Human drug metabolism genes in parathion- and estrogen-treated breast cells

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Abstract. Environmental chemicals may be involved in the etiology of breast cancer. Among them, organophosphorous compounds are the most widely used pesticides because of their extensive use in agriculture, medicine and industry. The risk of breast cancer is associated with prolonged exposure to female hormones and is attributed to estrogen since prolonged stimulation by steroid hormones may increase cell division. The aim of the present study was to identify the differentially expressed genes encoding enzymes that are important to drug transport and metabolism in parathion- and estrogen-treated human breast epithelial cell lines using cDNA microarrays. MCF-l0F, an immortalized human breast epithelial cell line was treated with parathion and estrogen, either alone or in combination, and malignant cells were developed through a series of sequential steps. Differential expression from the drug metabolism gene array showed that 17 genes were found to be altered either by parathion or estrogen alone, or the combination of both. Among the genes altered by parathion in comparison to the control were CHST5, CHST6 and CHST7 (sulfotransferases); CYP2F1, CYP3A7 and CYP4F3 (CYPs); GSTP1, GSTT2 and MGST1 (GSTs); MT1X (metallothionein); TPMT (methyltransferase); UGT1A1 and UGT2B (UDP glycosyltransferases). The same genes were down-regulated in estrogen alone including several metallothioneins (MT1A, MT1E, MT1H, MT1L and MT2A). The combination of parathion and estrogen induced down-regulation of three sulfotransferases, CYP2F1 and CYP4F3, MGST1, all metallothioneins and TPMT genes. There was no change in CYP3A7, GSTP1, GSTT2, UGT1A1 and UGT2B genes in the presence of both substances. It can be concluded from this study that organophosphorous pesticides such as parathion in the presence of estradiol induced changes in human drug metabolism gene expression in breast cells.

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Introduction

Environmental chemicals may be involved in the etiology of breast cancer (1-3). Organophosphorous compounds are the most widely used pesticides by virtue of their biodegradable nature and short persistence. Such compounds are of great interest because of their extensive use in agriculture, medicine and industry. Pesticides seem to be serious threats to humans (4-9). The relationships between chemical pesticides and the incidence of cancer have been previously reviewed (7). The organophosphorous compounds are causally linked with non-Hodgkin's lymphoma and leukemia (8). In animal studies, many pesticides have been proven to be carcinogenic; e.g., organochlorines such as DDT, chlordane and lindane are tumor promoters (8). On the other hand, the risk of breast cancer is also associated with prolonged exposure to female hormones (10-13). Among these hormonal influences, a leading role is attributed to estrogen since prolonged stimulation by steroid hormones may increase cell division, increasing the risk of breast cancer (10-13).

Differential expression from the drug metabolism gene array includes functional gene groups related to *Drug transporter* such as metallothioneins and P-glycoprotein family; *Phase I metabolizing enzymes* such as cytochrome P450 (CYPs) and *Phase II metabolizing* enzymes such as acetyltransferases, epoxide hydrolases, glutathione S-transferases (GSTs), methyltransferases, sulfotransferases and UDP glycosyltransferases (14-18). Metallothionein (MT) is a sulfhydryl- and cysteine-rich protein found in micro-organisms, plants and all invertebrate and vertebrate animals (14). MTs are a group of ubiquitous low-molecular-weight proteins that have functional roles in cell growth, repair and differentiation. MTs are implicated primarily in metal ion detoxification, as they are essential for the protection of cells against the toxicity of cadmium, mercury and copper. MT is a stress-response protein and free radical scavenger and it is related to inflammation and cellular protection from reactive forms of oxygen, ionizing radiation, pharmacological agents and mutagens (14).

Human cytochrome P450 2E1 (CYP2E1) is a *Phase I metabolizing* enzyme. It is involved in the biotransformation

of xenobiotics and endogenous substrates (15). Inter-individual genetic polymorphisms of the CYP2E1 gene are associated with different cancer diseases. Mammalian cytosolic GSTs form a super family consisting of four distinct families, named α, μ, π and θ. The μ (GSTM1) and θ (GSTT1) members of the GST multigene family are candidate cancer susceptibility genes because of their ability to regulate the conjugation of carcinogenic compounds to excretable hydrophilic metabolites (16-18). Worldwide, organophosphate insecticides result in numerous poisonings each year. The organo(thio)phosphate esters are one of the most widely used classes of insecticides. In insects, GSTs play an important role in insecticide resistance. GST enzymes mediate exposure to cytotoxic and genotoxic agents and may be involved in cancer susceptibility (14-18).

An *in vivo* rat mammary tumor model has already been established induced by eserine and the organophosphorous pesticides parathion and malathion, possibly through acetyl cholinesterase inhibition (19) and by 17ß estradiol (20). The identification of factors involved in cell proliferation and transformation has been facilitated by *in vivo* studies as well as *in vitro* by using various human epithelial cell lines (21-27). Since there is little or no information available on pesticide-induced breast cancer, an *in vitro* breast transformation model provides an opportunity for studying breast carcinogenesis.

The aim of the present study was to identify the differentially expressed human drug metabolism genes encoding enzymes that are important to drug transport and metabolism in an immortalized human breast epithelial cell line, MCF-l0F (21) treated with parathion and estrogen, either alone or in combination by using cDNA microarrays.

Materials and methods

Cells. The MCF-10F cells were used in their 41st passage. Cells were cultured with DMEM/F-12 (1:1) medium supplemented with antibiotics [100 U/ml penicillin, 100 μg/ml streptomycin, 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) 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-10F cells continuously treated with parathion at 100 ng/ml (parathion-ethyl) (Sigma-Aldrich, St. Louis, MO) for 20 passages, iii) MCF-l0F continuously treated with estrogen at 10^{-8} M (E2) (Sigma-Aldrich) and iv) the combination of both.

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) (Roche Pharm., 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. Each sample was then purified following established procedures (28). The purified RNA sample was first measured by a spectrophotometer (the ratio of absorbance reading at 260 nm/280 nm at least 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 the established procedure (28).

cDNA expression array. The GE Array Q Series Human Drug Metabolism Gene Array includes genes that encode enzymes important for *Drug Transport*, *Phase I metabolism*, and *Phase II metabolism* (SuperArray, Bethesda, MD). It is designed to profile gene expression of a panel of 96 key genes associated with drug metabolism. The purified mRNAs were used for the synthesis of cDNA probes with Biotin-16-dUTP (Roche Pharm.). 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 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-dUTP, 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 was 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 one 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 (29). 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 (GEAhyb solution with a heat-denatured sheared salmon sperm DNA at a final concentration of 100 μg/ml) (SuperArray) and mixed gently for a few sec. Prehybridization was continued at 60˚C for 1 to 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 the GEAprehyb solution and kept at 60˚C. Then the GEAprehyb solution was replaced by GEAhyb solution and incubation was continued overnight and 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 time with gentle agitation and then washed twice with solution 2 (0.1X sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate) at 60˚C for 15 min each time with gentle agitation.

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 the 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 each washing and rinsed twice with 3 ml of rinsing Buffer G. Then the membrane was covered with 1.0 ml of CDP-Star chemiluminescent substrate to incubate at room temperature for 2 to 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 hybridization signals on the expression array membranes was carried out by exposing the autoradiographic film in a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA), and then estimated both with the ImageQuant (Molecular Dynamics) and ScanAnalyzer program (Eisen Lab, Lawrence Berkeley National 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 (29,30). 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 signals were 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/ScanAnalyzer and were then separately scanned and compared with housekeeping genes so the chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of 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 ≥2.5-fold versus control were used. The median background was subtracted and signals that were <2.5-fold 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 change in fold indicated whether a gene exhibited increased, decreased, or unchanged expression, based on statistical criteria (30).

Results

Expression of drug metabolism genes in the parental MCF-10F and transformed cells induced by the various treatments was ascertained using a human drug metabolism gene array. MCF-10F cells treated with parathion, E2, and parathion plus E2 in comparison to the control MCF-10F were studied to analyze the expression profile of 96 genes involved in human drug metabolism regulation. The origin and phenotypic characteristics of cells used in these studies are shown in Table I. The parental MCF-10F cell line was treated with

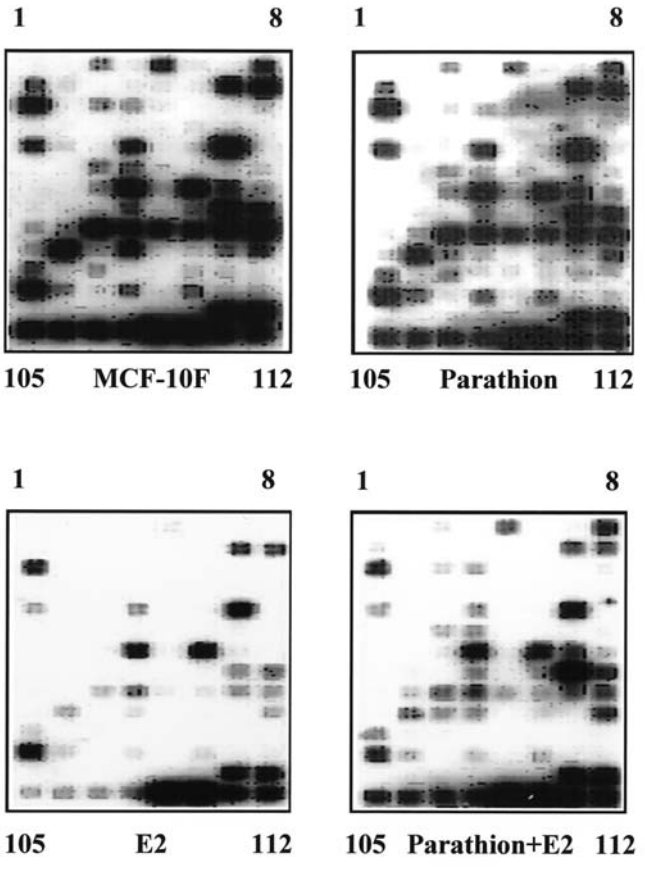

Figure 1. Human Drug Metabolism Gene Array. Differentially expressed genes of human drug metabolism in the parental MCF-10F (top left), MCF-10F plus parathion (top right), MCF-10F plus estrogen (E2) (bottom left) and MCF-10F plus parathion and E2 (bottom right) cells.

parathion, E2, and the combination of parathion and E2 for 20 passages in culture. The anchorage independence capability of treated cells was analyzed. MCF-10F cells did not form colonies in agar, and colony-forming efficiency scored 21 days after plating in agar fluctuated from 1-3% in the presence of parathion, E2, and the combination of both (31,32). The invasive characteristics of the control and treated MCF-10F cells were scored 20 h after plating onto matrigel basement membranes using Boyden's chambers. The control MCF-10F cell line and E2-treated cells showed no invasive capabilities, and there was no significant difference between these two groups. Addition of parathion to the growth medium enhanced the invasive phenotype of cells in comparison to the control and E2-treated cells. Cells treated with parathion alone and parathion combined with E2 had greater invasive capability than the control MCF-10F and E2-treated cells. However, no significant difference was observed between the cells treated with parathion alone and the cells treated with parathion combined with E2 (31,32).

Results indicated that among the various human drug metabolism genes present in the array, 17 genes were altered either by parathion, E2, or the combination of both as seen in Fig. 1 and Table IIA and B. The functional gene groups altered by the effect of the pesticide and E2 were sulfotransferases, cytochromes P450 (CYPs) (subfamily IIF polypeptide 1, subfamily IIIA polypeptide 7, subfamily IVF polypeptide 3), glutathione S-transferases (GSTs), metallo-

Table I. Origin and phenotypic characteristics of the cell lines.

Cells	Origin	AIA	IA
MCF-10F	MCF-10F parental cells	-	-
Parathion[a]	MCF-10F treated with parathion	+	+
Estrogen (E2)[b]	MCF-10F treated with estrogen	-	-
Parathion + E2[c]	MCF-10F treated with combination	+	+

MCF-10F cells were treated with [a]100 mg/ml parathion during 20 passages; [b]estrogen (10^{-8} M) (E2) during 20 passages; and [c]combination of parathion and E2 for 20 passages. AIA, Anchorage independence colony-forming efficiency in soft agar. IA, 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 number of cells that crossed the filters; -, lack of anchorage independent growth and invasiveness.

Table II. Human drug metabolism genes.

A

Position in array	Name	Symbol	E2	P	P + E2
15	Carbohydrate (N-acetylglucosamine 6-0) sulphotransferase 5	CHST5	↓	↑	↓
16	Carbohydrate (N-acetylglucosamine 6-0) sulphotransferase 6	CHST6	↓	↑	↓
17	Carbohydrate (N-acetylglucosamine 6-0) sulphotransferase 7	CHST7	↓	↑	↓
33	Cytochrome P450, subfamily IIF, polypeptide 1	CYP2F1	↓	↑	↓
36	Cytochrome P450, subfamily IIIA, polypeptide 7	CYP3A7	↓	↑	(-)
39	Cytochrome P450, subfamily IVF, polypeptide 3	CYP4F3	↓	↑	↓
52	Glutathione S-transferase π 1	GSTP1	↓	↑	(-)
54	Glutathione S-transferase θ 2	GSTT2	↓	↑	(-)
63	Glutathione S-transferase microsomal	MGST1	↓	↑	↓

B

Position in array	Name	Symbol	E2	P	P + E2
67	Metallothionein 1A (functional)	MT1A	↓	(-)	↓
68	Metallothionein IE (functional)	MT1E	↓	(-)	↓
69	Metallothionein 1G	MT1G	↓	(-)	↓
70	Metallothionein 1H	MT1H	↓	(-)	↓
71	Metallothionein 1L	MT1L	↓	(-)	↓
72	Metallothionein 2A	MT2A	↓	(-)	↓
74	Metallothionein IX	MTIX	↓	↑	↓
89	Thiopurine S-methyltransferase	TPMT	↓	↑	↓
92	UDP glycosyltransferase 1 family, polypeptide Al	UGT1A1	↓	↑	(-)
94	UDP glycosyltransferaee 2 family, polypeptide B	UGT2B	↓	↑	(-)

↑ Up-regulated expression; ↓ down-regulated expression; (-) no change in expression of that gene with respect to the control MCF-10F. Note, both up-regulation and down-regulation of gene expression over 2.5- to 5-fold alterations were taken into consideration. P, parathion; E2, estrogen.

thioneins (MTs), methyltransferase and UDP glycosyltransferases.

Among the genes altered by parathion in comparison to control were CHST5, CHST6 and CHST7 (sulfotransferases); CYP2F1, CYP3A7 and CYP4F3 (CYPs); GSTP1, GSTT2 and MGST1 (GSTs); MT1X (metallothionein); TPMT (methyltransferase); UGT1A1 and UGT2B (UDP glycosyltransferases). The enzyme gene expression was 2.5- to 5-fold up-regulated in the parathion-treated group in comparison to the control. The same genes were 2.5- to 5-fold down-regulated in E2 alone including several metallothioneins (MT1A, MT1E, MT1H, MT1L and MT2A). The combination of parathion and E2 induced down-regulation of three sulfotransferases, CYP2F1 and CYP4F3, MGST1, all metallothioneins and TPMT genes. There was no change in CYP3A7, GSTP1, GSTT2, UGT1A1 and UGT2B genes in the presence of both substances.

Discussion

Differentially expressed drug metabolism genes were evaluated in parathion- and E2-*in vitro*-transformed breast cells. Pesticides are currently used to improve agricultural production (5-8). However, insecticides have been classified as carcinogens by the International Agency for Research on Cancer in humans (33-35). The human data are limited due to the restricted number of studies examining individual pesticides. However, the human population is exposed not only to pesticides but also to a mixture of estrogenic or estrogen-like agents. Epidemiological and experimental evidence has implicated estrogens in the etiology of breast cancer (33-35). It is known that estrogens are associated with carcinogenic events in both humans and animals (36,37).

The effect of estrogens in breast cancer remains unclear. Molecular biomarkers are essential to analyze the progression of breast cancer and to identify genes involved in the process of neoplastic transformation induced by several agents, among which pesticides and E2 are very important. The present study analyzed the presence of several molecular biomarkers in a simple array by using transformed cells. 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 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 (31,32) *in vitro* showed that parathion and E2 alone and the combination of both were capable of altering cell proliferation and inducing transformation of the MCF-10F cell line. The increase in cell proliferation rate indicated by a shorter doubling time, anchorage independent growth and *in vitro* invasive capability suggest a very aggressive phenotype. Treatment of parathion alone or its combination with E2 exhibited significant invasive capabilities as compared to the control cells.

Molecular biomarkers of Phase II enzymes have been studied in relation to the risk of cancer. Cytosolic sulfotransferase enzymes catalyze the sulfation of a large variety of drugs and endogenous substances. Results indicated that CHST5, CHST6 and CHST7 (sulfotransferase) genes were up-regulated by parathion and down-regulated by E2. The combination of parathion and E2 induced down-regulation of three sulfotransferase genes. A high frequency of SULT1A1 has been identified in patients with breast cancer (18).

Expression of several CYP genes was up-regulated by the effect of parathion whereas the same genes were down-regulated in the presence of E2 alone. The combination of parathion and E2 induced down-regulation of CYP2F1 and CYP4F3. There was no change in the CYP3A7 gene in the presence of both substances. These changes suggest that these substances may also have the potency to cause malignant transformation of breast epithelial cells through modulation of expression of such genes. It has been previously reported that the CYPs play a critical role in the oxidative metabolism activation and detoxification of a variety of endogenous and exogenous compounds influencing the biological effects of estrogens.

DNA damage by steroidal estrogens via catechol estrogen metabolites has been reported in relation to the carcinogenic activity of 4-hydroxyestradiol (4-CE), comparable to that of E2 in the hamster kidney tumor model (36-40). The 2-hydroxylation (2-CE) of steroidal estrogens is the major metabolic oxidation of estrogenic hormones in most mammalian species (41-43). Such oxidation was catalyzed in the human or hamster liver by cytochrome P450 3A4 enzymes (42-49), whereas cytochrome P450 1A1 enzymes are the predominant estrogen 2-hydroxylases in extrahepatic tissues (44-47). In humans, the predominant conversion of E2 to 4-CE has been detected in benign and malignant mammary tumors and normal mammary tissue (40). The human estrogen-4-hydroxylase activity has been identified as cytochrome P450 1B1, a novel extrahepatic isozyme detected specifically in mammary tissue, ovary, adrenal gland, uterus and several other tissues (48-50).

These studies showed that GST enzymes, such as the GSTP1, GSTT2 and MGST1 genes were up-regulated in the parathion-treated cells and down-regulated in the E2-treated group in comparison to the control. The combination of parathion and E2 induced down-regulation of three MGST1 genes. There was no change in the GSTP1 and GSTT2 genes in the presence of both substances. Nascimiento et al (16) and Rebbeck (17) have indicated an association between genotypes of Phase II enzymes and cancer risk by epidemiological studies. The molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 has indicated cancer susceptibility (17). A possible influence of the glutathione S-transferase GSTTI null genotype on the age of onset of sporadic colorectal adenocarcinoma has been reported (16). The high frequency of the GSTTI null genotype in patients diagnosed before the age of 60 years suggested that this genotype influences the age of disease onset. Variant alleles in GST, UDP glucuronosyltransferase (UGT) have been used as molecular genetic biomarkers of risk. The GSTM μ 1 has been associated with an increased risk of colorectal cancer, lung cancer, bladder cancer and GSTP π 1 with prostate cancer.

Notably, the only functional gene altered by the effect of parathion among the metallothioneins was metallothionein 1X. Its gene expression was up-regulated by parathion in

comparison to the control whereas the same gene was down-regulated in E2 alone and in the presence of the combination of both. However, it has only been previously reported that metallothionein 2A expression is associated with cell proliferation in breast cancer (14).

Conjugation with UDP (uridindiphosphate) by UGT (glucuronidation) is one of the major routes of elimination and detoxification of drugs and endogenous compounds. Carcinogens and their reactive metabolites may also be glucuronidated to harmless intermediates that can be eliminated from the body. The UGT1A1 and UGT2B genes were up-regulated by parathion and down-regulated by E2. However, the combination of parathion and E2 induced no change in these genes. Dalhoff *et al* (18) have identified clinical studies using molecular genetic biomarkers of Phase II enzymes as markers of susceptibility to the development of cancer since UGT1A1 and UGT2B were increased in ovarian cancer. Therefore, the UGT1A1 and UGT2B genes seem to be reliable molecular biomarkers for the risk of cancer. It can be concluded from this study that organophosphorous pesticides such as parathion in the presence of estradiol induce changes in human drug metabolism gene expression in breast cells such as sulfotransferases, cytochromes P450, glutathione S-transferases, metallothioneins, and methyltransferase.

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