DISCUSSION
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Breast cancer is one of the most lethal female cancers in the world (He and Chen 2013). The precise etiological factors for breast cancers are still not clear. It has been indicated that different dietary factors partially account for the difference in the incidence of breast cancer among Caucasian, Hispanic and Asian women (Catsburg et al. 2015; Murtaugh et al. 2008; Ursin et al. 1999). However, Chlebowski et al. (2005) suggested that differences in breast cancer incidence rates in most of the racial/ethnic groups can be largely explained by difference in risk factors. In India also the racial differences in breast cancer incidence and mortality exist throughout different populations (Shrivastava et al. 2015; Gajalakshmi et al. 2007). As far as the genetics of breast cancer is concerned, it is a well-established fact that the BRCA1 mutations are prevalent in breast cancers in high-incidence, low-incidence and racially diverse populations (Eachkoti et al. 2007).

BRCA1 has been mostly studied for their contribution in familial breast cancer through germline mutation (Gabai-Kapara et al. 2014). Interestingly, there has been a trend to find out the role of BRCA1 in non-familial or sporadic breast cancers. Loss of heterozygocity or homozygotic mutation in BRCA1 and BRCA 2 may contribute to sporadic breast cancer (De Leeneer et al. 2012). Methylation of BRCA1 also contributes to the epigenetic masking of the expression of the gene, which results in an allelic imbalance at BRCA1 locus.
Also, phenotypic similarities between *BRCA1* methylated and familial *BRCA1* tumors have been reported (Birgisdottir et al. 2006)

Soumittra *et al.* (2009) from Tamil Nadu has been reported 12 *BRCA1* mutations among which 6 were novel in hereditary breast cancer patients. A founder Jewish Ashkenazi mutation in *BRCA1* (185 Del AG) was studied by Mary *et al.* (2010) in breast cancer patients at a tertiary care hospital. But they failed to find any patient with such mutation. Eight sequence variants were reported by Vijayalakshmi *et al.* (2011) from Andhra Pradesh (AP). Among them one was novel deleterious frame-shift mutation (c.2717insA) and one was novel polymorphism (c.1400A>G) in exon 11 while another mutation was intronic (intron 1) variant (base1822C>T) of *BRCA1*. All the reported mutations found in exon 11 fall in DNA binding domain of BRCA1 protein (Vijayalakshmi *et al.* 2011). A Tamil family has been studied for a deletion of nucleotide “T” at np 1307 codon 396 which cause a frame shift mutation in *BRCA1* and a loss of BRCT domain of BRCA1 protein (Gajalakshmi *et al.* 2007). Another hospital based population study from Bangalore reports for three novel mutations in the coding region of *BRCA1* which leads to the frame shifts (Vaidyanathan *et al.* 2009). Saxena *et al.* (2002) had identified two novel splice variants (331+1G>T; 4476+2T>C) in *BRCA1* gene in a North Indian population. However, the information regarding the contribution of these *BRCA1* mutations in the incidence of and predisposition to breast cancer in ethnic South Indian population, particularly in AP is lacking. There are a few
records for the studies about the \textit{BRCA1} variation in South India populations. Moreover, most of them are exploring the coding region of the gene. For study of \textit{BRCA1} promoter region variations no record has been found.

A recent study of Chinese breast cancer patients advocated that the minor allele “T” of rs11655505 in the BRCA1 promoter (c.2265C/T) enhances promoter activity. Thus BRCA1 promoter variation rs11655505 (c.2265C/T) has a protective effect on breast cancer risk (Chan \textit{et al.} 2009). Based on the studies of Chan \textit{et al.} Verderio \textit{et al.} (2010) genotyped rs11655505 in 2912 female breast cancer cases and 2783 unaffected female controls from larger Caucasian breast cancer studies (Australia, Germany and Italy). But they did not find evidence for an association between rs11655505 and breast cancer risk. Their study failed to confirm a role of rs11655505 in breast cancer risk. However, they recommended further studies to determine if there is a weak association between this SNP and breast cancer risk (Verderio \textit{et al.} 2010).

To our knowledge present study of rs11655505 (c.2265C/T) in breast cancer in South Indian population of AP is the first study on promoter region SNP of \textit{BRCA1}. Odds Ratio (OR) (CI=95%) was found to be 0.96 (0.69-1.34) for CT genotype and 1.30 (0.83-2.04) TT genotype and no significant association was found between rs11655505 (c.2265C/T) and breast cancer risk. To elucidate the possible risk in premenopausal, menopausal, familial and non-familial cancers, data from the patients were categorized accordingly and further analyzed statistically. Again there were no associations between rs11655505
(c.2265C/T) and breast cancer risk in different groups. Only menopausal patients group showed a significant association with the rs11655505 (c.2265C/T) where there was a protective effect of minor allele T on breast cancer predisposition. As in the control group TT genotypes accounted for 18.82% and 12.94% in menopausal patients (Table 5).

Test of two models TT+CT vs. CC and CC+CT vs. TT were performed as described earlier (Yang and Chen et al. 2011). In total patients-control analysis it was found that CC+CT vs. TT model was not significantly associated with breast cancer risk ($p=0.15$) and OR=1.33 (0.88-2.00). Rest other groups did not shown any association between breast cancer risk and either of the models except menopausal patients where OR for TT+CT vs. CC and CC+CT vs. TT were $1.08 (0.67-1.72); p=0.01$ and $2.12 (1.08-4.21); p=0.004$ (Table-8).

It was concluded that neither CC+CT vs. TT and TT+CT vs. CC models not the minor allele T were associated to the breast cancer risk in premenopausal, familial and non-familial cancers patients. These findings are in agreement with that of Verderio et al. (2010). However, the variation rs11655505 (c.2265C/T) showed a protective effect on menopausal breast cancer. Hence, this finding was in partial agreement with Chan et al. (2009).

Since the results of genotyping study did not find any significant associations between rs11655505 (c.2265C/T)-a promoter region SNP and breast cancer risk. Another study was undertaken to check if there is any
association between the SNP, promoter methylation and BRCA1 gene expression in sporadic breast cancer.

Although, familial breast cancer syndromes are rare in sporadic breast cancer (King et al. 2003; Fackenthal and Olopade, 2007), generally it is believed that the genes accounting for familial breast cancer play an important role in sporadic breast cancer too. Therefore, it was speculated that somatic BRCA1 mutations would contribute to sporadic breast cancer. But Futreal et al. (1994) reported that somatic BRCA1 gene mutations have not been found in sporadic breast tumors. Birgisdottir et al. (2006) have also been reported the same. But recently, polymerase chain reaction (PCR)-direct sequencing assay and analysis of 144 Chinese sporadic breast cancer women patient revealed that a small subset of sporadic breast cancers do harbor BRCA1 somatic mutations (Zhang et al. 2012). Loss of heterozygocity or homozygotic mutation in BRCA1 and2 may contribute to sporadic breast cancer (De Leeneer et al. 2012). Even after the findings of Zhang et al. (2012) and De Leeneer et al. (2012) there is a lack of ample evidence for the involvement of BRCA1 mutations in predisposition of sporadic breast cancer. It could be one of the reasons that involvement of BRCA1 in sporadic breast cancer is doubtful.

An alternative mechanism, hypermethylation of CpG islands in the promoter region of the gene is known to be strongly associated with gene silencing. After the discovery of the fact that DNA methyltransferase can pass on the methylation pattern of the promoter to the daughter cells conserving the
overall pattern of methylated CpG-islands, the methylation patterns of genes in tumor tissues of virtually all types of cancer, including breast carcinoma have been found to differ extensively from that of the corresponding normal tissues (Herman and Baylin, 2003). These epigenetic alterations may be global genomic hypomethylation as well as non-random hypermethylation of normally unmethylated CpG islands in the promoter (Jones and Baylin, 2002).

*Death-associated protein kinase1 (DAPK1)* is an important tumor suppressor gene. DNA methylation can inactivate genes. Also, it has been reported that there is strong association between *DAPK1* promoter methylation and cervical cancer. *DAPK1* promoter methylation may be a biomarker during cervical carcinogenesis that might serve as an early indication of cervical cancer (Xiong *et al.* 2014). The putative tumor suppressor *RASSF1A* promoter methylation has been reported for strong association with colorectal cancer (Wang *et al.* 2014). Chen *et al.* (2014) also described an association between *P16* gene promoter and colorectal cancer. In the similar fashion, association of *BRCA1* promoter with the breast cancer has been reported by Hsu *et al.* (2013) and Sharma *et al.* (2014).

*BRCA1* has been proven for their important role in familial breast cancer. Also, no frequent detection of *BRCA1* mutations in sporadic breast cancers made DNA methylation induced silencing an attractive mechanism for *BRCA1* silencing in sporadic breast cancer cases. *BRCA1* methylation has been found in sporadic breast cancers but it is not a frequent event and it is possible that
BRCA1 methylation is most common in rare subtypes of basal-like origin of sporadic tumors. Sporadic and inherited breast tumors have overall similar methylation profiles but BRCA1 tumors have reduced methylation of certain non-familial genes and have a phenotype associated with basal-like carcinomas (Jovanovic et al. 2010). Otani et al. (2014) studied 15 tumors reportedly BRCA1 methylated promoter and the paired adjacent normal tissues. They found 9 out of 15 normal breast tissue harbored BRCA1 promoter methylation in at least one site. Hence they suggested that a small proportion of normal breast epithelial cells with BRCA1 promoter methylation can be precursor cells from which BRCA1-methylated breast tumors may originate. A population based study revealed that methylation of BRCA1 promoter is associated with unfavorable prognosis in women with early-stage breast cancer, in Taiwan (Hsu et al. 2013). Moreover, Sharma et al. (2014) advocated in favor of the prognostic value of BRCA1 promoter methylation in case of triple negative early-stage breast cancer. In our study we found 9 (31.03%) out of 29 sporadic breast tumors have BRCA1 promoter methylated (Table 2). This finding shows a partial agreement with earlier discussed findings.

Van Erik et al. (2012) on the basis of their finding, proposed that DNA methylation regulates the gene expression in addition to the classical model of gene regulation. DNA methylation of homeobox gene HoxA5 was demonstrated to be a cause of decreased gene expression during tumor progression (Watson et al. 2004). Liu et al. (2014) also, advocated that methylation of DNA silence the
gene and may cause the disease in the similar fashion as of the mutational gene inactivation. *BRCA1* promoter hypermethylation has been reported for the decreased gene expression and vice-versa (Matros et al. 2005; Birgisdottir et al. 2006). In our study, we found that 29 tumor samples 9 (31.03%) had *BRCA1* promoter methylated whereas out of 26 normal biopsy samples 4 (15.38%) were methylated. Interestingly, among the 9 *BRCA1* promoter methylated tumor samples 8 (88.88%) showed a decreased gene expression while 1 (11.11%) was with normal *BRCA1* expression. Contrarily, out of 20 tumor samples with *BRCA1* promoter methylated 5 (25%) had a decreased expression of gene whereas 15 (75%) where normal for *BRCA1* gene expression (Table 13). This finding finds itself in the agreement with that of Watson et al. (2004), Liu et al. (2014) Matros et al. (2005) Birgisdottir et al. (2006)

The age and the menopausal status of patients have been reported to be associated with *BRCA1* promoter methylation (Bosviel et al. 2012; Wong et al. 2011). Pathologically estrogens have been associated with a higher risk for breast and endometrial cancer and hormone dependence of breast cancers is correlated with tumor progression and patient prognosis. In the beginning most of the breast cancers are positive for estrogen receptor (ER), later on their growth could be stimulated by estrogens and inhibited by antiestrogens. DNA methylation of the estrogen receptor 1 (*ESR1*) and Progesterone (*PGR*) promoters has been considered as a possible mode of the development of ER-negative tumors in cell lines as well as primary tumors. Hypermethylation
has been regarded as a possible cause of ER loss (Weigel and deConinck, 1993). However, clinical data remains contradictory. Lapidus et al. (1996) found hypermethylation of the ER promoter region in tumors, but other groups such as Hori et al. (1999) have detected no correlation between gene methylation pattern and ER gene expression in breast tumors. In summary, current evidence suggests that there is no clear link between \textit{ESR1} methylation and ER status. Johannsson et al. (1997) reported that BRCA1-associated breast cancers are more likely to be of high grade or ER negative. Moreover, Catteau et al. (1999) have been reported methylateion of \textit{BRCA1} promoter in 11% breast cancer and 5% ovarian cancer. Also, suggested that methylation of the \textit{BRCA1} promoter region is strongly correlated with lack of estrogen and progesterone receptor expression. In present study, out of 9 tumors with methylated \textit{BRCA1} promoter, 7 were ER positive and 2 were ER negative. Interestingly, out of 20 sporadic tumors having unmethylated status for \textit{BRCA1} promoter 6 were ER negative and 14 were ER positive. Statistically, this study also advocate that the metylation of \textit{BRCA1} promoter is associated with ER negative status and unmethylation of \textit{BRCA1} promoter is associated with ER positive status \((p=0.04)\) (Table 2). Hence, our findings are in agreement with the findings of Johannsson et al. (1997) and Catteau et al. (1999).

Otani et al. (2014) suggested that the progesterone receptor (PR) status-negative is most likely associated with \textit{BRCA1} promoter methylation. In our finding, although there has been no statistically significant association
between BRCA1 promoter methylation and the PR status, however out of 9 tumor samples BRCA1 promoter methylated, 6 (66.66%) were PR negative while among 20 tumor samples BRCA1 promoter unmethylated 8 (40%) were PR negative (Table 2). Hence, this indicates an inclination for association between the BRCA1 promoter methylation and PR negative status of tumors.

Hsu et al. (2013) and Otani et al. (2014) in their meta-analytical study suggested that HRE negative status is associated with BRCA1 promoter methylation in breast cancer. Although our study failed to find a significant association between BRCA1 promoter methylation, however we observed 5 (55.55%) out of 9 tumor samples with BRCA1 methylated were negative for HER status while 12 (66.66%) out of 20 tumor samples with BRCA1 promoter unmethylation were HER negative. Our finding shows an inclination of HER negative status towards unmethylated BRCA1 promoter (Table 2). Hence, this finding seems to be not in the agreement with above.

To the best of our knowledge, association of menopausal status with the BRCA1 promoter methylation has not been reported. In our study, 5 (55.55%) out of 9 tumor samples with BRCA1 methylated were pre-menopausal while 12 (66.66%) out of 20 tumor samples with BRCA1 promoter unmethylation were pre-menopausal. Hence, our finding shows an inclination of pre-menopausal status towards unmethylated BRCA1 promoter (Table 2).

In addition, a single nucleotide polymorphism (SNP) also can alter the expression of gene, particularly if it takes place in a promoter region of gene
van’t Veer et al. 2002). As far as the sporadic breast cancer is concerned, two
BRCA1 promoter SNPs, rs799907 (c.-2613G/C) and rs799906 (c.-2004G/A) did
not show any association with the predisposition of disease (Yu et al. 2004).
Another meta-populations study reported no association between rs11655505
(c.-2265C/T) and breast cancer risk (Freedman et al. 2005). Interestingly, in
Chinese population rs11655505 (c.-2265C/T) variation is associated with breast
cancer occurrence. The same study also reported that the minor allele T is
associated with the increased BRCA1 expression (Verderio et al. 2010). No
significant association has been found between rs11655505 (c.2265C/T)
variants with the predisposition of sporadic breast cancer in this study.
An increased allelic frequency of T allele was seen in tumor samples (Table9).
This finding is in agreement with that of Verderio et al. (2010) and
contradictory with that of Chan et al. (2009) (Verderio et al. 2010; Chan et al.
2009).

To our knowledge, for the first time this study reports the methylation
status of BRCA1 promoter and gene expression level among different genotypes
of rs11655505 (c.2265C/T) in sporadic breast cancer tumor and biopsy samples.
The number of methylated and unmethylated samples did not show any
significant difference in any of the genotypes (CC, CT and TT). CT genotype
was found to be associated significantly with the low expression of BRCA1
gene in tumor samples (p=0.04) (Table12). The statistical analysis to deduce the
effect of methylation status on the gene expression did not show any significant difference between group of tumor and normal biopsy samples (Table 13).

In order to find out the association of $BRCA1$ rs11655505 (c.-2265C/T) variants and gene expression level with methylation status of promoter MLR was performed for tumor and normal biopsy samples, separately. None of the $BRCA1$ genotypes were found associated with the methylated status in tumor samples as well as in normal biopsy samples. However, only the tumor samples showed a significant association between the methylated $BRCA1$ promoter and lower expression of gene (OR 25.09, CI 95% 2.17-297.5, $P$=0.01). This finding was in agreement with that of Wei et al. (2005) where they reported decreased copy number of $BRCA1$ in sporadic breast tumor samples.

Present study suggests that CT genotype of rs11655505 (c.-2265C/T) is significantly associated with decreased expression of $BRCA1$ gene in sporadic breast cancer (Table-4); methylated status of $BRCA1$ gene promoter is associated with decreased expression of the gene in tumor samples but not in normal biopsy samples (Table14). Interestingly, the methylated status was significantly associated with the ER negative tumor samples.