Detection of chromosomal aberrations by comparative genomic hybridization during transformation of human breast epithelial cells *in vitro*

G.A. BALOGH[1], I.H. RUSSO[1], B.R. BALSARA[2] and J. RUSSO[1]

[1]Breast Cancer Research Laboratory, [2]Human Genetics Program,
Fox Chase Cancer Center, Philadelphia, PA 19111, USA

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Abstract. Breast cancer is the most frequent malignancy in women. It is well recognized that tumorigenesis is a multistep process resulting from the accumulation of sequential genetic alterations. In breast cancers LOH has been described on one or both arms of multiple chromosomes. Comparative genomic hybridization (CGH) analysis was performed to identify chromosomal imbalances in the breast epithelial cells (HBEC). We have used a human *in vitro-in vivo* system in which the environmental carcinogen benz(a)pyrene (BP) and the c-Ha-ras oncogene were utilized for inducing *in vitro* transformation of HBEC. Immortal MCF-10F cells were treated with BP which resulted in the transformed cell line BP-1 that was further enhanced by transfection with the c-Ha-ras to generate the cell line BP-1-Tras. This cell line is tumorigenic when injected in severe combined immunodeficient (SCID) mice, generating the tumor cell line BP-1-Tras T J#4. Our comparative genomic hybridization analysis indicates that the most overrepresented segment after cell transformation and in the BP-1, BP-1-Tras and in the tumor cell line were 1p (80%), 5q21-ter (80%), 8q24.1 (90%) and Xq27-28 (60%). DNA sequence amplification at 10p14-15 was observed in BP-1-Tras T J#4 cells. Allelic losses of chromosome 4, 8p11-21 and 15q11-12, occur after cell transformation and are maintained consistently during tumorigenesis.

Introduction

Many genomic alterations, such as DNA amplification and loss of genetic material, which may involve tumor suppressor genes, have been found in breast cancer in at least 11 chromosomes (1-18). The detection of these genetic alterations in various tumor stages has provided evidence that primary and secondary events accumulate and hence contribute to stepwise neoplastic progression. The fact that the alterations are complex makes it difficult to precisely determine whether these genomic changes are responsible for the initiation and/or the progression of the disease, or whether they are the consequence of chromosomal destabilization caused by tumor cell proliferation (17,18). It is expected that specific types of genetic alterations identify essential steps in the initiation and/or progression of cancer. We used comparative genomic hybridization (CGH), a technique that has been utilized extensively to document both genetic gains and losses in human cancer, for determining whether somatic genetic aberrations occurred in our *in vitro-in vivo* system that recapitulates the initiation and progression of the disease (19-21). In our model, transformation of the normal immortalized human breast epithelial cells (HBEC) MCF-10F (22,23) was induced by *in vitro* treatment with the chemical carcinogen benz(a)pyrene (B[a]P) (24). After clonal selection the cells exhibited anchorage-independent growth, colony formation in agar methocel, and loss of ductulogenic capacity in collagen gel. c-Ha-ras transfection intensified the expression of transformation phenotypes, generating BP-1-Tras, a tumorigenic cell line (25). Injection of these cells to severe combined immunodeficient (SCID) mice generated the tumor cell line BP-1-Tras T J#4 (21). DNA analysis by comparative genomic hybridization (CGH) revealed that phenotypically transformed cells exhibited the same type of genomic alterations observed in spontaneous malignancies. A sequence of genomic changes was manifested during *in vitro* transformation and was maintained during the tumorigenesis process, with significant chromosomal losses on 4p/q and 15q11-12.

Materials and methods

Cell lines. For these experiments we used the spontaneously immortalized human breast epithelial cell line MCF-10F, its human breast epithelial origin has been confirmed by genetic, cytogenetic, ultrastructural, and phenotypic characteristics, which are identical to the mortal MCF-10M

Correspondence to: Dr Jose Russo, Breast Cancer Research Laboratory, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA
E-mail: j_russo@fccc.edu

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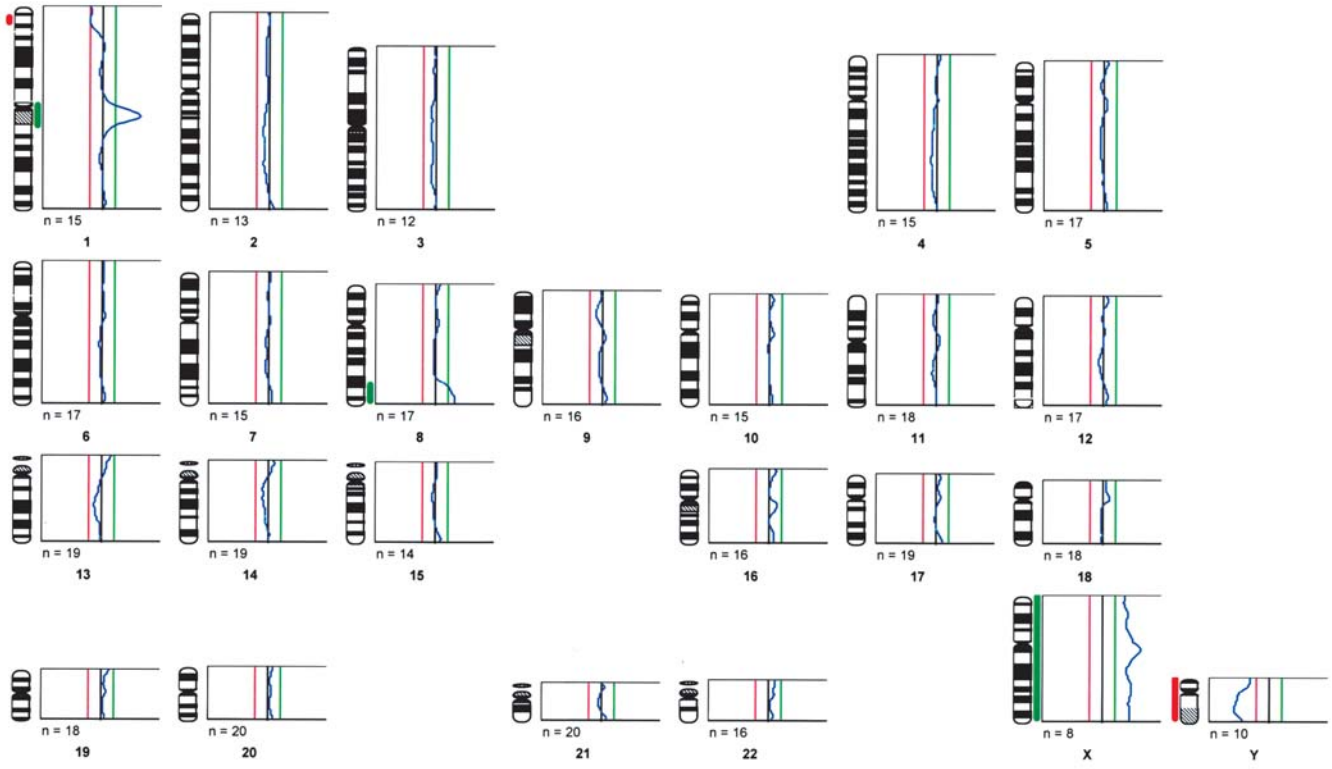

Figure 1. CGH profile of the MCF-10F cell line. The chromosomes and the gain and loss are indicated in green and red, respectively. The three vertical lines represent the balance state (control) and the lower (left) and the upper (right) thresholds used as diagnostic cutoff values for losses and gains, respectively.

cells from which they were derived, except for the fact that the immortal cells are pseudodiploid and express minimal chromosomal alterations (22,23). We also used the B[a]P transformed tumorigenic cell line BP-1Tras (24) and the tumor-derived cell line BP-1-Tras J#4 (21). All cell lines are cultured in our laboratory in DMEM medium under standard conditions at 37˚C in a O_2/CO_2 atmosphere.

DNA isolation. DNA was extracted from the cells in culture when they were 70-80% confluent. The cells were treated with lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) and 200 μg/ml proteinase K was added and incubated at 65˚C for 15 min with gentle agitation. The samples were cooled down on ice and treated with 100 μg/ml RNase at 37˚C for 30 min. The DNA was purified with a phenol extraction (pH 8.0) followed by chloroform:isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75 M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. The DNA was used for comparative genome hybridization (CGH).

Comparative genome hybridization (CGH). Protocols for DNA labeling and hybridization were performed as previously described (26). Gray-level images of fluorescence were captured with a Zeiss (Thorndale, NY) microscope connected to a cooled, charge-coupled-device camera (Photometrics, Tucson, AZ). Digital image analysis was performed using the Quips software (Vysis, Downers Grove, IL). The threshold was set at 0.8 and 1.2 for losses and gains, respectively. The mean values of individual ratio profiles were calculated from at least 7 metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms.

Table I. Genetic imbalances in HBEC cells.

Cell line	Gains	Losses
MCF-10F	8q24.1	
BP-1	1p	4
	5q21-ter 8q24	8p11-21
	13q33	15q11-12
	Xq27-28	
BP-1-Tras	1p	4
	5q21-ter	8p11-21
	8q24.1	15q11-12
	13q33-34	
	Xq27-28	
BP-1-Tras T J#4	1p	4
	5q21-ter	8p11-21
	6q25-27	13q11-13
	8q24.1	15q11-12
	18q11.3	
	20p11, 20q13	

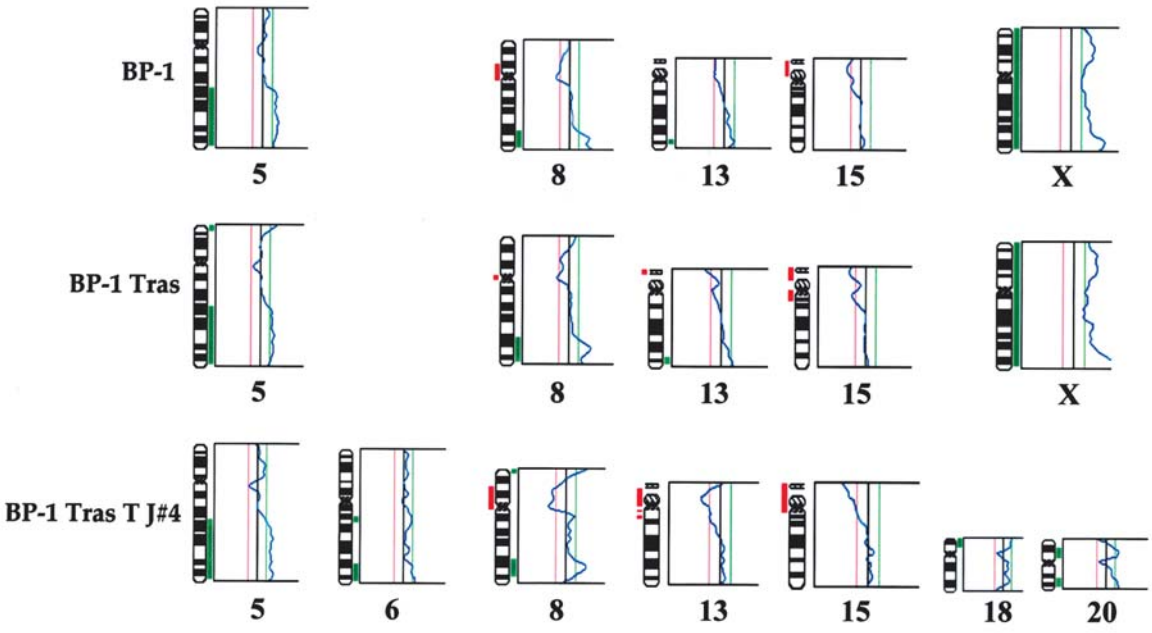

Figure 2. CGH profile of the transformed BP-1, BP-1-Tras and BP-1-Tras T J#4 Cell lines.

Results

The immortal cell line (MCF-10F) showed a normal profile with gain only in the chromosome band 8q24 (Fig. 1). There are, however, multiple genomic alterations in the transformed, tumorigenic and tumor cell lines (Table I and Fig. 2). The transformed cell line BP-1 and the tumorigenic line BP-1-Tras had several more genomic imbalances compared to MCF-10F cells. These include gains of 1p, 5q21-qter, 13q33, Xq27-28, amplification of 8q24, loss of chromosome 4, 15q11-12, and chromosome bands 8q11-21. Amplification at 8q24.1 was more pronounced in the tumorigenic cell line BP-1-Tras than in BP-1 cells. The tumor cell line had additional changes which include gains of 6q25-27, 18p11.3, 20p11 and 20q13, loss of chromosome bands 13q11-13 was also an additional change (Table I and Fig. 2). Allelic losses of chromosome 4, 8p11-21 and 15q11-12, occur after cell transformation and are maintained consistently during tumorigenesis (Table I and Fig. 2).

Discussion

In the present work, using comparative genomic hybridization analyses, we observed that during the process of cell transformation and tumorigenesis the gains of DNA sequences were more common than losses, with the most frequent gains at 8q24.1, 1p, 5q21-ter, 13q33-34 and Xq37-38. Of significance is the loss of both arms of chromosome 4, 8p11-21 and 15q11-12, which occur after cell transformation and were maintained consistently during the process of tumorigenesis involving one or more tumor suppressor genes which are frequently inactivated in several types of cancers.

The gain in the HBEC cells in the 8q24.1 was found in all the cells, including the MCF-10F cells. The gain in the 8p region has been observed in many other human tumors (27). In 80% of the HBEC cells, gain of 8q24.1 was accompanied by loss of 8p11-21, suggesting the presence of an isochromosome of 8q. In fact, multiple copies of i(8)(q10) have been reported in several karyotypic studies of human lung cancer (28). Highest incidence at 8q24 were reported in lung cancer and lung cell cultures (29-32). The over-expression of 8q24.2 was reported in high grade cancers of breast and prostate (33). Also DNA amplification in the region 8q22-ter was reported in primary breast cancer tumors and their cell lines (34). Furthermore, amplification of MYC (8q24) has been associated with proliferation and unfavorable prognosis (35-37). Nephroblastoma overexpressed (NOV) gene are located in the specific region 8q24.1. Over expression of a member of cysteine rich 61/connective tissue growth factor/nephroblastoma over-expressed (CYR61/CTG/NOV) family growth regulators in invasive and metastatic human breast cancer cells has also been reported (38). It is known that CYR61 mediated diverse roles in development, cell proliferation and tumorigenesis (38). It was demonstrated that NOV protein associated with Notch 1 extracellular domain and inhibits myoblast differentiation via Notch signalling pathway (39). However, the exact mechanism by which this protein promotes carcinogenesis and aggressive breast cancer phenotype are still largely unknown.

Gain of 1p was the second most common genomic imbalance observed in all HBEC transformed cells. Genomic gains clustered to chromosome arm 1p were reported in 17% of human breast cancer in young women (40). In region 1p are 1,204 genes, such as the tumor suppressor protein p73; genes related with DNA repair like growth arrest and DNA-damage-inducible, α (1p31.2-p31.1), mutS homolog 4 (*E. coli*) (1p31), DnaJ (Hsp40) homolog, subfamily B, member 4 (1p31.3); genes related with apoptosis such as tumor necrosis factor receptor superfamily, member 18 (1p36.3), DNA fragmentation factor, 45 kDa, α polypeptide (1p36.3-p36.2), caspase-9, apoptosis-related cysteine protease (1p36.3-p36.1).

The next gain that was observed in this study was in chromosome 5q21-ter, which was observed consistently

from the transformed cell to the tumor cell line. This genomic aberration does not appear in the immortal cell MCF-10F. Additional changes in the tumor cell line BP-1-Tras were loss of 15q11-12 and gain at 5p15.3. In the cell line BP-1-Tras T J#4 we have observed loss of 13q11-14, gain of 6q25-27, 18p11.3, 20p11 and 20q13.

Another important abnormality found during the transformation process of MCF-10F cells by BP is the loss of the entire chromosome 4, 8p11-21 and 15q11-12. Several previously published reports have documented that allelic losses at chromosome 4 are present in several tumor types (41-48), although breast cancers have not been studied in detail. At least three discrete regions of frequent loss in 4q 33-34, 4q 25-26 and 4p 15.1-15.3 (48) have been identified in mesotheliomas and squamous cell carcinomas (49). It was reported that in the cultured tumor (breast cancer cell lines) and their corresponding tumors, the size of the deletions in chromosome 4 were similar (49). From the 20 regions of the whole chromosome 4 a total of 952 genes were expressed and reported to the human data base (BLAST The human Genome, http://www.ncbi.nih.gov/mapview), including one tumor suppressor gene, FAT tumor suppressor homolog 1 (4q34-35) directly related to breast carcinogenesis.

We also found a common loss in chromosome 8p11-21 after cell transformation, this region contains 194 genes (http://www.ncbi.nih.gov/mapview), including genes such as the bone morphogenetic protein 1, tumor necrosis factor receptor superfamily members 10b, 10a, 10c, 10d, gonadotropin-releasing hormone 1 (LHRH), cell division cycle associated 2, and clusterin or testosterone-repressed prostate message TRPM2. From these results we conclude that the loss of genes involved in chromosome 4 and in the region 8p11-21 are involved in the breast carcinogenesis process. Of interest is that the BP effects are irreversible in the HBEC and the same aberrations are maintained during the tumorigenesis process.

In conclusion, our panel of breast cancer cell lines provides a powerful tool for studying molecular and cytogenetic changes related to tumorigenesis. In addition to the histopathological and biochemical features reported previously (21,25). DNA copy number changes detected in these cell lines will provide valuable data for investigation of tumor progression *in vitro-in vivo* and for more detailed mapping and isolation of genes implicated in breast cancer.

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