CHAPTER 1

Defining Minimal Lost Region (MLR) On 17p13 And 17q21-23 By Low Resolution LOH Mapping In Sporadic Breast Tumors And Their Correlation With Clinical Parameters.
1.1 Introduction

i. Breast cancer incidence

Global scenario: Breast cancer, the most common malignancy in women world wide (Ferlay et al., 2001), has become the second largest cause of death due to cancer in women between the ages of 35 and 54, after lung cancer. It has been reported in the year 2000; that breast cancer resulted in an estimated 189,000 deaths in developed countries and 184,000 deaths in developing countries, accounting for 16 and 12 percent, respectively, of all cancer deaths in women (Ferlay et al., 2001). Latest estimates suggest that more than 1,050,000 new breast cancer cases occur worldwide annually, with nearly 580,000 cases occurring in developed countries and the remainder in developing countries thus, making it first among all cancers affecting women throughout the world (World Cancer Report 2003) [Fig 1.1]. The age-standardized incidence of breast cancer generally is found to be lower in developing countries than in developed countries (23.1 versus 63.2 per 100,000 women) although incidence rates vary widely between and within countries [Fig.1.2] (Ferlay et al., 2001).

Indian scenario: According to National Cancer Registry programme (1994), the crude incidence rates of cancer in India varied between 57.5 and 78.6 per 100,000 men; and between 57.7 and 89.7 per 100,000 women in urban areas. The age standardized incidence rates ranged from 98.7 to 138.3 per 100,000 men; and from 108.0 to 143.4 per 100,000 women in urban areas. (ICMR, India-report of nineties, published in the year 2001). According to NCRP (National cancer registry programme based on urban population, 2001), the estimated number of new cancers diagnosed in India every year was between 700-900,000 (Murthy et al., 1990; Yeole et al., 1992). Incidence of breast cancer in Indian women is not as high as in western countries (23.5 vs 90.7) but it is the most common female cancer reported (Saxena et al., 2002). According to Delhi region cancer registry from 1990-1996, breast cancer was number one cancer affecting women [Fig 1.3] with the incidence of 5511 per 100,000 (21.3%) (http://icmr.nic.in/ncrp/). The age-adjusted rate (AAR) was 28.1 for Delhi region [Table 1.1].

Although the causes and natural history of breast cancer remains unclear, epidemiological research has uncovered genetic, biological, environmental, and life style risk factors for the disease. Moreover, the situation is complexed in the context of Indian population,
Fig.1.1 Most prevalent cancers worldwide, expressed as thousands of person diagnosed with cancer in a study of five years (1995-2000). Breast cancer is seen as the most prevalent cancer in females world wide (3,860,000). (Source: World Cancer Report, 2003)

Fig.1.2 The world wide incidence of age-standardized breast cancer. Developing countries show less incidence in comparison to developed countries. (Source: Ferlay et al., 2001)
which shows great diversity in terms of ethnicity, religion, and cast groups. The
differences in incidence of breast cancer in India could precisely be attributed to some
extent to social and cultural factors and differential insults due to environmental exposures.
However, there is little information available on the possible role of genetic factors to
cause breast cancer, which are sporadic in nature. Regionally, incidence rates have been
reported to range from 18.8% in Trivandrum to 26.2% in Bombay (Mumbai), while
amongst religious groups; it is highest for Parsi women, attaining rates that are 2.1, 1.7 and
1.4 times higher than for Hindu, Muslim and Christian women, respectively (Jussawala et
al., 1977). The genetic background of cancer predisposition, tumor initiation, and the
malignant transformation ultimately leading to metastasis are all widely addressed topics,
and understanding of the genetic defects underlying these fundamental processes is
expected to unravel new possibilities for cancer treatment and prevention.

According to Comings et al. (2003 and references their in), 5-10% of the cases of breast
cancer in women can be attributed to inheritance, whereas 90-95% are sporadic, that
appear randomly with no clear, known predetermined genetic susceptibility.

ii. Breast cancer biology

(Source: Breast Imaging 2nd ed. 1998, by Daneal B. Konans)

Anatomy of breast: The breast is a modified gland of skin. It is derived primarily from the
epidermal layer. The female breast is composed of 15-20 sections, called lobes. Each lobe
ends in many smaller lobules. These lobules further end in dozens of tiny bulbs, which
produce milk during lactation. The three components, lobes, lobules, and bulbs, are all
linked together by thin tubes called ducts. All the small ducts eventually come together to
form larger ducts, which empty to the outside through the nipple [Fig.1.4a].

Development of breast: Breast is one of the few organs of the body that is not completely
developed at birth. It reaches full differentiation only under the stimulus of the hormones
of pregnancy and lactation. During the first trimester of intra-uterine growth the primitive
epidermal bud in the embryo begins to produce cords of epithelium that penetrate down in
to the dermis [Fig.1.4b]. The full term fetus shows the network of branching ducts. The
lobules appear only after adolescence. After puberty, the breast is composed of lobular
structures that reflect different stages of development. The development of the breast is
influenced by a myriad of hormones and growth factors. A major role is played by ovarian
Fig. 1.4a Anatomy of breast and the nature and sites involved in cancer.

Fig. 1.4b Developmental stages of breast. (Source: Wiseman et al., 2002)
steroid hormones, mainly estrogen, which is considered to promote the proliferation of both the normal and the neoplastic breast epithelium.

Types of breast cancer: The majority of breast cancers can be classified into one of the following categories, histopathologically [Fig.1.4a]: infiltrating ductal carcinoma, infiltrating lobular carcinoma, ductal carcinoma in situ, lobular carcinoma in situ, inflammatory carcinoma, Paget's disease, and cystosarcoma phyllodes. There are other tumors of the breast, such as angiosarcoma, squamous cell cancer and lymphoma which are quite rare.

a. Infiltrating ductal carcinoma: Infiltrating ductal carcinoma involves the cells which form the ducts of the breast. It is the most common form of breast cancer, comprising about 65-85% of all cases. On a mammogram, invasive ductal carcinoma is usually found as an irregular mass, or as a group of small white irregular dots called microcalcifications, or a combination of both. It may also appear as a lump in the breast. On physical examination, this lump usually feels much harder or firmer than other benign lumps in the breast.

b. Infiltrating lobular carcinoma: Infiltrating lobular carcinoma comprises of 5 to 10 percent of breast cancers. This type of breast cancer can appear similar to infiltrating ductal carcinoma on mammography, but on examination of the breast there is usually not a hard mass, but rather a vague thickening of the breast tissue. Lobular carcinoma can occur in more than one site in the breast (multicentric) or in both breasts simultaneously (bilateral).

c. Ductal carcinoma in situ (DCIS): Ductal carcinoma in situ (DCIS) is a pre-invasive form of breast cancer. It is commonly seen in association with an invasive breast cancer. There is usually no lump associated with it when it occurs without an invasive cancer. On mammography, there may be fine microcalcifications, which can signal its presence. DCIS is frequently multifocal, i.e. it is located in more than one area of the breast. Approximately one-third of DCIS cases are multifocal.

d. Lobular carcinoma in situ (LCIS): Lobular carcinoma in situ (LCIS) is usually encountered as an incidental finding in a breast biopsy. It has no symptoms, and has no
characteristic pattern on mammography. It has been found to occur in multiple sites in the same breast in 40 to 90% of cases. The risk of developing an invasive cancer of the breast with LCIS is approximately 1% per year. The invasive cancer that develops has about an equal chance of being in either breast regardless as to which breast the LCIS was initially found. A large percentage (38%) of women with LCIS may not develop an invasive cancer until more than 20 years after the initial diagnosis.

e. Inflammatory carcinoma: Inflammatory carcinoma of the breast is a sub-type of infiltrating ductal carcinoma, but is named for its typical clinical presentation. The breast becomes red, swollen, and warm, and the skin becomes quite thickened, as if it were infected. This appearance is due to the rapid growth of the cancer, which blocks the lymphatics in the breast, causing it to swell and appear infected. In 90% of the cases at the time of diagnosis, the cancer has already spread to the lymph nodes.

f. Paget's disease: Paget's disease of the breast accounts for about one to four percent of all breast cancers. It occurs typically as a crusting and scaling of the nipple. It can be mistaken for a benign skin condition unless there is a high index of suspicion.

g. Cystosarcoma phyllodes: Cystosarcoma phyllodes are a firm tumor that resembles a benign fibroadenoma. This cancer is very different when compared with other cancers of the breast. It seldom spreads to the lymph nodes, but can metastasize to other parts of the body through bloodstream.

iii. Chromosome 17 and Breast cancer Genetics: Questions unanswered

Cytogenetic studies have suggested the involvement of chromosome 17 in breast cancer, although not all studies agree on the significance. Studies on chromosomal aberration in cancer show balanced and unbalanced structural changes in cancerous cells (Mitelman et al., 2003; http://cgap.nci.nih.gov/Chromosomes/Mitelman). In a study, chromosome 17 monosomy was found as one of the most common numerical changes in near diploid breast tumors, suggesting that it represented an early event, while another study found chromosome 17 monosomy only in complex karyotypes of breast tumors, suggesting the loss to represent only secondary genome instability (Thompson et al., 1993, Pandis et al., 1995). A number of studies have shown that the introduction of chromosome 17 by micro cell-mediated chromosome transfer (MMCT) could produce growth arre...
this chromosome (Murakami et al., 1995; Plummer et al., 1997). There has been a functional evidence of another metastasis-suppressor-gene on chromosome 17q. This has been concluded from experiments using microcell mediated chromosome transfer (MMCT) of a part of chromosome 17p ter-q23 into rat mammary and prostate carcinoma cells which resulted in suppression of metastasis, independent of NM23 expression (Rinker-Schaeffer et al., 1994).

Several studies on chromosome 17 have shown that at least two regions of loss of heterozygosity (LOH) on the short arm (p), one at the p53 locus and the other distal to it; and at least three regions of LOH on the long arm (q), both proximal and distal to the BRCA1 locus, are involved in the development of breast cancer (Kirchweger et al., 1994; Nagai et al., 1995; Niederacher et al., 1997; Coles et al., 1990; Cornelis et al., 1994; Plummer et al., 1997). The region distal to the BRCA1 locus has been poorly investigated even though several studies suggest at least two regions of LOH in the telomeric part of 17q (Kirchweger et al., 1994; Nagai et al., 1995; Niederacher et al., 1997). Only one study (Plummer et al., 1997) has used dense markers in this region and reported three common regions of LOH distal to BRCA1, one at 17q22, the second at 17q23-24 and the third at 17q25.

Chromosome 17 is gene rich and hence at times it has been at the very center of genetic and biological interest. The G+C content is 45% and varies between 35% and 55% as assessed in 100 kb windows, which is higher than the figures for most other chromosomes (Pavlicek et al., 2002). Many genes are also associated with CpG islands, that is, regions rich in unmethylated CpG dinucleotides at the 5' ends. The number of such CpG islands is higher on chromosome 17 than on most other chromosomes. Chromosome 17 has over 74000 single nucleotide polymorphism (SNPs), which may be a reflection of the C+G content being above average (Wadelius et al., 2003). Several genes on chromosome 17 are associated with cancer such as TP53, BRCA2, HIC1, RARA etc.

In breast cancer research, a significant leap forward was taken with the identification of the two major breast cancer susceptibility genes, BRCA1 (17q21) and BRCA2 (13q) (Miki et al., 1994; Wooster et al., 1995). These genes have been known now to have important functions in DNA double-strand break (DSB) repair (De la Torre et al., 2003; Liu & West 2002 and refs. therein). Genetic linkage and mutation analysis in the past showed that the lifetime risk of breast cancer increased notably in people carrying
Fig. 1.1 Most prevalent cancers worldwide, expressed as thousands of person diagnosed with cancer in a study of five years (1995-2000). Breast cancer is seen as the most prevalent cancer in females worldwide (3,860,000). (Source: World Cancer Report, 2003)

Fig. 1.2 The worldwide incidence of age-standardized breast cancer. Developing countries show less incidence in comparison to developed countries. (Source: Ferlay et al., 2001)
iv. LOH mapping to study Minimal lost region (MLR)

Various approaches can be used to detect genes that may be tumorigenic when altered. Cytogenetic analyses have been used to locate distinct chromosomal regions that might harbor such genes contributing to tumorigenesis. Fine-scale molecular mapping of altered regions is needed to locate such genes precisely and this can be done by studying allelic gain or loss of heterozygosity (LOH) defined as a loss of one allele at a constitutional (germ line) heterozygous locus [Fig.1.5].

According to Knudson's 'two-hit' hypothesis, the inactivation of a TSG requires two independent events (Knudson 1971, 75). This hypothesis was experimentally confirmed in subsequent studies by the demonstration of loss of heterozygosity at the RB1 locus in retinoblastoma patients carrying a germ line mutation of the RB1 gene (Cavenee et al., 1983; Godbout et al., 1983). Loss of heterozygosity (LOH) within a certain narrow chromosomal region is usually recognized as a hallmark of a putative TSG (Cavenee et al., 1983). If one functional allele is inactivated as a result of chromosomal deletion, for example, the other copy can be subsequently silenced by either a genetic or an epigenetic alteration. However, if one of the alleles harbors an inherited mutation, even a single event can be sufficient to unmask the two inactivating alteration and thus result in biallelic silencing of the tumor suppressor gene (Kinzler & Vogelstein 1997).

Loss of heterozygosity analysis of solid tumors have not only enabled the delineation of specific minimally lost regions (MLR) as the likely locations of critical TSGs but also provided the molecular portrait of the pattern of accumulation of genetic alterations in a multistep progression of cancer (Vogelstein et al., 1988; 1989; Fearon et al., 1990; Lasko et al., 1991; Yokota et al., 1993; Thiagalingam et al., 2001). Confirmed TSGs could have been isolated by either linkage studies or LOH analysis of sporadic tumors because the MLRs almost always point to the map position of critical TSGs involved in different types of cancers (Wooster et al., 1995; Karp et al., 1995; Williams et al., 1996; Casey et al., 1996; Harris et al., 1996; Hahn et al., 1996; Thiagalingam et al., 1996; Schutte et al., 1996; Uchida et al., 1996; Li et al., 1997; Virmani et al., 1998; Elo et al., 2001). A TSG involved in a sporadic cancer could be a familial gene for a different cancer or a familial TSG could be non-familial for another type of cancer.
Fig. 1.5 Schematic representation of loss of heterozygosity (LOH) events in tumors in comparison to constitutional status at same locus in matching blood DNA.
**LOH Mechanisms**: Different mechanisms have been proposed which could contribute to [Fig. 1.6] the loss of heterozygosity (LOH) phenotype. (a) Localized loss of one allele or gene in a highly specific manner, accomplished by a simple deletion resulting from two double strand breaks or double mitotic recombination involving the homologous chromosomal arms. When this occurs, the unaffected genetic materials remain contiguous. If the loss involves a specific gene, it can be regarded as a gene conversion. Allele specific or gene specific probes and flanking probes would be necessary to detect such alterations. Although these micro-deletions are difficult and laborious to find because of the enormous amount of effort required to analyze numerous tumor samples. Two reports, one analyzing the NF1 locus on chromosome 17q in neuro-fibromas and the other delineating a highly specific localized LOH on chromosome 1p for colon cancers substantiate the claim that interstitial deletions targeting a single gene or allele could occur (Thiagalingam et al., 2001; Serra et al., 2001). Additionally, highly specific localized homozygous deletions observed within a gene or locus also indicate that a double hit could occur simply by targeted loss of genetic material (Thiagalingam et al., 1996; Friend et al., 1986; Schutte et al., 1995; Hahn et al., 1996). (b) Extensive loss of genetic material involving a portion of or an entire chromosomal arm could be accomplished by a double-strand break with the loss of genetic material distal to the break, a single mitotic recombination involving the homologous pair of chromosome, or reciprocal or non-reciprocal translocation. Mitotic recombination is the result of a reciprocal exchange of genetic material between non-sister chromatids of homologous or non-homologous chromosome in mitotic cell as detected by substitution of contiguous markers with reference to an established marker (Cavenee et al., 1983; Vogelstein et al., 1988; Thiagalingam et al., 2002; Virmani et al., 1998; Hahn et al., 1996; Cropp et al., 1990; Varella-Garcia et al., 1998; Zhu et al., 1992).

Loss of a whole chromosome is also accomplished by non-disjunction defects in chromosome segregation (Schutte et al., 1996; Baker et al., 1989). However, the existence of multiple copies of chromosome in tumor cells deemed to have lost one member of the homologous pair by reduplication of the remaining chromosome leading to homozygosity (Thiagalingam et al., 2001; Varella-Garcia et al., 1998; De NooiJ-Van Dalen et al., 1998; Knuutila et al., 1999). Other than these, additional mechanisms of LOH were reported (Bauer et al., 2001).
Fig. 1.6 Schematic representation of the different mechanisms contributing to the loss of heterozygosity (LOH).

**Top left:** In heritable disease, a recessive defect (labeled “t”) at the tumor locus (TMR) is inherited, giving a genotype of t/+ in all cells. The predisposition is unmasked by loss of wild type of alleles, by one of the chromosomal mechanism mentioned, followed by other alterations during malignant progression.

**Lower left:** In sporadic disease, somatic mutation at the TMR locus results in formation of a predisposed precursor cell. Predisposition is unmasked in mechanistically similar ways as in heritable disease.

**Right:** Predicted results using polymorphic markers or probes for loci proximal (A) and distal (B) to the tumor (TMR) locus. The Normal DNA is informative for these markers (Lanes N) but in tumors (lane T) loss of heterozygosity is observed except when localized changes occur. Loss is confirmed by quantitation of the signal on autoradiogram. Modified from (Lasko et al., 1991)
LOH Molecular players: The LOH or genomic instability could be elicited due to failure of cellular functions to maintain the genetic integrity of the genome by faithful DNA replication, DNA damage repair, telomere protection, segregation of chromosome at mitosis or unscheduled recombination. The two categories of chromosomal abnormalities that involve the portion of a chromosome or an entire chromosome could be considered derived from defect in caretaker genes or chromosome segregator gene respectively.

A caretaker gene is any gene required to maintain the integrity of DNA during the processes such as DNA replication, repair, or recombination; telomere maintenance and protection; and chromosome packaging or to protect the DNA from nucleases and other adversities of intracellular physiological by product (Kinzler et al., 1997; Yu et al., 1999; Heck et al., 1997; Kelly et al., 2000; Bishop et al., 2000; Artandi et al., 2000).

The chromosome segregator genes are those required to mediate orderly disjunction of sister chromatids to the daughter cells during mitotic cell division. These genes could include all those involved in determining the centromere structure, sister chromatid cohesion and the anaphase promoting complex, and others required for the proper assembly of the bipolar spindle apparatus to ensure faithful segregation of genetic information (Hirano et al., 1999; Nasmyth et al., 2000; Saffery et al., 2000; Tyler Smith et al., 2000; Mitchison et al., 2001).

LOH as Prognostic and diagnostic Marker: Prognostic markers are useful in the management of breast cancer patients to be able to predict the clinical course of the disease at the time of primary treatment, in order to choose the optimal treatment for individual patients (Ferno et al., 1998). Currently some biological markers, including TP53, BCL2, ERBB2, cyclin expression, DNA ploidy, and markers of proliferation have been used for prognosis (Ravaioli et al., 1998). The predictive ability of these molecular markers for individual patients is still the subject of controversy whereas the conventional prognostic factors, such as lymph node status, tumor size, histology grade, and hormone receptor status, remain unsurpassed as prognostic indicator (Ferno et al., 1998; Henderson et al., 1998; Ravaioli et al., 1998).

The clinical value of loss of heterozygosity data as markers has already been established for diagnosis and prognosis of cancer. A higher frequency of consistent LOH at defined
chromosomal regions critical for specific cancers has made it useful and reliable DNA marker for diagnosis and prognosis, regardless of whether the target gene has been identified (Sidransky et al., 1997; Cairns et al., 1999; Nicholl et al., 1999; Dong et al., 2001). The loss of heterozygosity analyses also lead in elucidating the genetic pathways in the evolution of breast carcinoma in situ (Fulvia-Ferabegoli et al., 2002).

The LOH studies have played a great role in the evaluating the response of chemotherapy and radiotherapy treatment to disease. Prediction of postoperative outcomes among patients who have undergone surgery for breast cancer has increased in importance, in view of the variety of postoperative adjuvant therapies that are now available. In their long term study Hirano et al. (2001) correlated allelic losses at different loci 3p25.1, 8p22, 13q12, 17p13.3 and 22q13 with postoperative recurrence and found allelic losses at these loci to be negative prognostic indicator.

LOH and Gene mapping: The presence of cancer genes on specific chromosome was first suggested by karyotype analysis showing nonrandom cytogenetic abnormalities. Cancer with its somatic alteration characteristics provides novel opportunities for gene identification by using molecular analysis of the allelic pattern of tumor DNAs with polymorphic markers. Inactivating mutations include point mutations, loss of chromosomal material, gene conversion, or mitotic recombination or deletion (Knudson 1978; Cavenee et al., 1983). Therefore, chromosomal regions that frequently exhibit allelic losses are expected to harbor putative tumor suppressor genes (Sato et al., 1990). The mechanism of somatic hyper mutability provides sufficient justification for identifying predisposition genes by studying somatic mutation inactivation of candidate gene region (Solomon et al., 1991). There are precedents for carrying out such studies, which derive from Mendelian cancer syndromes; The PTEN gene causing cowden’s syndrome and the DPC4 /SMAD4 gene causing juvenile polyposis were both originally identified by mapping somatic homozygous deletion in cancer (Hahn et al., 1996, Li et al., 1997).

At present, loss of heterozygosity (LOH) using highly polymorphic microsatellite markers is the most common methodology employed for the localization of sites in the genome with a high probability for the presence of candidate TSGs. In order to identify new cancer related genes, first generation of genome wide loss of heterozygosity studies were
performed in early nineties (Larsson et al., 1990; Andersen et al., 1992) however only few markers per chromosome were used. A second generation of allelotyping was performed by taking advantage of the availability of thousands of markers developed and mapped (Gyapay et al., 1994; Dib et al., 1996) and more than 50 potential region of deletion were identified (Kerangueven, et al., 1997). The complete sequencing of human genome and sequence map offers attractive ways to identify the new tumor suppressor genes or cancer related genes by defining minimal lost region (MLR), which could be determined by using the polymorphic markers such as Simple tandem repeats (STS markers) by studying loss of heterozygosity (LOH). LOH mapping could be applied to both sporadic and familial form of disease.

v. Loss of heterozygosity (LOH) at 17p & 17q region in breast tumors

Allelic losses at different chromosomal arms such as 1q, 3p, 6q, 7q, 9q, 11q, 15q, 10q, 17q, 18q and X have been reported by Sato et al. (1990). It was observed that the transfer of a human chromosomal region 17p13 into the TP53 positive breast cancer cell line Cal51 resulted in loss of tumourigenicity of the transfected cells (Theile et al., 1995). Allelic loss of the short arm of chromosome 17 is among the most frequently deleted genetic alterations in human breast cancer (Bieche et al., 1995). Loss of heterozygosity on chromosome 17p has been reported in 40-60% of sporadic breast carcinomas (Sato et al., 1990; Stack et al., 1995; Phelan et al., 1998). On 17p13, there are two independent regions of LOH, one spanning the TP53 locus (17p13.1) and the other involving a more distal region at 17p13.3, implying the existence of at least another TSG distal to TP53 (Seitz et al., 2001). Studies also reported commonly deleted regions at 17p13, distal to TP53 in other type of human cancer. Philips et al. (1996) and Schultz et al. (1996) found the smallest region of overlapping deletions to be between D17S28 and D17S5 in ovarian cancer. Lung cancers are also shown to carry commonly deleted regions from 17p13.3 between D17S379 and D17S5 (56% LOH) and around a more telomeric region (D17S1866/D17S695) (Konishi et al., 1998). Commonly deleted regions at 17p13 were also defined for neural (Biegel et al., 1992; Cogen et al., 1992) and cervical tumors (Park et al., 1995) around the D17S34 locus and for urothelial (Bernues et al., 1999) and cervical cancer (Park et al., 1995) around the D17S5 locus. To date, a number of genes, mapping to 17p13 have been suggested as potential TSGs. One such candidate gene, HIC-1 (hypermethylated in cancer-1) was isolated by the cloning of a chromosomal fragment in close proximity to the D17S5 locus. HIC-1 is proposed to be commonly inactivated by hypermethylation (Makos et al., 1992). Two other candidate TSGs, OVCA1 and OVCA2
were cloned from a region located between D17S5 and D17S28 (Schultz et al., 1996). Another gene, the CRK proto-oncogene, homologous to SH2/SH3 domain-containing intracellular signal transduction proteins was mapped in proximity to D17S28 (Matsuda et al., 1992). In addition, replication protein A (RPA1), a ubiquitous eukaryotic single strand binding protein essential for DNA replication, recombination and repair, was localized to 17p13.3. Seitz et al., (2001) defined seven commonly deleted relevant regions on chromosome 17p13.3 region. These data provide evidence for putative TSGs at 17p13 that might be implicated not only in breast cancer but also in a number of other malignancies.

The loss of 17p13 region were also found to be associated with clinicopathological characteristic in breast cancer such as high proliferative capacity (Chen, 1991; Merlo, 1992) high risk of recurrence (Nagai, et al., 1994) and metastasis to lymph nodes (Takita, et al., 1992). This association could become relevant in disease prognosis, as it was observed by Emi et al., (1999). They found that, allelic losses at 17p13.3 loci could serve as a negative prognostic indicator.

Allelic imbalances have also been reported in chromosome 17q21-24 regions in various cancers (Lerebours et al., 2002; Dai et al., 2001; O'Neil et al., 2001; Sirchia et al., 2000; Forozan et al., 2000; Kitamura et al., 2000; Abujiang et al., 1998). The chromosomal region 17q21 harbors the BRCA1, a tumor suppressor (Miki et al., 1994) and NM23 a metastasis-suppressor genes (Backer et al., 1993). Two NM23 genes on 17q22 have been reported NM23-H1 (Steeg et al., 1988) and its homologue NM23-H2 (Stahl et al., 1991). The NM23 locus has shown the 72% genomic alteration in colorectal cancer (Berney et al., 2000). The association of BRCA1 gene with susceptibility to breast and ovarian cancer has been strongly supported (Miki et al., 1994; Neuhausen et al., 1996). However the effect of BRCA1 in families with BRCA1 linkage is probably not limited to breast and ovarian cancer, and it could influence the risk for other tumors such as colon cancer (Ford et al., 1994). Interestingly, LOH without point mutation at the BRCA1 locus occurs in a high proportion of sporadic breast carcinomas, suggesting that other genes at 17q21 might be involved in breast cancer and eventually in other tumors in families with germ line BRCA1 mutations (Cropp et al., 1994). Some tumors, such as prostate adenocarcinoma and squamous cell carcinoma of the esophagus, have been screened on the basis of LOH in the 17q21 region (Gao et al., 1995) For prostate tumors, a high rate of LOH was found, mainly at the D17S855 and D17S856 loci suggesting that the BRCA1 gene or some other unidentified tumor suppressor gene located in this region could be implicated in the
pathogenesis of this malignancy (Gao et al., 1995). The chromosomal region 17q23 initially was found to be amplified in breast cancer based on genome wide copy number analysis by comparative genome hybridization (CGH) (Kallioniemi et al., 1994; Merlo et al., 1992). The 17q23 amplification is seen in 20% of primary breast cancer by CGH, and it seems to be more common in high grade tumors and in tumors from BRCA1 or BRCA2 mutation carriers (Moore et al., 1999; Tirkkonen et al., 1997) and this amplification is associated with poor prognosis of breast cancer patients (Barlund et al., 2000), suggesting the genes affected by this amplification may have a crucial role in breast cancer progression.

Copy number gains at 17q23 by CGH also have been reported in tumors of the brain (Vandesompele et al., 1998; Weber et al., 1997) lung (Ried et al., 1994; Schwendel et al., 1997) bladder (Richter et al., 1997) testis (Korn et al., 1996) and liver (Marchio et al., 1997) indicating that genes located at 17q23 may contribute to the development of other tumor types as well. Five genes (RPS6KB1, RAD51C, PAT1, APPBP2, SIGMA1B and TBX2) have been implicated as putative target genes for the 17q23 amplification (Barlund et al., 2000; Couch et al., 1999; Wu et al., 2000).

The loss of heterozygosity (LOH) have not only provided a view of the genetic abnormalities of complex disease such as cancer but also stimulated the drive to obtain a better understanding of the various cellular processes such as DNA repair, replication, recombination, telomere maintenance and cell division by providing excellent, highly discernible visual examples resulting from their defects.

vi. Simple tandem repeat alterations (Microsatellite instability: MSI) in breast cancer.

Chromosomal analysis of malignant tumors established the aneuploidy as cardinal feature of neoplastic cells. A novel mechanism of tumorigenesis came into picture in early 1990 studying on the hereditary non polyposis colon cancer (HNPCC) syndrome (Aaltonen et al., 1993) that is not characterized by gross aneuploidy but instead by inactivation of the DNA mismatch repair system, (which corrects base pair mismatches arising during DNA replication) leading to a hyper mutable state in which simple repetitive DNA sequences (SSR) are unstable during DNA replication termed as mutator phenotype (Replication error; RER). Tumors with the replication error phenotype are said to display micro
satellite instability phenotype [Fig. 1.7]. Microsatellites are scattered throughout the genome including non-coding and coding regions of various genes.

A consensus panel of five microsatellite markers was proposed to distinguish high frequency MSI (MSI-H; two or more unstable markers), Low frequency MSI (MSI-L; one unstable marker) and microsatellite stability (MSS: no unstable Markers) in colorectal cancer (Boland et al., 1998). Tumors showing MSI are generally near diploid and are at low stage of development. Inactivation of both the alleles of mismatch repair gene MSH2 or MLH1 is necessary to generate the high MSI phenotype in HNPCC patients. It has been observed that RER phenotype is associated with site specific tumors, it is frequently observed in endometrium carcinoma (Bubb et al., 1996), Gastric adenocarcinoma (Yokozaki et al., 1999; Habano et al., 2000) and a lesser incidence is observed in ovarian tumors, melanoma and gliomas.

Replication errors, RER are key features of hereditary non-polyposis colorectal cancer (HNPCC) and indicators of defects in the DNA mismatch repair genes (Parson et al., 1993). Breast cancer is rarely associated with the RER phenotype. This type of mutation has been reported to occur in female breast cancer at frequencies ranging from zero to over 80% in different cohorts (Siah et al., 2000). The positive family history of breast tumors could show the RER phenotype as reported by Glebov et al. (1994). In their study RER was seen in five of 14 (35%) cases of sporadic breast tumors and 15 of 18, (83%) in the familial tumors. Another study revealed RER at a single locus on chromosome 11p15.5 in 20 of 69 (Karnik et al., 1995) 29% primary breast tumors analyzed. In independent studies RER has been reported in 5%, 5 of 93 and 8%, 8 of 100 of primary breast tumors, respectively (Jonsson et al., 1995; Toyama et al., 1996). Bergthorsson et al. (1995) suggested three explanations for the appearance of replication error in sporadic breast cancer: First, the instability observed is not caused by mismatch repair gene defects, but rather reflects the ability of this repair system to work in the environment provided by the tumor cell. Secondly, sporadic RER tumors could be caused by mild mutations, and perhaps in different genes. Finally, sporadic breast tumors may have a defective mismatch repair system but need an additional environmental or genetic co-factor to result in a strong RER phenotype. The breast cancer cases with RER do not seem to be part of a HNPCC syndrome since a family history of colorectal cancer growth is not detected in relatives (Huiping et al., 1999) it can be concluded that RER is a rare somatic event during human breast carcinogenesis.
Fig. 1.7 Schematic representation of microsatellite instability (MSI) phenotype observed in tumors using polymorphic markers (Microsatellite markers) and PCR typing.
1.2 Materials & Methods

i. Chemicals and Biochemicals

Acrylamide (Sigma), Acetic acid, Acetone, Ammonium persulphate, Agaragar (Qualigens), Agarose (Pronadisa, Roche), Ampicillin, bis-Acrylamide (Sigma), â-Mercaptoethanol (Sigma), Boric acid (Qualigens), Bromophenol blue, Calcium chloride dihydrate (Sigma), Chloroform (Qualigens), Diethyl pyrocarbonate (Sigma), DNA molecular weight markers (1 kb ladder, 100bp ladder, 100 bp ladder (Promega)), 20 bp ladder (Sigma), Ethylene diamine tetra acetate (EDTA)(Qualigens), Ethanol (MERCK and Bengal Chemicals),Ethidium bromide (Sigma), Formaldeyde (Qualigens), Formamide (Sigma), Glycerol, Glutaraldehyde, Hydrochloric acid (SD Fine), Isoamyl alchohol, Isopropanol (Qualigens), Manganese chloride (Sigma) ,Methanol, Magnisium chloride (Qualigens), Phenol (Qualigens), Proteinase K (Sigma), Potassium chloride (Qualigens), Potassium dihydrogen phosphate, Disodium hydrogen phosphate(Qualigens), RNase A(Sigma), Sodium acetate, Sodium bicarbonate, Sodium carbonate, Sodium chloride, Sodium dodecyl sulfate (Sigma), Sodium hydroxide, Sucrose( Qualigens), Trisodium citrate(Qualigens), Tris buffer (Sigma), TEMED (Sigma), Tryptone (Pronadisa), Triton -X- 100( Sigma),Tween 20 (Sigma), Urea, xylene cyanol (Sigma), Yeast extract (Pronadisa).

PCR primers: STS marker primers used in this study was 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.

Taq polymerase enzyme and dNTPs: The Taq polymerase enzyme and dNTPs were procured from the Banglore Genei, India and Promega Inc. (USA).

Radioactive material: α³²P (3000Ci/mMol, 10 µCi/µl) was procured from Amersham Pharmacia

ii. Blood and Tumor Tissue Samples

In this study, breast cancer patients admitted in Rotary Cancer Hospital (IRCH), AIIMS, and New Delhi, were the source of the sample. The study subjects consisted of 34 sporadic breast cancer patients who underwent surgery from 1997-2000. The breast tumors were
removed by either mastectomy or lumpectomies from untreated patients in various stages of cancer. The tumor samples were dissected quickly to remove normal tissue and stored at -80°C. Both peripheral blood and the tissue samples [Table 1.2] were collected from all the patients with informed consent.

iii. High Molecular Weight Genomic DNA Isolation from Peripheral Blood Samples
DNA was isolated from peripheral blood lymphocytes using the conventional phenol chloroform method (Kunkal et al., 1977). 5 ml of non-heparinised blood was layered over 45 ml of lysis buffer (see appendix) for 15 minutes on ice, which was intermittently mixed. It was then centrifuged at 2800 rpm at 4°C for 20 min. The pellet thus obtained after centrifugation was gently resuspended in 4.5 ml of digestion buffer. Proteinase K solution was then added to the suspension to make the final concentration of 100 μg/ml. The lysed cell suspension was gently swirled overnight at 37°C. Deproteinization was carried out by extracting the cell suspension twice with an equal volume of Phenol: Chloroform: Isoamylalcohol: 24:24:1 at 5000 rpm for 20 minutes at room temperature and then twice with only Chloroform: Isoamylalcohol: 24:1 at similar conditions as specified above. High molecular weight DNA was precipitated by adding 1/10th volume of 3 M sodium acetate pH 5.2 and 1 1/2 volume of chilled Isopropanol to the final aqueous phase. DNA was spooled by gently swirling the tube and collected with a broad mouthed pasteur pipette in an eppendorf tube in pure ethanol which was later washed with 70% ethanol. Finally, it was dried and dissolved in 100 μl of sterile water/TE. DNA was quantified using the dual beam UV spectrophotometer at wavelength 260 nm and 280 nm and amount of DNA was calculated using the formula (A 260 nm X dilution factor X 50) = DNA ug/ml. The purity of the DNA was checked by taking the ratio at A260/280, the ratio being >1.5 for pure DNA. The quality of DNA was checked by running an aliquot of DNA sample in 0.8 % agarose gel followed by EtBr staining.

iv. High Molecular Weight DNA Isolation from Tumour Tissue
Cryo-preserved tumour tissue were taken directly in tissue lysis buffer (except Proteinase K) and chopped to fine pieces and minced followed by addition of Proteinase K and incubated at 65 °C for 10 min. with gentle shaking. The sample was then left overnight with gentle shaking at 37 °C in a water bath. Deproteinization and precipitation was carried out in similar fashion as in the case of blood samples.
Table 1.2 Age and disease status of cases collected for study from: IRCH; AIIMS, New Delhi (1997-2000).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Stage</th>
<th>Type</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T_3N_1M_x</td>
<td>Carcinoma</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>T_3N_1M_x</td>
<td>Carcinoma</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>T_xN_0M_x</td>
<td>Carcinoma</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>T_3N_1M_0</td>
<td>Infiltrating lobular carcinoma</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>62</td>
</tr>
<tr>
<td>6</td>
<td>T_2N_1M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>55</td>
</tr>
<tr>
<td>7</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>T_3N_1M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>36</td>
</tr>
<tr>
<td>13</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>40</td>
</tr>
<tr>
<td>14</td>
<td>T_3N_1M_0</td>
<td>Carcinoma</td>
<td>35</td>
</tr>
<tr>
<td>15</td>
<td>T_1N_2M_0</td>
<td>Carcinoma</td>
<td>59</td>
</tr>
<tr>
<td>16</td>
<td>T_1N_2M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>42</td>
</tr>
<tr>
<td>17</td>
<td>T_1N_2M_0</td>
<td>Carcinoma</td>
<td>45</td>
</tr>
<tr>
<td>18</td>
<td>T_1N_2M_0</td>
<td>Carcinoma</td>
<td>48</td>
</tr>
<tr>
<td>19</td>
<td>T_1N_2M_0</td>
<td>Infiltrating lobular carcinoma</td>
<td>45</td>
</tr>
<tr>
<td>20</td>
<td>T_1N_2M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>60</td>
</tr>
<tr>
<td>21</td>
<td>T_1N_2M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>65</td>
</tr>
<tr>
<td>22</td>
<td>T_1N_2M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>40</td>
</tr>
<tr>
<td>23</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>38</td>
</tr>
<tr>
<td>24</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>26</td>
</tr>
<tr>
<td>25</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>48</td>
</tr>
<tr>
<td>26</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>49</td>
</tr>
<tr>
<td>27</td>
<td>T_2N_1M_0</td>
<td>Infiltrating lobular carcinoma</td>
<td>42</td>
</tr>
<tr>
<td>28</td>
<td>T_2N_1M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>32</td>
</tr>
<tr>
<td>29</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>65</td>
</tr>
<tr>
<td>31</td>
<td>T_2N_1M_0</td>
<td>Infiltrating ductal carcinoma</td>
<td>50</td>
</tr>
<tr>
<td>32</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>60</td>
</tr>
<tr>
<td>33</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>35</td>
</tr>
<tr>
<td>34</td>
<td>T_2N_1M_0</td>
<td>Carcinoma</td>
<td>36</td>
</tr>
</tbody>
</table>
v. *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.

vi. *PCR amplification of STS markers*

The STS markers used in this study were chosen from the GDB database (http://gdb.org/). The information on the marker heterozygosity and their GDB accession no. is mentioned in the Table 1.3. The detail information about each marker regarding sequences, allele sizes and their Gene bank Accession number are provided in Tables 1.4 to 1.9. The position of markers on the cytogenetic map [Fig. 1.8] and the primer sequence is provided in the Table 1.10.

The standardization of PCR reaction for specific amplification was carried out by varying concentrations of MgCl₂, target DNA and annealing temperature. The PCR conditions used for specific pairs of primer are mentioned in Table 1.10.

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 a ³²P dCTP, 0.5 unit of Taq Pol. enzyme, 1mM of MgCl₂, 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 30 sec, 60- 52°C, 72 °C for 30 sec and a final extension at 72°C for 5 minutes.

vii *Loss of heterozygosity analysis*

The genomic DNA isolated from blood and matching tumors was amplified by PCR using a 12.5µl reaction mixture. The PCR mix was consisted of 12.5 picomole of each primer (Forward and Reverse), 200 nM of dNTP with a ³²P dCTP, 0.5 unit of Taq Pol. enzyme, 1mM of MgCl₂, and 1x PCR buffer. Thermal cycling was performed on PTC-100 Programmable Thermal Controller (MJ Research Inc.) using the following conditions:
Table 1.3 Details of STS markers studied for low-resolution mapping, selected from http://gdb.org/ database.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer name</th>
<th>Type of sequence Amplified</th>
<th>GDB Accession ID</th>
<th>Maximum Heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S5</td>
<td>YNZ22-1/YNZ22-2</td>
<td>VNTR</td>
<td>GDB: 178624</td>
<td>0.8625</td>
</tr>
<tr>
<td>D17S379</td>
<td>D17S379.PCR1.1/ D17S379.PCR1.2</td>
<td>Dinucleotide</td>
<td>GDB: 197041</td>
<td>0.8099</td>
</tr>
<tr>
<td>D17S855</td>
<td>AFM248yg9a/ AFM248yg9m</td>
<td>Dinucleotide</td>
<td>GDB: 192761</td>
<td>0.8220</td>
</tr>
<tr>
<td>D17S934</td>
<td>AFM256vb9a/ AFM256vb9m</td>
<td>Dinucleotide</td>
<td>GDB: 199561</td>
<td>0.8412</td>
</tr>
<tr>
<td>D17S787</td>
<td>AFM095tc5a/ AFM095tc5m</td>
<td>Dinucleotide</td>
<td>GDB: 187960</td>
<td>0.8169</td>
</tr>
<tr>
<td>D17S948</td>
<td>AFM291ve9a/ AFM291ve9m</td>
<td>Dinucleotide</td>
<td>GDB: 199827</td>
<td>0.8300</td>
</tr>
</tbody>
</table>
Table 1.4  D17S5 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S5</td>
<td>Hypervariable number tandem repeats</td>
<td>M21143 J03056</td>
</tr>
<tr>
<td>Locus YNZ22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequence of amplified region

```
cgaagagtgaagtgcacaggagggcaaggcgggccctcaccctgccctgggttcgagggcagggctgtgagaccctccctttacagaagcaatgagggcttgaggag
Ggggttaggggccctgggctggggcaggctgtgcagacccctccctttacagaagcaatgagggcttgaggag
Gcaatgaggctttgaggagggggttaggggccctgggctggggcagggctgtgagaccctccctttacagaagcaatgagggcttgaggag
Ggagcctccttacagaagcaatgagggcttgaggaggggttaggggccctgggctggggcagggctgtgagaccctccctttacagaagcaatgagggcttgaggag
Tgggctggggcagggccttgagacccctccctttacagaagcaatgagggcttgaggag
Gaggaggggttaggggccagtaagttactttggaggacggagatgtggggggagcctgaagaataaagactgtg
```

D17S5 polymorphisms

<table>
<thead>
<tr>
<th>Alleles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size bp</td>
<td>168</td>
<td>238</td>
<td>308</td>
<td>378</td>
<td>448</td>
<td>518</td>
<td>588</td>
<td>658</td>
<td>728</td>
<td>798</td>
<td>868</td>
<td>938</td>
<td>1.008</td>
<td>1.078</td>
<td>1.148</td>
<td>1.218</td>
<td>1.288</td>
</tr>
<tr>
<td>Freq</td>
<td>.06</td>
<td>.17</td>
<td>.14</td>
<td>.26</td>
<td>.05</td>
<td>.05</td>
<td>.01</td>
<td>.04</td>
<td>.07</td>
<td>.06</td>
<td>.03</td>
<td>.04</td>
<td>.01</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
<td>.00</td>
</tr>
</tbody>
</table>
Table 1.5 D17S379 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S379</td>
<td>Dinucleotide repeats (CA)n</td>
<td>X69879</td>
</tr>
</tbody>
</table>

Sequence of amplified region

```
gaccacatctgtcctcacctgtgaggagcctgcccctccctccctagcc
Cttccagcctgggacacacacacacacacacacacacacacacacaca
Cgacgcacagcagcagcactctcctctcctgctgctttaccttttgat
Gtcccagttggccccctggtggaggagctcctacctccgggaggagggaga
Ggttggtctctgggggccaagaaaggcagggatgcctggagggtaac
Tggggccaccatgaaccctctctccagaaaaagctgctttctcccccata
Ccgggtcaccacccccaaaccacccagagtgccctttttacagtgagg
actcg
```

D17S379 polymorphism

<table>
<thead>
<tr>
<th>Alleles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>362</td>
<td>360</td>
<td>358</td>
<td>356</td>
<td>354</td>
<td>352</td>
<td>350</td>
<td>348</td>
<td>342bp</td>
</tr>
<tr>
<td>Freq.</td>
<td>.07</td>
<td>.01</td>
<td>.13</td>
<td>.16</td>
<td>.04</td>
<td>.24</td>
<td>.28</td>
<td>.07</td>
<td>.01</td>
</tr>
</tbody>
</table>
Table 1.6 D17S855 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Markers Information

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S855</td>
<td>Dinucleotide repeats (CA)n</td>
<td>Z23813</td>
</tr>
</tbody>
</table>

Sequence of amplified region

```
ggatggccttttagaaagtggtcaccctccccctttanagacagacggacaga
Aacacacacacacacaccaaacacacacacacacacacacacacacacactctttactt
 taccaccagagtgaaaagaatggcagtaggacaagtctgtg
```

D17S855 polymorphism

<table>
<thead>
<tr>
<th>Alleles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>155bp</td>
<td>153</td>
<td>151</td>
<td>149</td>
<td>147</td>
<td>145</td>
<td>143</td>
</tr>
<tr>
<td>Freq.</td>
<td>0.05</td>
<td>0.19</td>
<td>0.18</td>
<td>0.15</td>
<td>0.12</td>
<td>0.26</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 1.7 D17S934 Marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S934</td>
<td>Dinucleotide Repeats (CA)n</td>
<td>Z23831</td>
</tr>
</tbody>
</table>

Sequence of amplified region

tctgaatggcccttggtaatatgctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctctc..
Table 1.8 D17S787 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S787</td>
<td>Dinucleotide Repeats (CA)n</td>
<td>Z16558</td>
</tr>
</tbody>
</table>

Sequence of amplified region

```
tggtctcactatagcaacctatcttttctctggtttctgtgtgcctcaagtac
Tggctgcatctgtatctctcaatgtccttcagattaacacacacacac
acacacacacacacacacacacacacacatacccttcaaaaaaggtatca
```

D17S787 polymorphism

<table>
<thead>
<tr>
<th>Alleles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>138</td>
<td>140</td>
<td>142</td>
<td>148</td>
<td>152</td>
<td>154</td>
<td>160</td>
<td>164</td>
<td>166bp</td>
</tr>
<tr>
<td>Freq.</td>
<td>0.12</td>
<td>0.32</td>
<td>0.17</td>
<td>0.01</td>
<td>0.17</td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Table 1.9 D17S948 marker details; clone name, amplified sequence, allele numbers, size and frequency in Caucasians.

Markers Information

<table>
<thead>
<tr>
<th>Name of marker</th>
<th>Type of sequence amplified</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S948</td>
<td>Dinucleotide Repeats (CA)n</td>
<td>Z24102</td>
</tr>
</tbody>
</table>

Sequence of amplified region

```
gtctctgtccttaggagtta cattttnatgagaggtctctctctctct
Gtctctctctctctcaca cacaacacacacacacacacacacacacacacacacacacaca
cacaacacacacacaaca aagaaatgggcaagatata
```

D17S948 polymorphism

<table>
<thead>
<tr>
<th>Alleles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size bp</td>
<td>143</td>
<td>125</td>
<td>139</td>
<td>131</td>
<td>149</td>
<td>129</td>
<td>127</td>
<td>141</td>
<td>147</td>
<td>145</td>
<td>133</td>
</tr>
<tr>
<td>Freq.</td>
<td>.26</td>
<td>.07</td>
<td>.01</td>
<td>.03</td>
<td>.01</td>
<td>.25</td>
<td>.08</td>
<td>.03</td>
<td>.14</td>
<td>.05</td>
<td>.01</td>
</tr>
</tbody>
</table>
Fig. 1.8 Cytogenetic location of syntenic STR markers on chromosome 17p13 and 17q21-23 regions used for study.
Table 1.10 Primer sequences and PCR conditions of the STR markers used for low-resolution mapping.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Primers sequence</th>
<th>Tm</th>
<th>Ta</th>
<th>MgCl₂ Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S5</td>
<td>17p13.3</td>
<td>F 5'-CACAGTCTTTATCTTACGCG-3' R 5'-CGAAGAGTGAGTGACAGG-3'</td>
<td>62 (20 mer)</td>
<td>60 (21 mer)</td>
<td>1mM</td>
</tr>
<tr>
<td>D17S379</td>
<td>17p13.3</td>
<td>F 5'-GACCAACTGCTTGCTTCACCTGT-3' R 5'-CGAGTCCTCACTGTAAACAAGG-3'</td>
<td>68 (22mer)</td>
<td>66 (22mer)</td>
<td>1mM</td>
</tr>
<tr>
<td>D17S855</td>
<td>17q21</td>
<td>F 5'-GGATGGCCTTTATTAGAAAGTG-3' R 5'-ACACAGACTTGTCCTACTG-3'</td>
<td>62 (21mer)</td>
<td>64 (21 mer)</td>
<td>1mM</td>
</tr>
<tr>
<td>D17S934</td>
<td>17q21</td>
<td>F 5'-TCTGAATGCCCCTTGG-3' R 5'-TCTCTGTAAGGTGGGTG-3'</td>
<td>50 (16mer)</td>
<td>56 (18mer)</td>
<td>1mM</td>
</tr>
<tr>
<td>D17S787</td>
<td>17q22</td>
<td>F 5'-TGTCCTAATATGACC-3' R 5'-TTGATACCTTTGAGGGG-3'</td>
<td>58 (20mer)</td>
<td>56 (20mer)</td>
<td>1mM</td>
</tr>
<tr>
<td>D17S948</td>
<td>17q23</td>
<td>F 5'-GTCTGTCTTTAGGGTTTA-3' R 5'-TATCTTGGCCATTTCTTG-3'</td>
<td>60 (22mer)</td>
<td>52 (19mer)</td>
<td>1mM</td>
</tr>
</tbody>
</table>

bp= base pair length  
Tm= 2 (A+T) + 4 (G+C)  
Ta= Primer annealing Temperature
Initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 sec, 60-52°C, 72°C for 30 sec and a final extension at 72°C for 5 minutes. The amplified products were mixed with SSLP dye (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 1mM EDTA), denatured at 94°C for 5 min, snap chilled and electrophoresed through 8% denaturing polyacrylamide gels (8M Urea, 29:1 Acrylamide: Bis-acrylamide). The gels were dried and exposed to intensify plate of phosphorimager (FujiFilm: BAS1800, FLA 5000). Tumor samples showing 50% reduction in the intensity of the upper or lower allele in comparison with the corresponding blood samples were considered lost and scored for LOH. Cases showing allelic loss were repeated to confirm the change. Care was taken that an equal concentration and amount of DNA was run both from lymphocyte and tumor samples. The informative cases were scored for the marker and the number of the informative cases showing the loss of allele (LOH) or change of repeat alteration (MSI) recorded.

viii. Statistical analysis
The differences in the LOH frequencies between the markers were tested by the chi-square or Fisher's exact test (two tailed). The same test was extended for the evaluation of association between LOH and the clinico-pathological variables. The analysis was performed for: i) each marker, ii) each of LOH region, and for iii) the whole 17p13 and 17q21-23 regions. The LOH frequency for each of the LOH region was also calculated by combining the markers in the representative region. For all statistical tests we used a comparison related significance level of 0.05 (5%). The statistical analysis was done by using SPSS version10 package.
1.3 Results

Six polymorphic marker loci, two from short arm of chromosome 17 (17p13.3) and four from the long arm which span the 17q21-23 region, were analyzed for the loss of heterozygosity (LOH) and microsatellite instability (MSI) status in sporadic breast tumors. Table 1.11 provides the clinicopathological status of all the cases studied and Fig. 1.9 and 1.10 depict the representative examples of LOH and MSI observed in the studied sporadic breast cancer cases. The results of allelic loss (LOH) and microsatellite instability (MSI), using six markers, are summarized in Fig. 1.11.

Overall, among 34 sporadic breast tumors 82% (28/34) cases showed allelic loss (LOH) at least at one locus out of the six studied loci. Analysing each locus separately, only in informative cases, it was observed that: 25% (8/32) showed an allelic loss at D17S5 locus, 56% (13/23) at D17S379 locus, 26% (7/26) at D17S855 locus, 54% (13/24) at D17S934 locus, 26% (9/34) at D17S787 locus and 24% (7/29) at D17S948 locus. Whereas, LOH was observed in 41% (14/34) cases at two or more loci. The two loci D17S379 and D17S934 showed a high frequency of loss of heterozygosity (LOH) in the studied cases. Both these loci shared the 41% (7/17) of informative cases for showing loss of heterozygosity (LOH). Marker D17S787 along with D17S934 presented with a 47% (16/34) loss of these two loci amongst all the studied cases [Fig. 1.11 and 1.12].

The overall LOH observed was 48% (16/33) at 17p13 region and 64% (22/34) at 17q21-23 region. The two loci on 17p13.3 region (D17S5, D17S379) shared 25% (5/20) of informative cases displaying LOH when analysed together [Figs.1.11 and 1.12].

In addition to LOH the overall microsatellite instability (MSI) observed at both the regions (17p13 + 17q21-23) was 50% (17/34) at least at one locus out of the six studied loci. The overall MSI observed was 20% (7/34) and 35% (12/34) at 17p13 and 17q21-23 regions, respectively [Fig.1.12]. The percentage of MSI at each marker locus was as follows: 12% (4/32) for D17S5; 6% (2/31) for D17S379; and 6% (2/31) for D17S855. The marker D17S934 did not show the microsatellite instability (MSI) in any of the studied cases. It was further observed that marker D17S787 and D17S948 displayed instability (MSI) in 35% (12/34) and 10% (3/30) of the sporadic tumors, respectively [Fig.1.11].
Table 1.11 Clinico-pathological features and number of the patients studied.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>26-65</td>
</tr>
<tr>
<td>Mean</td>
<td>46</td>
</tr>
<tr>
<td>Median</td>
<td>45</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td><strong>Pathological T stage</strong></td>
<td></td>
</tr>
<tr>
<td>Tx</td>
<td>5</td>
</tr>
<tr>
<td>T1</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>9</td>
</tr>
<tr>
<td>T3</td>
<td>7</td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
</tr>
<tr>
<td><strong>Pathological N stage</strong></td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td>1</td>
</tr>
<tr>
<td>N0</td>
<td>12</td>
</tr>
<tr>
<td>N1</td>
<td>14</td>
</tr>
<tr>
<td>N2</td>
<td>7</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td></td>
</tr>
<tr>
<td>Node negative</td>
<td>13</td>
</tr>
<tr>
<td>Node positive</td>
<td>21</td>
</tr>
</tbody>
</table>
Fig. 1.9 Representative results of loss of heterozygosity (LOH) observed at the studied loci (using six STS markers) in sporadic breast cancer patients.
Fig. 1.10 Representative results in autoradiograms of the microsatellite instability (MSI) observed at the studied loci (using STS markers) in sporadic breast cancer patients.
Fig. 1.11 Comprehensive results of LOH and MSI observed in 34 cases at all the six chromosome 17 marker loci.
Fig. 1.12 Percentage of loss of heterozygosity (LOH) and microsatellite instability (MSI) observed at studied loci.
The association of LOH and MSI between individual markers turned out to be non-significant for most of the markers. However, when MSI at D17S787 was compared with the MSI at D17S948, it was observed to be significantly associated (p<0.021) [Table 1.12a]. To find out if there was a relationship between LOH and MSI, the statistical analysis showed that the loss of heterozygosity (LOH) of D17S787 marker was close to a significant association with the overall microsatellite instability (MSI) at 17p13+17q21-23 region (p<0.059) [Table 1.12 b]. Same was found true for the LOH at 17p13 when compared with MSI at 17p13 + 17q21-23 and LOH at 17p13 + 17q21-23 in comparison to MSI at 17p13 [Tables 1.12 c,d]. The LOH at 17p13 + 17q21-23 regions, however, was significantly associated with the overall MSI at 17p13+17q21-23 regions (p<0.009) [Table 1.12 e].

An analysis of the association between loss of heterozygosity (LOH) at the studied loci with the clinicopathological parameters such as; stages of tumor and their nodal positivity or negativity showed that the loss of D17S948 marker was significantly associated with the stage III tumors (p<0.030) [Table 1.13a]. No other marker showed any association with different clinical stages of tumors. The LOH involving different markers, both on the short and long arm of chromosome-17, did not show any association with either the node positive or node negative tumor status. Though the combined loss of the two markers, D17S5+D17S787, was very close to show a significant association with the stage III tumors (p<0.59) [Table 1.13b]. It was observed that association between microsatellite instability (MSI) at D17S787 locus and stage II tumors was highly significant (p<0.009) [Table 1.14 a]. The stage II tumors further showed an association with the microsatellite instability (MSI) observed in the combined markers, D17S379 + D17S787, (p<0.006) and with the MSI in the 17q21-23 region (p<0.006) [Table 1.14 b, c].
Table 1.12 Association between LOH and MSI at 17p13 and/or 17q21-23.

a) Association between MSI at D17S787 and D17S948 locus.

<table>
<thead>
<tr>
<th>MSI at D17S787 * MSI at D17S948 Crosstabulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>MSI at D17S787</td>
</tr>
<tr>
<td>MSI-</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.021)

b) Association between LOH at D17S787 and MSI at 17p13+ 17q21-23 regions.

<table>
<thead>
<tr>
<th>LOH at D17S787</th>
<th>MSI+</th>
<th>MSI-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH+</td>
<td>2</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>LOH-</td>
<td>15</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>17</td>
<td>34</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.059)

c) Association between LOH at 17p13 and MSI at 17p13+ 17q21-23 regions.

<table>
<thead>
<tr>
<th>LOH at 17p13</th>
<th>MSI+</th>
<th>MSI-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH+</td>
<td>5</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>LOH-</td>
<td>11</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>17</td>
<td>33</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.057)
d) Association between LOH at 17p13+ 17q21-23 and MSI at 17p13 regions.

**Crosstab**

<table>
<thead>
<tr>
<th>LOH at 17p13+17q21-23</th>
<th>MSI at 17p13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH+</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>MSI-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>28</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.053)

e) Association between LOH and MSI at 17p13+ 17q21-23 regions.

**Crosstab**

<table>
<thead>
<tr>
<th>LOH at 17p13+17q21-23</th>
<th>MSI at 17p13+17q21-23</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH+</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>LOH-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value P<0.009

**Table 1.13** Association of LOH at 17p13 and/or 17q21-23 regions with clinical variables.

a) Association between stage III tumors and LOH at D17S948 locus.

**Crosstab**

<table>
<thead>
<tr>
<th>Stage III</th>
<th>LOH at D17S948</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH+</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>LOH-</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>22</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.030)
b) Association between stage III tumors with LOH at D17S5+ D17S787 regions.

Crosstab

<table>
<thead>
<tr>
<th></th>
<th>LOH at D17S5+D17S787</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>LOH+</td>
<td>LOH-</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>LOH-</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>21</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.059)

Table 1.14 Association of MSI at 17p13 and/or 17q21-23 regions with clinical variables.

a) Association between stage II tumors with MSI at D17S787 locus.

Crosstab

<table>
<thead>
<tr>
<th></th>
<th>MSI at D17S787</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE_I</td>
<td>MSI+</td>
<td>MSI-</td>
</tr>
<tr>
<td>MSI+</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>MSI-</td>
<td>12</td>
<td>22</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.009)

b) Association between stage II tumors with MSI at D17S379+ D17S787 loci.

Crosstab

<table>
<thead>
<tr>
<th></th>
<th>MSI at D17S379 + D17S787</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE_II</td>
<td>MSI+</td>
<td>MSI-</td>
</tr>
<tr>
<td>MSI+</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>MSI-</td>
<td>13</td>
<td>21</td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.006)
c) Association between stage II tumors with MSI at 17q21-23 region.

<table>
<thead>
<tr>
<th>Count</th>
<th>MSI at 17q21-23</th>
<th>MSI+</th>
<th>MSI-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE_II MSI+</td>
<td>9</td>
<td>9</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>MSI-</td>
<td>13</td>
<td>12</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>21</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Fisher exact test two tailed p value (p<0.006)
1.4 Discussion

Human cancer arises by a combination of genetic changes including activation of cellular oncogenes and inactivation of tumor suppressor genes (TSGs) with an influence from a variety of, known and unknown, environmental factors. One of the prominent approaches to unravel the involvement of new genomic sequences / genes has been to study the allelic loss by loss of heterozygosity (LOH) and genomic instability in specific regions of chromosomes. Different chromosome and chromosomal regions and their numerical and structural aberrations have routinely been reported in cancer cells (Mitelman et al., 2003). Chromosomal regions demonstrating a high rate of loss of genetic material in cancer cells invariably have been found to harbor putative TSGs. Alterations in one of the chromosomes, chromosome 17, are being reported in various cancers such as, Barett’s oesophageal adenocarcinoma (Dunn et al., 2003), urothelial cell carcinoma (Santos et al., 2003), alveolar soft part sarcoma (Uppal et al., 2003), neuroblastoma (Suspiro et al., 2003; Saito-Ohara et al., 2003), lung cancer (Konishi et al., 2003), glioma (Van Dartel et al., 2003), hepatocellular carcinoma (Zhao et al., 2003), osteosarcoma (Van Dartel et al., 2002), leukemia (Doneda et al., 2003), and also breast carcinoma (Watters et al., 2003). Chromosome 17 is also known to harbor two important tumor suppressor genes, TP53 (17p13) and BRCA1 (17q21). Both susceptibility genes including another gene, BRCA2 at 13q12-13, are infrequent targets for somatic inactivation in breast tumors (Esteller et al., 2000; Sullivan et al., 2002). The germline mutations in both BRCA1 and BRCA2 have been implicated in approx. 10% of the breast cancer cases with familial history. The absence of any tangible involvement of a gene or group of genes in the majority, approx. 90%, of sporadic cases has directed the attention of researchers to explore different chromosomes and chromosomal regions for the presence of relevant gene(s). Chromosome 17 has been one of the choices since it was observed that in MCF-7 cells, in vitro growth suppression could be achieved by introduction of a normal chromosome 17 by micro-cell mediated chromosome transfer (MMCT) (Casey et al., 1993). The functional role of chromosome 17 and the TSGs located on it in cancer came from in-vitro suppression of transformed phenotype of human breast epithelial cells following introduction of chromosome 17. This provided further support to the importance of chromosome 17 in breast cancer pathogenesis.

In this study genetic alterations at 17p13.3 and 17q21-24 were studied in breast tumors by allelo-typing with six STS repeat markers (two on 17p13.3 and four on 17q21-23 region). The aim was to identify a most prevalent region of allelic loss amongst the studied regions.
The aim was to identify a most prevalent region of allelic loss amongst the studied regions to fine map the region at a low resolution, in sporadic breast tumors. The two loci, one at 17p13.3 (D17S379) and the other at 17q21-23 region (D17S934), showed a significantly high allelic loss (LOH) of 56 % and 54%, respectively, when compared to other four markers used in this study. However, the informativeness increased considerably when the frequency of LOH for all the six markers was analysed together. It was observed that 82% of the 34 sporadic breast cancer patients showed LOH at least at one out of the six loci studied. An analysis between the LOH at 17p13+17q21-23 and MSI in the short and long arm regions together, showed a statistically highly significant association (p<0.009) which later guided the study to choose a specific region from the 17q arm for surveying the flanking regions for gene prediction in the second chapter (CHAPTER 2) of this presentation.

Chromosome 17p has been reported among the most frequently deleted regions in a variety of human malignancies, such as, lung cancer (Konishi et al., 2003), prostate cancer (Dumur et al., 2003), hepatocellular carcinoma (Zhao et al., 2003), thyroid cancer (Farrand et al., 2003), and breast cancer (Niederacher et al., 1997; Lou et al 2002). It has been reported that 73% of the breast tumors exhibited loss of heterozygosity (LOH) (Seitz et al., 2001) in 17p13 region. In this study, breast tumors with matching lymphocytic DNA of 34 patients were examined for LOH of the short arm of chromosome 17 at band 13.3 at two polymorphic loci (D17S5 and D17S379). The D17S5 and D17S379 markers of 17p13.3 region showed 25% and 56% loss in informative cases, respectively. The locus D17S5 maps close to TP53 on the short arm of chromosome 17 and at this locus previously, a high frequency of allelic loss (as high as 60%) had been detected in sporadic (Mackay et al., 1998) as well as in familial breast tumors (Lindblom et al., 1993). A reasonably high frequency (48%) of allelic loss was observed when both the loci were combined for analysis in sporadic breast tumors. The present observation and other reports (Konishi et al., 2003; Zhao et al., 2003; Guan et al., 2003; Zhao et al., 2001; Hoff et al., 2000; Tsuchiya et al., 2000; Sarkar et al., 2000; Konishi et al., 1998) provide the evidence that this region (17p13.3) could harbor important tumor suppressor gene(s), whose inactivation may contribute to tumor development or progression. These genes are distinct from TP53 and are located within the distal portion of the 17p chromosome arm. The observed loss at 17p13.3 does not reflect a general telomeric instability of chromosome arm (White et al., 1996). The frequency of 17p13.3 LOH reported here might actually be an underestimate of the prevalence of breast cancer allele loss at this locus. Since, in the
present study no micro-dissection was attempted, LOH might have been masked by the presence of normal cells in tumor samples.

Both 17p13.3 markers, D17S5 and D17S379 studied for LOH, covered a 2 Mb region on the build 33 sequence map. This region harbors putative tumor suppressor L132/Rox, identified by marker D17S379, and has a helix-loop-helix structure characteristic of many transcription factors and binds DNA by forming hetero-dimers with Max (Meroni et al., 1997). Two other genes OVCA1 and OVCA2 have been isolated from a cosmid clone containing the closely linked marker D17S5 that spans a minimal region of allele loss in ovarian cancer (Schultz et al., 1996). The HIC-1 gene has also been cloned from a region flanking the D17S5 marker and has been suggested as a candidate tumor suppressor gene because of its hypermethylation and transcriptional silencing in multiple common types of human cancers, including breast cancer (Makos et al., 1995; Hoff et al., 2001). However, to date neither inactivating mutations have been found in HIC-1 nor its tumor suppressor activity has been confirmed by functional complementation. However, it is possible that the hypermethylation in the HIC-1 region and the subsequent silencing of the gene may also affect other genes in this region (Nicoll 2001). In the recent past a novel putative tumor suppressor gene (C17orf25) from the deleted region was localized on chromosome 17p13.3 in hepatocellular carcinoma (Qin et al., 2001). The transfection of C17orf25 into the hepato-cellular carcinoma cell SMMC7721 and its over expression inhibited the cell growth. This gene could be the potent target in the sporadic breast cancer as well.

In this study, the 17p13.3 region flanked by D17S5 and D17S379 markers was observed to be involved in LOH, and this was independent of point mutations in the TP53 gene (data not shown, an ongoing study in the laboratory). The association between D17S5 and D17S379 for LOH was found to be non-significant, suggesting an independent deletion of each of the marker without an influence on each other. The D17S379 marker loss was shown to be associated with advanced glioblastoma multiforme (Chattopadhyay et al., 1997) and lung cancer (Tsuchiya et al., 2000). The present study could not demonstrate any association between LOH of 17p13.3 or independent loss of each marker (D17S5 and D17S379) with tumor stage, lymph node status. Liscia et al., (1999) had also reported the importance of association of LOH with both these markers but observed no correlation with clinico-pathological parameters. However, Seitz et al., (2001) reported a significant association between LOH at 17p13.3 and tumor size, grade, proliferative activity and oestrogen receptor, but ruled out an association with the lymph node and metastasic status.
Yet another study (Nagahata et al., 2002) reported the significant association between the LOH at 17p13.3 region and the lymph-node metastasis (p<0.0478) in a cohort of 504 patients who had undergone surgery for breast cancer. It was proposed that this could serve as a negative prognostic indicator. Although the correlation of 17p13.3 LOH with disease prognosis has been seen by others in breast cancer (Liscia et al., 1999; Emi et al., 1999; Nagai et al., 1994), which was not attempted in this study due to long term, follow up requirement of patients. Moreover, the focus of our study was to explore a minimal lost region (MLR) for further fine mapping of 17p and 17q regions by bioinformatics tools coupled with experimental support. Based on the results of LOH at 17p13.3 in sporadic breast cancer patients, it could be suggested that the tumors from such patients could be evaluated with such markers for prognosis, though the significance of this remains to be established. The LOH at 17p13.3 have been associated with negative as well as positive prognosis. Hirano et al., (2001) reported that those cases which showed the loss at 17p13.3 region showed significant association with the postoperative recurrence. The significance of LOH at 17p.13.3 as prognostic marker was also evaluated in other cancers. Nakayama et al., (2003) observed that ovarian cancer patients who were treated with cisplatin drug and carried LOH at 17p13.3 region in their tumors showed the reduced survival rate in comparison to patients who retained the heterozygosity at this region (17p13.3). The 5-year mortality rates for patients exhibiting allele loss and patients with allele retention was 55% and 40%, respectively, for 17p13.3 (P<0.0489) (Nakayama et al., 2003).

The studied sporadic breast tumors also showed microsatellite instability (MSI), reflecting a probable defect in mismatch repair system. Both the markers, D17S5 and D17S379, showed the microsatellite instability (MSI) in 12% and 6% of the studied tumors, respectively. The percentage of the MSI phenotypetype at 17p13.3 region was 20%. Tumors which presented with MSI in 17p13.3 region did not show any association with any stage, in this study, although the MSI phenotype was shown to correlate with clinicopathological parameters and disease prognosis in other cancers (Guo et al., 1999; Diep et al., 2003; Watanabe et al., 2003). It has been reported that microsatellite instability is uncommon in breast tumor (Anbazhagan et al., 1999). However, the presence of the MSI phenotype at 17p13.3 region suggested the existence of a ‘mutator’ phenotype in the studied tumors probably due to a defect in mismatch repair gene or gene expression in the studied tumors (Huang et al., 1999).
The long arm of chromosome 17 has also been reported as a frequent target of cancer associated genetic anomalies with recurrent chromosomal breakpoints, structural-balanced and unbalanced rearrangements (Mitelman et al., 2003). A number of known cancer genes have been localized on chromosome 17q, among which ERBB2, BRCA1, NME1 have been shown to be involved most frequently in breast cancer (http://www.infobiogen.fr/services/chromcancer/). The ERBB2, localized at 17q12-q21, has been known to amplify in high-grade tumors (Courjal et al., 1997). The position of BRCA1 and NME1 at 17q21 corresponds to regions of chromosomal losses in breast cancer (Cropp et al., 1993; Niederacher et al., 1997). The introduction of the long arm of human chromosome 17 through microcell-mediated chromosome transfer has shown the suppression of malignant phenotype of the prostate cancer cell line PPC-1, indicating the possible presence of a tumor suppressor gene (TSG) on chromosome 17q (Murakami et al., 1995). The 17q21-23 regions have been shown to suffer from the allelic losses in: breast cancer (Plummer et al., 1997; Niederacher et al., 1997; Silva et al., 1999; Rio et al., 1999; Dumur et al., 2003), prostate cancer (Clark et al., 2003) and colon cancer (Garcia-Patino et al., 1997). These conclusions were drawn on the basis of loss of heterozygosity (LOH) analysis. The same region was also reported to be amplified in the Fallopian tube carcinoma (Snijders et al., 2003), neuroblastoma (Matthay et al., 2003), hepatocellular carcinoma (Kim et al., 2003; Crawley et al., 2002), cervical cancer (Harris et al., 2003), pancreatic cancer (Mahlamaki et al., 2002) and breast cancer (Monni et al., 2001) studied by comparative genomic hybridization (CGH) analysis. Since the resolution and the sensitivity of LOH is considered to be more than the CGH (Lakhani et al., 2001), this study chose to use loss of heterozygosity (LOH) parameter at four STS marker loci in this region of the 17 chromosome to narrow down to a minimal lost region (MLR) within the area.

Genetic alterations were studied on 17q arm in sporadic breast tumors at four syntenic STS repeat markers (D17S855-D17S934-D17D787-D17S948), between 17q21-23 regions, covering an approximately 18 Mb region on build 33 sequence map. It was observed that D17S855 marker was lost in 26 % of informative sporadic breast tumors. This marker incidentally is intragenic to BRCA1 gene. Other studies have reported the varying frequency (Johnson et al., 2002; Yamashita et al., 1996; Rouba et al., 2000; Ando et al., 2000) of loss of D17S855 marker in breast tumors. Flanking region (200-400 Kb region) to D17S855 marker, which includes the BRCA1 gene, has been reported to be subjected to a recombination suppression mechanism, showing nearly complete linkage
disequilibrium for a series of common biallelic polymorphisms, all of them with rarer allele frequency close to 0.4 (Osorio et al., 2003). It has been observed in Spanish population that individuals with specific allele of D17S855 (139 and 141 bp) carry BRCA1 germ line mutation frequently than controls (54% vs. 31%, p = 0.0004) (de la Hoya et al., 2002), suggesting a role of the specific allele in conferring high risk. The association between the LOH at this locus and clinicopathological variables were found to be non-significant as well. There are studies which have reported a significant association of LOH in BRCA1 locus and at D17S855 locus with clinico-pathological variables (Silva et al., 1999; Regitnig et al., 2002). Silva et al. (1999) observed LOH in the BRCA1 region in 47% of tumors and correlated significantly with estrogen receptor content (p = 0.025), progesterone receptors (p = 0.004), higher grade (p = 0.0008), peritumoral vessel invasion (p = 0.001), and lymph node metastases (p = 0.002). Fukino et al., (1999) reported allelic loss on 17q (D17S934 locus and TOC locus) in primary tumors. Associations were observed between the solid-tubular histologic type (P = 0.0129) and in estrogen-negative and progesterone-negative tumors (P = 0.0281 and 0.0196).

Among the other studied markers from the 17q region, D17S934 marker showed a high frequency of loss (LOH) (54%) in our studied cases. Previous reports on D17S934 locus had observed LOH in 45% of primary breast tumors (Fukino et al., 1999). The alteration at D17S934 locus is not reported frequently but this marker locus has been linked to the Gordon's syndrome (GS), a salt-sensitive, hyperkalaemic, familial hypertension syndrome which may masquerade in milder forms as essential hypertension (O'Shaughnessy et al., 1998). The absence of microsatellite instability at D17S934 locus, in this study, with a high frequency of loss, made the somatic alteration (LOH) at D17S934 locus important to explore the locus further. The flanking markers on either side of D17S934 locus (D17S855 and D17S787, D17S948) also showed a loss (LOH) thus, suggesting the importance of the whole region (17q21-23) in sporadic breast cancer pathogenesis.

The loss of D17S787 marker and D17S948 was previously reported in the high grade of breast tumors (Plummer et al., 1997). The Markers on 17q21-24 regions were also evaluated for the microsatellite instability phenotype. Marker D17S855, D17S787 and D17S948 showed MSI in 10%, 35% and 9% of the studied tumors, respectively. The MSI at D17S787 locus was significantly associated with the stage II tumors in this study (p<0.009), suggesting that the MSI at D17S787 locus could have acted as an early event in the studied tumors. Although there is no report of MSI at D17S787 locus in breast tumors.
but this marker's potential has been exploited as diagnostic marker along with other marker loci in colorectal cancer (de Leon et al., 1999). Previously the D17S787 region has been found to be duplicated in renal cell tumors (RCT). Balint et al., (1999) reported the presence of trisomy of chromosome 17 in association with RCT and mapped in approximately 300 kb genomic sequences flanked by the two markers D17S787 and D17S1799 for possible presence of a putative gene associated with RCT. It was further observed in this study that the loss (LOH) of D17S948 marker was significantly (p<0.030) associated with the stage III tumors. The same marker, D17S948, also exhibited the instability (MSI), supporting further the conclusions drawn of the 17q21-23 region to be very prone to genomic loss or instability.

Although the sample size in this study was small, nevertheless, the main aim of the study was to focus on a specific chromosome region for in-silico analysis and carry out ab-initio gene prediction, based on the initial observations of high LOH and MSI in specific regions of chromosome 17. It was interesting to find high MSI in sporadic breast cancer cases, the phenotype not reported frequently in the sporadic breast tumors, suggesting the existence of a mutator phenotype in our cases, which could have arisen due to a defect in the mismatch repair pathway (Benachenhou et al., 1999). Observation of a significant correlation between the MSI and LOH phenotypes for some of the markers studied and a correlation of these with the stage II and III of sporadic form of breast cancer was again interesting. To conclude, the observations made in this study suggested that D17S934 and the D17S787 marker regions in the long arm of chromosome 17 were important to investigate further to fine map the region for localization of a putative tumor suppressor gene. The two minimal deleted (MLR) regions (I-D17S5-D17S379; II- D17S934-D17S787) observed in this study became the focus for in-silico mapping of the region. The availability of the high-resolution human genome sequence map made the job easy and this became the part of this work in the form of the second chapter (CHAPTER 2). Based on the results from the first chapter, it was further realized, to investigate the relevant and non-relevant candidate gene regions to understand the germline status of these genes and evaluate generalized genomic instability by somatic mutation analysis as a part of CHAPTER 3, since most of the study samples were advanced stage tumors and showed genomic instability which correlated with the marker status mentioned in detail already.
1.5 References


Harris CC. p53 tumor suppressor gene: from the basic research laboratory to the clinic—an abridged historical perspective. *Carcinogenesis* 1996; 17:1187-98.


Richards RI, Sutherland GR. Simple repeat DNA is not replicated simply. Nat Genet. 1994; 6: 114-6


Siah SP, Quinn DM, Bennett GD, Casey G, Flower RL, Suthers G and Rudzki Z. Microsatellite instability markers in breast cancer: a review and study showing MSI was not detected at ‘BAT 25’ and ‘BAT 26’ microsatellite markers in early-onset breast cancer. Breast Cancer Res.


