

Discussion

Discussion

Breast cancer is now the leading cause of cancer death among females in economically developing countries, a shift from the previous decade during which the most common cause of cancer death was cervical cancer (Jemal *et. al.*, 2011). Cancer is characterized by uncontrolled cell proliferation or cell's failure to obey the normal rules of growth and proliferation due to accumulation of abnormalities in the genes that control cellular growth and differentiation. Variety of genes has been implicated in the development of cancer, which usually occurs due to inactivation, over-expression or altered signalling of genes. Recent studies demonstrate that initiation and progression of cancer is controlled by both genetic and epigenetic events. Unlike genetic alterations, which are almost impossible to reverse, epigenetic aberrations are potentially reversible. First introduced by C.H. Waddington in 1939 to name "the causal interactions between genes and their products, which bring the phenotype into being," epigenetics was later defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence. The best-known epigenetic marker is DNA methylation. The initial finding of global hypomethylation of DNA in human tumors was soon followed by the identification of hypermethylated tumor-suppressor genes. These and other demonstrations of how epigenetic changes can modify gene expression have led to human epigenome projects which study epigenetic changes in cancer cells by three powerful diagnostic applications such as classification markers, sensitive detection markers, and risk assessment markers that offer unique prospects for potential cure of the disease.

While many studies have identified cancer causing oncogenes and tumor suppressor genes that mark the transformation of cells from several tissue types, such as colon, pancreas and lung, comparable studies in breast cancer have met with limited success. This reflects the difficulty in finding genetic and epigenetic alterations that are present in a significant proportion of breast cancers and the phenotypic and genetic heterogeneity of breast cancer itself. Out of these, genes which are found frequently silenced in association with promoter methylation as a result of epigenetic abnormalities could effectively be considered as diagnostic and therapeutic tool for disease management.

BRCA1 (Breast cancer susceptibility gene1), p16 gene, E-cadherin 1 (CDH1), HIC-1 (Hypermethylated in cancer-1) and GSTPi (Glutathione S- transferase Pi) are few of the genes that are often implicated with epigenetics of cancer in general and breast cancer in particular. Therefore, in the present study these five tumor suppressor genes have been investigated to see if the methylation pattern could serve as reliable biomarker for progression or prognosis of breast cancer.

1. Promoter hypermethylation correlates with clinicoepidemiological risk factors for breast cancer

In present study, the age of patients ranged from 39-79 with a mean age of 56(SD \pm 10.28) years. The most frequent age group that affected by breast cancer in our study is found to be 50-59 years. Also, these appears to be not much change in age distribution of breast cancer in India in the last one decade though many breast cancer cases are seen to be in early age group of 25 to 35 years. It indicates majority of breast cancer cases are found to be in postmenopausal women as in USA. There are several factors which may be responsible for susceptibility to breast cancer including socioeconomic status (Devesa *et al.*, 1980; Liu *et al.*, 1988; Yost *et al.*, 2001), life style, smoking (Palmer *et al.*, 1993; Couch *et al.*, 2001), dietary factors (Hunter *et al.*, 1996; Smith-Warner *et al.*, 2001) and obesity etc. Besides these epidemiological and environmental factors, various genetic and epigenetic factors play important role in breast carcinogenesis. Epigenetic silencing of tumor suppressor genes by methylation of promoter region of such genes or through histone modification plays an important role in pathogenesis of many types of cancers, including breast cancer. Several studies have reported tumor related genetic and epigenetic alterations in serum, plasma, ductal lavage fluids and fine needle aspirates of breast cancer patients (Muller *et al.*, 2003; Laird, 2003; Krassenstein *et al.*, 2004; Dulaimi *et al.*, 2004; Shukla *et al.*, 2006). The aim of this study was to determine whether the methylation status of genes with a putative role in breast cancer were associated with distinctive pathological characteristics including prognosis.

Promoter methylation of several genes increases with age in normal tissues, although the mechanism of age-related methylation is unknown (Ahuja and Issa *et al.*, 2000). Several factors may modulate age-related methylation, such as exogenous carcinogens,

endogenously generated reactive oxygen species, and genetic differences in individual susceptibility (Ahuja and Issa *et. al.*, 2000). These factors with age related methylation contributes to carcinogen. Clinical outcomes in breast cancer often require prolonged follow up and prospective methylation based clinical studies are limited, nevertheless, even in this limited period methylation status of GSTP1 and BRCA1 in tumors was associated with significant risk for recurrence in breast cancer patients. Previous report had also shown significant association of GSTP1 CpG islands with age related hypermethylation with poor prognosis in breast cancers (Arai *et. al.*, 2006).

Another gene p16, is involved in cell cycle and perturbations in the cell cycle are the most frequent events in human cancers including breast cancer. Defects in many of the genes that regulate the cell cycle have been implicated in cancer development and progression. The pRb (pRb/p16INK4a/ cyclin D1) and p53 (p14ARF/mdm2/p53) pathways are the two main cell-cycle regulatory pathways frequently targeted in tumorigenesis, and the alterations occurring in each pathway depend on the tumor type. Virtually all human tumors show deregulation of either the pRb or p53 pathway, or both pathways simultaneously (Stewart *et. al.*, 2001; Macaluso *et. al.*, 2005). p16 is the most commonly inactivated tumor suppressor gene in human cancer and is a key component of the pRb pathway. p16 is a CDK inhibitor that form complexes with CDK4, CDK6, and D-type cyclins to arrest cell cycle's progression from G1 to S phase (Sherr CJ *et. al.*, 1996). Hypermethylation of p16 gene has been reported to be an early and critical step in breast cancer development (Holst CR *et. al.*, 2003; Tlsty TD *et. al.*, 2004).

In our study, p16 promoter was found to be methylated in 27.58% of the cases which lies in the reported range by other authors from India. Raish *et. al.*, (2009) have reported 31.9% prompter methylation of p16 in breast cancer in northern India. Similarly Sharma *et. al.*, (2007) reported p16 to be hypermethylated in 44% of north Indian sporadic breast cancer patients. Various studies done world over on the promoter methylation of p16 gene report the frequency to vary between 15-55% (Woodcock *et al.*, 1999; Raish M *et. al.*, 2009; Esteller M *et. al.*, 2001; Jing F *et. al.*, 2008; Herman *et. al.*, 2009; Voyatzi *et. al.*, 2010). Our results are also in concordance with other authors.

Besides breast cancer, methylation of *p16* has also been observed in lung cancer (25–60%) (Kim DH *et. al.*, 2001), colorectal carcinoma (5–25%), esophageal squamous cell carcinoma (40%) (Xing E *et. al.*, 1999). These findings imply that p16 might play an important role in the initiation and progression of lesions and carcinoma that can act as a crucial event in cell transformation and tumorigenesis.

The other interesting gene under investigation was E-cadherin (CDH1), an extremely important molecule in cell-cell adhesion for epithelial tissues. It is localized on the surfaces of epithelial cells in regions of cell-cell contact known as adheren junctions (Gumbiner BM *et. al.*, 1996). It is essential for the formation and maintenance of epithelia (Gallin WJ *et. al.*, 1987). Besides its role in normal cells, this highly conserved gene can play a major role in malignant transformation, and tumor development and progression. The suppression of E-cadherin expression is regarded as one of the main molecular events responsible for dysfunction in cell-cell adhesion. Most tumors have abnormal cellular architecture, and loss of tissue integrity can lead to local invasion. Thus, loss of function of E-cadherin tumor suppressor protein correlates with increased invasiveness and metastasis of tumors (Vleminckx K *et. al.*, 1991), and hence referred to as “suppressor of invasion” gene.

CDH1 gene is one of the most frequently inactivated genes by methylation in sporadic breast cancer patients (Parrela P *et. al.*, 2004), and it can be analyzed in DNA samples obtained from plasma of invasive breast cancer patients (Hu XC *et. al.*, 2003), from fine needle aspirates from breast lesions (Jerônimo C *et. al.*, 2003), and in sentinel lymph node biopsies (Shinozaki M *et. al.*, 2005).

In present study we observed methylation of CDH1 gene promoter in 29/87 (33.33%) of cases. Earlier reports from India vary from 15% to 50% (Vishwanathan *et. al.*, 2006; Prasad *et. al.*, 2008). Other studies done world over report CDH1 methylation of to be 19% to as high as 80% (Toyota M *et. al.*, 1999; Nass S.J. *et. al.* 2000; Esteller M *et. al.*, 2001; Jeronimo C *et. al.*, 2003; Caldeira JR *et. al.*, 2006; Li S *et. al.*, 2006). These findings suggest that CDH1 promoter methylation is a most common molecular event associated with the pathogenesis of breast cancer. Frequency of *CDH1* methylation has been found to be maximal (84%) in esopharyngeal carcinoma (Esteller M *et. al.*, 2001), while it is as low as only 8% in lung cancer (Zochbauer-Muller S *et. al.*, 2001).

HIC-1 (Hypermethylated in cancer-1) is a zinc-finger transcription factor which act as a tumor suppressor gene and found to be aberrantly methylated in many types of human cancers with better prognosis [Prostate cancers (Morton *et al.*, 1996); Non-small cell lung cancers (Hayashi *et al.*, 2001); Breast cancers (Fujii *et al.*, 1998); liver cancers (Kanai *et al.*, 1999) and Esophageal cancers (Eads *et al.*, 2001)]. In the present study, we observed hypermethylation of HIC-1 gene in 32% cases of breast cancer as compared to control sample where no methylation was found. This could be due to loss of heterozygosity or allelic imbalance that may attribute as an early event in epigenesis of cancer. In agreement with our observation, Parella and colleagues (Parella *et al.*, 2004) have also demonstrated hypermethylation and down-regulation of HIC-1 gene. 50 % hypermethylation of HIC1 was found in 40-49% age group of breast cancer patients. Also, 48% premenopausal women with breast cancer showed promoter hypermethylation of HIC1 gene.

Similarly, GST-pi (Glutathione S- transferase pi) comprise a family of iso-enzymes expressed in normal tissues at varying levels and provide protection to mammalian cells against electrophilic metabolites of carcinogens and reactive oxygen species. Previous studies have shown that the CpG-rich promoter region of the π -class gene GST-pi is methylated at single restriction sites in the majority of prostate cancer with poor prognosis (Song JZ *et al.*, 2002). The frequency of GSTP1 promoter hypermethylation in IDCs (28%) was found to be higher as reported previously. The frequency given in other studies of breast varied between 13 to 30% (Arai *et al.*, 2006; Esteller M *et al.*, 1998; Hoque MO *et al.*, 2006; Parella P *et al.*, 2004; Shinozaki *et al.*, 2005). Several studies have demonstrated that GSTP1 expression in breast cancer ranges anywhere from 21% to 78% and one of the main mechanisms of loss of its expression is DNA methylation (Ronneberg *et al.*, 2008). Consistent with previous reports, we observed a significant association between GSTP1 CpG islands hypermethylation and tumor size as well as advanced tumor stage (Shinozaki *et al.*, 2005; Arai *et al.*, 2006). These observations indicate that breast tumors with GSTP1 CpG islands hypermethylation have a biologically aggressive phenotype.

However, little is known about epigenetic silencing of HIC-1 and GST-pi gene by promoter hypermethylation in the precursors of breast cancer.

Breast cancer gene 1 (BRCA1) encodes a multifunctional protein involved in DNA repair, cell cycle control, protein ubiquitinylation, and chromatin remodeling (Rahhan R *et. al.*, 2007). Approximately 26% of sporadic breast cancer cases display hypermethylation of the BRCA1 promoter in our study. These tumors are mainly estrogen receptor (ER)-, progesterone receptor (PR)- and HER2-positive. Although, Hansmann *et. al.*, (2013) showed 20% hypermethylation in sporadic breast cancer cases in ER, PR and Her2/neu negative cases. The incidence of BRCA1 methylation has previously been reported to be higher in breast tumors (Birgisdottir *et. al.*, 2006). Since all our patients are of the infiltrating ductal type, hypermethylation of BRCA1 was somewhat lower to that of current literature.

2. Promoter hypermethylation correlates with lymph node status of breast cancer patients

Breast cancer is one of the most common malignancies with a high mortality rate among women (Jemal *et al.*, 2009). Breast cancer, a heterogeneous disease, presents various pathological signs such as axillary lymph node metastasis which is associated with a high risk of recurrence and considered as an important prognosis factor in the early stages of the disease (Lindahl *et al.*, 2000; Reintgen *et al.*, 2000; Barekati *et. al.*, 2012). Invasion and metastasis are two important hallmarks of malignant tumors associated with complex genetic and epigenetic alterations that allow tumors to disseminate throughout lymphatics or blood vessels, giving rise to the colonization and growth of metastatic cells in distant organs.

Out of 87 cases, 42 cases were pathologically negative for axillary lymph nodes, while 45 cases were having positive axillary lymph nodes. p16 promoter methylation was found in 35% lymph node positive cases.

GSTP1 hypermethylation was found to be significantly associated with lymph-node metastasis. These observations indicated that breast tumors with GSTP1 CpG-island hypermethylation may possess a biologically aggressive phenotype, as suggested in

previous studies in Asian and Caucasian breast cancers (Shinozaki *et al.*, 2005; Arai *et al.*, 2006), and other cancers, such as prostate (Bernardini *et al.*, 2004) and endometrial cancer (Chan *et al.*, 2005). However, few research reports have concluded that GSTP1 hypermethylation was not associated with lymph-node metastasis in breast cancers of Asian and Caucasian women (Lee *et al.*, 2007; Pongtheerat *et al.*, 2012). Previously, it was reported that GSTP1 hypermethylation correlates with LN metastasis (Shinozaki M, *et al.*, 2005; Sunami *et al.*, 2008). Similarly in this study, GSTP1 hypermethylation was found to be more frequent in the Lymph node positive group than in the negative group in ER positive cases. In Lymph node positive group, p16 hypermethylation was found to be significantly ($P < 0.036$) in ER-positive than in ER-negative breast tumors (**Table R9**). HIC1 showed higher methylation frequency of PR positive cases having node negative status. P16 and GSTP1 showed higher frequency in node positive cases suggesting that level of methylation is also increasing during the progression in breast cancers cases.

3. Promoter hypermethylation of specific gene(s) correlates with hormonal receptor status of breast cancer patients

During the past several years, new molecular biomarkers have been discovered that are important targets for the diagnosis and therapy of breast cancer (Widschwendter *et al.*, 2004, D'Andrea *et al.*, 2007). ER, PR and HER2/neu are important prognostic biomarkers and therapeutic targets in primary breast cancer. ER-negative tumors appear to be more malignant (Kepple J *et al.*, 2006, Ma H *et al.*, 2006, Rusiecki JA *et al.*, 2005, Giacinti L *et al.*, 2006), resulting in a poorer prognosis than with ER-positive tumors (Duffy MJ *et al.*, 2006, Schiff R *et al.*, 2005, Leu YW *et al.*, 2004). ER, PR, and HER2/neu have proven to be very successful biomarkers that accurately predict benefit to treatment with tamoxifen trastuzumab and Herceptin respectively. However, it has proven more difficult to identify further biomarkers that could help predict response to specific chemotherapy drugs to tailor current chemotherapy regimens in an effort to gain maximum benefit with minimal exposure to unnecessary drugs.

The present study was conducted to identify differences in epigenetic events related to ER expression by infiltrating breast cancer. To date, few studies have rigorously assessed ER-negative and ER-positive primary breast tumors for epigenetic differences. We focused on the epigenetic differences between ER-positive and ER-negative breast cancers, and used tumor specimens for patient age, tumor size, nodal status, and presence or absence of distant metastasis. This sampling enabled rigorous analysis, and the results imply that epigenetic features of ER-positive tumors are different from those of ER-negative tumors and same is the case for PR and HER2/neu positive and negative tumors. Furthermore, we observed a significant difference in methylation status of genes p16 and GSTPi between the ER-positive and ER-negative groups (**Table R7**). In contrast, Li and colleagues (Li S *et al.*, 2006) reported that ER-positive patients exhibited a higher frequency of p16 methylation and a lower frequency of CDH1 methylation than did ER-negative patients which was not found significant. We also found no significant difference in the methylation frequencies of GSTPi, HIC1 and BRCA1 between ER-positive and ER-negative groups. The discrepancy may also have resulted from difference in the approach to the particular specimens assessed. Because methylation and ER status change with tumor progression (Yang *et al.*, 2004), It is important to take precaution when sampling ER-positive and ER-negative tumors to evaluate epigenetic changes with clinical outcomes.

To clarify when differences in methylation status between ER-positive and ER-negative tumors occur, we compared differences in methylation status between early tumor stage (T1) and later tumor stages (T2/T3) subgroups. The ER-positive group exhibited more frequent hypermethylation of GSTP1 gene ($p < 0.07$). (**Table R7**) Moreover, the ratio of methylation frequency differs between T1 and T2/T3 stage subgroups (**Table R8**). This observation indicates that the difference in methylation patterns change when breast tumors progress from T1 to T2/T3 stage. Previously, it is reported that *GSTP1* hypermethylation correlates with LN metastasis (Shinozaki M, *et al.*, 2005). Similarly in this study, GSTP1 hypermethylation was found to be more frequent in the Lymph node positive group than in the negative group in ER positive cases. Sunami *et al.*, 2008 demonstrated similar findings in case of GSTP1 gene. In Lymph node positive group,

p16 hypermethylation was found to be more common in ER-positive than in ER-negative tumors significantly ($p < 0.036$). (**Table R9**) Furthermore, the difference in methylation status of HIC1, BRCA1 and CDH1 genes between the ER-positive and ER negative groups can be recognized in early stages of cancer, such as the T1 or N0 stages. These findings suggest that ER expression may influence epigenetic changes in early stages of breast cancer.

Progesterone receptor (PR) is another important prognostic marker for breast cancer. PR gene over-expression is identified in 15% to 25% of invasive breast carcinomas, and its absence is related to metastatic potential and poor survival (Francis G *et al.*, 2006, Salmon DJ *et al.*, 1989). In our study, PR over-expression was identified in 43% of breast cancers, and was independent of ER status (**Table R1**) Hypermethylation of GSTP1 was found significant with PR status of women with breast cancer ($p = 0.05$) (**Table R11**). GSTP1 was found to be methylated more in PR positive cases. CDH1 gene also showed significant hypermethylation ($p = 0.04$) as it was found to be methylated in PR positive cases frequently. p16 gene showed a significant hypermethylation in later tumor stages relative to PR status ($p = 0.05$) (**Table R12**). The methylation frequency of p16 gene was significantly higher in PR positive cases in early stage tumors ($p = 0.03$) while in later stage tumors p16 hypermethylation was found more in PR negative cases. This suggests that level of methylation of p16 gene is increased during the progression of breast cancer and it affects the severity of the disease. BRCA1 also showed higher methylation frequency in PR positive cases which was not significant. HIC1 also showed higher methylation frequency of PR positive cases having node negative status. CDH1 and GSTP1 showed higher frequency of methylation in node positive cases suggesting that level of methylation is also increasing during the progression in breast cancers cases.

A negative relation between ER status and HER2/neu over-expression has been documented by others (Schiff R *et al.*, 2005, Huang HJ *et al.*, 2005, Isari A *et al.*, 1999). One of the plausible explanations for the lack of difference found in the frequency of HER2/neu-positive tumors between the ER-negative and ER positive groups is that patients included in the present study were relatively young (average age 56 years). According to Huang and coworkers, in women younger than 45 years the inverse

association between ER and HER2/neu was not apparent (Huang *et al.*, 2005). Of our sample population, 30% of patients were under 45 years old, and this age distribution may explain why we could not detect an inverse relation between ER and HER2/neu status. We demonstrated low methylation frequency of GSTP1 and HIC1 gene in HER2/neu positive group. However, regarding gene methylation and HER2/neu status, Eiji Sunami demonstrated that HIC1, GSTP1, and CDH1 were frequently methylated in the HER2/neu over-expressed group (Sunami *et al.*, 2008). Although we have found higher frequency of methylation of CDH1 gene in HER2 neu overexpressed group. **(Table R15)** The HER-2/neu proto-oncogene is amplified and as a result over expressed in 25% to 30% of human breast cancer and is usually associated with tumour aggressiveness and poor prognosis (Salmon DJ *et al.*, 1987). These findings indicate that there are epigenetic differences between HER2/neu breast tumors and these differences are independent on ER status. However, we have found a significant correlation of GSTPi gene hypermethylation between ER+/PR+ cases and triple negative cases ($p=0.02$). **(Table R18)** This shows that GSTPi hypermethylation is associated with good prognosis of invasive breast cancer. CDH1 and BRCA1 gene hypermethylation was found more in ER/PR positive cases suggesting these genes are also associated with good prognosis of the disease. However, HIC1 gene showed higher methylation frequency in triple negative breast cancer which suggests its association with bad prognosis of the invasive breast cancer patients.

Out of 87 breast tumor cases, hypermethylation of the HIC-1 gene was observed in 27% (12/44) cases of ER positive samples while 37% (16/43) cases showed hypermethylation in ER negative samples and 72% (32/44) and 62% (27/43) cases showed no methylation pattern irrespective of ER status. **(Table R7)** We have also correlated the above findings with PR status in the same cases and found that among 87 breast cases, 36% (14/38) were found to be hypermethylated in PR positive cases while 28% (12/49) cases showed hypermethylation in PR negative cases. **(Table R11)** So, On the basis of clinico-pathological data for ER/PR, we found no significant correlation between HIC-1 methylation and ER/PR status. Although reported literature correlate HIC-1 hypermethylation with good prognosis in other cancers (Nicoll G *et al.*, 2001) but it remains to be examined in large number of samples if similar relationship can be

established in the breast cancer. Further, we have analyzed HIC1 hypermethylation relative to HER2/neu status. In HER2/neu positive cases, HIC1 hypermethylation was found in 29% (11/37) cases while 34% hypermethylation was observed in HER2/neu negative breast cancer cases. HIC1 hypermethylation was not found significant in HER2 neu overexpressed cases. **(Table R15)**

On the contrary, hypermethylation of GST-pi gene was observed in only 14% breast cancer cases of ER negative samples whereas only 29% ER positive breast cancer cases were found to be hypermethylated. **(Table R7)** In context with PR status, it was found that in 32% of cases in which hypermethylation was found were having PR positive status whereas 14% hypermethylated cases were found to be PR negative. **(Table R11)** Thus it is evident from the present study that GST-pi hypermethylation is present in more no. of cases with tumor samples having ER negative and PR positive status. Although, we have showed that 14% of breast cancer have GST-pi hypermethylation and this hypermethylation may be involved in poor prognosis which could be validated with large number of samples.

In the present study we have observed BRCA1 gene promoters were methylated in 26.43% of cases. Recently, Nicholas C. Hsu *et al.* (2013) demonstrated 36% BRCA1 methylation in breast tumors. Chi-square analysis revealed that there was no significant difference between status of BRCA1 promoter methylation with age, tumor size, lymph node status, stage, histological grade, ER, PR, and HER-2 positivity.

Nass *et al.*, (2000) had reported that coincident methylation in both CpG islands of *CDHI* and the estrogen receptor (ER) gene increases with advancing disease, suggesting that malignant progression of ductal carcinomas involves the accumulation of multiple epigenetic hits. This association was confirmed by Parrella *et al.*, (2004) which identified other methylated genes associated with estrogen receptor promoter hypermethylation.

We have found more methylation frequency in ER negative breast tumors which is relevant to these studies. Thus, it seems likely that the accumulation of epigenetic

changes may contribute to the diminished expression of key genes during the mammary carcinogenesis process.

4. Contribution of promoter hypermethylation in silencing of specific genes expression in breast carcinogenesis

Aberrant methylation of the promoter region is considered one of the major mechanisms for the silencing of cancer-related genes, resulting in down-regulation of the gene expression. It has been demonstrated that CpG island hypermethylation is implicated in the loss of expression of a variety of critical tumor suppressor and growth regulatory genes distributed in several categories including cell cycle regulating, steroid receptors, tumor susceptibility, carcinogen detoxification, cell adhesion and inhibitors of matrix metalloproteinases (MMPs) (Yang *et. al.*, 2001, Szyf *et. al.*, 2004).

Our results of 33% breast cancer cases being positive for methylation of CDH1 gene are higher than other studied genes and suggest that the promoter methylation of CDH1 gene is more frequent and occur early in breast carcinogenesis (**Table R2**). Studies have shown that aberrant E-cadherin expression is associated with the acquisition of invasiveness and more advanced tumor stage for many other cancers including breast cancer (Rasbridge SA *et. al.*, 1993; Oka H *et. al.*, 1993; Palacios J *et. al.*, 1995; Kowalski *et. al.*, 2003). We have found relevant methylation frequency of CDH1 gene in early stage tumors in ER negative cases. This correlates well with our study population in which ~56% (49/87) samples were in stage II or advanced stage.

We observed a high frequency of cases with CDH1 promoter hypermethylation and reduced expression of the estrogen receptor; 43 IDCs showed a decrease in ER levels and 15 of them presented CDH1 hypermethylation (**Table R7**). CDH1 hypermethylation was observed in 12 out of 49 cases that showed reduced expression of PR protein (**Table R11**). Additionally we detected a complete absence of ER expression in 8 out of 15 (53.33) IDC samples showing methylation of the CDH1 gene. Also, PR expression was absent in 8 out of 12 (66.66%) IDC samples showing hypermethylation of CDH1 gene and HER2/neu expression was absent in 8 out of 15 cases (**Table R15**). These data

support the hypothesis that disruption of the maintenance mechanisms of the methylation pattern could result in distinct hypermethylation profiles of primary breast cancer with tumor subsets characterized by reduced expression studies have routinely revealed that loss of expression of E-cadherin and ER exhibits heterogeneity. Similar to our findings, early studies have demonstrated that the heterogeneous patterns of CpG island methylation parallel the heterogeneous loss of both ER and E-cadherin expression in breast tumors (39,40). Thus, it seems likely that the accumulation of epigenetic changes may contribute to the diminished expression of key genes for the mammary carcinogenesis process. Reduced levels of E-cadherin protein were observed in 17 out of 29 cases showing hypermethylation of CDH1 gene (**Table R31**). Most of these cases showed decreased levels of ER, PR and HER2/neu protein.

While various mechanisms like loss of heterozygosity and mutational inactivation have been proposed and held responsible for this gene silencing (Bex G *et al.*, 1995), our results of high methylation of CDH1 promoter further suggest that hypermethylation must be playing an important role in silencing of this gene. Differences in methylation frequency could also be the result of differences in geographic distribution or due to small sample size (n=87) in the present study.

The familial breast cancer gene 1 (BRCA1) is an important component of the cellular DNA repair machinery; its inactivation by promoter methylation is being increasingly implicated in many human cancers, including sporadic breast cancers and ovarian tumors (Jacinto and Esteller *et. al.*, 2007; Tapia *et. al.*, 2008). In our study, immunohistochemical analysis showed that loss of BRCA1 protein was not uniformly associated with BRCA1 hypermethylation. Overall, reduced expression of BRCA1 was observed in 38% of our samples. It is clear from the low frequency of abnormal methylation of the BRCA1 promoter region, that this is not the sole mechanism accounting for the loss or reduced expression of BRCA1 protein. Several studies have demonstrated that mutations, LOH and deletions can also repress the expression of BRCA1 in invasive sporadic breast tumors (Birgisdottir *et. al.*, 2006). The Indian breast cancers show infrequent BRCA1 mutations and a large proportion of triple-negative breast cancers in comparison with the Western population. Hence, the association of

BRCA1 methylation with triple-negative breast cancers is likely to impact the understanding of genesis of these tumors and their management in future studies. Sporadic breast tumors with BRCA1 promoter methylation have been reported to be ER α and PR negative (Mirza *et al.*, 2007). Loss of ER- α expression has been associated with aberrant CpG island hypermethylation in breast cancer cell lines and tumors (Leu *et al.*, 2004; Giacinti *et al.*, 2006) and shown to modulate breast cancer progression (Shinozaki *et al.*, 2005). Diverse DNA methylation and gene expression profiles have been shown to correlate with differential adaptation of breast cancer cells to the antiestrogens, tamoxifen and fulvestrant (Fan *et al.*, 2006). Indian women often have biologically aggressive breast carcinomas as shown by lower incidence of estrogen receptor positive and progesterone receptor positive tumors and higher incidence of Her 2/neu positivity (Chopra, 2001; Saxena *et al.*, 2005).

Clinicopathologically, basal like cancers are triple-negative breast cancers; about 85% of triple negative breast tumor phenotypes are deemed to be basal-like subtypes. In the present study also, significant association was observed between loss of BRCA1 protein expression and ER α , PR negativity and triple negative phenotype. Other recent studies have also suggested that there is a fundamental similarity between BRCA1 deficiency and basal-like breast cancer (Miyoshi *et al.*, 2008).

To evaluate the effect of DNA methylation on protein expression, immunohistochemical staining of BRCA1 was carried out. Consistent with the methylation status of BRCA1 promoter, the expression levels of BRCA1 protein were reduced in tumors with methylated genes. **(Table R31)** BRCA1 promoter hypermethylation has been implicated as one of the mechanisms of loss of gene expression and has been identified in 9–32% of unselected sporadic breast cancer (Esteller *et al.*, 2000, Bal A *et al.*, 2012, Xu *et al.*, 1995, Catteau *et al.*, 1999, Krop *et al.*, 2003, Birgisdottir *et al.*, 2006). The incidence of BRCA1 methylation has previously been reported to be higher in breast tumors of infiltrating ductal type (Birgisdottir V *et al.*, 2006). Since all our patients are of the infiltrating ductal type, this finding was somewhat comparable to that of current literature. BRCA1 protein expression was found to be absent or markedly decreased in the majority of the BRCA1 methylated tumors, suggesting epigenetic gene silencing in these tumors (Birgisdottir V *et al.*, 2006). Breast cancers with BRCA1 promoter methylation

also showed decreased expression of ER (Bal A *et al.*, 2012) and basal-like phenotype (Bal A *et al.*, 2012). Our results indicated that BRCA1 promoter methylation correlated significantly with triple-negative breast cancer ($p < 0.041$). Genetic differences among different ethnicities/races may account for disparities in breast cancer susceptibility (Fong M *et al.*, 2006). Aberrant gene promoter methylation has also been shown to be affected by ethnicity in breast cancer (Mehrotra *et al.*, 2004). Increased understanding of the genetic/epigenetic abnormality together with the ethnic factors/differences involved in the pathogenesis of breast cancer is crucial and may provide a basis for detection and treatment. Whether the phenomenon we observed in this study are due to ethnicity or etiology remained to be determine in larger studies\ that include breast cancer patients of different ethnicities/races.

While many studies have identified oncogenes and tumor suppressor genes that mark the transformation of cells from several tissue types, such as colon, pancreas and lung, comparable studies in breast cancer have met with limited success. This reflects the difficulty in finding genetic and epigenetic alterations that are present in a significant proportion of breast cancers and the phenotypic and genetic heterogeneity of breast cancer itself. Out of these, genes which are found frequently silenced in association with promoter methylation as a result of epigenetic abnormalities could effectively be considered as diagnostic and therapeutic tool for disease management. HIC-1 (Hypermethylated in cancer-1) and GST-Pi (Glutathione S- transferase Pi) are few of these genes related with epigenetics of cancer in general and breast cancer in particular. HIC-1 (Hypermethylated in cancer-1) is a zinc-finger transcription factor which act as a tumor suppressor gene and found to be aberrantly methylated in many types of human cancers with better prognosis [Prostate cancers (Morton *et al.*, 1996); Non-small cell lung cancers (Hayashi *et al.*, 2001); Breast cancers (Fujii *et al.*, 1998); liver cancers (Kanai *et al.*, 1999) and Esophageal cancers (Eads *et al.*, 2001)].

Similarly, GST-pi (Glutathione S- transferase pi) comprise a family of iso-enzymes expressed in normal tissues at varying levels and provide protection to mammalian cells against electrophilic metabolites of carcinogens and reactive oxygen species. Previous

studies have shown that the CpG-rich promoter region of the α -class gene GST-pi is methylated at single restriction sites in the majority of prostate cancer with poor prognosis (Song JZ *et. al.*, 2002). However, little is known about epigenetic silencing of HIC-1 and GST-pi gene by promoter hypermethylation in the precursors of breast cancer. However, hypermethylation of the GSTP1 promoter has been associated with gene silencing in prostate cancer and kidney cancer (Lee *et. al.*, 1994; Brooks *et. al.*, 1998; Cairns *et. al.*, 2001; Jerónimo *et. al.*, 2002; Dulaimi *et. al.*, 2004). Previous studies reported that GSTP1 promoter hypermethylation is associated with loss of GSTPi protein expression, as measured by immunohistochemistry (Arai *et. al.*, 2006, Chan *et. al.*, 2005, Zhong *et. al.*, 2002). In this study, we have demonstrated a significant correlation between the promoter hypermethylation of GSTP1 with the data obtained from immunohistochemical analyses. Promoter hypermethylation of GSTP1 resulted in loss of GSTP1 protein expression in samples with methylation. These data provide evidence that GSTP1 promoter hypermethylation is a major mechanism involved in GSTP1 gene inactivation, resulting in impaired GSTP1 function during breast cancer development. The inconsistency between GSTP1 promoter hypermethylation and protein expression has been reported previously (Arai *et. al.*, 2006, Chan *et. al.*, 2005, Esteller *et. al.*, 1998, Zhong *et. al.*, 2002).

Hypermethylation status is correlated with significant downregulation of GSTP1 expression. The frequency of GSTP1 methylation was higher in tumors with reduced-GSTP1 expression (56.81%) than in tumors with normal or high GSTP1 expression (43%; $P < 0.04$) in ER Positive cases. These data indicate that GSTP1 inactivation through CpG hypermethylation is common in breast carcinomas and may contribute to aggressive progression of carcinogenesis.

Almost all the cases showing hypermethylation in GST-pi (19/87) as well as HIC-1 gene (28 of 87), were found to be of stage II tumor while only five cases were stage III tumor. Thus, by correlating all the molecular data, we could presume that loss of function of GST-pi and HIC-1 gene along with hypermethylation status may play an early event in the development of breast carcinogenesis could be a marker for better patient prognosis. Future investigations are required with large sample size to determine whether

methylation status is actually responsible for the causes or the consequence of the disease.

Nass J *et al.*, (2000) reported previously that coincident methylation in both CpG islands of CDH1 and the estrogen receptor gene increases with advancing disease, suggesting that malignant progression of ductal carcinomas involves the accumulation of multiple epigenetic hits. This association was confirmed by Parrela (Parrela *et al.*, 2004) which identified other methylated genes associated with estrogen receptor promoter hypermethylation. Several reports have demonstrated the association either of the *CDH1* gene methylation (Nass J *et al.*, 2000; Parrela *et al.*, 2004; Hu XC *et al.*, 2003; Jerônimo C *et al.*, 2003; Shinozaki M *et al.*, 2005) or the abnormal expression of E-cadherin in breast cancer progression (Ásgeirsson KS *et al.*, 2000). However, there are a limited number of studies directly correlating *CDH1* methylation and E-cadherin expression in the same sample of breast carcinomas (Ásgeirsson KS *et al.*, 2000). In our study, *CDH1* promoter methylation was not uniformly associated with the loss of E-cadherin expression. Overall, reduced expression of E-cadherin (moderate, weak or absence of staining in cell membranes) was observed in 85% of our samples. Although not statistically significant, the intensity of E-cadherin staining tended to diminish with methylation of the *CDH1* promoter region. The dynamic nature of epigenetic regulation including changes in DNA methylation patterns, in expression and/or function of *trans*/acting factors and chromatin mediated effects, could explain the lack of uniformity in the *CDH1* hypermethylation and the loss of E-cadherin expression observed at the immunohistochemical level.

A candidate tumor suppressor gene, *HIC1* was identified because of its association with a CpG rich region, or methylated and transcriptionally inactivated in several common types of human cancers, including breast cancer cell lines (Makos-Waales *et al.*, 1995).

We find that hypermethylation of *HIC1* gene and probable absence of expression of this gene, are characteristic of 28% of primary breast cancers. Our present study demonstrates frequent methylation and transcriptional inactivation of *HIC1* in breast cancer. It suggests that *HIC1* could well be an early tumor suppressor inactivation event in breast cancer pathogenesis.

GSTP1 is known to play a role in detoxification of potential carcinogens (Lee *et. al.*, 1994). Breast epithelial cells with lack the expression of GSTP1 is supposed to suffer from DNA damage more easily upon exposure to carcinogens. Loss of GSTP1 expression is observed in approximately two thirds of breast cancers, which indicates its potential role in breast carcinogenesis (Ari *et. al.*, 2006). GSTP1 promoter hypermethylation appears likely to be responsible for absence of GSTP1 expression (Chan QK *et. al.*, 2005). Previous studies have described the significance of GSTP1 promoter hypermethylation in invasive breast cancer (Arai *et. al.*, 2006; Esteller M *et. al.*, 1998; Hoque MO *et. al.*, 2006; Parella P *et. al.*, 2004; Shinozaki *et. al.*, 2005). To determine when, in the breast cancer, promoter hypermethylation of the GSTP1 gene begins to play role, we analyzed the promoter hypermethylation of GSTP1 in invasive breast cancer as well as its adjacent normal lesions. We found that GSTP1 promoter methylation is an early event in breast carcinogenesis.

The frequency of GSTP1 promoter hypermethylation in IDCs (28%) was found to be higher as reported previously. The frequency given in other studies of breast varied between 13 and 30% (Arai *et. al.*, 2006; Esteller M *et. al.*, 1998; Hoque MO *et. al.*, 2006; Parella P *et. al.*, 2004; Shinozaki *et. al.*, 2005).

Previous studies reported that GSTP1 promoter hypermethylation is associated with loss of GSTP1 protein expression, measured by immunohistochemistry (Arai *et. al.*, 2006; Lee *et. al.*, 1994; Zhong S *et. al.*, 2002). In this study we find significant correlation between the promoter hypermethylation of GSTP1 as revealed by immunohistochemical analysis. Promoter hypermethylation of GSTP1 resulted in loss of GSTP1 protein expression in samples with methylation. These data provide evidence that GSTP1 gene inactivation, resulting in impaired GSTP1 function during breast cancer development. While GSTP1 promoter hypermethylation status correlated well with GSTP1 protein expression. Ogino *et. al.*, (2006) found that tumors that show low levels of methylation in the gene promoters do not silence protein expression

Promoter hypermethylation and loss of GSTP1 expression in IDCs provide opportunities for breast cancer prevention strategies including restoration of GSTP1 function via treatment with inhibition of promoter methylation.

In summary, GSTP1 promoter hypermethylation was found to be progressively elevated along the continuum from normal breast tissue to invasive breast cancer. GSTP1 promoter hypermethylation was associated with loss of GSTp1 expression. Our results suggest that GSTP1 promoter hypermethylation is an early event in breast carcinogenesis and appears to functionally silence GSTP1 expression.

While immunohistochemical studies demonstrated that reduced or absence of E-cadherin expression is also common in infiltrating ductal carcinomas (IDCs) (Palmisano *et. al.*, 2000), in majority of these genes, CDH1 mutations were rare or absent. Recently, it was demonstrated that epigenetic silencing of the gene CDH1 by CpG island methylation of its promoter region, occurs in some human breast cancer cell lines, as well as in unselected primary breast cancer (Schulmn *et. al.*, 2005). Nass *et. al.*, 2006) demonstrated that hypermethylation of the CDH1 promoter region was evident in 30% of in situ ductal carcinomas and increased to 60% in IDCs. In this study, we reported aberrant methylation of the 5' CpG island of the CDH1 gene associated with reduced levels of E-cadherin expression in breast cancer.

Reduced expression of E-cadherin is regarded as one of the main molecular events involved in the dysfunction of the cell-cell adhesion system, triggering cancer invasion and metastasis (Debies *et al.*, 2001). In our study, we correlated the hypermethylation at the CDH1 promoter and the E-cadherin expression levels determined by immunohistological analysis in 87 primary breast cancers.

Several reports have demonstrated the association either of the CDH1 gene methylation (Steele *et. al.*, 2005) or the abnormal expression of E-cadherin in breast cancer progression. However, there is a limited number of studies directly correlating CDH1 methylation and E-cadherin expression in the same sample of breast carcinomas (Graff *et. al.*, 2000). In our studies CDH1 promoter methylation was not uniformly associated with loss of E-cadherin expression. Overall reduced expression of E-cadherin (moderate, weak or absence of staining in cell membrane) was observed in 58.6% of our samples.

Although not statistically significant, the intensity of E-cadherin staining intended to diminish with methylation of the CDH1 promoter region. Several studies have demonstrated that E-cadherin expression may be repressed by mechanisms other than promoter hypermethylation, such as allele loss (LOH), gene mutation, changes in chromatin structure, and alteration of specific transcription pathways regulating the expression of CDH1 gene. Histopathological analysis has revealed inconsistencies in the correlation of E-cadherin expression and prognosis in breast cancer.

5. Epigenetic silencing influences prognostic behavior in breast cancer

Breast cancer is heterogenous. With the availability of an increasing number of therapy options, it is important to identify ways to predict individual tumor response to given therapy. It is also crucial to streamline treatment and spare patients from receiving often toxic and expensive therapies that are not likely to be effective. The methylation status of many genes and microRNAs are likely to be important for prognosis. Here we found that BRCA1, p16, CDH1, GSTP1 and HIC1 are good indicators of prognosis. Recent studies indicate that BRCA1 methylation is an important marker for prognosis. The magnitude of the disease of functional BRCA1 methylation correlates with disease prognosis. (Mirza S *et. al.*, 2007; Vincent-Solomon A *et. al.*, 2007). Tumors with BRCA1 mutations are usually more likely to be higher-grade, poorly differentiated, highly proliferative, estrogen and progesterone receptor negative, and harbor p53 mutations. The presence or the absence of ER expression is another important prognostic indicator for survival (Campan m *et l.*, 2006; Giacinti L *et. l.*, 2006). ER negative tumors are unresponsive to antiestrogens, more likely to have a more aggressive clinical course, more likely to be poorly differentiated, have a higher histological grade and are associated with higher recurrence rate and decreased overall survival. Approximately 66% of breast cancers express ER. A fraction of breast cancers that are initially ER-positive lose ER expression during tumor progression, but it is unclear if this is due to methylation or other causes (Giacinti L *et. al.*, 2006).

Epigenetic alterations are clearly involved in breast cancer initiation and progression. Early studies focused on single gene important in prognosis and prediction, but newer genome-wide methods are identifying many genes whose regulation is epigenetically

altered during breast cancer progression. Detection of hypermethylation in specific genes could be used as a form of surveillance to detect early stage breast cancer, however future studies may find that addition of multiple genes and the inclusion of histone alteration to predictive panels may improve sensitivity and specificity. In addition to the use of epigenetic alterations as means of screening, epigenetic alterations in a tumor or adjacent tissues may also help clinicians in determining prognosis and treatment in breast cancer patients. As we understand specific epigenetic alterations contributing to breast tumorigenesis and prognosis, these discoveries will lead to significant advances for breast cancer treatment. Therapeutics that target methylation and histone modifications in breast cancer already exist. Newer versions of these drugs are likely to play an important role in future clinical treatment. Since epigenetic modifications can also be used as biomarkers, targeted therapies may some day be used as preventative measures.

Several studies demonstrate that epigenetic silencing of E-cadherin gene by 5'CpG methylation occurs in some human breast cancer cell line as well as 50% of unselected primary breast cancer (Graff et al., 1995, Hiraguri et al., 1998), metastatic progression and decreased patient survival (Bringuir et al., 1993)

A significant positive correlation exists between HIC1 hyper-methylation and loss of its expression that may lead to gene silencing, a hallmark of epigenetic event in breast cancer. Fujii *et al.*,(1996) suggested that methylation-associated inactivation of HIC-1 could well be an early tumor suppressor inactivation event in breast cancer pathogenesis.

We also observed hypermethylation of GST-pi gene in 22% breast cancer cases of tumor sample as compared to control samples which showed unmethylation pattern. In contrast, protein expression study revealed down-regulation of GST-pi in tumor cases with ER/PR negative status while it was found to be very high in healthy controls. A significant positive correlation exists between GST-pi hyper-methylation and loss of its expression that may lead to gene silencing, a hallmark of epigenetic event in breast cancer.

The CDH1 (16q22.1) gene encodes the transmembrane glycoprotein E-cadherin that is important in maintaining hemophilic cell-cell adhesion in epithelial tissues (Overduin *et al.*, 1995).The cadherins are the family of Ca²⁺ dependent adhesion molecules that function in cell recognition and tissue morphogenesis. Alterations in E-cadherin expression have been related in several cancer types including breast cancer and

correlated with pathological features such as poor tumor differentiation, infiltrative growth, lymph node metastasis and decreased patient survival. (Hirohashi *et. al.*, 1998 Debbis *et. al.*, 2001)

Aberrant methylation of the promoter region is considered one of the major mechanisms for the silencing of cancer related genes, resulting in downregulation of gene expression. It has been demonstrated that CpG island hypermethylation is implicated in the loss of expression of a variety of critical tumor suppressor and growth regulatory genes distributed in several categories including cell cycle regulation, steroid receptors, tumor susceptibility, carcinogen detoxification, cell adhesion and inhibitors of matrix metalloproteinases (Yng *et. al.*, 2001). In breast cancer CDH1 has been reported in ~30% of insitu ductal carcinomas and increased substantially to nearly 60% in metastatic tumors (Parella *et. al.*, 2004).

Furthermore CDH1 gene is one of the most frequently inactivated by methylation in sporadic breast cancer (Parella *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 (Hu XC *et. al.*, 2003) and in sentinel node metastasis. These findings suggest that CDH1 promoter methylation is an important event associated with pathogenesis of breast cancer.

In the present study, the methylation pattern of the CDH1 gene was not correlated with the age of the patients at diagnosis suggesting that they are not due to age related methylation changes (Liu *et. al.*, 2003) but probably is correlated with the deregulation of the methyltransferase activity during tumor progression (De Mrzo *et. al.*, 1999).

Nass *et. al.*, (2006) reported previously that coincident methylation in both CpG islands of CDH1 and the estrogen receptor gene increases with advancing disease, suggesting that malignant progression of ductal carcinomas involves the accumulation of multiple epigenetic hits. This association was confirmed by Parella *et. al.*, in 2004 which identified other methylated genes associated with ER promoter hypermethylation.

The potential reversibility of epigenetic states offers exciting opportunities for novel cancer drugs that can reactivate epigenetically silenced tumor suppressor genes. If epigenetic changes occur in precursor lesions of cancer, these changes may be targets for

chemoprevention. GSTP1 detoxify carcinogens by conjugating them with glutathione. It is suggested that GSTP1 plays an important role in the prevention of the development of cancer upon exposure to carcinogens (Lee *et. al.*, 1994).