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Research Article

Cancer Prevention Research

Differential Gene Regulation and Tumor-Inhibitory Activities of Alpha-, Delta-, and Gamma-Tocopherols in Estrogen-Mediated Mammary Carcinogenesis

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Abstract

Despite experimental evidence elucidating the antitumor activities of tocopherols, clinical trials with α-tocopherol (α-T) have failed to demonstrate its beneficial effects in cancer prevention. This study compared the chemopreventive efficacy of individual tocopherols (α-, δ-, and γ-T) and a γ-T–rich tocopherol mixture (γ-TmT) in the August-Copenhagen Irish (ACI) rat model of estrogen-mediated mammary cancer. Female ACI rats receiving 17β-estradiol (E2) implants were administered with 0.2% α-T, δ-T, γ-T, or γ-TmT for 30 weeks. Although α-T had no significant effects on mammary tumor growth in ACI rats, δ-T, γ-T, and γ-TmT reduced mammary tumor volume by 51% ($P < 0.05$), 60% ($P < 0.01$), and 59% ($P < 0.01$), respectively. Immunohistochemical analysis revealed that δ-T, γ-T, and γ-TmT reduced levels of the cell proliferation marker, proliferating cell nuclear antigen, in the rat mammary tumors. To gain further insight into the biological functions of different forms of tocopherols, RNA-seq analysis of the tumors was performed. Treatment with γ-T induced robust gene expression changes in the mammary tumors of ACI rats. Ingenuity Pathway Analysis identified "Cancer" as a top disease pathway and "Tumor growth" and "Metastasis" as the top signaling pathways modulated by γ-T. Although the results need further functional validation, this study presents an unbiased attempt to understand the differences between biological activities of individual forms of tocopherols at the whole transcriptome level. In conclusion, δ-T and γ-T have superior cancer preventive properties compared to α-T in the prevention of estrogen-mediated mammary carcinogenesis. *Cancer Prev Res; 1–10. ©2017 AACR.*

Introduction

Breast cancer is one of the most frequently diagnosed malignancies in women worldwide and the second leading cause of cancer-related mortality among women in the United States (1). Estrogens are known to be critical factors in the etiology of breast cancer (2). Both estrogen receptor (ER)–dependent and ER-independent mechanisms have been implicated in the initiation and progression of mammary cancer (2–4). Although activation of ERα enhances cell proliferation and accumulation of mutations resulting from replicative errors (2, 4), genotoxic estrogen metabolites can also induce neoplastic transformation (3, 4). Understanding the mechanisms underlying estrogen-mediated carcinogenesis could lead to the development of effective strategies for the prevention and treatment of breast cancer.

Recent studies shed light on the role of natural products in the inhibition of estrogen-dependent breast cancer (5, 6). Dietary components and bioactive natural compounds have been reported to inhibit mammary carcinogenesis by reduction of estrogen-induced oxidative stress as well as downregulation of ER-mediated signaling (7). Tocopherols, members of the vitamin E family present in the diet, have been demonstrated to exert chemopreventive effects in preclinical models of ER-positive breast cancer (8–12) as well as lung, colon, and prostate cancers (13–16).

Tocopherols are a group of fat-soluble phenolic compounds consisting of a chromanol ring and a saturated phytyl side chain (17). Depending on the number and position of methyl groups on the chromanol ring, tocopherols are designated as α, β, δ, and γ (17). α-Tocopherol (T) is trimethylated at the 5-, 7-, and 8-positions of the chromanol ring, β-T is dimethylated at the 5- and 8-positions, γ-T is dimethylated at the 7- and 8-positions, whereas δ-T is monomethylated at the 8-position (17). Structural differences in the chromanol ring are thought to be responsible for the variation in biological activity of each individual form of tocopherol. α-T has superior antioxidant activity, whereas the unmethylated carbon atoms at 5-position of the chromanol ring

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make δ- and γ-T more effective in trapping reactive nitrogen species (18). Although tocopherols have been proposed to reduce the risk of cancer due to their antioxidant properties (19, 20), large-scale chemoprevention clinical trials with α-T have provided inconsistent conclusions (21–23). Thus, detailed investigation of the biological activities and anticancer properties of the different forms of tocopherols are fundamental to future intervention studies with tocopherols.

γ-T is the most abundant form of tocopherol in the U.S. diet, being 3 to 5 times more abundant than α- or δ-T, whereas β-T is present in minute amounts (24). Tocopherols are mostly found in vegetable oils such as soybean, corn, and cottonseed oil (24). γ-TmT is a naturally occurring tocopherol mixture rich in γ-T obtained as a byproduct in the distillation of vegetable oil (25). γ-TmT has been shown to inhibit estrogen-induced mammary tumor growth in August-Copenhagen Irish (ACI) rats as well as MCF-7 xenografted immunodeficient mice (12). We have previously reported that δ-T, γ-T, and γ-TmT suppress *N*-methyl-*N*-nitrosourea (NMU)–induced mammary tumor growth in Sprague–Dawley rats (9, 10). Recently, the tumor-inhibitory effects of δ-T, γ-T, and γ-TmT in MCF-7 xenografts supplemented with estrogen have been demonstrated (26). ACI rats exhibit 80% to 100% tumor incidence upon prolonged exposure to estrogen (27), providing a physiologically relevant model for long-term cancer prevention studies. This study utilized ACI rats to evaluate the comparative chemopreventive efficacy of α-, δ-, γ-T, and γ-TmT in estrogen-mediated mammary carcinogenesis.

High-throughput RNA sequencing (RNA-seq) has revolutionized transcriptome profiling, enabling relatively precise quantification of transcript levels in biological samples (28). Currently, RNA-seq is extensively used to analyze differential gene expression in tissues subjected to different treatment conditions. For an unbiased understanding of the mechanisms underlying the anticancer activity of the different forms of tocopherols, RNA-seq analysis was performed on mammary tumors from ACI rats treated with α-, δ-, γ-T, and γ-TmT. Our analysis identified the top genes and biological networks modulated by individual tocopherols at the whole transcriptome level, providing new insights into their differential chemopreventive activities.

Materials and Methods

Diets

Natural γ-TmT was obtained from BASF Corporation (Covi-ox T-90, Batch number 0008778732). It contained 56.1% γ-T, 22.3% δ-T, 11.5% α-T, and 1.2% β-T. γ-T was purified to ≥97% from γ-TmT with no detectable α- and δ-T. α- and δ-T were purified from commercial grade α-T (T3634) and δ-T (T2028; Sigma-Aldrich) to ≥97% purity with no other detectable forms of tocopherol. A CombiFlash Companion XL automated flash chromatographic system (Teledyne ISCO) with a RediSep Rf Gold high-performance flash silica gel column (20–40 μm in particle size) was used for the purification. Semipurified AIN-93M diet obtained from Research Diets, Inc. was used as the control diet. Experimental diets were prepared by adding 0.2% each of α-, δ-, γ-T, and γ-TmT to the AIN-93M diet.

Animals and experimental procedures

Female ACI rats were purchased from Harlan Laboratories at 6 to 7 weeks of age. After 2 weeks of acclimatization, the rats were subcutaneously implanted with silastic tubing filled with 9 mg of 17β-estradiol (E2; Sigma-Aldrich) or sham implants, following a previously described method (29). Rats receiving sham implants were fed with control diet. E2-implanted rats were fed with control diet or diets containing 0.2% α-, δ-, γ-T, or γ-TmT. Diets were administered from the day of E2 implantation. Rats were sacrificed at 30 weeks after treatment. Each treatment group included 27 animals. Body weight of the rats was measured weekly, and they were palpated for mammary tumors weekly starting from 18 weeks after E2 implantation. Blood was collected at necropsy and serum stored at −80°C. Mammary tumors were snap frozen in liquid nitrogen or fixed in 10% formalin for further analysis. All animal studies were approved by the Institutional Review Board for the Animal Care and Facilities Committee at Rutgers, the State University of New Jersey (Protocol Number: 03-024).

Analysis of tocopherol levels in the rat serum

The levels of tocopherols (α, δ, and γ) and their metabolites in rat serum were analyzed by high-performance liquid chromatography using previously described methods (16).

Immunohistochemical analysis

Mammary tumors were fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μm thickness. Sections were incubated overnight at 4°C with antibody to proliferating cell nuclear antigen (PCNA; 1:4,000; M 0879, Dako), followed by incubation with biotinylated secondary antibody and avidin/biotin peroxidase complex. The sections were then stained with 3′-diaminobenzamine substrate and counterstained with Modified Harris Hematoxylin. Tumor sections from three different animals per treatment group were stained. Representative images were taken randomly, and nuclear staining was quantified using an Aperio ScanScope by counting at least 15,000 cells per slide.

RNA-seq analysis

Total RNA was extracted from rat mammary tumors ($n = 4$/group), and the quality was assessed. The samples were subjected to cDNA library construction and Illumina sequencing (NextGen 75 bp pair-end) yielding 75 million reads per sample. Quality control on raw reads was performed using FastQC (30). Good quality reads were aligned to the rat reference genome (rn6) using Bowtie2, and differential expression of transcripts was detected using Cuffdiff (31). Analysis of pathways and gene networks of the expression data was performed with QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) for genes with P value ≤ 0.001 (FDR < 0.05). IPA is a widely used bioinformatics tool to analyze biological molecule interactions, including miRNA, mRNA, and proteins. Pathways with $P < 0.001$ were found to be significantly differentially expressed. The RNA-Seq datasets described in this study have been deposited in the NCBI Gene Expression Omnibus with accession number GSE 103646.

mRNA expression analysis using qPCR

RNA was extracted from frozen mammary tumors. Reverse transcription and qPCR was performed as previously reported (32). Labeled primers were used for chemokine (C-X-C motif) receptor 2 (CXCR2), insulin-like growth factor binding protein 3 (IGFBP3), serpin peptidase inhibitor, clade A, member 1 (SERPINA), Cbp/P300-interacting transactivator with glu/asp-rich carboxy-terminal domain, 1 (CITED1), mesothelin (MSLN), fermitin family member 1 (FERMT1), extracellular matrix protein

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1 (ECM1), insulin-like growth factor 1 (IGF1), matrix metalloproteinase 13 (MMP13), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Tumor-free survival (TFS), or time to appearance of the first tumor, was estimated by the Kaplan–Meier method. The log-rank test was used to assess the homogeneity of TFS between different treatment groups. Tumor multiplicity was analyzed using the log-linear model (Poisson regression). Statistical significance was evaluated using one-way ANOVA followed by Dunnett multiple comparison *post hoc* test, preserving the overall type-1 error at the 5% level. The data are represented as ± SE. Differences were considered statistically significant when $P < 0.05$.

Results

Administration of α-, δ-, γ-T, and γ-TmT increases the serum levels of tocopherols and their metabolites in ACI rats

To determine the bioavailability of tocopherols in the experimental animals, the levels of α-, δ-, γ-T, and their respective short-chain carboxyethyl hydroxychroman (CEHC) metabolite were measured in the serum of the rats (Table 1). Administration of diets enriched with α-, δ-, and γ-T significantly increased the serum concentrations of the corresponding tocopherols by 2-, 81-, and 78-fold, respectively. In the γ-TmT–treated group, δ- and γ-T levels increased by 30- and 16-fold, respectively. Treatment with α-, δ-, and γ-T also led to significant increase in the serum levels of respective CEHC metabolites, by 82-, 204-, and 102-fold, respectively. γ-TmT supplementation increased the α-, δ-, and γ-CEHC concentrations by 3-, 61-, and 36-fold, respectively. Tocopherols are transferred to the blood by α-Tocopherol transfer protein (α-TTP). The affinity of α-TTP is highest for α-T, followed by γ-T and δ-T. Because hepatic α-TTP selectively facilitates the transfer of α-T from liver to blood, α-T is the most abundant tocopherol in blood. Because α-T is already abundant in the blood, supplementation with α-T did not cause a dramatic fold increase in the serum levels of α-T. As most of α-T is transported to blood, only a small percentage of α-T is metabolized. On the other hand, because relatively lower amounts of γ-T and δ-T are transferred into the blood, γ-T and δ-T are more extensively degraded in the liver and their side-chain degradation metabolites are more abundant than those of α-T. Hence, the levels of γ- and δ-CEHC metabolites in the rat serum are higher than that of α-CEHC.

Dietary δ-T, γ-T, and γ-TmT inhibit estradiol-induced mammary tumorigenesis in ACI rats

Female ACI rats implanted with E2 were fed with control diet or diets supplemented with 0.2% α-, δ-, γ-T, or γ-TmT for 30 weeks. TFS for each treatment group was estimated (Fig. 1A). Although no overall difference in TFS time was observed between the E2 control and tocopherol-treated groups (Fig. 1A), the median time to the appearance of first tumor was 25 weeks in the E2 control group and 26 weeks in the 0.2% α-, δ-, γ-T–, and γ-TmT–treated groups. Administration of 0.2% α-, δ-, γ-T, and γ-TmT inhibited mammary tumor growth (Fig. 1B). Final tumor measurements revealed that 0.2% α-, δ-, γ-T, and γ-TmT reduced tumor volume by 28%, 51% ($P < 0.05$), 60% ($P < 0.01$), and 59% ($P < 0.01$), respectively. δ-T, γ-T, and γ-TmT significantly inhibited E2-induced mammary tumor growth in the ACI rats, whereas α-T had no significant effect on tumor reduction (Fig. 1C). Although γ-T inhibited tumor growth more effectively than δ-T, the difference between γ-T and δ-T was not statistically significant. At the end of the 30-week study, the overall tumor multiplicity of the E2 control was not significantly different from the groups treated with 0.2% α-, δ-, γ-T, or γ-TmT (Fig. 1D). Therefore, tocopherols may act by slowing down tumor growth rather than blocking initiation of tumor development. In comparison with the negative control, the average body weights of rats at 30 weeks were not affected by E2 or any form of tocopherol treatment, indicating that none of the treatments were toxic for the given duration (Fig. 1E).

Effects of α-, δ-, γ-T, and γ-TmT treatment on cell proliferation in ACI rats

Tumor growth data showed that δ-T, γ-T, and γ-TmT suppressed estrogen-mediated mammary tumor growth in ACI rats, whereas α-T had no inhibitory effects. To investigate if such differences in tumor inhibition could be attributed to the effects of α-, δ-, γ-T, and γ-TmT on cell proliferation, immunohistochemical analysis of the cell proliferation marker, PCNA, was performed in the mammary tumors of ACI rats (Fig. 2). α-T treatment had no significant effect on the levels of PCNA in mammary tumors. However, δ-, γ-T, and γ-TmT reduced PCNA levels in the mammary tumors of rats by 63% ($P < 0.05$), 69% ($P < 0.05$), and 65% ($P < 0.05$), respectively.

Overview of differentially expressed genes in response to α-, δ-, and γ-T treatment in the mammary tumors of ACI rats

In order to determine the effects of different forms of tocopherol treatment on the whole transcriptome, we compared global gene expression profiles of mammary tumors from the E2 control group with those of α-, δ-, and γ-T–treated groups. RNA-seq analysis was performed on four tumor samples per treatment group. For the γ-T–treated group, one outlier was detected after RNA-seq analysis. Hence, all further analysis was performed on four samples each from α- and δ-T and three from the γ-T group. Figure 3A shows a heatmap of gene expression changes across four treatment groups, E2, α-T, δ-T, and γ-T based on 61 of 256 differentially expressed genes. Treatment with α-, δ-, and γ-T upregulated or downregulated a large number of genes with

Table 1. Analysis of tocopherols and their metabolites in the serum of ACI rats

Treatment	α-T (μmol/L)	δ-T (μmol/L)	γ-T (μmol/L)	α-CEHC (μmol/L)	δ-CEHC (μmol/L)	γ-CEHC (μmol/L)
Negative control	28.4 ± 3.7	0.08 ± 0.03	0.30 ± 0.10	0.2 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
E2 control	34.8 ± 8.0	0.05 ± 0.02	0.30 ± 0.10	0.3 ± 0.0	0.9 ± 0.2	1.3 ± 0.4
E2 + 0.2% α-T	74.5 ± 8.7**	0.06 ± 0.01	0.02 ± 0.00	24.6 ± 13.8**	1.2 ± 0.5	2.6 ± 1.4
E2 + 0.2% δ-T	27.9 ± 0.6	4.06 ± 0.50 ***	0.30 ± 0.01	0.3 ± 0.1	184.1 ± 20.7***	2.7 ± 0.7
E2 + 0.2% γ-T	27.0 ± 1.3	0.10 ± 0.01 ***	23.60 ± 2.80***	0.3 ± 0.1	1.5 ± 0.2	133.3 ± 13.9***
E2 + 0.2% γ-TmT	34.3 ± 1.7	1.50 ± 0.40 **	4.80 ± 1.00**	0.9 ± 0.2*	55.4 ± 7.9***	47.3 ± 11.9**

NOTE: The effects of α-, δ-, γ-T, and γ-TmT supplementation on the levels of α-, δ-, and γ-T and their short-chain CEHC metabolite in the serum (μmol/L) of ACI rats were analyzed at 30 weeks. *P* values are compared with the E2 control. Statistical significance, *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

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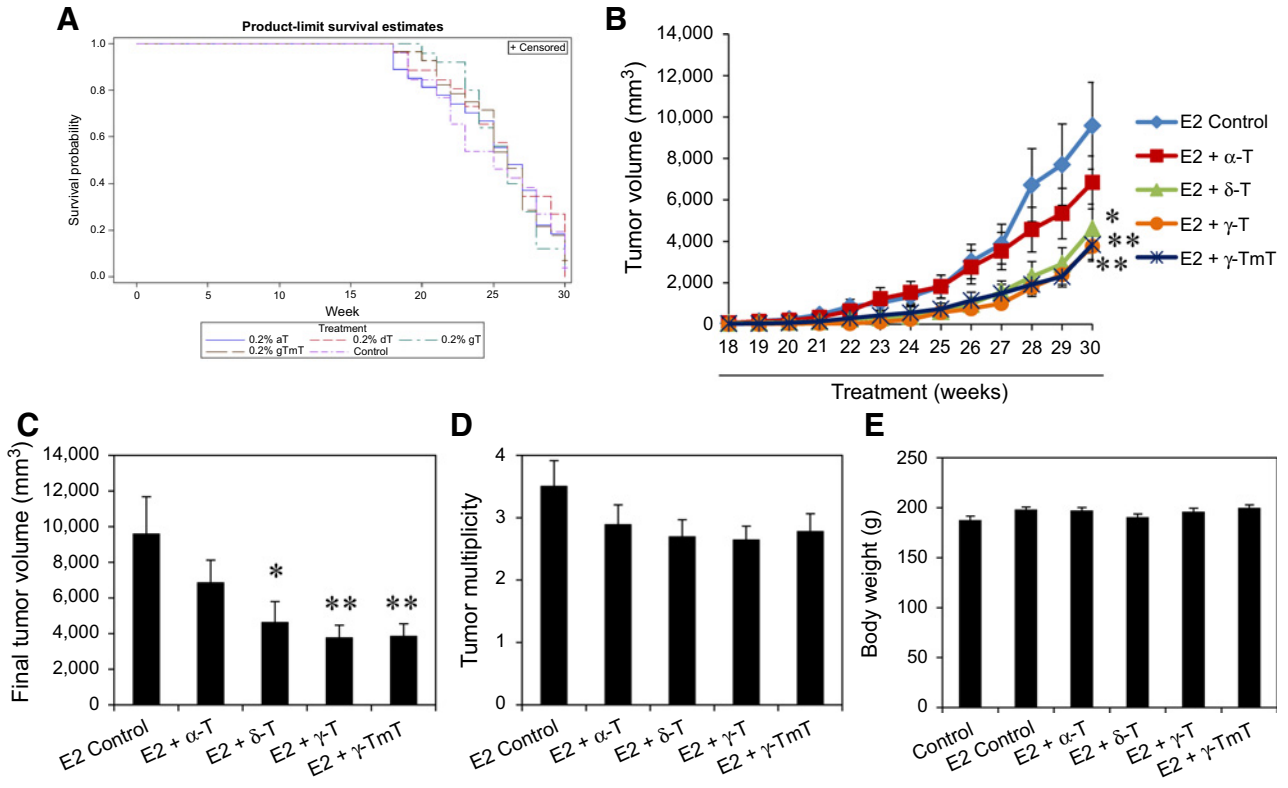

Figure 1.

δ-T, γ-T, and γ-TmT inhibit estrogen-induced mammary tumorigenesis in ACI rats. ACI rats implanted with E2 were fed with control diet or diet containing 0.2% α-, δ-, γ-T, or γ-TmT for 30 weeks ($n = 27$/group). **A,** The TFS curve of each treatment group is shown. **B,** Average tumor volume of the different treatment groups at weekly time points starting from 18 weeks is shown. **C,** Average final tumor volume of each treatment group at 30 weeks is shown. **D,** Average tumor multiplicity of each treatment group at 30 weeks is shown. **E,** Average body weight of each treatment group at 30 weeks is shown. Statistical significance, *, $P < 0.05$; **, $P < 0.01$.

respect to E2 control. We identified 47 differentially regulated genes (5 upregulated and 42 downregulated) in the α-T–treated group, 51 (34 upregulated and 17 downregulated) in the δ-T–treated group, and 192 (72 upregulated and 120 downregulated) in the γ-T–treated group. Overall, the data showed that γ-T induced more significant gene expression changes in the ACI rat mammary tumors compared with α-T and δ-T. Of the 72 genes upregulated by γ-T, 5 were also upregulated by δ-T (FCGBP,

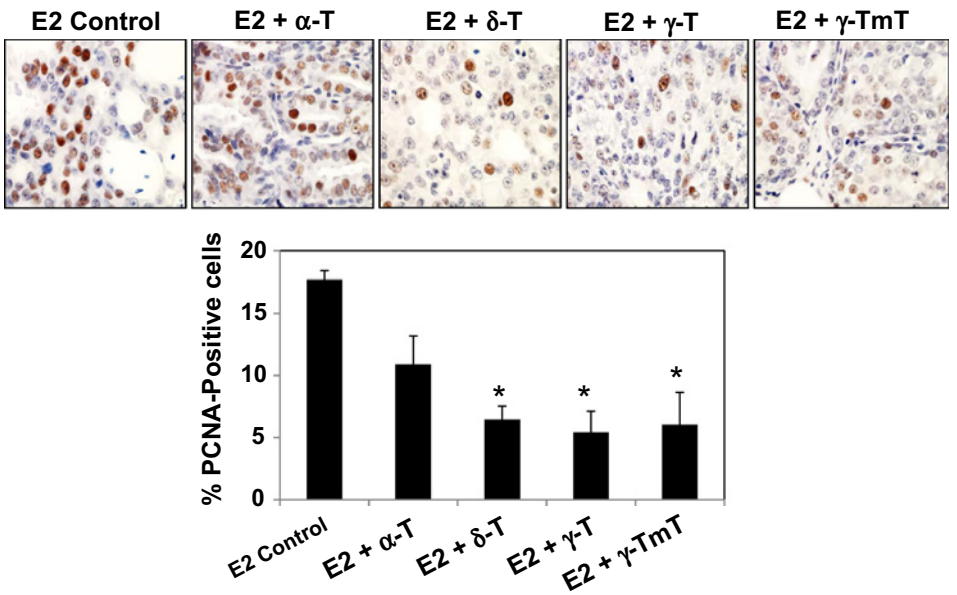

Figure 2.

Effect of α-, δ-, γ-T, and γ-TmT treatment on cell proliferation of mammary tumors from E2-treated ACI rats. Representative images of PCNA staining in the mammary tumors of ACI rats (×40), and quantification of nuclear PCNA staining is shown ($n = 3$/group). Statistical significance, *, $P < 0.05$.

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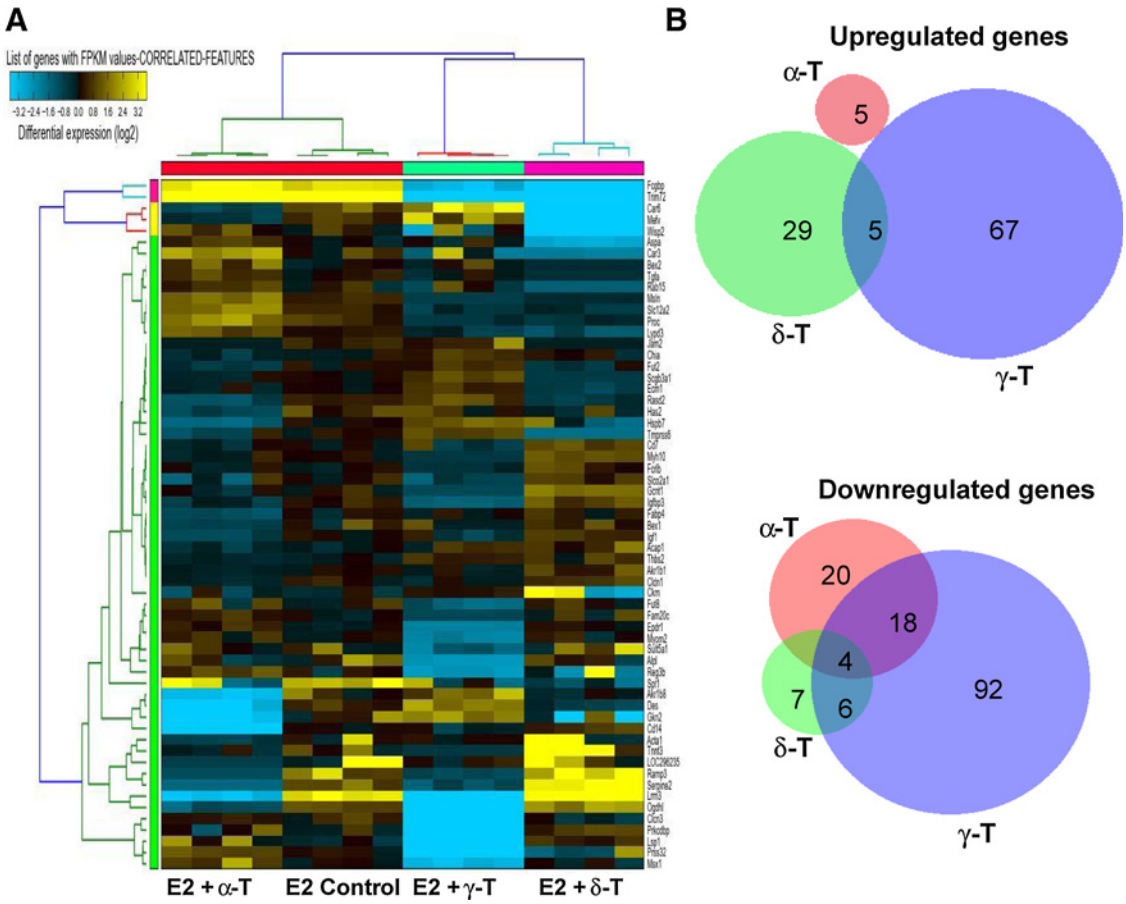

Figure 3.

Overview of genes regulated by the different tocopherols. **A,** The clustered heatmap of 15 samples based on 61 of 256 differentially expressed genes across 4 groups (E2 control, α-, δ-, γ-T). **B,** Venn diagrams comparing the genes upregulated and downregulated by treatment with α-, δ-, and γ-T compared with E2 control.

S100B, JAM2, WFDC3, and RPLP2). The genes upregulated by α-T were unique, sharing no common genes with δ- or γ-T (Fig. 3B). Among the 120 genes downregulated by γ-T, 6 were downregulated by δ-T (CITED1, ANKS1B, RASD2, ENDOU, CPA3, and MRVI1) and 18 by α-T. Four genes (PLAU, CMA1, PPP1R1A, and FERMT1) were downregulated by α-, δ- and γ-T (Fig. 3B).

The main gene networks and canonical pathways modulated by γ-T treatment

Tumor inhibition data showed that γ-T is the most effective form of tocopherol in suppressing E2-mediated mammary tumor growth. Further, RNA-seq analysis demonstrated that γ-T had the most profound influence on tumor transcriptome, when compared with α- or δ-T. Considering the superior chemopreventive activity of γ-T, in-depth analysis was done on RNA-seq data from the γ-T–treated group to investigate the possible biological functions and pathways regulated by γ-T. Using the IPA software (www.qiagen.com/ingenuity), we performed core analysis of all the genes significantly upregulated or downregulated by γ-T in comparison with the E2 control group. This analysis identified "Cancer" and "Organismal Injury and Abnormalities" as the top two disease and disorder pathways modulated by γ-T (Fig. 4A). Further analysis revealed that the effect of γ-T on the Cancer network involved genes related to different cellular functions such as tumor growth (IGFBP3, TGFA, COL18A1, PLAU, and TNFRSF9) and metastasis (ANGTPL4, PDLIM3, SERPINA1, and IL2RB; Fig. 4B and Supplementary Fig. S1).

Effect of γ-T treatment on gene expression in the mammary tumors of ACI rats

IPA analysis revealed that γ-T treatment had a robust effect on genes commonly involved in cancer. To gain a better understanding of the mechanism by which γ-T inhibited mammary tumor growth, the genes differentially regulated by γ-T were further analyzed. The top differentially expressed genes in response to γ-T treatment are reported in Table 2. γ-T modulated the expression of genes involved in different cellular functions which can be broadly classified as follows:

Cell proliferation, apoptosis, and signal transduction. γ-T downregulated the expression of KLHDC8A, FERIL4, CXCR2, and IGFBP-3, whereas the expression of SCGB3A1, G0S2, and SFRP-1 was upregulated.

Serine protease inhibitors. The expression of SERPINA1 and SERPINB9 was downregulated by γ-T.

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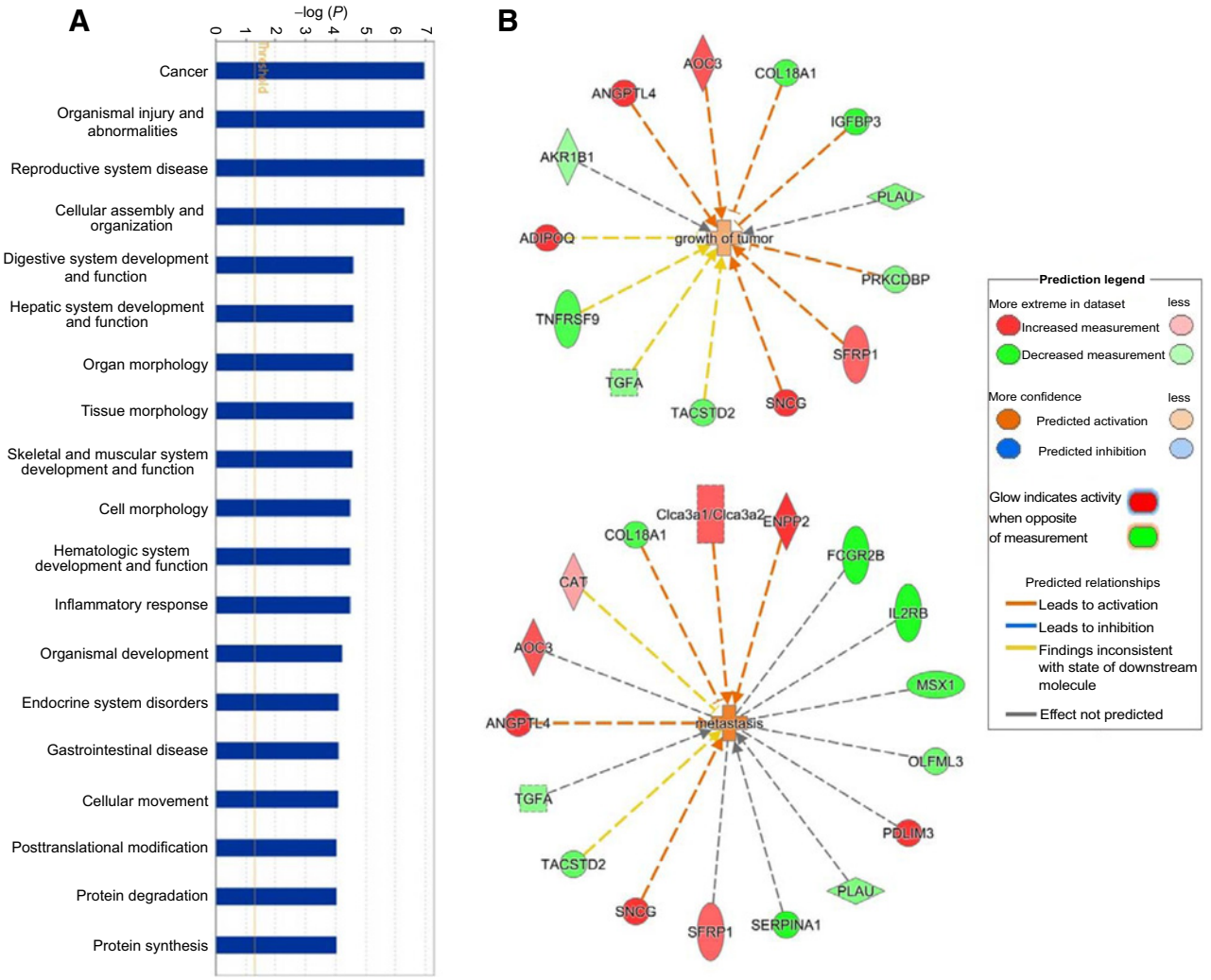

Figure 4.

Treatment with γ-T differentially regulates genes involved in various signaling pathways. **A,** IPA showing the top disease and disorder gene networks modulated by treatment with γ-T. **B,** Network map created by IPA showing the genes differentially regulated by γ-T and their functions.

Transcription. Expression of the transcriptional coactivator CITED1 was downregulated in response to γ-T treatment.

Cell adhesion and extracellular matrix components. γ-T downregulated the expression of MSLN, FERMT1, ECM1, and CLDN1, whereas PDLIM3 and JAM2 were upregulated.

Immune response and growth factors. Expression of genes involved in immune response, such as SFTPD, IL2RB, TPSBP2, and TNFRSF9, and the growth factors IGF1 and WISP2 were downregulated upon γ-T treatment.

Angiogenesis and metastasis. γ-T downregulated the angiogenic marker ESM1 and the metastatic marker MMP13.

Others. Gene expressions of the enzymes CMA1, CPA3, HDC, the regulator of lipid metabolism, ADIPOQ, and the ribosomal component RPLP2 were modulated by γ-T treatment.

Nine genes belonging to the different cellular pathways regulated by γ-T were randomly selected, and their mRNA expression levels were determined by qPCR (Fig. 5). The results further confirmed the downregulation of these genes in response to γ-T treatment in support of the RNA-seq analysis.

Discussion

Tocopherols have been known to reduce the risk of cancer (33). However, clinical trials with α-T, the most abundant form of tocopherol in human tissues, have failed to demonstrate its cancer preventive properties (21). Previously, we reported that the natural tocopherol mixture, γ-TmT, rich in γ-T, inhibited E2-mediated mammary tumor growth in ACI rats at the doses of 0.3% and 0.5% (12). The current study assessed the chemopreventive potential of individual forms of tocopherols in estrogen-mediated breast cancer. The anticancer activities of a single dose (0.2%) of α-, δ-, γ-T, and γ-TmT were tested using the ACI rat model. Based

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Table 2. Genes regulated by γ-T treatment

Gene symbol	Gene description	Log_2 (fold change)	*P* value
Genes downregulated by γ-T			
Cell proliferation, apoptosis, signal transduction			
KLHDC8A	Kelch domain containing 8A	−5.5	5.00E−05
FERIL4	Fer-1 like family member 4, pseudogene	−4.0	5.00E−04
CXCR2	Chemokine (C-X-C motif) receptor 2	−3.8	1.00E−04
IGFBP3	Insulin-like growth factor binding protein 3	−2.9	5.00E−05
Serine protease inhibitor			
SERPINA1	Serpin peptidase inhibitor, clade A, member 1	−6.6	5.00E−05
SERPINB9	Serpin peptidase inhibitor, clade B, member 9	−1.5	8.00E−04
Transcription			
CITED1	Cbp/P300-interacting transactivator with glu/asp-rich carboxy terminal domain, 1	−4.4	5.00E−05
Cell adhesion, extracellular matrix			
MSLN	Mesothelin	−3.3	5.00E−05
FERMT1	Fermitin family member 1	−3.3	5.00E−05
ECM1	Extracellular matrix protein 1	−2.3	5.00E−04
CLDN1	Claudin 1	−2.1	5.00E−05
Immune response, growth factors			
SFTPD	Surfactant protein D	−5.6	5.00E−05
IL2RB	Interleukin 2 receptor, beta	−5.5	5.00E−05
TPSB2	Tryptase beta 2	−3.9	5.00E−05
IGF1	Insulin-like growth factor 1	−3.8	1.00E−04
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	−2.4	5.00E−05
WISP2	Wnt-1–inducible signaling pathway protein 2	−3.1	7.50E−04
Angiogenesis, metastasis			
MMP13	Matrix metalloproteinase 13	−2.1	6.50E−04
ESM1	Endothelial cell–specific molecule 1	−2.0	7.00E−04
Others			
CMA1	Chymase 1	−5.2	5.00E−05
CPA3	Carboxypeptidase A3	−4.2	5.00E−05
HDC	Histidine decarboxylase	−4.2	1.00E−04
Genes upregulated by γ-T			
Cell proliferation, apoptosis, signal transduction			
SCGB3A1	Secretoglobin, family 3A, member 1	Below detection limit	5.00E−05
G0S2	G_0–G_1 switch 2	2.3	5.00E−05
SFRP-1	Secreted frizzled-related protein 1	2.3	5.00E−05
Cell adhesion, extracellular matrix, cytoskeleton			
PDLIM3	PDZ and LIM domain 3	5.5	5.00E−05
JAM2	Junctional adhesion molecule 2	2.2	5.00E−05
Lipid metabolism			
ADIPOQ	Adiponectin, C1Q, and collagen domain containing	4.4	5.00E−05
Others			
RPLP2	Ribosomal protein, large, P2	Below detection limit	5.00E−05

on the results of previous studies, the dose of 0.2% was chosen to evaluate the relative activities of the different tocopherols. The 30-week study revealed that δ-T, γ-T, and γ-TmT inhibited mammary tumorigenesis in ACI rats, whereas α-T had not significant inhibitory effect (Fig. 1). γ-T reduced E2-induced mammary tumor growth most effectively, followed by γ-TmT and δ-T. This finding is in accordance with previous studies which have reported the superior cancer-preventive properties of δ-T and γ-T over α-T in colon cancer (14) and NMU-induced mammary cancer (9). Supporting the tumor inhibition results, δ-T, γ-T, and γ-TmT inhibited cell proliferation in E2-treated mammary tumors, as evident from the reduced percentage of PCNA positive cells, whereas α-T did not (Fig. 2).

This study reports for the first time the transcriptomic analysis of mammary tumors from ACI rats treated with α-, δ-, and γ-T (Fig. 3). α-T, which did not significantly inhibit the growth of rat mammary tumors, had minimal effect on the transcriptome (47 differentially expressed genes). Moreover, the fact that the 5 genes upregulated by α-T were unique, sharing no common genes with δ- or γ-T, could account for its reduced tumor-inhibitory activity. δ-T, with moderate tumor inhibitory potential, modulated the expression of 51 genes. γ-T, which was most effective in inhibiting mammary tumorigenesis, had the most profound influence on the rat transcriptome, leading to the differential expression of 192 genes. Of these 192 genes, 159 were unique to γ-T. IPA revealed γ-T as the only form of tocopherol which modulated "Cancer" as the top disease pathway (Fig. 4). Recent studies indicate a correlation between the chemopreventive/therapeutic potential of compounds and their effects on differential gene expression. Treatment of pancreatic cancer cells with single agents metformin and aspirin as well as their combination revealed that the combination inhibited cell growth more effectively and modulated the expression of a larger number of genes compared with the single agents (34). Thus, the dramatic effect of γ-T treatment on the rat tumor transcriptome could be linked to its superior chemopreventive activity.

γ-T regulated the expression of genes related to different cellular functions (Table 2). Of the top genes downregulated by γ-T treatment, KLHDC8A has been reported to be overexpressed in aggressive gliomas which have lost dependence on mutant EGFR

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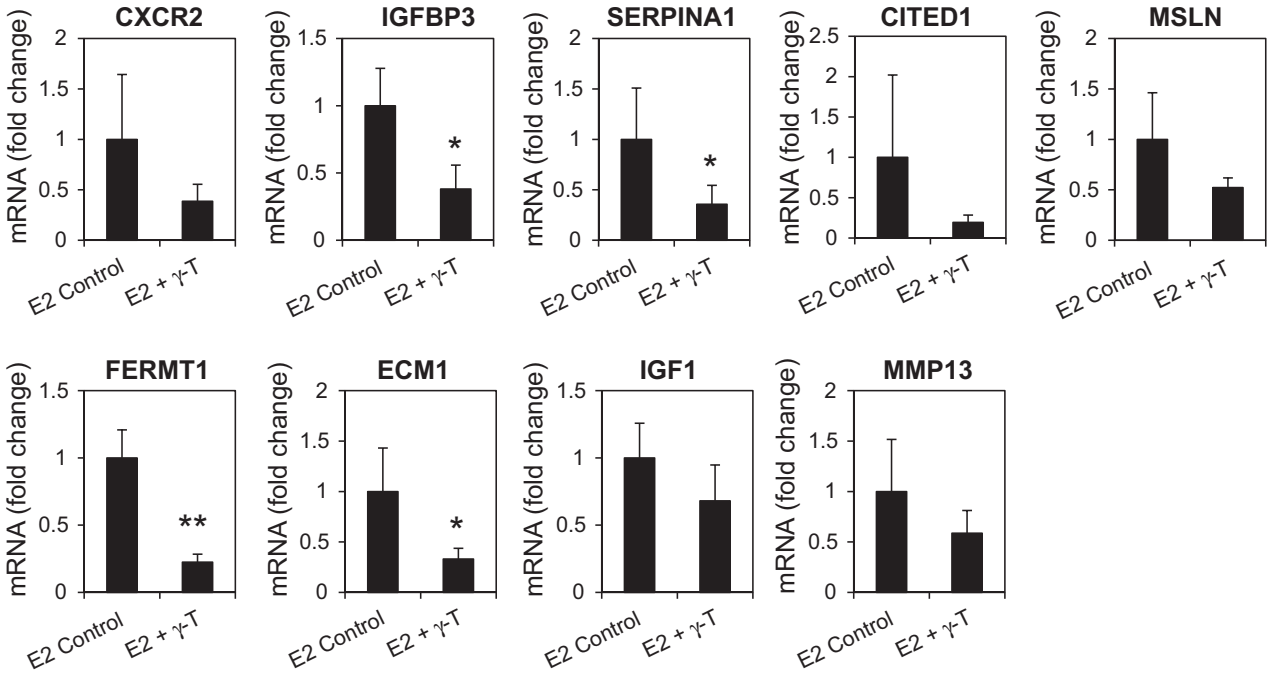

Figure 5.

Validation of RNA-seq analysis by qPCR. Nine genes were randomly selected from the different cellular pathways modulated by γ-T, and their mRNA levels in the mammary tumors of ACI rats were determined by qPCR (n = 6–8/group).

(35). However, its role in breast cancer is still unknown. CXCR2 is a G-protein–coupled receptor which binds CXC chemokines, namely CXCL1-3 and 5-8, to trigger their function. Treatment with γ-T downregulated CXCR2 gene expression. CXCR2 has been associated with poor outcomes for different cancers through its effects on migration, invasion, and angiogenesis (36). In breast cancer, targeting CXCR2 has been reported to enhance chemotherapeutic response and inhibit tumor growth, angiogenesis, and lung metastasis *in vivo* (37). Thus, inhibition of CXCR2 by γ-T could be critical to its chemopreventive potential. γ-T was also found to downregulate IGFBP3, which can exert prosurvival or proapoptotic effects on tumor cells depending on cell type and context (38). γ-T reduced expression of the serine protease inhibitor, SERPINA1, and the transcriptional coregulator, CITED1. Recently, SERPINA1 has been reported to be a direct ER target gene (39). CITED1 is a nuclear protein that binds directly to ERα and activates ER-mediated transcription (40). Therefore, downregulation of SERPINA1 and CITED1 could be indicative of selective inhibition of ER-dependent transcription by γ-T. In future studies, functional validation of these genes modulating ER function could help in improved understanding of the mechanism by which γ-T suppresses E2-mediated mammary tumor growth.

γ-T downregulated gene expression of the cell adhesion molecules MSLN and FERMT1. MSLN is a tumor differentiation antigen that is highly expressed in several human cancers such as pancreatic, lung, ovarian, and triple-negative breast cancer. MSLN is an important target for cancer immunotherapy (41). Although γ-T reduced MSLN expression, the significance of targeting MSLN in E2-dependent breast cancer is yet unknown. FERMT1 regulates integrin functions and has been implicated in breast cancer growth and lung metastasis (42). ECM1 is a marker for tumorigenesis associated with tumor recurrence in breast cancer. It promotes epithelial-to-mesenchymal transition and maintenance of breast cancer stem cells (43). Inhibition of FERMT1 and ECM1 by γ-T might open new opportunities for prevention of cancer progression and metastasis by a natural, dietary compound.

SFTPD, a component of innate immune response responsible for maintaining lung homeostasis, was downregulated by γ-T. Recently, SFTPD has been reported to inhibit lung cancer progression by binding to EGFR for suppression of EGF signaling (44). However, the role of SFTPD in breast cancer has not been studied. γ-T reduced the expression of IGF1. IGF1, acting through type 1 insulin-like growth factor receptor (IGF-1R), regulates multiple aspects of breast cancer–like cell proliferation, survival, and metastasis (45). High levels of circulating IGF1 have been correlated with increased risk of breast cancer (45), and drugs targeting the IGF axis are being tested in clinical trials. Inhibition of IGF1 by γ-T is particularly significant in an E2-dependent model of mammary cancer because of the cross-talk that exists between ER and IGF signaling pathways (46). Blocking of IGF action by γ-T could inhibit the activity of ER. γ-T also downregulated expression of the metastatic biomarker, MMP13. Elevated levels of MMP13 have been associated with decreased overall survival and osteolytic bone metastasis in breast cancer (47). Thus, by suppressing MMP13, γ-T may inhibit breast cancer metastasis.

Among the genes upregulated by γ-T, SCGB3A1, also known as HIN1, was the most dramatically induced. SCGB3A1 is silenced in a substantial fraction of breast cancers due to methylation, suggesting a tumor-suppressive function. It inhibits cell growth, invasion, and Akt activation (48). γ-T upregulated the tumor-suppressor G0S2 which has been reported to inhibit oncogenic

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transformation through repression of Myc activity (49). Thus, treatment of ACI rats with γ-T regulated the expression of genes that control cell proliferation, ER-dependent signaling, apoptosis, tumor progression, and metastasis.

To summarize, this study demonstrated that δ-T, γ-T, and γ-TmT are potent inhibitors of E2-mediated mammary tumorigenesis in ACI rats, with γ-T exhibiting the maximum anticancer activity. Tocopherols, especially γ-T, exerted dramatic effects on the rat transcriptome, regulating the expression of genes involved in cell proliferation, metastasis, and tumor progression. These findings provide new insights into the anticancer activities of γ-T, mediated via antiproliferative effects, distinct from its widely reported antioxidant effects. In conclusion, δ-T and γ-T have superior cancer-preventive properties compared with α-T in E2-dependent mammary cancer and deserve more attention in future chemoprevention studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Differential Gene Regulation and Tumor-Inhibitory Activities of Alpha-, Delta-, and Gamma-Tocopherols in Estrogen-Mediated Mammary Carcinogenesis

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