Introduction

INTRODUCTION

Cancer is a multi-step process during which cells acquire genetic or epigenetic alterations that drive the progressive alterations and transformation of normal cells into highly malignant cells. Self-sufficiency in growth, insensitivity to anti-growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis are signatures of transformed cells (Hanahan and Weinberg *et. al.,* 2010). These causal factors may act together or in sequence to initiate or promote carcinogenesis. The unregulated growth of cancerous cells is caused by damage to DNA, resulting in mutations in genes that encode for proteins involved in cell cycle regulation. Thus cancer is a genetic and/or epigenetic disease caused by mutations, by environmental carcinogenic agents and/or by epigenetic changes. Almost all organs and cell types can succumb to oncogenic transformation, giving rise to cancer. For a long time cancer has been considered as a group of diseases driven by genetic modifications including chromosomal translocations, gene amplifications or point mutations in oncogenes and/or tumor suppressor genes or DNA repair genes. However, it is now becoming increasingly clear that epigenetic changes that occur without alteration in DNA sequence, such as DNA methylation and histone tail modifications convey an altered gene expression pattern which can also lead to cancer progression.

Cancer is one of the most dreaded diseases of humankind characterized by uncontrolled growth of cells. The unregulated growth that characterizes cancer is caused by damage to DNA, resulting in mutations to genes that encode for proteins controlling cell division. As the average age along with cancer-related deaths in many countries are steadily rising, cancer will be one of the most common causes of death in the 21st century. Almost all organs and cell types can succumb to oncogenic transformation, giving rise to cancer.

Cancer is a multi-step process requiring several changes in the genetic and molecular machinery of a cell. These changes may be also brought about by infection(s) caused by a single or multiple pathogenic agent(s) or damage to the genetic material by environmental factors which may cause sequential mutations in the cancer predisposing/

growth regulating genes. There are at least three types of cellular genes such as oncogenes, DNA repair genes and tumor suppressor genes, which have been strongly implicated in the development of human cancers. Activation of oncogenes by molecular and chromosomal rearrangements in co-ordination with cellular transcription factors, mutation in tumor suppressor genes or interaction of tumor suppressor gene products with viral oncoproteins, loss of function of DNA repair genes along with several other etiological factors may contribute to the development of cancer.

According to recent IARC report, worldwide incidence of cancer was 12.7 million new cases with a mortality rate of 7.6 million cases in 2008. In India, cancer incidence was 9, 48, 900 (new cases) with mortality of 6, 33, 500 cases in 2008 (Ferlay *et. al.,* 2008 GLOBOCAN). The most common types of cancers that affects humans are lung cancer, oral cancer, breast cancer, gastrointestinal cancer, cervical cancer, endometrial, kidney and thyroid cancer. In India, most common cancers in males are lung, oral cavity, esophagus and stomach whereas, cervical cancer has been found to be a leading cause of cancer related deaths in females followed by breast cancer (Das *et. al.,* 2008).

Breast cancer remains the most common malignancy in women worldwide and is the leading cause of cancer-related mortality in females with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers), and ranks second overall (10.9% of all cancers). It is now the most common cancer both in developed and developing regions (Ferlay *et. al.,* 2008 GLOBOCAN). Incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.9 per 100,000 women in Western Europe, and are higher (greater than 80 per 100,000) in developed regions of the world (except Japan) and low (less than 40 per 100,000) in most of the developing regions. The range of mortality rates is much less (approximately 6-19 per 100,000) because of the more favourable survival of breast cancer in (high-incidence) developed regions. In India, breast cancer is the second most common malignancy among women with respect to incidence, frequency, and mortality (Yeole *et. al.,* 2008). According to recent IARC report, annual incidence of breast cancer in India was 115, 251 with a mortality of 53, 592 (Ferlay *et. al.,* 2008 GLOBOCAN). Till recently cervical cancer had the highest incidence in India but recently it has been

overtaken by breast cancer as the most commonly diagnosed cancer among women particularly in urban area of India (Pal and Mittal *et. al.,* 2004).

Overall, there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons alive with cancer (within 3 years of diagnosis). The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million) and colorectal (1 million); the most common causes of cancer death are lung cancer (1.18 million deaths), stomach cancer (700,000 deaths), and liver cancer (598,000) deaths. The most prevalent cancer in the world is breast cancer (4.4 million survivor's upto 5 years following diagnosis) (Parkin *et. al.,* 2005).

In India, although the nationwide figures on cancer incidence, prevalence and mortality are not available, data from six population-based and five hospital-based cancer registries established under National Cancer Registry Program (NCRP, 2005) of Indian Council of Medical Research (ICMR) suggest that cancer of breast is one of the most prevalent cancers second to cancer of uterine cervix in women (Mitra *et. al.,* 1989; Rao *et. al.,* 1998). But as to recent report of NCRP, breast cancer is the leading cancer in metropolitan cities Chennai, Mumbai and Delhi.

EPIGENETICS AND CANCER

Epigenetics refers to a heritable trait caused by changes other than those in the primary sequence of a genome. This particularly involves change in the ability of a gene to transcribe itself; it may either up-regulate or down-regulate the transcription of epigenetically altered genes. The major mediators of such epigenetic alterations are changes in methylation status of CpG islands in promoter region of tumor suppressor genes, acetylation or deacetylation of specific amino acid residues in histone tails and tightly regulated targeted degradation of mRNAs using mi-RNAs. It is of great interest in cancer related studies as a cause and potential cure for the disease which is an explicit result of over-expression of certain genes (oncogenes) while silencing of others (tumor suppressor genes) that are involved in crucial cell signalling pathways regulating cell growth, proliferation and cell death. Several tumor suppressor and other cancer genes

have been found to be hypermethylated in cancer but unmethylated in normal cells, e.g., RAS association domain family protein 1A (RASSF1A), adenomatous polyposis coli (APC), and death associated protein kinase (DAP-kinase) genes (Esteller *et. al*., 2001; Lehmann *et. al*., 2002; Dammann *et. al*., 2001; Jin *et. al*., 2001). Out of these, one class of genes frequently found silenced in association with promoter methylation in cancer are HIC-1(Hypermethylated in cancer-1) and GST-Pi (Glutathione S- transferase Pi), BRCA1, p16 and E-cadherin.

A number of oncogenes as well as tumor suppressor genes in the development and prognosis of breast cancer continue to be the subject of intensive investigation. Among the most studied tumor suppressor genes, retinoblastoma susceptibility gene Rb and nuclear phosphoprotein gene p53 have been strongly implicated in various types of tumors (Lee *et. al.,* 1990; Lane and Benchimol *et. al.,* 1990; Green *et. al.,* 1989). However, the list of tumor supressor genes found to be frequently methylated in many tumors is constantly growing with new TSGs with hypermethylated promoter regions being found and there have been attempts to characterize the methylation profile of different tumors (Esteller *et. al.,* 2001).

The genetic and epigenetic alterations that initiate and drive tumorigenesis are promising targets for early detection because they may precede clinically obvious cancer, can be detected at sensitive levels, may be specific to tumor cells, and can potentially provide information about the prognosis and treatment of the disease (Sidransky *et. al.,* 2002; Laird *et. al.,* 2003). CpG islands located in promoter regions of genes are normally unmethylated. In cancer cells, aberrant hypermethylation of these promoter regions is associated with transcriptional silencing. Hypermethylation is therefore an alternative mechanism for inactivation of tumor suppressor genes (Baylin *et. al.,* 1998; Jones *et. al.,* 1999). Because gene hypermethylation has been found to be a common and early alteration in many tumor types (Belinsky *et. al.,* 1998; Esteller *et. al.,* 2000; Esteller *et. al.,* 2001), including breast (Lehmann *et. al.,* 2002; Holst *et. al.,* 2003), it has emerged as a promising target for detection strategies in clinical specimens (Sidransky *et. al.,* 2002; Laird *et. al.,* 2003).

Epigenetics is of interest in cancer related studies as a reputed cause and potential cure for the disease which is an explicit result of over-expression of certain genes (oncogenes) while silencing of others (tumor supressor genes). Since such genes are mostly involved in crucial cell signalling pathways regulating cell growth, proliferation and cell death etc, their altered expression profiles often progressively lead to this life threatening condition.

One class of genes frequently silenced in association with promoter methylation can actually foster carcinogenesis by leading to genetic instability in cells with the resultant accumulation of gene mutations. This effect involves epigenetic silencing of genes that normally act to prevent or correct DNA damage (Jones *et. al.,* 2002). For example, one of the most commonly suppressed genes in epithelial cancers, lymphomas, and lymphocytic anemias is the tumor suppressing gene *p16INK4a.* Experimental models have shown that the epigenetic suppression of this gene early in the cell growth process results in the bypass of key control mechanisms that ordinarily trigger cell senescence or death, (Foster *et. al.,* 1998) and this then allows for the accrual of chromosomal abnormalities and gene mutations. The loss of the gene *GST-Pi*, a nearly universal event in prostate cancer, predisposes cells to oxidative damage, especially at adenines (Lee *et. al.,* 1994). The loss of a DNA repair gene, *O6-methylguanine-DNA methyltransferase (MGMT)*, which prevents G to A transitions, occurs early in the course of colon cancer and results in the accumulation of these transitions in important regulatory genes such as *K-RAS* and *p53* (Jones *et. al.,* 2002). Of the 10% to 15% of patients with colon cancer who have microsatellite instability, approximately 70% to 80% exhibit epigenetic gene silencing of a mismatch repair gene, *MLH1* (Kane *et. al.,* 1997; Herman *et. al.,* 1998). In addition, methylated cytosine is directly mutagenic, undergoing spontaneous C to T transitions (Coulondre *et. al.,* 1978).

Of special interest here are the tumor supressor genes like CDH1, BRCA1, RAR-B2, APC, HIC1, GSTP1, $p16^{ink4a}$, p53, and many more. As their name suggests, these genes when functioning normally prevent the formation of tumor by keeping the cell division cycle in tight check. When such genes are silenced as is the case in most tumors, unregulated proliferation of cells results. Thus epigenetic changes correlated with

development of cancer. However it is still a matter of debate whether these are causing cancer or are result of it.

BRCA1 gene

In 1990, the first breast cancer susceptibility gene BRCA1 was localized to chromosome 17q12-21 by linkage analysis of multiple families affected by early onset breast and ovarian cancer and cloned 4 years later. BRCA1 is large spread over 80 kb genomic DNA composed of 24 exons, 22 codings and 2 non-coding exons that are transcribed into a 7.8 kb mRNA encodes a protein containing 1863 amino acids (Miki *et. al.,*1994; Smith *et. al.,* 1996). The approximate molecular mass of the BRCA1 protein is 220 kDa (ilki *et. al.,* 1994). The BRCA1 gene bears no homology with other genes, with the exception of a RING finger motif at the amino-terminal end. In other proteins such a motif has been shown to interact with nucleic acids and to form protein complexes, suggesting a role of BRCA1 in transcription. Nuclear localization sequence in exon 11, and a conserved acidic carboxy terminus, the BRCT (BRCA1 carboxyl terminal) domain. To date, more than 800 different mutations in the BRCA1 gene have been reported. The majority of these are frameshift or nonsense mutations located throughout the gene. BRCA1 gene contains N-terminal RING finger domain and two C-terminal BRCT (BRCA1-C-Terminal) domain, both involved in protein-protein interactions. Exon 11 of BRCA1 gene contains over 60% of protein and encodes two putative localization signals, also contain a domain that interacts with RAD51, a homology of e. coli rec. a involved in DNA damage repair. BRCA1 was associated and co-purified with RNA polymerase complex, and interacts with RNA helicase a in transcription. BRCA1 has little effect with transcription controlled by USF, Jun, fos, or ga14.

Several BRCA1 mutation founders have been identified with; the two most common are 185delAG and 5382insC, which account for approximately 10% of all the BRCA1 mutation (Couch and Weber, 1996). Germline mutation in BRCA1 has been detected in approximately 80-90% of familial breast/ovarian cancer and about 45-50% of familial breast cancer alone (Alberg and Helzlsouer *et. al.,* 1997; Paterson *et al.,* 1998).

p16 gene

The p16 gene encodes a cyclin-dependent kinase inhibitor, $p16^{ink4A}$, which regulates the transition from G1- to S-phase via its effect on Rb phosphorylation (Liggett & Sidransky *et. al.,* 1998). *p16* silencing has been proposed as a possible contributor to breast tumorigenesis (Foster *et. al.,* 1998). Tumor suppressor gene p16, plays an important role during the carcinogenesis of esophageal, gastric, breast and colorectal carcinomas (Zhou X *et. al.,* 1994; Toyota M *et. al.,* 1999; Nakayama H *et. al.,* 2002; Munot K *et. al.,* 2006; Jing F *et. al.,* 2007; Sharma G *et. al.,* 2007; Di Vinci A *et. al.,* 2005). Recently, p16 has been found to horbor promoter hypermethylation associated with a loss of protein expression in cancer cells. In fact, the presence of p16 gene promoter methylation has been demonstrated frequently in gastric cancers, and this methylation was useful as a molecular target for tumor cell detection in the serum (Hibi K *et. al.,* 2001). Methylation profile of p16INK4A promoter differed in each cancer type. 5-aza-dC induced growth inhibition might be resulted from the release of methylation silenced cell cycle regulatory gene p16INK4A. There are significant differences in the regulatory response to DNA methylation in different genes including tumor suppressor gene and proto-oncogenes (Fang JY *et. al.,* 2003). Quantification of epigenetic changes in genes like p16INK4A in peripheral blood is useful for the detection and monitoring of hepatocellular carcinoma (Wong IH *et. al.,* 2003). Methylation of CDH1 and CDH13 has been suggested a possible marker for cervical cancer risk assessment (Widschwendter A *et. al.,* 2004). p16, E-cad, DAPK, and MGMT etc. have been shown to be frequently methylated in head and neck squamous cell carcinoma (Righini CA *et. al.,* 2007).

E-cadherin gene

The E-cadherin gene encodes a cell-surface adhesion protein that is important in maintaining homophilic cell–cell adhesion in epithelial tissues (Ilyas & Tomlinson *et. al.,*

1997). Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer (Oka *et. al.* 1992). In breast cancer, CDH1 promoter methylation has been reported in approximately 30% of in-situ ductal carcinomas and increased substantially to nearly 60% in metastatic tumours (Nass SJ *et. al.,* 2000). Furthermore, this gene is one of the most frequently inactivated by methylation in sporadic breast cancer (Parrela P *et. al.,* 2004), as well in DNA samples obtained from plasma of invasive breast cancer patients (Hu XC *et. al.,* 2003), from fine needle washings from breast lesions (Jeronimo C *et. al.,* 2003) and in sentinel lymph node metastasis (Shinozaki M *et. al.,* 2005). These findings suggested that CDH1 promoter methylation is an important event associated with the pathogenesis of breast cancer.

Several reports have demonstrated the association either of the CDH1 gene methylation (Nass SJ *et. al.,* 2000; Parrela P *et. al.,* 2004; Hu XC *et. al.,* 2003; Jeronimo C *et. al.,* 2003; Shinozaki M *et. al.,* 2005) or the abnormal expression of E-cadherin in breast cancer progression (Asgeirsson KS, *et. al.,* 2000). However, there are limited number of studies directly correlating CDH1 methylation and E-cadherin expression in the same sample of breast carcinomas (Graff JR *et. al.,* 2000). The E-cadherin expression may be repressed by mechanisms other than promoter hypermethylation, such as allele loss (LOH), gene mutation, changes in chromatin structure (Henning G *et. al.,* 1996) and alterations of specific transcription pathways regulating the expression of CDH1 gene (Hajra KM *et. al.,* 1999; Peinado H *et. al.,* 2004).

The phenomenon of methylation is also being targeted for anti cancer therapy. Several methylatin inhibitors, histone deacetylase inhibitors and demethylating agents are undergoing trials for their therapeutic effects (Yang *et. al.,* 2001; Szyf *et. al.,* 2004).

HIC1 gene

HIC-1 (Hypermethylated in cancer-1) as it name implied, was originally isolated as a new candidate tumor suppressor gene located at 17p13.3 and frequently targeted for

allelic loss and CpG island hypermethylated in human cancers (Wales *et. al*., 1995). HIC1 encodes a transcription factor associating an N-terminal BTB/POZ domain to five C-terminal Krüppel-like C2H2 zinc finger motifs. There is some evidence from human tumor specimens to suggest that epigenetic HIC-1 silencing events predispose tissues to tumorigenesis and have better prognosis value in different cancers such as prostate cancers (Morton *et. al*.,1996); non-small cell lung cancers (Eguchi *et. al*., 1997; Hayashi *et. al*., 2001); breast cancers (Fujii *et. al*., 1998); gastric and liver cancers (Kanai *et. al*., 1998; Kanai *et al*., 1999); esophageal cancers (Huang *et al*., 2000; Eads et al., 2001) and human male non-seminomatous germ cell tumors (Koul *et. al*., 2002). Epigenetic silencing of HIC1 has been also very frequently observed in the most common malignant brain tumor of childhood medulloblastoma (Rood *et. al*., 2002; Waha *et. al*., 2003) and the glial malignancy, ependymoma (Waha *et. al*., 2004). Further characterizing HIC1 function and its direct transcriptional target swould not only help to understand its role as a tumor suppressor gene and provide new insights into epigenetics and transcriptional repression in general but would also open new therapeutic approaches to major human cancers.

GST-Pi gene

GST-Pi (Glutathione S- transferase Pi) GSTs' are a family of enzymes implicated in the detoxification of a wide range of xenobiotics and chemotherapeutic agents. GSTs catalyze the conjugation of glutathione with electrophilic compounds including carcinogens and exogenous drugs, resulting in less toxic and more readily excreted metabolites. There are four distinct classes (α, μ, θ and π) of isozymes in the GST superfamily, each encoded by a different gene at different loci and with peculiar structural and functional characteristics. The pi-class GST (GST-pi) is of particular interest to the study of cancer biology. GST-pi is expressed in normal tissues at varying levels in different cell types, and abnormal GST-pi activity and expression have been reported in a wide range of tumors including those of the breast and kidney (Cairn J *et.*

al., 1992; Silvestrini R e*t. al*., 1997). GST-pi is encoded by the GSTPI gene located in chromosome 11. The 5' region of GST-pi contains a CpG island, and in cancer cells, the hypermethylation of the CG-rich area in the promoter region of tumor suppressor genes correlates with its loss of transcription, as demonstrated for many tumor suppressor genes. Recently, hypermethylation of regulatory sequences at GST-pi associated with the loss of GST-pi expression has been found in the vast majority of human prostate carcinomas with poor prognosis (Lee WH *et. al*., 1994). However, little is known about epigenetic silencing of GST-pi gene by promoter hypermethylation in the precursors of breast cancer and other tumor types. Consequently, the significance of alterations in GST-pi promoter hypermethylation status during multistage carcinogenesis opens new avenues for cancer chemoprevention based on the inhibition or reversal of epigenetic alterations before the onset of cancer and could pave the way for the use of DNA methylation as cancer biomarkers for better patient prognosis.

Considering the potential role of promoter hypermethylation in silencing of tumor suppressor genes in breast cancer, the following study has been designed with the following objectives:

1. **Analysis of promoter methylation of tumor suppressor genes, BRCA1, E-cadherin, p16, GSTPi and HIC-1 in sporadic breast cancer patients in comparison to controls.**
2. **To study the expression of BRCA1, E-cadherin, p16, GSTPi and HIC-1 proteins in breast cancer patients and controls by Western blotting and their in situ correlation by Immunohistochemistry.**
3. **To correlate the promoter methylation status and gene expression level with clinico-pathological attributes of the breast cancer patients.**