Cancer genes induced by malathion and parathion in the presence of estrogen in breast cells

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Abstract. The identification of genes involved in the process of neoplastic transformation is essential for analyzing the progression of breast cancer when induced by endogenous and exogenous agents, among which are the estrogens and the organophosphorous pesticides, respectively. It is important to consider the impact of such substances when they are present in combination. *In vitro* experimental models are needed in order to understand breast carcinogenesis. The aim of this work was to examine the effect of 17ß estradiol (estrogen) combined with either malathion or parathion on the transformation of human breast epithelial cells *in vitro*. Results showed that estrogen combined with either malathion or parathion altered cell proliferation and induced cell transformation as well as exhibited significant invasive capabilities as compared to the control MCF-10F cell line. Several genes were up-regulated by the effect of all of the treatments, such as the cyclins, *cyclin D1* and *cyclin-dependent kinase 4*, *IGFBP3* and *IGFBP5*, and *keratin 18*. The *c-Ha-ras* oncogene was up-regulated by the effect of malathion alone and with the combination of estrogen and either malathion or parathion. The *DVL1* gene was up-regulated only with malathion alone and the combination of parathion with estrogen. Expression of the *HSP 27*, *MCM2* and *TP53* inducible protein 3 genes was up-regulated with malathion alone and with the combination of estrogen and either malathion or parathion while *TP53* (Li-Fraumeni syndrome) was up-regulated by estrogen alone and malathion alone. Thus, we suggest that pesticides and estrogens affect human breast cells inducing molecular changes indicative of transformation.

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Introduction

Cancer of the breast is the most common form of malignant disease occurring among women of the western world, and endogenous factors and environmental chemicals seem to be involved in the etiology of this disease. Insecticides have been classified as carcinogens by the International Agency for Research on Cancer in humans (1-3), but human data are limited due to the restricted number of studies examining individual pesticides. Epidemiological and experimental evidence has implicated estrogens in the etiology of breast cancer (4). The association between breast cancer and prolonged exposure to estrogens also suggests that this hormone plays a role in breast cancer (5). Among the hormonal influences a leading role is attributed to estrogen since prolonged stimulation by steroid hormones may increase cell division, thus increasing the risk of breast cancer (5).

Many studies have reported an association between human cancer and exposure to the organophosphorous pesticides such as malathion and parathion, which have been used to improve crop production and are currently used to control mosquito plagues (6,7).

Cancer occurs at the molecular level due to alterations in genes, and among them *c-Ha-ras* seems to be important in the signal transduction pathways in human malignancies. Ras belongs to the family of guanine nucleotide proteins, and the proteins (H-, K- and N-Ras p21) are members of a much larger super-family of related proteins (8,9).

Cell cycle progression is controlled, in part, by a family of cyclin proteins and cyclin-dependent kinases (Cdks). Several Cdk proteins have been identified in complex with D-type cyclins, and among these Cdk2, Cdk8 and Cdk4 are suggested to regulate cell growth during the G1 phase of the cell cycle (10-13). Mammalian homologues of the *Drosophila dishevelled* (*Dsh*) gene have been identified, including *DVL1*, *DVL2* and *DVL3*. The mammalian dishevelled proteins contain three homologous domains, two of which are unrelated to any other known protein. The third region is homologous to the discs-large homology domain of *Drosophila* discs-large-1, a tumor suppressor protein (14).

The heat shock proteins (HSPs) comprise a group of highly conserved, abundantly expressed proteins with diverse functions, including the assembly and sequestering of multi-protein complexes, transportation of nascent polypeptide

chains across cellular membranes and regulation of protein folding 90 and 27 (15-19). The insulin-like growth factor-binding proteins or IGFBPs are a family of homologous proteins that have co-evolved with the IGFs (20-26). They serve not only as shuttle molecules for the soluble IGFs, but also regulate the IGF signaling system.

Cytokeratins comprise a diverse group of intermediate filament proteins (IFPs) that are expressed as pairs in both keratinized and non-keratinized epithelial tissue. Cytokeratins play a critical role in differentiation and tissue specialization and function to maintain the overall structural integrity of epithelial cells (27). Cytokeratins have been found to be useful markers of tissue differentiation which is directly applicable to the characterization of malignant tumors. The mini-chromosome maintenance (MCM) family of proteins, including MCM2, are regulators of DNA replication which act to ensure replication occurs only once in the cell cycle (28,29). The p53 is a tumor suppressor gene located on chromosome 17p13. Mutations in the p53 gene have been found in ~20-50% of human cancers (30). This gene has 11 exons, and a p53 mutation occurs in exons 4-8 in human cancers (~95%). The 53-kDa nuclear phosphoprotein binds to large T antigen (31).

The causative factors for breast carcinogenesis remain an enigma. The human population is exposed not only to pesticides but also to a mixture of estrogenic or estrogen-like agents. Thus, the objective of this study was to determine the effects of 17ß estradiol (E2), malathion, parathion, and the combination of either malathion or parathion with E2 on cell transformation of MCF-10F, an immortalized human breast epithelial cell line *in vitro*.

Materials and methods

Cells. The MCF-10F was used in their 44th passage. Cells were cultured with DMEM/F-12 (1:1) medium supplemented with antibiotics [100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B, (all from Life Technologies, Grand Island, NY)] and 10 µg/ml and 5% equine serum (Biofluids, Rockville, MD), 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO, USA) and 0.02 µg/ml epidermal growth factor (Collaborative Research, Bedford, MA) were added. The cells used in these experiments were: i) control MCF-10F; ii) MCF-l0F continually treated with estrogen (E2) at 10^{-8} M (Sigma-Aldrich, St. Louis, MO, USA); iii) MCF-10F cells treated continuously either with malathion (32,33) or parathion (100 ng/ml) (parathion-ethyl); and iv) the combination of E2 with malathion or parathion.

cDNA expression array. We used the commercially available Oligo GEArray® Human Cancer Microarray (SuperArray, Bioscience Corp., Bethesda, MD). It was designed to profile gene expression of several genes.

Isolation and purification of total RNA and mRNA. Total RNA was isolated from both the control MCF-10F and treated cells with Trizol reagent (Invitrogen Corp., Long Island, NY). Each sample comprising 500 µg of total RNA was treated with 5 µl of DNase I (10 U/µl) (Boehringer Mannheim, Indianapolis, IN) for 60 min at 37˚C. Then 10X Termination Mix (0.1 M EDTA, pH 8.0 and 1 mg/ml glycogen) (Clontech, CA) was used to stop the reaction. The purified RNA sample (34) was first measured by a spectrophotometer (the ratio of absorbance reading at 260 nm/280 nm >1.8) and then electrophoresed on denaturing formaldehyde/agarose/ethidium bromide gel. Each sample of 500 µg of purified total RNA was then subjected to polyA$^+$ RNA analysis with the Oligotex mRNA Purification Kit (Qiagen Inc., Valencia, CA). PolyA$^+$ RNA was then purified following an established procedure (34). The purified mRNAs were used for the synthesis of cDNA probes with Biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN). The annealing mixture was prepared by mixing ~1.0-5.0 µg of mRNA with 3 µl of Buffer A (GE primer mix) (SuperArray), and the final volume was adjusted to 10 µl. The mixture was then incubated in a preheated thermal cycler at 70˚C for 3 min, cooled to 42˚C and kept at that temperature for 2 min. Then 10 µl of the RT cocktail was prepared by mixing 4 µl of 5X Buffer BN [for 50 µl 10X Buffer, we added 1 µl of 1 M DTT and 50 µl of 10X dNTP mix (5 mM dATP, dCTP, dGTP and 500 µM dTTP)], 2 µl of Biotin-16-UTP, 2 µl of RNase-free H_2O, 1 µl of RNase Inhibitor (Promega Corp., Madison, WI) and 1 µl of MMLV Reverse Transcriptase (Promega Corp.). The RT cocktail was then warmed at 42˚C for 1 min and slowly mixed with 10 µl of pre-warmed annealing mixture. Incubation continued at 42˚C for 90 min, and then the labeled cDNA probe was denatured by heating at 94˚C for 5 min, and quickly chilled on ice. cDNA probes were prepared from each of these and hybridized to the respective membranes. Experiments using the same mRNA preparation were repeated two or three times, and measurable median-normalized expression values of each gene were compared to avoid false-positive signals (35).

Differential hybridization of cDNA expression array. Each array membrane was pre-wetted with 5 ml of deionized water and incubated at 60˚C for 5 min. It was then replaced with 2 ml of pre-warmed (60˚C) GEAprehyb solution with a heat-denatured sheared salmon sperm DNA at a final concentration of 100 µg/ml (SuperArray) and mixed gently for a few seconds. Pre-hybridization was continued at 60˚C for 1-2 h with continuous gentle agitation. An approximate 0.75-ml solution of GEAhyb was prepared by adding the entire volume of denatured cDNA probe into GEAprehyb solution and kept at 60˚C. Then GEAprehyb solution was replaced by GEAhyb solution and incubation was continued overnight with hybridization at 60˚C with continuous gentle agitation. Subsequently, array membranes were washed twice in wash solution 1 (2X sodium chloride sodium citrate and 1% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation and then twice with solution 2 (0.1X sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation. To assess the reproducibility of the hybridization array assays, pair-wise comparisons between array data sets for each cell line were tested by repeated hybridization, and the mRNAs prepared in different lots were analyzed in scatter plots with multiple regressions as previously described (35,36). In each case, expression levels of 95% of the genes had repeated values that were within 2-fold of each other (35).

Chemiluminescent detection of cDNA probes. After discarding the last wash, 2 ml of GEAblocking solution was added to each membrane and incubated for 40 min at room temperature with continuous agitation. Then binding buffer was prepared by diluting alkaline phosphatase-conjugated streptavidin (AP) with 1X buffer F (SuperArray) in a 1:7500 dilution. GEAblocking solution was replaced by 2 ml of binding buffer and incubated for 10 min with continuous but gentle agitation. Then the membrane was washed 4 times with 4 ml of 1X binding buffer F for 5 min in each washing and rinsed twice with 3 ml of rinsing buffer G (SuperArray). Then the membrane was covered with 1.0 ml of CDP-Star chemiluminescent substrate to incubate at room temperature for 2-5 min. It was then exposed to X-ray film (Kodak BioMax MS Film; Kodak Corp., Rochester, NY) with a corresponding intensifying screen at room temperature for multiple exposures of 1-5 min.

Quantification of array hybridization. Quantification of hybridization signals of the array membranes was carried out by exposing the autoradiographic film in a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA) and was then estimated both with the ImageQuant (Molecular Dynamics) and ScanAnalyze programs (Eisen Lab). Volume quantification was performed by calculating the volume under the surface created by a three-dimensional plot of pixel locations and pixel values as described (34,35). All raw signal intensities were corrected for background by subtracting the signal intensity of a negative control or blank. They were also normalized to that of a housekeeping gene. These corrected, normalized signals were then used to estimate the relative abundance of particular transcripts. To delineate the potential signal interference between adjacent strong hybridization signals, equal-sized ellipses were drawn around each signal area (hybridization spots) by using the software ImageQuant/ScanAnalyze and were then separately scanned and compared with the housekeeping genes so the chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of the different housekeeping genes from multiple autoradiographic exposures between different hybridization experiments was conducted by taking the average signals of each of the housekeeping genes. Data from the concentration spots that were ≥2.5-fold versus the control were used. The median background was subtracted, and signals that were minor to 2.5-fold below were considered too low to accurately measure and were omitted from the analysis. Signals for each individual gene were also normalized to the geometric mean of the expression level of that gene across the set of membranes being compared. Mean signals were calculated from quadruplicate measurable spots, or if three of the four spots were measurable. Then the changes in fold indicated whether a gene exhibited increased, decreased, or unchanged expression. These were based on statistical criteria (36).

Results

This study established the effects *in vitro* of E2 and the organophosphorous pesticide either malathion or parathion, alone or in combination with E2 on cell transformation of MCF-10F. Previous studies have shown that E2 in combination with parathion induced malignant transformation of MCF-10F as indicated by increased cell proliferation and invasive capabilities (32,33) in comparison to the control MCF-10F cell line. The present results showed that E2 in combination with either pesticide induced malignant transformation of MCF-10F. The malignant feature was confirmed by anchorage independency and invasive capabilities. Table I shows the anchorage independence capability of MCF-10F-treated cells as well as the invasive characteristics scored 20 h after plating onto matrigel basement membranes using Boyden's chambers. RNA samples of the control and MCF-10F-treated cells were analyzed in a human cancer array to analyze the expression profile of 408 genes.

Table I. Origin and phenotypic characteristics of cell lines.

Cell lines	Origin	AI	I
MCF-10F	MCF-10F parental cells	-	-
Estrogen (E2)[a]	MCF-10F treated with estrogen	-	-
Malathion[b]	MCF-10F treated with malathion	+	+
Parathion[c]	MCF-10F treated with parathion	+	+
Malathion + E2[d]	MCF-10F treated with combination	+	+
Parathion + E2[e]	MCF-10F treated with combination	+	+

MCF-10F cells treated with [a]17ß estradiol (10^{-8} M) during 20 passages; [b]malathion (0.5 μg/ml) during 20 passages; [c]parathion (100 ng/ml) during 20 passages; [d]the combination of E2 and marathion for 20 passages; and [e]the combination of E2 and parathion for 20 passages. AI, anchorage independence colony-forming efficiency in soft agar. I, invasion assay - invasive characteristics of the control and MCF-10F-treated cells scored 20 h after plating onto matrigel basement membranes using Boyden's chambers. +, The results in relation to anchorage independent growth and the number of cells that crossed the filters; -, lack of anchorage independent growth and invasiveness.

The genes represented in the human cancer microarray used in this study included functional gene groupings related to apoptosis, cell cycle, cell growth and differentiation, signal transduction pathway and other cancer-related genes. Of the 408 genes examined, 17 were involved in the regulation of human cancer. Fig. 1 and Table II show the genes modified by E2 and pesticide exposure in the breast cells. The *c-Ha-ras* oncogene was up-regulated in the presence of malathion alone and the combination of E2 with either malathion or parathion.

The cyclins, *cyclin D1* and *cyclin-dependent kinase 4* genes, were up-regulated by all of the treatments. *DVL1* was up-regulated by the effect of malathion alone and by the combination of E2 with parathion. *DVL1* gene expression was up-regulated only with malathion alone and the combination of E2 with parathion.

HSP 27 expression was up-regulated by the effect of malathion alone and by the combination of E2 with either malathion or parathion. The *HSP 90* genes were up-regulated by the effect of the different treatments. *IGFBP3* and *IGFBP5* expression was up-regulated by the effect of the different treatments. *Keratin 18* expression was up-regulated by the effect of the different treatments. MCM2 was up-

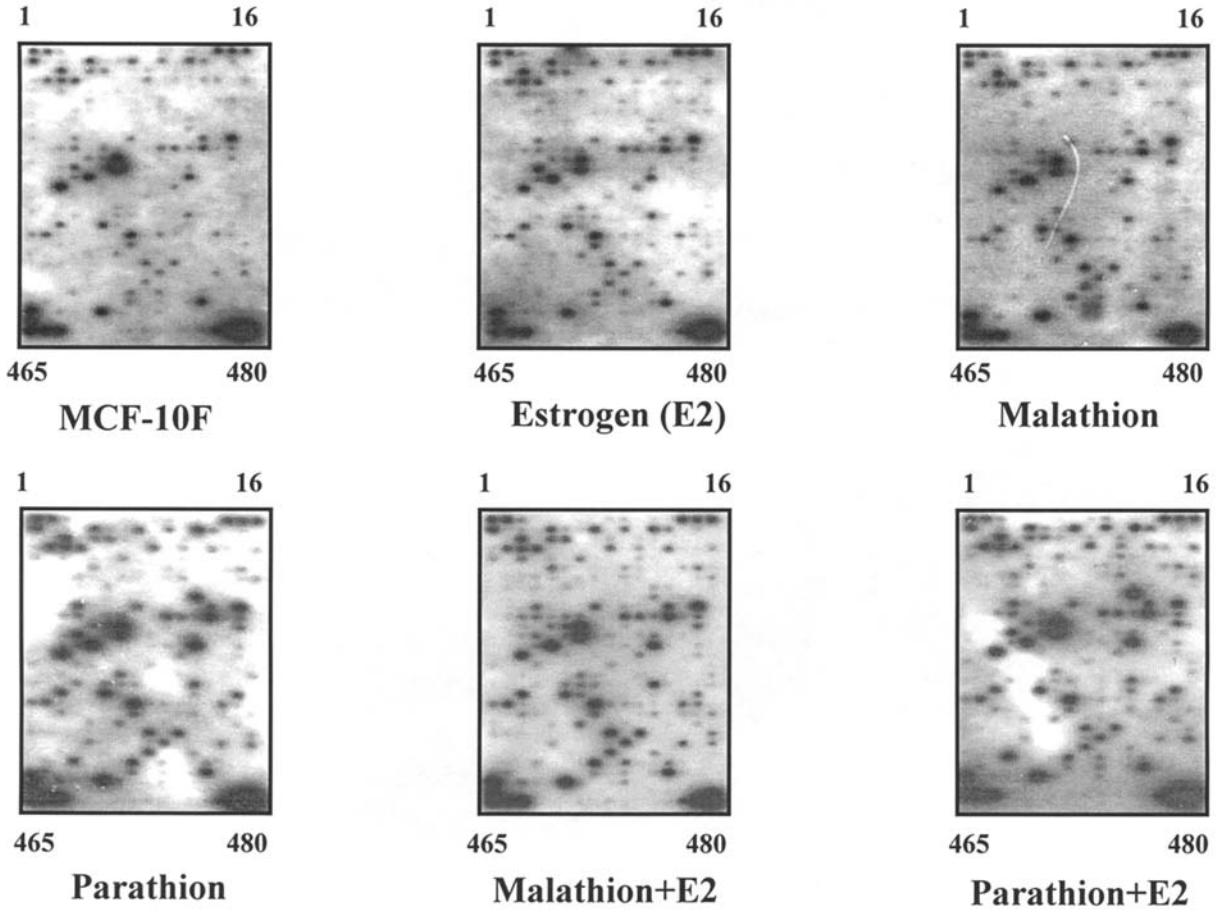

Figure 1. Differential gene expression of estrogen- and pesticide-treated breast epithelial cells. Expression of human oligo cancer microarray in the parental MCF-10F, estrogen-, malathion-, parathion-, malathion plus estrogen- and parathion plus estrogen-treated cells. The expression profile was generated using the Oligo GEArray® human cancer microarray (cat. no. OHS-802). The image was recorded by a CCD camera-based imager.

regulated by the effect of the combination of E2 with either malathion or parathion. *TP53* inducible protein 3 was up-regulated by the effect of the combination of E2 with either malathion or parathion. However, *TP53* (Li-Fraumeni syndrome) was up-regulated only by either E2 or malathion alone.

Discussion

Differentially expressed cancer array genes were evaluated in transformed human breast epithelial cells by the effects of estrogen and organophosphorous pesticides malathion and parathion *in vitro*. It is known that estrogens are associated with carcinogenic events in both humans and animals (1-3), and the effect of estrogens in breast cancer remains unclear. Experimental studies have demonstrated that mammary cancer is a hormone-dependent multi-step process that can be induced by a variety of compounds and mechanisms. Therefore, it is necessary to consider the impact of the combined effects of both pesticides and estrogens. *In vivo* and *in vitro* experimental models are needed in order to understand the effects of these compounds in breast carcinogenesis. Previous studies *in vitro* showed that estrogen (33) combined with parathion (32,33) altered cell proliferation and induced transformation of the MCF-10F cell line. The increase in the cell proliferation rate indicated by a shorter doubling time, anchorage independent growth and *in vitro* invasive capability suggested a very aggressive phenotype. Treatment of parathion alone or its combination with estrogen exhibited significant invasive capabilities as compared to control cells.

Identifying the genes involved in the process of neoplastic transformation is essential for analyzing the progression of breast cancer when induced by several agents. Among such agents endogenous substances such as estrogen and the organophosphorous pesticides were deemed important for study. As a result, the *c-Ha-ras* oncogene was upregulated by the effect of malathion alone and by the combination of E2 with either malathion or parathion. The *c-Ha-ras* oncogene (8,9) seems to be important in signal transduction pathways and mutations in many human malignancies.

The cyclins, *cyclin D1* and *cyclin-dependent kinase 4* genes, were up-regulated by the effects of the different treatments. Cell cycle progression is controlled in part by a family of cyclin proteins, and Cdks have been identified in complex with D-type cyclins which seem to regulate cell growth during the G1 phase of the cell cycle. Notably, the Cdk genes were up-regulated in the array, and it is known that these proteins play a role in the phosphorylation of key substrates involved in each phase of cell cycle progression

Table II. Cancer gene array.

PA	UniGene	Symbol	Description	E2	M	P	M+E2	P+E2
7	Hs.446504	ABL1	V-abl Abelson murine leucemia viral oncogene homolog 1	↑				
10	Hs.75589	ACP2	Acid phosphatase 2, lysomal					↑
17	Hs.311640	RPS27A	Ribosomal protein S27a				↑	↑
21	Hs.1239	ANPEP	Alanyl (membrana) aminopeptidase (aminopeptidase M, microsomal aminopeptidase, CD13, p150)	↑		↑	↑	↑
24	Hs.433892	AP2M1	Adaptor-related protein complex 2, mu 1 subunit		↑		↑	↑
28	Hs.179735	ARHC	Ras homolog gene family, member C		↑	↓	↑	↑
35	Hs.181243	ATF4	Activation transcription factor 4 (tax-responsive enhancer element B67)	↑	↑	↑	↑	
37	Hs.406510	ATP5B	ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide	↑			↑	
38	Hs.409140	ATP5O	ATP synthase, H^+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	↑			↑	
43	Hs.171825	BHLHB2	Basic helix-loop-helix domain containing, class B, 2					↑
51	Hs.356181	CAPN1	Calpain 1, (mu/1) large subunit	↑	↑	↑	↑	↑
52	Hs.388469	CAPNS1	Calpain, small subunit 1		↑			
57	Hs.371468	CCND1	Cyclin D1	↑	↑	↑	↑	↑
61	Hs.1600	CCT5	Chaperonin containing TCP1, subunit 5 (ε)				↑	↑
64	Hs.278573	CD59	CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344)					↑
65	Hs.82906	CDC20	CDC20 cell division cycle 20 homolog (*S. cerevisiae)*					↑
67	Hs.153752	CDC25B	Cell division cycle 25B	↑			↑	
71	Hs.95577	CDK4	Cyclin-dependent kinase 4	↑	↑	↑	↑	↑
75	Hs.370771	CDKN1A	Cyclin-dependent kinase inhibitor 1 (p21, Cip1)		↑		↑	↑
85	Hs.135471	CIB1	Calcium and integrin binding 1 (calmyrin)		↑		↑	↑
95	Hs.70312	COX7A2	Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)		↑		↑	↑
100	Hs.181390	CSNK1G2	Casein kinase 1, γ 2	↑	↑	↑		↑
107	Hs.8867	CYR61	Cysteine-rich, angiogenic inducer, 61			↑		↑
112	Hs.11806	DHCR7	7-dehydrocholesterol reductase	↑	↑	↑	↑	↑
116	Hs.74375	DVL1	Dishevelled, dsh homolog 1 (*Drosophila*)		↑			↑
118	Hs.96055	E2F1	E2F transcription factor 1	↑	↑	↑	↑	
124	Hs.171596	EPHA2	EphA2	↑	↑		↑	
140	Hs.418138	FN1	Fibronectin 1		↑	↑	↑	↑
148	Hs.433670	FTL	Ferritin, light polypeptide			↑	↑	↑
152	Hs.169744	G22P1	Thyroid autoantigen 70 kDa (Ku antigen)	↑	↑	↑	↑	↑
157	Hs.157307	GNAS	GNAS complex locus		↑	↑	↑	↑
159	Hs.5662	GNB2L1	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	↑	↑	↑	↑	↑
170	Hs.89525	HDGF	Hepatoma-derived growth factor (high-mobility group protein 1-like)				↑	↑
171	Hs.274485	HLA-C	Major histocompadaility complex, class I, C					↑
173	Hs.57301	HMGA1	High mobility group AT-hook 1	↑	↑	↑	↑	↑
181	Hs.48516	B2M	ß-2-microglobulin		↓	↑	↑	↑
183	Hs.76067	HSPB1	Heat shock 27 kDa protein 1		↑		↑	↑
191	Hs.76095	IER3	Immediate early response 3		↑	↑	↑	↑
196	Hs.450230	IGFBP3	Insulin-like growth factor binding protein 3	↑	↑	↑	↑	↑
199	Hs.512226	IGFBP5	Insulin-like growth factor binding protein 5	↑	↑	↑	↑	↑
204	Hs.265829	ITGA3	Integrin, α 3 (antigen CD49C, α 3 subunit of VLA-3 receptor)				↑	↑
212	Hs.2780	JUND	Jun D proto-oncogene	↑				↑
213	Hs.446608	K-ALPHA-1	Tubulin, α, ubiquitous	↑	↑	↑	↑	↑
220	Hs.406013	KRT18	Keratin 18		↑	↑	↑	↑
223	Hs.122645	LAMB1	Laminin, ß 1					↑
227	Hs.204238	LCN2	Lipocalin 2 (oncogen 24p3)	↓	↓	↓	↓	↓
235	Hs.366546	MAP2K2	Mitogen-activated protein kinase kinase 2	↑	↓	↓	↑	↑
244	Hs.57101	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin (*S. cerevisiae*)				↑	↑
265	Hs.437922	MYCL1	V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)			↑	↑	↑
279	Hs.433416	NME2	Non-metastatic cells 2, protein (NM23A)	↑	↑	↑	↑	↑

Table II. Continued.

PA	UniGene	Symbol	Description	E2	M	P	M+E2	P+E2
300	Hs.1976	PDGFB	Platelet-derived growth factor ß polypeptide (simian sarcoma viral (v-sis) oncogene homolog)		↑	↑	↑	↑
305	Hs.288856	PFDN5	Prefoldin 5	↑	↑	↑	↑	↑
308	Hs.85701	PIK3CA	Phosphoinositide-3-kinase, catalytic, α polypeptide	↑	↑	↑	↑	↑
316	Hs.387667	PPARG	Peroxisome proliferative activated receptor, γ			↑		↑
319	Hs.83383	PRDX4	Peroxiredoxin 4	↑	↑	↑	↑	↑
328	Hs.459927	PTMA	Prothymosin, α (gene sequence 28)	↑	↓	↑	↑	↑
336	Hs.865	RAP1A	RAP1A, member of RAS oncogene family			↓		
343	Hs.444499	REA	Repressor of estrogen receptor activity	↑	↑	↑	↑	↓
351	Hs.406532	RPN2	Ribophorin II					↑
361	Hs.184510	SFN	Stratifin	↑	↑	↑	↑	↑
363	Hs.74335	HSPCB	Heat shock 90 kDa protein 1, ß	↑	↑	↑	↑	↑
368	Hs.112058	SIVA	CD27-binding (Siva) protein				↑	
378	Hs.74335	HSPCB	Heat shock 90 kDa protein 1, ß	↑	↑	↑	↑	↑
382	Hs.443914	SOD1	Superoxide dismutase 1, soluble (amyotrophic later sclerosis 1 (adult))		↑		↑	
384	Hs.31439	SPINT2	Serine protease inhibitor, Kunitz type, 2		↑	↑		↑
393	Hs.74335	HSPCB	Heat shock 90 kDa protein 1, ß	↑	↑	↑	↑	↑
396	Hs.231411	TBRG4	Transforming growth factor ß regulador 4	↑		↑	↑	↑
404	Hs.421496	421496	Transforming growth factor, ß-induced, 68 kDa			↑		
408	Hs.74335	HSPCB	Heat shock 90 kDa protein, ß	↑	↑	↑	↑	↑
410	Hs.446641	TIMP1	TIMP metallopeptidase inhibitor 1	↑	↓		↑	↑
413	Hs.164457	TK1	Thymidine kinase 1, soluble				↑	
424	Hs.408312	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	↑	↑			
426	Hs.50649	TP5313	Tumor protein p53 inducible protein 3				↑	↑
429	Hs.374596	TPT1	Tumor protein, translationally-controlled 1	↑	↑	↑	↑	↑
438	Hs.183704	UBC	Ubiquitin C	↑	↑	↑	↑	↑
442	Hs.404814	VDAC1	Voltage-dependent anion channel 1				↑	↑

PA, Position in array. ↑ Up-regulated expression; ↓ down-regulated expression of the gene with respect to the control MCF-10F cells. E2, estrogen; M, marathion; P, parathion.

(10-13). The *DVL1* gene was also up-regulated with malathion alone and with the combination of parathion and estrogen. Dvl1 is one of the mammalian dishevelled proteins considered as a tumor suppressor protein. Dsh is a component of the frizzled signaling pathway. Both mammalian dishevelled and frizzled proteins are components of the Wnt signaling pathway (14).

The gene of *HSP 27* protein expression was up-regulated with malathion alone and with the combination of E2 and malathion or parathion. Since the heat shock proteins (HSPs) are a group of highly conserved, abundantly expressed proteins with diverse functions, including the assembly and sequestering of multi-protein complexes, transportation of nascent polypeptide chains across cellular membranes and regulation of protein folding (15-19), it is possible that they play an important role in the presence of estrogens and pesticides. HSPs, also known as molecular chaperones, fall into six general families and among them HSP 90 and HSP 27, are constitutively expressed cytoplasmic proteins that co-localize to the nucleus upon stress induced by insult. Hormones, heat and cytokines are among the factors that stimulate the synthesis of HSP 27. *In vitro*, HSP 27 becomes highly phosphorylated following exposure to stress. The discovery that HSP 27 was regulated by estrogen has led to studies establishing a relationship between HSP 27 and breast cancer (15-19).

Both the *IGFBP3* and *IGFBP5* genes were up-regulated by the effects of all of the different treatments. The IGFBPs are a family of homologous proteins that have co-evolved with the IGFs (20-26). They serve not only as shuttle molecules for the soluble IGFs, but also regulate the IGF signaling system. Physical association of the IGFBPs with IGF influences the bioavailability of the growth factors, as well as their concentration and distribution in the extracellular environment. In addition, the IGFBPs appear to have biological activity independent of the IGFs. Seven IGFBPs have been described and each differ in their tissue distribution, half-lives and modulation of IGF interactions with their receptors (20-26). It is important to consider that the IGFBPs serve not only as shuttle molecules for the soluble IGFs, but also confer a level of regulation to the IGF signaling system. Physical association of the IGFBPs with IGF allows availability of growth factors, as well as their distribution in the extracellular environment.

The *keratin 18* gene was up-regulated by the effect of all of the treatments. It is well known that cytokeratins are useful markers of tissue differentiation applicable to the characterization of malignant tumors. Thus, cytokeratin 18 is expressed in a majority of adenocarcinomas and basal cell carcinomas (27). *MCM2* was up-regulated by the effect of the combination of E2 with either malathion or parathion. The MCM family of proteins, including MCM2 regulate DNA replication to occur only once in the cell cycle. It has been reported that expression of MCM proteins increases during cell growth, peaking at the G1 to S phase. The MCM proteins are regulated by E2F transcription factors, which induce MCM expression, and by protein kinases that interact with MCM proteins to maintain the post-replicative state of the cell. MCM2/MCM4 complexes function as substrates for Cdc2/cyclin B *in vitro*. Cleavage of MCM3, which can be prevented by caspase inhibitors, results in the inactivation of the MCM complex during apoptosis. A complex composed of MCM4, MCM6 and MCM7 has been shown to be involved in DNA helicase activity (28,29).

TP53 inducible protein 3 was up-regulated by the effect of the combination of E2 with either malathion or parathion while *TP53* (Li-Fraumeni syndrome) was up-regulated only by E2 alone and malathion alone. The p53 is a tumor suppressor gene located on chromosome 17p13. Mutations in the p53 gene have been found in ~20-50% of human cancers. Mutant p53 can act as a dominant oncogene, whereas wild-type p53 is a recessive tumor suppressor gene (30,31).

The present study suggests that E2 and the organo-phosphorous pesticides induced changes in gene expression in breast cells influencing the process of carcinogenesis. Since the causative factors for breast carcinogenesis remain an enigma and the human population is exposed not only to pesticides but also to a mixture of estrogenic or estrogen-like agents, investigation into the effects of E2 alone and in combination with either malathion or parathion on cell transformation of breast epithelial cells plays a pivotal role.

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