug development. The EGFR signaling network activates several pathways involved in the G1-S transition as well as disables proapoptotic molecules, thus leading to deregulated proliferation and enhanced tumor cell survival (18). Gefitinib (Iressa) is a synthetic anilinoquinazoline tyrosine kinase inhibitor selective for EGFR that can effectively block the tumorigenic potential that arises from the EGF signaling pathway. Recent studies have shown that gefitinib prevents estrogen receptor-α-negative tumor formation in MMTV-ErbB-2 mice (19). Our statistical analysis revealed 491 genes to be differentially expressed \((P < 0.05)\) between untreated p53-null mammary epithelium and gefitinib treatment (Fig. 1A). Among these transcripts, 252 were up-modulated and 239 were down-modulated by gefitinib treatment (see Supplementary Table S1). GO annotation of the 491 differentially expressed genes showed that \(\sim16\%\) of the transcripts are involved in cell cycle/proliferation and apoptosis/cell differentiation, \(12\%\) are related to signal transduction/transcriptional regulation, and \(8\%\) are related to cell adhesion/migration and cytoskeleton organization (Fig. 1B). Table 1 shows the most highly deregulated transcripts by gefitinib treatment in p53-null mammary epithelium (fold change \(\geq7\); \(P < 0.01\)).

**Celecoxib (Cox-2 inhibitor) treatment**

Cox-2 is one of the rate-limiting enzymes in converting free arachidonic acid to PGG2. Cox-2 is up-regulated in response to tumor promoters, growth factors, and cytokines, and it is responsive to various oncogenes such as v-src, v-Ha-ras, Wnt1, and HER-2/neu (20). Cox-2 is overexpressed in \(\sim40\%\) of breast cancers including in situ lesions. Celecoxib, a selective Cox-2 inhibitor, has been tested for its ability as chemopreventive agent, shown to significantly reduce the incidence of mammary tumors formation in some transgenic mouse models (20). Our statistical analysis revealed 200 genes to be differentially expressed \((P < 0.05)\) between p53-null mammary epithelium from vehicle-treated versus celecoxib-treated mice (Fig. 1A). Among these transcripts, 117 were up-modulated and 83 were down-modulated by celecoxib treatment (see Supplementary Table S1). GO annotation of the 200 differentially expressed genes showed that \(\sim18\%\) of the transcripts are involved in apoptosis/cell differentiation and cell cycle/proliferation (Fig. 1B). Table 1 shows the most highly deregulated transcripts by celecoxib treatment in p53-null mammary epithelium (fold change \(\geq7\); \(P < 0.01\)).

**Three-way comparison of genes deregulated by the tested chemopreventive agents**

To identify a common core of effector genes among the three chemopreventive agents, we performed a three-way comparison of the above-described SAGE data sets. Among the three treatments, a total of 835 genes were identified as deregulated in p53-null mammary epithelium obtained from treated mice. Eighty-seven genes were identified as commonly deregulated by more than one of the chemopreventive agents (Fig. 2A; see Supplementary Table S2). Thirty-four genes were identified as co-deregulated in bexarotene and gefitinib treatments, representing a nonrandom significant number of overlapping genes based on normal approximation to the binomial distribution \((P < 0.001)\); Fig. 2B). Forty-six genes were co-deregulated in gefitinib and celecoxib treatments \((P < 0.001)\), and 17 genes were identified between bexarotene and celecoxib treatments \((P < 0.001)\); Fig. 2B). Only five genes were identified...
as co-deregulated by all three treatments; these are the common up-regulation of TCDD-inducible poly(ADP-ribose) polymerase (Tiparp), cysteine-rich protein 1 (Crip1), glutamate-ammonia ligase (Glul) and down-regulation of tumor protein translationally controlled 1 (Tpt1) and ribosomal protein S4 (Rps4x). GO annotation of the 87 commonly deregulated genes showed that 13% of the transcripts are involved in cell cycle/proliferation, 13% are related to signal transduction/transcriptional regulation, 13% are related to cell adhesion/cytoskeleton organization, and 10% are related to extracellular matrix/proteolysis (Fig. 2C).

Transcriptomic changes relevant to p53-null mammary mouse tumor development

To identify the deregulated genes of relevance to tumorigenesis, we compared the SAGE profiles of the chemopreventive agents with genes identified as differentially expressed in p53-null mouse mammary tumors. We identified 574 differentially expressed genes (P < 0.05) when comparing SAGE data from the p53-null mammary tumors versus p53-null normal mammary epithelium (Fig. 3A). Among the 574 transcripts, 224 were up-modulated and 350 were down-modulated transcripts in p53-null mammary tumors (see Supplementary Table S3).

Bexarotene treatment of p53-null “normal” mammary epithelium affects the expression of 44 transcripts commonly deregulated in p53-null mammary tumors. Among these transcripts, 26 were up-modulated and 18 were down-modulated in p53-null mammary epithelium in opposite way to how the same transcripts are affected in p53-null mammary tumors (Fig. 3B). Gefitinib and celecoxib treatment of p53-null mammary epithelium affects the expression of 44 and
Table 1. Most highly deregulated transcripts in mammary epithelium from p53-null transgenic mice for each chemopreventive treatment assessed (fold change ≥7; \( P < 0.01 \))

<table>
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<th>Tag</th>
<th>Gene</th>
<th>Description</th>
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<th>Fold change*</th>
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<td>GTTGTGCTGTA</td>
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<td>Junction plakoglobin</td>
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<td>Tissue inhibitor of metalloproteinase 2</td>
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</tr>
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<td>CCGAGTCCTCT</td>
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<td>CAATAAAACA</td>
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<td>Stno</td>
<td>Strawberry notch homologue (Drosophila)</td>
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<td>Ptdn5</td>
<td>Prefoldin 5</td>
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<td>Down syndrome critical region homologue 1 (human)</td>
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<td>ATPase, Ca(^{2+}) transporting</td>
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<td>Ywhab</td>
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*Up-regulated transcripts for each treatment are represented by positive fold changes and down-regulated transcripts are represented by negative fold changes.
Fig. 2. Co-occurring differentially expressed genes among bexarotene, gefitinib and celecoxib treatments in p53-null normal mammary epithelium. Eighty-seven genes were identified modulated by more than one treatment. A, heat map of the 87 deregulated transcripts. Color scale at the bottom depicts the approximate fold change in expression for each transcript and library relative to control mammary gland. Negative fold change (e.g., transcripts with decreased expression in bexarotene treatment) is represented in green, and positive fold change (e.g., transcripts with overexpression in bexarotene treatment) in red. Aquamarine bars on the left indicate co-occurring transcripts modulated both by bexarotene and gefitinib treatments. B, Venn diagram showing the overlap between transcripts modulated by bexarotene, gefitinib, and celecoxib treatments. Statistical analysis showed a significant number of overlapping genes between treatments ($P < 0.001$). Hatched area with blue lines, number of genes commonly modulated by both bexarotene and gefitinib treatments. C, GO classification of the 87 transcripts deregulated by the chemopreventive treatments.
Fig. 3. Transcripts identified as deregulated in p53-null mammary tumors that were observed to be modulated in the opposite direction as the result of treatment with chemopreventive agents in normal mammary epithelium (i.e., up in tumors, down in the treated epithelium, or vice versa). A, scatter-plot representation of differentially expressed genes between p53-null normal epithelium and p53-null tumors SAGE libraries (P < 0.05). B, heat maps of the transcripts modulated in the opposite direction in tumors versus treated normal epithelium: p53-null tumors (black cluster) and bexarotene-treated normal p53-null mice epithelium (aquamarine cluster), gefitinib-treated (fuchsia cluster), and celecoxib-treated (orange cluster). Color scale at the bottom depicts the approximate fold change in expression for each transcript and library relative to control mammary gland. Negative fold change is represented in green, and positive fold change in red.
20 transcripts, respectively, that are also deregulated in p53-null mammary tumors (Fig. 3B). Among these transcripts, 32 genes were up-modulated by gefitinib treatment (12 down-modulated) and 19 genes were up-modulated by celecoxib treatment (1 down-modulated transcripts) in opposite way to how the same transcripts are affected in p53-null mammary tumors (Fig. 3B).

Although transcripts modulated by the three chemopreventive agents share significant overlap, bexarotene and gefitinib treatments affect the expression of more transcripts (44 genes each one) deregulated in p53-null mammary tumors compared with celecoxib treatment (20 genes). Interestingly, both bexarotene and gefitinib, at 100 mg/kg dose, were effective antitumorigenic agents in the p53-null mammary model, reducing tumor incidence by 75% and 50%, respectively, in virgin mice (8). On the other hand, celecoxib treatment did not affect tumorigenicity in either the virgin or hormone-stimulated mice.

The heat maps shown in Fig. 2A and Table 2 display 34 transcripts commonly deregulated (in the same direction) by the

<table>
<thead>
<tr>
<th>Tag</th>
<th>Gene</th>
<th>Description</th>
<th>Fold change*</th>
<th>B</th>
<th>G</th>
<th>T</th>
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<tr>
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<td></td>
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<tr>
<td>CATCTGTATT</td>
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<td>GGTTTTGTTT</td>
<td>Wsb1</td>
<td>WD repeat and SOCS box-containing 1</td>
<td>6.2</td>
<td>↑↑</td>
<td>-</td>
<td>-</td>
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<td>GGATATGTGG</td>
<td>Egr1</td>
<td>Early growth response 1</td>
<td>2.1</td>
<td>↑↑</td>
<td>-</td>
<td>↓</td>
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<tr>
<td>GCGCCCTCC</td>
<td>Ccl21b</td>
<td>Chemokine (C-C motif) ligand 21b</td>
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<td>↓</td>
<td>1</td>
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<td>GATTCTGTGC</td>
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<td>Guanine nucleotide binding protein y5</td>
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<tr>
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<td>Ptn</td>
<td>Pleiotrophin</td>
<td>2.6</td>
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<td>↑↑</td>
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<td>Dip3β</td>
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<td>GCCAAACCAA</td>
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<td>CDC-like kinase 1</td>
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<td>-</td>
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<tr>
<td>Cytoskeleton organization/extracellular matrix remodeling</td>
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<tr>
<td>CTCCTGGAACA</td>
<td>Gsn</td>
<td>Gelsolin</td>
<td>3.1</td>
<td>↑↑</td>
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<td>TTAACCTCTGA</td>
<td>Prelp</td>
<td>Proline arginine-rich and leucine-rich repeat</td>
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<td>↑↑</td>
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<td>-</td>
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<tr>
<td>CCCTGAGTCC</td>
<td>Actb</td>
<td>Actin, β, cytoplasmic</td>
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<td>↑↑</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>Ctss</td>
<td>Cathepsin S</td>
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<td>Calpain 12</td>
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<td>Csrp1</td>
<td>Cysteine and glycine-rich protein 1</td>
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<td>↓↓</td>
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<td>-</td>
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<td>Protein metabolism</td>
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<td></td>
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<td>TTACCATTCG</td>
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<td>-</td>
<td>↓</td>
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<td>TGACCCCGGGG</td>
<td>Uba52</td>
<td>Ubiquitin A-52 residue ribosomal protein</td>
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<td>TGGTGTAAGA</td>
<td>Hspa5</td>
<td>Heat shock 70-kDa protein 5</td>
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<tr>
<td>TGGGTGGTCT</td>
<td>Tpt1</td>
<td>Tumor protein translationally controlled 1</td>
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<tr>
<td>GTGAAACTAA</td>
<td>Rps4x</td>
<td>Ribosomal protein S4, X-linked</td>
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<td>CTATAAAGC</td>
<td>Fau</td>
<td>Finkel-Biskis-Reilly murine sarcoma virus</td>
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<td>↓↓</td>
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<td>Miscellaneous</td>
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<td>GAGGACTGCCC</td>
<td>Ly6e</td>
<td>Lymphocyte antigen 6 complex, locus E</td>
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<td>↑↑</td>
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<tr>
<td>CCCTTCTCTCT</td>
<td>Hba-a1</td>
<td>Hemoglobin α, adult chain 1</td>
<td>3.3</td>
<td>↑↑</td>
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<tr>
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<td>RIKEN cDNA 1500012F01 gene</td>
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<tr>
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<td>1</td>
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<td>Triosephosphate isomerase 1</td>
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<td>1</td>
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<tr>
<td>CTATAAAGAA</td>
<td>Cox4I</td>
<td>Cytochrome c oxidase subunit IV isoform 1</td>
<td>-2.1</td>
<td>↓↓</td>
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<tr>
<td>TAAAGCAAAA</td>
<td>Hist1h2bc</td>
<td>Histone 1, H2bc</td>
<td>-1.9</td>
<td>↓↓</td>
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</tbody>
</table>

Abbreviations: B, bexarotene treatment; G, gefitinib treatment; T, p53-null mammary tumors.

*Up-regulated transcripts are represented by positive average fold changes and down-regulated transcripts are represented by negative average fold changes among bexarotene and gefitinib treatments.
bexarotene and gefitinib treatments. Within this list, we find some genes on which little is known as well as genes previously described to be associated with human breast cancer, such as Fos-like antigen 2 (Fosl2), early growth response 1 (Egr1), gelsolin (Gsn), and tumor protein translationally controlled 1 (Tpt1), among others.

Within the functional group of transcriptional regulation, we find the transcription factor Fosl2 (also known as Fra2), a Fos family member, among the most prominently up-regulated by both chemopreventive compounds. Interestingly, an antioxidant promoter, the phenolic antioxidant tert-butylhydroquinone, was reported to induce expression of Fra2 (Fosl2) as well as Fra1. Furthermore, the authors concluded that inhibitory activator protein-1 complexes composed of Jun-Fra heterodimers, induced by tert-butylhydroquinone, antagonize the transcriptional effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, which are mediated by Jun-Fos heterodimers (21). Similarly, inhibition of interleukin-6-stimulated cell growth of human myeloma and mouse hybridoma cells was shown to be associated with increased expression of Fra2 protein (22). Fra2 has also been associated with differentiation in epidermis, and exogenous expression of Fra-2 (Fosl2) repressed activator protein-1 transcriptional activity in 12-O-tetradecanoylphorbol-13-acetate--treated keratinocytes and plays an opposing role to that of Fos (23). In ovary, expression of Fra2 and JunD is induced and maintained by luteinizing hormone with the transition of proliferating granulosa cells to terminally differentiated, nondividing luteal cells (24). Perhaps the observed up-regulation of Fosl2 in mammary gland epithelium of animals exposed to the effective chemopreventive agents is conducive to tilting the balance for the formation of activator protein-1 complexes with growth inhibitory properties.

Also within the group of transcriptional regulators, we find Egr1, a member of the immediate early gene group of transcription factors in a family that includes the tumor suppressor WT1. Egr1 is rapidly and transiently expressed after stimulation of cells with serum, growth factors, phorbol ester tumor promoters, or ionizing and nonionizing irradiation (25). Human Egr1 plays an important role in cell growth, differentiation, and development. Huang et al. (26) showed that the suppressive activity of Egr1 is applicable to several different types of human tumor cell lines including breast carcinoma, glioblastoma, osteogenic sarcoma, and fibrosarcoma (26). It was previously shown that Egr1 acts like a tumor suppressor gene, with its expression repressed in breast carcinomas. Recently, it was reported that the Egr1 gene is deleted in estrogen receptor--negative human breast carcinomas (27). Interestingly, we detected significant up-modulation of Egr1 gene expression in p53-null mammary epithelium of mice treated with bexarotene and gefitinib.

Among other transcripts of interest in cancer detected in our study and within the functional group associated with the cytoskeleton, we observed that both bexarotene and gefitinib treatment significantly up-modulate gelsolin expression in p53-null normal mammary epithelium. Gelsolin encodes a calcium-dependent protein that regulates actin filament length. This protein is suggested to play critical roles in actin cytoskeleton organization, cell motility, and apoptosis. Interestingly, and in agreement with our findings, loss of gelsolin expression is one of the most frequently alterations in mammary cancer across species (9, 28). Approximately 70% of human invasive breast carcinomas and 56% of ductal carcinomas in situ were reported to be deficient in the gelsolin protein expression (29, 30). It is very intriguing to observe that mammary epithelia from mice treated with the two most effective chemopreventive agents in our study displayed increase in gelsolin expression.

Cysteine cathepsins are a family of lysosomal proteases that have recently emerged as important players in cancer and have been involved in apoptosis, angiogenesis, cell proliferation, and invasion (31). The expression and activity levels of some cysteine cathepsins are commonly up-regulated in human and mouse cancers. Increased levels of cathepsin D, cathepsin B, and cathepsin L have been reported to be indicators of aggressive tumor behavior in human breast tumors (32). Recently, an important role was shown for Cathepsin S (Ctss) in regulating angiogenesis and tumor growth in a genetically engineered mouse model of pancreatic cancer (33). We observed significant up-modulation of Ctss expression in p53-null mammary tumors compared with normal mammary epithelium (fold change, 3.6). More importantly, Ctss gene expression was significantly down-modulated in p53-null mammary epithelium of mice treated with bexarotene and gefitinib (average fold change, −5.1; Table 2). Intriguingly, we also observed another cysteine protease, calpain 12 (Capn12), to be very significantly down-regulated (average fold change, −6; Table 2). In general, calpains are cysteine proteases involved in a variety of cellular processes including apoptosis, cell division, modulation of integrin-cytoskeletal interactions, and synaptic plasticity (34); however, no information is available on the specific role of Capn12.

Among genes related to protein modifications, Tiparp (also known as PARP1/Parp7) encodes a poly(ADP-ribose) polymerase that catalyzes the transfer of the ADP-ribose moiety from its substrate NAD⁺ to a limited number of proteins involved in chromatin architecture, DNA repair, and DNA metabolism. Poly(ADP-ribosylation) is a posttranslational modification of nuclear proteins in response to DNA damage that activates the base excision repair machinery (35). The generation of poly(ADP-ribose) polymerase--deficient mice showed the importance of poly(ADP-ribose) polymerase in the maintenance of genomic integrity due to its function in base excision repair (36, 37). In our study, we detected that treatment with all three chemopreventive agents, bexarotene, celecoxib, and gefitinib, up-modulates Tiparp gene expression in p53-null mammary epithelium (Table 2). On the other hand, we observed significant down-modulation of Tiparp expression when comparing p53-null mammary tumors with normal mammary epithelium. Perhaps, treatment with chemopreventive agents, such as those here studied, increases DNA repair activity in mammary epithelium at preneoplastic stages, and a biomarker of this increased activity is the observed Tiparp overexpression.

In summary, our analyses of differentially expressed genes in mammary epithelium of mice exposed to each chemopreventive agent revealed significant similarities across treatments. These results are particularly relevant in light of the findings of Medina et al. (8), in which bexarotene and gefitinib were observed to be effective as chemopreventive agents in the p53-null mammary epithelium cancer model, whereas celecoxib did not show any preventive effect. Most importantly,
the comprehensive comparison of gene expression profiles allowed us to identify a substantial set of transcripts that behave almost identically in mammary epithelia from mice exposed exclusively to the effective antitumorigenic agents (bexarotene and gefitinib), thus generating a gene expression signature that could be a biomarker of chemopreventive effectiveness in this model. Furthermore, our data provide insight into the molecular bases at play distinguishing the effective from the ineffective chemopreventive interventions and of relevance in mammary tumor development. Not surprisingly, bexarotene and gefitinib seem to exert their chemopreventive activity by affecting multiple cellular pathways, such as modulating the expression of genes related to cell proliferation, cytoskeleton, and extracellular matrix remodeling. A somewhat surprising but important observation is that these agents modulate cell adhesion and protein biosynthesis pathways, in addition to the more expected cell proliferation and apoptotic pathways.

Further studies will be required focusing on the functional characterization and mechanistic aspects of key cellular pathways identified by our gene expression analysis. The pathways of interest can be first experimentally tested in the described mouse model and in the future may become targets of interest for translational research.

Disclosure of Potential Conflicts of Interest

R.P. Bissonnette: Ligand Pharmaceutical employee. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank AstraZeneca for kindly providing the compound gefitinib to perform the studies here described.

References

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Martin C. Abba, Yuhui Hu, Carla C. Levy, et al.


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