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Tocotrienol-treated MCF-7 Human Breast Cancer Cells Show Down-regulation of API5 and Up-regulation of MIG6 Genes

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Abstract. *Background: Tocotrienols belong to the vitamin E family and have multiple anticancer effects, such as antiproliferative, antioxidant, pro-apoptosis and antimetastatic. This study aimed to identify the genes that are regulated in human breast cancer cells following exposure to various isomers of vitamin E as these may be potential targets for the treatment of breast cancer. Materials and Methods: Gene expression profiling was performed with MCF-7 cells at inhibitory conditions of IC_{50} using Illumina's Sentrix Array Human-6 BeadChips. The expression levels of selected differentially expressed genes were verified by quantitative real-time-PCR (qRT-PCR). Results: The treatment with tocotrienol-rich palm oil fraction (TRF), α-tocopherol and isomers of tocotrienols (α, γ, and δ) altered the expression of several genes that code for proteins involved in the regulation of immune response, tumour growth and metastatic suppression, apoptotic signalling, transcription, protein biosynthesis regulation and many others. Conclusion: Treatment of human MCF-7 cells with tocotrienol isomers causes the down-regulation of the API5 gene and up-regulation of the MIG6 gene and the differential expression of other genes reported to play a key role in breast cancer biology.*

Breast cancer is the second most common type of cancer in the World after lung cancer and the fifth most common cause of cancer death. Breast cancer is also the most common female malignancy in Malaysia and worldwide (1, 2). Chemoprevention of breast cancer, using natural and synthetic compounds to intervene in the early precancerous stages of carcinogenesis before invasion begins, could be a measure to reduce breast cancer risk for women at high risk of developing the disease. One group of such natural compound that has recently gained intense interest among nutritionists, health professionals and researchers is the tocotrienols. Tocotrienols together with tocopherols, has each of which, four isomers α, β, γ, and δ (3), are collectively known as vitamin E, or tocochromanols (4). Tocotrienols and tocopherols have substituted methyl groups at an identical position on the chroman ring and differ only in their side-chains. Tocotrienols have unsaturated isoprenoid side-chains with double bonds in the 3', 7' and 11' positions, while tocopherols have saturated phytyl carbon chains. Tocopherols exist only as free chromanols in nature, whilst tocotrienols can also occur naturally in esterified forms (5). Tocopherols are abundant in common vegetables and nuts, while tocotrienols can be found in rice bran, wheat germ and most abundantly in palm oil (6, 7). Previous studies have well defined the major physiological activity of vitamin E, which is its anti-oxidant role and protective effects against lipid peroxidation in biological membranes (8), with α-tocopherol having the most activity of all the vitamin E isomers. However, the unique effect of tocotrienol was later identified when several lines of evidence supported the beneficial effects of tocotrienols on inhibiting tumour development (9). Tocotrienol-treated mice showed a significant elongation in tumour latency, while tocopherol had no effect (10). Other remarkable biological and physiological properties of tocotrienols which include potential blood cholesterol lowering and cardioprotective effects, efficient antioxidant activity in biological systems, and possible anticancer and neuroprotective effects (11) differing from those of tocopherols have been further identified. Previous studies have also reported that tocotrienol treatment of cultured cells led to apoptosis (12-14), protection from oxidative damage of neuronal cells (15) and anti-proliferative effects (16, 17).

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Key Words: Vitamin E, tocotrienols, gene expression, apoptosis inhibitors, EGFR inhibitor, breast cancer, API5, MIG6.

Table I. *Primers of GAPDH, β-actin (housekeeping genes) and 2 genes selected from the microarray data used for quantitative real time-PCR.*

Gene name	Accession no.	Forward and reverse primer
GAPDH	NM_002046.3	5'-AGG TGA AGG TCG GAG TCA AC-3'
		5'-AGG GAT CTC GCT CCT GGA A-3'
β-Actin	NM_001101.3	5'-ACA GAG CCT CGC CTT TGC CG-3'
		5'-TGG GCC TCG TCG CCC ACA TA-3'
API5	NM_006595.3	5'-GGG GTT TGT GGA GTG TTT AAT G-3'
		5'-AGC TCA TGG TCT TTT GGA ATG T-3'
MIG6	NM_018948.3	5'-GGG GTA CGT TAG ACT CAG ATG G-3'
		5'-AAG GAT GTG AAA ATC GGA ACA C-3'

API5: Apoptosis inhibitor 5; MIG6: mitogen-induced gene 6.

Table II. *The IC_{50} values for TRF, individual isomers of tocotrienol and α-tocopherol in MCF-7 cells after 72 hours of culture.*

Test compound	Concentration range tested	IC_{50} (72 hours)
Tocotrienol-rich fraction (TRF)	0-20 μg/mL	10.86
Gamma-tocotrienol (γ-T3)		7.71
Delta-tocotrienol (δ-T3)		7.61
Alpha-tocotrienol (α-T3)		12.4
Alpha-tocopherol (α-T)		Not achieved
Alpha-tocopherol (α-T)	20-100 μg/mL	50.2

Microarray technology is widely used to examine physiologically relevant gene expression profiles of a multitude of cells and tissues. This technology is based on the hybridization of RNA from tissues or cells to either cDNA or oligonucleotides immobilized on a glass chip. Cutting-edge technology, Illumina Beadchips microarray probing over 48,000 mRNA species was used to investigate the effects of a tocotrienol-rich fraction from palm oil, tocotrienol isomers and α-tocopherol on gene expression in human breast cancer cells.

Materials and Methods

Vitamin E. The individual fractions of tocotrienols (α, γ, and δ) were obtained from Davos Life Science Pte Ltd (Singapore). The α-tocopherol (αT) was obtained from Sigma® Chemical Company (Croydon, England) at a purity of approximately 99%. Extraction of TRF from palm oil has been described previously (6). Briefly, the palm oil fatty acid distillate is converted into methyl esters by esterification. The methyl esters are then removed, leaving a vitamin E concentrate. This is further concentrated by crystallization and passed through an ion-exchange column to give 60-70% pure vitamin E. Further purification is achieved by washing and then drying the concentrate followed by a molecular distillation stage. The TRF was obtained from the Malaysian Palm Oil Board, Selangor, Malaysia. The final purity of vitamin E in the TRF preparation was 95-99%, with a composition of (wt/wt) α-tocopherol 32%, and tocotrienols (α, γ and δ) 68%.

Cell lines and culture conditions. The hormone-dependent MCF-7 McGrath human breast cancer cells were kindly provided by Malaysian Palm Oil Board (Selangor, Malaysia). Stock cells were grown in T75 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum (FCS), 1% penicillin-streptomycin, 1% L-glutamine and 10^{-8} M oestradiol in a humidified atmosphere of 5% carbon dioxide in air at 37˚C. Stock solutions of TRF, tocotrienol (α, γ and δ) isomers and αT were prepared in DMSO at a concentration of 10 mg/mL. For the cell proliferation studies, the stock solutions of the various vitamin E isomers were diluted in phenol-red-free RPMI-1640 medium supplemented with 5% dextran-charcoal-treated FCS (DCFCS) to final concentrations of 2-20 μg/mL in the test medium.

Establishment of IC_{50} values. The MCF-7 oestrogen-positive cells (0.5×10^6) were cultured in a 24-well plate with 2-20 μg/mL of TRF, αT, αT3, δT3 or γT3 for 72 hours. A Coulter particle counter (Beckman Inc., Brea, CA, USA) was used to count the viable cells. To perform the cell viability count, the vitamin E-treated and control (untreated) MCF-7 cells were washed with 0.9% NaCl to remove the non-adherent dead cells. The cells were then lysed in 0.5 ml 0.01 M HEPES buffer containing 1.5 mM $MgCl_2$ and two drops of Zap-Oglobin II lytic reagent for 15 min. The nuclei released were counted in isoton using an automated particle counter (Beckman Inc.). The IC_{50} value for each treatment was calculated from the cell viability graph normalized against the control (untreated).

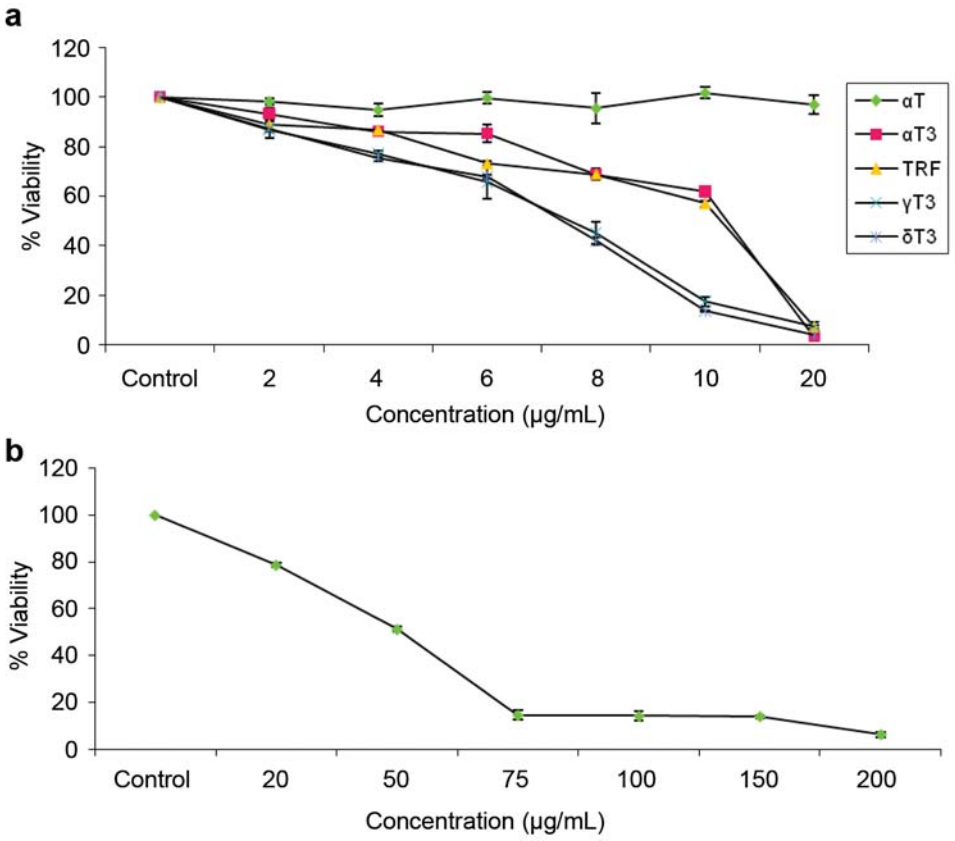

Figure 1. *a) Dose-response inhibition of MCF-7 cell proliferation by TRF, individual isomers of tocotrienol (αT3, γT3 and δT3) and α-tocopherol (αT). b) Dose-response inhibition of MCF-7 cell proliferation by higher concentrations of αT. Data is expressed as percent viability based on untreated (control) samples. A significant difference (p<0.05) was observed between TRF and α-T treatments as well as the individual tocotrienol isomers (αT3, γT3 and δT3) and α-T.*

RNA extraction and quantification. The MCF-7 cells were maintained in DMEM in culture flasks. When the cells reached 70% confluence, the vitamin E isomers (TRF, αT3, δT3, γT3 or αT) were added to the cultures at their respective IC_{50} concentration. After 72 hours of culture, the total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technology Inc., Carlsbad, CA, USA). The RNA sample was then treated with DNase I, RNaseOUT reagent (Invitrogen Life Technology Inc.) to remove RNases. Finally, a cleanup step using the QIAGEN RNeasy mini kit columns (Qiagen Gmbh, Hilden, Germany) was performed to obtain a better yield for *in vitro* transcription labelling. The quality of the total RNA was determined by gel analysis using an automated RNA analysis electrophoresis system (Bio-Rad Experion Bioanalyzer, Bio-Rad Laborotories, Selangor, Malaysia). Only samples with high quality RNA with minimal degradation and clear 28S/18S ribosomal bands were used in the microarray step. The RNA concentration was determined using a NanoDrop spectrophotometer. A replicate of five treated samples and one control (untreated) sample were used for the microarray experiment.

Probe labelling and Illumina Sentrix BeadChip array hybridization. Biotin-labelled complementary DNA (cRNA) samples for hybridization on Illumina sentrix human-6 arrays (Illumina Inc., San Diego, CA, USA) were prepared according to the recommended sample labelling procedure. In brief, 500 ng of total RNA was used for the cDNA synthesis, followed by the amplification/labelling step (*in vitro* transcription) to synthesize biotin-labelled cRNA according to the MessageAmp II aRNA Amplification kit (Ambion, Inc., Austin, TX, USA). A cRNA purification step was carried out to remove enzymes, salts and unincorporated nucleotides. The cRNA of each treatment was loaded into Illumina's Sentrix Array Human-6 BeadChip (Illumina Inc.). Control and treated samples were technically duplicated (two replications) using two beadchips and all the procedures (labelling, loading, hybridization and so on) were carried out simultaneously by a single researcher on the same day.

BeadChips scanning and quality control. Illumina's Sentrix Array Human-6 BeadChips, with each BeadChip comprising six microarrays on a glass slide, were scanned using an Illumina BeadChip Scanner (Illumina Inc.) to detect significant signal intensity difference of 48k genes (~24k RefSeq genes + ~24k unigenes) transcripts between the control and the various treatments. The Illumina microarray quality was determined by BeadStudio V3 software (Illumina Inc.). Annotation files for each chip were added into the BeadStudio program and quality control analysis for the microarray data was performed by image viewing of the

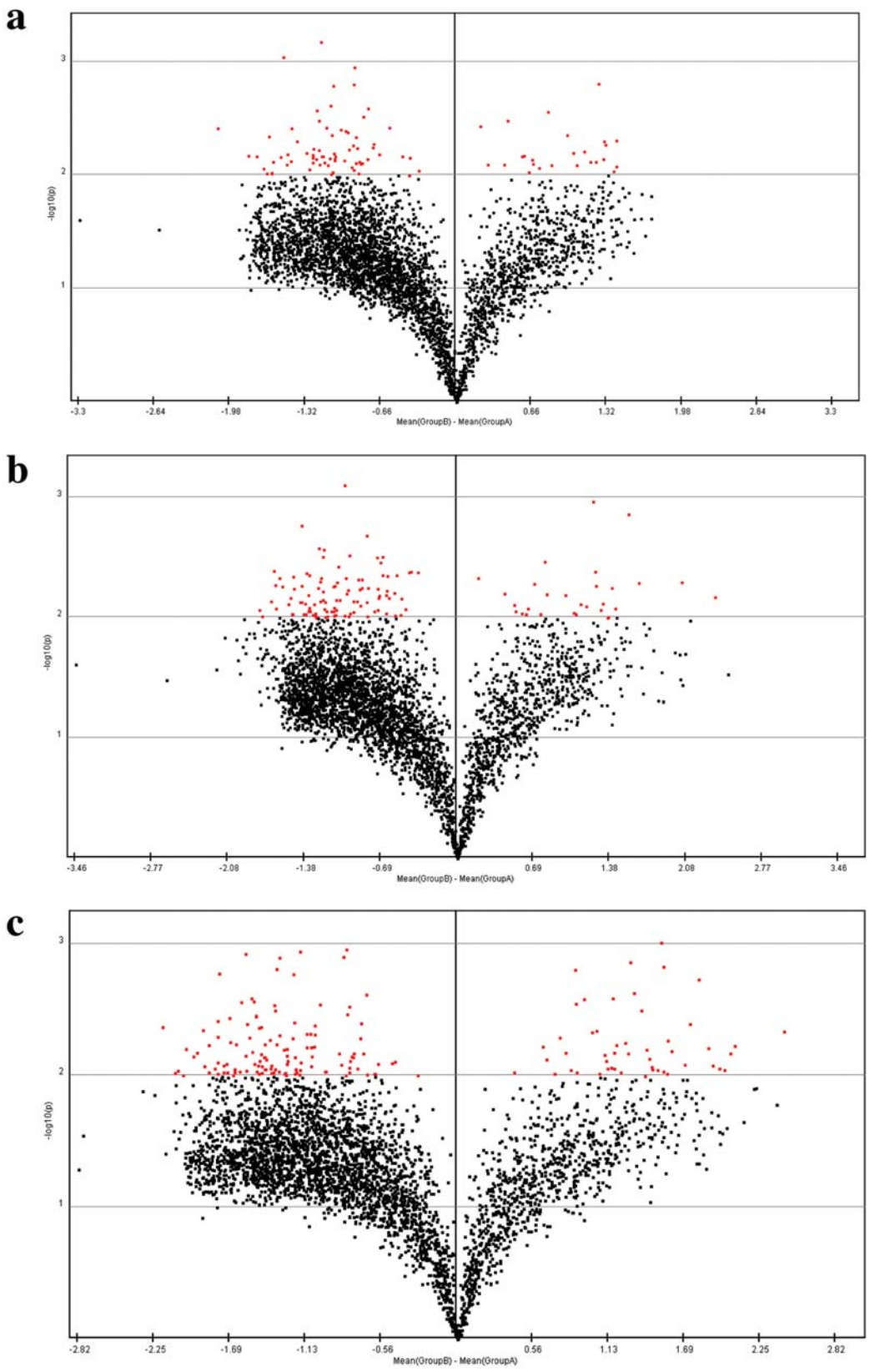

Figure 2. *Continued*

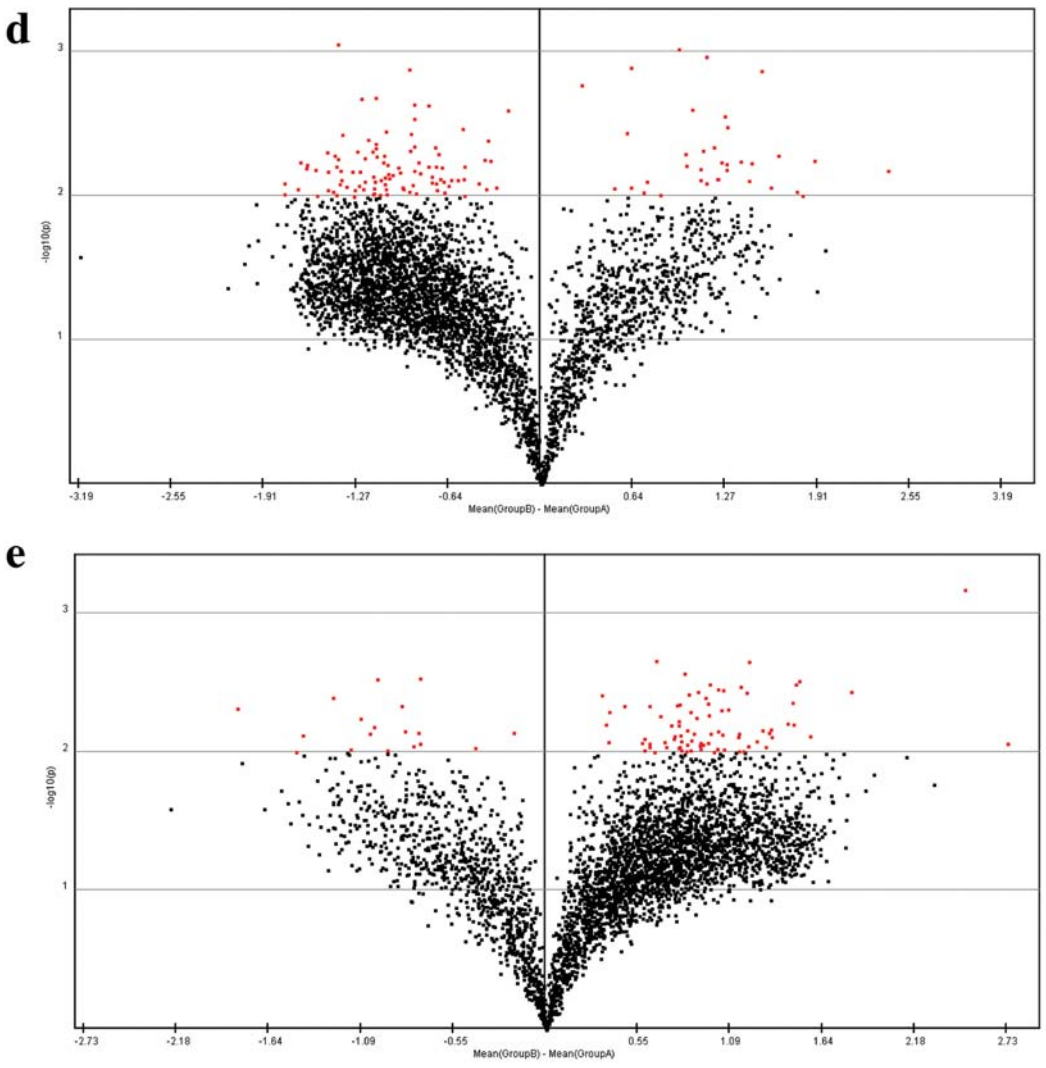

Figure 2. *Differential expression of genes between control (group B) and treatments (group A) were determined using Student's t-test with level of significance at p≤0.01: a) α-tocopherol, b)TRF, c) α-tocotrienol, d) γ-tocotrienol and e) δ-tocotrienol.*

corresponding scan and incorporated control bead analysis (housekeeping, hybridization, signal generation and background). Arrays with overall intensity outliers from the majority of arrays (caused by poor hybridization conditions or poor imaging) were excluded from further analysis. The Illumina BeadStudio was used to generate an output file of signal intensities for each bead type on an unlogged scale. BeadStudio GX V3.0 was used to generate a single file describing the signal intensity and detection (*p*-value) of all the arrays in the experiment with one row for each gene in the experiment. The expression matrix generated by BeadStudio was used for advanced statistical analysis.

Data acquisition and statistical method. The microarray data analysis of the present study was carried out using MultiExperiment Viewer (MeV) software of TM4 suite (http://www.tm4.org) (18). For data processing and normalization, the signal intensity and detection (*p*-value) data was generated for 48,687 transcripts along with their functional description. A subset of 3,986 transcripts was prepared, based on the criteria of 'detection value' computed by BeadStudio for each of the transcripts. One minus the '*p*-value' (1-p) was computed from the background model characterizing the chance that the signal (of target sequence) was distinguishable from the negative controls within the bead array chip. Every detection value of more than or equal to 0.99 was interpreted as 'signal present', whereas every detection value of less than 0.99 was interpreted as 'signal not detectable' by the array. Each of the 3,986 transcripts had a detection value within the range of 0.99-1.00 across the control and all treatments, thus balanced data was obtained. The subset data were normalized using the global mean intensity normalization method. Differential expression of the transcripts (or genes) between the control and the treatments were determined using the Student *t*-test (between subjects) with level of

significance at $p \leq 0.01$. Comparative analysis between the control and treated samples was conducted separately for each individual compound, including fold change (average signal intensity treated/average signal intensity control). If the calculated *p*-value for a gene was less than or equal to the user-input alpha (critical *p*-value), the gene is considered significant ($p \leq 0.01$). In the between-subjects design, the samples were assigned to one of two groups (group A treated, group B untreated) and genes that had significantly different mean $\log_2$ expression ratios between the two groups were assigned to one cluster, while the genes that were not significantly different between the two groups were assigned to another cluster. For the between-subjects *t*-test, the Welch *t*-test for small samples with unequal variances in the two groups was applied (19). The SAM method which estimates the false discovery rate (FDR) was used to pick out significant genes based on differential expression between replicates of the samples. This method excludes the proportion of genes likely to have been incorrectly identified by chance as being significant. The distribution of the test statistic, thresholds for significance (through the tuning parameter delta) was set after looking at the distribution. Genes were considered as 'positively significant', if their mean expression in group B was significantly higher than that of their mean expression in group A. Groups A and B were a pair of treatments of testing hypothesis. Genes were considered as 'negatively significant', if the mean expression in group A was significantly in excess that of their mean expression in group B. *t*-Test significant genes were divided into positive (up-regulation) and negative (down-regulation) expression using the threshold delta value at FDR value of zero (20).

Quantitative real-time PCR. Quantitative RT-PCR analysis was performed on selected genes, detected at higher fold-change and significance value ($p \leq 0.001$) in the microarray *t*-test and SAM analysis to validate the microarray results using the set of primers reported in Table I. The total RNA samples (DNase and RNase digested and QIAGEN cleaned up) previously used as a starting material for the microarray experiment was also used for the qRT-PCR experiment. The qRT-PCR was performed on an iCycler® from Bio-Rad using a SuperScript III Platinum SYBR Green one-step qRT-PCR kit ((Invitrogen Life Technology Inc.). The total RNA was diluted accordingly and 5 μL of 20 ng of the sample was pipetted into a 96-well reaction plate. Then 20 μL of master mix which comprised of SYBR Green one-step enzyme mix (SuperScript III RT, Platinum Taq DNA polymerase and RNaseOut), 2× SYBR Green reaction mix (SYBR Green I dye, 6 mM $MgSO_4$, 0.4 mM dNTP mix, buffers and stabilizers) and the primers were added. Non-template control (NTC) and non-enzyme control (No-RT) wells were also added for each different primer. The reactions were assayed in triplicate and performed in a final volume of 25 μL. The relative gene mRNA levels were normalized against β-actin and *GAPDH* housekeeping genes and a difference in expression of 2-fold or more between the experimental and control samples were considered to be significant. The iQ5 software (Bio-Rad Laboratories, Foster City, CA, USA) was used to plot and generate normalized fold expression for the gene of interest.

Results

Effects of TRF, α-tocopherol and tocotrienol isomers on the proliferation of MCF-7 cells. The tocotrienol isomers were able to inhibit proliferation of the MCF-7 cells ($p<0.05$) and reduce the cell numbers below plating density (Figure 1a). In contrast, αT had no effect on the growth of MCF-7 cells at any concentration up to 20 μg/mL. The IC_{50} value for αT could only be determined when higher concentrations (25-200 μg/mL) were used (Figure 1b). The order of potency for the compounds tested was found to be δT3<γT3<TRF <αT3<αT (Table II).

Effects of TRF, α-tocopherol and individual isomers of tocotrienols (α, γ, δ) on gene expression profiling at IC_{50}. The gene distributions of the subset of 3,986 genes were visually represented by volcano plots (Figure 2). The vitamin E-treatments modulated the expression of 96 genes in αT, 132 genes in TRF, 176 in αT3, 134 in γT3 and 99 in δT3-treated samples as compared with the untreated control ($p \leq 0.01$). Fifty genes in αT (25 up-regulated and 25 down-regulated), 57 genes in TRF (28 up-regulated and 29 down-regulated), 99 genes in αT3 (49 up-regulated and 50 down-regulated), 69 genes in γT3 (34 up-regulated and 35 down-regulated and 35 genes in δT3 (18 up-regulated and 17 down-regulated) treated samples expression differed from the untreated control ($p \leq 0.01$). The genes that had a fold-change ≥2.0 for up-regulated genes (Table III) and ≥0.5 for down-regulated genes (Table IV) were selected. Within this group, 26 genes in αT (12 up-regulated and 14 down-regulated), 36 genes in TRF (18 up-regulated and 18 down-regulated), 64 genes in αT3 (27 up-regulated and 37 down-regulated), 47 genes in γT3 (20 up-regulated and 27 down-regulated) and 23 genes in δT3 (14 up-regulated and 9 down-regulated) treated samples differed in expression as compared with the control samples ($p \leq 0.01$).

Categories of genes regulated by TRF, α-tocopherol and tocotrienol isomers. Treatment of the MCF-7 cells with the vitamin E compounds altered the expression of genes that code for proteins involved in immune response, tumour and metastasis suppressors, apoptotic signalling, transcription factors, protein biosynthesis regulators and others. The genes responsible for modulating immune response function were interferon-induced transmembrane protein 3 (*IFITM3*) by TRF, interferon-induced transmembrane protein 2 (*IFITM2*) by αT, TRF, αT3 and γT3, ferritin heavy polypeptide-1 (*FTH1*) by γT3 and collagen type IV alpha 3 by δT3. These genes were up-regulated (≥2.0 fold) compared with the control (Table III). The largest functional group of genes that was modulated by all the treatment was that encoding for protein biosynthesis where 16 genes were found to be up-regulated. In all the treatments, about 15 genes involved in regulation of transcription functions were found to be down-regulated compared with control (Table IV). Within a sub-sample of genes that are highly pertinent to breast cancer biology, two genes that cause negative regulation of apoptosis (anti-apoptosis) *API5* and *ICH1* were found to be down-regulated (>0.5) in the samples treated with γT3

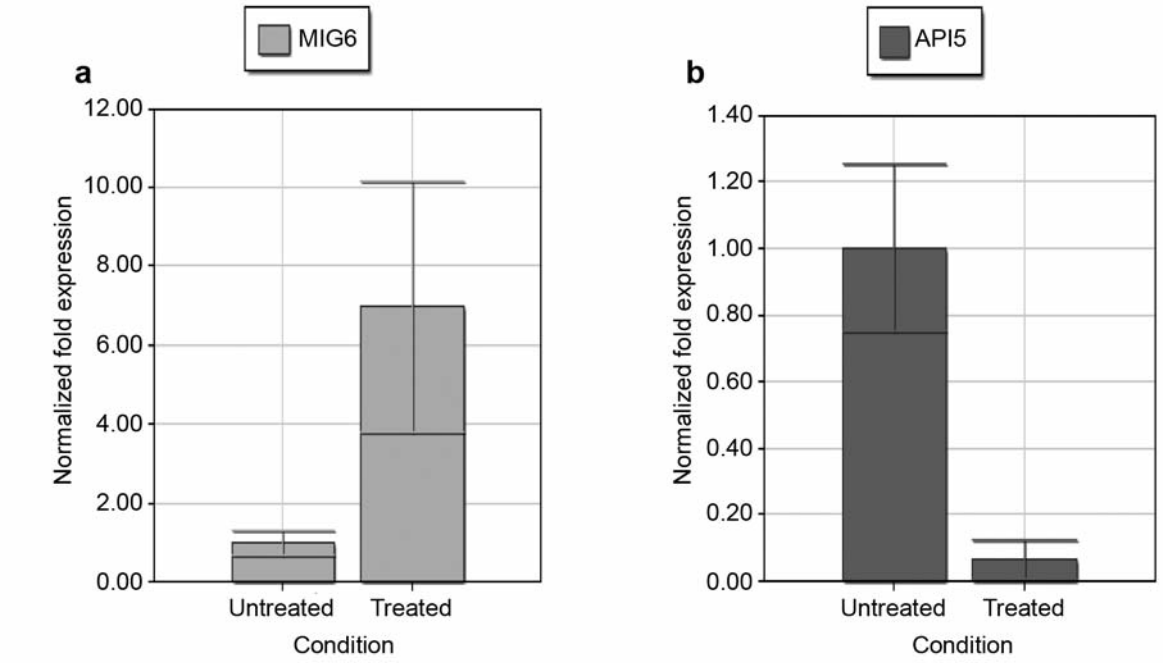

Figure 3. *Quantitative real-time PCR confirmed the a) up-regulation of MIG6 expression in MCF-7 cells treated with δ-tocotrienol and the b) down-regulation of API5 expression in the MCF-7 cells treated with γ-tocotrienol. The relative gene mRNA levels were normalized against the expression of the β-actin and GAPDH housekeeping genes. The difference in expression of 2-fold or more between experimental and control samples were considered to be significant.*

compared with control. The expression of tumour suppressor genes such as *MIG6* and *MIG9* were up-regulated in the αT3, yT3 or δT3 treated samples compared with controls.

Validation studies. The expression of one up-regulated (*MIG6*) and one down-regulated (*API5*) gene was analyzed by qRT-PCR. The qRT-PCR results confirmed the expression trends observed in the microarray data for the two transcripts (Figure 3). The expression of the reference housekeeping genes, *GAPDH* and β-actin, remained unchanged with treatment.

Discussion

Vitamin E exerted widespread effects in the MCF-7 cells and regulated (up and down) genes that have been implicated to have a role in breast cancer biology. In general, all the treatments exerted direct inhibitory effect on cell growth, in line with previous studies which reported that tocotrienols induced apoptosis in human and mouse cancer cell lines (20-22). In addition, previous studies have also shown that the anti-proliferative effect was greater in cells treated with γ- and δ-tocotrienols (15, 23) which was also confirmed here (Figure 1a and 1b). The identified genes encompassed a broad range of functional categories. The most important included are *API5* and *ICH1* whose high expression has been correlated to increased risk of many carcinomas (24, 25). Defects in the apoptotic pathway have been reported to facilitate tumour progression, by rendering cancer cells resistant to death mechanisms relevant to metastasis (26). In addition, dysregulation of apoptotic pathways can also contribute to neoplastic diseases by preventing or delaying normal cell turnover, thus promoting cell accumulation. The *API5* gene has been shown to inhibit programmed cell death in growth factors-deprived cells (27). This gene is also frequently up-regulated in tumour cells with potent anti-apoptotic action, mediated *via* suppression of apoptosis (28). API5 overexpression has been reported to induce cervical tumour cell invasiveness, and to occur in some metastatic lymph node tissues (28), raising the possibility that it may be a metastatic oncogene. In addition, the expression of this gene has been linked to poor prognosis in non–small cell lung cancer and squamous cell carcinoma (29). Inhibition of API5 has been suggested as a possible route for developing anti tumour agents (30). In the present study, γT3-treatment down-regulated the expression of the *API5* gene showing that γ-tocotrienol is a powerful apoptosis modulating agent. The inhibition of API5 function might offer a possible mechanism for antitumour exploitation.

The mitogen-inducible gene 6 (*MIG6*) is a negative feedback regulator of receptors for tyrosine kinases. The expression of this gene was markedly down-regulated in human breast carcinomas, correlating with reduced overall survival of breast

Table III. *The most significant (p<0.01) known genes differentially up-regulated (≥2 fold) in tocotrienol-rich fraction (TRF), α-tocopherol (αT) and tocotrienol isomers (αT3, γT3 and δT3) treated MCF-7 cells when compared with untreated control MCF-7 cells.*

GO Term and gene name	Treatment	Fold change
Protein biosynthesis		
Ribosomal protein S2 (*RPS2*)	αT3	2.85
Ribosomal protein S6 (*RPS6*)	αT3	2.17
Ribosomal protein L11 (*RPL11*)	αT3	2.81
Ribosomal Protein S14 (*RPLS14*)	αT, TRF, γT3	2.17, 2.47, 2.87
Ribosomal protein L30 (*RPL30*)	αT3	2.90
Ribosomal Protein L31 (*RPL31*)	αT, γT3	2.26, 2.44
Ribosomal protein L35 (*RPL35*)	αT3	2.37
Ribosomal protein L8 (*RPL8*)	αT, TRF, αT3, γT3	2.38, 2.34, 3.32, 2.37
Ribosomal protein S9 (*RPL9*)	TRF	2.25
Ribosomal protein S29 (*RPS29*)	γT3	2.93
Ribosomal protein S30 (*RPS30*)	αT, γT3	2.65, 2.74
Ribosomal protein S9 (*RPS9*)	αT3. γT3	2.89, 2.20
Ribosomal protein LP2 (*RPLP2*)	TRF	2.39
Ribosomal protein L18a (*RPL18A*)	αT3	3.96
Ribosomal protein S19 (*RPS19*)	αT3, γT3	2.17, 2.63
Glutamyl-prolyl-tRNA synthetase (*EPRS*)	δT3	2.10
Cell adhesion		
Claudin 3 (*CDLN3*)	αT, αT3	2.45, 4.08
Non-metastatic cells 2, protein (*NM23B*)	αT3, γT3	2.58, 2.14
Immune response		
Interferon induced transmembrane protein 2 (*IFITM2*)	αT, αT3, TRF, γT3	2.60, 3.65, 3.12, 3.11
Interferon induced transmembrane Protein 3 (*IFITM3*)	TRF	2.57
Ferritin, heavy polypeptide 1 (*FTH1*)	γT3	3.00
Collagen, type IV, alpha 3 (*CERT*)	δT3	2.95
Tumor suppressor		
Migration-inducing gene 9 (*MIG9*)	αT3, γT3	5.37, 5.39
Mitogen-induced gene 6 (*MIG6*)	δT3	5.55**
Glioma tumor suppressor candidate region gene 2 (*GLCSTR2*)	TRF, αT3	2.49, 2.69
Nucleosome assembly		
Histone 2 (*HIST2*)	α-T, TRF	2.08, 2.08
Histone 1 (*HIST1*)	αT3	2.14
Electron transport		
Cytochrome *c* oxidase subunit 7c (*COX7C*)	αT, TRF, γT3	2.65, 2.15, 2.42
Nucleotide and nucleic acid metabolism		
Thymocyte nuclear protein 1 (*THYN1*)	αT	2.04
Deoxyguanosine kinase (*DGUOK*)	αT	2.18
Ion transport		
ATP synthase 5 (*ATP5I*)	TRF	2.58
ATPase, lysosomal accessory protein 1 (*ATPLAP1*)	αT3	2.08
ATP synthase, isoform 2 (*ATP2*)	TRF	2.93
Protein Binding and modification		
Small nuclear ribonucleoprotein polypeptides B (*SNRPB*)	TRF	2.70
Finkel-Biskis-Reilly murine sarcoma virus (*FAU1*)	αT3, γT3	2.95, 2.33
Tight junction protein 1 (zona occludens 1) (*TJP1*)	δT3	2.76
Fatty acid metabolism		
Dodecenoyl-coenzyme A delta isomerise (*DCI*)	TRF, αT3, γT3	3.01, 2.38, 2.28
Protein phosphatase inhibitor activity		
Protein phosphatase 1, regulatory (inhibitor)	TRF, αT3	2.64, 2.65
Ribosomal protein S27a (*RPS27A*)	αT3	2.37
Cell proliferation		
Interferon stimulated exonuclease gene (*CD25*)	TRF, αT3, γT3	4.10, 4.18, 3.69
Cell signalling		
Growth differentiation factor 15 (*GDF15*)	TRF, αT3	5.05, 3.85
Proteolysis and peptidolysis		
Cathepsin L (*CTSL*)	TRF, αT3	2.47, 2.24
Intracellular signalling		
Cornichon homolog 4 (Drosophila) (*CNIH4*)	TRF	2.11

Table III. *Continued*

Table III. *Continued*

GO Term and gene name	Treatment	Fold change
Pseudouridine synthesis		
Nucleolar protein 10 (*NOP10*)	αT3	2.41
Nucleolar protein 7 (*NOL7*)	γT3	2.32
Hydrolase activity		
Stromal cell-derived factor 2-like 1 (*SDF2L1*)	αT3	2.24
Defense response		
Trefoil factor 3 (intestinal) (*TFF3*)	αT3	2.32
Chloride transport		
Chloride channel 7 (*CLCN7*)	αT3	2.27
Signal transduction		
S100 calcium binding protein A11 (*S100A11*)	γT3	3.49
Zyxin (*ZYX*)	δT3	2.22
Nucleotide binding		
Chromosome 14 open reading frame (*DC50*)	γT3	3.00
Kinesin family member 14 (*KIF14*)	δT3	2.27
Intracellular signaling cascade		
Cornichon homolog 4 (Drosophila) (*CNIH4*)	γT3	2.14
Intracellular Protein transport		
Translocase of outer mitochondrial membrane 7 (*TOM7*)	γT3	2.59
Vesicle docking protein p115 (*VDP*)	δT3	2.86
Ligatin (*LGTN*)	δT3	2.57
UDP-glucose metabolism		
UDP-glucose pyrophosphorylase 2 (*UGP2*)	δT3	2.02
Wnt receptor signaling pathway		
Casein kinase 1, alpha 1 (*CSNK1A1*)	δT3	3.48
Amino acid metabolism		
Solute carrier family 7A5, (*SLC7A5*)	δT3	2.94
Actin binding		
Spectrin, beta, non-erythrocytic 1 (*SPTBN1*)	δT3	2.82
Triacylglycerol metabolism		
Pancreatic lipase-related protein 2 (*PNLIPRP2*)	δT3	2.74
Regulation of transcription		
Polymerase (RNA) 1 polypeptide D (*POLR1D*)	αT, αT3, γT3	2.34, 2.48, 2.41
Nuclear factor (erythroid-derived 2)-like 2 (*NRF2*)	δT3	2.29
Zinc Ion Binding		
Zinc finger, AN1-type domain 2A (*ZNFAN12A*)	αT3	2.05
Biological Process Unknown		
Chromosome 11 open reading frame 10 (*C11ORF10*)	αT3	2.72

**Gene expression is significantly ($p \leq 0.001$) up-regulated. Observation has been validated by qRT-PCR.

cancer patients (31, 32) and it has been described to be mutated in different human carcinomas (33-35). A recent study has shown that MIG6 expression is reduced in skin, breast, pancreatic and ovarian carcinomas (36). A possible role of MIG6 as a tumour suppressor was indicated by MIG6-mediated inhibition of EGFR overexpression induced transformation of Rat1 cells (37). Therefore, MIG6 gene was identified as a novel negative feedback regulator of the epidermal growth factor receptor (EGFR) and potential tumour suppressor (38). The loss of MIG6 in breast cancer may thus be a marker of the process toward malignancy. The up-regulation of the MIG6 such as shown by δT3 in the present study in turn may suppress the EGFR functions on breast cancer.

The microarray approach used in the present study showed that treatment of MCF-7 cells with TRF, α-tocopherol or tocotrienols isomers (α, γ, δ) produced a genome wide effect on a higher number of genes covering various molecular functions. A recent study has reported that TRF has immunostimulatory effects and potential clinical benefits to enhance immune response to vaccines (39). Another finding showed that daily supplementation of palm TRF can induce a strong cell-mediated immune response, *i.e.*, T-helper-1 (Th1) response, which would be beneficial to fight viral infections and cancer (40). Hence, there is growing evidence to show that tocotrienols modulate a comprehensive range of transcriptional response of genes pertinent to different types

Table IV. *The most significant (p<0.01) known genes differentially down-regulated (≥0.5 fold) in tocotrienol-rich fraction (TRF), α-tocopherol (αT) and tocotrienol isomers (αT3, δT3 and γT3) treated compared with untreated MCF-7 cells.*

GO Term and gene name	Treatment	Fold change
Nucleic acid and nucleotide binding		
Splicing factor, arginine/serine-rich (*SRP46*)	αT, TRF	0.47, 0.39
Oligonucleotide/oligosaccharide-binding fold containing 2A II (*OBFC2A*)	αT, TRF	0.37, 0.46
Nucleolin (*C23*)	TRF, γT3	0.35, 0.36
Phospolipid biosynthesis		
Selenoprotein I (*SELI*)	αT, TRF	0.43, 0.46
Regulation of transcription		
SMAD family member 4 (*SMAD4*)	αT	0.43
Bobby sox homolog (Drosophila) (*BBX*)	αT, TRF, γT3	0.42, 0.36, 0.37
Zinc finger protein 161 homolog (*AF5*)	αT, αT3	0.45, 0.35
BTAF1 RNA polymerase II (*MOT1*)	αT, TRF	0.47, 0.43
NF-kappa B repressing factor (*NKRF*)	TRF	0.47
Bromodomain adjacent to zinc finger domain, 1A (*BAZ1A*)	TRF	0.32
Cullin-associated and neddylation-dissociated 1 (*TIP120*)	αT3	0.29
BCL6 co-repressor (*BCOR*)	αT3, γT3	0.39, 0.43
Nuclear receptor interacting protein 1 (*NRIP1*)	αT3	0.29
Zinc finger protein 91 (*HPF7, HTF10*)	γT3	0.36
CCR4-NOT transcription complex (*CAF1*)	γT3	0.39
Domain adjacent to zinc finger domain (*ACF1*)	γT3	0.42
BolA-like 2 (*BOLA2*)	δT3	0.45
Zinc finger DHHC-type containing 23 (*ZDHHC23*)	γT3	0.38
Thymocyte nuclear protein 1 (*THYN1*)	δT3	0.49
Cell cycle: mitotic spindle check point		
TTK protein kinase (*TTK*)	αT	0.35
BUB3 budding uninhibited by benzimidazoles (*BUB3L*)	αT, αT3	0.23, 0.22
Polo-like kinase 4 (Drosophila) (*PLK4*)	γT3	0.37
Cell division, differentiation and growth		
Septin 11 (*SEPT11*)	αT	0.47
Four and a half LIM domains 1 (*FHL1*)	γT3	0.34
Zinc ion binding		
Zinc finger protein 161 homolog (*ZF5*)	αT	0.45
Zinc binding alcohol dehydrogenase 2 (*ZADH2*)	αT, γT3	0.43, 0.38
Signal transduction		
Phosphodiesterase 8A (*PDE8A*)	αT	0.32
Phosphodiesterase 5A (*PDE5A*)	αT3	0.31
Linked *N*-acetylglucosamine (*GlcNAc*)	αT3	0.39
Protein phosphatase 1, regulatory (inhibitor) 12A (*PP1R12A*)	αT3	0.29
Protein phosphatase 4, regulatory subunit 1 (*PP4R1*)	αT3	0.39
Phosphodiesterase 8A (*PDE8A*)	αT3	0.27
Electron transport		
Pericentriolar material 1 (*PCM1*)	TRF	0.39
Nicotinamide nucleotide transhydrogenase (*NNT*)	αT3	0.30
Cytochrome c oxidase subunit 7c (*COX7C*)	δT3	0.37
Intracellular protein transport		
RAP2A, member of RAS oncogene family (*KREV*)	TRF, αT3, γT3	0.37, 0.37, 0.44
Muscle development		
Calmodulin-like 4 (*CALML4*)	TRF, αT3	0.38
Muscleblind-like (Drosophila) 1 (*MBNL1*)	αT3	0.35
Circulation		
Fer-1-like 3 (*FER1L3*)	TRF	0.42
Protein ubiqutination		
Histone acetyltransferase (monocytic leukemia) 3 (*MYST3*)	TRF	0.50
H3 histone, family 3A (*H3F3A*)	δT3	0.42
RNA processing		
Dicer1, Dcr-1 homolog (Drosophila) (*DICER1*)	TRF, αT3	0.32, 0.31
Nucleotide and nucleic acid metabolism		
Hypothetical protein (*FLJ10379*)	TRF, αT3	0.42, 0.34
Deoxyguanosine kinase (*DGUOK*)	δT3	0.48
Hypothetical protein (LOC84792)	γT3	0.47

Table IV. *Continued*

Table IV. *Continued*

GO Term and gene name	Treatment	Fold change
tRNA processing		
Pseudouridylate synthase 7 (*PUS7*)	TRF	0.47
Insulin receptor binding		
Pleckstrin homology domain interacting protein (*PHIP*)	TRF	0.33
Chromatin assembly or disassembly		
Chromobox homolog 1 (HP1 beta homolog Drosophila) (*CBX1*)	TRF	0.43
DNA replication		
Polymerase (DNA directed), theta (*POLQ*)	αT3	0.31
Nucleocytoplasmic transport		
Nucleoporin 205 kDa (*NUP205*)	αT3	0.43
Ubiquitin-dependent protein catabolism		
Ubiquitin specific peptidase 15 (*USP15*)	αT3	0.30
Protein binding, folding and modification		
BTB (POZ) domain containing 7 (*BTBD7*)	αT3	0.35
BTB (POZ) domain containing 1 (*BTBD1*)	αT3	0.33
Protein-L-isoaspartate (D-aspartate)		
O-Methyltransferase domain containing 2 (*PCMTD2*)	αT3	0.23
SCC-112 protein (*SCC-112*)	γT3	0.38
Ubiquitously-expressed transcript (*UXT*)	δT3	0.45
Denticleless homolog (Drosophila) (*DTL*)	γT3	0.33
Folliculin interacting protein 1 (*FNIP1*)	γT3	0.45
Protein amino acid phosphorylation		
Maternal embryonic leucine zipper kinase (*MELK*)	αT3	0.24
DNA binding		
Ligand-dependent corepressor (*MLR2*)	αT3, γT3	0.32, 0.40
Regulation of translation		
Death-associated protein 4 (*DAP4*)	αT3, γT3	0.36, 0.45
NMD3 homolog (*S. cerevisiae*) (*NMD3*)	γT3	0.32
Receptor activity		
Lin-9 homolog (*C. elegans*) (*LIN9*)	αT3	0.29
Protein-nucleus import		
Exportin 1 (CRM1 homolog, yeast) (*XPO1*)	αT3	0.34
ATP Binding		
Isoleucine-tRNA synthetase 2 (*IARS2*)	αT3	0.40
Protein kinase activity		
Bone morphogenetic protein receptor 2 (*BMR2*)	αT3	0.24
Membrane		
Golgi phosphoprotein 3 (*GOPP1*)	αT3	0.43
Calcium ion binding		
TTK protein kinase (*TTK*)	αT3	0.26
S100 calcium binding protein A16 (*S100A16*)	αT	0.50
TBC1 domain family (*MDR1*)	αT3	0.28
Oxidoreductase activity		
Co-proporphyrinogen oxidase (*CPOX*)	αT3	0.36
Cell adhesion		
Thrombospondin 1 (*THBS1*)	αT3	0.25
Fibronectin type III domain containing 3A (*FNDC3A*)	γT3	0.41
Galactose metabolism		
Galactokinase 2 (*GALK2*)	αT3	0.29
Regulation of GTPase activity		
Development and differentiation enhancing factor 2 (*DDEF2*)	αT3	0.37
Intracellular signaling cascade		
Syndecan binding protein (*SDCBP*)	γT3	0.34
DNA repair		
RAD51 associated protein 1 (*RAD51AP1*)	γT3	0.29
Metabolism		
ATPase, Ca2+ transporting, type 2C member 1 (*ATP2C1*)	γT3	0.45
Dehydrogenase E1 and transketolase domain 1 (DHTKD1)	γT3	0.39

Table IV. *Continued*

Table IV. *Continued*

GO Term and gene name	Treatment	Fold change
Protein amino acid phosphorylation		
Poly A-specific ribonuclease (*PAN3*)	αT3, γT3	0.40, 0.45
Cytokinesis		
Anillin (*ANLN*)	γT3	0.29
Anti apoptosis		
Apoptosis inhibitor 5 (API5)	γT3	0.44**
Caspase 2, apoptosis-related cysteine peptidase (ICH1)	γT3	0.36
Lipid metabolism		
Solute carrier family 27member A2 (SLC27A2)	δT3	0.45
Actin nucleation		
Enah/Vasp-like (EVL)	δT3	0.36
Proteolysis and peptidolysis		
Kallikrein 11 (KLK11)	δT3	0.28

**Gene expression is significantly ($p \leq 0.001$) down-regulated. Observation has been validated by qRT-PCR.

of cancer. The tocotrienol down-regulation of *API5* and up-regulation of *MIG6*, both verified by qRT-PCR in the present study, offer a potentially promising role for tocotrienols targeting the signal pathways of breast cancer risk-associated genes for a future chemopreventative programme.

The identification of effective concentrations of vitamin E compounds that affect gene or protein expression is an important goal in developing cancer-specific gene target therapies that should, in theory, have little or no toxicity to normal cells. The IC_{50} concentration was chosen in this study as the toxicity level of tocotrienols has not been fully established to date. Using the microarray approach a number of candidate genes pertinent to breast cancer biology were identified. The delineation of the roles of these genes in breast tumourigenesis will have implications for breast cancer management, as such genes could serve as potentially useful therapeutic targets.

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