

# Apoptotic activity of 5-fluorouracil in breast cancer cells transformed by low doses of ionizing $\alpha$ -particle radiation

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**Abstract.** Globally, breast cancer in women is the leading cause of cancer death. This fact has generated an interest to obtain insight into breast tumorigenesis and also to develop drugs to control the disease. Ras is a proto-oncogene that is activated as a response to extracellular signals. As a member of the Ras GTPase superfamily, Rho-A is an oncogenic and a critical component of signaling pathways leading to downstream gene regulation. In chemotherapy, apoptosis is the predominant mechanism by which cancer cells die. However, even when the apoptotic machinery remains intact, survival signaling may antagonize the cell death by signals. The aim of this study was to evaluate 5-fluorouracil (5-FU) in cells transformed by low doses of ionizing  $\alpha$ -particle radiation, in breast cancer cell lines on these genes, as well as apoptotic activity. We used two cell lines from an *in vitro* experimental breast cancer model. The MCF-10F and Tumor2 cell lines. MCF-10F was exposed to low doses of high linear energy transfer (LET)  $\alpha$ -particles radiation (150 keV/ $\mu$ m). Tumor2, is a malignant and tumorigenic cell line obtained from Alpha5 (60cGy+E/60cGy+E) injected into the nude mice. Results indicated that 5-FU decreased *H-ras*, *Rho-A*, *p53*, *Stat1* and increased *Bax* gene expression in Tumor2 and decreased *Rac1*, *Rho-A*, *NF- $\kappa$ B* and increased *Bax* and caspase-3 protein expression in Tumor2. 5-FU decreased *H-ras*, *Bcl-xL* and *NF- $\kappa$ B* and increased *Bax* gene expression. 5-FU decreased *Rac1*, *Rho-A* protein expression and increased *Bax* and caspase-3 protein expression in MDA-MB-231. Flow cytometry indicated 21.5% of cell death in the control MCF-10F and 80% in Tumor2 cell lines. It can be concluded that 5-FU may exert apoptotic activity in breast cancer cells transformed by low doses of ionizing  $\alpha$ -particles *in vitro* regulating genes of Ras family and related to apoptosis such as *Bax*, *Bcl-xL* and *NF- $\kappa$ B* expression.

## Introduction

Globally, breast cancer in women is the leading cause of cancer death with 1,383,500 estimated new cases each year (1). This fact has generated an interest to obtain insight into breast tumorigenesis and also to develop drugs that effectively combat the disease. Currently, women with advanced breast cancer develop metastases which account significantly for morbidity and mortality. Ras is a proto-oncogene that is activated transiently as a response to extracellular signals such as growth factors, cytokines, and hormones which stimulate cell surface receptors (2). Approximately 90% of the activating mutations have been found in codons 12 (wild-type GGT) and 13 (wild-type GGC) of exon 1 identifying these codons as hot-spot mutation points. The most frequently observed types of mutations are G→A transitions and G→T transversions (3,4).

As a member of the Ras GTPase superfamily (5), Rho-A is an oncogenic and a critical component of signaling pathways leading to downstream gene regulation (6-9). Rho family proteins are prominent members of the well-known Ras superfamily of small GTPases that can cycle between inactive GDP-bound state and active GTP-bound state and that exhibit intrinsic GTPase activities (10-12). Rho-A is frequently over-expressed in human cancer (13). In terms of function, several Rho GTPases have been shown to regulate diverse signal transduction pathways and are involved in a variety of biological processes, including cell morphology (14,15), motility (16), proliferation (17) and apoptosis (18,19). Recently, a number of studies has shown that Rho-A expression was upregulated in a group of malignancies, including breast cancer, colon cancer, lung cancer, and ovarian cancer (20-24) and that the expression level of Rho-A seemed to be positively correlated with the progress of these carcinomas, suggesting that Rho-A may play an important role in tumorigenesis and tumor progression.

*Rac1*, belonging to the Rho family, is a Ras-related small GTPase. Its activity is responsible for the regulation of diverse cellular behaviors including, formation of cortical actin-containing membrane ruffles, and induction of gene expression programs (25). *Rac1* activity is implicated in various steps of oncogenesis including initiation, progression, invasion and metastasis (26,27).

*p53*, considered as the 'guardian of the genome', is the most frequently mutated gene in human malignancies such as cancer, it is found inactivated in ~50% of tumors of any

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location and histological type (generally, point mutations of one allele and deletion of the other allele). Present in an inactive form in normal cells, p53 becomes fully functional when activated in response to cell stress (either oncogenic or genotoxic stress). p53 activation leads to the upregulation of various target genes responsible for cell cycle arrest or apoptotic cell death, depending on the cellular environment. Due to its crucial tumor suppressor activity, TP53 thus appears to be an appealing target for gene therapy or pharmacological intervention in cancer treatment (28).

The signal transducers and activators of transcription (Stats) belong to a family of seven cytoplasmic proteins that function as signal messengers and transcription factors participating in cellular responses to cytokines and growth factors. Stat1 is deficient or inactive in many types of human tumors whereas some tumors have activated Stat1. Whether Stat1 affects tumor growth and metastasis is unclear (29-31).

Apoptosis (programmed cell death), is a process of cellular destruction that is required for the development and homeostasis of multicellular organisms (32). Apoptosis is characterized by cell shrinkage, condensation of nuclei and internucleosomal degradation of DNA. Cells defective in apoptosis tend to survive with excess DNA damage and thus lead to carcinogenesis by accumulating mutations (33). In chemotherapy, apoptosis is the predominant mechanism by which cancer cells die. However, even when the apoptotic machinery remains intact, survival signaling may antagonize the cell death by signals, such as growth factor, steroid hormone, neuropeptide and the activation of phosphatidylinositol 3-kinase and Akt (34,35). In view of recent findings, specific patterns of resistance to chemotherapy can occur depending on the genetic or epigenetic abnormalities of the cancer cells (36,37).

The Bax gene, a member of the Bcl-2 family and an apoptosis promoter, regulates the release of cytochrome *c* from mitochondria (38), and its forced expression is known to lead to the activation of caspases and to programmed cell death (39,40). However, it is controversial whether caspases are required for Bax-induced apoptosis. Both caspase-dependent cell death (41,42) and caspase-independent cell death (43) mediated by Bax have been reported. Several caspase-3-like proteases exist and it is even uncertain whether caspase-3 is absolutely required in Bax-mediated cell death (44). Bcl-xL, one of several additional proteins with sequence homology to Bcl-2, is 233 amino acid protein with 43% sequence identity with Bcl-2 that suppresses cell death (45).

Caspase-3 is a member of the cysteine protease family, which plays a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins. Caspase-3 is the most widely studied of the effector caspases, it can be activated by diverse death-inducing signals, including the chemotherapeutic agents. It plays a key role in both the death receptor pathway, initiated by caspase-8, and the mitochondrial pathway, involving caspase-9. In addition, several studies have shown that caspase-3 activation is required for apoptosis induction in response to chemotherapeutic drugs e.g., taxanes, 5-fluorouracil (5-FU) and doxorubicin (46-48).

NF- $\kappa$ B has been implicated in many inflammatory and malignant diseases, such as breast cancer. NF- $\kappa$ B transcription factors play a crucial role in oncogenesis (49). NF- $\kappa$ B

is aberrantly activated in a wide range of human cancers, in which it promotes survival and malignancy by upregulating anti-apoptotic genes (50).

5-FU is a pyrimidine analog and is the most widely used chemotherapeutic agent for the treatment of a variety of solid cancers. Its mechanism of action has been attributed to the production of cytotoxic metabolites incorporated into RNA and DNA and inhibiting thymidylate synthase, finally leading to cell cycle arrest and apoptosis in cancer cells (51). The aim of this study was to evaluate 5-FU in cells transformed by low doses of ionizing radiation  $\alpha$ -particles in breast cancer cell lines (52) on apoptotic activity.

## Materials and methods

**Breast cancer cell lines.** The immortalized breast cell line, MCF-10F (ATCC, Manassas, VA, USA) retains all the characteristics of normal epithelium *in vitro*, including anchorage-dependence, non-invasiveness and non-tumorigenicity in nude mice. This cell line was grown in DMEM/F-12 (1:1) medium supplemented with antibiotics 100 U/ml penicillin, 2.5  $\mu$ g/ml amphotericin B, 100  $\mu$ g/ml streptomycin (all from Life Technologies, Grand Island, NY, USA) and 0.5  $\mu$ g/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 10  $\mu$ g/ml and 5% equine serum (Biofluids, Rockville, MD, USA), and 0.02  $\mu$ g/ml epidermal growth factor (Collaborative Research, Bedford, MA, USA). We used two cell lines from an *in vitro* experimental breast cancer model, the MCF-10F and Tumor2 cells. This model consisted of human breast epithelial cells in different stages of transformation (52). In brief, MCF-10F was exposed to low doses of high linear energy transfer (LET)  $\alpha$ -particle radiation (150 keV/ $\mu$ m) and subsequent growth in the presence or absence of 17 $\beta$ -estradiol at 10<sup>-8</sup> M (E) (Sigma-Aldrich) was evaluated. Tumor2, is a malignant and tumorigenic cell line obtained from Alpha5 (60cGy+E/60cGy+E) injected into the nude mice given rise to this cell line (52). The cells were incubated at 37°C with 5% CO<sub>2</sub> up to 70% of confluence. The other cell line used was MDA-MB-231, a metastatic human breast cancer cell line obtained from ATCC® HTB-26™ and grown in RPMI supplemented with 10% fetal bovine serum.

**Cell viability assay.** The cytotoxic effect of 5-FU on cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in breast cancer cell lines. Briefly, cells were seeded in 24-well culture plates at a density of 5x10<sup>4</sup> cells/well. After cells were attached, the cells were treated with 5-FU at different concentrations ranging from 0-5  $\mu$ M. The concentration of DMSO was 0.01% (v/v). The plates were incubated at 37°C with 5% CO<sub>2</sub> for 48 h. The control cells received the vehicle only. After 48-h incubation, the medium was removed, and 0.5  $\mu$ mol/l MTT was added into the wells. After another 4 h, 150  $\mu$ l DMSO was added into each well to dissolve the crystal. The absorbance was read at 570 nm on a microplate reader (Autobio Labtec Instruments, Zhengzhou, China). The drug concentration yielding 50% cell inhibition (LD<sub>50</sub>) was determined. The treatment groups were compared with the control group and the results were expressed as percentage of viable cells. All experiments were performed in triplicate.

Table I. Selected primers for target genes to develop cDNA probes.

Gene name	Product length (bp) <sup>a</sup>	Primer sequence <sup>b</sup>
<i>H-ras</i>	112	1-CCAGTACAGGGAGCAGAT 1'-GAGCCTGCCGAGATTCCACA
<i>Rho-A</i>	140	1-CCATCATCCTGGTTGGGAAT 1'-CATGTACCCAAAAGCGCCA
<i>p53</i>	128	1-CCTCAGCATCTTATCCGAGTGG 1'-TGGATGGTGGTACAGTCAGAGC
<i>Bcl-xL</i>	211	1-CTGAATCGGAGATGGAGACC 1'-TGGGATGTCAGGTCAGTAA
<i>Bax</i>	143	1-GCGAGTGTCTCAAGCGCATC 1'-CCAGTTGAAGTTGCCGTCAGAA
<i>NF-κB (RelA)</i>	114	1-ATCTGCCGAGTGAACCGAAACT 1'-CCAGCCTGGTCCCGTGAAA
<i>β-actin</i>	569	1-ACTACCTCATGAAGATCCTC 1'-TAGAAGCATTTGCCGTGGACGATGG

<sup>a</sup>Length of cDNA product amplified by gene-specific RT-qPCR analysis. <sup>b</sup>PCR primer sequences used to generate a product of the indicated size, listed in 5'-3' orientation. 1, forward; 1', reverse.

**Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were determined using a UV spectrophotometer. Total RNA was reverse transcribed into cDNA using High capacity cDNA Reverse Transcription kit and 10 units of RNase inhibitor (both from Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. A CFX 96 Touch Real-Time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA, USA) was used with an aliquot of cDNA (2 μl) in 20 μl qPCR reaction containing SYBR-Green PCR Master Mix (Agilent, La Jolla, CA, USA) for measurement of target genes such as *c-Ha-ras*, *Rho-A*, *NF-κB*, *Bcl-xL*, *Bax*, *p53* and *β-actin* was used as reference to obtain the relative fold-change for target genes using the comparative Ct method and using Bio-Rad CFX Manager 2.1 software. Relative expression was always normalized to the average in normal breast cells. Table I shows the primers for the genes selected to develop cDNA probes.

**Western blot analysis.** Cells were lysed with 1 ml lysis buffer (pH 7.2) (Tris Base (50 mM), NaCl (100 mM), EDTA (1 mM), orthovanadate (1 mM), PMSF (1 mM), Triton X-100 (0.1%) and centrifuged (13,200 rpm x 15 min). The supernatant with cellular proteins were dissolved in SDS-PAGE sample solution (60 mM) Tris, pH 6.5, 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 20% (w/v) SDS, and 0.025% (w/v) bromophenol blue and denatured by boiling (2x5 min), and vortex mixing (2x30 seg). The total amount of protein was 30 μg in each lane with standard protein markers (Bio-Rad Laboratories). After fractionation by SDS-PAGE on

gels (7x14 cm), proteins were electro-blotted onto PVDF membrane (Amersham Biosciences, Buckinghamshire, UK) using a blotting apparatus (Bio-Rad Laboratories). Prestained SDS-PAGE (Standards) blots were blocked for 2 h in 10% defatted dry milk-TBS-0.1% Tween-20 and then incubated for 2 h at room temperature with corresponding primary antibodies (1:200) Rac1 (sc-217), Rho-A (sc-418), Stat1 (sc-417), caspase-3 (sc-7148), Bax (sc-7480), NF-κB (sc-53744) and β-actin (sc-47778) followed by incubation with secondary peroxidase-conjugated mouse IgG (1:5,000) (Cell Signaling Technology, Danvers, MA, USA) in 5% defatted dry milk-TBS-0.1% Tween-20. All steps were performed at room temperature, and blots were rinsed between incubation steps with TBS-0.1% Tween-20. Cell blots were probed with mouse anti β-actin antibody as control. Immunoreactive bands were visualized by using the ECL<sup>TM</sup> Western Blotting Detection Reagent detection method (Amersham, Dübendorf, Switzerland) and exposure of the membrane to X-ray film. Protein determination was performed using the Bicinchoninic Acid Method (Bio-Rad Laboratories) and BSA as the standards. Experiments were performed in triplicate.

**Apoptosis assay.** Annexin V, a Ca<sup>2+</sup>-dependent phospholipid binding protein, has a strong binding affinity for phosphatidylserine (PS) which is inside of cell membrane in normal cells and is transferred to the surface during the early stage of cell apoptosis. Thus, apoptotic cells were quantified using the Annexin V-FITC apoptosis detection kit (Beckman Coulter, Fullerton, CA, USA) after cells were treated with 5-FU at 2 μM for 48 h. MCF-10F and Tumor2 cell lines were cultured until 70% confluent, then 5-FU with indicated concentrations was added. After 48 h, cells were trypsinized and washed twice with cold PBS, and then resuspended in 1X binding buffer with 10 μl of Annexin V-FITC and 20 μl of 7-amino-actinomycin D (7-AAD, a nucleic acid dye) at 1x10<sup>6</sup> cells/ml in a total volume of 100 μl. Cells were gently mixed and incubated in the dark for 15 min at room temperature. A quantity of 1X binding buffer (400 μl) was then added to a clean test tube and the number of apoptotic cells was quantified using a flow cytometer (Beckman Coulter FC500 Flow Cytometry System; Beckman Coulter) within 1 h. Cells that stain positive for Annexin V-FITC and negative for 7-AAD are undergoing apoptosis; cells that stain positive for both Annexin V-FITC and 7-AAD are either in the endstage of apoptosis, are undergoing necrosis, or are already dead; cells that stain negative for both Annexin V-FITC and 7-AAD are alive and not undergoing apoptosis. Analysis was performed by Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter). All experiments were performed at least three times.

**Statistical analysis.** Data are expressed as the average ± standard error of the mean (SEM). Comparisons of multiple groups were performed between treated groups and controls carried out by ANOVA and Dunnet's test. P-values of p<0.05 and p<0.01 were considered to be significant. Lethal dose at 50% (LD<sub>50</sub>) was calculated by a non-linear regression curve using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Assays were performed at least three times independently.

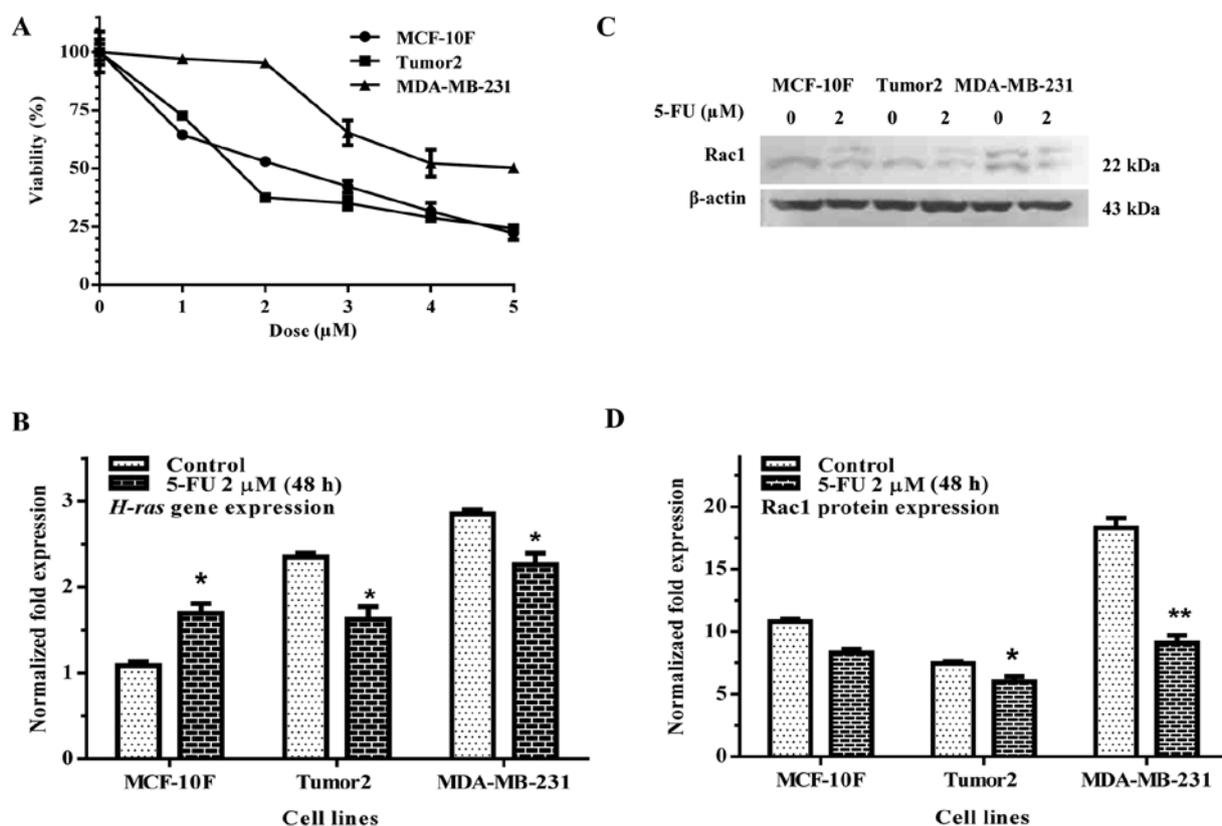


Figure 1. (A) Effect of 5-FU on cell viability. MCF-10F, Tumor2 and MDA-MB-231 cell lines were treated with different doses of 5-FU (from 0 to 5  $\mu$ M) and incubated for 48 h, and determined by using the MTT assay and an automated cell counter. (B) *H-ras* gene expression and (C and D) Rac1 protein expression were evaluated. Bars represent the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  vs. control group and their counterparts.  $\beta$ -actin was used as an endogenous control. Data represent the means  $\pm$  SEM of three independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  vs. counterparts.

## Results

MTT assay was carried out to evaluate the metabolic activity of living cells as indicator of viability in MCF-10F, Tumor2 and MDA-MB-231 cell lines and to determine the dose to be used in the experiments. Concentration range of 0-5  $\mu$ M was used of 5-FU for 48 h to calculate that LD<sub>50</sub> values for all cell lines tested. Results in Fig. 1A showed that the mean LD<sub>50</sub> was at 2  $\mu$ M after 48 h. Therefore, all the following experiments were carried out with this concentration of 5-FU.

Ras family is related to cell proliferation in cancer cells. *H-ras* gene expression was studied by RT-qPCR. Results of the experiments indicated that 5-FU significantly decreased *H-ras* gene expression in Tumor2 and MDA-MB-231 cell lines (Fig. 1B). Rac1 (Fig. 1C and D) protein expression was decreased in Tumor2 and MDA-MB-231 cells ( $p < 0.05$  and  $p < 0.01$ ) in comparison with its counterpart.

Rho-A is member of Ras family known to regulate the actin cytoskeleton and it is distributed in the nuclei of cancer cells. Rho-A gene and protein expression were studied by RT-qPCR and western blot analysis, respectively. Results of the experiments indicated that 5-FU significantly decreased Rho-A gene expression and protein expression of the Tumor2 cells ( $p < 0.01$ ) in comparison with its counterpart, however, the MDA-MB-231 cells were not altered (Fig. 2A-C).

Analysis of gene expression indicated that 5-FU decreased p53 in Tumor2 cells in comparison to its counterparts. However, MDA-MB-231 cells showed an increase in gene expression in

comparison with their counterparts (Fig. 2D). Fig. 2E and F show Stat1 protein expression. There was no effect on Stat1 either in Tumor2 or in MDA-MB-231 cells.

The apoptotic activity of 5-FU on MCF-10F, Tumor2 and MDA-MB-231 cell lines were analyzed. Results indicated that *Bcl-xL* (Fig. 3A) gene expression significantly decreased in MDA-MB-231 with regard to its counterpart ( $p < 0.01$ ). However, there was no effect in Tumor2 cells. 5-FU significantly increased caspase-3 protein expression in Tumor2 and MDA-MB-231 cells in comparison to its counterparts (Fig. 3B and C). It also increased Bax gene (Fig. 3D) and protein (Fig. 3E and F) expression in Tumor2 and MDA-MB-231 cell lines. Apoptotic cells were also measured by flow cytometry, the results indicated 21.5% of cell death in the control MCF-10F and 80% in Tumor2 cells (Fig. 4).

The activation of NF- $\kappa$ B is frequently observed in breast cancer cells. 5-FU significantly decreased NF- $\kappa$ B gene expression in MDA-MB-231 but not in Tumor2 in comparison to its counterparts (Fig. 5A). 5-FU also decreased protein expression in Tumor2 cell, but not in MDA-MB-231 cells (Fig. 5B-D), where we observed and increase in the expression in both subunits p105 and p50 kDa as shown in Fig. 5B.

## Discussion

Breast cancer is one of the most common causes of cancer-related death among women (1). 5-FU is frequently used to treat

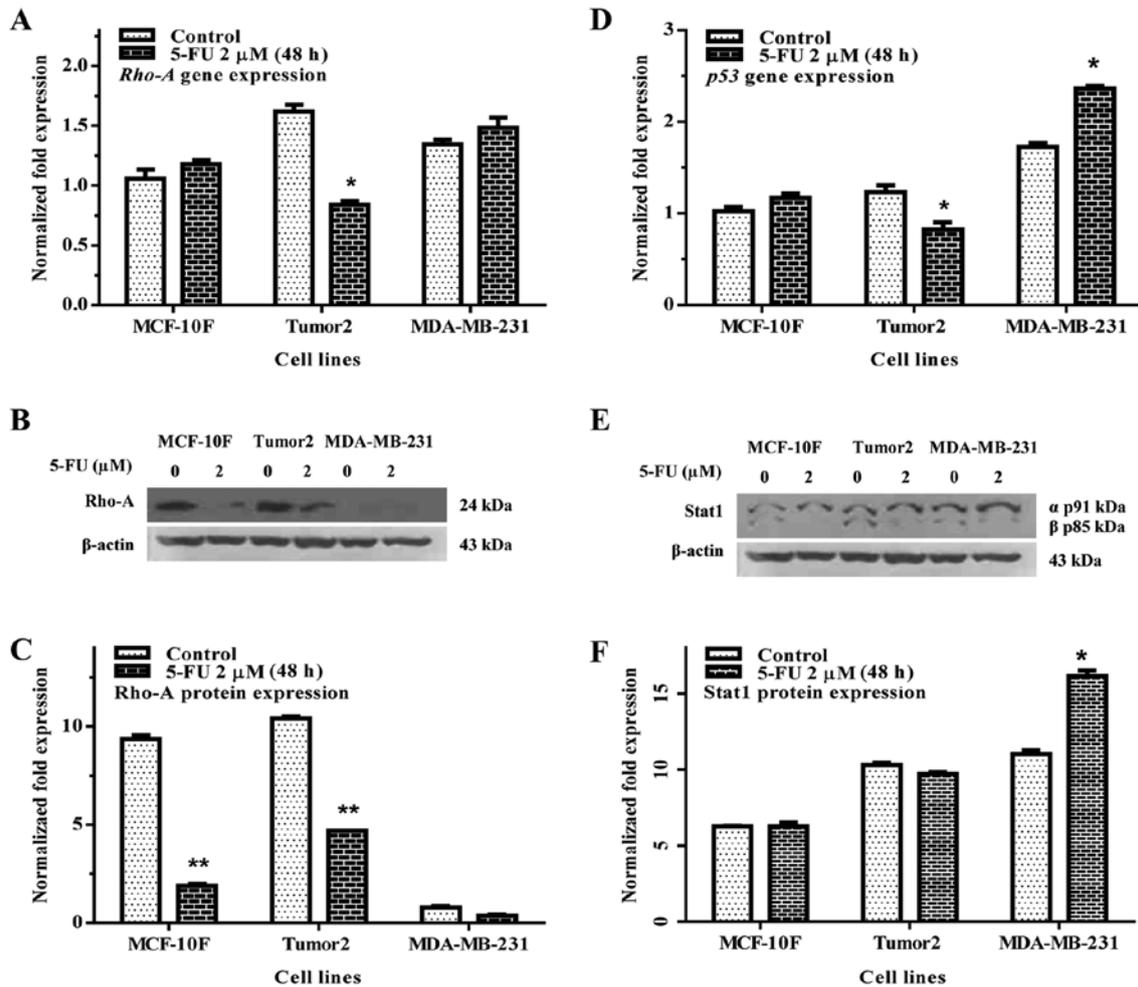


Figure 2. Rho-A gene and protein expression: Effect of 5-FU in MCF-10F, Tumor2 and MDA-MB-231 cell lines (A-C) determined by RT-qPCR and western blot analyses, respectively.  $\beta$ -actin was used as control for loading. (D) *p53* gene expression and (E and F) Stat1 protein expression were evaluated. Band density of the specific protein was analyzed with Adobe Photoshop program software and the results were expressed as average density to  $\beta$ -actin. Graphs represent the relative grade of luminescence to assess the protein level of the cell lines. Bars represent the mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  vs. counterpart.

breast cancer. This agent can inhibit breast cancer progression by a variety of different mechanisms such as apoptosis by affecting cell death pathways. Therefore, several clinical trials are currently under investigation to overcome drug resistance due to modulation of apoptosis (51). In the present study, the *in vitro* effects of 5-FU in breast cancer cell lines were evaluated by several parameters. 5-FU showed a direct apoptotic activity in breast cancer cell lines, which is in agreement with results from previous studies (51).

5-FU decreased *H-ras* gene and protein expression in Tumor2 and MDA-MB-231 cell lines in comparison to its counterparts and MCF-10F. Authors have demonstrated that resistance to 5-FU may result from low levels of GTPase-activating proteins, such as N-ras and H-ras in tumor cells (20). 5-FU has been shown to be a highly effective inhibitor of human cell proliferation by inactivating the Ras/ERK pathway (20,21). The effects of H-ras on cell motility appeared to be through activation of a MAP kinase cascade, presumably via the Ras effector Raf (24).

Rac1 is responsible for Ras-induced phenotype changes by regulating motility mammary epithelial cells (53). Our results have shown that 5-FU significantly decreased Rac1 protein

expression in Tumor2 and MDA-MB-231 cells. Rac is related to a profound change in cell phenotype such as motility, invasiveness, and resistance to apoptosis or the ability to adapt to environmental changes and continue to invade successfully (54). Anti-apoptotic activity of Rac has been indicated, although the molecular mechanism through which Rac inactivation promotes apoptosis has yet to be elucidated (55).

Previous studies have highlighted the role of signal transduction pathways controlled by the Rho family of small GTPases (21). 5-FU decreased Rho-A gene and protein expression in Tumor2 cell line in comparison to their counterparts. It is of interest to note that MDA-MB-231 were not altered by this chemotherapeutic drug which is highly resistant. The inhibition of Rho proteins may provide a possibility to reduce metastasis and apoptosis. Recent studies have indicated that 5-FU induced apoptotic effects in myeloma cells *in vitro* (19-27).

*p53* acts as a transcription regulator and has been shown to block the entry of DNA-damaged cells into the S-phase and also to trigger an apoptotic pathway in many transformed cells by inducing the expression of a set of genes related to the control of cell proliferation (28). The present results indicated that *p53* gene expression decreased by 5-FU in Tumor2 in

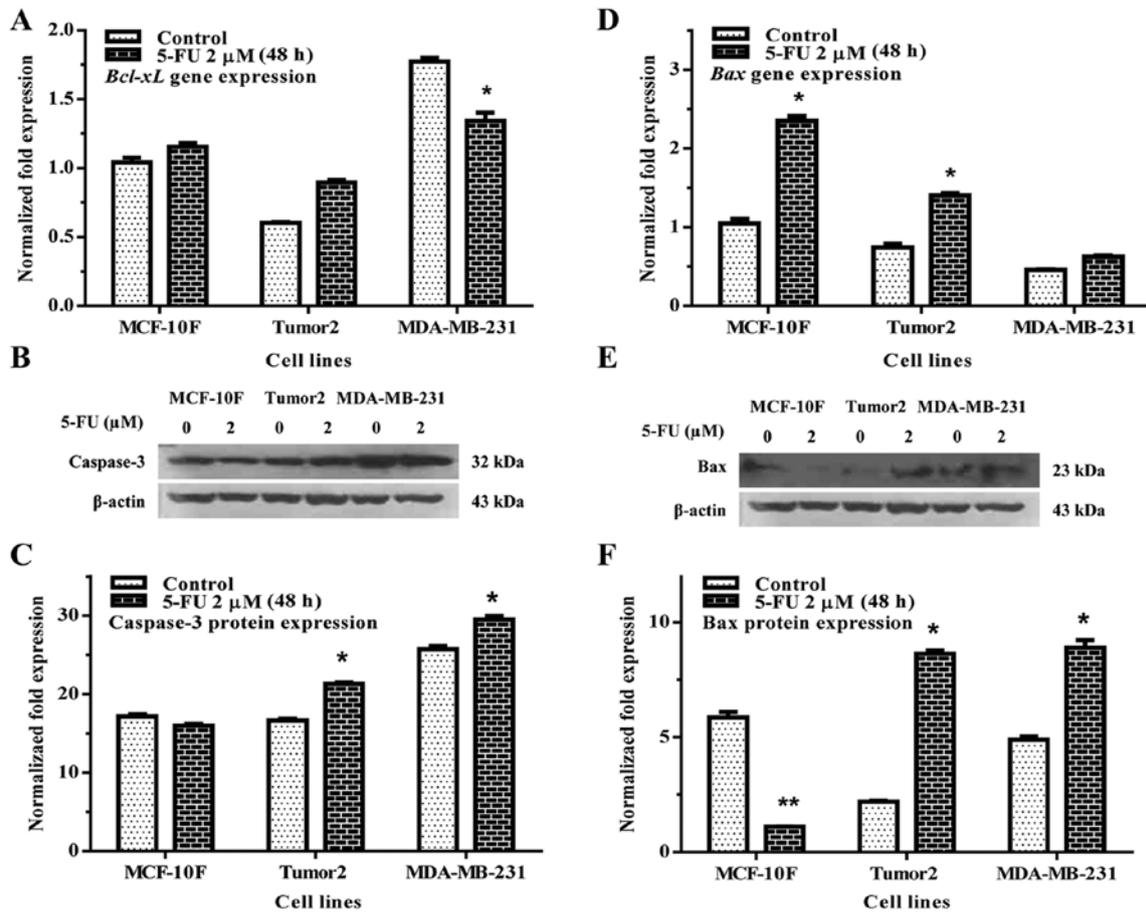


Figure 3. Effect of 5-FU on (A) *Bcl-xL* and (D) *Bax* gene expression by RT-qPCR and (C) caspase-3 and (E and F) Bax protein expression by western blotting analysis in MCF-10F, Tumor2 and MDA-MB-231 cell lines.  $\beta$ -actin was used as an endogenous control gene. Bars represent the mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$  and \*\* $P < 0.01$  vs. counterparts.

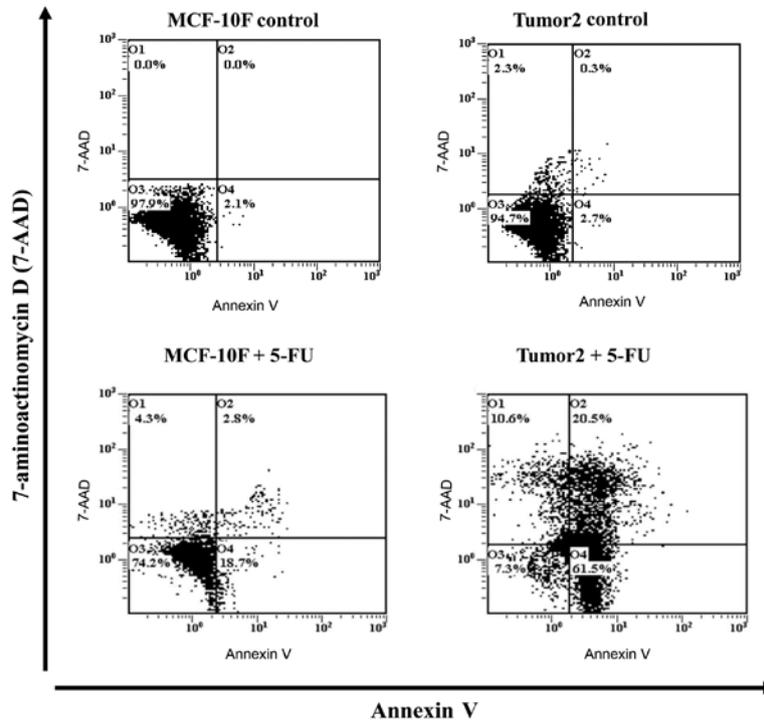


Figure 4. Flow cytometric analysis for apoptosis of breast cancer cells: apoptotic effects of 5-FU (2  $\mu$ M/48 h) were evaluated in MCF-10F and Tumor2 cell lines compared with their counterparts. Upper left quadrant (O1) indicates cells undergoing necrosis; upper right quadrant (O2), cells at the end stage of apoptosis; lower left quadrant (O3), cells that are viable, or no measurable apoptosis and lower right quadrant (O4), cells undergoing apoptosis. The percentage of the cells in apoptosis was determined by CXP software analysis.

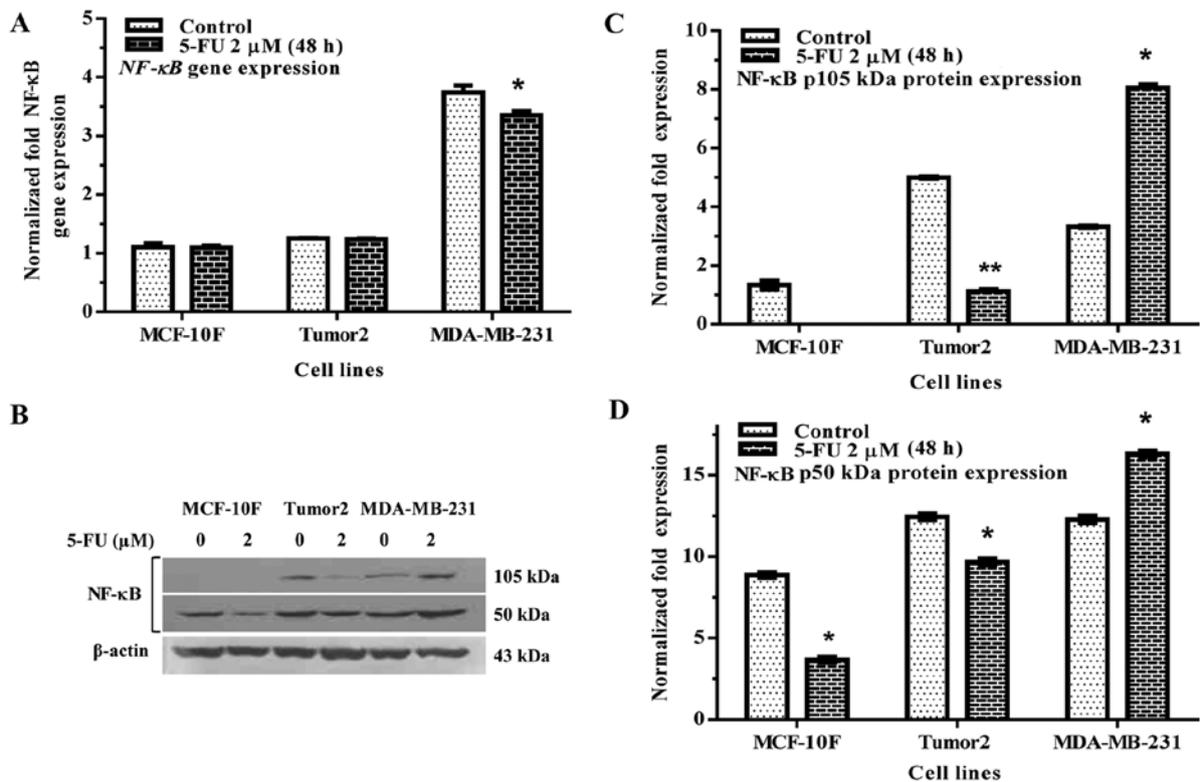


Figure 5. Effect of 5-FU on (A-D) *NF-κB* gene and protein expression in MCF-10F, Tumor2 and MDA-MB-231 cell lines by RT-qPCR and western blotting analysis.  $\beta$ -actin was used as an endogenous control gene. Bars represent the mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$  vs. counterparts.

comparison to its counterpart. Others, have showed that 5-FU induces apoptosis of human gastric cancer cells via wild-type p53 gene expression (56) which is consistent with our results. In addition to the high levels of anti-apoptotic Bcl-2 and Bcl-xL proteins combined with a low level of Bax were correlated to high 5-FU resistance of wild-type p53 cell lines (57). 5-FU did not affect p53 gene expression in MDA-MB-231 cell line; however, this cell line as well as T47D, or SKBR-3 with GnRH-p53 in combination with 5-FU significantly enhanced p53-activated apoptotic signals including BAX translocation to mitochondria, and activated caspase-3. Intratumoral injection of the GnRH-p53 protein inhibited MDA-MB-231 xenograft growth and induced p53-mediated apoptosis in the tumors (58).

Stat1 participates in regulation of tumor angiogenesis, growth, and metastasis (29). Our results did not show any significant difference in Stat1 protein expression with the treatment of 5-FU in Tumor2 and MDA-MB-231. Stat1 has been shown to be associated with cell growth modulation and cell death signaling (59). This implied that Stat1 may have a modulatory role in cell death signaling when tumor cell growth is blocked by another Stat such as Stat3 inhibition (59).

The caspases, a family of cysteine proteases, are major mediators of the execution phase of apoptosis; possibly by direct activation of the death receptor or following mitochondrial changes (57,58). The cytotoxic effect of 5-FU induced apoptosis in cancer cells. Our results showed that 5-FU significantly increased caspase-3 expression in Tumor2 and MDA-MB-231 cell lines suggesting activation of apoptosis. Other authors have confirmed that 5-FU induced increased activity of caspase-3 and -8 (57,58).

NF- $\kappa$ B is an important signaling pathway involved in chemoresistance induced by 5-FU. Constitutive activation of NF- $\kappa$ B is observed in several cancer cells and such activation results in the control of a signaling network, which includes the expression of anti-apoptotic genes, cell cycle regulatory genes and genes encoding cell surface receptors. The activation of NF- $\kappa$ B is frequently observed in breast cancer cells. The present study shows that 5-FU decreased NF- $\kappa$ B gene expression in MDA-MB-231. 5-FU also decreased protein expression in Tumor2 cell line in comparison to its counterparts. It has been indicated that inhibition of inducible NF- $\kappa$ B activity reduces chemoresistance to 5-FU in human stomach cancer cell line (60). Other studies have shown that downregulation of NF- $\kappa$ B was able to enhance therapeutic efficacy of 5-FU (60-63).

The regulation of the genes by NF- $\kappa$ B is related to apoptosis (60) since it is a key positive regulator of cancer cell proliferation and survival. It has the ability to transcriptionally activate many pro-survival and anti-apoptotic genes such as Bax and Bcl-xL (64). In the present study, 5-FU decreased genes related to apoptosis such as Bcl-xL in Tumor2 cell line. It can be concluded that 5-FU may exert apoptotic activity in breast cancer cells transformed by low doses of ionizing  $\alpha$ -particles *in vitro* regulating Bax and Bcl-xL and NF- $\kappa$ B expression, respectively.

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