



## MOLECULAR ALTERATIONS IN TUMORIGENIC HUMAN BRONCHIAL AND BREAST EPITHELIAL CELLS INDUCED BY HIGH LET RADIATION

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### ABSTRACT

Carcinogenesis is a multi-stage process with sequence of genetic events governing the phenotypic expression of a series of transformation steps leading to the development of metastatic cancer. In the present study, immortalized human bronchial (BEP2D) and breast (MCF-10F) cells were irradiated with graded doses of either 150 keV/ $\mu\text{m}$  alpha particles or 1 GeV/nucleon  $^{56}\text{Fe}$  ions. Transformed cells developed through a series of successive steps before becoming tumorigenic in nude mice. Cell fusion studies indicated that radiation-induced tumorigenic phenotype in BEP2D cells could be completely suppressed by fusion with non-tumorigenic BEP2D cells. The differential expressions of known genes between tumorigenic bronchial and breast cells induced by alpha particles and their respective control cultures were compared using cDNA expression array. Among the 11 genes identified to be differentially expressed in BEP2D cells, three (*DCC*, *DNA-PK* and *p21<sup>CIP1</sup>*) were shown to be consistently down-regulated by 2 to 4 fold in all the 5 tumor cell lines examined. In contrast, their expressions in the fusion cell lines were comparable to control BEP2D cells. Similarly, expression levels of a series of genes were found to be altered in a step-wise manner among tumorigenic MCF-10F cells. The results are highly suggestive that functional alterations of these genes may be causally related to the carcinogenic process. © 2001 COSPAR. Published by Elsevier Science Ltd. All rights reserved.

### INTRODUCTION

Carcinogenesis is a progressive process involving a number of mutational events with each mutation conferring additional growth advantage for the clonal expansion of genotypically altered cells (Fearon and Vogelstein, 1990). Although ionizing radiation is a well-established human carcinogen and is known to induce both lung and breast cancer, the underlying cellular and molecular mechanism of radiation carcinogenesis remain unknown. The carcinogenic risk for human epithelial cells after exposure to low LET radiation has been estimated to be in the range of  $10^{-12}$  per cell per Gy based on the breast cancer incidence among Japanese A-bomb survivors (Hei *et al.*, 1996), an incidence too low to be reproduced under any laboratory setting. Immortalized human lung and breast epithelial cell cultures offer the next

best alternatives as useful models in assessing the various phenotypic and genotypic changes leading to malignancies after exposure to ionizing radiation. Although these cells are immortalized either spontaneously (MCF-10F human breast) or through viral transduction (BEP2D human bronchial), they are phenotypically normal and do not express any transformed characteristics such as anchorage independent growth and tumorigenicity in nude mice. After exposure to either 150 keV/ $\mu\text{m}$   $\alpha$  particles or 1 GeV/ nucleon  $^{56}\text{Fe}$  ions, transformed cells arise through a series of sequential stages including altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity, and metastasis (Figure 1, Hei *et al.*, 1994, 1998, Calaf and Hei, 2000).

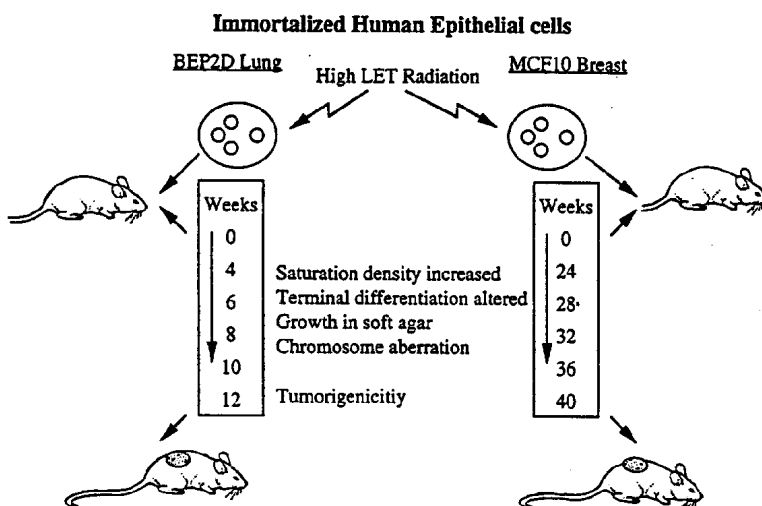


Fig.1 Schematic diagram illustrating the multistep neoplastic transformation process in both immortalized human bronchial and breast epithelial cells irradiated with a single 60 cGy dose of  $\alpha$  particles. Irradiated cells need to undergo successive passages with the concomitant accumulation of additional mutagenic changes before tumorigenicity can be demonstrated. The time scale necessary for breast epithelial cells to undergo neoplastic changes is significantly longer than comparatively treated lung cells.

Previous studies have shown that a single 60 cGy dose of these high LET radiations can induce neoplastic transformation of the BEP2D cells in a step-wise fashion at a frequency of  $\sim 10^{-7}$  (Hei *et al.*, 1996). The immortalization step, therefore, increases the transformation yield of primary human epithelial cells by more than a million fold. It should be noted that, while the majority of agar-positive BEP2D clones are non-tumorigenic, they all demonstrated the propensity to resist serum-induced terminal differentiation. In addition, each preceding stage represents a necessary, yet insufficient step towards the later, more malignant phase. Tumorigenic BEP2D cells show no mutation in any of the *ras* oncogenes (Hei *et al.*, 1994, 1996b). Results of cell fusion studies between  $\alpha$  particle-induced tumorigenic and parental BEP2D cells demonstrated that the tumorigenic phenotypic could be completely suppressed by fusion with non-tumorigenic control cells (Zhao *et al.*, 2000). These data indicate that non-tumorigenic BEP2D cells complement the loss of putative suppressor element among tumorigenic cells and suggest that loss of suppressor gene(s) as an important mechanism of radiation carcinogenesis. Since BEP2D cells express both E6 and E7 viral proteins (Willey *et al.*, 1991), these

data suggest that abnormal p53 and Rb functions are insufficient for tumorigenic conversion in these cells and additional genetic factors and cellular events are required.

### USE OF cDNA EXPRESSION ARRAYS TO COMPARE DIFFERENTIALLY EXPRESSED GENES IN RADIATION INDUCED TUMORIGENIC CELL LINES

Identification of changes in the gene expression profile in radiation-induced transformed cells at various stages of the neoplastic process will lead to a better understanding of the mechanism of radiation carcinogenesis. A recently developed technique, cDNA expression array, allows the large-scale comparison of multiple genes in a single hybridization. The assay has the advantage of providing rapid and immediate information on interested genes as well as the functions of their proteins. The Clontech cDNA expression array (Clontech Laboratories, Palo Alto, CA) contains six quadrants, which cover different categories of genes, including oncogenes, tumor suppressor genes, intracellular signal transduction modulators, DNA synthesis and repair genes, transcription factors, and receptors and growth factors genes. Briefly,  $^{32}\text{P}$ -labelled cDNAs are prepared by reverse transcription using mRNA from control and tumorigenic BEP2D or MCF-10F cells in the presence of gene-specific primers and  $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ . The probes are then purified using spin columns to remove any unincorporated nucleotides. After pre-hybridization, hybridization is usually carried out overnight and the signals are analyzed by autoradiography and further quantified by phosphorimaging (ImageQuant software). The expression levels of human  $\beta$ -actin and G3PDH housekeeping genes are used as standards for normalizing the expression levels of other genes. Each cDNA of the 588 preset genes are spotted in duplicate on the nylon membranes as shown in Figure 2.

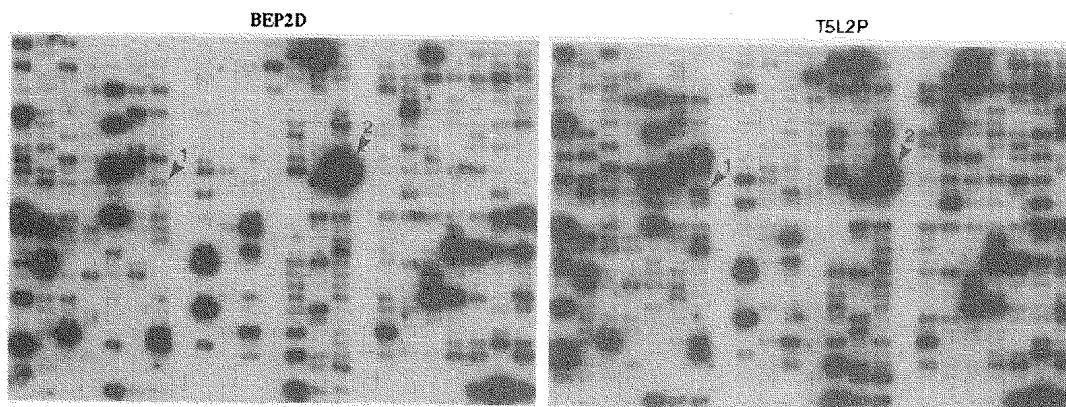


Fig. 2 Differential gene expression in a representative tumorigenic BEP2D cells induced by a single 60 cGy dose of  $\alpha$  particles (T5L2P, right hand panel) relative to control BEP2D cells (left hand panel). The labeled cDNAs were hybridized to the array overnight at  $65^{\circ}\text{C}$ . The image was captured on X-ray film after exposure for 2 days at  $-80^{\circ}\text{C}$ . Arrows indicate the location of the cDNA spots on the membrane for 1) *cdc 25B* and 2) *HSP 27*.

Since the incidence of false positives among cDNA expression analyses could be as high as 15-20%, it is essential to double-check the message level using Northern blotting. Table I lists the differentially expressed genes and their relative mRNA expression levels pooled from 5 tumorigenic BEP2D cell lines induced by  $\alpha$  particles. Expression levels of the individual genes were compared relative to the level of the  $\beta$ -actin gene from the same membrane. Among the 11 genes initially identified as differentially expressed, two of which, *c-myc* and fibronectin could not be confirmed when subsequently analyzed by Northern blotting. Several cell growth and differentiation genes were found to be down-regulated in the tumorigenic BEP2D cells relative to their corresponding controls. These included the *DCC* tumor suppressor gene, the DNA dependent protein kinase gene, the differentiation marker cytokeratin *CK14*, and the cell cycle modulator *p21<sup>CIP1</sup>* gene. In contrast, 3 genes were found to be up-regulated in the tumorigenic cell lines by an average of 2-3 fold. These included *c-fos*, *NF $\kappa$ B*, and the M-phase inducer gene *CDC-25B* (Table I).

**Table I.**

List of Differentially Expressed Genes Between Tumorigenic and Control BEP2D Cells Induced by  $\alpha$  particles Based on mRNA Values

Gene	Expression levels
<i>DCC</i>	↓ 2.1 ± 0.2
<i>DNA-PK/ Ku70</i>	↓ 2.2 ± 0.5
<i>HSP 70</i>	↓ 4.9 ± 2.3
<i>Cytokeratin 14</i>	↓ 5.0 ± 0.8
<i>DNKN1A (p21<sup>CIP1</sup>)</i>	↓ 4.4 ± 2.1
<i><math>\alpha</math>-catenin</i>	↓ 2.2 ± 0.5
<i>CDC 25B (M-phase inducer)</i>	↑ 3.3 ± 1.2
<i>c-fos</i>	↑ 3.0 ± 0.9
<i>NF<math>\kappa</math>B</i>	↑ 1.8 ± 0.5
<i>c-myc</i>	No change
<i>Fibronectin</i>	No change

## CORRELATION OF GENE EXPRESSION TO SPECIFIC PHENOTYPIC STAGES OF THE NEOPLATIC PROCESS IN HUMAN BREAST EPITHELIAL CELLS

The human breast epithelial cell line MCF-10F, spontaneously-immortalized and derived from the breast tissue of a 36-year-old female undergoing mastectomy for fibrocystic disease, is unique in the sense that it has the morphological characteristics of normal breast epithelial cells. These characteristics include dome formation in confluent cultures, three-dimensional growth in collagen gel, dependence upon hormonal and growth factors for optimal growth *in vitro* (Soule *et al.*, 1990). In addition, these cells are anchorage dependent, non-invasive, and non-tumorigenic in nude mice (Calaf *et al.*, 1995). There is recent evidence that MCF-10F cells can be malignantly transformed with two 60 cGy doses of  $\alpha$  particles (Calaf and Hei, 2000). Transformed cells produce progressively growing tumors in nude mice some 10 months post-irradiation with a latency period of 3-4 months. Interestingly, irradiated

MCF-10F cells follow a similar step-wise, phenotypically altered pattern as that of the bronchial epithelial cells before becoming tumorigenic. However, the time scale for the neoplastic process is 3-4 fold longer than that of BEP2D cells (Figure 1). The availability of this model provides a unique opportunity to delineate the genetic alterations associated with specific stages of the neoplastic process in human breast epithelial cells.

Figure 3 shows the differentially expressed genes identified using cDNA expression arrays between control and transformed MCF-10F cells at different stage of the neoplastic process. A total of 14 genes were found to be altered in transformed MCF-10F cells that acquired anchorage independent growth but not tumorigenicity. Eleven of these genes were found to be up-regulated in levels ranging from 2 to 4 fold relative to controls. Two of these genes, *RbA* and *EB1*, were found to be down-regulated instead. As the cells progressed and acquired the malignant phenotype such that they were readily tumorigenic in either nude or SCID mice, an additional 11 genes were found to be altered. These late-expression genes were all shown to be up-regulated in levels ranging from 2 to 3 fold.

### Alterations in Gene Expression with Phenotypic Progression in MCF-10F Cells Induced by High LET Radiation

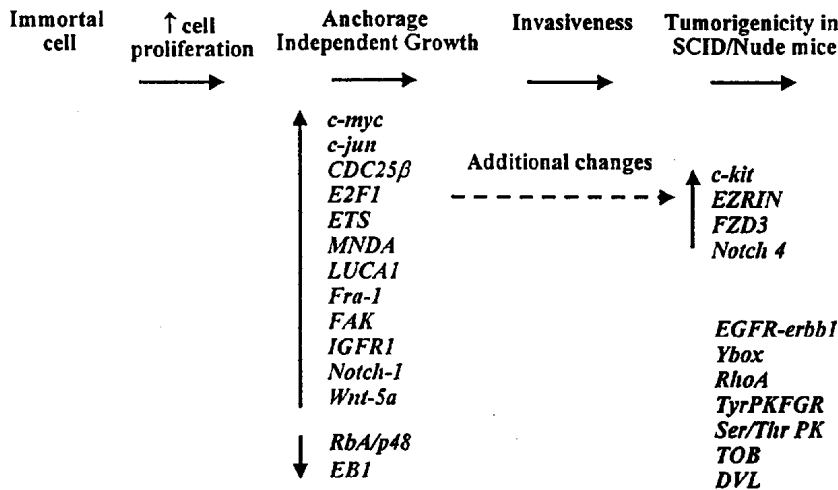


Fig. 3 Differentially expressed genes as a function of transformation stages in MCF-10F cells.

To correlate alterations in gene expression level with the corresponding protein expression in transformed and tumorigenic MCF-10F cells, immunohistochemical staining coupled with confocal microscopy were used. Briefly, exponentially growing cells at similar passage numbers were plated on chamber slides until they reached 70% confluent. After staining with the primary and then with Rhodamine -conjugated secondary antibodies, the cultures were visualized using a laser scanning confocal microscope. The fluorescent images were quantified using an image analysis software. Figure 4 shows the changes in expression levels of various cell cycle and apoptosis regulating proteins as a function of the phenotypic progression in irradiated MCF-10F cells. An increase in protein expression of c-MYC, c-JUN, c-FOS and proliferating cell nuclear antigen (PCNA) were found early in the transformation process. The increased expression levels of these proteins persisted in transformed MCF-

10F cells that acquired the tumorigenic phenotype. Likewise, the expression levels of several pro-apoptotic proteins such as BCL2 and BAK decreased early in the transformation process. In contrast, increased expression levels of H-RAS, p16 and RAD51 appeared to occur late in the neoplastic progression of irradiated MCF-10F cells.

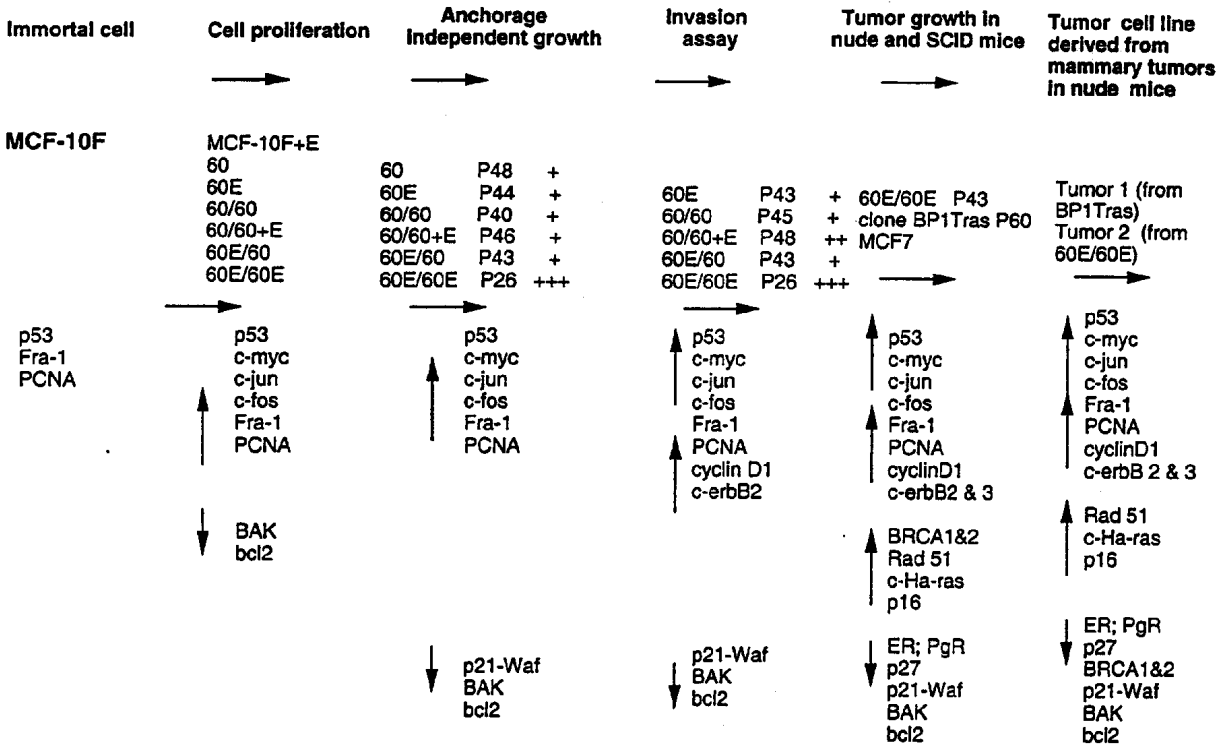


Fig. 4 Schematic diagram that illustrates the gradual phenotypic and molecular alterations in MCF-10F cells irradiated with high LET radiation. Exponentially growing cells were irradiated with either a single or double doses of 60 cGy dose of high LET  $\alpha$  particles with or without concurrent treatment with estrogen (E). The corresponding passage numbers (P) of the various cultures attending the phenotypic changes were shown (For detail please refer to Calaf and Hei, 2000).

### HIGH LET RADIATION CARCINOGENESIS AND DIFFERENTIAL GENE EXPRESSION

Identification of the step-wise molecular alterations in carcinogenesis is one of the major goals in cancer research. The cDNA array provides a useful method to screen differentially expressed genes between control and tumor cells. Although our present study identified a number of differentially expressed genes between tumorigenic and control cells in both the human bronchial BEP2D and the human breast MCF-10F cells induced by high LET radiation, the nature of the interaction of these genes in modulating the malignant phenotype is not clear. It is likely that concerted efforts of many genes rather than loss of a single suppressor function is critical for the tumorigenic phenotype. For example, a reduced expression level of *DNA-PK* may contribute to genetic instability of transformed BEP2D cells.

Subsequent down-regulation of the E-cadherin-catenin complex may enhance invasiveness of transformed cells. The reduced expressions of *DCC* might collaborate with the other genes in malignant progression by promoting genomic instability and increase susceptibility to oxidative damage. Evidence for this line of thought comes from the cell fusion studies which indicate that loss of tumorigenic potential is accompanied by a re-expression of these genes to the control levels.

A number of genes are found to be down regulated in both the tumorigenic MCF-10F as well as BEP2D cells. The *p21<sup>CIP1</sup>* is a cyclin-dependent kinase inhibitor that can effectively inhibit CDK2, CDK4 and CDK6 kinases and is capable of inducing cell cycle arrest in G1 when overexpressed. Although *p21<sup>CIP1</sup>* is induced by p53 in response to radiation, *p21<sup>CIP1</sup>* can be expressed at high level in p53 negative cells, indicating the existence of p53-independent mechanisms to regulate *p21<sup>CIP1</sup>* expression (Harper *et al.*, 1993). This observation is consistent with the fact that wild type p53 is expressed in very low level in both MCF-10F and BEP2D cells due to the presence of a point mutation in the *p53* gene in the former and the presence of E6 oncoprotein in the later. The down-regulation of *p21<sup>CIP1</sup>* has been shown to increase CDC2 and CDK2 kinase activity and promote cell cycle and cellular proliferation (Crow *et al.*, 1998). Reduced protein levels of *p21<sup>CIP1</sup>* have been reported in many human cancers and may contribute to the malignant progression (Pinyol *et al.*, 1997).

Another gene which is up-regulated in both the tumorigenic breast and bronchial epithelial cells is the M-phase inducer gene *CDC25*. The *CDC25* family of mammalian phosphatases includes 3 homologs: *CDC25A*, *CDC25B* and *CDC25C*, which can activate cyclin-dependent kinases by removing inhibitory phosphates from tyrosine and threonine residues. The *CDC25* functions at G1/S phase and at the G2/M phase (Galaktinov and Beach, 1991). The overexpression of *CDC25B* was observed in many breast and head and lung cancers (Wu *et al.*, 1998). The gene cooperates with H-*ras* or loss of RB1 in oncogenic transformation, and high expression of *CDC25B* in primary breast cancers correlates with less favorable prognosis and survival (Galaktinov *et al.*, 1995). Reduced *CDC 25B* mRNA level and deregulated protein expression by antisense oligonucleotides result in S-phase delay and anti-proliferative effects in synchronized HeLa cells (Garner-Hamrick and Fisher, 1998). These data indicates that *CDC25B* possess oncogenic properties and is consistent with our observation that *CDC25B* was up-regulated 3 fold in both tumorigenic MCF-10F and BEP2D cells, suggesting that an overexpression of *CDC25B* may play an important role in radiation carcinogenesis.

Although *c-myc* expression was found to be unchanged in tumorigenic BEP2D cells, the gene was overexpressed in MCF-10F cells in a progressive manner. The result was consistent with the observation that c-MYC expression could be induced by ionizing radiation during either stimulation of cellular proliferation or transformation (Mothersill *et al.*, 1994). Furthermore, C-MYC has been shown to be overexpressed in many breast tumor samples (Shiu *et al.*, 1998) and its expression is critical for the progression of both hormone dependent as well as hormone independent breast cancer cells (Field and Spandidos, 1990). Since the expression of c-MYC protein is an early event, an overexpression of the gene as a result of amplification or mutation probably plays a causal role in breast carcinogenesis.

**ACKNOWLEDGMENT:** Work supported by NIH grants CA 49062, ES 07890, CA/NASA 73946, and Research Resource Center grant RR 11623.

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