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# Methylation of *E-cadherin* and *hMLH1* genes in Indian sporadic breast carcinomas

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Hypermethylation of promoter regions leading to inactivation of tumor suppressor genes is a common event in the progression of several tumor types. We have employed a novel restriction digestion based multiplex PCR assay to analyse the methylation status of promoter regions of tumor suppressor genes (*p16, hMLH1, MGMT* and *E-cadherin*) in sporadic breast carcinomas of Indian women. The present results indicated the absence of hypermethylation in promoter region of *p16* and *MGMT* genes. However, 6 of the 19 (31.6%) sporadic breast carcinomas showed hypermethylation in the promoters of two of the genes analysed; three in *hMLH1* and another three in *E-cad*. Since our earlier studies have shown lack of genetic alterations such as missense mutations and deletions in the tumor associated genes-*p16, ras* and $p14^{ARF}$ in sporadic breast tumors, the epigenetic alterations of the two genes reported in the present study could be of interest and might be among the events in the genesis/progression of sporadic breast carcinomas.

**Keyword**: Breast carcinoma, *E-cadherin*, *hMLH1* gene, Indian sporadic carcinoma, Methylation

Breast cancer is one of the most prevalent forms of cancer in Indian women that is associated with postmenopausal effect in women[1,2]. Genetic factors that are implicated in breast cancer include well-studied mutations in tumor suppressor genes – *BRCA1* and *BRCA2*[3]. Other risk factors include environment, obesity, familial incidence and reproductive history. Epigenetic mechanisms like hypermethylation of promoter regions have been implicated to play a major role in the genesis and progression of several cancer types by transcriptional repression of tumor suppressor genes[4,5]. We have studied four genes known to be involved in the initiation and progression of tumors – *p16*, a CDK inhibitor and a key component of the Rb pathway in cell cycle regulation; *hMLH1*, the human homologue of bacterial *Mut L*, involved in mismatch repair; *MGMT*, the gene involved in repair of methylated guanosine residues formed as a result of modification by alkylating carcinogens and *E-cadherin* (E-card), which is involved in homotypic cell-cell adhesion and associated with tumor metastasis. The promoter regions of these genes are known to be inactivated by methylation in various human cancers[5]. Therefore we have assessed the methylation status of these genes in sporadic breast carcinomas of Indian women. We have used a restriction digestion, multiplex PCR based assay that is reliable, yet simple enough to be used in clinical diagnosis[6]. A consensus PCR condition was developed which could, using the listed primers, simultaneously detect the methylation status of promoter regions of several genes. We designed primers encompassing the promoter regions within 500 bp of the exon 1, which were known CpG islands verified using the CpG plot program of the EMBL-EBI website. These regions were known to be susceptible to hypermethylation inactivation and using the assay we have observed substantial methylation in the promoter regions of the *hMLH1* and *E-cadherin* genes in sporadic breast carcinomas.

## Materials and Methods

Genomic DNA (3 μg) was digested initially with 5 U of *Hpa II* restriction enzyme (Amersham Pharmacia Biotech) in 1x supplied buffer L for 2 hr, at 37°C. The reaction was refreshed with 10 U of *Hpa II* and incubated again for 2 hr. A phenol chloroform extraction was performed immediately after the restriction digestion to inactivate enzyme activity.

Placental DNA was methylated *in vitro* using Sss 1 methylase (New England Biolabs) according to manufacturer's instructions in a reaction consisting of, 10x buffer (*p*H 7.9 at 25°C) 10 μl; 32 m*M* S-adenosyl

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methionine 0.5 µl; DNA 6 µg; Sss1 methylase (4000 U/ml) 2 µl and water to make to 100 µl. The methylation mixture was incubated at 37°C and DNA was extracted immediately using phenol-chloroform-isoamylalcohol**.**

Semi-nested multiplex PCR's were carried out in a 10 µl reaction using 50 ng of template DNA, 1.5 m*M*, $MgCl_2$; 1x; PCR buffer; 200 µ*M*, dNTPs, 5.0%, DMSO; 0.2 µ*M* each of sense, nested, and 0.4 µ*M* of antisense primers and 0.25 U of Promega Taq DNA polymerase. The primer sequences for the methylation assay were as described earlier[6]. The PCR conditions for the assay used for detecting deletions of $p14^{ARF}$ were as reported earlier[7].

The consensus thermal cycling conditions optimized to amplify all the amplicons for detection of methylation in the candidate genes simultaneously were as described previously[6]. It was done as - initial denaturation at 95°C for 3 min followed by touch-down cycling at 95°C for 30 sec; 62°C for 1 min (incubation temperature was reduced by 0.5°C each cycle up to 54°C and incubation time by 1.0 sec each cycle) and 72°C for 30 sec. This was followed by additional 15 cycles at 95°C for 30 sec, 54°C for 45 sec and 72°C for 30 sec.

## Results

We used a modified methylation sensitive restriction digestion based multiplex PCR assay to detect methylation at specific CpG sites of promoter regions as described earlier[6]. The strategy involved digestion of the total genomic DNA with methylation sensitive restriction enzyme (*Hpa II*) followed by a semi-nested multiplexed PCR using primers designed for the promoter region, the outer primers amplifying a product encompassing a single restriction site for the enzyme while the product generated by the semi-nested primer was not affected by the restriction digestion (Fig. 1). Appearance of two bands using enzyme treated genomic DNA as template indicated methylation of CpG site, while a single band indicated unmethylated DNA (Fig. 1). We verified the efficacy of the assay in detecting methylation status of the

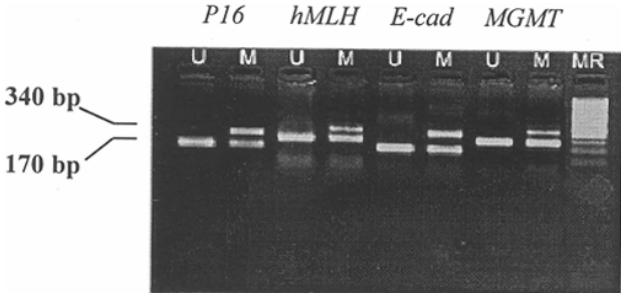


Fig. 2 — Cleavage pattern of *in vitro* methylated DNAs. The figure shows PCR products electrophoresed in 2% agarose gel. [Lane 1, 2-*p16*; lane 3, 4-*hMLH1*; lane 5, 6-*E-cad*; lane 7, 8-*MGMT*; lane 9, 100 BP DNA ladder. (U, placental DNA (unmethylated) digested with *Hpa II*; M, *in vitro* methylated placental DNA digested with *Hpa II*)]

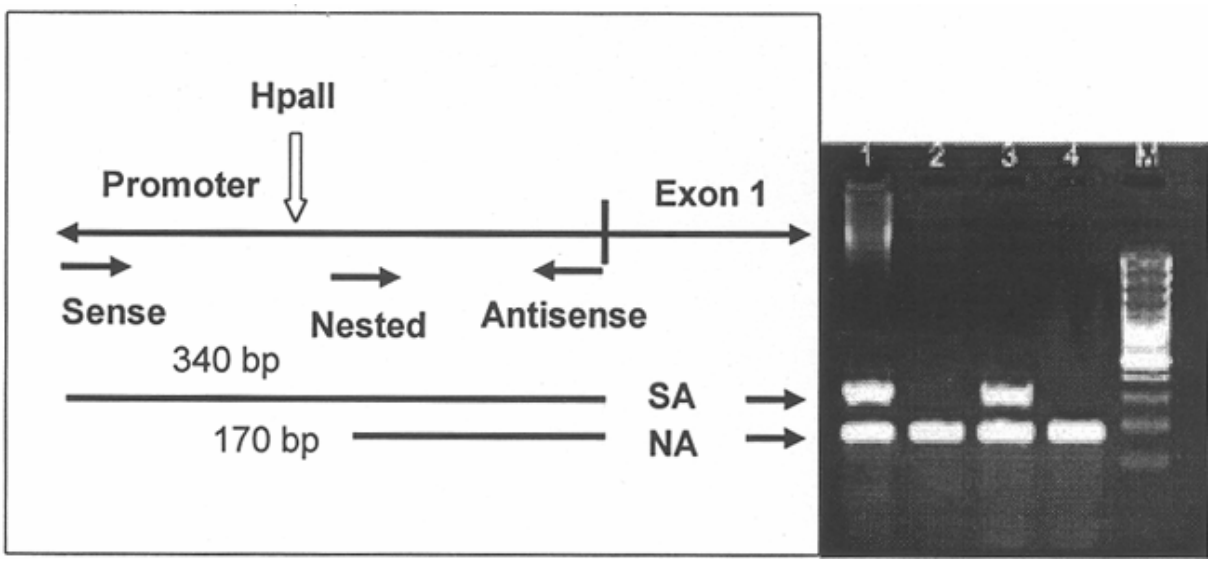


Fig. 1—Multiplex-PCR strategy to detect methylation. The figure shows positioning of the sense, antisense and nested primers in the promoter region of the gene studied for hypermethylation. SA, (340 bp) product generated by sense and antisense primers. NA, (170 bp) product generated by nested and antisense primers. The inset gel picture shows the PCR products generated after digestion with *Hpa II* methylation sensitive restriction enzyme (lanes 1, 3) when the DNA is methylated; lanes 2, 4 show the pattern when the DNA is not methylated, M, 100 DNA bp ladder.

promoter regions of *p16, hMLH1, MGMT* and *E-cadherin* genes using *in vitro* methylated (*Sss1* methylase treated) human placental DNA as control. The results showed the efficacy of the assay in detecting methylated promoter regions of the four genes included in the study (Fig. 2).

Complete restriction digestion of the genomic DNA is very important in the outcome of the assay and this was verified using the *Hpa II* digested DNA as templates in a PCR reaction using primers for the human protein phosphatase gene promoter region which encompassed a single *Hpa II* site. Absence of amplicons in the *Hpa II* digested DNA compared to a control undigested DNA indicated complete digestion of the genomic DNA samples used in this study (data not shown).

Using this modified protocol we found that the promoter regions of the *p16* tumor suppressor gene and the $O^6$-methyl guanine methyl transferase, (*MGMT*) gene were not methylated in the 19 sporadic breast carcinomas (Fig. 3 a,b). In contrast, 3 tumors

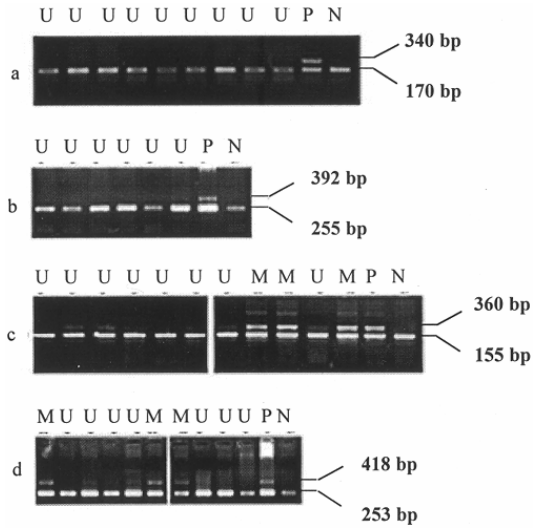


Fig. 3 — Methylation detection in breast carcinoma samples. PCR products generated after digestion with *Hpa II* methylation sensitive restriction enzyme. [(a) – *p.16*; (b) – *MGMT*; (c) – *hMLH1* and (d) – *E-cadherin.* U – unmethylated; M – methylated; P – positive control (*in vitro* methylated placental DNA); N – negative control (placental DNA)].

Table 1 — Methylation analysis of four tumor associated genes-*p16, hMLH1*, *MGMT* and *E-cadherin* in sporadic breast carcinomas.

| Sample | Age | TNM Stage | Epigenetic alterations | | | Genetic alterations[16] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Promoter hypermethylation | | | Mutations | | | Deletions |
| | | | *hMLH1* | MGMT p16 | *E-cadherin* | *p53* | Fhit | *p16 Ras* | *p14/ARF* |
| B1 | 30 | ---- | Unmeth | Unmeth | Meth | Nil | + | Nil | Nil |
| B2 | 52 | $T_3N_1M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B3 | ---- | ---- | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B4 | 45 | $T_4N_1M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B5 | 48 | $T_2N_0M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B6 | 40 | $T_{4B}N_1M_0$ | Unmeth | Unmeth | Meth | Nil | Nil | Nil | Nil |
| B7 | 32 | $T_{4B}N_2M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B8 | 32 | $T_{4B}N_1M_0$ | Unmeth | Unmeth | Unmeth | + | Nil | Nil | Nil |
| B9 | 54 | $T_XN_1M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B10 | 50 | $T_{4C}N_1M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B11 | 39 | $T_4N_1M_0$ | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B12 | 37 | ---- | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B13 | 32 | ---- | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B14 | 44 | ---- | Unmeth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B15 | 60 | $T_2N_0M_0$ | Unmeth | Unmeth | Meth | Nil | + | Nil | Nil |
| B16 | 58 | ---- | Meth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B17 | 41 | $T_3N_2M_0$ | Meth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |
| B18 | 28 | $T_2N_1M_0$ | Unmeth | Unmeth | Unmeth | ++ | Nil | Nil | Nil |
| B19 | 53 | $T_3N_1M_0$ | Meth | Unmeth | Unmeth | Nil | Nil | Nil | Nil |

Meth – Methylated; Unmeth – Unmethylated;
Nil – No alterations; + Mutation; ++ Double mutation

showed hypermethylation in the promoter of *hMLH1* and another three had methylation in the *E-cad* gene promoter. Taken together these results indicated hypermethylation in 31.6% (6 out of 19) of sporadic breast carcinomas analysed in the present study. (Fig. 3 c, d; and Table 1).

## Discussion

The modified strategy and design of a single 'consensus' PCR condition to detect methylation in four genes using the same reaction conditions reported in the present study make this method to be a valuable and simple procedure for use in routine clinical diagnosis. The restriction digestion using the methylation sensitive restriction enzyme was found to be complete by an assay using the human protein phosphatase gene. Further, none of the cases studied were simultaneously positive for methylation of all the genes studied using the same *Hpa II* digested DNA samples indicating the absence of false positives. In comparison with the conventional bisulfite modification method to detect methylation, our method may be less sensitive. However using cloned *p16* promoter region we determined that the assay could detect methylation even if 20% of the given sequences were methylated (data not shown). The advantage of our method is its simplicity, so that it can be used for quick screening for methylation in large number of samples. We have also used this method to assess hypermethylation of tumor associated genes in oral cancer[6]. We have not analysed *BRCA1* and *BRCA2* genes for mutation since mutations in these genes are rare events in sporadic breast carcinomas[8].

It has been shown that promoter hypermethylation of the *p16* tumor suppressor gene is not a major event leading to its inactivation in breast cancers[9]. In accordance with earlier reports on the status of inactivation of the *MGMT* gene by promoter hypermethylation[10], our results indicated that hypermethylation inactivation of this gene did not occur in breast carcinoma, though it is a common event in several primary human neoplasms. The involvement of *E-cadherin* alterations in breast cancers have already been documented including LOH in the chromosomal region encompassing this gene and inactivating mutations have also been observed although with low frequency[11]. Hypermethylation of promoter region of *E-cadherin* is also likely to be a frequently occurring event involved in downregulation of the expression of this gene in sporadic breast cancers[12,13]. In the light of these studies, it was interesting to note that 3 of the 19 sporadic breast carcinomas analysed in the present study showed hypermethylation in this gene.

Frequent loss of heterozygosity and allelic deletions in *hMLH1* loci are implicated in the genesis of sporadic breast carcinoma[14]. Murata *et al*.[15] have reported that 31% of 32 breast carcinomas analysed are hypermethylated; the extent of methylation is even higher in the subset of tumors that exhibit microsatellite instability. Our report showed that the promoter region of this gene was hypermethylated in atleast 16% of sporadic breast carcinomas of Indian women. Our data indicated the possible inactivation by promoter hypermethylation of *hMLH1* and *E-cadherin* genes in a substantial subset (31.6%) of breast carcinoma cases. It was interesting to note that the hypermethylation of *hMLH1* and *E-cad* genes were non-overlapping suggesting that epigenetic silencing of one of these genes might be sufficient in the development of sporadic breast carcinomas.

Comparison of methylation profile of *hMLH1* and *E-cadherin* with data published from this laboratory on *p53* mutations in the same cohort of samples indicated methylation events in the four genes studied in the present study to be exclusive of *p53* missense mutations[16]. Two samples with mutations in *fhit* gene, were methylated and no mutations were detected in the *ras* and *p16* genes (Table 1).

$p14^{ARF}$ is another gene that is widely reported to be lost/inactivated in cancers and we have found deletions of the exon 1β of this gene in oral squamous cell carcinomas[7]; no deletion of this gene was observed in the breast carcinomas analyzed in the present study. We were unable to obtain biopsies of early stage breast carcinomas. The clinical data presented in Table 1 indicated that the samples analysed were of the midstage, well before metastasis. Therefore, we could not say whether hypermethylation of *hMLH1* and *E-cad* promoters were early or late events, but it could be concluded that they occurred before the terminal stage of metastasis since none of the tumors analysed by us were metastatic. Our data indicated that methylation of tumor associated genes could be among the events that could lead to progression/maintenance of the breast cancer phenotype even in the absence of inactivating/activating mutations in a tumor suppressor gene or oncogene (Table 1).

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