Microsatellite instability and loss of heterozygosity in chromosomes 9 and 16 in human breast epithelial cells transformed by chemical carcinogens

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Microsatellite instability (MSI) and loss of heterozygosity (LOH) in chromosomes 9 and 16 have been reported in human breast cancers. In order to determine whether changes in these chromosomes play a role in the initiation and progression of this disease, we performed microsatellite polymorphism analyses in human breast epithelial cells (HBEC) transformed by chemical carcinogens, an in vitro system that recapitulates various stages of neoplastic transformation. In this experimental system, we studied the mortal HBEC MCF-10M, immortal MCF-10F cells, derived from MCF-10M cells, and clones derived from MCF-10F cells treated with benzo[a]pyrene (B[a]P) (BP1 and BP1-E) and 7,12-dimethylbenz[a]anthracene (DMBA) (D3 and D3-1). The four clones of transformed cells were injected into severe combined immunodeficient (SCID) mice. Only BP1-E cells induced the formation of tumors, designated BP1-E-Tp cells. These cells originated six additional tumors, designated BP1-E-TF no. 1 through TF no. 6. Microsatellite analyses were carried out using five markers for chromosome 9 and 20 for chromosome 16. There was no evidence of MSI or LOH in clones BP1 and BP1-E when compared with the MCF-10M and MCF-10F cells, whereas BP1-E-Tp cells and BP1-E-TF no. 1–TF no. 6 tumors exhibited MSI at loci p23 and p21, and LOH at p21–22 of chromosome 9. They also exhibited MSI and LOH at multiple loci of both the short and long arms of chromosome 16, i.e., p13.13, p13.3, p12, q12.1, q12.2, q23 and q24, to which putative tumor suppressor genes have been localized. Clones D3 and D3-1 exhibited no genomic changes in chromosome 9, but did show MSI at locus q12.1 of chromosome 16 using marker D16S285. Although the cells treated with DMBA expressed early phenotypes of neoplastic transformation, they were not tumorigenic, and also manifested fewer changes than the tumorigenic BP1-E-Tp cells and the tumors BP1-E-Tf. The changes in chromosomes 9 and 16 observed in these latter ones indicated an association with the expression of tumorigenesis, which represents a late event in the progression of the neoplastic transformation of HBEC. Of interest was the observation that HBEC transformed by chemical carcinogens in vitro express genomic changes similar to those found in spontaneous breast carcinomas.

Introduction

The etiology of breast cancer, the most frequent malignancy diagnosed in women in the Western world, remains unidentified, despite intensive investigations (1). Chemical compounds such as the polycyclic aromatic hydrocarbons benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA) are known to be carcinogenic in experimental animal models (2–5); they have also been implicated to be of etiologic importance in various human cancers (6). Since genomic instability, which has been well documented in colon cancer (7), has also been considered to be involved in the evolution of breast cancer, from precursor lesions to metastatic cancer (8–11), we performed microsatellite polymorphism analyses in an in vitro system in which human breast epithelial cells (HBEC) transformed by chemical carcinogens recapitulate various stages of neoplastic transformation (12,13). Previous studies performed in our laboratory have shown that the transformation of MCF-10F cells with chemical carcinogens in vitro is manifested at phenotypic, functional and genotypic levels (12–17). Genotypic alterations were observed in the immortalization of MCF-10F cells, which exhibited a point mutation in exon 7 of the tumor suppressor gene p53 (14). The expression of colony formation in agar methocel and anchorage independence were both associated with loss of heterozygosity (LOH) and point mutations in codons 12 and 61 of the c-Ha-ras gene (15). These early stages of expression of cell transformation were accompanied by amplification of c-neu and c-myc oncogenes and rearrangement of int-2 (16). The expression of the tumorigenic phenotype was associated with LOH in the telomeric portion of chromosome 17, and overexpression of the mdm2 gene (17).

Since microsatellite instability in chromosome 16q has been detected in atypical ductal hyperplasia (ADH), which is considered to be a precursor lesion (8–11), in a frequency similar to that found in high and low nuclear grade ductal carcinoma in situ (DCIS) and invasive carcinoma (18,19), and in addition, LOH on the long arm of chromosome 16 has been found to be correlated with the occurrence of distant metastases (20,21), we selected this chromosome for analysis in our in vitro system. Another chromosome reported to exhibit breakpoints and deletions in breast cancer is the chromosome 9, on which the tumor suppressor gene cyclin dependent kinase-4 inhibitor CDKN2 (MTRp16) has been mapped to locus p21 (22). Although primary tumors and cultured breast cancer cell lines exhibit frequent LOH or MSI on the short arm of chromosome 9 (9p21-22), CDKN2 (MTRp16) has not been reported to be mutated in primary breast carcinomas, but only in cancer cell lines and in immortalized human breast epithelial cells (22,23). Collectively, these data indicate that

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Materials and methods

Cells and tumors

The spontaneously immortalized human breast epithelial cells MCF-10F, which originated from the mortal cells MCF-10M (26,27), when treated with the chemical carcinogens BP and DMBA gave rise to clones BP1 and BP1E, and D3 and D3-1, respectively (12,13). The tumorigenic potential of the four clones of transformed cells was tested by inoculation into SCID mice. Only BP1E cells at passage 22, when inoculated into SCID mice gave origin to a tumor after 101 days of injection; it was designated BP1E-Tp, which originated the BP1E-Tp cell line. BP1E-Tp cells in their 20th passage were injected into six 35–45-day-old female virgin SCID mice (10^7 cells/animal) in the right thoracic mammary gland. Six mammary carcinomas developed; they were designated BP1E-Tf no. 1 through no. 6 (Figure 1). BP1E-Tf tumors had a shorter latency (7 days) as well as a shorter period (21 days) to reach a diameter of 2 cm than the BP1E-Tp tumor (12), indicating that these tumors exhibited a more aggressive behavior, and thus would represent a more advanced stage in the process of neoplastic progression. DNA was isolated from the following cell lines and tumors: MCF-10M cells at passages 22 and 29, MCF-10F cells obtained at their 85th passage from the American Type Culture Collection (ATCC) (Rockville, MD) and at the 130th passage of a line maintained in our laboratory, BP1 cells at passages 27 and 52, BP1E cells at passages 23 and 60, BP1E-Tp cells at passage 6, BP1E-Tf no. 1, 2, 3, 4, 5 and 6, D3 cells at passages 36 and 61 and D3-1 cells at passages 39 and 70.

DNA extraction

For extracting high molecular weight DNA, cells were lysed in 10 ml of TNE (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA) with 500 mg/ml proteinase K and 1% sodium dodecyl sulfate (SDS), and incubated at 55°C overnight with constant gentle agitation. Following two extractions with phenol:chloroform (1:1 volume), the aqueous layer was adjusted to 0.75 M ammonium acetate, and DNA was spooled from two volumes of 100% ethanol, washed extensively in 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Microsatellite instability and loss of heterozygosity analysis

Polymerase chain reaction (PCR) was performed using primer sets obtained from Research Genetics (Huntsville, AL) or generously provided by Dr. A. Godwin (Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA). Five polymorphic microsatellite markers for chromosome 9 and 20 for chromosome 16 were analyzed (Figures 2 and 3). PCR was performed using 40 ng of template DNA, 5 µM of each paired primer, 1×

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Tag buffer (Perkin—Elmer, NJ) in addition to 0.1 µCi/reaction of [α-33P]dCTP (3000 Ci/mM) (Dupont/New England Nuclear, Boston, MA) in a total volume of 5 µl. The PCR reaction consisted of an initial denaturation step, followed by 35 cycles at 94°C for 40 s, the appropriate annealing temperature for 30 s, and 72°C for 30 s, extended at 72°C for 3 min. Each PCR reaction product was diluted 1:1 with loading buffer (95% formamide, 10 mM EDTA, pH 8.0, 0.1% xylene cyanol (w/v) and denatured at 95°C for 5 min. A 4 µl aliquot was loaded and electrophoresed on a 6% polyacrylamide gel at 60 W for 2 h. The gels were dried and exposed to Kodak X-omat-AR film (Eastman Kodak Co., Rochester, NY) at ~80°C with intensifying screens. The presence of LOH and MSI was evaluated depending upon detection of a loss and/or a shift or expansion of electrophoretic bands (28–30) where the intensity of a signal in one allele was diminished approximately one half or more of its normal intensity in relation to the remaining alleles (23).

Results

Chromosome 9

Of all the cell lines and clones tested, MCF-10F, BP1, BP1E, D3 and D3-1 cells did not exhibit any changes when compared with their parental cell line MCF-10M with any of the markers analyzed in this study (Figure 2). DNA obtained from BP1E-Tp cells and BP1E-Tf no. 1, 2, 3, 4, 5 and 6 tumors showed MSI at loci 9p23, 9p21 and LOH at locus 9p21-22 of chromosome 9 when tested with microsatellite markers D9S199, D9S171 and D9S157, but did not show any changes with microsatellite markers D9S169 and D9S165 (Figures 2 and 4). These observations led us to conclude that MSI and LOH occurring in the telomeric portion of the short arm of chromosome 9 were late events in carcinogenesis, since those changes were present only in samples, which upon transformation with the chemical carcinogen B[a]P, had progressed to expressing the tumorigenic phenotype.

Chromosome 16

When tested for LOH and MSI utilizing 10 microsatellite markers for the short arm and 10 for the long arm of chromosome 16, no changes were detected in MCF-10F, BP1 and BP1E cells when compared with their parental cell line MCF-10M (Figure 3). D3 and D3-1 cells showed MSI at the 16q12.1 locus using marker D16S285 (Figures 3 and 5). DNA obtained from BP1E-Tp cells and BP1E-Tf no. 1 through to Tf no. 6 tumors showed LOH and MSI at various loci of chromosome 16. The short arm of chromosome 16 showed MSI and/or LOH at loci p13.13 (D16S423, D16S404), p13.3 (D16S418), p13.1 (D16S405) and p12 (D16S420, D16S541) (Figure 3). The long arm of chromosome 16 showed MSI and/or LOH at loci q12.1 (D16S415), q12.2 (D16S389), q21 (D16S265), q22 (D16S398), q23 (D16S395) and q24 (D16S422, D16S449, D16S305) (Figure 3). LOH and MSI were detected more frequently in the telomeric regions of both the short and long arms of chromosome 16.

Since PCR amplification can sometimes generate spurious bands, each experiment in our study was repeated at least three times and the results obtained were consistent. Three to four passages from each cell line in this model were used to reduce the risk of PCR artifact and technical mishap. LOH and MSI detected in multiple tumors induced by BP1E-Tp cells were consistent with those found in tumor induced by BP1E cells in SCID mice, which confirmed the integrity of biological tumor development, thus allowing us to rule out the possibility of having detected random genetic alterations.

Discussion

Results reported here show that the chemical carcinogens BP and DMBA, which in vitro induce the transformation of human breast epithelial cells, also induce LOH and MSI in the telomeric portions of the short arm of chromosome 9 and in both the short and long arms of chromosome 16. The largest number of genomic alterations occurred in cells expressing the most advanced stages of neoplastic transformation, i.e. tumorigenesis in a heterologous host. Thus, BP1E-Tp cells and the tumors BP1E-Tf no. 1 through no. 6 all expressed MSI at loci 9p23 and 9p21 and LOH at locus 9p21-22 of chromosome 9, as well as a greater number of genomic alterations in both the short and the long arms of chromosome.
16. The clones D3 and D3-1, derived from DMBA treated cells, which express anchorage independence and invasiveness in a matrigel in vitro system, but are not tumorigenic in SCID mice (12,13), exhibited only MSI at locus q12.1 of chromosome 16, but chromosome 9 was not affected.

The short arm of chromosome 9, which has been shown to undergo hemizygous and homozygous deletions in a variety of tumors and cell lines, including breast cancer (23,31–38), contains at locus p21-22 the cyclin dependent kinase-4 inhibitor (CDKN2) gene. The protein product of this gene, commonly referred to as p16, is an inhibitor of the cell cycle. LOH and MSI on chromosome 9p have been reported in ~60% of primary breast carcinomas (23,31,33). Using five polymorphic markers that span the regions 9p23 to 9p13-q13 we found MSI at loci 9p23 and 9p21, and LOH at locus 9p21-p22 in BP1E-Tp cells and in all BP1E-Tf tumors. The changes induced by the chemical carcinogen in vitro treatment of the immortalized human breast epithelial cells MCF-10F involved the same regions reported in primary breast carcinomas (38), and in the human breast epithelial cell line 184A1, which has been immortalized by treatment with B(a)P (23). In our experimental system, however, LOH involving p21-22 locus was detected as a late phenomenon, associated with the expression of the tumorigenic phenotype, since clones BP-1 and BP1E, which were not tumorigenic, did not exhibit either LOH or MSI with any of the markers tested. The finding that BP induced LOH at loci 9p21-22 suggests that the tumor suppressor gene located in that area, found to be involved in breast, lung and ovarian neoplasms (39,40), might also be affected by this chemical carcinogen.

Our findings indicate that human breast epithelial cells transformed with a chemical carcinogen in vitro exhibit multiple sites of LOH and MSI in both the short and the long arms of chromosome 16 which coincide with the genomic alterations most frequently reported in human breast carcinomas (41,42). We detected MSI at locus p13.3 of chromosome 16 in the DNA of BP1E-Tp cells and in the six BP1E-Tf no. 1–no. 6 tumors, in a region in which an excision repair gene has been located (43,44). These observations support the concept that alterations in the mismatch repair (MMR) process are of importance for the emergence of the tumorigenic phenotype in the heterologous host. In vivo rodent studies have shown that MSI is present in some chemically induced tumors (45,46). It is likely that chemically-induced mutations in one of the MMR genes are responsible for maintaining the microsatellite instability. Therefore, it is plausible that the changes we observed have been induced by or are the consequence of changes induced by the chemical carcinogen in this human breast epithelial cell line.

Although LOH on 16q occurs commonly in breast cancer at very early developmental stages, it is also found in both highly aggressive and in relatively low-grade tumors (22,47,48). In ADH the incidence of LOH for 16q (D16S413) is similar to that found in ductal carcinoma in situ of high and low nuclear grade and in invasive ductal carcinoma (47,48). Lobular carcinoma in situ and ductal carcinoma in situ have been shown to exhibit the same LOH for 16q, indicating the same evolutionary pattern (49). The similarities in genomic changes detected in sporadic breast cancers and in HBE transformed in vitro by chemical carcinogens raises the possibility that environmentally relevant chemicals may be involved in the induction of breast cancer. Our observations also indicate that the genomic changes induced in chromosome 16 by this treatment appear in association with the emergence of the tumorigenic phenotype, since they were first observed in tumors induced in SCID mice by BP1E cells and BP1E-Tp cells. Alterations induced in 16q by chemical carcinogens might be related to alterations in several genes located in those regions (41,50–54), such as E-cadherin on 16q22.1 (55,56), 2-M-cadherin mapped to 16q24 (57), a tentative suppressor of tumor invasion, cell adhesion regulator (CAR) mapped to 16q24.3 (58), breast basic conserved gene or BBC1 (59), and renal dipeptidase (DPEP1) mapped to 16q24.32 (60), that have been suggested to be tumor suppressor genes.

The relatively high frequency of LOH and MSI in the telomeric region of 16q in BP1E-Tp cells and in the tumors BP1E-Tf no. 1–no. 6 suggested that a tumor suppressor gene may reside in this region which has already been indicated for sporadic breast cancer (42,61,62). We observed MSI at 16q12.1 in both DMBA treated non-tumorigenic cells D3 and D3-1 and BP treated tumorigenic BP1E-Tp cells and in the tumors BP1E-Tf no. 1–no. 6. This region is of interest since a breakpoint at the 16q12.1 locus and two break reciprocal translocations between chromosomes 5 and 16 have been described in association with the Townes–Brock syndrome (TBS), a rare autosomal dominant entity characterized by developmental abnormalities (60).

Human breast cancer is a diverse disease that presents with a broad spectrum of clinical and pathological characteristics. These diverse characteristics may be a reflection of the multiplicity and heterogeneity of the molecular mechanisms involved in its genesis and progression. The detection of LOH and MSI on both chromosomes 9 and 16 in human breast epithelial cells transformed with chemical carcinogens in vitro in the same regions found in primary breast cancers gives relevance to this model, suggesting that it provides an adequate experimental system for testing the functionality of these putative tumor suppressor genes in the initiation and progression of breast cancer.

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References


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