Summary and Conclusion

SUMMARY

Breast cancer is the leading cause of cancer death among females in economically developing countries, a shift from the past 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. Epigenetics is 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 studies 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.

Several 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.

We examined promoter methylation status and alterations in expression levels of five specific but most common tumor suppressor genes viz BRCA1, p16, GSTPi, HIC1 and CDH1 and explored their relationship with various clinicopathological and well known prognostic factors associated with breast carcinogenesis. Our study has shown the prognostic value of promoter methylation that is responsible for epigenetic modification of these above genes in breast cancer. In the present study, the incidence of carcinoma breast was found to be more in postmenopausal women (68.96%). 48% of carcinoma breast had no regional lymph nodes metastasis (N0). 52% had metastasis to movable lymph nodes (N1) and had hard, fixed axillary lymph nodes (N2) and no metastasis to ipsilateral internal mammary region (N3). Majority of the patients (56%) presented stage II disease.

The present study encompasses the following major findings:

1. Hypermethylation of GSTP1 gene was observed in 21% of breast cancer cases while expression was downregulated in 47% breast cancer cases as compared to normal controls. Hypermethylation of GSTP1 was found significant with PR status of women with breast cancer (p=0.05). GSTP1 was found to be methylated more in PR positive and ER negative cases whereas only 29% ER positive breast cancer cases were found to be hypermethylated. 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. Thus it is evident from the present study that GST1 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 GST1 hypermethylation and this hypermethylation may be involved in poor prognosis which could be validated with large number of samples.

2. There are epigenetic differences observed between HER2/neu breast tumors and these differences are independent on ER status. However, we have found a significant correlation of GSTP1 gene hypermethylation between ER+/PR+ cases and triple negative

cases (p=0.02). This shows that GSTP1 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.

3. Hypermethylation of the HIC1 gene was observed in 32% (28/87) in breast cancer cases while the protein expression was downregulated in 53% breast cancer cases as compared to controls. Hypermethylation of HIC1 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. 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. 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.

4. CDH1 gene was hypermethylated in 33% (29/87) in breast cancer cases while the protein expression was downregulated in 58.62% breast cancer cases as compared to controls. 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. CDH1 hypermethylation was observed in 12 out of 49 cases that showed reduced expression of PR protein. 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. CDH1 gene also showed significant hypermethylation (p=0.04) as it was found to be methylated in PR positive cases frequently.

5. Promoter hypermethylation of the p16 gene was observed in 27% (24/87) in breast cancer cases and decreased protein expression was observed in 29.16% breast cancer cases. 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 effects the severity of the disease. p16 gene also showed a significant hypermethylation in later tumor stages relative to PR status ($p<0.05$).

6. BRCA1 promoter hypermethylation was observed in 26% (23/87) in breast cancer cases while the protein expression was downregulated in 30.43 % breast cancer cases compared to normal controls. BRCA1 also showed higher methylation frequency in PR positive cases which was not significant. Immunohistochemical analysis showed that loss of BRCA1 protein was not uniformly associated with BRCA1 hypermethylation. 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. Interestingly statistically significant association was found between BRCA1 promoter methylation and triple negative breast cancer patients ($p<0.041$).

7. It is found that down-regulation of protein expression of all the five genes studied viz. GSTP1, CDH1, HIC1, p16 and BRCA1 is always found to be significantly higher than the frequency of hypermethylation that silence expression of genes. It suggests that promoter hypermethylation is not the sole mechanism which is responsible for loss of expression of genes. These appear existence of yet other pathways that contribute to functional silencing of tumor suppressor genes during the process of carcinogenesis.

8. We have compared promoter methylation of these five genes between triple positive (ER+/PR+/HER2 neu+) and triple negative (ER-/PR-/HER2 neu-) breast cancer

cases and we found HIC1 (100%) and CDH1 (80%) shows significant a higher level of methylation in triple positive cases which shows better prognosis while in triple negative cases which shows worst prognosis of these genes were less methylation. On the basis of the above findings it is suggested that HIC1 and CDH1 may serve as potential biomarkers for prognostication and better management of breast cancer.

9. Similarily We have compared promoter methylation of these five genes between ER+/PR+ positive and ER-/PR- negative breast cancer cases and we found CDH1 (52.63%) HIC1 (36.84%) shows higher level of methylation in ER+/PR+ positive cases which are known to show better prognosis while in ER-/PR- negative cases these genes showed much less methylation and showed bad prognosis. On the basis of these above findings we conclude that CDH1 and HIC1 may be used as a biomarker for the early diagnosis and prognostication of breast cancer.

CONCLUSION

Promoter hypermethylation of important tumor suppressor genes BRCA1, p16, CDH1, HIC1 and GSTPi is found to be common in sporadic breast cancer

Down-regulated expression of the five different but the most common tumor suppressor proteins in majority of primary invasive breast carcinomas in comparison to corresponding normal tissues and their correlation with promoter hypermethylation of these genes and their increase with the increasing severity of breast lesions indicate that epigenetic silencing of genes plays a pivotal role in breast tumorigenesis and they may serve as early diagnostic and/or prognostic biomarker for better treatment/management of breast cancer.

GSTP1 showed significant downregulation in ER positive cases which showed good prognosis of the disease. We suggest that GSTP1 could be considered as a potential biomarker for the diagnosis, prognostication and treatment of this disease. By correlating all the 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 and could serve as a biomarker for better patient prognosis.

It is suggested that abnormal methylation of CDH1 gene occurs in high frequencies in infiltrating breast cancers associated with a decrease in CDH1 expression in a subgroup of cases characterized by expression of other important genes to the mammary carcinogenesis process, probably due to disruption of the mechanism of maintenance of DNA methylation in tumoral cells.

The correlation between CDH1 hypermethylation and the reduced expression of ER, PR and HER2/neu is indirect evidence connecting hormonal receptors function, transcriptional repression, and E-cadherin expression in breast cancer.

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and we found HIC1 and CDH1 shows significantly a higher level of methylation in triple positive cases which shows better prognosis while in triple negative cases which shows worst prognosis, these genes were less methylated. CDH1 and HIC1 shows higher level of methylation in ER+/PR+ positive cases which are known to show better prognosis while in ER-/PR- negative cases these genes showed much less methylation and bad prognosis. On the basis of the above findings it is suggested that HIC1 and CDH1 may serve as potential biomarkers for prognostication and better management of breast cancer.

Along with other molecular genetic markers, the methylation profile may be employed in early diagnostics and prognostication of breast cancer. Standard marker sets established for the most common malignancies will allow efficient screening, early diagnosis, detection of micro metastasis, and development of effective treatment strategies.