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ORIGINAL PAPER

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Immunochemical analysis of protein expression in breast epithelial cells transformed by estrogens and high linear energy transfer (LET) radiation

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Abstract Breast cancer is a complex disease involving numerous genetic aberrations. Immunochemical analysis of protein expression is presented in a human breast epithelial cell line neoplastically transformed by high linear energy transfer (LET) α particle radiation in the presence of 17β estradiol (E) and in the parental human breast epithelial cell line (MCF-10F) which served as a non-tumorigenic control. The aim of this work was to determine the levels of mRNA and protein expression in control and transformed cells at various stages of the neoplastic process. The levels of mRNA and protein expression of PCNA, c-fos, JNK2 and Fra-1 were increased in the transformed cell line compared to the levels in non-tumorigenic control cells. The transforming factor Rho A was significantly increased only in the tumor cell line. Furthermore, the levels of mRNA and protein expression of ErbB2 were significantly increased in the transformed cell line and in tumor cells derived from the transformed cells after injecting them into nude mice. A decrease in RbA/p48 protein expression and mRNA levels was observed in cells treated with double doses of α particle radiation in the presence of estrogen, regardless of tumorigenicity. Such expression was lower than that in the control untreated MCF-10F cells. In summary, these studies show that estrogen and high LET-radiation induce changes in oncoprotein expression and mRNA levels of human breast cell lines. These changes are indicative of a cascade of events that characterize the process of cell transformation in breast cancer. These results provide evidence that multiple steps with consecutive changes are involved when normal cells become tumorigenic cells as a result of α particle irradiation and estrogen treatments.

Keywords Protein expression · Breast cancer – estrogens · Radiation · Northern blot

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Introduction

Epidemiological and experimental cancer studies strongly suggest that multiple genetic changes are required in the tumorigenic process. An increase in gene copies by amplification of specific DNA sequences occur frequently in tumor cells but not often in normal cells. The malignant progression of breast cancer involves a transition from a near normal process of controlled cell proliferation and survival, in early stages of this disease, to a highly abnormal regulation of these processes in metastatic disease. This progression often correlates with an increase in genetic/genomic instability (Schwab et al. 1984; Janocko et al. 1995; Shackney and Shankey 1997; Ingvarsson 1999). One of the hallmarks of genomic instability is gene amplification, often an amplification in the expression level of a normal gene. Among those genes that appear to be affected in breast cancer include the proliferating cell nuclear antigen (PCNA); proto-oncogenes such as *JUNK2 (JNK2), c-fos, FRA-1, ErbB2, transforming factor RhoA* and a tumor suppressor gene such as *Rb*.

It is probably that uncontrolled cell proliferation is an important first step in tumorigenesis and many tumor cells have altered expression of cell cycle related genes. Proliferative cell nuclear antigen (PCNA) is a 36 kDa non-histone intranuclear protein that serves as a cofactor for polymerase delta during the DNA synthesis stage of the cell cycle (Bravo et al. 1987; Waseem and Lane

1990; Woods et al. 1991). Detection of PCNA has considerable practical implication in revealing the proliferative activity of cells. It is synthesized in early G1 and S phases of the cell cycle and is strongly associated with the nuclear region where DNA synthesis takes place (Bravo et al. 1987; Waseem and Lane 1990; Woods et al. 1991; Balajee and Geard, 2001). Furthermore, PCNA immunocytochemistry has been used effectively as an alternative to traditional methods of evaluating the proliferation rate of tumors (Moriki et al. 1995; Alexiev 1996; Hall et al. 1990; Niewiadomska et al. 1998).

The *c-fos* and *c-jun* proto-oncogenes are members of the helix-loop/leucine zipper super-family (Sambucetti and Curran 1986; Bohman et al. 1987; Cohen et al 1989; Alkhalaf and Murphy 1992; Bamberger et al. 1999; Smith et al. 1999) and over-expression of their proteins have been detected in various types of cancers. *Fra-1*, a gene closely related to *c-fos*, is an early gene, the expression of which is induced by growth factors and mitogens (Philips et al. 1998). The *c-fos* and *Fra-1* form heterodimers with c-jun proteins that function as positive and negative transcription factors, by binding to specific DNA sequences [AP sites] (Cohen et al 1989). Members of the *c-fos* gene family, including *c-fos* and *Fra-1*, encode nuclear phosphoproteins that are rapidly and transiently induced by a variety of xenobiotic agents. These events function as transcriptional regulators for several genes (Cohen et al 1989). Another early responsive oncogene, JUNK2, a member of the MAP kinase family, designated c-jun NH_2-terminal kinase (JNK2), has also been identified (Derijard et al. 1994). It is activated by dual phosphorylation at the Thr-Pro-Tyr sequence during response to ultraviolet light and functions to phosphorylate *c-jun* at the amino terminal of serine regulatory sites (Philips et al. 1998).

In general, *RB* gene inactivation is very common in human breast carcinomas and various cell lines derived from human breast carcinomas (Horowitz et al. 1990). The *RB* gene is defective in all retinoblastomas as well as in other types of cancers (Bookstein et al. 1990). The *Rb gene* functions as a signal transducer, connecting the cell cycle clock with transcriptional mechanisms that mediate progression through the first two-thirds of G1 phase (Qian et al. 1993). *Rb* binds to double-stranded DNA in a non sequence-specific manner.

The *Neu* proto-oncogene, also known as *ErbB2, EGFR-2*, or *HER2*, is a member of a family of transmembrane receptor tyrosine kinases. *ErbB2* amplification is generally associated with high levels of ErbB*2* protein expression in breast tumors (Slamon et al. 1987; Di Fiore et al. 1987; Bacus et al. 1990; Paik et al. 1991; Kerns et al. 1993; Alimandi et al. 1995; Schonborn et al. 1995; Ceccarelli et al. 1999). It has been reported in approximately 30% of breast carcinomas. ErbB*2* protein levels have been proposed to be useful predictors of disease prognosis (Slamon et al. 1987).

Relatively few genes have been shown to directly affect the metastatic phenotype of breast cancer epithelial cells in vivo. The Rho family of proteins, including the Rho, Rac and Cdc42 subfamilies, are related to the small GTP binding proteins Ras and regulated diverse biological processes including gene transcription, cytoskeletal organization, cell proliferation and transformation. The Ras p21 family of guanine nucleotide proteins has been widely studied in view of its apparent role in signal transduction pathway and high frequency of mutations in human malignancies (Barbacid 1987; Morris et al. 1989). It is clear that the Ras protein (H-, K- and N- Ras p21) are members of a much larger superfamily of related protein (Chardin 1988). There are five mammalian Rho protein (Rho A, B, C, 7 and 8) are approximately 30% homologous to Ras and are expressed in a wide range of cell types (Maduale and Axel 1985; Yeramian et al. 1987; Olofsson et al. 1988; Adamson et al. 1992).

The role of estrogens in breast cancer and other cancers has been extensively analyzed (Dickson and Lippman 1988, 1995; Dickson 1999; Pasqualini et al. 1992). Breast epithelial cell division stimulated by prolonged hormonal treatment may increase the risk of breast cancer by increasing the risk of genetic errors (Dickson and Lippman 1988, 1995; Dickson 1999). It is known that the development of breast cancer involves many types of genes that need to be activated or inactivated in order to promote malignancy. However, the sequential steps leading to the alteration of genes that result in breast cancer are not clear. Gene amplification appears to be a late event in tumor progression and is mainly observed in cancer cells that have acquired genomic instability.

The objective of our study was to examine cell cycle proteins, i.e. *PCNA*, proto-oncogenes and tumor suppressor genes associated with breast cancer, i.e*., c-fos*, JNK2, *Fra-1*, *RbA/p48*, *ErbB2* and *transforming factor Rho A* using the spontaneously immortalized human breast epithelial cell line MCF-10F (Soule et al. 1990). Such genes were chosen because they are associated with cell cycle regulation, oncogenes and tumor supressor gene function and DNA replication. It is widely accepted that malignant transformation involves genetic changes that alter common regulatory mechanisms, resulting in uncontrolled cell proliferation.

The MCF-10F cell line was derived from subcutaneous mastectomy tissue obtained from a 36-year-old, parous, premenopausal woman with fibrocystic disease. It was initially developed as a mortal cell line (MCF-10M), which became senescent when transferred serially in 1.05 mM calcium. Such cells spontaneously gave rise to two immortal sublines, MCF10A (attached cells) and MCF-10F, (floating cells) which, to date, have been maintained in culture for many years. The media that sustains these cells is Dulbecco's modified essential medium and Ham's F-12 medium either with the customary calcium concentration of 1.05 mM (DMEM-High) or 0.04 mM calcium (DMEM-Low). Electron microscopy shows that both MCF-10F and MCF-10A have characteristic luminal ductal cells, although the concentration of calcium exerts a substantial influence on morphology. Cells grown in low calcium are

spherical whereas those growing in high calcium are cuboidal. MCF-10F has a near diploid karyotype and in vitro retains many characteristics of normal breast epithelium (Soule et al. 1990; Tait et al. 1990; Calaf et al. 2000; 2001). These cells also are anchorage dependent, non-invasive and non-tumorigenic in nude mice (Calaf and Hei 2000). The MCF-10F cell line was neoplastically transformed by a double dose of 60 cGy α particles in the presence of estrogen and subsequently showed gradual phenotypic and genetic changes, including altered morphology, increased cell proliferation, anchorage independency, invasive capabilities and tumorigenicity in nude mouse models (Calaf and Hei 2000). The identification of genes and their protein products that are critically involved in breast cancer is of crucial importance in understanding this disease. Since there is very little information available on radiation-induced breast cancer, an in vitro breast transformation model utilizing epithelial cells at different stages of neoplastic development provides a unique opportunity to delineate alterations involved in protein expression and genes related to this disease.

Materials and methods

Cell lines

The irradiated MCF-10F models were developed by irradiation with graded doses of 150 KeV/um α particles accelerated with the 4 MeV van de Graaf accelerator at the Columbia University Radiological Research Facilities (Calaf and Hei 2000). The following cell line was used as controls: a non-irradiated MCF-10F cell line. The experimental design is summarized in Table 2. The experimental cell lines were: a non-irradiated MCF-10F cell line treated with estrogen (E) (10^{-8} M), (MCF-10F + E) (Sigma-Aldrich, St. Louis, MO, USA). MCF-10F cells irradiated with a single dose of 60 cGy α particles (Alpha 1). Alpha 1 cell line subsequently treated with a second dose of radiation, 60 cGy/ 60 cGy (Alpha 3). Cell lines treated with one or double doses of radiation were cultured in the presence of E, 60 cGy + E, (Alpha 2) and 60 cGy/60 cGy + E (Alpha 4). Such cells were anchorage independent but non-tumorigenic in nude mice. The 60 cGy + E/60 cGy + E cell line (Alpha 5) was developed as follows: MCF-10F cell line was irradiated once, then treated with E for ten passages then subsequently irradiated again, and then treated with E for another ten passages. This cell line displayed anchorage independent growth, invasiveness and was tumorigenic in nude and SCID mice. The Tumor 2 cell line was derived from mammary tumors formed in nude mice after injection of the Alpha 5 cell line (Calaf and Hei 2000). Protein expression and RNA determination were measured after 30 passages in order to have non-transient transformed cell lines. Then studies were performed in a stable population. Experiments were repeated three times with cell lines of similar passages.

Light microscopy

Protein expression was evaluated by peroxidase immunochemical staining. Exponentially growing control cells and irradiated cells were plated on a four well glass chamber slide (Nunc Inc., Naperville, IL, USA). Cells were allowed to grow at a density of 1×10^4 cells in 1 ml of medium for 2–3 days until they reached 70% confluency. The cells were incubated with 1% H_2O_2 in methanol for 30 min, in order to block endogenous peroxidase, washed twice with a buffer solution, and fixed with buffered paraformaldehyde in PBS, pH 7.4, at room temperature. Subsequently, cell cultures were then covered with normal horse serum for 30 min at room temperature. Cultures were then washed once and incubated with the corresponding antibodies at a 1:500 dilution overnight at 4°C. The following antibodies were used: PCNA (PC10; sc56; mouse monoclonal), c-myc (9E10; sc40; mouse monoclonal), c-jun (H79; cs1694; rabbit polyclonal), JNK2 (D2; sc7345; mouse monoclonal), c-fos (4-10G; sc413; mouse monoclonal), Fra-1 (R20; sc605; rabbit polyclonal), RbA/p48 (C15; sc50; rabbit polyclonal), ErbB-2 (9G6; sc08; mouse monoclonal) and transforming factor Rho A (sc-418; mouse monoclonal antibody). All antibodies were from Biotechnology Inc., (Santa Cruz, CA, USA). The protein expression in the different cell lines was determined by using the avidin–biotin–horseradish immunoperoxidase peroxidase complex (Standard ABC kit; Vector, Burlingame, CA, USA). 3,3′-Diaminobenzidine (DAB) (Sigma-Aldrich) was used as a chromogen. For negative controls, duplicate samples were immunostained without exposure to the primary antibody or substituted with pre-immune serum. The extent of peroxidase staining was determined by calculating the percentage of positively stained cells in the population (from 0 to 100%) and 0, +1, +2, and +3 in relation to staining intensity. All numerical data were calculated as means plus and minus error standards. Statistical analysis was done with the *F*-test (Randomized Block) and comparisons between groups with the Bonferroni *t*-test with significance at p value < 0.05.

Confocal microscopy

To corroborated results of immunoperoxidase techniques, protein expression was also evaluated by immunofluorescent staining coupled with confocal microscopy. Exponentially growing control cells and irradiated cells, at similar passage numbers, were plated on a four well glass chamber slide (Nunc Inc.). Cells were allowed to grow at a density of 1×10^4 cells in 1 ml of medium for 2–3 days until they reached 70% confluency. The cells were incubated with 1% H_2O_2 in methanol for 30 min, in order to block endogenous peroxidase, washed twice with a buffer solution, and fixed with buffered paraformaldehyde in PBS, pH 7.4, at room temperature. Subsequently, cell cultures were then

covered with normal horse serum for 30 min at room temperature. Cultures were then washed once and incubated with the corresponding antibodies at a 1:500 dilution overnight at 4°C. Then cultures were washed in a buffer solution and incubated for 60 min at room temperature with anti-mouse or rabbit Rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Lab., West Grove, PA, USA) at a 1:1,000 dilution. Following several washes with a buffer solution, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Controls included cultures stained with either the primary or secondary antibodies alone, to monitor the background staining. Cells were viewed on a Zeiss Axiovert 100 TV microscope (Carl Zeiss) using a 40× 11.3 NA objective lens equipped with a laser scanning confocal attachment (LSM 410 Carl Zeiss). To excite the Rhodamine, an argon/krypton mixed gas laser (488 nm) was used. Fluorescent images were collected in black and white and changed to green color by Photoshop. Composite images were quantified by using Adobe Photoshop, version 5.0. A semi-quantitative estimation of the area and the intensity of the staining of the cells present in the culture dishes were based on the relative staining of protein expression by the control and by the transformed cells. The number of immunoreactive cells (30 cells/field, ×400) was counted in several randomly selected microscopy fields per sample as previously described (Calaf and Hei 2000). All numerical data were calculated as means and error standards. Statistical analysis was done with the *F*-test (randomized block) and comparisons between groups with the Bonferroni *t*-test with significance at p value < 0.05.

Preparation of cDNA probes and Northern hybridization analysis

Total RNA was isolated (Roy et al. 2001a) from both the control and transformed cell lines with Trizol solution (Gibco-BRL, Long Island, NY, USA). Each sample, comprising 500 µg of total RNA, was treated with 5 µl of DNAse I (10 units/µl) (Boehringer Mannheim, Indianapolis, IN, USA) for 60 min at 37°C. Then 10X Termination Mix (0.1 M EDTA, pH 8.0, 1 mg/ml glycogen) (Clontech, Palo Alto, CA, USA) was used to stop the reaction. Each sample was then purified following established procedures (Sambrook et al. 1999). The amount of each purified RNA sample was first measured by a spectrophotometer and then electrophoresed on denaturing formaldehyde/agarose/ethidium bromide gel to check its quality.

A sample of 0.5–1 µg of total RNA was used for first strand cDNA synthesis with the Advantage RT-for-PCR Kit (Clontech) with oligo $(dT)_{18}$ primer. Approximately 100 ng of the first-strand cDNA synthesis product was used for carrying out RT-PCRs with gene-specific primers, with an initial denaturation at 94°C for 4 min followed by 35 cycles. Each cycle comprised denaturation at 94°C for 30 s annealing at 65°C for 1 min, and extension at 68°C for 1 min, with a 5-min final extension at 68°C. The PCR product was run on a 1.2% agarose gel. DNA bands were eluted from the gel and purified by using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA). These gene specific double stranded bands were used as a probe in Northern blotting by radioactive label (α-[^{32} P] dATP) label with the Multiprime DNA Labelling Systems (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) and subjected to Northern hybridization analysis.

For preparing northern blot filters total RNA was electrophoresed in a 1% (w/v) agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N) (Amersham-Pharmacia Biotech, Piscataway, NJ) (Sambrook et al., 1999). RNA transfer was confirmed by visualization of ethidium bromide stained RNA under UV light. Blots were UV cross-linked and stored at 4°C until hybridization was carried out. Every lane was equally loaded with 10 µg. Human β-Actin (Clontech), in contrast was loaded with 20 µg and was used as a control to get a clearer resolution of the housekeeping genes which, ultimately were compared with the expression of other genes. The blot was then exposed to Kodak X-OMAT AR film at −80°C for 24 h. Intensity was assessed by a densitometer scanner and evaluated by a computer program (Molecular Dynamics, NJ). The primers for genes selected to develop cDNA probes are shown in Table 1.

Results

Table 2 summarizes the anchorage independent growth, invasion and tumorigenic assays of the cell lines examined in these studies. Anchorage independent growth and invasiveness were observed in only those cell lines that received double doses of α particle irradiation either in the presence or absence of E (Alpha 3 and Alpha 5, and Tumor 2). Such cells formed agar-positive clones only after 25 passages in culture. Mammary tumor developed in nude mice, after cell line injection, in only the cell line that was treated with two sequential α particle + estrogen treatments (Alpha 5). Injections of these cell lines derived from mice mammary tumors also formed mammary tumors in similar animals (Tumor 2). Thus differences in the phenotypic expression of cell anchorage independent growth, cell invasion and cell tumorigenicity in nude and/or SCID mice are clearly seen in these cell lines.

Figures 1, 2 and Table 3 provide the quantification of peroxidase staining (Fig. 1, Table 3) and immunofluorescent images (Fig. 2) of PCNA, c-myc, c-jun, JNK2, c-fos, Fra-1, RbA/p48, and ErbB2 protein expression. PCNA protein expression of α-particle-irradiated MCF-10F cells treated with or without E was quantified by peroxidase and fluorescent stain imaging. Using exponentially growing cell cultures, the PCNA protein expression level was over-expressed in the non-tumori-

Table 1 Primers for genes selected to develop cDNA probes

GAN[a]	Gene name	Product length (bp)[b]	Primer sequence[c]
X57800	*PCNA* (proliferating cell nuclear antigen)	475	1-GAACCTTGTTTTGATGGTAGTCATATGTGA 1′-TCTTCATCCTCGATCTTGGG
V00568	*c-myc* proto-oncogene	222	1-GCTCCTGGCAAAAGGTCAGAGTCTGG 1′-GGGGCTGGTGCATTTTCGGTTGTTGC
J04111	*c-jun* oncogene	460	1-TAGGGTGGAGTCTCCATGGTGACGGGCGGG 1′-ATTGTGACAGAATGCTGTTATAAATATTCA
AF052468	*JNK2 (mitogen activated* protein kinase)	534	1-GAGCGACAGTAAATGTGACAGTCAGTTTTA 1′-AGAGGATTAAGAGATAACTTCAGAAGCAGC
V01512	*c-fos oncogene*	320	1-AATTGGGGATGGGGGTAGGGGCATTCCT 1′-GATAGGGTTTTGCTGTGTCACCCAGGCTGG
X16707	*Fra -1* (fos related antigen)	280	1-CCCTGCCGCCCTGTACCTTGTATC 1′-AGACATTGGCTAGGGTGGCATCTGCA
X74262	*Rbbp4*	211	1-AGAGTGCAACCCAGACTTGCGTCTCC 1′-CCAGGAAACATCTTCTACTACTGCCG
M11730	*ErbB2*	480	1-CTGGAATGGGAAGCA 1′-GCCAGCAAAGAAATCTTAGACGT
G15871	β *-Actin(ACTB)*	220	1-ACCGAAGCCCCTCTTAACCC 1′-GTATGGCTGACACCATCACC

[a]Gen Bank accession number
[b]Length of cDNA product amplified by gene-specific RT-PCR analysis
[c]PCR primer sequences used to generate a product of the indicated size, listed in 5′ to 3′ orientation.
1 forward; *1′* reverse

genic as well as in irradiated and estrogen-treated tumorigenic cell lines when compared with the control MCF-10F cell line. Among the MCF-10F cell lines exposed to 60 cGy (Alpha 1) and double doses of 60 cGy α particles (Alpha 3) there was a moderate increase in protein expression, in comparison to the control MCF-10F cell line. Whereas, the tumorigenic Alpha 5 and the Tumor 2 cell lines had a significantly ($P < 0.05$) higher protein expression of this biomarker than the control cell line and the other groups. The granular distribution was homogeneous among the cells (Fig. 3). These images show the intensity and distribution of protein expression in the nucleus of the control MCF-10F cell line; the tumorigenic cell line (Alpha 5) and the Tumor 2 cell line. The granular distribution was remarkably greater in Alpha 5 and Tumor 2 cell lines than in the control cell line.

An increase in c-*myc* and c-*jun* protein expression was detected in all the irradiated populations compared with control MCF-10F cells. This increase occurred irrespective of E treatment (Fig. 1, Table 3). There was no significant difference in c-myc expression between cells irradiated with either a single 60 cGy dose (Alpha 1) or with a double dose of α-particles (Alpha 3) in comparison to the control MCF-10F cell line. Those cells irradiated with a double dose of radiation had significantly higher ($P < 0.05$) c-jun expression levels than those cells irradiated only once and the controls. However, the tumorigenic and tumor cell lines had higher protein expression than the other cell lines.

Table 2 Origin and phenotypic characteristics of cell lines

Cell lines	Origin	*AIA*	*IA*	*TA*
MCF-10F	MCF-10F parental cells	−	−	−
MCF-10F + E	MCF-10F treated with 17β estradiol 10^{-8} M	−	−	−
Alpha 1	MCF-10F cell line irradiated once with 60 cGy: 60 cGy cell line	−	−	−
Alpha 2	Alpha 1 cell line treated with E: 60 cGy + E cell line	−	−	−
Alpha 3	Alpha 1 cell line irradiated again with 60 cGy: 60 cGy/60 cGy	+	+	−
Alpha 4	Alpha 1 cell line irradiated again with 60 cGy and treated again with E: 60 cGy/60 cGy + E cell line	+	+	−
Alpha 5	Alpha 2 cell line irradiated again with 60 cGy and treated again with E 60 cGy + E/60 cGy + E cell line	++	++	++
Tumor 2	Cell line derived from mammary tumors formed in nude mice after injection with the Alpha 5 cell line	++	++	++

AIA Anchorage Independent Assay: colony-forming efficiency in agar fluctuated from 1 to 3%
IA Invasion Assay: invasive characteristics of control and irradiated MCF-10F cells scored 20 h after plating onto matrigel basement membranes using modified Boyden's chambers constructed with multi-well cell culture plates and cell culture inserts
Positive signs represent the results in relation to the number of cells that crossed the filters

TA Tumorigenic Assay: tumors formed in nude mice. Average of 6 animals/group
− No anchorage independent growth, invasiveness or formation of tumors in the nude mice
+ Anchorage independent growth, invasiveness and formation of tumors in the nude mice

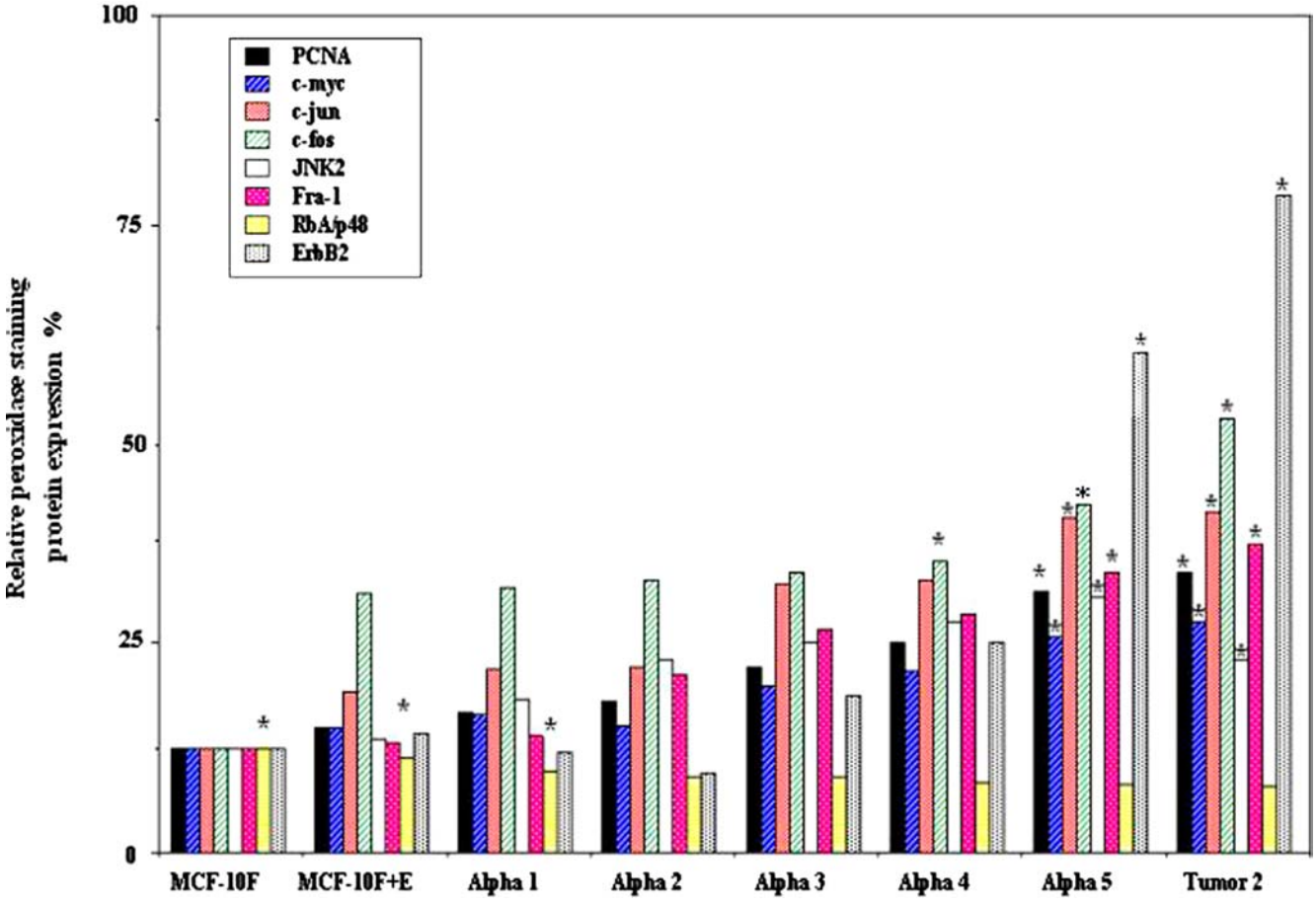

Fig. 1 Average relative amounts of peroxidase staining of protein expressed of PCNA, c-fos, JNK2, Fra-1, RbA/p48, and ErbB2 in MCF-10F, MCF-10F + E, Alpha 1, Alpha 2, Alpha 3, Alpha 4, Alpha 5 and Tumor 2 cell lines. *Bars* in the figure indicate mean ± standard error of the mean. $^*P < 0.05$ versus MCF-10F and other groups. The extent of peroxidase staining was determined by calculating the percentage of positively stained cells in the population (from 0 to 100%)

There was no significant difference between JNK2 protein irradiated with single or double doses of αparticles. However, there was a significant increase in each of them in comparison to controls. The tumorigenic and tumor cell lines had higher JNK2 protein expression than the other cell lines. The intensity and distribution of JNK2 protein expressions in control MCF-10F, Alpha 5 and Tumor 2 cells stained by peroxidase and fluorescent staining can be seen in Fig. 3.

MCF-10F cell lines exposed to a single dose of α particles, either in the presence or absence of E, expressed c-fos protein levels similar to the control MCF-10F cell line (Figs. 1, 2, Table 3). However, an increase in c-fos protein expression was detected in MCF-10F cell lines receiving double doses of α particles compared to control MCF-10F cell line. The E treatment after double doses of 60 cGy significantly ($P < 0.05$) enhanced c-fos expression compared to cells irradiated only once. The c-fos expression in the Alpha 5 and the Tumor 2 cell lines was significantly ($P < 0.05$) higher than the control MCF-10F cell line. In Fig. 3, representative peroxidase and confocal images of c-fos protein expression of MCF-10F, and Tumor 2 cell lines can be seen.

The Fra-1 expression level among MCF-10F cell lines exposed once to α-particles was similar to those of control MCF-10F cell lines (Figs. 1, 2, Table 3). However, estrogen increased such expression. It was evident that those cells receiving a double dose of α-particles had significantly higher expression levels of Fra-1 than cells irradiated only once. The E treatment *after* two doses of 60 cGy α-particles did not enhance Fra-1 expression. However, the non-tumorigenic cell line Alpha 4, and the tumorigenic cell line Alpha 5 and Tumor 2 cell line showed an expression level that was significantly greater ($P < 0.05$) than those irradiated once, irrespective of the E treatment, and the MCF-10F and MCF-10F + E cell lines. Representative images of Fra-1 after peroxidase and immunofluorescent staining show the intensity and distribution of protein expression in the nucleus of control MCF-10F cell line, the tumorigenic Alpha 5 and Tumor 2 cell lines. The granular distribution was greater in the two tumorigenic cell lines than in the control MCF-10F as seen in Fig. 4.

Decreased RbA/p48 protein expression was detected in all the irradiated cell lines, when compared with the control MCF-10F cell line (Figs. 1, 2, Table 3). There

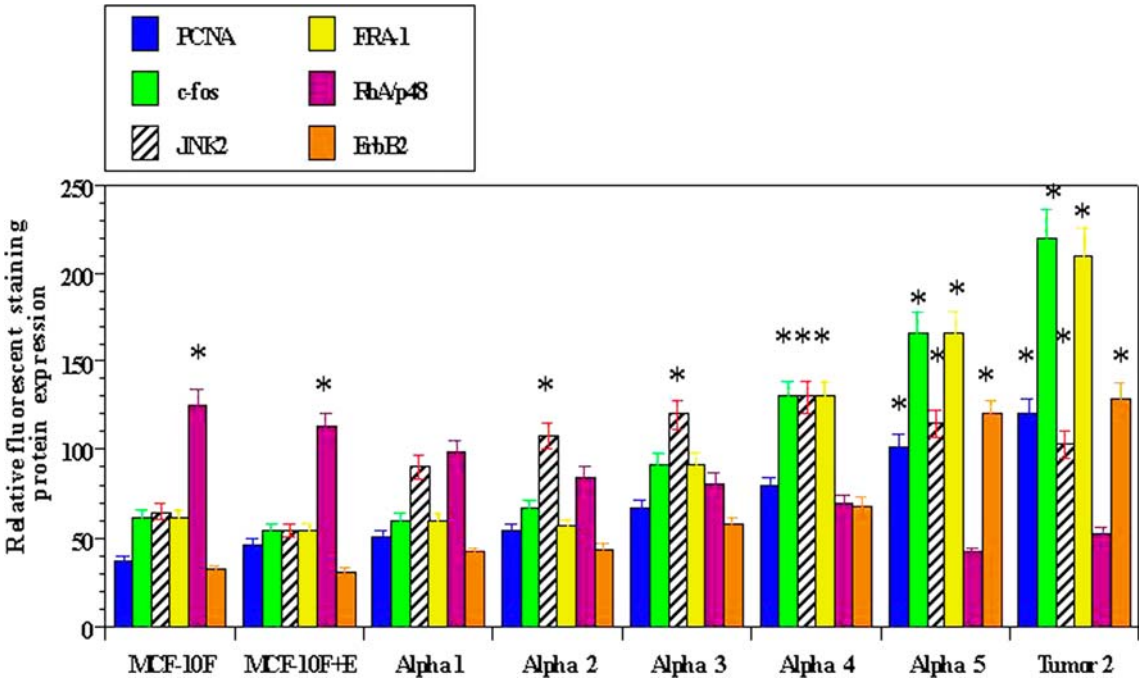

Fig. 2 Average relative amounts of immunofluorescent staining of protein expressed by cells for PCNA, c-fos, JNK2, Fra-1, RbA/p48, and ErbB2 in MCF-10F, MCF-10F + E, Alpha 1, Alpha 2, Alpha 3, Alpha 4, Alpha 5 and Tumor 2 cell lines. *Bars* in the figure indicate mean ± standard error of the mean. $*P < 0.05$ versus MCF-10F and other groups

was a significant decrease ($P < 0.05$) in the levels of RbA/p48 expression between cells irradiated with double 60 cGy doses of α particles (Alpha 3). There was also a decrease in levels of RbA/p48 expression in the presence of E in Alpha 5 and Tumor 2 cells in comparison to the MCF-10F cell line. There was no significant difference in the levels of RbA/p48 protein expression between cells irradiated with a single dose of α particles and the MCF-10F cell line. The decrease in the RbA/p48 levels of imaging staining of MCF-10F, Alpha 5 and Tumor 2 cell lines can be seen in Fig. 4.

The cell lines treated with E and those irradiated with single or double doses of 60 cGy α particles also exhibited a higher ErbB2 protein expression but this was not significantly different from the controls (Figs. 1, 2, Table 3). However, the tumorigenic Alpha 5 and the Tumor 2 cell lines showed ErbB2 protein expression levels significantly ($P < 0.05$) greater than the non-tumorigenic cell lines and the control. Such intensity can be appreciated in cell membranes using the peroxidase technique in the Alpha 5 cell line and the Tumor 2 cell line as seen in Fig. 4.

The cell lines treated with E and those irradiated with single or double doses of 60 cGy α particles also exhibited a higher transforming factor RhoA protein expression than the controls (Fig. 5a). However, Tumor 2 cell line showed significantly ($P < 0.05$) higher transforming factor RhoA protein expression than the tumorigenic cell line Alpha 5 and the control MCF-10F. Such intensity can be appreciated in the nucleus in Tumor 2 cell line by using the peroxidase (abc) and immunofluorescent techniques (Fig. 5b).

Table 3 Immunochemical analysis of protein expression in breast epithelial cells transformed by estrogen and high LET radiation

Cell lines	PCNA[a]	c-myc[a]	c-jun[a]	JNK2[a]	c-fos[a]	Fra-1[a]	RbA/p48[a]	ErbB2[a]
MCF-10F	+1	+1	+1	+2	+1	+2	+3	0
MCF-10F + E	+1	+1	+1	+2	+1	+2	+2	+1
Alpha 1	+1	+1	+1	+2	+2	+2	+2	+1
Alpha 2	+1	+1	+1	+2	+2	+3	+2	+1
Alpha 3	+1	+1	+2	+3	+2	+3	+2	+1
Alpha 4	+2	+1	+2	+3	+2	+3	+2	+2
Alpha 5	+3	+3	+3	+3	+3	+3	+1	+3
Tumor 2	+3	+3	+3	+3	+3	+3	+1	+3

[a] Immunochemical detection of PCNA, c-myc, c-jun, JNK2, c-fos, Fra1, RbA/p48, ErbB2 protein expression in control and irradiated MCF-10F cells. The extent of peroxidase staining was determined by calculating the percentage of positively stained cells in the population (from 0 to 100%)

Staining intensity of reaction expressed as: *0* negative, *+1* weakly positive, *+2* moderately positive, *+3* strongly positive

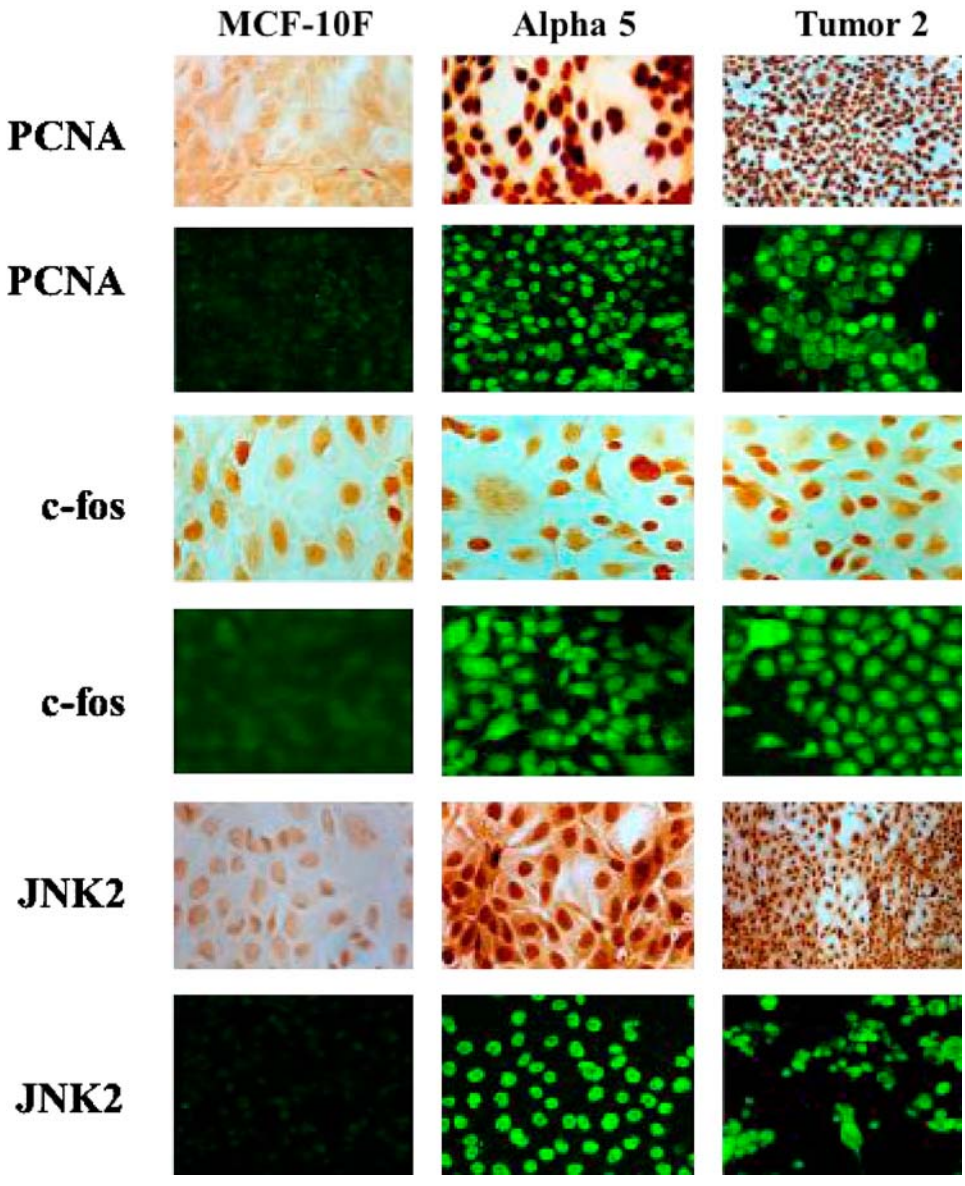

Fig. 3 Representative peroxidase and fluorescence in confocal microscopical images of PCNA, c-fos and JNK2 protein expression in MCF-10F, Alpha 5 and Tumor 2 cell lines

Altered mRNA levels of *PCNA, c-myc, c-jun, JNK2, c-fos*, *Fra-1*, *Rbbp4* and *ErbB-2* in MCF-10F, MCF-10F + E, Alpha 1, Alpha 2, Alpha 3, Alpha 4, Alpha 5 and Tumor 2 cell lines are shown in Fig. 6. Results indicated that radiation and estrogen induce altered mRNA expression levels in these genes. Of these eight genes, seven (*PCNA, c-myc, c-jun, JNK2, c-fos, Fra-1, and ErbB2)* showed up-regulation, and one them, *Rbbp4* gene, showed down-regulation at each of the stages of neoplastic transformation.

Discussion

A series of changes in protein and mRNA expression levels of various oncogenes and tumor suppressor genes in MCF-10F cell lines were identified in this study. These alterations correspond to ongoing phenotypic changes associated with tumor progression following exposure of the MCF-10F cell line to α particle irradiation and/or treatment with estrogen. Protein expression was altered in the earlier stages of progression to tumorigenesis while others were altered only in the tumorigenic cell lines and in those cells originating from a tumor formed in the nude mice (Calaf and Hei 2000). Protein expression and mRNA levels from PCNA, c-fos, JNK2, Fra-1, RbA/p48, ErbB2 and transforming RhoA were modified in their protein expression in these treated cell lines. Our present study demonstrates that exposure of the MCF-10F cell line to double doses of α particles, followed by exposure to estrogen treatment for several passages, named Alpha 5 results in more complex protein expression than in the control cell line and in cell lines treated with a single dose of radiation without or with estrogen as Alpha 1 and Alpha 2, respectively.

We have previously analyzed protein expression of several cell cycle regulators, oncogenes and tumor sup-

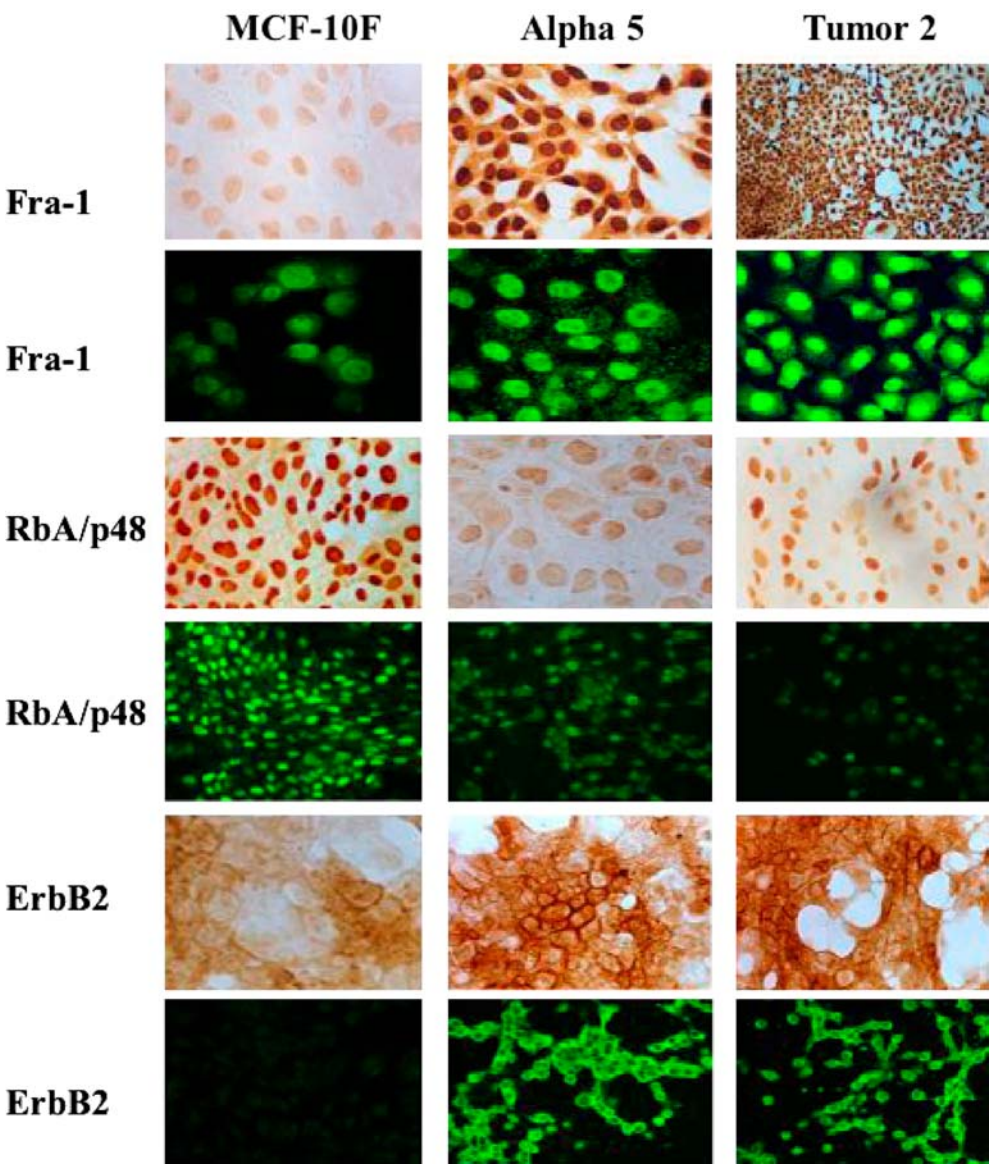

Fig. 4 Representative peroxidase and fluorescence in confocal microscopical images of FRA-1, RbA/p48, and ErbB2 protein expression in MCF-10F, Alpha 5 and Tumor 2 cell lines

pressor genes associated with breast cancer and others such as BRCA1, BRCA2, Rad51 (Calaf and Hei 2000), c-myc, c-Ha-ras, c-jun and mutant p53 oncoprotein expression (Calaf and Hei 2001). Pre-neoplastic and neoplastic human breast epithelial cells induced by high-LET α particles and estrogen allows us to examine various aspects of protein expression and provides a basis for understanding the complex interaction of genes and hormones.

The present results show that PCNA is over-expressed in the non-tumorigenic as well as in the irradiated and estrogen-treated tumorigenic MCF-10F cell line which when compared with the control MCF-10F cell line. PCNA has been used as an index of cell proliferation in breast cells by staining normal tissue adjacent to epithelial neoplasm (Alexiev 1996; Hall et al. 1990; Calaf et al. 1995). These observations could be explained by an altered regulation of PCNA gene expression by autocrine or paracrine growth factors. Such mechanisms may result in an increase in mRNA levels and consequently an increase in the protein level of PCNA in neoplastic cells.

Cell cycle regulators studied in these models confirmed the importance of understanding not only oncogenes and tumor suppressor genes but also cell cycle events that occur in parallel. The protein expression and the mRNA levels of c-fos among MCF-10F cell lines exposed to αparticles once, either in the presence or absence of E, was similar to the control MCF-10F cell line. An increased level of protein expression was detected in MCF-10F cell line receiving double doses of α particle radiation compared with control MCF-10F cell line. E treatment after double doses of 60 cGy also enhanced c-fos expression and mRNA levels even more than in those cell lines irradiated only once. In the tumorigenic and the tumor-derived cell lines similar phenomena was observed when compared with the control MCF-10F cell line.

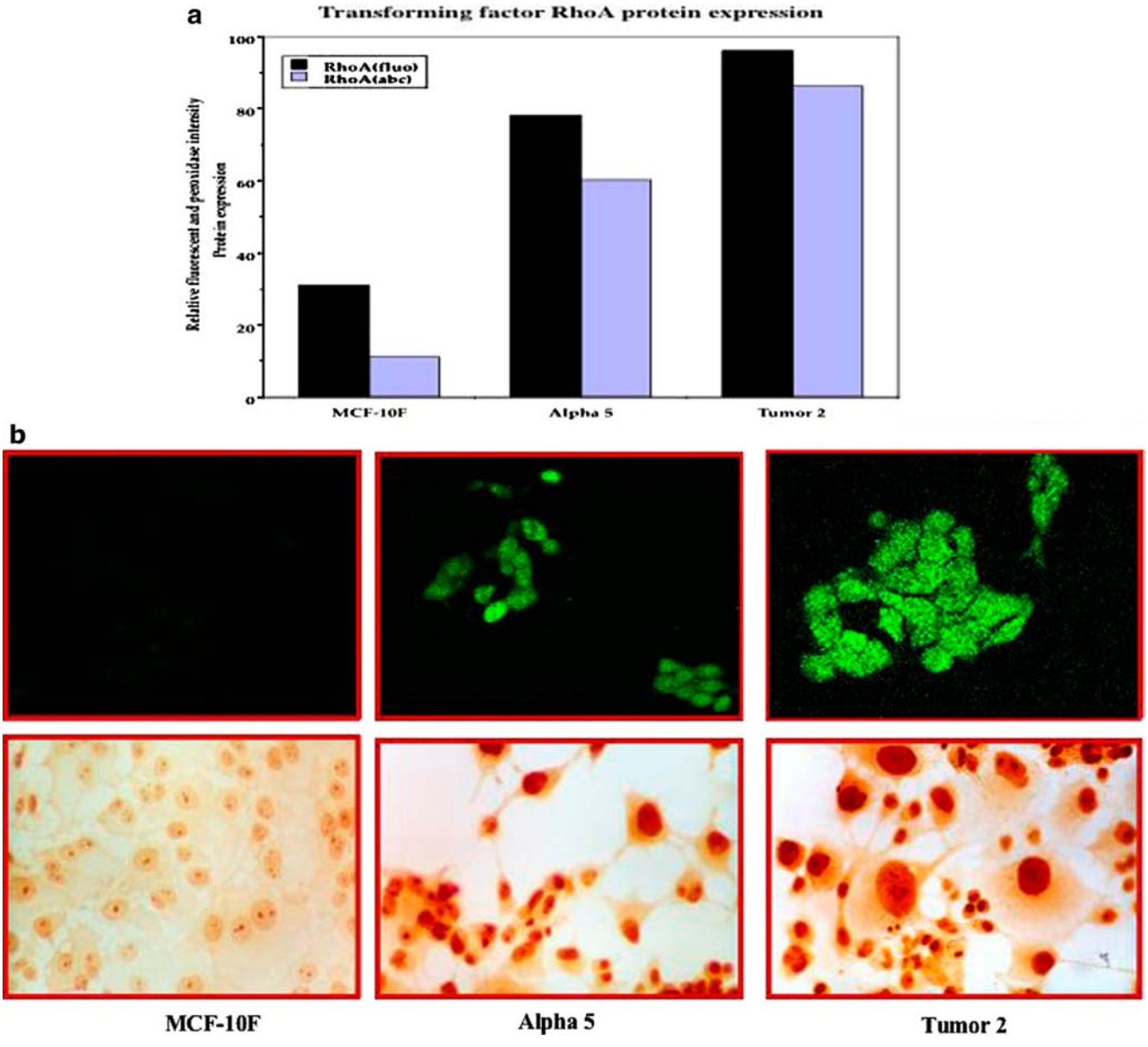

Fig. 5 **a** Average relative amounts of immunofluorescent staining of protein expressed by cells for transforming factor RhoA in MCF-10F, Alpha 5 and Tumor 2 cell lines. *Bars* in the figure indicate mean ± standard error of the mean. $*P < 0.05$ versus MCF-10F and other groups. **b** Representative immunofluorescence images from confocal microscope of transforming RhoA protein expression in MCF-10F, Alpha 5 and Tumor 2 cell lines

Protein expression of c-myc and c-jun quantified by peroxidase imaging corroborated immunofluorescent results previously reported (Calaf and Hei 2000). Our studies have shown that c-myc and c-jun expression increased in the transformed cell lines at all stages of transformation, right up to the tumorigenic stage of transformation. Protein products of at least three nuclear-oncogenes, *c-myc, c-fos, and c-jun*, appear to be induced by both estrogen and progesterone in various breast cancers (Dickson and Lippman 1995). A substantial body of evidence supports a causal link between the induction of these genes and the proliferation process. Thus, nuclear proto-oncogenes represent a unifying mechanistic link between the actions of growth-promoting steroids and cell growth in diverse tissues.

Interestingly, an increased in JNK2 protein expression was found during the first stages of cell transformation. An increase in JNK2 protein expression was more evident in MCF-10F cell lines irradiated with a single and a double dose of α particles, either in the presence or absence of E, in comparison to the control MCF-10F cell line. Furthermore, there was no significant difference among those irradiated with single or double doses of α particles, or with the tumorigenic and the Tumor 2 cell lines. The JNK2 mRNA levels were also higher in the single and double doses of estrogen and irradiated cell lines compared to the control MCF-10F and the MCF-10F + E cell lines. Various authors (Monno et al. 2000) have shown that specific stimuli use

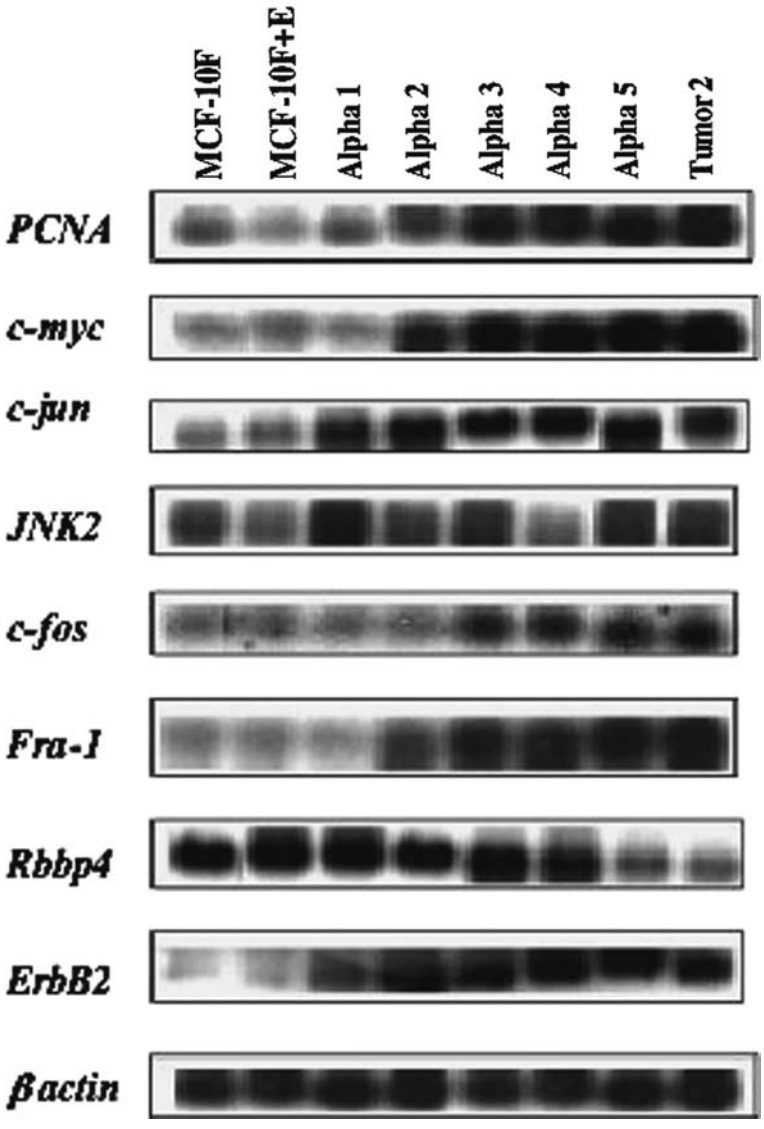

Fig. 6 Northern blot analysis of *PCNA, c-myc, c-jun, JNK2, c-fos, FRA-1, Rbbp4, ErbB2* mRNA expression of MCF-10F, MCF-10F + E, Alpha 1, Alpha 2, Alpha 3, Alpha 4, Alpha 5 and Tumor 2 cell lines. Human β-actin was used as a control to confirm similar loading in each sample

different mechanisms to recruit MAP kinases to regulate the c-jun N-terminal kinase (JNKs) pathways. For instance it has been shown that insulin growth factor activates the JNKs in the MCF-7 breast cancer cell line, suggesting that JNK may contribute to the mediation of alterations in gene expression and, induce possible cell growth of transformed cells.

Estrogen influenced the *Fra-1* gene expression both at protein and mRNA level among the irradiated MCF-10F cell lines. It was evident that those cells receiving a double dose of α-particles had significantly higher expression levels of Fra-1 than cells irradiated only once. However, the Alpha 4 non-tumorigenic cell line and the tumorigenic Alpha 5 and Tumor 2 cell lines showed an expression level that was significantly greater than those irradiated only once.

Our previous findings (Roy et al. 2001a) indicated that high LET radiation altered various oncogenes and tumor suppressor genes in this established model by studying gene expression through a cDNA expression array (Roy et al. 2001a). We reported that several genes were altered at various different stages of the neoplastic process while only a few of them were altered exclusively at the tumorigenic stage (Roy et al. 2001a, 2003). The results showed that out of a total of 190 genes, about 40 genes were differentially expressed due to an initial dose of high LET radiation. The cDNA expression array identified that *c-myc* and *Fra-1* were among the genes differentially expressed at all stages of the transformation process. The *c-myc* and *Fra-1* genes appear to be up regulated, as it was also demonstrated by the protein expression in the established model (Calaf and Hei 2001). Others (Philips et al. 1998) have also shown that Fra-1 expression level of AP-1 protein is modulated by estradiol in breast cells. Others have considered that both *c-jun and Fra-1* are crucial mediators of the ras-transformation of NIH3T3 clones (Mechta et al. 1997). Thus, it is possible that high Fra-1 protein expression could have also influenced these effects.

It is also interesting to note the sequential down-regulation of RbA/p48 protein expression and mRNA levels at all stages of neoplastic transformation right up to the tumor stage. A decreased RbA/p48 protein expression was detected in cells irradiated with single and double 60 cGy dose of α particles, in the presence of E, in the tumorigenic cell line Alpha 5 and in the Tumor 2 cells compared to the control MCF-10F cell line. Results from cDNA expression array also confirmed that *Rbbp4* was down regulated among the genes differentially expressed at all stages of the neoplastic transformation process (Roy et al. 2001a). Other authors have shown that the protein product of RbA/p48 can regulate transcription both in vivo and in vitro by interacting with cell cycle regulators as cdc2 kinase and mammalian D-type cyclin (Nielsen et al. 1997). Thus, down regulation of *Rbbp4* could indicate a loss of tumor suppressor function, which ultimately may lead to neoplastic transformation of normal cells.

The tumorigenic Alpha 5 cell line and the Tumor 2 cell lines showed ErbB2 protein expression levels that were greater than the non-tumorigenic cell lines and the control MCF-10F. The tumorigenic cell line Alpha 5 showed aberrant expression of ErbB2 oncoproteins at levels similar to that seen in tumors derived from these same cell lines, following injecting into nude mice. These two cell lines had different morphology than the control MCF-10F cell line, being aggregated as ''mini-foci'' of cells as seen with confocal microscopy. Since no change in ErbB2 expression was observed in the non-tumorigenic irradiated cell lines, such suggests that these oncoproteins may play a key role in the late stages of breast tumorigenesis. It has been reported that ErbB2 oncoprotein may be involved in the malignant transformation of breast tissues (Slamon et al. 1987; Alimandi et al. 1995).

High cellular proliferative activity and over-expression of the ErbB2 oncoprotein has been receiving much attention because of its potential as a prognostic indicator for breast carcinoma. The prognostic value of ErbB*2* oncogene amplification has also been compared with conventional histopathological factors used in breast cancer (Slamon et al. 1987; Van De Vijver et al. 1988; Bacus et al. 1990; Kerns et al. 1993). Alterations in ErbB2 protein expression in our irradiated and estrogen treated MCF-10Fcell lines may complement the over-expression of the c-myc, c-jun, c-Ha-ras oncogene and the p53 tumor suppressor gene detected in these cell lines (Calaf and Hei 2000; 2001). These results imply that biological cooperation between ErbB2 and other oncogenes may occur in human malignancy as it has been demonstrated with several human breast tumor cell lines (Bacus et al. 1990). Breast tumor formation in cells treated with double doses of 60 cGy αparticles in the presence of E and subsequently injected into nude mice suggests that tumorigenicity may be related to the high expression of many oncogenes. Several investigators have shown that cooperation of more than one oncogene in breast tumorigenesis may explain how breast cancer develops and progresses (Schwab et al. 1984; Janocko et al. 1995; Shackney and Shankey 1997; Ingvarsson et al. 1999). Changes observed during the process of MCF-10F cell transformation after single or double doses of radiation and estrogen treatments paralleled the progressive changes at the molecular level indicating a cascade of molecular events responsible for breast tumor formation.

Genetic alterations were also related to breast tumorigenesis analyzed by allelic imbalances [loss of heterozigosity (LOH) and microsatellite instability (MSI)] in our MCF-10F model with various markers of chromosomes 6, 17, 11, 8 (Roy et al. 2001b, 2003). A progressive degree of MSI and LOH were detected at chromosome 6q, 11p, 11q, 17p and 17q in 3 of the stages (Alpha 3 and Alpha 5 and Tumor 2 derived from Alpha 5). Such changes suggest that LOH may be involved in specific stages of breast tumorigenesis. Furthermore, BRCA1 protein expression was also down regulated in Tumor 2 and there was a complete deletion in locus17q12-q21 (marker D17S579), the place where BRCA1 is located (Roy et al. 2001b). However, LOH and MSI were found in that particular locus before tumors occurred. The *c-jun* and *c-Ha-ras* mRNA was also increased in the Alpha 5 and Tumor 2 cells (Roy et al. 2001a, 2003).

It was interesting to observe that in the cDNA array several genes were altered exclusively only in the tumor stage of the neoplastic process (Roy et al. 2001a, 2003). Among them, the transforming factor Rho A protein expression was 1.2 times higher in the tumor cell line than in the MCF-10F control cells. It has been reported that members of the Rho family are key regulators of the actin cytoskeleton, particularly in relation to the cell shape changes and the adhesion dynamic that drive cell migration (Xie et al. 2005). It has also been reported an activation of the function of Rho A in relation to cell motility and morphology. The morphology of this tumor cell line is indicative of neoplastic transformation.

Epidemiology of cancer strongly suggests that as a cell progresses through various stages, resulting in a tumor, it requires the accumulation of several genetic lesions. It appears, therefore, that cooperation of several genes contributes to the development and progression of breast cancer. It is also probably that alterations in crucial nuclear factors that control the cell cycle can bypass the requirements for certain paracrine and endocrine growth factors. Another possibility is that deregulation of these genes will confer a selective advantage in the later stages of tumor development and progression. The transmission of the cancerous properties during cell division is not necessarily genetic, but it may involve epigenetic changes like methylation or imprinting as well. In summary, these studies provide that many cellular changes are needed before normal cells can become tumor cells and that several steps are involved in this process. Genes and their protein products that are altered at any of these exclusively at the tumorigenic stages should prove very useful information in our understanding of tumorigenic process.

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