Organophosphorous pesticides and estrogen induce transformation of breast cells affecting *p53* and *c-Ha-ras* genes

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Abstract. Cancer progression has been associated with an increase in genomic instability indicated by inactivation of tumor suppressor genes and activation of oncogenes. Epidemiological and experimental evidence has implicated estrogens in the etiology of breast cancer. To study environmental organophosphorous pesticides is of interest since evidence indicate that pesticides may enhance cell division, increasing the risk of breast cancer. The aim was to evaluate the effects of these pesticides, such as parathion and malathion in the presence of estrogen on malignant transformation as well as on genomic instability, that is in the frequency of loss of heterozygosity (LOH) and microsatellite instability (MSI). The MCF-10F immortalized human breast epithelial cell line, that was treated with parathion or malathion alone and in combination with estrogen was used. These studies indicated that either pesticide alone or in combination with estrogen induced malignant transformation as shown by anchorage-independent growth capability and invasive characteristics in comparison to control. Such malignant phenotypic characteristics were corroborated by significant ($P<0.05$) increase in p53 and c-Ha-ras protein expression. Results indicated different degrees of allelic imbalance in the form of LOH or MSI with different microsatellite markers. MSI was found in malathion and estrogen-treated cells with a marker used for *p53* tumor suppressor gene at loci 17p13.1. The same combination of substances presented MSI with a marker used for *c-Ha-ras* mapped in chromosome 11p14.1, as well as mutations in *c-Ha-ras* for codons 12 and 61. LOH was observed in codon 12 in the presence of estrogen or malathion alone. Parathion alone and combined with estrogen induced MSI in codon 61. It can be concluded that the organophosphorous pesticides parathion and malathion induced malignant transformation of breast cells through genomic instability altering *p53* and *c-Ha-ras*, considered pivotal to cancer process.

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Introduction

Breast cancer is the most common form of cancer among women. Environmental chemicals and endogenous factors seem to be involved in the etiology of this disease. There has been continued interest in environmental contaminants that may play a role in breast cancer risk (1). Organophosphorous pesticides, such as parathion and malathion have been extensively used to control mosquito plagues (2,3). Whether exposure to agricultural pesticide applications is related with breast cancer incidence in women is an unsolved question (4). Pesticides have been of great interest in etiologic breast cancer studies due to the fact that many pesticides or their metabolites mimic estrogen, which is known to increase breast cancer risk (5-7). Epidemiological and experimental evidence has implicated estrogens in the etiology of breast cancer (8,9). 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 (8,10,11).

p53 has been identified as an important tumor suppressor gene in normal cells (12-14). It has been reported that early loss of cell-cycle control in the presence of a mitogenic stimulus allows a cell to continue dividing (13). Wild-type *p53* plays a crucial role in maintaining genomic stability by allowing the repair of damaged DNA through induction of a transient G1 arrest or eliminating the damaged cells by triggering apoptosis (12). *Mutant p53 (mp53)* fails to mediate any of these effects. Uncontrolled proliferation in the absence of wild-type *p53* would expectedly yield a high level of genomic instability, which is a common abnormality detected in primary breast cancer (12,14). Wild-type *p53* is a recessive tumor suppressor gene, located on chromosome 17p13 (15) and it has been implicated in the control of cell cycle, DNA repair and synthesis, differentiation and apoptosis. After DNA damage normal cells stop in G1/S to allow time for repair.

mp53 act as dominant oncogene and cells with alteration in the *p53* gene do not stop in G1/S. Thus, DNA damage is not repaired and genomic instability appears with accumulation of deletions and amplifications (16,17). The *mp53* has been found in ~20-50% of human cancers (17).

The activation of oncogenes promotes cell growth and survival. Transforming genes of Harvey and Kirsten murine sarcoma viruses are three closely-related genes, *c-H-ras, K-ras, N-ras,* expressed in mammalian cells (18). The incidence of *ras* mutation varies between different types of cancer and appears to occur early in the multi-step process. Mutated *ras* genes occur with a high frequency in common human cancers, including adenocarcinoma of the lung, colorectal cancer, myeloid leukemia, adenocarcinoma of the pancreas (18,19). The expression of ras proto-oncogenes in relation to regulation and implications of the genes was analyzed in the development of human tumors and they summarized and discussed experimental results concerning the regulatory mechanisms involved in oncogenic transformation.

Activated *c-Ha-ras* genes, mapped to 11p15.5 (20), containing single substitutions in particular positions in the gene locus has been found in ~10% of human cancers examined (21,22). The mutation responsible for the gene activation was located in codons 12, 13, 61 or 17 (21,22). Activated *Ki-, c-Ha-* and *N-ras*, all with a point mutation in codons 12, 13 and 61, but 12 and 61 mutation are more pronounced compared to codon 13 (23). Elevated expression of the Harvey ras oncogene was reported in human malignant tumors of the breast as compared to their respective normal tissue (24).

In general, the activation of oncogenes and inactivation of tumor suppressor genes underlie carcinogenesis, and tumors develop through an accumulation of several genetic alterations. Loss of heterozygosity (LOH) is a change from a heterozygous to a homozygous state due to loss of the wild-type allele. Many studies have revealed that LOH at chromosome bands 1p36, 3p24-p25, 6q12-q16, 11p15, 11q22, 11q23, 13q21, 16q22, 17p21, and 17q25, plays important roles in breast cancer, and these genetic alterations have complex interactions (25-27). Microsatellite instability (MSI) was formerly known as replication error phenotype or ubiquitous somatic mutations. It is defined as an alteration in the microsatellite sequence length within tumor DNA when compared with normal DNA from an individual (28-30). MSI can be detected by demonstration of variability in the number of repeat units in selected microsatellite markers following amplification using polymerase chain reaction (PCR) between tumor and normal DNA (28). Microsatellite marker analysis has been used extensively to detect evidence of defective repair of DNA synthesis errors (31).

Cancer occurs at molecular level due to gene alterations. The passage from one pathologic lesion to another has been associated with different genetic events, including an overall increase in genomic instability as inactivation of tumor-suppressor genes *(p53)* and activation of oncogenes *(c-H-ras)* (32). The present study evaluated the effect of two organophosphorous pesticides, parathion and malathion in combination with estrogen assessing the genomic instability of *p53* and *c-H-ras* in MCF-10F, a spontaneously immortalized human breast epithelial cell line.

Materials and methods

Cells. MCF-10F cell line (33) used in these studies was maintained in culture media as described previously (34-36). The cells used in these experiments were: 1) MCF-10F (control); 2) MCF-l0F continually treated with 17ß estradiol (E) (10^{-8} M) (Sigma-Aldrich, St. Louis, MO); MCF-10F cells treated continuously with 3) parathion (P) (100 μl/ml) (parathion-ethyl); 4) parathion in combination with estrogen (PE); 5) malathion (2 μl/ml) (M); and 6) malathion in combination estrogen (ME). Previous studies (37) indicated that 17ß estradiol at 10^{-8} M dissolved in the culture media was the best stimulatory dose when analyzed from 10^{-6} to 10^{-10} M. The cell line named Alpha5, a tumorigenic cell line was used in this study for comparative purpose and it derives from an established *in vitro* human breast cancer epithelial cell line model (35). MCF-10F cells was treated with 60 cGy dose of α particles followed by estrogen treatment and exposed to a second dose of 60 cGy of α particles followed by estrogen (60cGy+E/60cGy+E).

Cell transformation assays. To test for cell growth in semi-solid medium anchorage-independent assay was carried out with cells in passage 20 after the treatments as previously described (35). After 2 weeks of incubation, colonies with >50 cells were counted under a dissecting microscope and the cloning efficiency was determined. Cell invasion assay was carried out using modified Boyden's chambers (Transwell; Costar, Cambridge, MA). The total number of cells that crossed the membrane was counted under a light microscope (34).

Quantification of protein expression. mp53 and c-Ha-Ras protein expression was assessed by immunofluorescent technique coupled with confocal microscopy as previously described (34,36,37). Quantification of protein expression was performed with a computational program. For Western blot hybridization analyses, whole-cell protein extracts were prepared (38) and antibodies against mp53 (sc-99) and *c-Ha-Ras* (sc-29) (both from Santa Cruz Biotechnology) were used in these studies. A secondary horseradish peroxidase-coupled anti-mouse was used to detect immunodetection of proteins. The chemiluminescent immunoblotting system ECL kit (Amersham Life Sc., IL) was used for detection and results were quantified by scanning the blots with a densitometer.

DNA isolation. Control and treated cells were extracted and purified according to standard procedure (38). All cell cultures were treated with 1 ml lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS) with 200 mg/ml proteinase K and 100 μg/ml RNAase and incubated overnight at 37˚C with constant gentle agitation (39). Then there were two extractions with a phenol l: chloroform (1:1) mixture and the aqueous layer was adjusted to 0.75 M ammonium acetate and DNA was spooled from 2 volumes of 100% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) as described (38).

Microsatellite polymorphic marker selection. Polymorphic dinucleotide (CA) n repeat microsatellite markers (Research Genetics, Huntsville, AL) were selected on the basis of their

Table I. Microsatellite polymorphic marker selection.

A, Oligonucleotide primers for PCR amplification of microsatellite markers for *p53* and *c-Ha-ras*

Locus	Map position[a]	Maximum heterozygosity	Type of sequence	Size range base pairs
TP53	17p13.1	0.90	Dinucleotide	103–135
HRAS1	11p15.5	0.90	Dinucleotide	244-261

B, Primers selected to amplify microsatellite sequences for *c-Ha-ras* and *p53* studies, listed in a 5'→3' orientation

Primer name	Primer sequence
TP53	ATCTACAGTCCCCCTTGCCG/ GCAACTGACCGTGCAAGTCA
HRAS1	TCACTGACCCTCTCCCTTGACACAG/ TCATGCTACAGCAGCCCCTCAAAGG

[a]Precise location of markers on respective chromosome arms.

maximum heterozygosity (>0.70) and their location near mapped, *p53* and *c-Ha-ras* (Table IA). The sequences and characteristics of microsatellite oligonucleotide primers were obtained from the GDB database (Table IB).

Mutations studies in codons 12 and 61 of c-Ha-ras. To determine whether organophosphorous and estrogen treatment resulted in point mutations in *c-Ha-ras* codons 12 and 61, cells were studied by direct sequencing of the amplified *c-Ha-ras* oncogene. The Exon 1 of the *c-Ha-ras* oncogene was amplified with forward (5'-CGA TGA CGG AAT ATA AGC TTG TGG TGGT-3') and reverse (5'-GTT CAC CTG TAC TGG TGG AAT TCC TCA AA-3') primers (Life Technologies, Inc., Grand Island, NY) to obtain the 545-bp PCR product for codon 12. Similarly, Exon 1 was amplified with forward (5'-ACG CCT GTC CTC CTG CAA GCT TCC TAC-3') and same reverse primers (Life Technologies, Inc.) to produce the 202-bp PCR product for codon 61. DNA amplifications were carried out in a PCR reaction volume of 30 μl containing 50-100 ng of genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTPs, 0.8 μM of each primer (Life Technologies, Inc.) and 0.75 U of AmpliTaq polymerase (Perkin-Elmer Corp., Foster City, CA). The genomic DNA was initially denatured for a 5-min pre-incubation period at 95˚C and thereafter subjected to 35 cycles comprising of denaturation at 94˚C for 45 sec, annealing at 60˚C for 1 min and extension for 1 min at 65˚C, followed by a 7-min final extension at 65˚C using the GeneAmp PCR System 2400 (Perkin-Elmer Corp.) (20,40).

Direct sequencing of amplified DNA fragments. Sequencing was carried out on original PCR products amplified by using the same *c-Ha-ras* codon 12 and 61 primers. PCR products were gel purified by electrophoresis on 1% agarose-TAE gel (Life Technologies Inc.) and eluted with 100 μl of elution buffer of QIAquick gel extraction kit (Qiagen Inc., CA). Sequencing was done by using automated sequencer ABI PRISM 3100 genetic analyzer (Applied Biosystems/Hitachi, Foster City, CA). Each fragment was sequenced at least three times to rule out contamination and PCR fidelity artifacts.

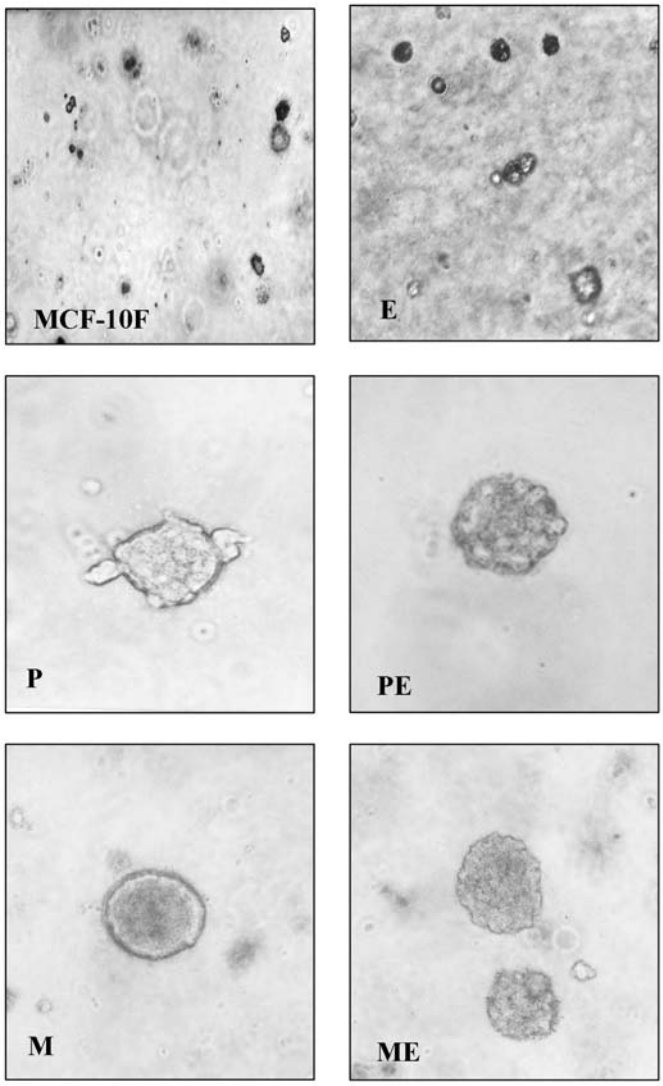

Figure 1. Representative images of anchorage-independency assay. Colony formation of MCF-10F-, E-, P-, PE-, and ME-treated cells.

Results

This study analyzed the effects *in vitro* of parathion and malathion, two organophosphorous pesticides, in combination with estrogen on cell transformation and genomic instability of MCF-10F. Phenotypic characteristics were assessed to determine malignant transformation of cells. These results indicated that P-, M-, PE- and ME-treated-cells were positive to anchorage-independent growth capability. The P-, M-, PE- and ME-treated cells formed agar-positive clones efficienly in 1% agar. The control and E-treated cells were unable to form colonies. Representative images of

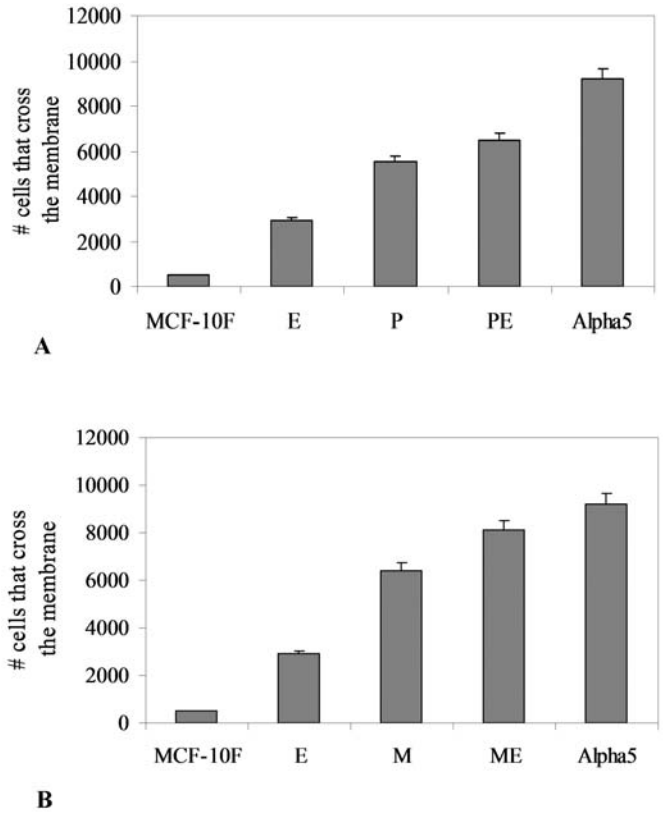

Figure 2. Invasive characteristics of (A) MCF-10F-, E-, P-, PE- and Alpha5-treated cells and (B) MCF-10F-, E-, M-, ME- and Alpha5-treated cells. Bars represent ± SD ($p<0.01$).

anchorage-independent growth of MCF-10F, E-, P-, M-, PE- and ME- treated cells are shown in Fig. 1.

Invasive capability of MCF-10F, E-, P-, M-, PE- and ME-treated cells and Alpha5 cell line are shown in Fig. 2A and B. Results indicated that P- and PE-treated cells had greater ($P<0.05$) invasive capability than control and either control or E-treated cells alone according to the number of cells that crossed the membranes (Fig. 2A). ME-treated cells had greater ($P<0.05$) invasive capability than control and either E- or M-treated cells according to the number of cells that crossed the membranes (Fig. 2B). The tumorigenic cell line Alpha5 had greater invasive capabilities than the combination of PE- and ME-treated cells.

Fig. 3A and B show the representative immunofluorescent images of confocal microscopy of mp53 and *c-Ha-ras* protein expression of MCF-10F, E-, P-, PE-treated cells and Alpha5 cell line. A significant ($P<0.05$) increase in protein expression was observed in PE-treated cells and Alpha5 cell line in comparison to MCF-10F or E-treated cells alone. Quantification of mp53 and *c-Ha-ras* protein expression detected by Western blot can be seen in Fig. 4A and B. Mutant p53 protein expression was significantly ($P<0.05$) increased in PE-treated cells in comparison to control or either E- or P-treated cells (Fig. 4A). Alpha5 cell line had similar protein expression to PE-treated cells. The c-Ha-ras protein expression was significantly ($P<0.05$) increased in PE-treated cells in comparison to control or either E- or P-treated cells (Fig. 4B). There was not significant difference between control and parathion-treated cell lines; however, PE-treated Alpha5 cell line had similar protein expression.

Different degrees of allelic imbalance in the form of LOH or MSI with different microsatellite markers are shown in Fig. 5A-D. Fig. 5A shows MSI in ME-treated cells with a marker used for *p53* tumor suppressor gene at loci 17p13.1. The marker used for *c-Ha-ras* mapped in chromosome 11p14.1 showed MSI in ME-treated cells (Fig. 5B). Mutations in *c-Ha-ras* for codons 12 and 61 can be seen in Fig. 5C and D. Fig. 5C showed LOH in genomic DNA for codon 12 in the E- and M-treated cells. Fig. 5D showed MSI in genomic DNA for codon 61 for P- and PE-treated cells and LOH for M-treated cells.

Discussion

In the 1980s, the International Agency for Research on Cancer conducted evaluations of the literature to assess the potential human carcinogenicity of malathion, concluding that there was limited evidence for the mutagenicity of malathion (41,42). Malathion or its metabolite malaoxon was not mutagenic in several Salmonella strains (43,44). The findings of both *in vitro* and *in vivo* studies of cytogenetic changes in humans and other mammals have been inconsistent (45-48). Some *in vivo* studies demonstrated that parathion and malathion results in disruption of endocrine system. Inhibition of progesterone secretion and poor conception occurred after malathion exposure at the onset of estrus in cattle (49).

Other studies showed significant disturbance in the ovarian cycle after treatment of rats with methyl parathion in the variability of the number of estrus cycles and the duration of each phase of the estrus cycle (50,51). The pesticide malathion gave similar results (52). In epidemiological studies malathion did not appear to be associated with increased risk for any of the cancers examined (53). However, other experimental evidence suggests that malathion or its derivatives may be carcinogenic. Blasiak *et al* (54) showed that malaoxon and isomalathion induced DNA damage. Both malaoxon and isomalathion are impurities found in commercial-grade malathion (55).

These studies indicated that the pesticide organophosphorous, either parathion or malathion, in combination with estrogen induced malignant phenotype of breast cells *in vitro*. However, only the combination of these organophosphorous and estrogen induced genomic instability as indicated by loss of heterozygosity and microsatellite instability considered pivotal to tumor growth. The phenotypic characteristics of parathion alone or parathion combined with estrogen, malathion, and combination of malathion and estrogen-treated cells were assessed to determine malignant transformation. Such cell lines formed agar-positive clones and had greater invasive capability than control and estrogen-treated cells. Analysis of mp53 and *c-Ha-ras* protein expression showed an increase in parathion and estrogen-treated cells similar to the tumorigenic cell line Alpha5 in comparison to control or estrogen-treated cells. We (36) previously showed an increase in mutant p53 oncoproteins in MCF-10F cells irradiated with a double dose of α-particles either in the presence or absence of estrogen and in a tumorigenic cell line in comparison with control MCF-10F cell line. These could be associated with a

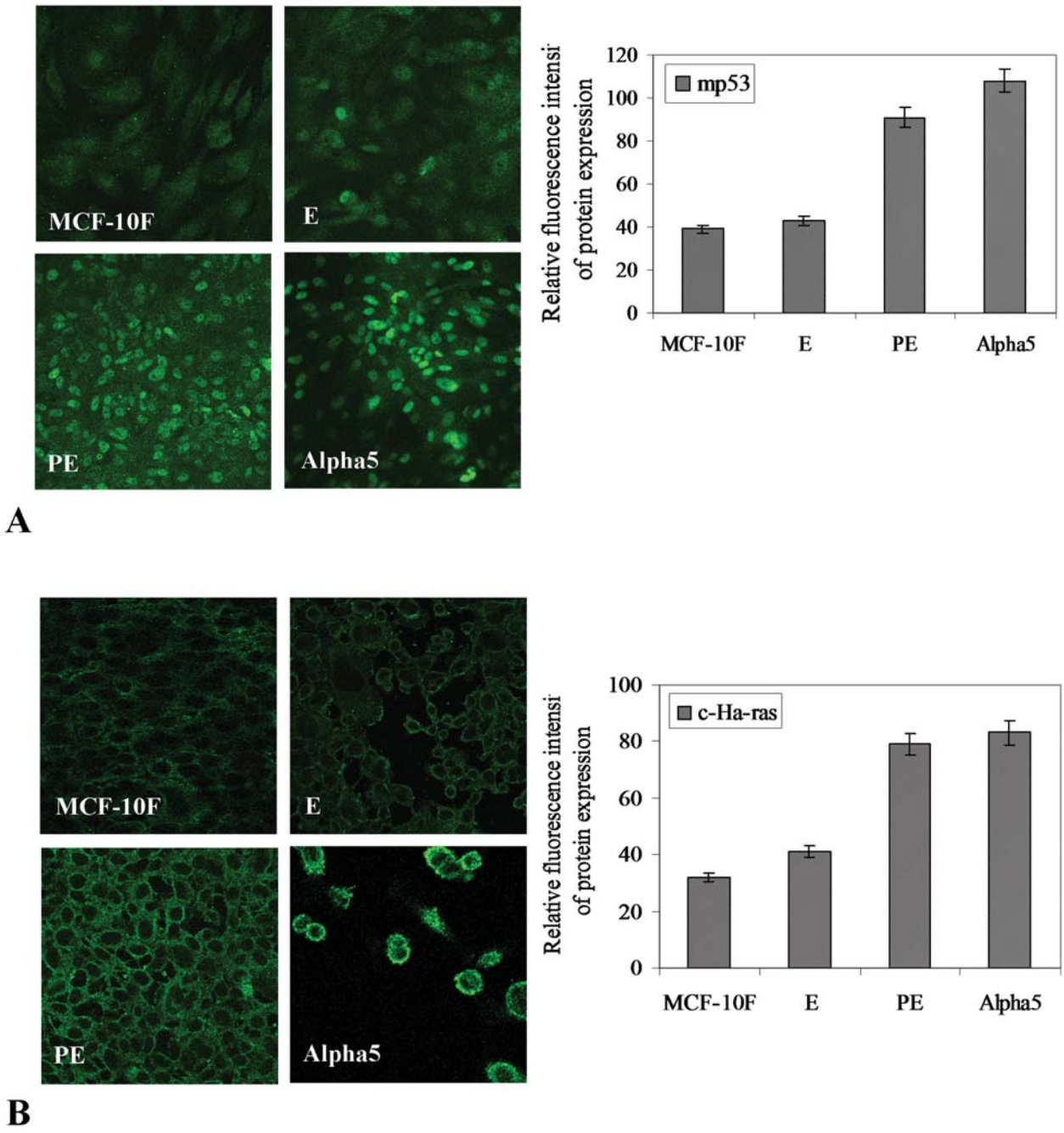

Figure 3. Representative images and quantification of protein expression done with immunofluorescence with confocal microscopy coupled with a computational program. (A) mp53 protein expression and (B) c-Ha-ras protein expression.

loss of control over DNA replication or mitotic errors and give rise to a further cascade of mutations. Previous studies (56) have shown that cells treated with parathion or malathion in combination with estrogen exhibited increased mutant p53, Rho-A, Rac-3, ß-catenin, epidermal growth factor receptor (EGFR), among others in comparison to control MCF-10F cell line when differential expression was analyzed with cDNA arrays.

Different degrees of allelic imbalance in the form of microsatellite instability with different microsatellite markers are reported in the present study. The combination of malathion and estrogen induced microsatellite instability with a marker used for *p53* tumor suppressor gene at loci 17p13.1. Aberrant expression of the p53 tumor suppressor gene is found in breast cancer reviewed by Kiaris and Spandidos (18). Mutations in p53 gene have been found in ~20-50% of human cancer (12,14). Cancers with microsatellite instability have a low number of mutations in p53 tumor suppressor gene, and are associated with loss of apoptosis in ductal breast carcinomas (58,59).

The marker used for *c-Ha-ras* mapped in chromosome 11p14.1 showed microsatellite instability in malathion and estrogen-treated cells. In the present study, loss of heterozygosity was found in parathion alone and combined with estrogen-treated cells, loss of heterozygosity in codon 12 in the estrogen alone and malathion alone-treated cells and micro-

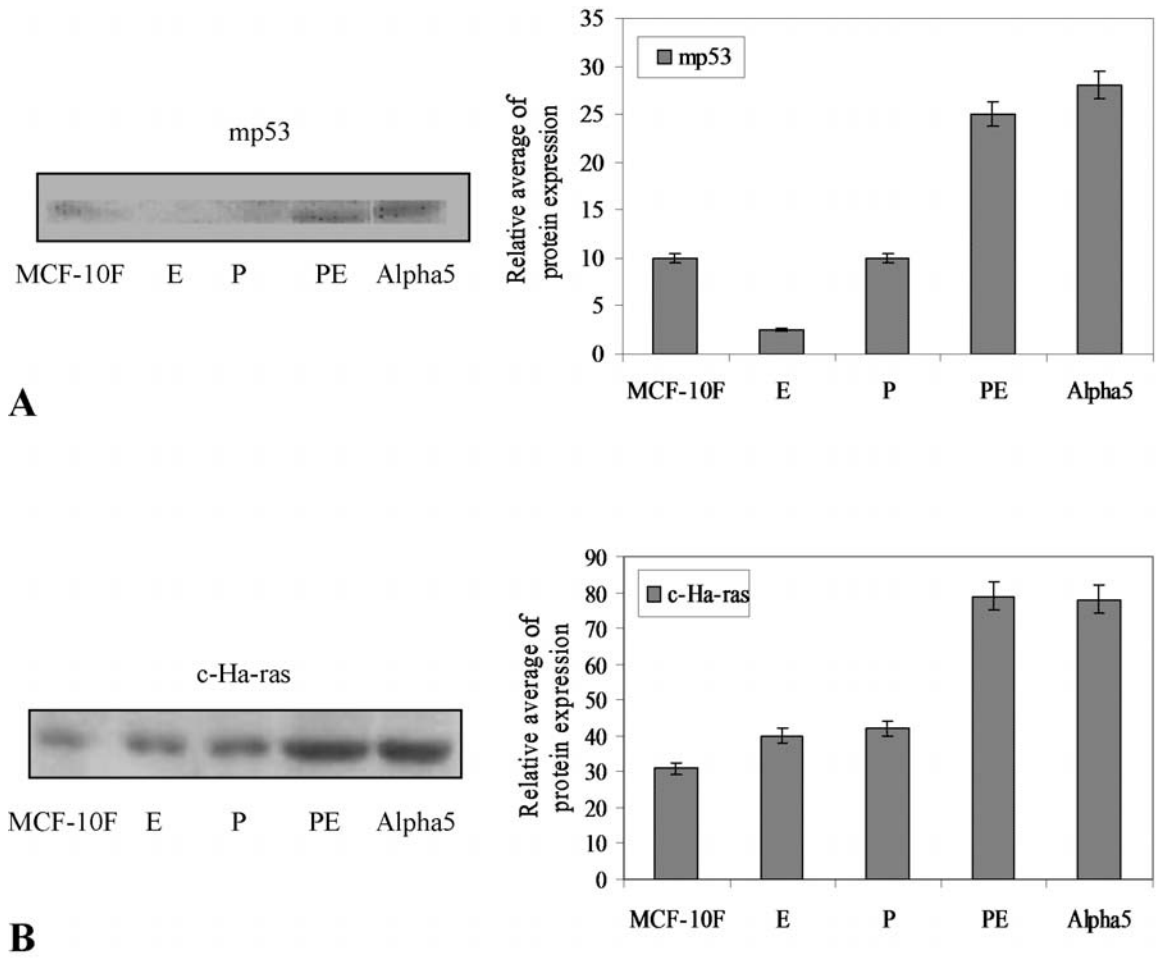

Figure 4. Image and quantification of protein expression with Western blot analysis of MCF-10F, P, E, PE and Alpha5 cells. (A) mp53 protein expression and (B) c-Ha-ras protein expression.

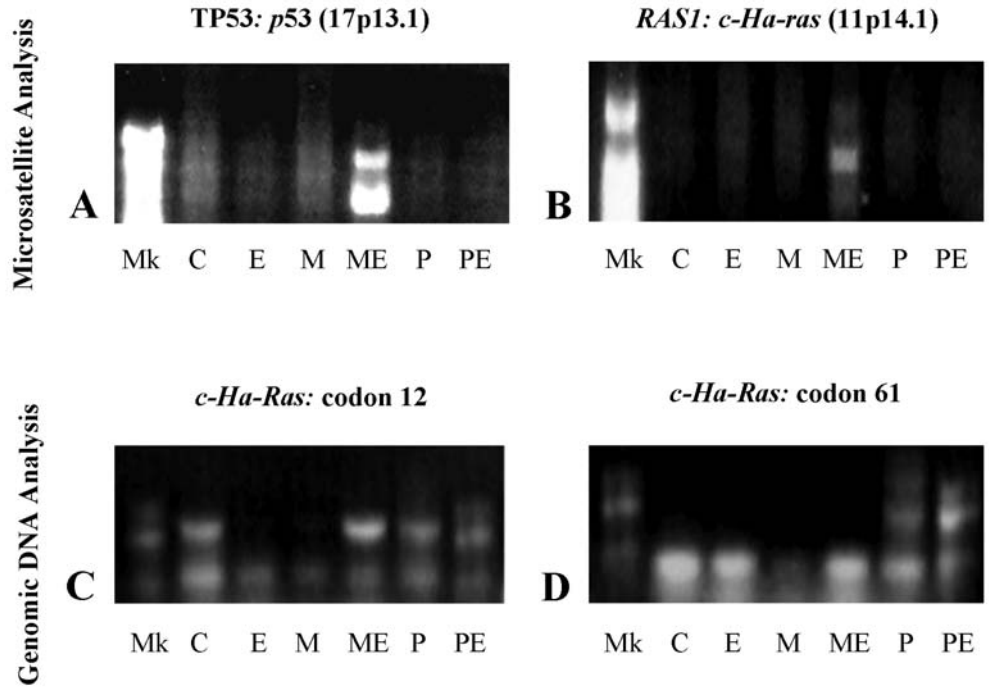

Figure 5. Microsatellite instability (MSI) and loss of heterozygosity (LOH) of MCF-10F, E, M, ME, P and PE of (A) *mp53* tumor suppressor gene located in chromosome17 and (B) *c-Ha-ras* oncogene located in chromosome 11. Genomic DNA for *c-Ha-ras* codon (C) 12 and (D) 61.

satellite instability in codon 61 of malathion in combination with estrogen-treated cells. We (60) previously reported *c-Ha-ras* allele loss and mutations in codons 12 and 61 induced by chemical carcinogens. We also previously determined the neoplastic phenotypes following treatment with benzo(a) pyrene (BP) and transfection with *c-Ha-ras* oncogene (61). Increased PCNA, Neu, ErbB-3 and cytokeratin 18 protein expression was observed in breast epithelial cells transformed with a chemical carcinogen and/or oncogene transfected that are not present in the MCF-10F.

Kiaris and Spandidos (18) indicated that overexpression of the Ras p21 protein can be found in different tissues according to numerous studies. Thus, breast tumors with higher Ras p21 protein expression than control was found in 60-70% of cases studied (62,63). In most cases, the somatic missense *Ras* mutations found in cancer cells introduce amino acid substitutions at positions 12, 13 and 61 (64). The incidence of allelic imbalance at different chromosomal levels was reported in our previous observations involving overexpression of *c-Ha-ras* oncogene (20,40). Oncogenic Ras proteins deregulate downstream effector pathways to confer the abnormal functional properties of cancer cells as deregulated cell growth and survival (64).

The specific genomic imbalances in microsatellite regions of specific genes seem to be important in cancer risk overall because tumor pathogenesis is in association of specific imbalances with disease prognosis and may be useful to apply therapy on specific targets. The knowledge of specific genetic changes and their biological consequences is critical to the understanding of breast cancer tumorigenesis. It can be concluded that the organophosphorous pesticide parathion and malathion induced malignant transformation of breast cells through genomic instability in the suppressor gene *p53* and the oncogene *c-Ha-ras*, considered pivotal in the cancer process.

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