CHARACTERIZATION OF MCF-7 CELL LINE WITH STS MARKERS LOCATED ON CHROMOSOME 16 AND 17 p AND q ARMS

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CERTIFICATE

This research work embodied in this thesis entitled, **"Characterization of MCF-7 cell line with STS markers located on chromosome 16 and 17 p and q arms"** has been carried out at the National Centre of Applied Human Genetics, School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full, for any other degree or diploma of any other University.

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CONTENTS

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Rahul Dev P. Malage

Abbreviations

β	Beta
α	Alpha
°C	Degree celsius
µg	Micro grams
µl	Micro liters
β-ME	β-mercaptoethanol
ADH	Atypical ductal hyperplasia
AI	Allelic imbalance
APS	Ammonium persulfate
Bis	N, N' methyl bis acrylamide
BPB	Bromo phenol blue
BLAST	Basic local algorithm search tool
bp	Base pair
BRCA1	Breast related cancer antigen-1
BRCA2	Breast related cancer antigen- 2
BSA	Bovine serum albumin
CAR	Cell adhesion regulator
cDNA	Complementary DNA
CGH	Comparative genomic hybridisation
CIN	Chromosomal instability
CpG	Cytosine- phosphate- guanine dinucleotide
DCIS	Ductal carcinoma in situ
DMBA	17, 12-dimethyl benz (a) anthracene
DNA	Deoxyribonuclic acid
dNTP	Deoxynucleoside triphosphate
ds	Double strand
DSB	Double strand break
E2	Estrogen
EDTA	Ethylene diamine tetra-acetic acid

ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERE	Esrtogen responsive element
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Fetal bovine serum
FCS	Fetal calf serum
GDB	Genome data bank
HBEC	Human breast epithelial cell
IDC	In-situ ductal carcinoma
IFN-γ	Interferon -γ
IGFBPs	Insulin like growth factor binding proteins
IGF-I	Insulin like growth factor
IRS-1,IR S-2	Insulin receptor subsrate 1,2
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
LCIS	Lobular carcinoma in situ
LOH	Loss of heterozygosity
M	Molar
M.W.	Molecular weight
MIN	Mutational instability
MLR	Minimal lost region
mM	Milli Molar
MMCT	Micro- cell mediated chromosome transfer
MMR	Mismatch repair
mRNA	messenger RNA
MSI	Microsatellite instability
NCBI	National center of biotechnological information
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	progesterone receptor
PTC-100	Programmable thermal controller
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SLS	Sodium lauryl sarcosine
SNPs	Single nucleotide polymorphisms
SSCP	Single strand conformation polymorphism
SSLP	Simple sequence length polymorphism
SSR	Simple sequence repeat
STR	Simple tandem repeat
STS	Sequence tagged site
TBE	Tris: Boric acid: EDTA solution
TBS	Towner-Brock syndrom
TE	Tris: EDTA solution
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β1	Transforming growth factor beta -1
TNF alpha	Tumor necrosis factor alpha
Tris	Tris (hydroxymethyl)-amino-methane
TSG	Tumor suppressor gene
UV	Ultraviolet
V	Volt
V/V	Volume/Volume
W/V	Weight/Volume
XC	Xylene cyanol
µl	Microliter

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Review of Literature

Characterization of MCF-7 cell line with STS markers located on chromosome 16 and 17 p and q arms

Introduction:

Cancer is a complex disease involving the activation of oncogenes and/or inactivation of tumor/growth suppressor genes. Oncogenes believed to be involved in cancer have been identified by means of amplification, chromosome translocation and *in-vitro* transformation assays (Bishop, 1991), while the identification of tumor suppressor genes has relied heavily on the study of hereditary cancers and loss of heterozygosity studies (Devilee and Cornelisse, 1994). Identifying the sites of losses, instability and rearrangement acquired as predominant chromosomal changes in cancer have greatly been helped by the recently accomplished project on human genome mapping and sequencing. The information available of the human genome sequences in chromosome specific manner has allowed to explore the experimentally observed regions of chromosomal loss for the probable presence of tumor suppressor gene(s) and also to study allelic imbalance using polymorphic STS markers (Lander et al., 2001; Venter et al., 2001).

Breast cancer is one of the most common neoplasms to affect women. Inherited mutations account for upto 10% of breast cancers, of which BRCA1 and BRCA2 may be mutated in the germ-line of half of such cases. Incidentally 90% of the breast cancer cases are sporadic and have not shown direct involvement of these two genes. In order to understand the biology of cancer in breast both in familial and sporadic cases, the work on MCF-7 cell line, a representative of metastatic breast epithelial carcinoma, has provided the advantage of studying different aspects of cancer biology on these cells in the past. However these cells have not been assessed for the allelic instability at different potential sites for tumor suppressor genes in chromosome 16 and 17.

Characteristics of MCF -7 Cell line

MCF -7 cell line is a representative of pleural effusion, adenocarcinoma of human mammary gland, of epithelial cell origin. The cell line was originally derived from a breast biopsy of metastatic site from 69-year-old Caucasian female. This cell line shows typical epitheloid morphology in culture and adherent growth *in-vitro*. The MCF -7 is an estrogen receptor positive cell line and retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes (Brandes et al., 1983). MCF-7 human breast cancer cells are known to be mitogenically responsive to insulin-like growth factors and estrogens, to secrete several insulin like growth factor binding proteins (IGFBPs), including BP-2, BP-4 and BP-5. Secretion of IGFBP's can be modulated by treatment with anti-estrogens (Pratt et al., 1993). It has also been seen that growth of human breast cancer MCF-7 cells arrested by serum starvation can be stimulated with recombinant human insulin-like growth factor-I (IGF-I) (Takahashi , et al., 1993). Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha) (Sugarman et al., 1985). Till date, MCF-7 has been characterized for various cellular processes such as growth and proliferation (Sugarman et al., 1985), differentiation (Bacus et al., 1990; Pratt et al., 1993), cell cycle regulation and apoptosis (Komarova et al., 1997).

MCF-7 cell line model for the studies on hormone dependence and tumor progression in mammary epithelium

MCF-7 cell line has been extensively studied to elucidate the mechanism by which hormones affect cell proliferation and protein synthesis. Among several estrogen receptor α positive cell lines, MCF-7 is one of the most frequently used cell line for functional observation of estrogen response. Estrogen metabolism is closely associated with the etiology and treatment of breast cancer. The growth of many breast neoplasms and breast cancer cell lines are stimulated by estrogens via the induction of growth-stimulatory peptides, hormone receptors, and oncogenic factors. Upon β estradiol (E2) binding, the estrogen receptor (ER) interacts with specific estrogen

responsive elements (ERE) in the promoters of these genes and modulates their transcription resulting in accelerated cell growth (Pietras et al., 1995). All breast cancers at some time in their development require estrogen stimulation for growth. This suggests that changes in the level of active estrogen or loss of estrogen responsiveness are important factors in the transformation to a malignant tumor (Santner et al., 1998). Estrogen plays a very important role in regulating the proliferation of breast tumors via the induction or suppression of key growth regulatory factors. Estrogen has been shown to be autoregulatory, down regulating its own receptor expression in MCF-7 cells (Saceda et al., 1988). Estrogen also, induces the expression of the PR (progesterone receptor) and inhibits expression of the potent growth suppressor TGF-β1 (Eckert et al., 1982; Knabbe et al., 1987; Read et al., 1988).

Estrogen mediated ER activity was seen in Reporter gene assay study in MCF-7 cell line and other ERα-positive cell lines such as T47-D, ZR75-1, BT474 which showed estrogen activated ERE-LacZ reporter gene in a dose dependent manner whereas no E2 stimulation of the ERE-LacZ reporter gene was evident in ERα negative cell lines (Singh et al., 2003). In presence of antiestrogen, ERα positive breast cancer cell lines failed to stimulate reporter gene activity which was similar to the activity observed in the absence of ligand, suggesting that approx 75% of the ERα positive patients would be likely to benefit from tamoxifen (Singh et al., 2003).

Role of STS markers in cancer

A basic view developing from studies of genomic instability is that the pathways involved in differentiation and development are disrupted during the development and progression of carcinogenesis. Cancer progression correlates with the substantial accumulation of genetic alterations in tumor cells either by amplification of oncogenes(C-myc, C- erbB-2) or by mutation, loss of function of tumor suppressor genes (p53, Rb) (Sato et al., 1990). According to Knudson's two-hit model, loss of function of TSG requires two phase process to inactivate both the alleles. Very often the loss of one allele due to the deletion of chromosomal region is involved in these

two events. This allelic alteration may occur either by 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. Frequent allelic losses have been found on different chromosomal arms (1p, 1q, 3p, 6q, 7q, 8p, 11p, 11q, 13q, 16q, 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 (Loeb et al., 1991; Parson et al., 1993), whereas LOH may indicate deletion of the remaining normal allele of a tumor suppressor gene. These deletions have been measured by the alterations of different microsatellite sequences associated with chromosomal arms. Due to their high reproducibility, hypervariable nature, and the relative ease of scoring by PCR, they are considered to be among the most powerful genetic markers in a wide variety of applications.

The existing model of carcinogenesis signifies that all human cancers have an unstable genome and that allelic imbalance is a very useful tool in assessing the level of genetic changes in the early stages of cancer progression. In breast cancer, many chromosomal loci have been shown to bear multiple regions of allelic imbalance. Among them chromosome 16 and 17 have received a great deal of attention as these contain several putative tumor suppressor genes.

Implication of chromosome 16 and 17 in breast cancer

Extensive analyses of breast tumors using cytogenetic and molecular genetic techniques have identified 16q and 17q as the most frequently changed chromosomes in human breast cancer (Bieche et al., 1995). Loss of 16q has been suggested to be an early event in breast tumorigenesis because 16q copy number alterations were found in atypical ductal hyperplasia (Gong et al., 2001), which represents the first clonal transformation event of ductal epithelial cells and in about 45% of pre-invasive DCIS (Vos et al., 1999). In breast cancer, loss of the entire chromosome 16 long arm is the most commonly observed LOH event. A study in the recent past of LOH in 712 breast

tumors concluded that there were three regions likely to harbor tumor suppressor genes, one at 16q22.1 and two at 16q24.3 (Cleton –Jansen et al., 2001). The E-cadherin gene has been identified as the likely LOH target at 16q22.1. E-cadherin gene has previously been found to be inactivated in invasive lobular carcinoma of the breast. Inactivation of E-cadherin is predominant event in LCIS and ILC (Kanai et al., 1995; Berx et al., 1995; Zhu X et al., 1996). Whereas, DCIS cases have shown expression of E-cadherin at the cell membrane (Vos et al., 1999). LOH in 16q is found in majority of well-differentiated DCIS and E-cadherin expression is always detectable in DCIS. However, in more frequent ductal breast carcinoma, mutations in E-cadherin have been described only in cell lines (Van de Watering et al., 2001), and a role of this gene as a tumor suppressor in ductal carcinoma is unclear. There probably is another unknown tumor suppressor gene responsible for the development of this type of DCIS.

The breast cancer, not only clinico-pathologically but also genetically, is a heterogeneous disease. The clinico-pathological heterogeneity is already manifest in its histologically recognizable precursor lesion, ductal carcinoma in situ (DCIS), which represents the proliferation of malignant cells within the ducts and lobules of the breast. Several histological classifications have been proposed (Scott et al., 1997). In all of these classifications DCIS is divided primarily on the basis of nuclear grade and/or necrosis. The classification differs to some degree, but all subdivide DCIS into three main subtypes; -well, -intermediately and poorly differentiated.

Poorly differentiated DCIS predominantly lack the estrogen (ER) and progesterone (PR) receptor; have a high proliferative rate, exhibit aneuploidy and an over expression of c-erbB2/neu and p53. In contrast well-differentiated DCIS are often ER- and PR-positive with low proliferative rate and rarely show c-erbB2/neu and p53 over expression. Previously done allelotyping studies, have shown that LOH on 16q and 17q are the most frequently found alterations (Radford et al., 1995). Further, Vos et al (1999), has reported in their study that LOH on chromosome 16q was present in 66% of well and intermediately differentiated DCIS and only in 39% of poorly differentiated DCIS. On chromosome 17, LOH was found in 70% of the poorly

differentiated DCIS and in 17% well differentiated DCIS. Mutations in p53 were also present in DCIS.

DCIS of the breast is known to be a pre-invasive stage of the breast cancer and is probably the precursor of infiltrating breast cancer. Thus, genetic alterations shown at this stage might indicate association with early event in malignancy or invasiveness. Specific chromosome arms more frequently affected by allelic losses and imbalances at pre-invasive stage of breast cancer, in particular, allelic losses affecting chromosome 16q as well as chromosome 17q and 7, appear to be early genomic abnormalities because these were observed in a significant number of DCIS lesion (C Marcelo Aldaz et al., 1995). LOH on chromosome 16q at various frequencies has been widely reported in breast, prostate, hepatoblastomas and Wilms tumor. In particular, two regions on chromosome 16q have been revealed to have a very high frequency of LOH in breast cancer, one maps to region 16q22.1 and the other to 16q24.2-ter. Taiping et al (1996), identified three distinct regions with high percentage (~70% above) of allelic losses among informative DCIS samples. Two of them agree with previously described areas at 16q21 at locus D16S400 and 16q24.2 at locus D16S402. However, the region between markers D16S515 and D16S504 were observed with high incidence of LOH. Within this region D16S518 locus was the most frequently affected, with 20 of 27 DCIS tumors (77%). These observations strongly suggest that a putative tumor suppressor gene or genes may be located at or near this locus. On the basis of partial YAC contig reported they were able to estimate that the minimum region with highest frequency of LOH is 2-3 Mb.

Allelic imbalance on chromosome 16 in breast epithelial cells transformed by chemical carcinogens.

The etiology of breast cancer, the most frequent malignancy diagnosed in women in the western world, remains unidentified despite intensive investigation (Parker et al., 1996). Chemical compounds such as polycyclic aromatic hydrocarbons, benzo (a) pyrene, 17, 12-dimethyl benz (a) anthracene (DMBA) are known to be carcinogenic in experimental animal models. They have also been implicated to be of

etiologic importance in various human cancers. Since genomic instability, which has been considered to be involved in the evolution of breast cancer from precursor lesions to metastatic cancer, microsatellite polymorphism analyses in *in-vitro* system in human breast epithelial cells (HBEC- transformed by chemical carcinogens), recapitulate various stages of neoplastic transformation.

Microsatellite instability in chromosome 16q has been detected in atypical ductal hyperplasia (ADH), which is considered to be a precursor lesion, in a frequency similar to that found in high and low nuclear grade ductal carcinoma in-situ (DCIS) and invasive carcinoma (Lakhani et al., 1995; Radford et al., 1995). In addition, LOH on the long arm of chromosome 16 has been found to be correlated with the occurrence of distant metastases (Lindblom et al., 1993). LOH on 16q occurs commonly in breast cancer at very early developmental stages, it is found in both highly aggressive and in relatively low-grade tumors. 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 invasive ductal carcinoma (Anderson et al., 1992; Stratton et al., 1995). Lobular carcinoma in-situ and ductal carcinoma in situ have been shown to exhibit the same LOH for 16q, indicating the same evolutionary pattern. The similarities in genomic changes detected in sporadic breast cancer and in HBEC transformed by chemical carcinogen raises the possibility that environmentally relevant chemicals may be involved in the induction of breast cancer.

Observations also indicate that the genomic changes induced in chromosome 16 by the carcinogen treatment appear in association with the emergence of tumorigenic phenotype since they were first observed in tumors induced in SCID mice by BP1E and BP1E-TP cells (Yuli Wu et al., 1997). Alterations induced in 16q by chemical carcinogens might be related to alterations in several genes located on those regions, such as E-cadherin on 16q22.1, M-cadherin mapped to 16q24, a tentative suppressor for tumor invasion, cell adhesion regulator (CAR) mapped to 16q24.3, breast basic conserved gene or BBCI and renal dipeptidase mapped to 16q24.32 that have been suggested to be tumor suppressor genes. The relatively high frequency of LOH and MSI in the telomeric regions of 16q in BP1E-Tp cells has suggested that a tumor

suppressor gene may reside in this region which has already been indicated for sporadic breast cancer. MSI at 16q12.1 in both DMBA treated non-tumorigenic cells and BP treated tumorigenic cells were observed. This region is of interest since a break point at the 16q12.1 and two-break reciprocal translocation between chromosome 5 and 16 have been described in association with the Towner-Brock syndrome (TBS), a rare autosomal dominant entity characterized by developmental abnormalities (Austruy et al., 1993).

Human breast epithelial cells transformed with a chemical carcinogen *in-vitro* exhibit multiple sites of LOH and MSI in both short and the long arms of chromosome 16 which coincide with the genomic alteration, most frequently reported in human breast carcinomas (Sato et al., 1990; Tsuda et al., 1994). MSI were detected at locus p13.3 of chromosome 16, a region in which an excision repair gene has been located (Thompson et al., 1995). 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. 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 observed resulted by the consequence of induction by the chemical carcinogen in this human breast epithelial cell line.

Among various gene abnormalities in breast cancer cells, loss of heterozygosity (LOH), or loss of one allele derived either from the maternal or paternal genome, on the long arm (16q) has been shown to occur in 50% of cases, regardless of the extent of tumor spread or the degree of cellular atypia (Lindblom et al., 1993; Tsuda et al., 1994). Such LOH can be examined in a small amount of DNA by the polymerase chain reaction (PCR) using oligo-nucleotide primers that encompass (AC)n repeat sequence located in the non coding DNA regions of the chromosome.These regions are very informative for detection of LOH in cancer tissue. A highly polymorphic (AC)n repeat region on the D16S305 locus on chromosome 16q24 was observed in the fine needle aspiration specimens from surgically resected materials. LOH was

observed in chromosome 16q in more than half of the cases where aspirated specimens were used. It seems highly probable to judge the specimen cancer positive, if LOH is found on 16q. A positive finding for LOH would provide meaningful supportive data for preoperative diagnosis of breast cancer (Hitoshi Tsuda et.al., 1996).

The long arm of chromosome 16 is also a target for LOH in other tumor types such as prostate, lung and hepatocellular cancer and rhabdomyosarcoma, suggesting that 16q harbors a multitumor suppressor gene. However, there is also a tumor type that show high LOH frequencies on other chromosome arms, but not on 16q eg., gastrointestinal tumors, indicating that 16q LOH is most likely a selected event.

Data on 16q LOH are further corroborated by several CGH studies on invasive and in-situ breast tumors. CGH show that the long arm of chromosome 16 is involved in physical deletion. Percentages are lower than those obtained with LOH studies with a mean of 25%. This can be attributed to the fact that LOH is also detected when mitotic recombination has occurred, a phenomenon that does not result in loss of copy number and consequently is not detected by CGH. The occurrence of LOH due to mitotic recombination strongly suggest that haploinsufficiency is unlikely to be the genetic mechanism of the TSG at 16q Many studies have attempted to identify the SRO's that are the target of LOH at chromosome 16q in breast cancer. At least three non-overlapping regions are required, of which 16q24.3 and 16q22.1 are most frequent.

Defining loss of heterozygosity of 16q in breast cancer cell lines

Loss of heterozygosity is demonstrated by the finding that a heterozygous genetic marker in the normal somatic tissue has lost one allele resulting in homozygosity in the tumor tissue. The region of LOH can be mapped using linked polymorphic markers. If by surveying a number of independent tumors a region of LOH is shown to be nonrandom, then this is considered to indicate the location of a tumor suppressor gene.

Two regions of the long arm of chromosome 16, 16q22.1 and 16q24.3 have been shown by numbers of LOH studies as a possible location of a breast cancer tumor

suppressor gene (Callen et al; 2002). Breast cancer cell lines are particularly useful for assessing candidate transcripts on the long arm of chromosome 16 as tumor suppressors. It is possible to indirectly assess the LOH by typing cell line with polymorphic microsatellite markers. LOH can be inferred when a series of linked polymorphic markers are all homozygous. Depending on the heterozygosity values of microsatellite markers, a statistical probability less than or equal to 0.001 was used to define LOH of adjacent markers. In practical terms this was usually equivalent to the finding that if five adjacent markers were homozygous, then LOH for this region was considered to exist.

A report from Callen et al (2002), showed combined microstellite typing together with dual colour fluorescence in-situ hybridization (FISH) using chromosome 16q painting and region specific probes to determine chromosome 16 LOH in 21 breast cancer lines and two non-tumorigenic breast epithelial cell lines. Tumorigenic breast cancer cell lines, BT-20, BT-474, BT-483, DU-4475, MDA-MB-175, -361, -415, MCF-7, UACC-893 and non tumorigenic breast epithelial cell lines HBL-100, MCF-12A showed number of loss of heterozygosity of chromosome 16q. All these cell lines showed heterozygosity for markers dispersed along the long arm of chromosome16. Many of these cell lines were characterized by particularly complex karyotypes eg. MDA-MB-361 did not posses a normal chromosome 16. Similarly, MCF-7 possesses two normal 16 and 6 marker chromosomes derived from chromosome 16.

MDA-MB-231, MDA-MB-468 showed LOH of entire chromosome 16 MDA-MB-231 had two chromosomes 16 of normal appearance, occasionally cells were seen with three. All 19 microsatellite markers typed on chromosome 16 were homozygous. MDA-MB-468 had one pair of chromosome 16 containing duplicated 16 material and a pair of derivatives from translocation involving chromosome 16. All 25 microsatellite markers tested were homozygous. Results for both cell lines are consistent with an origin of LOH by loss of one chromosome 16 homolog. In cell lines, MDA-MB-134, T47-D, ZR75-1, microsatellite markers on the short arm of

chromosome 16 were heterozygous while markers on the long arm were all homozygous.

Allelic Imbalance in chromosome 17

Chromosome 17 is one of the most frequently altered chromosomes in malignant breast cancer. Human chromosome 17 contains a number of genes and loci, believed to be involved in the pathogenesis of breast cancer. Four genes residing on chromosome 17 are known to be involved in breast cancer, BRCA1 (17q21), nm23 (17q22), Her2/neu (17q12), and p53 (17p13.1). The tumor suppressor gene p53 located on 17p13.1 is mutated in 15-35% of sporadic breast cancers (Greenblatt et al., 1994) and mutations in p53 may be associated with poor prognosis. Germ line mutation of p53 leads to the development of multiple cancer types, including breast cancer, as part of the rare Li-Fraumeni syndrom. The region of 17p distal to p53 (17p13.3) is believed to harbor one or more tumor suppressor genes based upon Loss of Heterozygosity (LOH) studies (Anderson et.

al., 1992). One of these regions may represent a new candidate tumor suppressor gene on 17p13.3 which is hypermethylated and under-expressed in a number of breast cancer cell lines. LOH analysis of sporadic breast tumors indicates at least one more tumor suppressor locus distal to HIC-1. Genes residing on 17q that are involved in breast cancer include the proto-oncogene Her2/neu (ERBB2) located at 17q12 that is amplified in upto 35% of breast cancers. Amplification is associated with poor prognosis, especially in lymphnode positive patients. Mutations in BRCA1 located at 17q21 are thought to be responsible for almost half of all inherited breast cancers. The role of BRCA1 in sporadic breast tumorigenesis is not clear. High rates of LOH at the BRCA1 locus have been reported in sporadic breast cancer; however mutations in BRCA1 coding regions have not been described. Down regulation of BRCA1 expression or nuclear exclusion of the BRCA1protein have been proposed as alternative mechanism of BRCA1 inactivation in sporadic breast tumors (Chen et al., 1995; Thompson et al., 1995), but these results have not been confirmed (Scully et al., 1996). The candidate breast cancer metastasis suppressor gene, nm23 (17q22), shows

an inverse correlation between gene expression and metastasis in breast tumors. In addition, transfection of nm23 gene into the highly metastatic breast cancer cell line MDA-MB-435, resulted in a significant reduction in metastatic potential *in-vivo*. There is also a functional evidence of a second metastasis suppressor gene on 17q (Plummer et al., 1997). Studies have shown that MMCT of chromosome 17pter-q23 into rat mammary and prostate carcinoma cells resulted in suppression of metastasis and this suppression was independent of nm23 expression. At least four regions of LOH have been reported on chromosome 17q in sporadic breast tumor. One region of LOH lies proximal to BRCA1 and another region distal. Two more regions of LOH have been found on distal 17q defined approximately by markers D17S74 and D17S4. It has previously been shown in studies of Plummer et al (1997) that the introduction of human chromosome 17 into the MCF-7 breast cancer cell line resulted in growth arrest within eight weeks. It was also reported that MCF-7 is structurally wild type for p53 and more importantly, that the over expression of a wild type p53 transgene is compatible with normal growth of MCF-7 cells. In addition MMCT of normal human Chromosome 13 has no effect on MCF-7 cells *in-vitro* or *in-vivo,* implying the presence of a novel growth suppressor gene on chromosome 17 and the growth suppressor activity localized to the region at 17q24-q25 (Plummer et al., 1997).

Amplificaton of oncogenes is another important genetic alteration in breast cancer. The most notable gain found was amplification of 17q with two distinguished regions 17q12 and 17q22-24. The oncogene that is amplified in the 17q12 region is c-erbB2/neu. The 17q22-q24 locus harbors as yet unknown gene that has been found to be amplified in breast cancer cell lines of invasive breast cancer by CGH (Kallioniemi et al., 1994).

Allelic imbalance on Chromosome17p arm

Breast cancer development is though to be the result of unmasking of one or more recessive tumor suppressor genes by somatic alterations (Knudson, 1985).
BRCA1 and BRCA2 are infrequent targets for somatic inactivation in sporadic breast cancer. TP53 is another gene also responsible for inherited predisposition to cancer

(Lancaster et al., 1996). P53 gene is mutated in about 22-27% of sporadic breast tumors (Sjorgen et al., 1996). Several studies have been published in an effort to estimate the frequency of somatic loss of wild type TP53 alleles. Most of these studies have initially used the highly informative D17S5 marker which maps close to TP53 on the short arm of chromosome 17. It later became clear that the D17S5 locus is actually located on band 17p13.3 approximately 20CM distal to the TP53 gene (Coles et al., 1990). At this locus a high frequency of allelic losses (approximately 60%) has been detected in sporadic and in familial breast carcinoma (Sato et al., 1990; Lindblom et al., 1993; Phelan et al., 1998). On 17p13 there are two independent regions of LOH, one spanning the TP53 locus at 17p13.3 and involving a more distal region, implying the existence of at least another TSG distal to TP53, further supported by the data obtained by various studies by cell transfection and allelotyping studies (Theile et al., 1995).

In breast carcinoma 17p13.3 LOH is independent of TP53 point mutations and is associated with a high S phase index (Merlo et al., 1994). LOH of D17S5 locus is associated with disease free survival in stage I-IV breast cancer patients (Nagai et al., 1994). Another marker D17S379 is linked to D17S5 but is located 200 Kbp proximal. D17S5 and D17S34/ABR cover 3Mb of DNA and do not detect interstitial deletions affecting smaller portions of the chromosome arm bordered by these markers. A study carried on primary breast carcinomas of 152 patients, showed 27.8% mutation in TP53 gene. The allelic loss in the 17p13.3–17pter region for four polymorphic loci namely, D17S5, D17S379, D17S34/ ABR was examined. All of the tumors that were found to be deleted at the D17S5 locus showed loss of the D17S379 marker. These results indicate that the 17p13.3-pter region bordered by loci D17S5/D17S379 and D17S34/ABR were frequently affected by LOH that is independent of point mutations in the p53 gene (Liscia et al., 1999).

The role of 17p13.3-pter LOH in breast cancer is stressed by the evidence of chromosomal alterations even in premalignant lesions such as mammary atypical hyperplasias and ductal carcinomas in-situ, in which the rate of allele losses have been reported to be higher than 35% (Radford et al., 1995). The association of LOH of

17p13.3-pter with malignancy provides only a circumstantial evidence of the presence of TSG.

The smallest common region of allelic loss in 17p defined by markers D17S5, D17S379, D17S34 /ABR harbors a number of recently cloned putative tumor suppressor genes. L132/Rox, a helix-loop-helix structure that is a characteristic of several transcription factors was identified by marker D17S379. OVCA 1 and OVCA2 have been isolated from a cosmid clone, containing the closely linked marker D17S5 that spans minimal region of allele loss in ovarian cancer (Schultz et al 1996). The HIC-1 gene has been cloned from a region flanking the D17S5 marker, found frequently hypermethylated in human cancers, including breast cancer. Loss of function, through somatic mutations of genes, mapping in the distal region of the 17p chromosome appears to be a crucial event in the development of and progression of sporadic breast cancer.

To date most of the LOH studies performed on 17p13 region have used limited number of markers. The recent study by Susane et al (2001), attempted to define more precisely the LOH pattern at 17p13.3 region with 16 micro satellite markers and the two RFLP markers .The results obtained suggest that there are at least seven commonly deleted regions on chromosome 17p13.3. The most frequently deleted regions were located between the markers D17S1831 and D17S1845 (56% LOH) at chromosomal location 17p13.3-17p13.2, between markers D17S1810 and D17S1832 (53% LOH), and between markers D17S938 and TP53 (55% LOH) at 17p13.1. Previous studies have also revealed common deleted regions at 17p13, distal to TP53. In other types of tumors such as ovarian cancer, a smallest region of overlapping deletions between D17S28 and D17S5 was found (Shultz et al., 1997). Lung cancers have been shown to carry independent commonly deleted regions between D17S379 and D17S5 (56% LOH). In LOH studies at 17p13.3 region between markers D17S5 and D17S34 showed high level of LOH in HCC cases. (Zhao et al., 2003).

In agreement with the various studies performed till date, LOH at 17p region has been observed in breast cancer, lung cancer, colon cancers, medulloblastoma, follicular thyroid carcinoma, leukemia and lymphomas (Baker et al., 1989; Mc Donald

et al., 1994; Phillips et al., 1996; Schultz et al., 1996; Grebe et al., 1997; Konishi et al., 1998; Sanker et al., 1998; Xintai Zhao et al., 2003). In total, these data provide an evidence for a putative tumor suppressor gene at 17p region that might be implicated not only in breast cancer but also in other tumor malignancies (Sussane et al., 2001).

Allelic imbalance on Chromosome 17q arm

Allelic imbalance (AI) may represent DNA amplification or loss of heterozygosity (LOH) of chromosomal material; in accordance with previous studies approximately 80% of allelic imbalance represents LOH (Devilee and Cornelisse, 1994). AI values under 24% probably represent background levels of instability of chromosome 17 in breast tumors (Plummer et al., 1997).

At least four regions of allelic imbalance have been described on chromosome arm 17q in sporadic breast tumors, one region of LOH located proximal to BRCA1, and another region which overlaps BRCA1. Further two regions are located on distal 17q (Devilee and Cornelisse, 1994; Kirchweger et al., 1994; Nagai et al., 1994). Previous investigations reported that AI on chromosome arm 17q correlate with ERBB2 amplification (Sato et al., 1991), high mitotic index (Nagai et al., 1994), ER negative tumors (Anderson et al., 1992), ER and PR positive tumors (Niederacher et al., 1997).

Plummer et al (1997) assessed allelic imbalance on chromosome region 17q12-qter by PCR analysis of 85 paired tumor/normal DNA using 21 STR markers. Allelic imbalance of all the markers studied was observed in 16 of 85 (19%) tumors, whereas a partial AI of 17q loci was observed in 24/85 (28%) samples. The frequency of AI of individual STR locus was between 20% and 35%. The highest region of AI were at D17S790/D17S787 (29% / 28%), D17S 808/D17S948 (35% / 34%), D17S968 (29%) and D17S939/D17S802 (32% / 34%).

Interestingly, D17S855, a marker intragenic to BRCA1, showed a very low rate of AI (21%), suggesting that inactivation of BRCA1 does not occur frequently in this tumor set. Common Regions of allelic imbalance were observed in tumors showing partial AI of chromosome 17 loci. Locus D17S800 showed an AI of 26% whereas in

one case imbalance at D17S800 with retention of D17S855, suggested the localization of a gene proximal to BRCA1. It is possible that some of the AI observed at D17S800 might represent amplification of the ERBB2 locus, or imbalance of another unidentified gene. Another common region was found at D17S787 with retention of D17S790 which is proximal to D17S787, as well as retention of D17S792 or D17S808 markers which are more distal to D17S787. A third common region defined by marker D17S948 on 17q23-q24 showed defined breaks in tumors in this region indicating that D17S948 may closely define the boundaries of this region of imbalance. The fourth common region of imbalance was located in the region composed by D17S937/D17S939/D17S802/D17S836 on 17q25. Yet, another region of AI was defined by markers D17S968 and D17S785.

The region of AI at D17S787 have been suggested to represent the areas previously reported near NM23 (Anderson et al 1992; Nagai et al., 1994; Niederacher et al., 1997). A number of studies have shown broad regions of AI located at 17q22-q24 region, including ITGB3(GP3A), D17S74 (CMM86) and the growth hormone locus (Sato et al., 1991.; Kirchweger et al., 1994). Two regions at locus D17S787, and D17S948 are located within this broad region which is further sub-divided into two more precise regions of AI. Analysis of flanking markers has put the estimates of the regions of AI to be 7CM at the D17S787 and 8CM at the D17S 948 locus (Plummer et al., 1997). Studies of Su-Ling Tseng et al (1997), on BRCA1 and adjacent loci on 17q by using five microsatellite markers; D17S250, D17S856, D17S855, D17S579, D17S588 mapping to 17q spanning the region of 17q11.2-21, including marker D17S855 which is within the BRCA1 gene, detected allelic loss on 17q . LOH was found in relatively high proportion (52%) on 17q. The locus D17S855 that is intragenic to BRCA1 showed 25.4% LOH. However genomic deletion among these loci displayed a dispersed pattern with LOH ranging form 21% to 35%. Collectively consistent and statistically significant proportion of allelic loss at every locus on 17q including BRCA1 (D17S855), was demonstrated.

BRCA1 and TOC are independent targets of allelic loss on 17q in primary breast cancer

Sporadic breast cancers often show allelic losses on the long arm of chromosome 17. BRCA1 locus lies at 17q21, whereas the TOC locus associated with esophageal cancer, lies at 17q25.1. Allelic losses in this region were observed by using microsatellite markers covering the relevant regions of 17q in 97of 178 (55%) primary breast tumors. Losses were most frequent at markers around the TOC locus (48% at D17S1839 and 43% at D17S1603). 45% allelic loss was found at D17S934 locus (Koichi et al., 1999). These results indicate that BRCA1 and TOC are independent targets of allelic loss on 17q in primary breast cancers, and that inactivation of the TOC locus in particular may play an important role in the genesis of sporadic breast tumors. In addition, association between LOH at 17q21 and ER negative and/or PR negative status has also been documented (Nagai et al., 1994). These findings obtained by employing microsatellite analyses suggests that inactivation of some genes residing on 17q by LOH might be related to the events that render cancer cells independent of hormonal control, during the development and progression of breast cancers.

Role of BRCA1 in breast cancer and Experimental models:

Approximately 90% of breast and/or ovarian cancer families show loss of function, germ line mutations in the tumor suppressor genes, BRCA1 and BRCA2 located at17q21 and 13q12-q13, respectively. Mutations in BRCA1 account for 3% of all breast cancer and further addition of 2% results from mutations in BRCA2. Breast cancer patients with BRCA1 germ line mutations very often demonstrate loss of the corresponding wild type allele.

Wild type BRCA1 protein has a vital role in cell cycle regulation. BRCA1 protein binds to various cellular proteins involved in cell cycle regulation including DNA repair protein Rad51, tumor suppressor protein p53, RNA Polymerase II holoenzyme, RNA Helicase A, CtBP-interacting protein, c-myc, BARD1, BRCA2 protein (Scully et al., 1996 ; Fraser et al., 2003). Mutations in BRCA1 may affect BRCA1 protein by disrupting its active conformation and thus affect its cellular activity that eventually

leads to progression towards malignancy. High rates of LOH at the BRCA1 locus have been reported in sporadic breast cancer; however germ line mutations in BRCA1 coding regions have been described in very few cases of sporadic breast cancer (Garcia-Patino et al., 1998). Whereas, no somatic mutations in BRCA1 have been identified without simultaneous germ line mutations, contrary to ovarian cancer, where single somatic mutations have been found in BRCA1. To investigate LOH of BRCA1 in sporadic breast cancer, locus 17q21 (D17S855, THRA1, D17S579) was very informative. D17S855, D17S579 loci were detected in the tumor-2 line (Roy et al., 2001). The interesting finding of this work was the promotional effect of the estrogen, along with the radiation, on tumorigenesis. None of the cell lines irradiated with the α particles underwent neoplastic transformation without estrogen co-treatment. These results indicated the importance of the estrogen in breast carcinoma (Roy et al., 2001).

In nude mice, tumor growth of MCF-7 breast cancer cells was found to be inhibited by transfection of wild type BRCA1 copy. Retroviral transfection study also showed that wild type BRCA1 inhibits growth of breast and ovarian cancer cells *in-vitro* (Holt et al., 1996). Another study showed that fibroblast expressing the BRCA1 antisense RNA, with corresponding low levels of endogenous BRCA1 protein, resulted in accelerated growth and tumor formation in nude mice (Rao et al., 1996). The data accumulated to date, strongly support a role of BRCA1 in breast cancer and its role in the control of breast neoplasia of sporadic origin (Fraser et al., 2003).

Studies on allelic imbalance in MCF-7 and other breast carcinoma cell lines

There is an evidence that introduction of normal human chromosome 17 into the breast cancer cell line MCF-7 by micro-cell mediated chromosome transfer (MMCT) results in growth arrest (Plummer et al., 1997). Similarly, suppression of tumorigenicity in nude mice was found after transfer of the long arm of chromosome 17 into the MDA-MB-231 tumorigenic cell line. In contrast the MDA-MB-231 cell line carrying the short arm of chromosome 17 retained its tumorigenicity with reduced tumor growth rate. These data suggest that chromosome 17 may contain at least one gene mapping to the long arm that suppress the tumorigenic phenotype.

On chromosome17, at least two major regions show frequent amplification in breast cancer i.e. 17q12.21 and 17q23. The 17q23 region shows gain on CGH analysis in approximately 20% of primary breast carcinomas, as well as in several breast carcinoma cell lines (Couch et al., 1999; Barlund et al., 2000; Monni et al., 2001). To date, number of genes in addition to ERBB2 that are over expressed and /or amplified in breast cancer have been mapped to 17q12-21. These genes includes GRB7, TOP2A, LASPI /MLN 50, and TRAF 4. As well as, a number of amplified and over expressed genes mapping to 17q23 have been suggested to play a role in breast carcinoma development including RPS6 KB1, APPBP2, MUL /TRIM37, TRAP 240 AND TBX2 (Barlund et al., 2000; Monni et al., 2001).

Information has been accumulated till date, on the distinct 17q12-q21 and 17q23 regions. There has been no high resolution expression array evaluation for the whole region of 17q12-q23 in the same clinical tumor samples, to address whether there is any potential relationship between genes located on 17q12-23 regions, in carcinogenesis. Amplification and over expression of three uncharacterized gene sequences mapping to the telomeric portions of 17q21.23 was found in tumor samples and in the MCF-7 cell line that indicate a possible role for these sequences in breast carcinoma. This further suggests that apart from amplification and over expression of ERBB2; 17q12.23 region harbors multiple genes whose DNA amplification and concomitant over-expression, either independently or synergistically promote breast carcionogensis.

Another cell line, MCF-10 is a spontaneously immortalized human breast epithelial cell line which has been derived from mortal human breast epithelial cell line MCF-10M and has a near diploid karyotype and is of luminal epithelial origin. These cells retain all the characteristics of normal epithelium *in-vitro*, including anchorage dependence, non-invasiveness and non-tumorigenicity in nude mice (Soule et al., 1990). Until recently, there were few human cell culture models available for the study of radiation carcinogenesis for a better understanding of the cellular and molecular changes associated with radiation induced breast carcinogenesis. Roy et al (2001), recently developed a transformation model based on a spontaneously

immortalized human breast epithelial cell line, MCF-10F, irradiated with graded doses of α particles in the presence of E2. This model was used for the examination of the incidence of allelic imbalance in chromosomes 6 and 17 by means of microsatellite markers which were selected based on their location near to known mapped tumor suppressor genes, oncogenes, or other cancer related genes and regions or near loci associated with cell cycle regulation, DNA replication, DNA repair or signal transduction protein genes. A variety of observations have been reported in literature for allelic imbalances scored in different chromosomes in the two cell lines related to breast tissue. For chromosome 6, loss of heterozygosity was found at 6q25-q27 (D6S220, D6S264, IGF 2R), at 6q23-q25 (D6S355), at 6q24 (D6S441 and UTRN) and at 6q24-q27 (ESR) and MSI at 6q27 (D6S281) in MCF-7 cell line when screened and compared with the MCF-10F cell line. For chromosome 17, seven alterations were found in comparison to MCF-10 cell line. Among them four alterations for LOH were screened at three loci: 17q11-q12 (D17S250), 17q12-q21 (D17S579, and D17S855) and 17p13.1 (TP53), and three alterations for MSI at two loci: 17q12-q21 (D17S558 and D17S1322) and 17p13.3 (D175849). Allelic imbalance on the long arm of chromosome 17 at locus 17q12-q21, for the markers D17S588, D17S857, D17S1322, D17S579, D17S846 and D17S855, were in accordance with the studies carried out by several other authors. It has been previously shown that among the various transformed cell lines, both BRCA1 at 17q 12-q21 and BRCA2 at 13q12-q13 showed altered protein expression.

Aims and Objectives

Aims and Objectives

The MCF-7 cells are established from a malignant cancer tissue and are already transformed. Any event of loss of a tumor suppressor gene (TSG) must already have occurred in these cells. If such a loss is either due to deletion of a region bearing such a TSG or due to mitotic recombination leading to homozygosity (LOH) of the region distal to the recombination point, then an analysis of the status of polymorphic microsatellite markers should reflect it. Since chromosome 16 and 17 have been implicated in breast cancer, it was proposed to choose specific regions in p and q arm of chromosome 16 and 17 and study nine STS markers for their genotypic status.

The objective therefore was to study the status of STS markers located on Chromosomes-16p&q and 17p&q in cultured MCF-7 cells. Chromosome-16 markers: D16S3082 located on short arm at p13.3 and D16S413, D16S305 located on long arm at q24.3 were used for the study. On chromosome 17, six microsatellite markers namely, D17S5 and D17S379 located on short arm at 17p13.3 and D17S787, D17S855, D17S934 and D17S948 located on long arm at 17q21-23 were used for allelotyping studies.

The rationale for studying the status of the polymorphic markers was also to characterize the MCF-7 cells for the genotypic status of some of the polymorphic markers in chromosome 16 and 17 for monitoring future LOH studies while transforming the cells further *in-vitro* neoplastically and look for potential 'TSG' locations.

1. Why not study expression of some known genes such as bax, bcl2, p53 and a few other housekeeping genes in this cell line and compare with expression of normal cells (not brain for me-p).

Stepwise 1, 2, 3 objectives might be considered.

Materials and Methods

Materials and Methods

Chemicals

Acrylamide (Sigma), Acetic acid, Acetone, Ammonium persulphate, Agarose (Pronadisa, Roche), Ampicillin, bis-Acrylamide (Sigma), β-Mercaptoethanol (Sigma), Boric acid (Qualigens), Bromophenol blue, Calcium chloride dihydrate (Sigma), Chloroform (Qualigens), Diethyl pyrocarbonate (Sigma), DNA molecular weight markers (100 bp ladder (Promega)), 20 bp ladder (Sigma), Ethylene diamine tetra acetate (EDTA) (Qualigens), Ethanol (MERCK and Bengal Chemicals),Ethidium bromide (Sigma), Formaldehyde (Qualigens), Formamide (Sigma), Glycerol, Glutaraldehyde, Hydrochloric acid (SD Fine), Isoamyl alchohol, Isopropanol (Qualigens),Methanol, Magnisium chloride (Qualigens), Phenol (Qualigens), Proteinase K (Sigma), Potassium chloride (Qualigens), Sodium acetate, Sodium bicarbonate, Sodium carbonate, Sodium chloride, Sodium dodecyl sulfate (Sigma), Sodium hydroxide, Tris buffer (Sigma), TEMED (Sigma),xylene cyanol (Sigma), DMEM (Gibco BRL),Peniciliin,Steptoycin (Sigma).

Culture and maintenance of MCF-7 Cell line

Human breast carcinoma cell line MCF-7, obtained from NCCS, Pune, India, were cultured in DMEM (Gibco BRL) supplemented with 10% Fetal Calf Serum (GibcoBRL), 100U/ml penicillin, and 100ug/ml streptomycin at 37^{0}C under 5% CO_2.

DNA isolation from MCF-7 cell line

(Laird P W Neuclic Acid Resarch 1991, 19: 4293)

Confulent MCF –7 cells in the 6th passage were trypsinized in 25cm^2 square tissue culture flask, taken out in 1.5 ml eppendorf tube with 1ml lysis buffer and incubated for 3 hours at 37°C. Equal volume of Isopropanol was added to the lysate. Samples were mixed and swirled properly for 10-20 minutes. The DNA was recovered by lifting aggregated precipitate from the solution using a disposable tip. Excess liquid

was dabbed off and the DNA was dispersed in a prelabled eppendorf tube containing, 20 to 200 µl of 1X TE (depending on the size of the precipitate) and kept overnight for dissolution.

PCR primers

STS marker primers used in this study were selected from the genome data bank (http://gdb.org/). The PCR primers were synthesized commercially (Biobasic Inc). The list of the primers and their sequences are mentioned in Methods section. The Taq polymerase enzyme and dNTPs were procured from Banglore genei.

Agarose Gel Electrophoresis

Agarose gel mixture was poured in gel casting tray fitted with a comb. It was allowed to polymerize for approximately 30 min. in the cold room or approximately for 60 min. at room temperature. The comb was removed after polymerization and the DNA samples were loaded. The samples were run at 120 volt in 0.5X TBE. The gel was then stained in EtBr (final concentration of 0.5 µg/ml) for 30 min. and visualized under UV illumination at a wavelength of 302 nm.

PCR amplification of STS markers

The STS markers used in this study were chosen from the GDB database (http://gdb.org/). The detail information about each marker regarding sequences, allele sizes and their Gene Bank Accession number are provided in Tables 1 to 9. The position of markers on the cytogentic map [Fig.1.a, b] and the primer sequence is provided in the Table 10.

The standardization of PCR reaction for specific amplification was carried out by varying concentrations of $MgCl_2$, target DNA and annealing temperature. The PCR conditions used for specific pairs of primer are mentioned in Table 10.

The PCR amplification of marker specific region was carried out in 25 µl of reaction volume by using 25-50 ng genomic DNA as target. The PCR mix consisted of 12.5 picomole of each primer (Forward and Reverse), 200 nM of dNTP with 0.5 unit

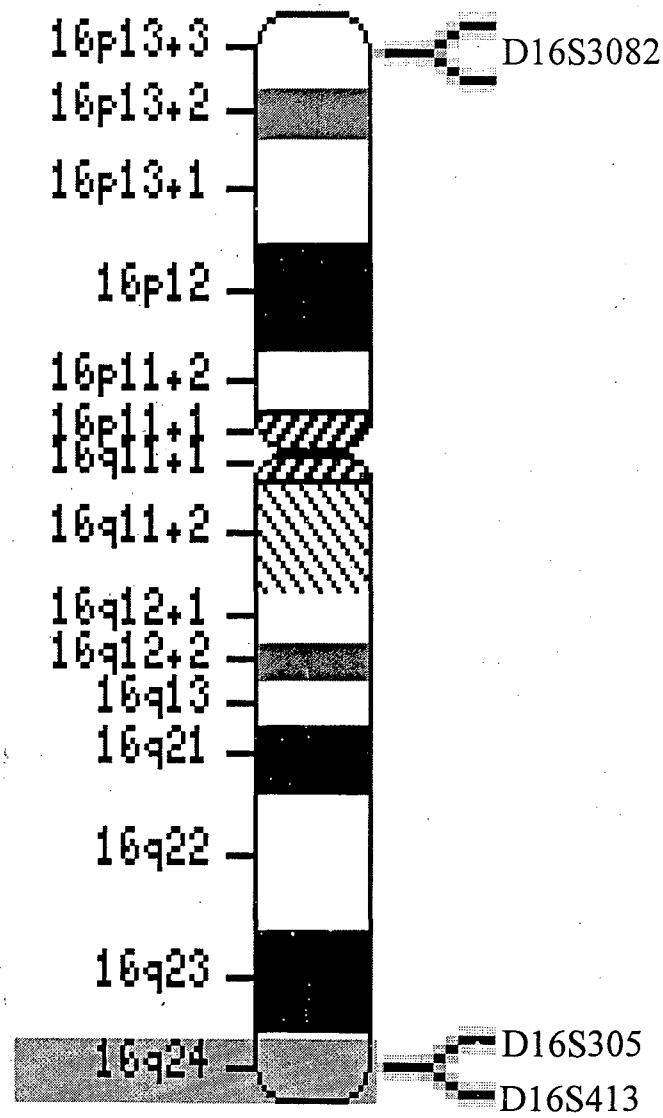

Fig 1a. ~~Depiction of~~ position of micro-satellite markers on chromosome 16

18

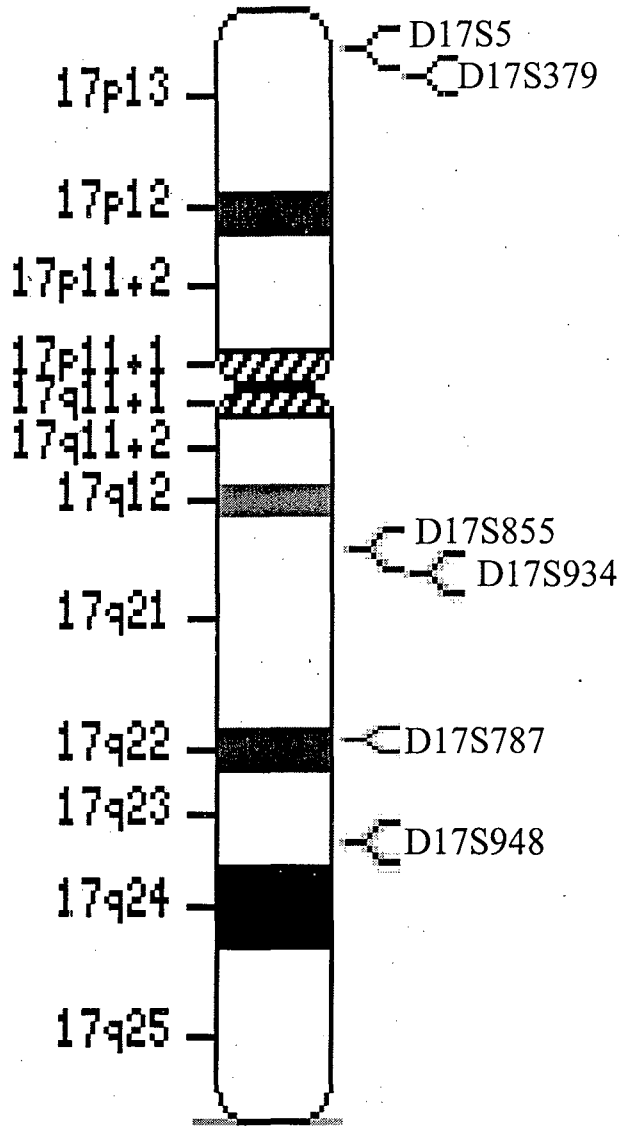

Fig 1b. Depiction of position of micro-satellite markers on chromosome 17

of Taq Pol. enzyme, 1-1.5mM of $MgCl_2$, and 1x PCR buffer. Thermal cycling was performed on PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following conditions: Initial denaturation at 94°C for 3 min., 30 cycles of 94°C for 1min, 52-65°C for 1min., 72 °C for 1 min. and a final extension at 72°C for 5-10 minutes.

Table 1: D16S 3082 marker details and amplified sequence.

D16S 3082

Name of marker	Type of sequence amplified	Accession no.
D16S3082	Dinucleotide Repeats (CA)n	Z53260

Sequence of amplified region

agcttcttca tgtnatcaga gaggacctgc ggaaataacg
gtgacactna gtctccatgt gtnaccttaa cctttcctcc
aggataattt atacacacac acacacacac acacacacac
acacacacac acacacacac agagacacat ggcagaggta
caggataaga ggagacagca gtcccaggag cccctcacag
cctggaaaga ccggccaggg ctgtcctcga aacgnctcag
aaagcgcttc tctcaggagc cggctcagct

Table 2: D16S413 marker details and amplified sequence.

D16S413

Name of marker	Type of sequence amplified	Accession no.
D16S413	Dinucleotide Repeats (CA)n	Z16839

Sequence of amplified region

agctaaggca ggagaatcac ttgaacccag gaggcggagg
ttgcagtgaa ctgagatcat accactgnac tccagcccga
gtaacaaaag catctcaaaa aaaaaaaaaa atacacacac
acacacacac acacacacac acacacacac acacacacac
atatatatat aaagnaaaag gaaatcttcg aacccacctg
tgaccaggaa gcccatcccc gccttggagt tgtcccgcct
ttctcggnag aaccaaaaaa taccttttcat gtactgactg
atgtctgcct gtaacttctg nccttgtagg aggtatgaac
tccagct

Table 3: D16S305 marker details.

D16S305

Name of marker	Type of sequence amplified	Accession no.
D16S305	Dinucleotide Repeats (CA)n	?

Sequences of Primers

F-5′CCTCCCAGGTTCAGGCAATTCTTCT -3′

R-5′TAGGCGACAGAGTGGGACTCCATTA-3′

Table 4: D17S5 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S5 Locus YNZ22	Hypervariable number tandem repeats	M21143 J03056

Sequence of amplified region

```
cgaagagtgaagtgcacaggagggcaaggcggtcctcaccctgcctgggct
Ggggcagggctgtgagaccctcccttacagaagcaatgagggcttgaggag
Ggggttaggggcctgggctggggcagggctgtgagaccctcccttacagaa
GcaaTgagggcttgaggaggggttaggggcctgggctggggcagggctgt
GagaccctCccttacagaagcaatgagggcttgaggaggggttaggggcc
Tgggctggggcagggctgtgagaccctcccttacagaagcaatgagggctt
Gaggaggggttaggggcagtaagttaacttgggaggcggatgtgggggaa
cgctgaagaataaagactgtg
```

D17S5 polymorphisms

Alleles	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Size bp	168	238	308	378	448	518	588	658	728	798	868	938	1.008	1.078	1.148	1.218	1.288
Freq.	.06	.17	.14	.26	.05	.05	.01.	.04	.07	.06	.03	.04	.01	.00	.000	.00	0.00

Table 5: D17S379 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S379	Dinucleotiderepeats (CA)n	X69879

Sequence of amplified region

gaccacatctgtcctcacctgtgggagcgcctggccctcctccctagcc
Cttccagcctgggacacacacacacacacacacacacacacacacaca
Cgcacgcacacgcacacatcttacctctcatgcgtgttttacctttgat
Gttcagagtggctcactggctgggagtccttacctcggggaggaggggga
Ggttggttccttgggggccaaagaaggcagggaatgcctggagggtaac
Tggggccaccatgaacccctttctccagaaaagctgcttctcccccat
Cccgggtcccacccccaaacccccagaggtggcccttgtttacagtgagg
actcg

D17S379 polymorphism

Alleles	1	2	3	4	5	6	7	8	9
Size	362	360	358	356	354	352	350	348	342 bp
Freq.	.07	.01	.13	.16	.04	.24	.28	.07	.01

Table 6: D17S855 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S855	Dinucleotide repeats (CA)n	Z23813

Sequence of amplified region

ggatggcctttagaaagtggtcaccctcccccttanagacagacggacaga
Aacacacacacacacaaacacacacacacacacacacacacactcttactt
Taccaccagagtgaaaagaaatggcagtaggacaagtctgtg size ?

D17S855 polymorphism

Alleles	1	2	3	4	5	6	7
Size	155bp	153	151	149	147	145	143
Freq.	0.05	0.19	0.18	0.15	0.12	0.26	0.05

Table 7: D17S934 Marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S934	Dinucleotide Repeats (CA)n	Z23831

Sequence of amplified region

tctgaatggcccttggtccatgcctctctccctctctcctccctcttcctca
Cttcctgacttcaggacctgttccctcctgctccctacaagcatgcatacac
Acacatacacacacacacacacacacacacacacacacacacacacgcactcat
accccacctcagatagga

D17S934 polymorphism

Alleles	1	2	3	4	5	6	7	8	9	10
Size	186	184	180	174	172	178	176	170	182	190
Freq.	.05	.05	.16	.19	.25	.03	.05	.14	.03	.01

Table 8: D17S787 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S787	Dinucleotide Repeats (CA)n	Z16558

Sequence of amplified region

```
tgggctcaactatatgaacctacttttctctggtttctgcttgctcaagtac
Tggctgcattggtatctcttcaatgccttcaagattaaacacacacacacac
acacacacacacacacaaacacacataccccttcaaaaaggtatcaa
```

D17S787 polymorphism

Alleles	1	2	3	4	5	6	7	8	9
Size	138	140	142	148	152	154	160	164	166

Table 9 : D17S948 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Name of marker	Type of sequence amplified	Accession no.
D17S948	Dinucleotide Repeats (CA)n	Z24102

Sequence of amplified region

gtctctgtccttagggagtttacattttnatgagagaggtctctctctctct
Gtctctctctctctctcacacacacacacaaacacacacacacacacacaca
Cacacacacacacacacacaagaaatgggcaaagatata

D17S948 polymorphism

Alleles	1	2	3	4	5	6	7	8	9	10	11
Size bp	143	125	139	131	149	129	127	141	147	145	133
Freq.	.26	.07	.01	.03	.01	.25	.08	.03	.14	.05	.01

Table 10: Primer sequences and PCR conditions of the STR markers used for allelotyping.

Locus	Location	Primers sequence (bp)	Tm (°C)	Ta (°C)	$MgCl_2$ Conc. (mM)
D16S 3082	16p13.3	F 5'- CCTGCGGAAATAACGGTGA -3' R 5'- GTTTCGAGGACAGCCCTGG -3'	58 62	60-62	1mM
D16S413	16q24.3	F 5'- ACTCCAGCCCGAGTAA -3' R 5'- GGTCACAGGTGGGTTC -3'	50 52	55-60	1.5 mM
D16S305	16q24.3	F-5' CCTCCCAGGTTCAGGCAATTCTTCT -3' R-5' TAGGCGACAGAGTGGGACTCCATTA-3'	76 76	60-64	1.5 mM
D17S5	17p13.3	F 5'-CACAGTCTTTATTCTTCAGCG-3' R 5'-CGAAGAGTGAAGTGCACAGG-3'	62 60	60-58	1mM
D17S379	17p13.3	F 5'-GACCACATCTGTCCTCACCTGT-3' R 5'-CGAGTCCTCACTGTAAACAAGG-3'	68 66	62-60	1mM

D17S855	17q21	F 5'-GGATGGCCTTTTAGAAAGTGG-3' R 5- ACACAGACTTGTCCTACTGCC-3'	62 64	60-58	1mM
D17S934	17q21	F 5'-TCTGAATGGCCCTTGG-3' R 5'-TCCTATCTGAGGTGGGGT-3'	50 56	58-55	1mM
D17S787	17q22	F 5'-TGGGCTCAACTATATGAACC-3' R 5'TTGATACCTTTTTGAAGGGG-3'	58 56	58-56	1mM
D17S948	17q23	F 5'-GTCTCTGTCCTTAGGGAGTTTA-3' R 5'-TATCTTTGCCCATTTCTTG-3'	60 52	55-53	1mM

bp= base pair length

Tm= 2 (A+T) + 4 (G+C)

Ta= Primer annealing Temperature

SSLP

Simple sequence length polymorphism, SSLP, by non-denaturing polyacrylamide gel electrophoresis: the PCR amplified products of DNA were mixed with loading dye and electrophoresis was carried out on a 12% polyacrylmide gel (19:1 acrylamide; bis-acrylamide) in 1xTBE buffer, at 120 volts over night at room temperature to investigate different length fragments. Following electrophoresis, gels were stained with ethidium bromide, visualized under UV light under transilluminator and finally silver stained.

Silver staining of polyacrylamide gels

Following the electrophoretic separation of the DNA according to the fragment size, the gels were kept in a fixative (10% ethanol + .5 ml acetic acid) for about 30 min. Then the gel was treated with 0.1% silver nitrate solution (freshly prepared-see Appendix) for fifteen minutes followed by rinsing with autoclaved water thrice. The (1.5% NaOH+ .4 ml formaldehyde) developer was added to the gel exposed till the bands appeared. Immediately after the appearance of the clear bands, the developer was neutralized by adding 0.75 % sodium carbonate. The silver stained gels were photographed and anlaysed.

Results

Results

Estrogen receptor positive breast carcinoma cell line, MCF-7 (Fig.2) was assessed for the microsatellite markers, located on chromosome 16 and 17 p and q arms.

On chromosome 16, three microsatellite markers namely, D16S3082 located on short arm at p13.3 and D16S413, D16S305 located on long arm at q24.3 were used for allelotyping studies. On chromosome 17, six microsatellite markers namely D17S5 and D17S379 located on short arm at 17p13.3 and D17S787, D17S855, D17S934 and D17S948 located on long arm at 17q21.23 were used for allelotyping studies.

A total of nine markers were used for the assessment of the genotypic status and the allelic variation on chromosome 16 and 17 in MCF-7 cells. Among these, seven markers showed heterozygosity whereas, two makers were homozygous (Fig.3a,b). On chromosome 16, D16S305 and D16S3082 markers were found to be in a heterozygous state whereas, D16S413 marker showed a homozygous profile.

On chromosome 17, D17S379, D17S787, D17S855, D17S934 and D17S948 markers were found to be heterozygous whereas, D17S5 marker was homozygous. Results of all the markers and their genotypic status are summarized in Table-11.

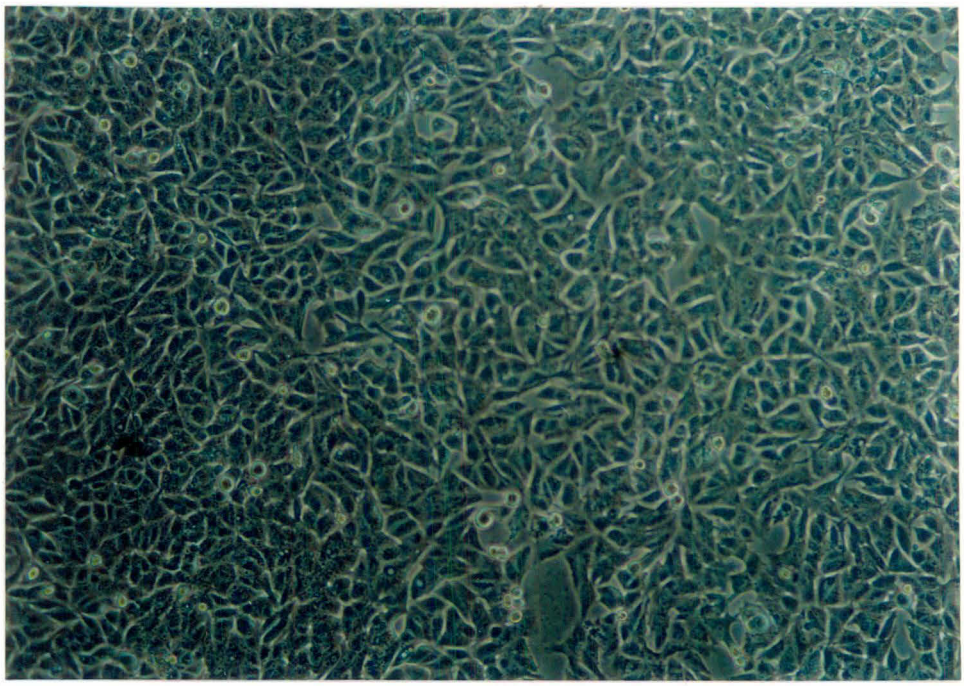

Fig 2. MCF-7 cells in culture showing epitheloid cell pattern.

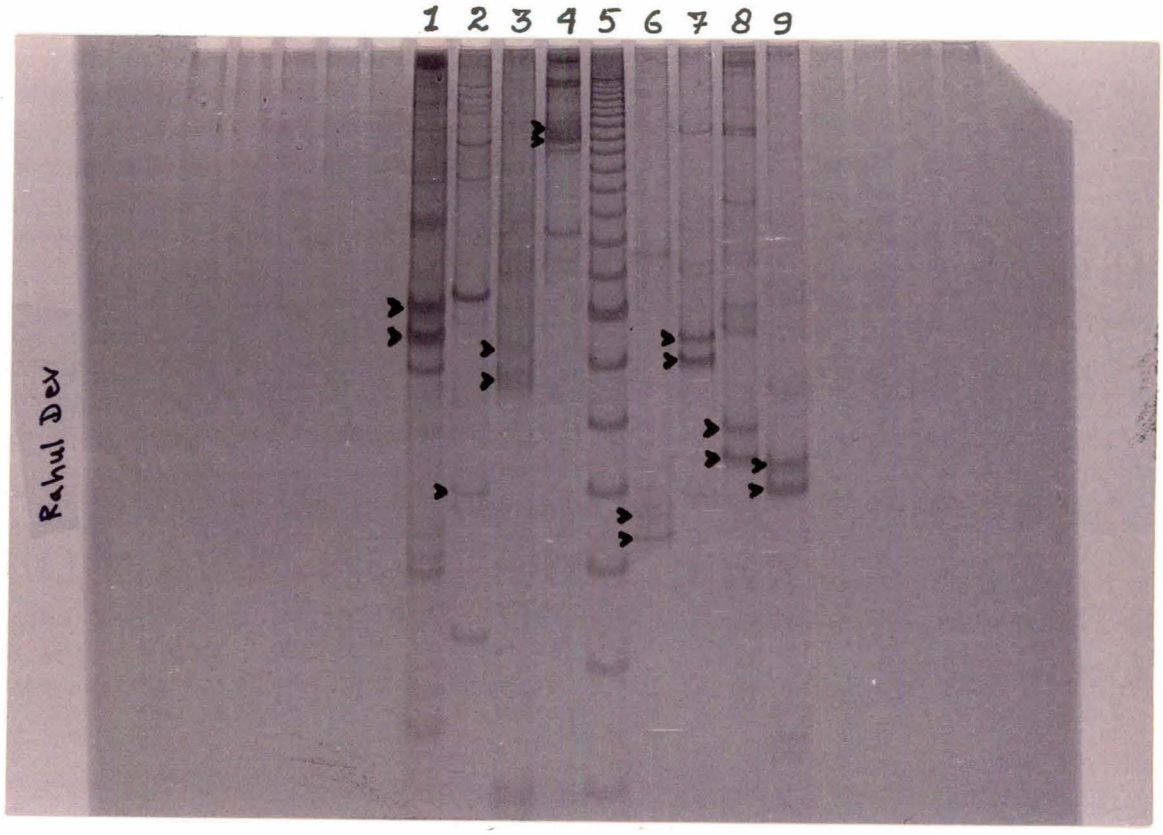

Fig 3a. SSLP profile of MCF-7 cells using different Microsatellite markers.
D16S3082 (LANE 1), D16S413 (LANE 2), D16S305 (LANE 3), D17S379 (LANE 4), 20 bp DNA ladder (LANE 5), D17S948 (LANE 6), D17S934 (LANE 7), D17S855 (LANE 8), D17S787 (LANE 9).

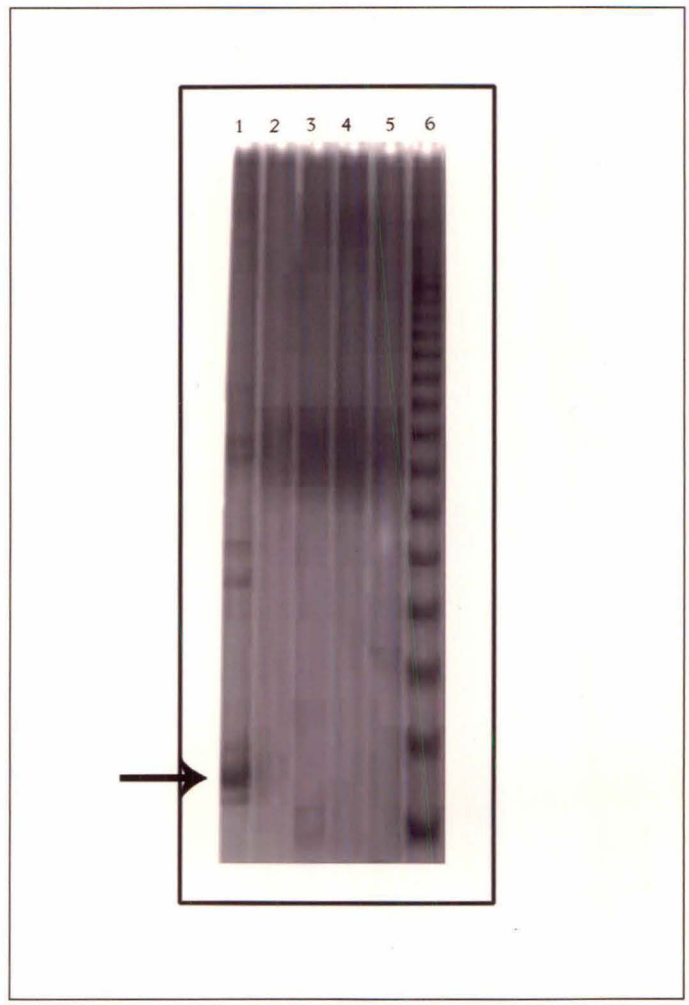

Fig 3b. SSLP profile of MCF-7 cells using different Microsatellite markers.
D17S5 (LANE 1), D17S948 (LANE 2), D17S 934 (LANE 3), D17S787 (LANE 4), D17S855 (LANE 5), 20bp DNA ladder, LANE 6).
Note: SSLP profile of all markers except D17S5 has been shown in fig 3a.

Table – 11: Allelotyping of MCF-7 cell line for microsatellite markers

Chromosome 16

Markers	Location	Number of alleles	Genotypic status
D16S3082	16p13.3	2	heterozygous
D16S305	16q24.3	2	heterozygous
D16S413	16q24.3	1	homozygous

Chromosome 17

Markers	Location	Number of alleles	Genotypic status
D17S5	17p13.3	1	homozygous
D17S379	17p13.3	2	heterozygous
D17S787	17q21.23	2	heterozygous
D17S855	17q21.23	2	heterozygous
D17S934	17q21.23	2	heterozygous
D17S948	17q21.23	2	heterozygous

Discussion

Discussion

MCF-7 cell line represents metastatic breast epithelial cell carcinoma. This cell line has provided the advantage of studying different aspects of cancer biology in the past. MCF-7 has been characterized for various fundamental cellular processes such as cell survival and proliferation (Sugarman et al., 1985), differentiation (Bacus et al., 1990; Pratt et al., 1993), cell cycle regulation and apoptosis (Komarova et al., 1997). This cell line can be a basic model for an understanding of various fundamental aspects of cancer genetics to know the functions of various tumor suppressor genes, oncogenes, and the consequences of their nonfunctional status due to various alterations at genetic level. Therefore a study of mirosatellite status of the cell line for the marker alleles in chromosome 16 and 17 at different potential sites for tumor suppressor genes, could be a useful proposition for future studies to understand the transformation process.

In this study emphasis was given to identify status of various STS marker loci on chromosome 16 and 17 for future studies on microsatellite instability (MSI) and loss of heterozygosity (LOH) in carcinogen exposed MCF-7 cell line. Seven out of the nine markers studied presented with a heterozygous genotype profile. Since the syntenic markers did not show a homozygous profile, it is apparent that neither marker loss nor mitotic recombination for the region proximal to these markers seems to have taken place in this cell line. Nevertheless, the basic information generated of the status of 9 polymorphic STS markers will provide a base for carrying out future experiments on another line of thought. Carcinogen exposed MCF-7 cells simultaneously assessed for their malignant status using single cell colony assays and nude mouse tumor formation assay, could now be evaluated for LOH status at the heterozygous marker loci regions on chromosome 16 and 17 to find if any of the heterozygous markers reported in this study show a loss preferentially and could harbor a new tumor suppressor gene in the region nearby.

In several studies it has been reported that genetic alteration on chromosome 16 and 17 are involved in breast malignancy. In particular, loss of 16q and 17q are the most frequently changed chromosomes in human breast cancer. Loss of 16q has been suggested to be an early event in breast tumorigenesis because 16q copy number alterations were found in atypical ductal hyperplasia (ADH) (Bieche et al., 1995; Vos et al., 1999; Gong et al., 2001). It was found that in ADH the incidence of LOH for 16q (D16S413) is similar to that in ductal carcinoma in-situ of high and low nuclear grade and invasive ductal carcinoma (Anderson et al., 1992; Stratton et al., 1995). Recent study suggests that, chromosome 16 long arm depicts the most commonly observed LOH event, in 712 breast tumors, with the conclusion that there were three regions likely to harbor tumor suppressor gene, one at 16q22.1 and two at 16q24.3 (Cleton–Jansen et al., 2001). Similarly, studies of Taiping et al (1996), identified three distinct regions with high percentage (~70% above) of allelic losses among informative DCIS samples. Two of them agree with previously described areas 16q21 at locus D16S400, 16q24.2 at locus D16S402. However, the region between markers D16S515 and D16S504 were observed with high incidence of LOH. Within this region D16S518 locus was the most frequently affected (77%).These observations strongly suggest that a putative tumor suppressor gene or genes may exist at or near this locus. On the basis of partial YAC contig, estimates of 2-3Mb of the minimum region with highest frequency of LOH were made. In another study, a total of 25samples with DCIS associated with IDC, greater than 10% LOH was found on chromosome region 16q22.1. Marker locus D16S241 did not show LOH, whereas D16S3028 showed 5.8% and D16S318 showed 40% LOH, however no LOH was observed at 16q 24.3 for locus D16S3023) in pure DCIS (Farabegoli et al., 2002).

In a study of Mitsuru Emi et al (1999), with the examination of 200 primary breast cancers for LOH, using more than 150 polymorphic microsatellite markers derived from the total human genome, resulted in the identification of several regions which corresponded to the location of known TSGs. In another study, among 18 markers examined, D16S413 at16q24.3, showed highest frequency of LOH (113 of the 197 tumors, 57%) (Mitsuru Emi et al., 1999). The E-cadherin gene has been identified

as the likely LOH target at 16q22.1. It was found inactivated in invasive lobular carcinoma of the breast. Inactivation of E-cadherin is predominant event in LCIS and ILC (Kanai et al., 1994; Berx et al., 1995; Zhu X et al., 1999). Whereas, in DCIS cases E-cadherin expression is always detectable (Vos et al., 1999). However, in ductal breast carcinoma, mutations in E-cadherin have been described only in cell lines (Van de Watering et al., 2001). Role of this gene as a tumor suppressor in ductal carcinoma is unclear. There could be as yet another unknown tumor suppressor gene responsible for the development of DCIS.

Breast cancer cell lines are particularly useful for assessing candidate transcripts on the long arm of chromosome 16 as tumor suppressors. It is possible to indirectly assess the LOH by typing cell line with polymorphic microsatellite markers. LOH can be inferred when a series of linked polymorphic markers are all homozygous. Depending on the heterozygosity values of microsatellite markers, a statistical probability less than or equal to 0.001 was used to define LOH of adjacent markers. In practical terms this was usually equivalent to the finding that if five adjacent markers were homozygous, then LOH for this region was considered to exist.

Although in this study, it was interesting to find the marker at 16q24.3 to be in a homozygous condition conforming to the expected observations after a possible recombination proximal to this site, as reflected by the heterozygous status of D16S305 marker, however, it remains to be confirmed by observing the homozygous status of more number of markers distal to D16S413. There could be a possible LOH at D16S413 locus leading to a homozygous profile as observed in this study.

A report from Callen et al (2002), showed combined microstellite typing together with dual colour fluorescence in-situ hybridization (FISH) using chromosome 16q painting and region specific probes to determine chromosome 16 LOH in 21 breast cancer cell lines and 2 non-tumorigenic breast epithelial cell lines. Tumorigenic breast cancer cell lines, BT-20, BT-474, BT-483, DU-4475, MDA-MB-175, -361, -415, MCF-7, UACC-893 and non tumorigenic breast epithelial cell lines, HBL-100, MCF-12A showed number of loss of heterozygosity of chromosome 16q. All these cell lines showed heterozygosity for markers dispersed along the long arm of

chromosome16. The allelic status of marker D16S3082 located at 16p13.3 has not been reported so far, in tumor samples as well as in cell lines. In MCF-7 cell line a heterozygous status for this marker locus (D16S3082) was found. The genotypic status of marker D16S305 located at16q24.3 was heterozygous that is consistent with studies of Callen et al (2002). But interestingly, the allelic status of marker D16S413 located at 16q24.3 by us was found to be homozygous which is contrary to the results of Callen et al (2002).

In various studies, LOH at 17p region has been observed in breast cancer, lung cancer, colon cancers, medulloblastoma, follicular thyroid carcinoma, leukemia and lymphomas (Baker et al., 1989; Mc Donald et al., 1994; Phillips et al., 1996; Schultz et al., 1996; Grebe et al., 1997; Konishi et al., 1998; Sanker et al., 1998; Zhao et al., 2003).These studies provide the extensive analysis of 17p region in various malignancies for a putative tumor suppressor gene that might be implicated not only in breast cancer but also in other tumor malignancies To estimate the frequency of somatic loss of wild type TP53 alleles, most of these studies have initially used the highly informative D17S5 marker that is thought to map close to TP53 on the short arm of chromosome 17. It later became clear that the D17S5 locus is actually located on band 17p13.3 approximately 20CM distal to the TP53 gene (Coles et al., 1990). At this locus a high frequency of allelic losses (approximately 60%) has been detected in sporadic and in familial breast carcinoma (Sato et al., 1990). A homozygous profile observed in our study of D17S5 marker, although interesting, needs further confirmation with respect to LOH at this locus or a possible mitotic recombination proximal to this marker locus.

Another marker D17S379 is linked to D17S5 but is located 200 Kbp proximal. D17S5 and D17S34/ABR cover 3Mb region. A study carried on primary breast carcinomas of 152 patients, showed 27.8% mutation in TP53 gene. The allelic loss in the 17p13.3–17pter region for four polymorphic loci namely, D17S5, D17S379, D17S34/ ABR, was examined. All of the tumors that were found to be deleted at the D17S5 locus showed loss of the D17S379 marker (Liscia et al., 1999).The smallest common region of allelic loss in 17p defined by markers D17S5, D17S379, D17S34

/ABR harbors a number of recently cloned putative tumor suppressor genes. L132/Rox, a helix-loop-helix structure that is a characteristic of several transcription factors was identified by marker D17S379. OVCA1 and OVCA2 are associated with closely linked marker D17S5 that spans minimal region of allele loss in ovarian cancer (Schultz et al 1996). The HIC-1 gene that is proximal to the D17S5 marker has been found frequently hypermethylated in breast cancer. Loss of function, through somatic mutations of genes, mapping in the distal region of the 17p chromosome appears to be a crucial event in the development of and progression of sporadic breast cancer.

D17S800 and D17S855 are located on chromosome 17q 21.12. A D17S855 map intragenic to the BRCA1 gene, whereas D17S800 is more centromeric (Bieche et al., 1995). Loss of BRCA1 markers has been correlated with high grade, and ER loss in DCIS and invasive breast carcinoma (Niederacher et al., 1997; Phelan et al., 1998). To investigate LOH of BRCA1 in sporadic breast cancer, locus 17q21 (D17S855, THRA1, D17S579) is very informative. D17S855, D17S579 loci were detected in the tumor-2 line.

Analysis of flanking markers, estimates the regions of Allelic imbalance to be 7CM at the D17S787 loci and 8CM at the D17S948 loci (Plummer et al., 1997). The region of Allelic imbalance at D17S787 may represent the areas previously reported near NM23 (Anderson et al 1992; Nagai et al., 1994; Niederacher et al., 1997).

Another common region localized to the region defined by marker D17S948 on 17q23-q24. Analysis of tumors showed defined breaks in this region indicates that D17S948 may closely define the boundaries of this region of imbalance. The fourth common region of imbalance was located in the region composed by D17S 937/D17S939/D17S802/D17S836 on 17q25. Another region of AI might be defined by markers D17S968 and D17S785 (Plummer et al., 1997).

D17S934 is one of the polymorphic loci located at17q21.23 region .It is commonly associated with deleted region at BRCA1 region in breast cancer. In esophageal cancer 45% allelic loss was found at D17S934 locus. In another study, LOH for the marker locus D17S934 at 17q21.1was found to be 14% (69/170) in breast tumors. Allelic loss at this locus along with other loci on chromosome 17, p and q arm

can serve as negative prognostic indicators to guide postoperative management of patients (Mitsuru Emi et al., 1999). In this study, however, in MCF -7 cell line all the short arm and long arm markers studied, except for D17S5, showed a heterozygous profile, indicating no LOH in this region in MCF-7 cells.

Conclusion

Conclusion

Breast cancer is one of the most common neoplasms to affect women. Inherited mutations account for upto 10% of breast cancers, of which BRCA1 and BRCA2 may be mutated in the germ-line of half of such cases. Incidentally 90% of the breast cancer cases are sporadic and have not shown direct involvement of these two genes. In order to understand the biology of cancer in breast both in familial and sporadic cases, the work on MCF-7 cell line, a representative of metastatic breast epithelial carcinoma, has provided the advantage of studying different aspects of cancer biology on these cells in the past. However these cells have not been assessed for the allelic instability at different potential sites for tumor suppressor genes in chromosome 16 and 17.

The objective of this study was to assess the status of STS markers located on Chromosomes-16p&q and 17p&q in cultured MCF-7 cells. Chromosome-16 markers: D16S3082 located on short arm at p13.3 and D16S413, D16S305 located on long arm at q24.3 were used for the study. On chromosome 17, six microsatellite markers namely, D17S5 and D17S379 located on short arm at 17p13.3 and D17S787, D17S855, D17S934 and D17S948 located on long arm at 17q21-23 were used for allelotyping studies. The rationale for studying the status of the polymorphic markers was also to characterize the MCF-7 cells for the genotypic status of some of the polymorphic markers in chromosome 16 and 17 for monitoring future LOH studies while transforming the cells further *in-vitro* neoplastically and look for potential 'TSG' locations.

Seven out of the nine markers studied presented with a heterozygous genotype profile. However, a homozygous profile observed in our study of D17S5 marker, although interesting, needs further confirmation with respect to LOH at this locus or a possible mitotic recombination proximal to this marker locus. There could be a possible LOH at D16S413 locus leading to a homozygous profile as observed in this study. A homozygous profile observed in our study of D17S5 marker, also is

interesting and needs further confirmation with respect to LOH at this locus or a possible mitotic recombination proximal to this marker locus.

Since the syntenic markers did not show a homozygous profile, especially in 17q region, it is apparent that neither marker loss nor mitotic recombination for the region proximal to these markers seems to have taken place in this cell line. Nevertheless, the basic information generated of the status of 9 polymorphic STS markers will provide a base for carrying out future experiments on another line of thought. Carcinogen exposed MCF-7 cells simultaneously assessed for their malignant status using single cell colony assays and nude mouse tumor formation assay, could now be evaluated for LOH status at the heterozygous marker loci regions on chromosome 16 and 17 to find if any of the heterozygous markers reported in this study show a loss preferentially and could harbor a new tumor suppressor gene in the region nearby.

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Appendix

Appendix

DNA isolation from Cell line

Lysis buffer (50 ml)

100mM Tris-HCl (pH 8.5)	5 ml
0.5 M EDTA	0.5 ml
5M NaCl	2 ml
10 % Sodium dodecyl sulphate	1ml
20mg/ml Proteinase K	0.25 ml
Autoclaved water	41.25 ml

Agarose gel Electrophoresis

10X Tris borate buffer (TBE)

Tris base	80 mM
Boric acid	40 mM
EDTA pH 8.3	2 mM

Agarose	0.8-2% in 1X TBE (W/V)
EtBr (Stock)	10 mg/ml (W/V)

LP Loading dye

Bromophenol blue	0.5mg
Xylene	0.5mg
Formamide	980ul

Tris EDTA buffer

Tris	25 mM
EDTA	10 mM pH 8.0

PBS (100ml) pH 7.2

Sodium chloride	0.8 gm
Potassium chloride	0.02 gm
di-Sodium hydrogen phosphate	0.144 gm
Potassium di-hydrogen phosphate	0.024 gm

SSLP analysis

Gel mix (20%)

Acrylamide	9.75 gm
Bis-acrylamide	0.25 gm
10x TBE	5ml
5ml	150u
TEMED	50 ul
10% APS	150 ul

Silver staining

Fixative (100ml)

Ethanol	10 ml
Acetic acid	0.5 ml
Water	85 ml

Silver nitrate Solution

Silver nitrate	0.1% (w/v)
Water	100 ml

Developer

Sodium Carbonate	3% (w/v)
Formaldehyde	0.05% (v/v)
Water	100 ml

Trypsin solution(100ml)

0.25 % Trypsin	250mg
0.02 % EDTA	20mg
0.1% Glucose	100mg
Saline	100ml