

# Tocotrienols and Gene Expression—Techniques Available

Kalanithi Nesaretnam\*

## INTRODUCTION

A mammalian diploid cell (a cell having two complete sets of chromosomes) expresses  $\sim 2 \times 10^4$  mRNAs (messenger ribonucleic acids) from the genome of  $1.40 \times 10^5$  genes. This well established observation implies the existence of complex mechanisms that selectively transcribe the cell-specific genes within the nucleus and coordinate the transcriptional processes with dynamic events in the extranuclear and extracellular milieu. The analysis of tocotrienol-sensitive changes in the expression of genes, therefore, is opening a window on the responsiveness of complex transcriptional machinery to a natural product. Tocotrienols are vitamin E isomers, and vitamin E tocopherols and tocotrienols are well recognized for their effective inhibition of lipid oxidation in foods and biological systems.

As vitamin E is synthesized only by plants, it is a very important dietary nutrient for humans and animals. Although tocopherols are generally present in nuts and in common vegetable oils, tocotrienols are found in high concentrations only in cereal grains and certain vegetable oils such as palm and rice bran oils. Tocotrienols have been the focus of active research in the last 10-15 years not just as secondary forms of vitamin E but also as unique nutritional compounds with unique antioxidant properties. The action of phytochemical compounds consumed as foods has long been investigated by clinical and academic researchers. For example, one of the tenets of the Saas Fee Declaration on the significance of antioxidants in preventive medicine (Packer and Fuchs, 1996) stated that "there is a need for further work at the fundamental scientific level..., which can be expected to lead to

more precise information (about antioxidant actions) being made available." Seven years later, the National Institute of Health's Centres for Dietary Supplements Research recognized that 16 million Americans consume supplements and botanical extracts and emphasized four areas of research. One of these was the identification and elucidation of their mechanism of action.

The consumption of vitamin E for prevention and treatment of human diseases is well documented. Recent work has shown that tocotrienols can exert direct inhibitory effects on cell growth in human breast cancer cell lines *in vitro* (Nesaretnam *et al.*, 1995; 2000) and that the inhibitory effects occurred irrespective of oestrogen receptor status of the cells (Nesaretnam *et al.*, 1998). The inhibitory effect on cell growth was more pronounced with  $\gamma$ - and  $\delta$ -tocotrienols. The mechanism of action is unknown, with previous data suggesting that the action is not due to antagonism of oestrogen action. Alpha-tocopherol

itself has no inhibitory action on breast cancer cell growth (Nesaretnam *et al.*, 1995; 1998; 2000).

Changes in the rates of cell proliferation and death play an important role in initiation and progression of chronic disease and utilize constitutive and inducible metabolic and signaling pathways. In the past decade, the techniques for simultaneous monitoring of changes in the expression of a large number of genes, possibly the entire expressed genome of a cell at mRNA and protein levels, have undergone a revolution. It is now possible to monitor changes in the entire expressed genome of a eukaryotic cell using gene chip technology (DeRisi *et al.*, 1997). We recently described the effect of tocotrienols on the gene expression profile of MCF-7 human breast cancer cells and on tumours derived from these cells when injected into athymic nude mice (Nesaretnam *et al.*, 2003). In the following pages, the results of applications of different techniques to define changes in the expression of tocotrienol sensitive genes in human breast cancer cells and tumours are described.

## DIFFERENTIAL DISPLAY OF mRNAs

Liang *et al.* (1994) described a polymerase chain reaction (PCR)-based method that enabled simultaneous evaluation of upregulated and downregulated expression of mammalian genes in a pair of differently treated cells. The method took advantage of random oligonucleotide primers,

\* Malaysian Palm Oil Board,  
P.O. Box 10620, 50720 Kuala Lumpur,  
Malaysia.

designed to hybridize to all of the cDNAs (complementary deoxyribonucleic acids) at sites distal to the oligo-dT tails prepared from mRNAs with four different oligo-dNT primers. The mRNA isolated from MCF-7 human breast cancer cells supplemented with tocotrienols showed changes in approximately 30 different transcripts after screening. *Figure 1* shows the differential display autoradiogram. The identities of these transcripts remain to be defined and are likely to be the mRNAs identified by cDNA arrays on nylon filters and oligonucleotide microarrays.

### cDNA Arrays of Genes or Expressed Sequence Tags (EST)

When EST are dotted on nylon membranes, they offer the advantage of rapid identification of the altered expressions of mRNAs in the presence of the compound

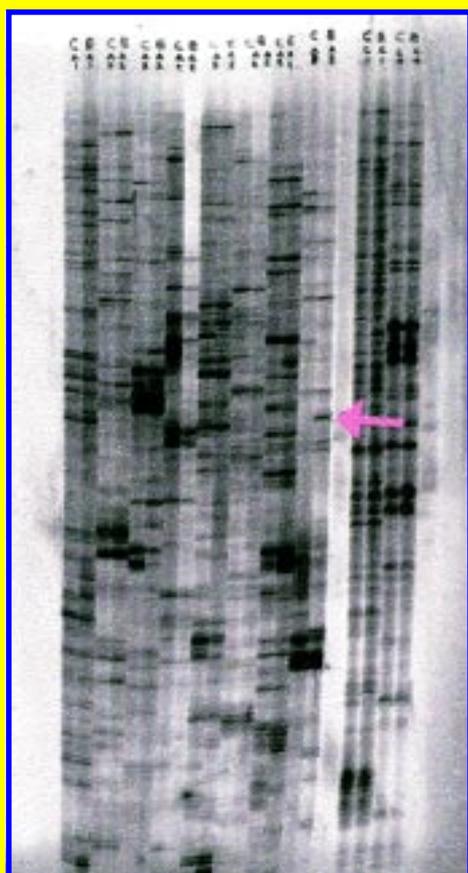
to be tested. In addition, a good selection of function-specific cDNA arrays are commercially available. One such cDNA array of cancer genes was used to screen the changes in the gene expression of MCF-7 human breast cancer cells in the presence of tocotrienols and MCF-7 tumours that arose from injecting the cells into athymic nude mice (*Figure 2*). The nude mice were supplemented with tocotrienols for 20 weeks. The array screened for 15 different functional classes of genes, including cell cycle regulators, growth regulators, apoptosis, oncogenes, immune system proteins, stress response proteins, and genes for DNA response and repair. Radiolabelled cDNA prepared from mRNA isolated from MCF-7 cells hybridized to 1200 genes on the array. An example of differential gene expression in MCF-7 human breast cancer cells in response to tocotrienols is

shown in *Figure 3*. The figure shows that the CD74 gene is upregulated in tumours of tocotrienol supplemented nude mice. *Table 1* shows the expression fold changes (upregulated genes show a fold change of  $>2$ , whilst downregulated genes show fold changes of  $<2$ ) in four genes. The results and function of two of these genes that were differentially regulated are explained.

The interferon-induced protein 1 (IFITM-1) is a membrane protein that has been isolated by differential screening on the basis of its alpha and gamma interferon inducibility in tumour cell lines. Interferons are antiproliferative proteins that affect the function of well-known cell cycle proteins such as c-myc. In the array analysis, a significant downregulation of c-myc gene expression in tumours from animals treated with tocotrienols, that correlates with the observed decreased tumorigenicity in tocotrienol-treated animals was observed. The c-myc oncogene has in fact been implicated in malignant progression in a variety of human tumours including breast cancer tumours and elevated expression of the gene has been associated with activation of the advanced neoplastic stage.

Cyclins are important in regulating the cell cycle through their formation of enzymatic complexes with various cyclin-dependent kinases. Cyclin b1 was found to be overexpressed in various human breast cancer cell lines and tumour tissues. The finding that the expression of cyclin b1 is downregulated by tocotrienols in MCF-7 cells is consistent with our previous results on the ability of these compounds to inhibit growth of tumour cells both *in vitro* and *in vivo*.

These data show that tocotrienols selectively alter the expression of broad functional groups of genes and support the data obtained by differential display of mRNA.



*Figure 1.* Differential display of mRNAs of MCF-7 cells treated with tocotrienols for 72 hr. Total RNA was isolated and subjected to polymerase chain reaction (PCR) amplification as described by Liang et al. (1994). The arrow shows the upregulation of mRNA fragment following incubation with tocotrienols.

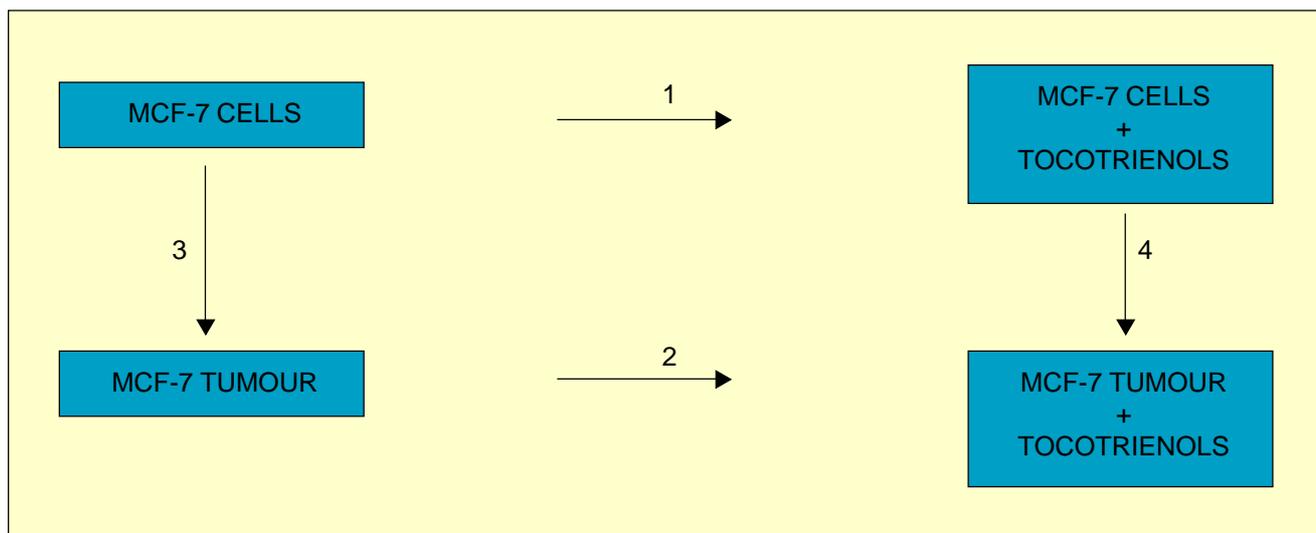


Figure 2. Array analyses (1, 2, 3, 4).

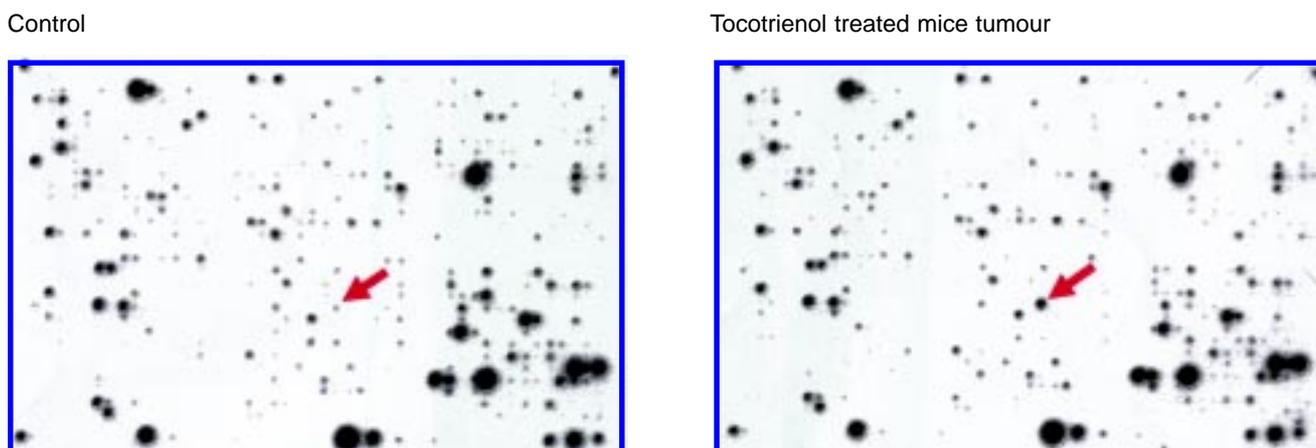


Figure 3. Array analysis of tocotrienol gene expression in tumours induced by MCF-7 injection in nude mice. Arrows show the differential expression of the CD74 gene by tocotrienols.

TABLE 1. EXPRESSION FOLD CHANGES

Genes	1	2	3	4
HLA-DR antigen-associated invariant subunit (CD74)	-	11.480	-	6.129
Interferon-inducible protein 1 (IFITM-1)	0.407	2.220	0.023	-
CyclinB1	0.398	-	-	4.113

Notes: 1 - Array analyses of MCF-7 cells control vs. tocotrienol treatment.  
 2 - Array analyses of MCF-7 tumour in control vs. tocotrienol treated mice tumour.  
 3 - Array analyses of MCF-7 cells control vs. MCF-7 tumour (control).  
 4 - Array analyses of MCF-7 cells treated with tocotrienols vs. tocotrienol treated mice tumour.

### DNA Microarray Chips

Microarray chips offer a more comprehensive assessment of the transcriptional response of a cell. The technique enables simultaneous screening of changes in the entire expressed genome (up to 20 000 mRNAs) of a cell in response to a variable such as a DNA damaging agent, or to a serum depletion or a botanical extract. Currently, there are two distinct techniques available for monitoring changes in gene expression. Each technique offers a large amount of data on qualitative and quantitative

changes in the gene expression profile of the cell under investigation.

One gene profiling technique, pioneered by Patrick Brown's and David Botstein's teams at Stanford University, utilizes PCR amplified fragments of cloned DNA (cDNA) dotted, robotically, onto a glass microscope slide. In a typical DNA microarray slide, up to 6000 different cDNAs can be dotted per slide; each dot is approximately 200 µm in diameter, containing approximately 10 ng of DNA. If DNAs are randomly selected from the entire human genome data bank of known human genes, then a typical experiment can screen approximately 10% of the expressed genome in the selected cell type. However, the response of an almost completely (90%) expressed genome to a stimulus can be screened if the cDNAs dotted onto the glass slides are obtained from the cDNA library prepared from the specific cell under investigation. The latter approach, therefore, requires the experimenter to follow an additional step of construction of the cell specific cDNA library representing every mRNA expressed in that cell. A DNA genearray generated by the dotting technique is used as a microarray probe to define changes in two populations of target cDNAs, each of which is specifically tagged with a distinct fluorescently labelled nucleotide. The fluorescent-

labelled target cDNAs from control and test cells, immobilized by specific hybridization to their complementary probes on the GeneChip, are visualized by a confocal laser scanner. Mathematical and statistical programs to obtain quantitative information on the number and amount of different mRNAs present in the sample analyse the fluorescent images obtained from the scanner. Such an analysis of data generates a cell-specific gene expression profile that can be compared with similar data from the same cell type under different physiological and pathological conditions.

### CONCLUSION

Several techniques for the analysis of gene expression at the level of mRNAs have uncovered complex effects of tocotrienols that cannot be entirely accounted for by their antioxidant properties. Analytical strategies aimed at defining whole-cell responses of gene expression at the level of transcription and translation (proteomics) will reveal novel molecular targets of tocotrienols. Data from such analysis are essential for more rational and targeted use of tocotrienols to improve health.

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