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RESEARCH ARTICLE

ANALYSIS OF EPIGENETIC ALTERATIONS IN THE PROMOTER REGIONS OF *TIMP3* AND *GSTP1* GENES IN SPORADIC BREAST CANCER

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ABSTRACT

Objective: Tissue Inhibitor of Metalloproteinases-3 (*TIMP3*) and Glutathione S-transferase P1 (*GSTP1*) are tumor suppressor genes, which play important role in regulation of extracellular matrix proteolysis and cellular detoxification from various xenobiotic drugs and carcinogens. Aberrant methylation of tumor suppressor gene at the promoter regions can inactivate its expression, which is important in the carcinogenesis of various cancer including breast cancer. Hence the present study was designed to determine the role of promoter methylation of *TIMP3* and *GSTP1* genes in sporadic breast cancer patients from South Indian population.
Materials and Methods: DNA methylation analyses of *TIMP3* and *GSTP1* gene were performed by methylation-specific polymerase chain reaction (MSP). Fifty biopsy samples of breast tumor and their corresponding non-malignant portions as controls were studied. mRNA expression analysis of these two genes were also done using real time PCR.
Results: Methylation of the *TIMP3* promoter was detected in 18% (9/50) and *GSTP1* promoter was detected in 20% (10/50) tumor samples. None of the normal tissues showed promoter hypermethylation in both the genes. The difference in methylation frequency between cancerous and normal tissue was statistically significant (p = 0.0029 and p =0.0013). *GSTP1* promoter methylation was positively associated with lymph node involvement (p = 0.034) and metastasis(p = 0.036). Any significant association was not found between *TIMP3* promoter hypermethylation and clincopathalogical parameters.
Conclusion: In conclusion, this study showed that promoter hypermethylation of *TIMP3* and *GSTP1* genes were associated with sporadic breast cancer patients from the South Indian population and may be useful as a new biomarker for breast cancer detection.

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INTRODUCTION

Tumor cells create an environment by interacting with surrounding cells and can promote tumor growth and protect the tumor from immune attack (Bissell *et al*., 2011). How cancer cells create their microenvironment to assist tumor growth and spreading is an area of intense investigation for more personalized treatment. It is clear that multiple strategies are involve in such reprogramming, among those are secreted growth factors and alterations to the extracellular matrix and cell-cell interactions (Pavlova and Thompson 2016). The extracellular matrix (ECM) control tissue and organ architecture, as well as the growth of tumor cells (Spence *et al*., 2007). Matrix metalloproteinases (MMPs) are ECM proteases and may be involved in carcinogenesis and metastasis (Comoglio and Trusolino 2005). MMPs can be synthesized by tumor cells, but are often produced by surrounding stromal cells, including fibroblasts and infiltrating inflammatory cells (Coussens *et al*., 2002). Function of matrix metaloproteinase is degradation of extracellular matrix and its activity is frequently increased in tumors (Anania *et al*., 2011). They can control cellular properties such as growth, death and migration and contribute to the invasion, promotion, angiogenesis, and metastasis in distant organ sites. The balance between activated matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) controls ECM activity (Brew and Nagase 2010). Tissue inhibitor of metalloproteinase-3 (*TIMP3*) gene is a tumor suppressor gene encodes a member of TIMP family protein TIMP3 protein inhibit the proteolytic activity of matrix metalloproteinases (Qi *et al*., 2003) and a potent

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inhibitor of angiogenesis. TIMP3 is a secreted protein, binds tightly to the extracellular matrix (Anania *et al*., 2011). Loss of *TIMP3* gene expression correlates with advanced-stage of cancer and poor prognosis in colorectal, breast, brain, bladder and particularly head and neck squamous cell carcinoma (HNSCC) (Jackson *et al*., 2017*)*. The *TIMP3* promoter is often methylated and its epigenetic silencing is characteristic of a pro-tumorigenic outcome (Hsu *et al*., 2012; Shin *et al*., 2012). In addition to these, proliferating cancer cells often alter the metabolic composition of the extracellular microenvironment as well. Glutathione S-transferases (GSTs) detoxifies several cytotoxic compounds and are the most important enzymes of the phase II metabolizing xenobiotic pathway (Negovan *et al*., 2017), which are involved in the metabolism of carcinogens, drugs, and reactive oxygen species (ROS) and plays a protective role against the oxidative damage of DNA (Tahara *et al*., 2011). GSTP1 enzymes is one of the glutathione S-transferases (GSTs) family, which catalyze the detoxification of endogenous and exogenous substances conjugating them with glutathione (GSH) (Laborde 2010). Glutathione S-transferase pi 1 (*GSTP1*) is a tumor suppressor gene and locate on chromosome 11q13 **(**Arai *et al*., 2006) and encodes GSTP1 enzymes. This enzyme interacts with several other factors (such as regulatory kinases) and modulates signaling pathways involved in cell proliferation, differentiation, and apoptosis. Altered expression of *GSTP1* gene and its correlation with the development of multidrug-resistance suggests additional roles for GSTP1 protein, which is influencing of metabolic and signaling pathways in cancer cells (Laborde, 2010).

Beyond glutathonylation and detofixication functions, GSTP1 also possess chaperone functions, regulation of nitric oxide pathways, control over various kinase signaling pathways (Zhang *et al*., 2014). For example, GSTP1 inhibits JNK (Jun N-terminal kinase) signaling and prevents downstream transcriptional activation of cell stress pathways (Okamura *et al*., 2015). GSTP1 has also been linked to many other functions in cancer and other human pathologies and even in drug addiction. *GSTP1* methylation is also frequently associated with tumor development or poor prognosis in a wide range of cancers such as neuroblastoma (Gumy-Pause *et al*., 2012), hepatocellular carcinoma (Li *et al*., 2015), endometrial (Fiolka and Zubor, 2013), breast (Fang *et al*., 2015), and prostate cancers (Goering *et al*., 2012; Martignano *et al*., 2016). Methylation associated *GSTP1* silencing, seems to be one of the first events to cause a preneoplastic phenotype to develop into a malignant phenotype (Schnekenburger *et al*., 2014). Although methylated *GSTP1* DNA is predominately reported as a marker of prostate cancer, Papadopoulu *et al*., (2006) indicated its prognostic impact in breast cancer also. In India 1.45 million (27%) women were detected with breast cancer for the year 2012, among those 70,218 died. Globally almost 1.67 million new breast cancer cases have been diagnosed in 2012 (25% of all cancers) (http://globocan.iarc.). According to World Health Organization (WHO) by 2020, 70% of all breast-cancer cases are predicted to be in developing countries like India. Although breast cancer survival has improved significantly within the last few decades, the assessment of individual risk factors remains of intense importance and may help in the decision making for a more tailored treatment approach in the near future. As such, the development of new molecular staging methods might represent a highly desirable approach for individual tumor therapy (Matuschek *et al*., 2010). The parallel analysis of different methylated markers takes into account the inter-individual variations of gene expression and methylation. We hypothesized that promoter hypermethylation of *TIMP3* and *GSTP1* gene may play a role in breast carcinogenesis in South Indian population. Even though, few previous reports have shown a correlation between promoter hypermethylation and reduction of *TIMP3* and *GSTP1* expression in breast cancer, however, these data still need to be confirmed. To our knowledge methylation analysis of these two genes are not yet done in South Indian population. So our aim was to analyze the promoter methylation status and mRNA expression of *TIMP3* and *GSTP1* gene in sporadic breast cancer patients from South India. MS-PCR was used to study the methylation status of *TIMP3* and *GSTP1* gene promoter and Real time PCR was done for expression analysis of *TIMP3* and *GSTP1* mRNA.

MATERIALS AND METHODS

Study population

This study included 50 sporadic breast cancer patients from South Indian population. Informed consent was obtained from all patients. The study was approved by the Institutional Ethics Committee for Biomedical Research, Bhagwan Mahavir Medical Research Centre and have been performed in accordance with the ethical standards as laid down in the 1964 declaration of Helsinki and its later amendments or comparable ethical standards. Demographic and Clinico-pathological data was collected by direct interviews in a structured Performa, and also with the help of co-investigator.

Criteria for selection of study group

Inclusion criteria: All patients were selected at the time of first diagnosis by the oncologists. All these patients were cases of confirmed breast cancer. None of these cases belonged to the category of co-morbidities. All the cases were above 30 years and not pregnant.

Exclusion criteria: All patients who were undergoing Chemotherapy were excluded. All those patients which were suffering from additional other diseases were also excluded.

Sample collection

Total 95 tissue samples (50 malignant and 45 corresponding adjacent non cancerous tissue areas) were collected from 50 patients with sporadic breast cancer from a tertiary surgical oncology department during 2014 January to 2016 July. The breast cancer patients ranged in the age group of 32 to 71 years, with a median age of 54.42 years. None of the studied cases had a hereditary form of breast cancer. Patients were classified on the basis of tumor size, nodal status, tumor stage etc. The samples collected were frozen immediately and stored at minus 80 °C until use.

DNA extraction

DNA extraction was performed from 0.01 - 0.02 g of tissue sample. In brief, the tissue was digested with cell lysis buffer and proteinase K solution (1mg/ml) at 55 °C for 4 hour. The DNA was purified with normal Phenol chloroform method and precipitated in ethyl alcohol. The isolated DNA was eluted in TE buffer and kept in -20 °C. Purity of the DNA was checked by nanodrop method.

Bisulphite modification and MSP

Purified DNA samples were bisulphite-converted using Methylcode bisulfite conversion kit (Invitrogen) according to the manufacturer's protocol. MS-PCR was performed using primers specific for methylated and unmethylated DNA for *TIMP3* and *GSTP1* gene. Primers were retrieved from http://medgen.ugent.be/methprimerdb and listed in Table 1. MS-PCR was performed using Invitrogen Amplitaq gold PCR master mix. 20 µl reaction mixtures contained 10 picomole primers, 1.5 µl template DNA, and 10 µl master mix. PCR condition was as follows- hot start at 95 °C for 10 min and the following cycling parameters: 35 cycles of 96 °C for 3 s, X °C for 20 s, 68 °C for 10 s, and 72 °C for 1 min, and 4 °C to cool. After amplification, PCR products were then loaded and electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under UV illumination. The presence of a product in the methylated or unmethylated reaction indicated the presence or absence of methylated or unmethylated promoter.

Real-time qRT–PCR for *TIMP3* and *GSTP1* mRNA expression

We extracted total RNA from tissue using trizol method. The cDNA synthesis kit (Invitrogen) was used for converting 1 µg of total RNA to cDNA according to the manufacturer's instructions. We selected glyceraldehyde-3-phosphate dehydrogenize (*GAPDH*) as an endogenous control. Real time-PCR of *TIMP3*, *GSTP1* and *GAPDH* genes performed using SYBR green assay by 7300 Real-Time PCR System (Applied Biosystems). Results are expressed as N-fold differences in *TIMP3* and *GSTP1* mRNA expression relative to the *GAPDH* mRNA and termed '$N_{TIMP3'}$ and N '$_{GSTP1'}$,were determined as'N_{TIMP3}' and 'N_{GSTP1}' ' = $2^{\Delta ct}$ sample, where the ΔCt value of the sample was determined by subtracting the Ct value of the *TIMP3* gene and *GSTP1* gene from the Ct value of the *GAPDH* gene. The 'N $_{GSTP1}$' and 'N_{TIMP3}'' values of the samples were subsequently normalized such that the median of the 'N $_{GSTP1}$' and '$N_{TIMP3'}$ values for the control was one.

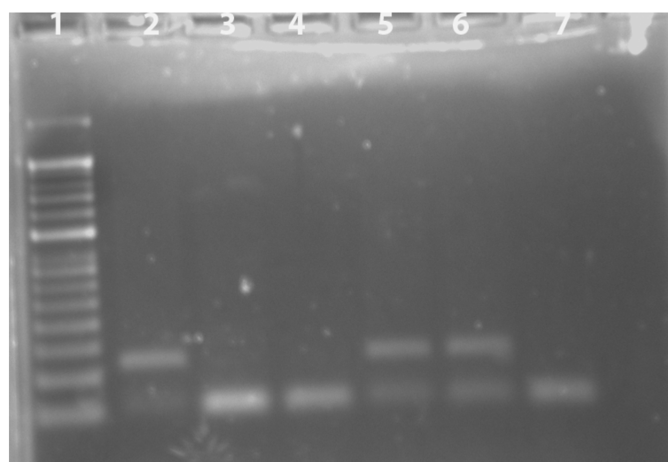
Statistical analysis

Statistical analyses were performed by using SPSS 16.0 software package and Graph Pad Prism 5.0 (Graph Pad Software Inc., La Jolla, CA, USA). The χ^2 test was used to determine associations between methylation of *TIMP3* and *GSTP1* gene promoter and various clinicopathological features of breast cancer. All *p* values were derived from two-tailed statistical tests. *p* values of < 0.05 (95 % significant level) were considered in this study. The distributions of *TIMP3* and *GSTP1* mRNA levels were characterized by median value. Relationships between *TIMP3* and *GSTP1* mRNA and clinicopathological parameters, were identified using nonparametric tests, Mann-Whitney test. Assaying relative gene expression between methylated and unmethylated promoter were also done by Mann-Whitney test. Significance level was set at $p < 0.05$ for all tests.

RESULTS

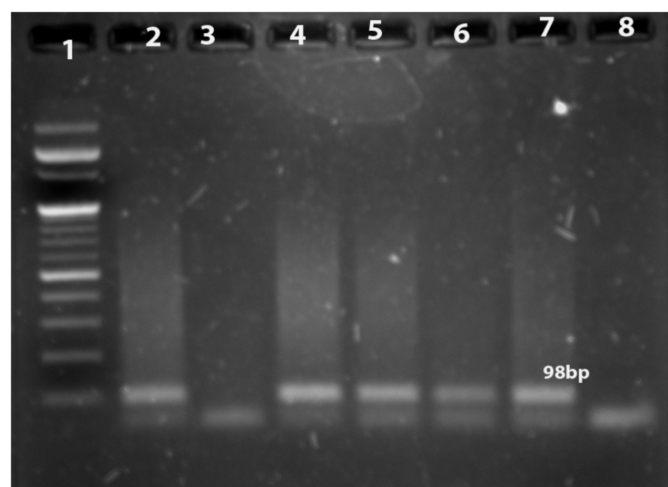
Detection of methylation in *TIMP3* and *GSTP1* genes using MS-PCR

Results of the *TIMP3* and *GSTP1* genes promoter methylation status in tumor and non tumorous tissue of Sporadic breast cancer and their relationship with clincopathalogical parameters are shown in Tables 2 and 3. The clinical characteristics of the 50 cancer patients at the time of surgery are summarized in Table 3. Among these patients, the medium age was 54 years (ranging from 32 to 71 years). We evaluated promoter methylation of *TIMP3* and *GSTP1* of tumor and normal tissue in the study group i.e. BC patients. Methylation of the *TIMP3* promoter was detected in 9 (18%) and *GSTP1* promoter was detected in 10 (20%) tumor samples out of the 50 tumors examined. Whereas none of the normal tissue sample shows promoter hypermethylation in both the genes. Fig. 1 and 2 shows representative methylation status of *TIMP3* and *GSTP1* promoter by methylation specific PCR. The difference in promoter methylation frequency between tumor and normal tissue for *TIMP3* and *GSTP1*gene was statistically significant (p = 0.0029 and p =0.0013). No significant association was found between *TIMP3* promoter hypermethylation with clincopathalogical parameters of breast cancer. Whereas a significant association was found between the *GSTP1* promoter hypermethylation and presence of lymph node (p = 0.034) and disease metastasis (p = 0.036) (Table 3).



Lane 1: 50 bp Ladder
Lane 2 and 6 :Unmethylated
Lane 5 :Methylated

Fig. 1. Representative methyl specific PCR of *TIMP3* promoter in breast cancer



Lane 1:100bp ladder
Lane 2,4,5, 6 and 7: Represents amplified with only methylated primer
Lane 3 and 8: Unmethylated

Fig. 2. Representative results of methylation‑specific PCR analysis of *GSTP1* in breast cancer patients

Table 1. Primer sequences for methylated and unmethylated DNA template

Gene	Primer sequence	Annealing temperature (◦C)	Amplicon size
GSTP1	Methylated specific		
	F5′-TTCGGGGTGTAGCGGTCGTC-3′ ,		98 bp
	R 5′-GCCCCAATACTAAATCACGACG-3	59	
	Unmethylated specific		
	UF 5′-GATGTTTGGGGTGTAGTGGTTGTT-3′		108 bp
	UR 5′-CCACCCCAATACTAAATCACAACA-3′		
TIMP3	Methylated specific		116bp
	F5′-CGTTTCGTTATTTTTTGTTTTCGGTTTC-3′		
	R 5′- CCG AAAACCCCGCCTCG-3		
	Unmethylated specific		
	F 5′- TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT - 3′	59	122bp
	R 5′- CCCCCAAAAACCCCACCTCA-3′		

Table 2. Comparison of promoter methylation of *TIMP3* and *GSTP1* genes in patients with breast cancer and controls

TIMP3	Patients (n=50)	Controls (n=45)	*p*-value	*GSTP1*	Patients (n=50)	Controls (n=45)	*p*-value
Methylated	9	0			10	0	
Unmethylated	41	45	0.0029		40	45	0.0013

Table 3. Associations between TIMP3 and *GSTP1* promoter methylation with clinicopathological features of breast cancer

Characteristics	Case n=50	*TIMP3* promoter methylation		*p*-value	*GSTP1* promoter methylation		*p*-value
		Present n=9	Absent n=41		Present n=10	Absent n=41	
Age(year)							
< 50years	20 (40%)	4(20%)	16(80 %)	0.764	4(20%)	16(80 %)	1
≥ 50 years	30 (60%)	5 (17 %)	25(83 %)		6 (20 %)	24(80 %)	
Histological type							
Non-ductal	3(6%)	1(33%)	2(67%)	0.476	1(33%)	2(67%)	0.552
Ductal	47(94%)	8(17%)	39(83%)		9(19%)	38(81%)	
Nodal involvement							
Negative	9 (18%)	3(67%)	6(33%)	0.186	0(0%)	9(100%)	
Positive	41 (92%)	6(15 %)	35(85%)		10(24 %)	31(76%)	0.034
TNM Stage							
I/II(early)	26(52%)	4 (15%)	22 (85%)	0.616	4 (15%)	22(85%)	0.395
III/IV(Advance)	24(48%)	5(21%)	19(79%)		6(25%)	18(75%)	
Metastasis							
Yes	3 (6%)	1(33 %)	2(67%)	0.509	2(67 %)	1(33%)	0.036
No	47 (94%)	8 (17 %)	39(83%)		8 (17 %)	39(83%)	
Tumor size							
≤20 mm	10 (20%)	1(10%)	9(90%)	0.436	2(20%)	8(80%)	0.971
>20 mm	40 (80%)	8 (20 %)	32(80%)		8(21%)	32(79%)	
Menopausal status							
Pre	11 (22 %)	3(15%)	8(85%)	0.383	2(18%)	9(82%)	0.863
Post	39 (78%)	6 (27 %)	33(73%)		8 (20 %)	31(80%)	

Table 4. Comparison of gene expression levels of *TIMP3* and *GSTP1* between with breast tumor and controls

Gene	N	Mean ± SD	p value(Mann-Whitney test)
TIMP3	case(17)	1.89±1.77	
	control(11)	1.95±1.32	0.66
GSTP1	case(17)	2.24± 1.47	
	control(11)	1.49±1.28	0.284

Table 5. Characteristics of the 17 breast tumors tested for *TIMP3* mRNA level

Characteristics	***TIMP3* mRNA expression relative to control**	***p*-value**
Case n=17	0.692 (0.132-5.28) **(**median range)	
Age(year)		
< 50years 5	0.255(0.132-0.69)	0.037
≥ 50 years 12	3.029 (0.166-5.28)	
Histological type		
Non-ductal 2	3.212(3.03-3.4)	0.294
Ductal15	0.68(0.13-5.28)	
Nodal involvement		
Negative 3	0.681(0.255-4.47)	0.953
Positive 14	1.74(0.13-5.28)	
TNM Stage		
I/II(early) 10	2.9(0.132-5.28)	0.314
III/IV(Advance) 7	0.681(0.167-3.22)	
Tumor size		
≤20 mm 4	3.21(0.208-5.284)	0.231
>20 mm 13	0.68(0.13-4.47)	
Menopausal status		
Pre 3	0.507(0.255-.692)	0.509
Post 14	2.9(0.13-5.28)	

Analysis of relative *TIMP3* and *GSTP1* gene expression

Analysis of relative gene expression ($2^{\Delta ct}$) for *TIMP3* and *GSTP1* mRNA between cases and controls was done by Mann-Whitney test. As shown in Table 4, *TIMP3* relative expression was 1.89±1.77 for cases (n= 17, range: 0.131-5.28) and 1.95±1.32 for controls (n = 11, range: 0.0027-3.94). The *GSTP1* data were 2.24± 1.47 for cases (n = 17, range: 0.00012-4.27) and 1.49±1.28 for controls (n = 11, range: 0.123-3.6). The difference was not statistically significant between tumor and normal tissue of breast cancer patients. Although methylated samples of both the gene shows lower mRNA expression compared to unmethylated sample but the value was not statistically significant. A significant association was found between the lower *TIMP3* mRNA and patient age below 50 (p=0.037) (Table 5).

DISCUSSION

Breast cancer arises from a multi-step process and occurs in multiple stages. The affected cell acquires a series of mutant gene products initiating a cascade of pathophysiological events which include continuous non-stoppable cell growth and increased angiogenesis, tissue invasion, and finally loss of genomic stability. The mechanism behind tumor development involves activation of protooncogene to oncogenes and also in many cases inactivation of tumor suppressor genes. It has been shown that along with, genetic alteration epigenetic alterations are also responsible for carcinogenesis in breast. Previous studies have focused on changes in gene expression that are inherited through meiosis and do not involve a change in DNA sequence but affect the expression and gene regulating function of DNA, mainly by chemical modification. Epigenetic mechanism is gaining increased attention from researchers of tumor formation processes because of its reversible nature. Alterations in epigenetic regulation mechanisms, such as promoter hypermethylation, are often involved decrease expression of tumor suppressor gene which are associated in tumor development, progression, and recurrence (Sarkar *et al*., 2013; Vecchio *et al*., 2013; Casadio *et al*., 2013; Martignano *et al*., 2016). Altered gene expression is often responsible for a transformed behaviour of tumor tissue and may distinguish tumor from healthy cells (Matuschek *et al*., 2010). The tissue inhibitors of metalloproteinase (*TIMPs*) are important tumor suppressor gene, whose protein product prevent degradation of the extracellular matrix by the metalloproteinases. TIMP metallopeptidase inhibitor 3 (TIMP3) is a member of TIMP family matrix-bound protein which regulates matrix composition by inhibiting matrix metalloproteinase that affects tumor growth, angiogenesis, invasion, and metastasis. TIMP-3's anti-angiogenic effects occur through direct binding to Vascular endothelial growth factor (VEGF) receptor 2 and acting as antagonist and therefore, blocking VEGF-A mitogenic effects and inhibition of proliferation, migration and tube formation of endothelial cells (ECs**)** (Qi and Apte ;2015). In addition, TIMP-3 inhibits several ADAMs (a disintegrin and metalloproteinase) such as tumor necrosis factor-a (TNF-a) convertase TACE (tumor necrosis factor-a-converting enzyme) and ADAM-17 (ADAM metallopeptidase domain 17) (Fata *et al*., 2001), which are crucial for controlling TNF-mediated inflammation (Mohammed *et al*., 2004). TIMP-3 also exhibits inhibitory activity of cell shedding of several molecules (L-selectin, syndecans 1 and 4, interleukin-6 (IL-6) receptor and c-MET) and cleavage of insulin-like growth factor-binding proteins 3 and 5 (Fata *et al*., 2001).

TIMP3 gene Silencing by promoter hypermethylation has been reported with poor prognosis in various human cancers such as kidney, brain, colon (Bachman *et al*., 1999), non-small cell lung (Zochbauer-Muller *et al*., 2001) and meningiomas (Barski *et al*., 2010). loss of heterozigosity on chromosome 22q, is frequently associated with loss of *TIMP3* gene expression, in various cancers like secondary glioblastoma (Nakamura *et al*., 2005) and clear renal cell carcinomas (Masson *et al*., 2010). Similarly, both promoter hypermethylation and LOH of the *TIMP3* allele were reported in human papilloma virus (HPV)-infected non-small-cell lung cancer (NSCLC)(Wu *et al*., 2012). Lower *TIMP3* expression was also reported in gastric cancer from non-neoplastic to metastatic lymph nodes (Guan *et al*., 2013) and endometrial carcinomas (stage I versus stage II–IV) (Catasus *et al*., 2013), because of hypermethylation in CpG islands. Hypermethylation of promoter of *TIMP3* gene may causes lower expression and subsequently cannot inhibit matrix metalloproteinase and other downstream protein and cell may become malignant. *TIMP3* promoter methylation was reported in 21% to 27% of breast cancer patients and in invasive ductal carcinomas that were associated with high tumor grading and lymph node metastasis (Bachman1999; Lui *et al*., 2005). Hoque *et al*., (2009) also found *TIMP3* promoter hyper methylation in ductal breast carcinoma. Kajabova *et al*., (2013) studied promoter methylation of *TIMP3* gene in both tumor and plasma sample and found 27.55% and 31.93% methylation frequency in breast cancer patients respectively. Zmetakova *et al*., (2013) also reported higher methylation levels in *TIMP3* genes in peripheral blood cell DNA of sporadic breast cancer patients but the value is below 15%. Our data also showed *TIMP3* promoter hypermethylation was present in 18% (9 out of 50) sporadic breast cancer patients from South Indian population which is consistent with previous data. However we did not find any significant association of hypermethylated *TIMP3* promoter with clinicopathogical characteristics.

GSTP1 enzyme conjugates the antioxidant tri-peptide glutathione with many toxic hydrophobic and electrophilic xenobiotics to facilitate their elimination from cell (Sawers *et al*., 2014). GSTP1 also inhibit c-Jun N-terminal kinase (JNK) through direct protein–protein interaction. Under cellular stress conditions such as, higher reactive oxygen stress GSTP1 has been shown to dimerize into larger aggregates and prevent binding to JNK, prevent JNK activation (Louie *et al*., 2016). JNK is a MAP (Mitogen activated protein) kinase involved in stress response, apoptosis, inflammation, and cellular differentiation and proliferation (Finazzi and Laborde 2010). Ultraviolet (UV) radiation, protein synthesis inhibitors, and a variety of stress stimuli can activate JNK that phosphorylates c-Jun, a component of the activator protein-1 (AP-1) transcription factor. This activation leads to induction of AP-1-dependent target genes involved in cell proliferation and cell death (Karin *et al*., 2005). Previous study demonstrated that the methylation level of *GSTP1* was significantly higher in breast cancer patients (6% to more than 75%) than controls (Klajic *et al*., 2013; Jung *et al*., 2013; Jeronimo *et al*., 2003; Shinozaki *et al*., 2005; Lee 2007; Pasquali *et al*., 2007; Saxena *et al*., 2012), which indicated its potential role in the etiology of breast cancer. Fang *et al*., (2015) did a meta analysis of 19 case control studies to find the role of *GSTP1* promoter methylation in the occurrence of breast cancer and its relationship with tumor stage and histological grade and found *GSTP1* promoter methylation probably plays an important role in breast carcinogenesis and conclude that aberrant *GSTP1* promoter

methylation could be a helpful biomarker for the early screening of breast cancer.

We also observed higher *GSTP1* promoter hypermethylation in breast tumor sample of our studied group which is 20% (10/50) and well within the previous reported frequency. Previous study of Saxena *et al*. (2012) demonstrate that presence of aberrant promoter hypermethylation in 34.4% breast cancer cases. But to our knowledge, the promoter methylation study of *TIMP3* and *GSTP1* genes in South Indian population with sporadic breast cancer was not done till date. This is the first report of methylation status of *TIMP3* and *GSTP1* genes in South Indian population with sporadic breast cancer.

Conclusion

This study shows that *TIMP3* and *GSTP1* promoter methylation is an epigenetic event related to breast cancer in South Indian population. In addition to this we also found a significant association of *GSTP1* promoter hypermethylation with lymph node positive patient and patients with metastasis. Therefore, *GSTP1* promoter hypermethylation might result in more aggressive behavior of breast cancer. Although statistically not significant but we found lower mRNA expression of both *TIMP3* and *GSTP1* genes in methylated samples. So we may conclude that hypermethylation of promoter region results lower expression of *TIMP3* and *GSTP1* gene, which may change microenvironment of cell and play an important role in carcinogenesis in our studied group. As we did not find any promoter hypermethylation in adjacent normal tissue of these two genes we may also conclude that hypermethylated promoter of *TIMP3* and *GSTP1* gene may serve as potential biomarkers in breast cancer, because DNA methylation markers could be more informative, as they are more stable than other RNA or protein-based markers. However, our study has some limitations, it has been focused on analysis of only 2 genes so, identification of further novel CpG islands that are specifically linked with breast cancer will be needed to create a panel of gene with higher sensitivity and specificity. Obviously, further studies are needed with large sample size and more number of gene to establish the role of hypermethylation in breast cancer progression and to create potential new biomarker series of risk prediction in breast cancer.

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Ethical approval: “All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

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