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Frequent allelic imbalance on chromosome 6 and 17 correlate with radiation-induced neoplastic transformation of human breast epithelial cells

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The development of human breast cancer is a complex multi-step process that depends on various exogenous and endogenous factors that modulate the transformation of normal human breast epithelial cells into neoplastic ones. Using a spontaneously-immortalized human breast epithelial (MCF-10F) cell line, we have shown previously that radiation, in combination with estrogen, induces a stepwise neoplastic transformation of this cell line. In the present study, we investigate the incidence of microsatellite instability and loss of heterozygosity using a battery of markers on chromosomes 6 and 17, we correlate the genetic alteration with the malignant transformation of the MCF-10F cell line ranging from altered morphology to increase in proliferative rate, anchorage independent growth and tumorigenicity in nude mice. Microsatellite markers were selected from the hot spot regions (6q21-q27, 17p12-p13.3 and 17q12-q21) of both chromosomes. We found that the frequency of allelic imbalance occurs at the different stages of tumor progression with a range of 21 to 50% depending on the marker studied. The relatively high rate of allele imbalance at all these loci suggests the presence and inactivation of one or more tumor suppressor genes in these regions. Thus, the present data will be useful for systematic studies to identify the cellular and molecular changes associated with radiation-induced breast carcinogenesis.

Introduction

The aetiology of breast cancer, the most frequently diagnosed malignancy in women in the western world, remains unidentified, despite intensive investigations (1). The progression of breast cancer follows a complex multi-step process that depends on various exogenous (socioeconomic situation, diet, breast irradiation, oral contraception, geography, etc.) and endogenous (hormonal imbalances and family history of breast cancer) factors that modulate the transformation of human breast epithelial cells to a neoplastic stage (2,3). There is evidence that multiple mechanisms account for genomic instability and mutation in neoplastic transformation. Neoplastic cells typically possess numerous genomic mutations and chromosomal aberrations, including point mutations, gene amplifications, deletions and replication errors. Acquisition of such genomic instability may represent an early step in the process of carcinogenesis (4,5). Accordingly, a large number of alterations have been identified at the genetic level in the multi-step carcinogenesis of breast cancer.

It has been established that cancer progression correlates with the accumulation of genetic alterations in tumor cells either by amplification of oncogenes (*c-myc*, *c-erbB-2*, *int-2* and *ccnd1*) or by mutation of tumor suppressor genes (*p53*, *Rb*) (6–8). According to Knudson's two-hit model, inactivation of a tumor suppressor gene requires a biphasic process to eliminate both alleles. Most frequently one of these two events involves the loss of one allele due to chromosomal deletion (9). This allelic alteration may occur either by a gross chromosomal aberrations (aneuploidy, deletions or amplifications), microsatellite instability (MSI) or loss of heterozygosity (LOH) at various stages of the neoplastic process. It is unclear whether allelic imbalance is the cause or the result of carcinogenesis but it is probably the most common genetic factor associated with cancer.

A basic tenet emerging from studies of genomic instability is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. In the progression of breast carcinogenesis, frequent allelic losses have been found on different chromosomal arms (1p, 1q, 3p, 6q, 7q, 8p, 11p, 11q, 13q, 17p, 17q, 18q and 22q) either in the form of MSI or LOH. The emergence of MSI may involve defects in DNA replication or mismatch repair (MMR) mechanisms (10,11), whereas LOH may indicate deletion of the remaining normal allele of a tumor suppressor gene(s) (12). These deletions have been measured by the alteration of different microsatellite sequences associated with those chromosomal arms. Due to their high reproducibility, hypervariable and co-dominant nature, and the relative ease of scoring by polymerase chain reaction (PCR), they are considered to be among the most powerful genetic markers in a wide variety of applications.

The existing model of carcinogenesis indicates that all human tumors have an unstable genome and that allelic imbalance is a very useful tool in assessing the level of genetic damage in the early stages of cancer progression. In breast cancer, several chromosomal loci have been shown to bear multiple regions of allelic imbalance (13). Among them chromosomes 6 and 17 have received a great deal of attention because they contain several putative tumor suppressor genes (14,15). Deletions on the long arm of chromosome 6 in breast cancer were originally revealed by cytogenetic analysis. Subsequently, molecular analysis using polymorphic genetic markers has also identified the presence of several domains of imbalance on 6q (16). LOH at the distal portion of 6q (6q21-q27) has been best documented by the cancer related genes *ESR* and *M6PR/IGF2R* map in this region (17,18). The presence of several target domains is supported by chromosome transfer experiments using all, or portions of, chromosome 6 in cancer cells, as revealed by the transfer of 6q21-q23 and/or 6q26-q27 which leads to inhibition of tumorigenicity of the MDA-MB-231 breast cancer cell line (19).

Abbreviations: DMEM, Dulbecco's modified Eagle medium; LOH, loss of heterozygosity; MSI, microsatellite instability; OD, optical density; PCR–SSCP, polymerase chain reaction–single strand conformation polymorphism; RER, replication error repair.

There is evidence that introduction of a normal human chromosome 17 into the breast cancer cell line MCF-7 by micro-cell mediated chromosome transfer (MMCT) results in growth arrest (20). Similarly, transfer of the long arm of chromosome 17 into the MDA-MB-231 tumorigenic cell line suppresses their tumorigenicity in nude mice. In contrast, the MDA-MB-231 cell line, carrying the short arm of chromosome 17, retained its tumorigenicity with a reduced tumor growth rate. These data suggest that at least one gene mapping to the long arm of chromosome 17 may suppress the tumorigenic phenotype (21).

Until recently, there were few human cell culture models available for the study of radiation carcinogenesis (22–24). To have a better understanding of the cellular and molecular changes associated with radiation-induced breast carcinogenesis, we have recently developed a transformation model based on a spontaneously-immortalized human breast epithelial (MCF-10F) cell line irradiated with graded doses of α-particles in the presence of 17β-estradiol (E) (24).

In the present study, we have utilized this model to examine the incidence of allelic imbalance in chromosomes 6 and 17 in the neoplastic progression of the MCF-10F cell line since the transformed MCF-10F cell line underwent a series of phenotypic stages before becoming tumorigenic in nude mice (24).

Materials and methods

Cell lines

The recently established radiation-induced breast carcinogenesis model based on the MCF-10F cell line was used in this study (24). The spontaneously immortalized human breast epithelial cell line MCF-10F was derived from mortal human breast epithelial cell line MCF-10M and has a near diploid karyotype and is of luminal epithelial origin (25). These cells retain all the characteristics of normal epithelium *in vitro*, including anchorage dependence, non-invasiveness and non-tumorigenicity in nude mice (24,26,27). Cells were cultured on DMEM/F-12 (1:1) medium 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, Rockville, MD), 0.5 μg/ml hydrocortisone (Sigma, St Louis, MO) and 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, MA). From such a model, the following cell lines were used as control (24): MCF-10F; MCF-10F treated with 17β-estradiol (E) (10^{-8} M) (Sigma), named MCF-10F + E. MCF-10F cells irradiated with a single dose of 60cGy α-particles (60 cGy) at 40–50 passages and a double dose of 60 cGy α-particles (60 cGy/60 cGy) at 45–50 (early) and 95–100 (late) passages were also utilized for this study. These cell lines were positive for anchorage independency but were not tumorigenic in nude mice. The only tumorigenic cell line used was the 60cGy cell line treated with estrogen and then subjected to a second 60 cGy dose of α-particle and estrogen treatment (60 cGy + E/60 cGy + E). This tumorigenic cell line was positive for anchorage independency and produced tumors in 3 out of 6 animals injected. Tumor-2, one of the three primary tumor cell lines originating from the tumorigenic cell line (60 cGy + E/60 cGy + E) was used in the present study (28). The MCF-7 cell line (29), a widely used breast cancer cell line, was also used as a positive control.

DNA isolation

All cell cultures were treated with 1 ml lysis buffer (100 mM NaCl, 20 mM Tris–HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 200 mg/ml proteinase K and 100 μg/ml RNAse and incubated overnight at 37°C with constant gentle agitation (30). The samples were then purified by extracting twice with 1 vol 1:1 (v/v) phenol:chloroform, the aqueous layer was then adjusted to 0.75 M ammonium acetate, followed by the addition of 2 vol 100% ethanol. DNA was spooled from the solution, dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) as previously described (31).

Microsatellite polymorphism marker selection

Twelve polymorphic dinucleotide $(CA)_n$ repeat microsatellite markers along with one tetranucleotide marker (Research Genetics, Huntsville, AL) from each of the following chromosomal sectors 6q21-q27, 17p12-p13.3 and 17q11-q23 (Figure 1a and b) were selected for the study based on their maximum heterozygosity (more than 0.70). The markers, listed in Table I, were selected based on their location near known mapped tumor suppressor genes, oncogenes or other cancer related genes and in regions or near loci associated with cell-cycle regulation, DNA replication, DNA repair or signal transduction protein genes. The sequences and characteristics of microsatellite oligonucleotide primers were obtained from the GDB database (http://www.gdb.org) and are summarized in Table II. We also tested D2S123 (2p16, 0.77, dinucleotide, 197–227 bp), a CA repeat marker linked to the *HMSH2* gene, mapped at 2p16, where LOH is rarely encountered (data not shown).

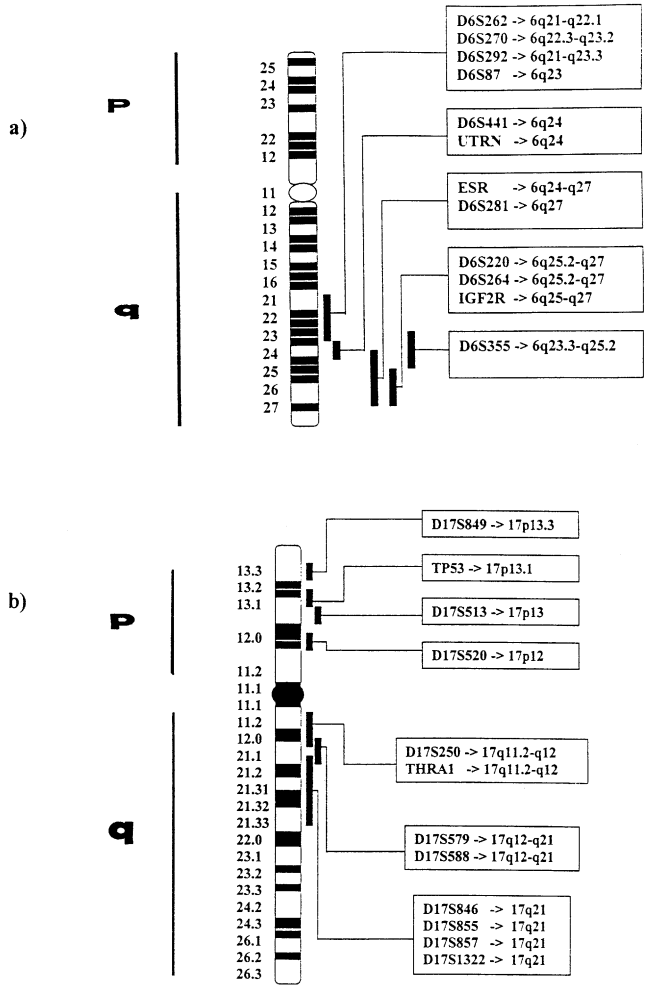

Fig. 1. Map of chromosomes 6 and 17 showing the putative positions of the $(CA)_n$ repeat microsatellite markers used in this study. Bold black vertical lines indicate regions of possible map positions of the markers.

PCR–SSCP analysis

Polymerase chain reaction–single strand conformation analysis (PCR–SSCP) was carried out in a total volume of 30 μl containing 50–100 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 200 μM each dNTP, 0.8 μM each primer (Research Genetics, Huntsville, AL) and 0.75 U AmpliTaq polymerase (Perkin-Elmer, Foster City, CA) (32). One of the primers was 5′ end-labeled with 3000 Ci/mmol [α-^{32}P]ATP (Amersham Pharmacia Biotech, Arlington Heights, IL) by T_4-polynucleotide kinase (Amersham Pharmacia Biotech). After a 5 min pre-incubation period at 94°C, DNA was amplified for 35 cycles comprising 45 s at 94°C, 45 s at 55°C, and 1 min at 72°C, followed by a 7 min final extension at 72°C using the GeneAmp^R PCR System 2400 (Perkin-Elmer, Foster City, CA). PCR products were diluted in an equal volume of denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue), denatured at 95°C for 5 min and then frozen at 4°C. An aliquot of approximately 2 μl was loaded and electrophoresed on a 6% polyacrylamide gel containing 8.3 M urea for 2–3 h at 40 W. The gel was fixed in 10% methanol–10% acetic acid, dried and exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) at –70°C with an intensifying screen for 12–16 h. The PCR

reaction was always repeated two to three times with different adjacent passages of these cell lines to get consistent results.

Assessment of allelic loss

Microsatellite instability and LOH were screened by PCR amplification of polymorphic microsatellite markers. MSI was defined as a shift of a specific allelic band or a change (increase or decrease) in the broadness of a specific allelic band in the autoradiograms, whereas LOH was defined as a total loss (complete deletion) or a 50% or more reduction in signal density of one of the heterozygous alleles in the autoradiograms. It was first scored by visual inspection of autoradiograms and then band intensity was quantitated in a Densitometric scanner (Model 300A, Molecular Dynamics) using IMAGE QUANT bioimage software (version 3.3; Molecular Dynamics). An optical density range of 0.01–4.0 was chosen (in OD units), whereas a resolution (spatial) of 100 points/cm was selected in both the *x* and *y* direction. The resolution (signal) was selected at 4096 levels (12-bit) of optical density.

Table I. Characteristics of $(CA)_n$ repeat markers on chromosomes 6 and 17 selected for the study of allelic imbalance

Locus	Map position[a]	Maximum heterozygosity	Type of sequence	Size range (base pairs)
D6S220	6q25.2-q27	0.68	Dinucleotide	175
D6S262	6q21-q22.1	0.84	Dinucleotide	167–173
D6S264	6q25.2-q27	0.71	Dinucleotide	108–122
D6S270	6q22.3-q23.2	0.77	Dinucleotide	141–157
D6S281	6q27	0.70	Dinucleotide	203–219
D6S292	6q21-q23.3	0.83	Dinucleotide	141–161
D6S355	6q23.3-q25.2	0.74	Dinucleotide	348–362
D6S441	6q24	0.86	Dinucleotide	162–186
D6S87	6q23	0.60	Dinucleotide	137–155
ESR	6q24-q27	0.82	Dinucleotide	–
IGF2R	6q25-q27	0.70	Dinucleotide	99–101
UTRN	6q24	0.80	Dinucleotide	143–163
D17S250	17q11.2-q12	0.91	Dinucleotide	151–169
D17S513	17p13	0.89	Dinucleotide	183–203
D17S520	17p12	0.85	Dinucleotide	130–144
D17S579	17q12-q21	0.87	Dinucleotide	111–133
D17S588	17q12-q21	0.85	Dinucleotide	154–174
D17S846	17q21	0.83	Tetranucleotide	215–255
D17S849	17p13.3	0.68	Dinucleotide	251–256
D17S855	17q21	0.82	Dinucleotide	145
D17S857	17q21	0.81	Dinucleotide	106–122
D17S1322	17q21	0.67	Dinucleotide	130
THRA1	17q11.2-q12	0.81	Dinucleotide	158–176
TP53	17p13.1	0.90	Dinucleotide	103–135

[a]Precise location of the markers on respective chromosomal arms.

Results

Twenty-four microsatellite markers for chromosomes 6 (6q21-q27) and 17 (17q11-q23 and 17p13.1-p13.3) were used to determine the frequency of allelic imbalance in irradiated, tumorigenic and tumor cell lines by PCR–SSCP. These allelic imbalances were more pronounced as the phenotypic characteristics of the cellular transformation progressed from early to late stage when the transformed cell lines became tumorigenic (24). The different degrees of allelic imbalance were expressed in the form of MSI or LOH. Some of the markers detected mainly MSI (e.g. D17S849 and D17S1322) whereas others detected mostly LOH (e.g. D6S292, IGF2R and D17S846). No imbalance was detected for any of the chromosomal markers examined in MCF-10F + E cells when compared with parental line.

Chromosome 6

A total of 12 microsatellite markers for chromosome 6 were utilized to detect allelic imbalance in irradiated, tumorigenic and tumor cell lines. The MCF-10F cell line was compared with the 60cGy cell line (Figure 2a), where a total of two loci were altered. Such alterations were in the form of MSI at loci 6q21-q23 (D6S292) and 6q25-q27 (D6S220) while no LOH was detected. When the cell lines 60cGy/60cGy (early) and MCF-10F were compared (Figure 2b), a total of four alterations were observed. Loss of heterozygosity was screened in these four loci at 6q21-q23 (D6S292), 6q25-q27 (D6S220), 6q24-q27 (ESR) and 6q24 (UTRN) but no MSI was detected. However, when cell lines 60cGy/60cGy (late) and MCF-10F were compared (Figure 2c), a total of seven alterations were

Table II. Sense and antisense primers of microsatellite markers used for the study of allelic imbalances of chromosomes 6 and 17

Locus	Primer name	Primer sequence[a]
D6S220	G119-1/G119-2	ACCAGGCACCCAAAACTGT/TTGGGATTTTGGCTGAGATC
D6S262	AFM059yd6a/AfM059yd6m	ATTCTTACTGCTGGAAAACCAT/GGAGCATAGTTACCCTTAAAATC
D6S264	AFM079zb7a/AFM079zb7m	AGCTGACTTTATGCTGTTCCT/TTTTCCATGCCCTTCTATCA
D6S270	AFM127xb2a/AFM127xb2m	GTGTAACTGATCTGAATGGTTCC/GTAGTGAAGCCTGGATGTGG
D6S281	AFM176xh8a/AFM176xh8m	CTGGTAGTGTCAGGCATGGC/CCTATGTTTCAGGCAAAGGC
D6S292	AFM203za9a/AFM203za9m	AATTCACAAGACACAATCTCAG/AGAACTAAAGTTGCCTGTTCNTGTA
D6S335	91696/91749	GGTTGATAGGTGCAGCAAACCAT/ TGAAAAGGTGCTGGCTTAATGCAT
D6S441	AFM269ze1a/AFM269ze1m	AACAATATTTGGTGACTGTTAAAGG/TGGACAAATTGATTAGGAAGTAAAG
D6S87	Mfd47CA/Mfd47GT	ACAGAGTGAGACCGTGTAAC/AGAGAAGCATCTCACTTAGT
ESR	ER1/ER2	CTGCAGCGCAGGTCTACGGTCAGACC/AGCAAGCTTCGGCGGCG GGTGCAGT
IGF2R	IGF2RPCR2.1/IGF2RPCR2.2	TTGCCGGCTGGTGAATTCAA/GTATCATGAGAACCTGAAGAG
UTRN	UTRN.PCR.1/UTRN.PCR.2	CCATTCAATATCTTTAAACGTAA/ATTTGGTAATTGCTCAAAAACT
D17S250	Mfd15CA/Mfd15GT	GGAAGAATCAAATAGACAAT/GCTGGCCATATATATATTTAAACC
D17S513	12G6CA/12G6GT	TTCACTGTGGGCTGCTGTC/TAAGAAAGGCTCCCACAAGCA
D17S520	Mfd144CA/Mfd144GT	GGAGAAAGTGATACAAGGGA/TAGTTAGATTAATACCCACC
D17S579	658/705	AGTCCTGTAGACAAAACCTG/CAGTTTCATACCAAGTTCCT
D17S588	42D6-CA/42D6-GT	CCTGGTCTAGGAAGAGTGTCA/GTGTAAGCATCTGTGTATACTAC
D17S846	2272A/2273A	TGCATACCTGTACTACTTCAG/TCCTTTGTTGCAGATTTCTTC
D17S849	AFM234.3a/AFM234.3m	CAATTCTGTTCTAAGATTATTTTGG/CTCTGGCTGAGGAGGC
D17S855	AFM248.9a/AFM248.9m	GGATGGCCTTTTAGAAAGTGG/ACACAGACTTGTCCTACTGCC
D17S857	OF1-1/OF1-1	TTTGTCTGCAAACATGGAGG/TCAGCTGAAGAGAAAAATGGC
D17S1322	s754-A/s754-B	CTAGCCTGGGCAACAAACGA/GCAGGAAGCAGGAATGGAAC
THRA1	THRA1-AC/THRA1-TG	CTGCGCTTTGCACTATTGGG/CGGGCAGCATAGCATTGCCT
TP53	TP53.B1/TP53.B2	ATCTACAGTCCCCCTTGCCG/GCAACTGACCGTGCAAGTCA

[a]PCR primers used to amplify microsatellite sequences listed in a 5′→3′ orientation.

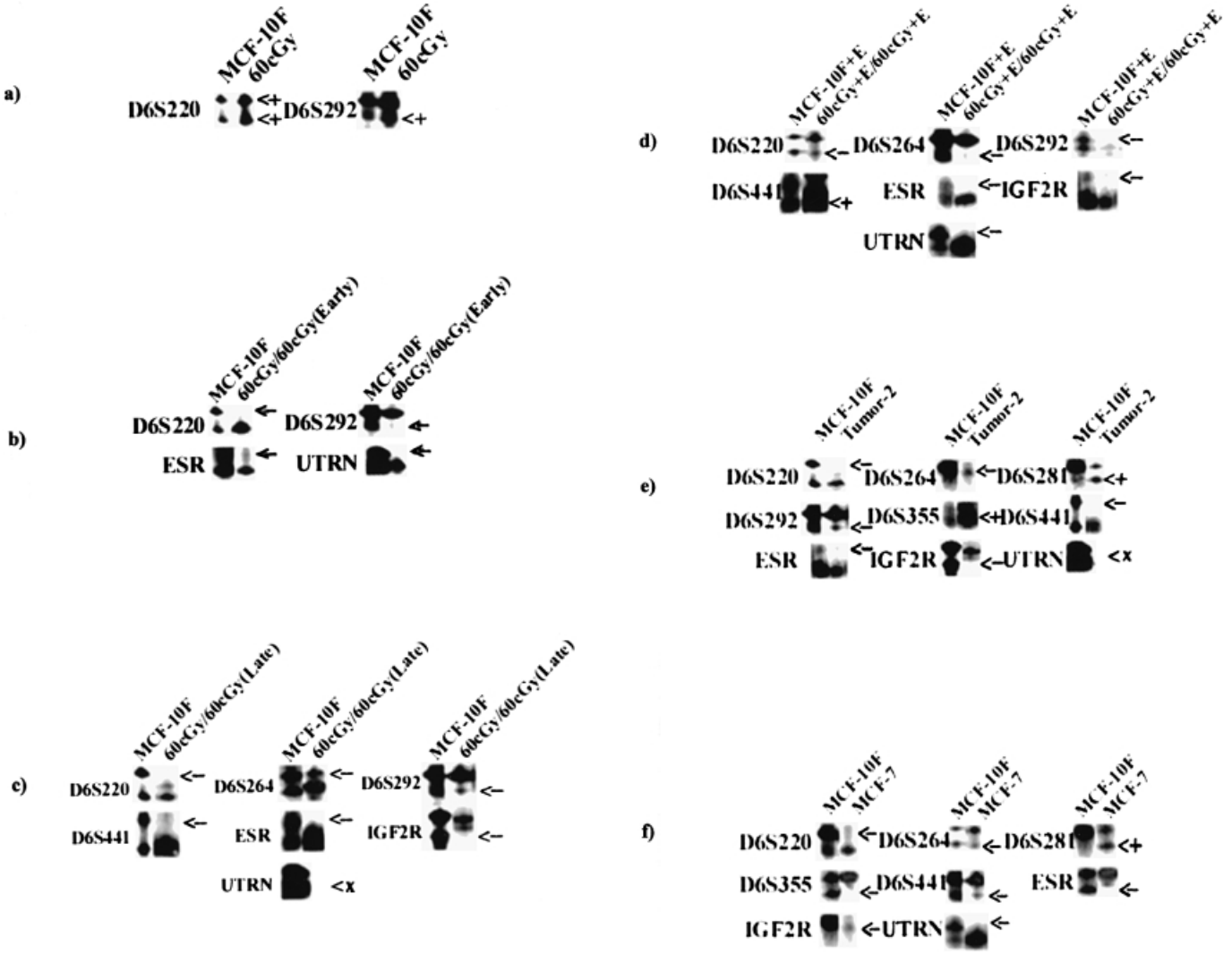

Fig. 2. Frequency of MSI and LOH screened at the respective loci of $(CA)_n$ repeat markers of chromosome 6 in irradiated, tumorigenic and tumor cell lines. Allele losses are indicated by arrowheads. (<–, Loss of heterozygosity; <+, microsatellite instability; <x, complete deletion).

detected, six for LOH and one complete deletion. Among those seven alterations, three were similar to early stage and the three additional LOHs were found in the 6q25-q27 (D6S264 and IGF2R) and 6q24 (D6S441) loci. A complete deletion at the 6q24 (UTRN) locus was also observed, which showed only LOH in the early stage.

Comparisons were also made between tumorigenic 60cGy + E/60cGy + E and MCF-10F + E cell lines (Figure 2d), which indicated a total of seven alterations, LOH in six and MSI in one. Loss of heterozygosity was observed at the 6q25-q27 (D6S220, D6S264 and IGF2R), 6q21-q23 (D6S292), 6q24-q27 (ESR) and 6q24 (UTRN) loci, whereas MSI was detected only at the 6q24 (D6S441) locus. Similarly, when the Tumor-2 cell line was compared with the MCF-10F cell line, nine alterations were identified (Figure 2e): six for LOH, two for MSI and one for complete deletion. Loss of heterozygosity was observed at the 6q25-q27 (D6S220, D6S264 and IGF2R), 6q21-q23 (D6S292), 6q24-q27 (ESR) and 6q24 (D6S441) loci and MSI at the 6q23-q25 (D6S355) and 6q27 (D6S281) loci. A complete deletion was detected only at the 6q24 (UTRN) locus. The MCF-7 cell line had a total of eight alterations when screened and compared with the MCF-10F cell line: seven for LOH and one for MSI. Loss of heterozygosity was found at the 6q25-q27 (D6S220, D6S264, IGF2R), 6q23-q25 (D6S355), 6q24 (D6S441 and UTRN) and 6q24-q27 (ESR) loci whereas 6q27 (D6S281) showed MSI (Figure 2f).

Chromosome 17

A total of 12 microsatellite markers were also utilized to detect MSI and LOH in both the short and long arms of chromosome 17. Different allelic imbalances were observed in almost 80–90% of the markers in 60cGy, 60cGy/60cGy (early and late), tumorigenic 60cGy + E/60cGy + E and Tumor-2 cell lines. The MCF-10F cell line was compared with 60cGy cell line (Figure 3a) and a total of two alterations were detected: one MSI at locus 17p13.3 (D17S849) and one LOH at locus 17q12-q21 (D17S579). When 60cGy/60cGy (early) and MCF-10F cell lines were compared (Figure 3b), a total of six alterations were screened. Among those six alterations were two for LOH and four for MSI. Loss of heterozygosity was observed at the 17q11-q12 (D17S250) and 17q12-q21 (D17S579) loci, and MSI at the 17q12-q21 (D17S588), 17q21 (D17S1322) and 17p13.1-p13.3 (D17S849 and TP53) loci. However, when the 60cGy/60cGy (late) cell line was compared with the MCF-10F cell line, a total of seven alterations were detected: two for LOH, four for MSI and one for complete deletion. Loss

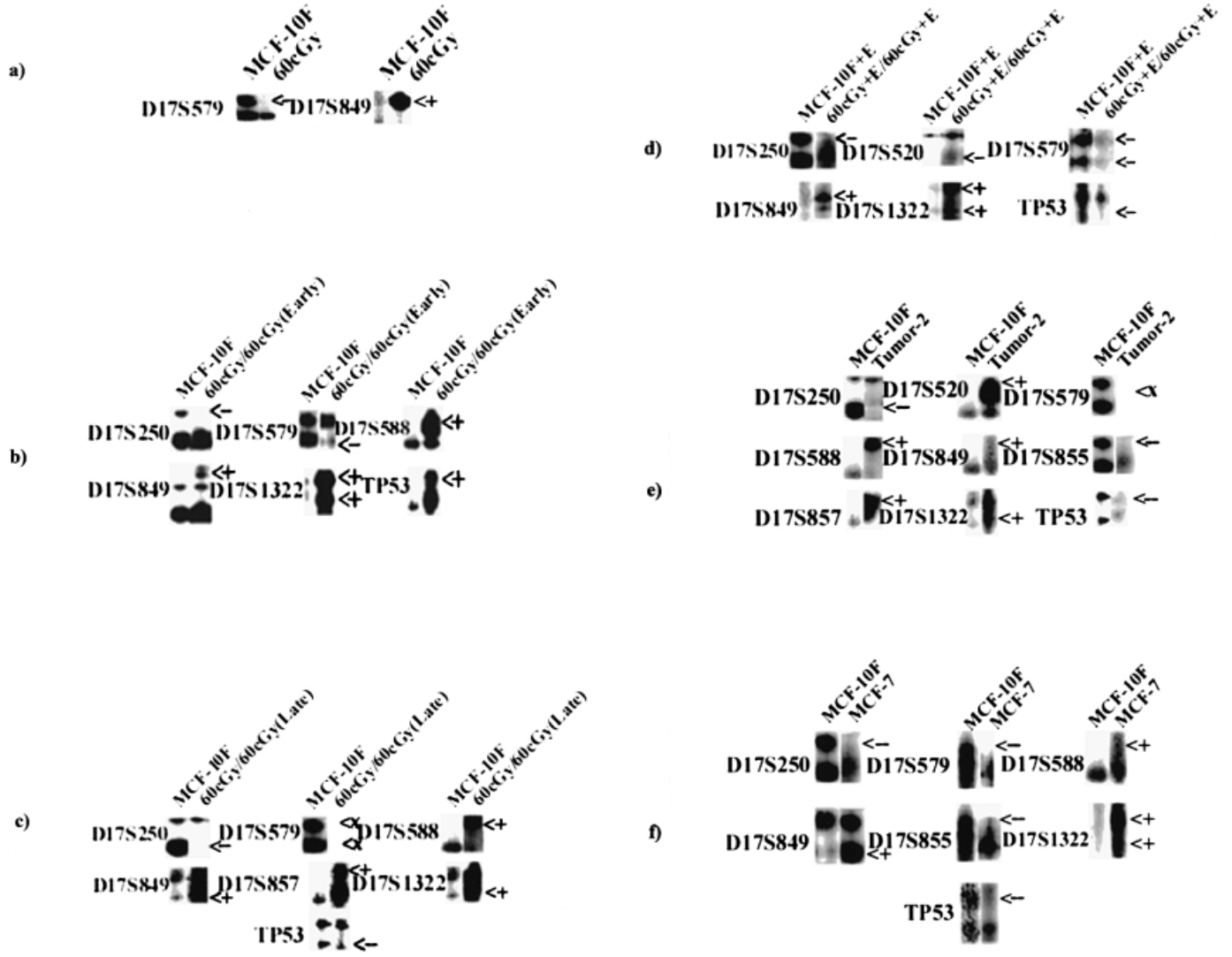

Fig. 3. Frequency of MSI and LOH screened at the respective loci of $(CA)_n$ repeat markers of chromosomes 17 in irradiated, tumorigenic and tumor cell lines. Allele losses are indicated by arrowheads (<-, loss of heterozygosity; <+, microsatellite instability; <x, complete deletion).

of heterozygosity was screened for at the 17q11-q12 (D17S250) and 17p13.1 (TP53) loci and MSI at the 17q12-q21 (D17S588), 17q21 (D17S857 and D17S1322) and 17p13.3 (D17S849) loci. The marker D17S579, which also belongs to locus 17q12-q21, showed complete deletion (Figure 3c).

Similarly, by comparing the tumorigenic 60cGy + E/60cGy + E cell line with MCF-10F + E(Figure 3d), a total of six alterations were identified, three each for LOH and MSI. Loss of heterozygosity was detected at the 17q11-q21 (D17S250 and D17S579) and 17p13.3 (TP53) loci whereas MSI was identified at the 17p12 (D17S520), 17p13.3 (D17S849) and 17q21 (D17S1322) loci. A total of nine alterations were screened for MSI and LOH when Tumor-2 cell line was compared with MCF-10F cell line (Figure 3e). LOH was observed in three, MSI in five and a complete deletion in one locus. LOH was observed at the 17q12-q21 (D17S250), 17q21 (D17S855) and 17p13.1 (TP53) loci, whereas MSI was observed at the 17q12-q21 (D17S588), 17q21 (D17S857 and D17S1322), 17p12 (D17S520) and 17p13.3 (D17S849) loci. The locus 17q12-q21, which also belongs to marker D17S579, showed complete deletion. MCF-7, the positive control tumor cell line, was also compared with respect to the MCF-10F cell line and seven alterations were detected. Among them, four alterations for LOH were screened at three loci, e.g. 17q11-q12 (D17S250), 17q12-q21 (D17S579 and D17S855) and 17p13.1 (TP53), and three alterations for MSI at two loci e.g. 17q12-q21 (D17S588 and D17S1322) and 17p13.3 (D17S849) (Figure 3f).

Discussion

Our present study demonstrates that MCF-10F cell lines exposed to double doses of α-particle radiation and treated with estrogen show a more complex pattern of allelic imbalance in various markers compared with cells treated with a single dose of radiation without estrogen. Cell lines treated with a double dose of radiation without estrogen and analysed with different passage numbers also showed a progressive change. However, a single 60 cGy dose of α-particles was also sufficient to induce persistent genetic changes in 4/24 of the marker loci examined, long before the cell reaches the tumorigenic stage.

During the process of cell transformation several phenotype changes were induced, such as anchorage independence and invasive capabilities, by single or double dose of radiation, either in the presence or absence of estrogen. Genetically, these changes were expressed in the form of LOH/MSI. The number of such alterations in a cell population seems to be a key event in tumorigenic progression. These results indicate that the changes were not necessarily due to the intrinsic level of genomic instability in these particular cell lines but due to cell division *per se*, therefore increasing the risk of genetic errors (33). Thus, the frequency of allelic imbalances were directly proportional to the dose of radiation, as well as on the passage number of the cells after irradiation, which seems to have a more deleterious effect when given in combination with estrogen.

A progressive degree of allelic imbalance (MSI and LOH) was detected at 6q, 17p and 17q at the early transformed stage (60 cGy), late transformed stage [60 cGy/60 cGy (early and late)], tumorigenic (60 cGy + E/60 cGy + E) and tumor cell lines established from the tumor nodule (Tumor-2) using specific microsatellite markers. There is also an increasing body of evidence indicating the existence of more than one growth-suppressing gene on the long arm of chromosome 6 and both arms of chromosome 17 (34–36). Aberrations in these markers are frequently associated with neoplastic changes due to their location in regions at, or near loci associated with, cell-cycle regulation, DNA replication, DNA repair or signal transduction protein genes (37–39), and their position in reference to mapped genes such as *MYB* (36) or the estrogen receptor (*ESR*) (39).

Our data suggest that LOH is more pronounced towards the telomeric region of 6q24-q27, whereas MSI is mainly concentrated in the 6q21-q23.3 region from late stage 60 cGy/60 cGy, tumorigenic 60 cGy + E/60 cGy + E and Tumor-2 cell lines. The existence of two possible regions, 6q21-q23 and 6q24-q27, in chromosome 6q associated with allelic imbalance (MSI and LOH) is in good agreement with previous findings in both ovarian and breast cancer (34,40,41). In addition, allelic losses at 6q24-q27 were of particular importance due to the localization of the estrogen receptor gene (*ESR*) in this region (39,42). Although the loss of *ESR* expression has negative consequences on the evolution of neoplastic progression, it is difficult to conclude that it is due to the loss of the gene itself and is most likely due to some other reason (43). Another potential target gene at loci 6q26-q27 is *IGF2R/M6PR* (insulin growth factor receptor 2 gene), which has been considered as a candidate for a tumor suppressor gene because its gene product binds to the survival factor *IGF2* without transducing a proliferation signal (44). Furthermore, mutations of this gene have also been reported in a small cohort of *in situ* carcinomas of the breast (45).

Since chromosome 17 is also a frequent target during breast-cancer progression, our study identified multiple foci of allelic alterations on both arms of chromosome 17. The loci 17p12-p13 and 17q12-q21 from both arms of chromosome 17, were more susceptible to MSI and LOH. This has been similar to the observation of various other studies on LOH in breast carcinogenesis (46). Molecular analysis suggests the location of the *p53* gene at 17p13.1 and, in addition, it is assumed that there may be at least three other tumor suppressor genes on chromosome 17 which frequently show LOH at 17q13.3 and 17q12-qter in breast tumors (47). This study revealed allelic imbalance on the long arm of chromosome 17 at locus 17q12-q21 for the markers D17S588, D17S857, D17S1322, D17S579, D17S846 and D17S855. Several authors (36,45) have reported the allelic loss of this locus during cancer progression.

BRCA1 (48) is a gene predisposing to hereditary breast and ovarian cancers and it has been mapped to locus 17q12-q21 of chromosome 17 by genetic linkage analysis (49,50). To investigate LOH of *BRCA1* in sporadic breast cancer, locus 17q21 (D17S855, THRA1, D17S579) is very informative. (D17S855 and D17S579) loci were detected in the tumor-2 line. It has been previously shown that among the various transformed human breast cell lines, both BRCA1 (17q12-q21) and BRCA2 (51) (13q12-q13) protein expression were altered (24). We are in the process of studying the various markers on chromosome 13 related to the *BRCA2* gene locus.

It is known that some of the LOH seen on chromosome 17 represent key events during cancer progression, such as those affecting *p53*, whereas others would represent secondary mutations bearing less selective advantage. We showed previously an increase in mutant p53 oncoproteins in MCF-10F cells irradiated with a double dose of α-particles either in the presence or absence of estrogen and in a tumorigenic cell line in comparison with control MCF-10F cell line (28). These could be associated with a loss of control over DNA replication or mitotic errors and give rise to a further cascade of mutations.

Since the complete loss of chromosome 17 seems to be rare, chromosomal non-disjunction does not appear to be a leading cause of LOH in this situation. Thus, MSI appears to be a novel molecular marker of carcinogenesis, reflecting RER induction by mismatch-repair genes. This DNA mismatch repair gene mutation led to activation of proto-oncogenes (such as *c-erbB2*, *c-myc*, *int-2*, etc.) or inactivation of tumor-suppressor genes (such as *p53*, *Rb*, etc.). Amplification and over-expression of various oncogenes and down-regulation of tumor-suppressor genes are associated with breast cancer progression and among them *c-erbB2* and *p53* are most important. They are generally associated with chromosome 17 (52). It is also now known that LOH at 17p13.3 is directly associated with the absence of the *PgR* gene (progesterone receptor) (46,47,49).

One interesting finding of this work is the promotional effect of estrogen, along with radiation, on tumorigenesis. None of the cell lines irradiated with either a single or double dose of α-particles underwent neoplastic transformation without estrogen co-treatment. These results indicate the importance of estrogen in breast tumor progression. It is now well established that estrogen might play a dual role in affecting breast cancer risk (53). On the one hand, there is evidence that estrogen might serve as a pre-initiators, initiators and promoters of breast cancer by DNA damage, genetic instability and mutations in cells (54) whereas, in contrast, it reduces breast cancer risk during pregnancy, prepubertal period and childhood (55).

Human breast cancer is a very complex and diverse disease that consists of a broad spectrum of clinical and pathological characteristics reflecting the multiplicity and heterogeneity of the molecular mechanisms involved in its genesis and progression. The array of genetic anomalies during tumor progression increases the probability of random rearrangements, which not only favors chromosomal disintegration, which leads to LOH, but at the same time, it also favors mitotic recombination, which leads to MSI. MSI has also been associated with enhanced expression of various oncoproteins during cancer progression. In this study, an estimate of the

overall incidence of alterations (LOH and amplifications) in the long arm of chromosome 6 and both arms of chromosome 17 was attempted during radiation-induced and estrogen treated breast carcinogenesis.

Multiple, sequentially occuring, allelic alterations were reported during the progression of breast cancer cell lines. The detection of allele loss on both chromosome 6 and 17 in human breast epithelial cells induced by radiation and estrogen treatment *in vitro* seem to be similar to regions of imbalance found in primary breast cancer, which highlights the relevance and usefulness of this model. Identification and characterization of these altered loci are also helpful for the systematic studies to identify the cellular and molecular changes associated with radiation-induced breast carcinogenesis.

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