MOLECULAR CARCINOGENESIS 31:192–203 (2001)

Profiling of Differentially Expressed Genes Induced by High Linear Energy Transfer Radiation in Breast Epithelial Cells

Debasish Roy,* Gloria Calaf, and Tom K. Hei

Center for Radiological Research, College of Physicians and Surgeons, Columbia University, New York, New York

Methods to define patterns of gene expression have applications in a wide range of biological systems. Several molecular biological techniques are used to study expression patterns during the neoplastic progression of breast epithelial cells. In the present study, differential expression of human oncogenes/tumor suppressor genes in human breast epithelial cell lines irradiated with low doses of high linear energy transfer radiation and treated with estrogen was assessed with cDNA expression arrays. Transformed and tumorigenic cell lines were compared with the control cell line to identify differentially expressed genes during tumorigenic progression. Autoradiographic analysis showed that of the 190 genes analyzed, 49 genes showed a high level of altered expression, and 12 genes had minor differences in expression levels. Among these 49 genes, 17 genes were altered at all stages of transformation, 21 were altered only at the early stage, and the remaining 11 were at the late stage of transformation to the tumorigenic stage of progression. Among the 11 late stage–associated genes, seven genes were altered exclusively in the tumorigenic cell lines and in Tumor-T. Of the 17 all-stage genes, six were randomly selected, and we confirmed their altered expression by gene-specific semiquantitative reverse transcription polymerase chain reaction, followed by Northern blot analysis. The results showed that the mRNA expression patterns of all these genes were consistent with the expression pattern seen on the array. Among these six genes, five genes, including c-*myc, puf, MNDA*, c-*yes,* and *Fra-1* showed upregulation, and the other gene, *RBA/p48,* showed downregulation in the transformed and tumorigenic cell lines compared with the control MCF-10F cell line. Investigation of these genes should help establish the molecular mechanisms of progression that are altered by radiation and estrogen treatment. A number of candidates reported here should be useful as biomarkers involved in breast carcinogenesis.

Key words: human breast epithelial cell; high linear energy transfer radiation; cDNA expression array; gene-specific reverse transcription polymerase chain reaction; Northern blot

INTRODUCTION

Breast cancer is the most common malignancy and the second major cause of cancer-related deaths among women in the United States [1]. Amplification of several proto-oncogenes, such as c-*myc,* has been shown to be a major risk factor for human familial breast cancer in different types of mammary carcinomas [2]. The development and progression of cancer and the experimental reversal of tumorigenicity are accompanied by complex changes in the patterns of gene expression [3,4]. The genetic alterations include loss of a functional suppressor gene or activation or enhanced expression of several cellular proto-oncogenes that have been implicated to varying degrees in the development and progression of human malignancies [5–8].

The biological heterogeneity of breast cancers may arise from many possible molecular changes in the neoplastic process, with variable efficiency at each step. This heterogeneity also may result from various mutations in the malignant growth process involving many genes and signaling pathways controlling cell proliferation, death, and differentiation [9,10]. Patterns of gene expression in cell lines and tissues have important applications in a variety of biological systems and are influenced by exposure to chemical carcinogens or by ionizing radiation [11,12].

At present, substantial information is available about chemically induced cellular transformation and differential gene expression in breast tissue [13,14], but little is known about such regulation by ionizing radiation, particularly high linear energy transfer (LET) radiation. Based on the study of Japanese atom bomb survivors, radiation has been shown to induce breast cancer; but the exact mechanism of radiation-induced carcinogenesis remains unknown [15]. For accurate risk assessment

*Correspondence to: Center for Radiological Research, Vanderbilt Clinic 11-218, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032.

Received 25 October 2000; Revised 20 April 2001; Accepted 16 May 2001

Abbreviations: LET, linear energy transfer; E, 17β-estradiol; PCR, polymerase chain reaction; RT, reverse transcription; ER, estrogen receptor.

of human exposure to high LET radiation, mainly by α particles, and to examine the molecular mechanisms involved in radiation-induced human breast carcinogenesis, we used a model system based on spontaneously immortalized human breast epithelial MCF-10F cell lines irradiated with graded doses of α particles and treated with 17β-estradiol (E) [12]. MCF-10F cell lines are unique in the sense that they have all the morphologic characteristics of normal breast epithelial cell lines [11,13,16]. With the help of this unique model system, it is possible to study different stages of cellular transformation by radiation- and estrogen-induced human breast epithelial cell lines.

The sequential process of cellular transformations also is initiated to different degrees by exposure to various carcinogens. Experimental studies have shown that high LET radiation, in general, is more effective at inducing mutagenic and oncogenic transformation in cultured cells than X-rays or γ-rays at equivalent doses [17,18]. In addition, high LET radiation, such as α particle radiation, can induce many biological effects by direct interaction with DNA [19]. It induces expression of certain DNA repair genes in some prokaryotic and lower eukaryotic cells and modulates the regulation of gene expression as well as the pattern of protein synthesis in mammalian cells [20,21].

To better understand radiation- and estrogen-induced breast carcinogenesis, we need to have a large body of information regarding the alteration of various genes involved in this process. To this end, we have applied a relatively new technique, differential gene expression through cDNA expression array [22], which can show the expression profiles of thousands of genes in a single experiment, providing clues to the functional role of many genes, including potentially important oncogenes and tumor suppressor genes [23]. This alteration in gene expression associated with a specific dose of radiation and estrogen treatment may be an efficient way to uncover clues to the specific molecular derangements that contribute to cancer progression and thus ultimately help identify the appropriate targets for therapeutic intervention [24,25]. Here we report the identification of a series of oncogenes and tumor suppressor genes whose expressions were altered with stepwise neoplastic transformation of human breast epithelial cells with respect to radiation and estrogen treatment.

MATERIALS AND METHODS

Cell Lines

The spontaneously immortalized human breast epithelial cell line MCF-10F was derived from the mortal human breast epithelial cell line MCF-10M [16]. MCF-10F cells have a near-diploid karyotype and are of luminal epithelial origin. These cells retain all the characteristics of the normal epithelium in vitro, including anchorage dependence, noninvasiveness, and nontumorigenicity in nude mice [11,13,16,26]. The recently established model developed to study breast carcinogenesis in vitro was used in these studies [12]. Cell lines were irradiated with graded doses of 150 keV/μm α particles, accelerated with the 4-MeV van de Graaf accelerator at the Columbia University Radiological Research Facilities, as described previously [12,27,28].

In the present study, MCF-10F cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM)/F12(1:1) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL amphotericin B, 10 μg/mL insulin (all from Life Technologies, Grand Island, NY), 5% equine serum (Biofluids Inc., Rockville, MD), 0.5 μg/mL hydrocortisone (Sigma Chemical Co., St. Louis, MO), and 0.02 μg/mL epidermal growth factor (Collaborative Research, Bedford, MA). The following cell lines were used as controls [12,29]: MCF-10F cell lines, and MCF-10F cell line treated with E (0.01 μM) (Sigma Chemical Co.), named MCF-10F + E. One experimental cell line used was the MCF-10F cell line irradiated with a double dose of 60 cGy of α particles, named 60/60 cGy (passage 48 as an early passage and passage 95 as a late passage). This cell line was anchorage independent but non-tumorigenic in nude mice. The MCF-10F cell line also was subjected to a double dose of 60 cGy of α particle and treated with E before each radiation exposure; this line was named 60 cGy + E/60 cGy + E (passage 45). This cell line was both anchorage independent and tumorigenic in nude mice and produced tumors in 3/6 animals injected. The Tumor-T (passage 25), one of the three primary tumor cell lines, was derived from this tumorigenic cell line.

Atlas Human Oncogene/Tumor Suppressor cDNA Expression Array

The Atlas Human Oncogene/Tumor Suppressor cDNA Expression Array membranes were purchased from Clontech (Palo Alto, CA) [30–32]. Each membrane contained cDNAs from 190 known oncogenes and tumor suppressor genes as well as nine housekeeping genes for control purposes. Each of these genes was amplified by polymerase chain reaction (PCR) with gene-specific primers, to generate 200- to 500-bp products. One hundred nanograms of each PCR product was spotted in duplicate onto a positively charged membrane.

Preparation of cDNA Probes From mRNA

Total RNA was isolated from both the control and transformed cell lines with TRIzol solution (Gibco-BRL, Long Island, NY). Each sample, comprising 500 μg of total RNA, was treated with 5 μL of DNAse I (10 U/μL) (Boehringer Mannheim, Indianapolis, IN) for

60 min at 37°C. Then 10× Termination Mix (0.1 M EDTA at pH 8.0 and 1 mg/mL glycogen) (Clontech) was used to stop the reaction. Each sample was then purified following established procedure [33]. 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. Each sample of 500 μg of purified total RNA then was subjected to polyA$^+$ RNA analysis with the Oligotex mRNA Purification Kit (QIAGEN Inc., Valencia, CA). About 1 μg of polyA$^+$ RNA from all the cell lines was separately mixed with 10× CDS Primer Mix (Clontech), and deionized water was added to a final volume of 3 μL.

The mixture was incubated in a preheated PCR thermal cycler at 70°C for 2 min; the temperature of the thermal cycler was reduced to 50°C, and the incubation was continued for another 2 min. Then 8 μL of the master mix (1× reaction buffer, 1× dNTP mix [for dATP label], 35 μCi of [α-^{32}P]dATP [3000 Ci/mmol, 10 mCi/mL], 100 mM dithiothreitol, and 50 U of Moloney murine leukemia virus reverse transcriptase [50 U/μL]) was added to all the tubes (already in the thermal cycler), and incubation continued at 50°C for another 25 min. Then 1 μL of 10× Termination Mix was added to stop the reaction. Purification of ^{32}P-labeled cDNA from unincorporated ^{32}P-labeled nucleotides and small (< 0.1 kb) cDNA fragments was performed with CHROMA SPIN-200 DEPC-H_20 columns (Clontech) that had been warmed according to the manufacturer's protocol. Then the elution profile was checked, and the required fraction was collected. The required fraction contained the maximum amount of ^{32}P-labeled cDNA to use as a probe for differential hybridization.

Differential Hybridization of Atlas Human Oncogene/Tumor Suppressor cDNA Expression Array

For the hybridization of cDNA probes to the Atlas Array membrane, 15 mL of ExpressHyb solution (Clontech) was preheated to 68°C for each sample and mixed with 1.5 mg of sheared salmon-sperm DNA (Sigma Chemical Co.). The mixture was kept at 68°C until used. Before addition of the sheared salmon-sperm DNA to the ExpressHyb solution, the mixture was denatured at 95–100°C for 5 min and chilled quickly on ice. The Atlas Array membrane was then wetted with deionized water and prehybridized with 10 mL of ExpressHyb mixture for each sample for 30 min, with continuous agitation at 68°C. Labeled cDNA probes (about 200 μL, 2–5 × 10^6 cpm) were mixed with one-tenth of the total volume (about 22 μL) of 10× denaturing solution (1 M NaOH and 10 mM EDTA) and incubated at 68°C for 20 min. Then 5 μL (1 μg/μL) of Cot1 DNA (Clontech) and an equal volume (about 225 μL) of 2× neutralizing solution (1 M NaH_2PO_4 at pH 7.0) were added to this mixture, and the incubation continued at 68°C for 10 min.

Probe solution was mixed with the remaining 5 mL of ExpressHyb solution to act as a hybridization solution. Prehybridization solution was replaced by hybridization solution, and hybridization continued for 18 h with continuous agitation at 68°C. Subsequently, array membranes were washed in wash solution 1 (2× sodium chloride sodium citrate and 1% sodium dodecyl sulfate) for 60 min, with continuous agitation at 68°C. After a second wash was performed at the same temperature, with continuous agitation for 60 min in wash solution 2 (0.1× sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate), array membranes were exposed to X-ray film (Kodak BioMax MS Film; Kodak Corp., Rochester, NY) with the corresponding intensifying screen at −70°C for 24–48 h.

In each cell line tested, mRNA was isolated and purified from different passages, and cDNA probes were prepared from each of them and hybridized to the respective membranes. To assess the reproducibility of the hybridization array assays, pairwise comparisons between array data sets for each cell line obtained by repeated hybridization and the mRNAs prepared in different lots were represented in scatter plots with multiple regression (Figure 1). In each case, expression levels of 95% of the genes had repeated values that were within twofold of each other. Experiments using the same mRNA preparation were repeated two or three times, and measurable, median-normalized expression values for each gene were compared to avoid false-positive signals. No significant differences were observed with cDNA probes generated from total RNA or mRNA.

The quality of the array images obtained with the Atlas Array from Clontech was normalized to the averaged signals of housekeeping genes by exactly following the *Atlas cDNA Expression Arrays Users Manual (PT3140-1)*. Atlas hybridization probes were sufficiently less complex than probes generated with oligo(dT) or random primers, which results in an approximately eightfold to tenfold increase in sensitivity with a concomitant reduction in the level of nonspecific background. The background hybridization signals were within the permissible range, but in some cases when they varied, values were adjusted by means of different exposure times.

Quantification of Array Hybridization

Quantification of hybridization signals on the expression array membranes was carried out from the autoradiographic film by estimating in a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA) with the ImageQuant program (Molecular Dynamics). Volume quantitation was performed by calculating the volume under the surface created by a three-dimensional plot of

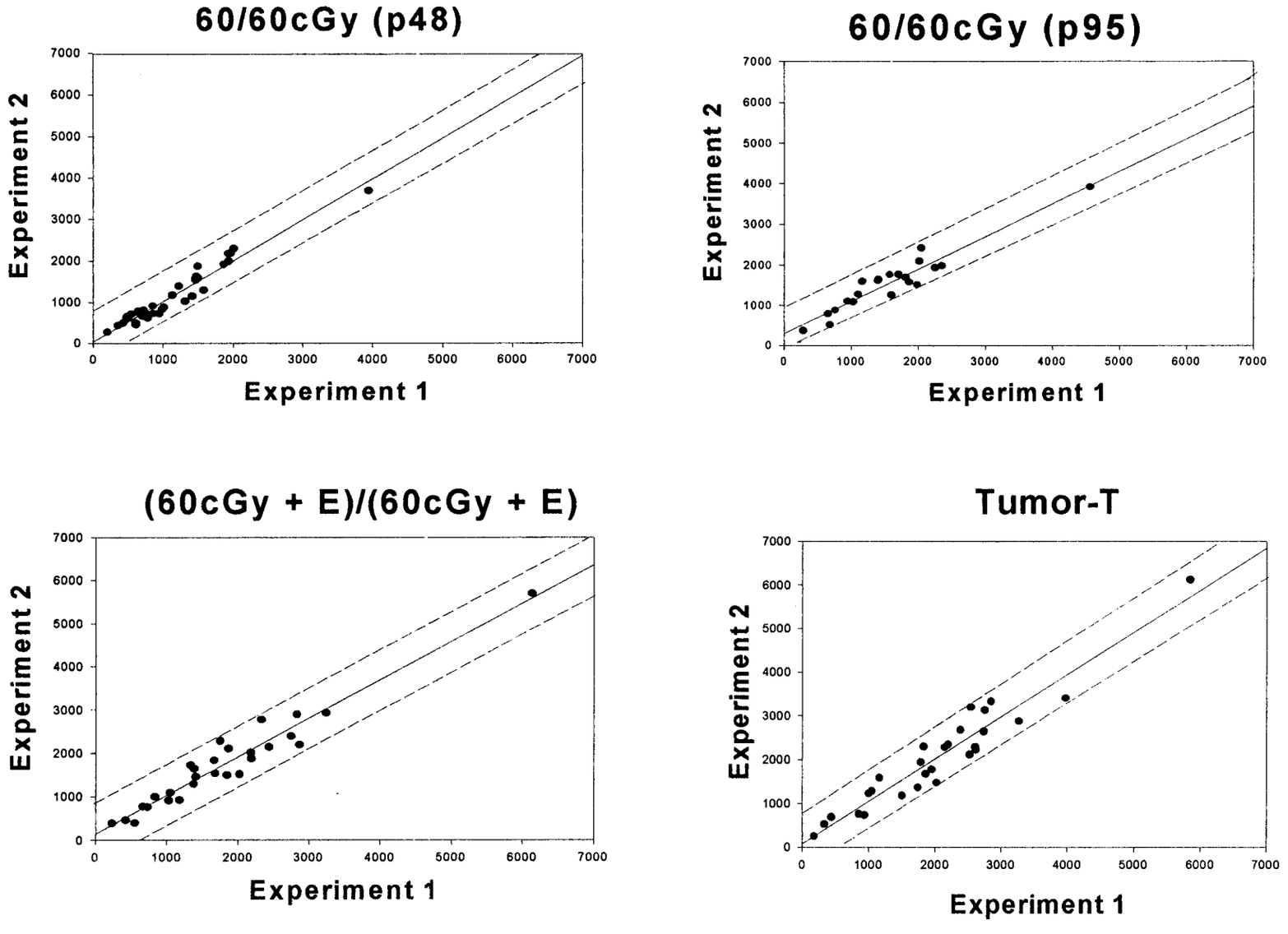

Figure 1. Reproducibility of the hybridization array assay. Scatter plot for each cell line from two experiments with different mRNA populations with different replicate membranes shown. Dotted lines indicate twofold reproducibility limits. Ninety-five percent of genes had repeated values that were within twofold of each other.

pixel locations and pixel values. An optical density range of 0.01–4.0 was chosen in optical density units, whereas spatial resolution was selected at 100 points/cm in both X and Y directions. Signal resolution was selected at 4096 levels (12-bit) of optical density. The Image Quant value of the intensity of hybridization of each gene of the array was given by Q. The ratios between the absolute values of irradiated and estrogen-treated tumorigenic cell lines (I) and the control MCF-10F (C) and MCF-10F + E (CE) cell lines (C) give us the value of Q that indicates either the upregulation (positive) or downregulation (negative) of gene expression.

To delineate the potential signal interference between adjacent strong hybridization signals, ellipses of equal size were drawn around each signal area (hybridization spots) with Image Quant software and were separately scanned and compared with housekeeping genes, so that the chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of different housekeeping genes from many autoradiographic exposures between different hybridization experiments was effected by averaging the signals of each of the housekeeping genes. Data from only higher concentration spots were used. The median background was subtracted, and signals that were < 1.5-fold above background levels were considered too low to measure accurately and were omitted from the analysis. Signals for individual genes also were normalized to the geometric mean of the expression level of that gene across the set of membranes being compared. Mean signals were calculated from quadruplicate measurable spots or if three of the four spots were measurable.

Construction of Gene-Specific Primers

For gene-specific reverse transcription (RT)–PCR and labeling of gene-specific probes, we used PCR primers (Genset Oligos, La Jolla, CA) to amplify the randomly selected six genes and human β-actin (Clontech) as a control amplifier set. Table 1 shows the base pair length of amplified cDNA of the six genes under study and the sequence of sense and antisense primers to amplify those cDNAs.

Gene-Specific RT-PCR Analysis

To confirm differential expression of the six genes under study, gene-specific probes were generated by a gene-specific RT-PCR technique [34]. Different amounts of cDNAs and various numbers of PCR cycles were used to generate gene-specific probes. A linear increase was noted in product generation in all cases except myeloid cell nuclear differentiation antigen (*MNDA*), for which the required amount of cDNA was double that of others (data not shown). Based on the findings of this experiment, we used 100 ng of cDNA (200 ng in the case of *MNDA*) and 35 cycles of PCR for amplification of the six genes by RT-PCR.

About 500 μg of total RNA was treated with 5 μL of DNAse I (10 U/μL) (Boehringer Mannheim, Indianapolis, IN) for 60 min at 37°C. The RNA was extracted and precipitated with 7.5 M ammonium acetate at pH 5.2. 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$(dT)_{18}$ and random hexamer primers. 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. Differentially expressed gene-specific DNA bands were eluted from the gel and purified with the help of the QIAquick Gel Extraction Kit (QIAGEN Inc.) (Figure 2). These gene-specific DNA bands were used as a probe in Northern blotting. They then were labeled with the Multiprime DNA Labelling Systems (Amersham Inc.) and subjected to Northern hybridization analysis (Figure 3). A human β-actin control amplifier set probe also was used in Northern hybridization to confirm similar expression in all the samples.

RESULTS

Several genes were detected with the Atlas Human Oncogene/Tumor Suppressor cDNA Expression Array. Expression of these genes was altered by radiation and estrogen treatment. These alterations were noted from early and late stages of transformation to the tumorigenic stage. The types of altered genes identified here by expression array elucidate the process of tumorigenic progression.

Altered expression of the genes fell into two main categories. Some genes were upregulated, whereas others were downregulated in the transformed and tumorigenic cell lines with respect to the control MCF-10F cell line. Of 190 oncogenes and tumor suppressor genes examined, 61 genes showed altered expression at different stages of progression: 49 genes were very prominent ($Q > \pm 1.60$), whereas 12 others showed minor differences in their expression ($Q < \pm 1.20$) (data not shown). The absolute gene expression values of the 49 genes in different irradiated tumorigenic and control cell lines are listed in Table 2, and their ratios are summarized in Table 3.

Of the 49 differentially expressed genes, 21 were altered only at the early stage of transformation. Seventeen genes were altered at all stages of transformation. Four genes were altered from the late stage of transformation to the tumorigenic stage

Table 1. Primers of Six Differentially Expressed Genes Selected for Gene-Specific RT-PCR Analysis

GAN*	Gene name	Product length†	Primer‡
V00568	c-*myc* Proto-oncogene protein	222 bp	1-GCTCCTGGCAAAAGGTCAGAGTCTGG 1′-GGGGCTGGTGCATTTTCGGTTGTTGC
L16785+ M36981	*NDK-B* [c-*myc* transcription factor (*puf*)]	282 bp	1-CCTTCATCGCCATCAAGCCGGACG 1′-GCTTTGAATCTGCTGGATTGGTCTCCC
M81750	Myeloid cell nuclear differentiation antigen (*MNDA*)	324 bp	1-CCACCGCAAGAAACAAACTGACATCGG 1′-TAAATGGCGCTGTTGCTTTCAGTACCAC
M15990	Proto-oncogene tyrosine protein kinase (c-*yes*)	351 bp	1-TGGTTGATATGGCTGCTCAGATTGCTGA 1′-AGACATTGGCTAGGGTGGCATCTGCA
X16707	*Fra-1* (fos-related antigen)	280 bp	1-CCCTGCCGCCCTGTACCTTGTATC 1′-AGACATTGGCTAGGGTGGCATCTGCA
X74262	*RBA/p48*	211 bp	1-AGAGTGCAACCCAGACTTGCGTCTCC 1′-CCAGGAAACATCTTCTACTACTGCCG

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

of progression, and the remaining seven genes were altered exclusively at the tumorigenic stage.

Among these 17 all-stage genes, 15 were upregulated and two were downregulated at early and late stages of transformation and in tumorigenic and Tumor-T cell lines compared with control MCF-10F cell line. The group of 15 upregulated genes included c-*myc,* insulin-like growth factor 1 receptor (*IGFR1*), *TEL,* c-*jun, puf, MNDA,* hyaluronidase (*luca1*), *Fra-1,* focal adhesion kinase (*fak*), *cdc25B,* glycogen synthase kinase 3 (*gsk3*), c-*myc* binding protein, E2F-1 pRB binding protein (*e2f1*), *Wnt-5a,* and Tyr-PK-YES (*c-yes*). The two downregulated genes were *RBA/p48* and *EB1* (Table 3). From these 15 genes, six ($Q > \pm 2.0$) were randomly selected (Table 1) and amplified by RT-PCR to use as a probe for Northern hybridization analysis (Figure 2). Four genes—c-*kit, EZRIN, FZD3,* and *Notch-4*—were upregulated at the late stage of transformation and in tumorigenic and Tumor-T cell lines, whereas the remaining seven genes—*EGFR/ERBB1, yboxbp1, RhoA,* Tyr-PK-FGR (*fgr*), *TOB, dvl* and Ser/Thr-PK (*pctaire 1*)—were upregulated only in tumorigenic and Tumor-T cell lines compared with control MCF-10F cell line (Table 3).

Six randomly selected genes were studied by Northern blot analysis, and all of them showed exactly the same expression pattern as seen with the Atlas Human cDNA Expression Array procedure (Figure 3). Of these six genes, five (*c-myc, puf, MNDA, c-yes,* and *Fra-1*) showed upregulation, and one (*RBA/p48*) showed downregulation at all stages of transformation to tumorigenic stages of progression. Ratios of gene expression values (Q) of all these

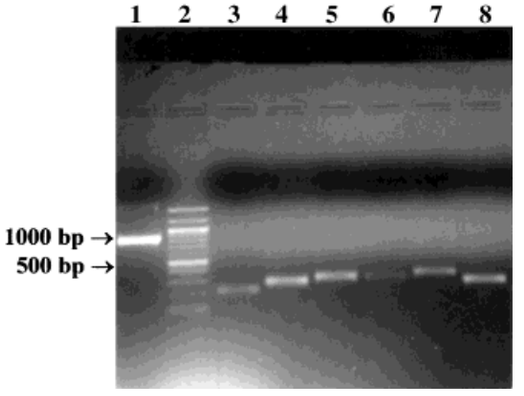

Figure 2. Gel electrophoresis pattern of six amplified gene fragments from gene-specific RT-PCR analysis required to generate gene-specific probes. 1: Human β-actin (983 bp); 2: 100-bp DNA ladder; 3: c-*myc* (222 bp); 4: *puf* (282 bp); 5: *MNDA* (324 bp); 6: c-*yes* (351 bp); 7: *Fra-1* (280 bp); 8: *RBA/p48* (211 bp).

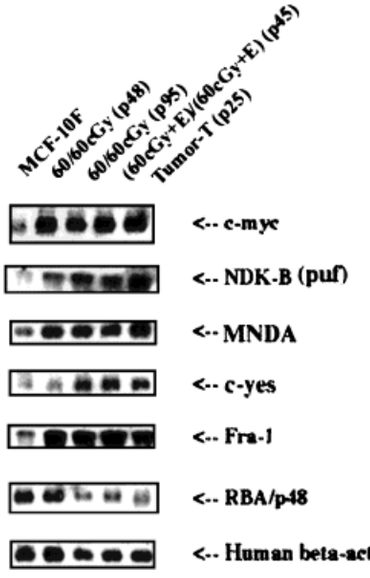

Figure 3. Northern blot analysis of gene-specific RT-PCR amplified fragments of six genes (c-*myc, puf, MNDA,* c-*yes, Fra-1* and *RBA/p48*) identified by differential hybridization of the cDNA expression array.

Table 2. Absolute Range of Expression Levels of Altered Genes Identified by Image Quant in Different cDNA Expression Array Experiments*

GenBank accession no.	Gene name	MCF-10F (C)	60/60 cGy (p48) (I_1)	60/60 cGy (p95) (I_2)	Tumor-T (I_τ)	MCF-10F + E (CE)	60 cGy + E/60 cGy + E (p45) (I_3)
X07282	Retinoic acid receptor β2	289.55	532.09				
K00650	c-*fos*	268.34	551.57				
M97935	*Stat1*	560.34	898.91				
U47686	*Stat5B*	541.79	925.35				
J02958	*met*	468.30	774.08				
M76125	*axl* (Tyr-pk-receptor-ufo)	346.11	711.38				
X03996	Pro-oncogene Tyr-pk *(src)*	278.06	453.90				
X66362	Ser/Thr-PK-*(pctaire-3)*	709.06	1176.58				
U07236	Pro-oncogene Tyr-pk *(lck)*	392.41	695.97				
U61262	Tumor supp. protein *(dcc)*	341.31	556.08				
M63167	*akt1 (rac-pk-α)*	484.58	249.83				
X12795	coup Tf	487.02	848.80				
X15218	*ski* oncogene	723.26	1186.53				
U17075	Cdkinase 4 inhibitor B *(cdk41)*	1026.2	1627.33				
M36089	*xrcc1*	431.34	793.08				
U22398	Cdkinase inhibitor 1C *(cdkI p57)*	859.17	1509.19				
M33294	Tumor necrosis factor receptor	421.44	785.02				
S85655	Prohibitin	799.83	409.20				
U49262	*(dvl)+(dvl3)*	431.13	721.01				
U43318	*frizzled 5*	335.64	529.70				
X74594	*RB2/p130*	411.01	716.45				
V00568	c-*myc* proto-oncogene protein	1123.0	2049.14	2231.16	3689.11	1103.1	3086.02
X04434	*IGFR1*	747.86	1306.38	1388.29	2063.02	731.60	1765.46
U11732	ETS-related protein *(TEL)*	879.99	1551.98	1671.26	2402.37	886.24	2094.24
J04111	c-*jun* proto-oncogene	371.01	625.84	723.46	1120.45	358.01	916.50
L16785	c-*myc (puf)*	2010.4	3758.70	4241.90	5989.80	2059.6	5869.80
M81750	*MNDA*	741.25	1289.72	1430.61	2312.70	760.02	2029.21
U03056	Hyaluronidase *(luca 1)*	1141.8	2009.64	2089.55	2911.69	1122.2	2547.53
X16707	*Fra-1 (fos-related antigen)*	400.3	752.56	824.61	1393.04	386.21	1073.66
U43408	Focal adhesion kinase *(fak)*	842.87	1422.89	1525.59	1744.74	856.22	1668.88
M81934	*cdc25B*	1046.2	1893.52	2165.67	3096.81	1054.3	2857.36
L40027	Glycogen synthase kinase 3	1080.7	675.45	345.28	220.10	1036.1	310.55
D89667	c-*myc* binding protein	1217.5	2167.78	2240.32	2873.46	1240.3	2567.46
M96577	E2F-1 pRB binding protein	1086.2	1976.99	2063.89	3084.97	1109.2	2529.09
L20861	*Wnt-5a*	911.12	1503.34	1713.05	2414.46	930.48	2009.83
M15990	*Tyr-pk (c-yes)*	1063.0	584.10	571.54	433.90	1025.3	453.69
X74262	*RBA/p48*	532.24	899.48	1053.83	1841.55	547.66	1511.37
U24166	*EB1*	847.67	1558.48	1763.15	2661.68	869.20	2285.99
X06182	c-*kit* proto-oncogene	627.71		1048.25	1870.54	663.40	1426.31
X51521	*EZRIN (villin 2)*	826.94		1554.57	2207.82	848.32	1746.88
U82169	*FZD3*	917.43		1697.19	2532.02	936.23	1984.74
U95299	*Notch-4*	726.26		1256.32	1764.66	776.10	1528.91
X00663/ U48722	*EGFR/ERBBI*	680.22			2299.14	691.40	1597.13
M83234	Ybox binding protein I	810.02			1547.13	790.33	1327.70
L25080	Transforming protein RhoA	568.15			1176.07	584.92	1046.97
M19722	Proto-oncogene Tyr-PK *(fgr)*	249.60			586.56	260.00	473.20
D38305	*TOB*	594.30			1331.20	572.80	979.48
U46461	*Disheveled homolog (dvl)*	442.26			827.02	459.10	743.74
X66363	*Ser/Thr-pk* (pctaire-1)	365.20			792.48	371.44	720.51

*C, volume intensity in control MCF-10F; CE, volume intensity in MCF10F + E; I_1, volume intensity in 60/60 cGy (p48); I_2, volume intensity in 60/60 cGy (p95); I_3, volume intensity in 60 cGy + E/60 cGy + E′ (p45); I_τ, volume intensity in Tumor-T. Each absolute value is an average of two to three array hybridization experiments and is expresed by volume intensity. Blank spaces indicate values similar to control, i.e., no change.

Table 3. Identification of Differentially Expressed Genes in Irradiated and Tumorigenic Breast (MCF-10F) Cell Lines

Gene name	MCF-10F	60/60 cGy ($Q = I_1/C$)*	60/60 cGy ($Q = I_2/C$)*	60 cGy + E/60 cGy + E ($Q = I_3/CE$)*	Tumor-T ($Q = I_t/C$)*
Retinoic acid receptor β2		(1.83)			
c-*fos*		(2.05)			
Stat1		(1.61)			
Stat5B		(1.71)			
met		(1.65)			
axl (Tyr-pkreceptor-ufo)		(2.05)			
Pro-oncogene Tyr-pk *(src)*		(1.64)			
Ser/Thr-pk (pctaire-3)		(1.65)			
Pro-oncogene Tyr-pk *(lck)*		(1.77)			
Tumor supp. protein *(dcc)*		(1.63)			
akt1 (rac-pk-alpha)		(1.74)			
*coup*Tf		(1.64)			
ski oncogene		(1.59)			
Cdkinase4 inhibitor B *(cdk4I)*		(1.83)			
xrcc1		(1.76)			
Cdkinase inhibitor 1C *(cdkI p57)*		(1.87)			
Tumor necrosis factor receptor		(1.68)			
Prohibitin		(1.56)			
(dvl)+(dvl3)		(1.74)			
frizzled 5		(−1.95)			
RB2/p130		(−1.94)			
c-*myc* proto-oncogene protein		(1.82)	(1.98)	(2.79)	(3.28)
IGFR1		(1.74)	(1.88)	(2.41)	(2.76)
ETS-related protein *(TEL)*		(1.76)	(1.90)	(2.38)	(2.73)
c-*jun* proto-oncogene		(1.68)	(1.95)	(2.56)	(3.02)
c-*myc (puf)*		(1.87)	(2.11)	(2.85)	(2.98)
MNDA		(1.74)	(1.93)	(2.67)	(3.12)
Hyaluronidase *(luca 1)*		(1.76)	(1.83)	(2.27)	(2.55)
Fra-1 (fos-related antigen)		(1.88)	(2.06)	(2.78)	(3.48)
Focal adhesion kinase *(fak)*		(1.68)	(1.81)	(1.98)	(2.07)
cdc25B		(1.80)	(2.07)	(2.71)	(2.96)
Glycogen synthase kinase 3		(1.78)	(1.84)	(2.07)	(2.36)
c-*myc* binding protein		(1.82)	(1.90)	(2.28)	(2.84)
E2F-1 pRB binding protein		(1.65)	(1.88)	(2.16)	(2.65)
Wnt-5a		(1.69)	(1.98)	(2.76)	(3.46)
Tyr-pk *(c-yes)*		(1.84)	(2.08)	(2.63)	(3.14)
RBA/p48		(−1.60)	(−3.13)	(−3.48)	(−4.91)
EB1		(−1.82)	(−1.86)	(−2.26)	(−2.45)
c-*kit* proto-oncogene			(1.67)	(2.15)	(2.98)
EZRIN (villin 2)			(1.88)	(2.06)	(2.67)
FZD3			(1.85)	(2.12)	(2.76)
Notch-4			(1.73)	(1.97)	(2.43)
EGFR/ERBBI				(2.31)	(3.38)
Ybox binding protein 1				(1.68)	(1.91)
Transforming protein RhoA				(1.79)	(2.07)
Proto-oncogene Tyr-pk *(fgr)*				(1.82)	(2.35)
TOB				(1.71)	(2.24)
Disheveled homolog *(dvl)*				(1.62)	(1.87)
Ser/Thr-pk *(pctaire-1)*				(1.94)	(2.17)

*Ratio (Q) of the Image Quant value of the volume intensity of irradiated (I) and control (C) cells. The (↑) sign indicates the upregulation and (↓/-) indicates the downregulation of gene expression. Blank spaces indicate the values similar to control, i.e., no change.

genes showed a gradual change (either upregulation or downregulation) moving from the transformed to the tumorigenic stage. Expression of c-*myc, puf, MNDA,* c-*yes,* and *Fra-1* genes was enhanced almost twofold at early and late stages of transformation, whereas expression was enhanced threefold or more in the tumorigenic and Tumor-T cell lines. Moreover, the expression of *RBA/p48* was suppressed about 1.5-fold at early stages of transformation to fourfold or more in tumorigenic and Tumor-T cell lines compared with the control MCF-10F cell line. The expression of the human *β-actin* control amplifier set, other housekeeping genes (ubiquitin, phospholipase A_2, tubulin alpha, ribosomal protein S9), and negative controls (*M13mp18[+]* strand *DNA*, λDNA, and pUC18) remained similar in all the cell lines.

DISCUSSION

Several cytologic and molecular biological techniques [35] can be used to identify the genetic changes involved in the progression of breast carcinogenesis. A differential hybridization technique in the form of a cDNA expression array has been used extensively to study gene expression [36]. In the present study, we used this technique to examine the effects of different known oncogenes and tumor suppressor genes in immortalized human breast epithelial cell lines, after they were subjected to different doses of high LET radiation and estrogen.

The association between breast cancer development and prolonged exposure to estrogens suggests that this hormone has a causative role in this disease. To examine this correlation, we treated the MCF-10F cell line with E, the most active estrogen, and assessed its effect on radiation-induced breast carcinogenesis. Although the parental MCF-10F cell line was negative for estrogen receptor (ER-α and ER-β), the tumorigenic cell lines in this model were found to be positive for these receptors [29]. These data indicate that changes in proliferative activity during the carcinogenesis process were mediated either by alteration in ER regulation or by an unknown non–receptor-mediated mechanism of action [37].

There is evidence that the increase in the ER-α/ER-β ratio commonly seen in primary breast tumors is due to an increase in ER-α and a corresponding decrease in ER-β mRNA expression [38]. We suggest that the role of ER-α–driven and ER-β–driven pathways or their interactions probably change during breast tumorigenesis. Thus, it is clear that ER-mediated regulation of gene expression plays many significant roles in normal and cancer cells [39], and the role of estrogen as an endogenous carcinogen must be further studied.

Comparable to the ongoing phenotypic changes associated with tumor progression in MCF-10F cell lines exposed to α particles and estrogen, a corresponding series of changes in the expression levels of various oncogenes and tumor suppressor genes also was identified. We found that among the 61 genes altered at different stages of the neoplastic process, only seven were detected exclusively at the tumorigenic stage. In all these cases, transformed cell lines were anchorage independent, but invasive behavior was seen only from the late stage of transformation to the tumorigenic phase [12].

From the 17 genes shown to be differentially expressed at all stages of the transformation process, six were randomly selected to confirm their differential expression with gene-specific RT-PCR and Northern blotting. Among them, the c-*myc* proto-oncogene was upregulated in all of our cell lines, and it has been reported to be amplified in breast cancer and is crucial for cell proliferation [40,41]. A previous study confirmed that the level of c-myc protein expression is higher in human breast epithelial cell lines transformed by high LET radiation and estrogen [42]. He et al. [43] have shown that c-*myc* is changed at the late stage in the dysregulated *Wnt/APC*/β-catenin pathway. Another altered gene, nucleoside diphosphate kinase B (*puf*), was found to be upregulated in our study. It stimulates gene transcription by acting as a c-*myc* transcription factor in cellular differentiation and tumor metastasis [44]. We also identified the elevated expression of c-*yes,* of the c-*src* family of protein kinases, at different stages of transformation. Muthuswamy and Muller [45] have shown that c-*yes* kinase activity is elevated in *neu*-induced mammary tumors. Thus, overexpression of this gene may be a suitable marker for detection of breast carcinogenesis.

It is also interesting to note the upregulation of the *Fra-1* gene at all stages of transformation right up to the tumorigenic stage of progression. Differential expression of *Fra-1* on human cutaneous epithelial cells by ultraviolet exposure and its role in retinoid action have been established [46,47]. Antisense research has shown the role of different genes of the AP-1 complex, including *Fra-1* and others, in neoplastic transformation [48,49]. Expression of another cell-cycle regulatory protein gene, *RBA/p48,* was downregulated in transformed and tumorigenic MCF-10F cell lines. It seems to coordinate cellular growth and differentiation and function as a tumor suppressor gene [50]. Several authors have shown that the protein product of *RBA/p48* can regulate transcription both in vivo and in vitro by interacting with the cell-cycle regulator *cdc2* kinase and mammalian D-type cyclins [51,52]. Thus, downregulation of *RBA/p48* could indicate a loss of tumor suppressor function that ultimately may lead to neoplastic progression in the cell. Our results also showed the elevated expression of the nuclear protein MNDA, but because not much is known

about its role in breast cancer progression, further study is needed.

Apart from the six genes already mentioned, the other 11 genes also were analyzed. Ten genes were upregulated, and the *EB1* gene was downregulated from the early stage of transformation to the tumorigenic stage of progression. Within this group of genes, *IGFR1* was overexpressed in all of our cell lines, and it has been shown [53] to synergize with E in stimulating the growth of human breast cancer in vitro. The *TEL* gene, a member of the family of ETS transcription factors, also was upregulated. It is known to be overexpressed in hyperplasia, in ductal carcinoma in situ, and in invasive breast carcinoma [54]. The elevated expression of the *luca1* and *fak* genes was consistent with the results of other breast cancer studies. This elevated expression has established their role in tumor formation and as signaling molecules in tumor cell invasion [55,56].

Enhanced expression of the *cdc25B* gene was noted in all of our cell lines. This finding may be important, because upregulation of this gene has been observed in various human cancers, including breast [57]. The overexpression of the *gsk3* gene identified here corroborated the results of other studies that have found that it interacts with the tumor suppressor genes *PTEN,* β-catenin, and E-cadherin in breast cancer progression [58,59]. The threefold to fourfold upregulation of the *e2f-1* and *Wnt5a* genes seen in the transformed and tumorigenic cell lines was also consistent with the results of other breast cancer studies [60,61].

All the genes upregulated at the late stage of transformation and the tumorigenic stage of progression were related to some membrane receptor–associated genes, such as the c-*kit* proto-oncogene, *EZRIN* (villin2), *FZD3,* and *Notch-4* genes. The proto-oncogene c-*kit* has been known to be overexpressed in breast tumors [62]; similarly, *EZRIN* is a family of cytoskeleton-associated membrane proteins directly regulated by estrogen in ER-containing breast cancer cell lines [63]. The *FZD3* and *Notch-4* genes are human transmembrane receptor proteins expressed predominantly in various organs [64,65], but further study is needed to identify their role in breast cancer progression.

Among the 49 genes studied, elevated expression of seven genes was seen exclusively at the tumorigenic stage of progression. Within these seven genes, it is interesting to note from our results that *EGFR/ERBB1* and proto-oncogene *fgr* were both altered; this finding confirmed what several authors have shown during breast cancer progression [66]. The *rhoA* gene also was overexpressed. The finding of elevated expression of the y-box binding protein 1 and *TOB* genes in tumorigenic cell lines was novel, in the sense that they are generally known to be associated with proliferation of liver and NIH-3T3 cells [67]. The *dvl* and *pctaire1* genes are widely expressed in various human organs, but there has been no report of their activity in breast cancer.

In conclusion, differential expression of various human oncogenes and tumor suppressor genes can elucidate the different stages of the transformation process, which is modified by such factors as radiation and estrogen treatment. Some genes were modified either at the early stage or at the late stage of transformation, whereas others were altered from the very early stage of transformation to the tumorigenic stage. Genes that were altered exclusively at the tumorigenic stage of progression might prove useful as biomarkers to detect tumor induction in breast carcinogenesis.

ACKNOWLEDGMENTS

This work was supported by funding from the National Institutes of Health grants CA 49062 (T.K.H.) and ES 07890 (T.K.H.) and the Avon Products Foundation Breast Center CU 51470301 institutional grant to Columbia University (G.C.).

REFERENCES

1. Miller BA, Ries LAG, Hankey BF, Kosary CL, Edwards BK. Cancer statistics review: 1973–1989. Bethesda, Md: National Cancer Institute; 1992. NIH Publication no. 92-2789.
2. Adnane J, Gaudray P, Simon MP, Simnoy-Lafontaine J, Jeanteur P, Theillet C. Proto-oncogene amplification and human breast tumor phenotype. Oncogene 1989;4(11): 1389–1395.
3. Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends Genet 1993;9:138–141.
4. Su Y, Ray ME, Lin T, et al. Reversion of monochromosome-mediated suppression of tumorigenicity in malignant melanoma by retroviral transduction. Cancer Res 1996; 56(14):3186–3191.
5. Dietrich CU, Pandis N, Teixeira MR, et al. Chromosome abnormalities in the benign hyperproliferative disorders of epithelial and stromal breast tissue. Int J Cancer 1995; 60:49–53.
6. Levine AJ. The tumor suppressor genes. Annu Rev Biochem 1993;62:623–651.
7. Weinberg RA. The molecular basis of oncogenes and tumor suppressor genes. Ann N Y Acad Sci 1995;758:331–338.
8. Hunter T. Oncoprotein networks. Cell 1997;88:333–346.
9. Smith HS, Lu Y, Deng G, et al. Molecular aspects of early stages of breast cancer progression. J Cell Biochem 1993;17G:144–152.
10. Dorosevich AE, Golubev OA, Abrosimov S, Bekhtereva IA. The role of cell communication in the pathogenesis of breast cancer. Vopr Onkol 1998;44(4):398–402.
11. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 1993;14(3): 483–492.
12. Calaf G, Hei TK. Establishment of a radiation- and estrogen-induced breast cancer model. Carcinogenesis 2000; 21(4):769–776.
13. Calaf G, Zhang PL, Alvarado ME, Estrada S, Russo J. c-Ha-ras oncogene enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. Int J Oncol 1995;6:5–11.
14. Bieche I, Lidereau R. Genetic alterations in breast cancer. Genes Chromosom Cancer 1995;14:227–251.
15. Krieger N. Exposure, susceptibility, and breast cancer risk: A hypothesis regarding exogenous carcinogens, breast tissue

development, and social gradients, including black/white differences, in breast cancer incidence. Breast Cancer Res Treat 1989;13:205–223.
16. Soule HD, Maloney TM, Wolman SR, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10A. Cancer Res 1990; 50:6075–6086.
17. Hei TK, Komatsu K, Hall EJ, Zaider M. Oncogenic transformation by charged particles of defined LET. Carcinogenesis 1988;9:747–750.
18. Hall EJ, Hei TK. Modulating factors in the expression of radiation induced oncogenic transformation. Environ Health Perspect 1990;88:149–155.
19. Zhu LX, Waldren CA, Vannais D, Hei TK. Cellular and molecular analysis of mutagenesis induced by charged particles of defined linear energy transfer. Radiat Res 1996; 145(3):251–259.
20. Hall EJ. Radiobiology for the radiologist. Philadelphia: Lippincott Williams and Wilkins; 2000; p 17–50.
21. Boothman DA, Bouvard I, Hughes EN. Identification and characterization of X-ray induced proteins in human cells. Cancer Res 1989;49(11):2871–2878.
22. Sehgal A, Boynton AL, Young RF, et al. Application of the differential hybridization of atlas human expression arrays technique in the identification of differentially expressed genes in human glioblastoma multiforme tumor tissue. J Surg Oncol 1998;67(4):234–241.
23. DeRisi J, Penland L, Brown PO, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet 1996;14:457–460.
24. Pietu G, Alibert O, Guichard V, et al. Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridization of a high density cDNA array. Genome Res 1996;6(6):492–503.
25. Debouck C, Goodfellow PN. DNA microarrays in drug discovery and development. Nat Genet 1999;21: (suppl 1). 48–50.
26. Calaf G, Russo J, Tait L, Estrada S, Alvarado ME. Morphological phenotypes in neoplastic progression of human breast epithelial cells. J Submicrosc Cytol Pathol 2000;32(1):83–96.
27. Ponnaiya B, Cornforth MN, Ullrich RL. Induction of chromosomal instability in human mammary cells by neutrons and gamma rays. Radiat Res 1997;147(3): 288–294.
28. Hei TK, Piao CHQ, Willey JC, Thomas S, Hall EJ. Malignant transformation of human bronchial epithelial cells by radon-simulated α-particles. Carcinogenesis 1994;15:4311–4317.
29. Singhal H, Guo L, Bradlow HL, Mittelman A, Tiwari RK. Endocrine characteristics of human breast epithelial cells, MCF-10F. Horm Res 1999;52(4):171–177.
30. Adams MD, Kerlavage AR, Fleischmann RD, et al. Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence. Nature 1995;377(suppl 6547):3–174.
31. Chalifour LE, Fahmy R, Holder EL, et al. A method for analysis of gene expression patterns. Anal Biochem 1994; 216:299–304.
32. Sebastian JL, Rigberg DA, Shrivatsan E, Revasova E, McFadden DM, Levingston EH. The use of large-scale cDNA analysis to profile differential gene expression in KYSE 410 human esophageal cancer cells after irradiation. Bull Exp Biol Med 2001;130(9):882–885.
33. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 1989.
34. Sehgal A, Keener C, Boynton AL, et al. Isolation and characterization of a novel gene from human glioblastoma multiforme tumor tissue. Int J Cancer 1997;71:565–572.
35. Diatchenko L, Lau Y-FC, Campbell AP, et al. Suppression subtractive hybridization: A method for generating differ entially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1996;93:6025–6030.
36. Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW. Parallel human genome analysis: Microarray-based expression monitoring of 1000 genes. Proc Natl Acad Sci U S A 1996;93(20):10614–10619.
37. Calaf GM, Roy D, Hei TK. Neoplastic progression in a radiation- and estrogen-induced breast cancer model. Abstract. Proceedings of the American Association for Cancer Research 2001;42:893–894.
38. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. Cancer Res 1998;51(15):3197–3201.
39. Dickson RB, Stancel GM. Estrogen receptor–mediated processes in normal and cancer cells. J Natl Cancer Inst Monogr 2000;27:135–145.
40. Nass SJ, Dickson RB. Defining a role for c-Myc in breast tumorigenesis. Breast Cancer Res Treat 1997;44:1–22.
41. Watson PH, Pon RT, Shiu RPC. Inhibition of c-Myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. Cancer Res 1991;51:3996–4000.
42. Calaf G, Hei TK. Oncoprotein expression in human breast epithelial cells transformed by high LET radiation. Int J Rad Biol 2000;77(1):31–40.
43. He T-C, Sparks AB, Rago C, et al. Identification of c-Myc as a target of the APC pathway. Science 1998;281:1509–1512.
44. Agou F, Raveh S, Mesnildrey S, Veron M. Single strand DNA specificity analysis of human nucleoside diphosphate kinase B. J Biol Chem 1999;274(28):19630–19638.
45. Muthuswamy SK, Muller WJ. Activation of Src family kinases in neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. Oncogene 1995;11(9): 1801–1810.
46. Ariizumi K, Bergstresser PR, Takashima A. Wavelength-specific induction of immediate early genes by ultraviolet radiation. J Dermatol Sci 1996;12(2):147–155.
47. Kaiser A, Brembeck FH, Marschall Z, Riecken EO, Weidenmann B, Rosewicz S. Fra-1: A novel target for retinoid action. FEBS Lett 1999;448(1):45–48.
48. Vallone D, Battista S, Pierantoni GM, et al. Neoplastic transformation of rat thyroid cells requires the junB and fra-1 gene induction which is dependent on the HMGI-C gene product. EMBO J 1997;16(17):5310–5321.
49. Smith LM, Wise SC, Hendricks DT, et al. c-Jun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. Oncogene 1999;18(44):6063–6070.
50. Bookstein R, Lee WH. Molecular genetics of the retinoblastoma suppressor gene. Crit Rev Oncog 1991;2(3):211–227.
51. Lin BT, Gruenwald S, Morla AO, Lee WH, Wang JY. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. EMBO J 1991;10(4):857–864.
52. Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J, Livingston DM. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell 1993;73(3):487–497.
53. Lee AV, Jackson JG, Gooch JL, et al. Enhancement of insulin-like growth factor signaling in human breast cancer: Estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. Mol Endocrinol 1999;13(5):787–796.
54. Edel MJ. Analysis of the TEL protein during tumour angiogenesis. Anticancer Res 1999;19(4B):2945–2951.
55. Bertrand P, Girard N, Duval C, et al. Increased hyaluronidase levels in breast tumor metastases. Int J Cancer 1997; 73(3):327–331.

56. Weiner TM, Liu ET, Craven RJ, Cance WG. Expression of focal adhesion kinase gene and invasive cancer. Lancet 1993;342(8878):1024–1025.
57. Yao Y, Slosberg ED, Wang L, et al. Increased susceptibility to carcinogen-induced mammary tumors in MMTV-Cdc25B transgenic mice. Oncogene 1999;18(37):5159–5166.
58. Lu Y, Lin YZ, LaPushin R, et al. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene 1999;18(50):7034–7045.
59. Li Y, Bharti A, Chen D, Gong J, Kufe D. Interaction of glycogen synthase kinase 3beta with the DF3/MUC1 carcinoma-associated antigen and beta-catenin. Mol Cell Biol 1998;18(12):7216–7224.
60. Zhang SY, Liu SC, Al-Saleem LF, et al. E2F-1: A proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 2000;9(4):395–401.
61. Lejeune S, Huguet EL, Hamby A, Poulsom R, Harris AL. Wnt5a cloning, expression, and up-regulation in human primary breast cancers. Clin Cancer Res 1995;1(2):215–222.
62. Hines SJ, Litz JS, Krystal GW. Coexpression of c-kit and stem cell factor in breast cancer results in enhanced sensitivity to members of the EGF family of growth factors. Breast Cancer Res Treat 1999;58(1):1–10.
63. Ediger TR, Kraus WL, Weinman EJ, Katzenellenbogen BS. Estrogen receptor regulation of the Na^+/H^+ exchange regulatory factor. Endocrinology 1999;140(7):2976–2982.
64. Wang YK, Samos CH, Peoples R, Perez-Jurado LA, Nusse R, Francke U. A novel human homologue of the *Drosophila* frizzled wnt receptor gene binds wingless protein and is in the Williams syndrome deletion at 7q11.23. Hum Mol Genet 1997;6(3):465–472.
65. Uyttendaele H, Marazzi G, Wu G, Yan Q, Sassoon D, Kitajewski J. Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development 1996;122(7):2251–2259.
66. Yamamoto T, Toyoshima K. The erbB-related proto-oncogenes encoding growth factor receptors. Gan To Kagaku Ryoho 1987;14(6 pt 2):2075–2082.
67. Matsuda S, Kawamura-Tsuzuku J, Ohsugi M, et al. Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. Oncogene 1996;12(4):705–713.