Development of a multiplex MethyLight assay for the detection of DAPK1 and SOX1 methylation in epithelial ovarian cancer in a north Indian population

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Ovarian cancer is the fourth most common cancer in women worldwide. It is very heterogeneous at the clinical, histopathological and molecular levels and is caused by the accumulation of genetic and epigenetic changes in regulatory genes. More than 90% of ovarian cancers are epithelial in origin. Ovarian cancer is typically asymptomatic in its early stages, and, due to difficulties in early detection, most ovarian cancers are diagnosed at an advanced stage. The positive predictive value of CA-125, a routinely used serum protein marker, is < 30%; therefore, for effective screening, there is a need to develop a marker with high sensitivity for early detection. Development of blood-based biomarkers that detect DNA methylation in cell-free tumor-specific DNA is now being considered as a potential approach for the early diagnosis of cancer. Our objective in this study was to develop an absolute quantitative method, the MethyLight assay, to detect the promoter methylation status of two tumor suppressor genes. We analyzed the methylation level of the promoter regions of these genes in 42 tumor samples using the MethyLight assay. SOX1 promoter methylation was significantly higher in cancer samples than in normal samples ($P = 0.011$), whereas this difference between cancer and normal samples was not significant for DAPK1 promoter methylation ($P = 0.18$), when analyzed separately in a singleplex assay, whereas the detection frequency and significance level increased several-fold when these genes were analyzed together in a multiplex assay ($P = 0.0004$). The sensitivity was found to be 62% and 83% for DAPK1 and SOX1, respectively, when analyzed separately in the singleplex assay, but increased to 90% in the multiplex assay when either or both of the SOX1 and the DAPK1 gene promoters showed methylation.

Key words: DNA methylation, epigenetic biomarker, MethyLight assay and ovarian cancer

INTRODUCTION

Ovarian cancer is the fourth most common cancer in women in India, with an annual occurrence of 26,834 new cases. Epithelial ovarian cancer (EOC) is the most common (about 85 to 90%) of all forms of ovarian carcinoma (Globocan 2012, globocan.iarc.fr). Ovarian cancers are very heterogeneous at the histopathological, clinical and

molecular levels. They are caused by the accumulation of genetic and epigenetic changes in regulatory genes (Hanahan and Weinberg, 2000). Among various epigenetic modifications, DNA methylation is common in a variety of tumors. Aberrant DNA methylation is a very stable repressive mark of DNA which is commonly associated with the loss of gene function (Antiquera and Bird, 1999). Numerous reports indicate that methylation at CpG islands in the promoter region is the major cause of silencing of many tumor suppressor genes, such as RASSF1A, DAPK1, p16 and GBGT1, in various cancers (Collins et al., 2006; Bammidi et al., 2012; Matoo et al., 2013; Shi et al., 2013; Bhagat et al., 2014; Jacob et al.,

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Abbreviations: PMR: percent methylation reference; CA-125: carbohydrate antigen 125

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2014). Its high stability and sensitivity of detection make DNA methylation an attractive clinical tool for the diagnosis of different cancers, prediction of potential malignancy progression and metastasis/recurrence of cancer.

No single gene in ovarian cancer has been identified to be methylated in most histological types, unlike prostate cancer in which GSTP1 is methylated in over 90% of cancers (Song et al., 2002). SFN, TMS1 and WT1 promoter methylation is more frequent in ovarian clear cell carcinomas than in other types of ovarian cancer, whereas invasive ovarian carcinomas exhibit a higher rate of RASSF1A, APC, GSTP1 and MGMT methylation than low malignancy potential tumors (Barton et al., 2008). Methylation patterns of various genes have been studied but no consistent result has yet been found to enable any gene to serve as a diagnostic biomarker for early detection of ovarian cancer. The aim of the current study was to analyze the methylation status of the promoter regions of the DAPK1 and SOX1 genes, well known as hypermethylated targets in epithelial ovarian cancer, with respect to an Indian population.

This study includes an analysis that simultaneously detects the methylation status of DAPK1 and SOX1 and quantifies the methylation level of these two genes through the MethyLight assay, which, provided that at least one of these genes is methylated, can be used for effective screening and surveillance of epithelial ovarian cancer. MethyLight is a sodium bisulfite-dependent, quantitative, fluorescence-based (TaqMan), real-time PCR method for sensitively detecting and quantifying DNA methylation in genomic DNA. This assay is capable of detecting methylated alleles in the presence of a 10,000-fold excess of unmethylated alleles.

MATERIALS AND METHODS

Tissue specimens The 42 ovarian tumor samples used in this study were obtained from patients who underwent surgery at Sir Sunderlal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Tissue samples were collected with the informed consent of the patient. The work plan was approved by the ethics committee of Motilal Nehru National Institute of Technology. Among the tumor samples collected, there were three clear cell, seven mucinous and thirty serous EOC samples. Seven normal human ovarian tissue samples were also collected. The tissue samples were stored at -80°C immediately after surgical resection until they were analyzed. Clinical information was collected from the patient's records and pathology reports.

DNA extraction and bisulfite treatment Total genomic DNA was extracted from frozen tissue specimens (10–50 mg) according to a standard protocol. Tissues were homogenized in 2 ml SET buffer containing 0.3 M sucrose,

25 mM Tris (pH 8.0) and 5 mM EDTA (pH 8.0). After centrifugation, the pellet was lysed with 1 ml lysis buffer (50 mM Tris (pH 8.0) 2.5 mM EDTA (pH 8.0), 100 mM NaCl and 10% SDS) and was subjected to proteinase K digestion (50 $\mu\text{g}/\text{ml}$) at 37°C for 12–14 h followed by phenol:chloroform:isoamyl alcohol extraction (25:24:1) at 25°C . Finally, DNA was precipitated with 1/30 volume of 3 M sodium acetate (pH 5.0) and two volumes of chilled absolute ethanol, and washed once with 70% ethanol. The DNA pellet was re-suspended in 100–200 μl of TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)) and then stored at 4°C until use. All DNA samples were bisulfite-converted using a BisulFlash DNA Modification kit (Epigentek, Farmingdale, NY).

MethyLight assay The MethyLight assay (quantitative real-time PCR) was performed in the ABI StepOne Plus system (Applied Biosystems, Foster City, CA). For each gene, a set of primers and a probe containing multiple CpG sites for bisulfite-converted DNA were designed within the putative CpG islands around the gene promoters. For the endogenous reference gene, COL2A1, the primers and probe were designed without any CpG dinucleotides. A singleplex MethyLight assay was performed in a 15- μl reaction volume containing $1 \times$ real-time PCR master mix (Takyon Rox Probe MasterMix dTTP Blue, Eurogentec), 330 nM probe, 450 nM primers of each gene, and 1 μl bisulfite-converted DNA templates. PCR was performed with an initial denaturation at 95°C for 4 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 35 s. Reactions were performed in duplicate with positive controls on each plate. A multiplex MethyLight assay was performed with same primers and probes, under the same conditions. Primer and probe sequences of the selected genes are listed in Table 1.

A positive standard was prepared as a control for methylated alleles of DAPK1 and SOX1. DNA from normal ovarian tissues was fully methylated by treatment with MSss1 methyltransferase (New England Biolabs, Ipswich, MA) in vitro, and was modified according to the sodium bisulfite conversion protocol.

PMR value calculation Percent methylation reference (PMR) values were used to define the percentage of fully methylated molecules at a specific locus. PMR values were calculated by the comparative Ct method, for which serial dilutions of bisulfite-treated universally methylated DNA were used to construct a relative standard curve for each gene. The relationship between the percentage of methylated DNA molecules and Ct was described as

$$\text{PMR} = 2^{-\Delta\Delta\text{Ct}} \times 100\%, \text{ where } \Delta\Delta\text{Ct} \\ = \Delta\text{Ct}_{(\text{Gene})} - \Delta\text{Ct}_{(\text{COL2A1})} = \left[\text{Ct}_{(\text{Gene})} - \text{Ct}_{(\text{COL2A1})} \right]_{(\text{Sample})} \\ - \left[\text{Ct}_{(\text{Gene})} - \text{Ct}_{(\text{COL2A1})} \right]_{(\text{MSss1-treated normal DNA})}$$

Table 1. Primer and probe sequences of selected genes for the MethyLight assay

Gene	Forward primer	Reverse primer	Probe
DAPK1	5'-TCGTCGTCGTTTCGGT TAGTT-3'	5'-TCCCTCCGAAACGCTAT CG-3'	5'-FAM- CGACCATAAACGCCAACGCCG-TAMRA-3'
COL2A1	5'-TCTAACAATTATAAAC TCCAACCACCAA-3'	5'-GGGAAGATGGGATAGA AGGGAATAT-3'	5'-NED- CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA- TAMRA-3'
SOX1	5'-CGTAGGAGGAAGGAG ATA-3'	5'-CCGAAAACGATACGC TAA-3'	5'-VIC- AATATACTATCTCCTCGCCGACCG-TAMRA-3'

Fig. 1. Amplification plots of the candidate biomarker genes DAPK1 and SOX1, and the control gene COL2A1, using (A) singleplex and (B) multiplex MethyLight assays. ΔR_n is defined as the cycle-to-cycle change in the reporter fluorescence signal normalized to a passive reference fluorescence signal.

The number of cycles at which the fluorescence signal crossed a detection threshold is referred to as Ct. Representative amplification plots for DAPK1 and SOX1 are illustrated in Fig. 1, A and B. Samples containing > 4% fully methylated molecules were designated as methylated, and samples containing $\leq 4\%$ were designated as unmethylated. A cut-off value of 4% gave the best discrimination between normal and tumor tissue.

Statistical analysis *P* values and methylation indices were calculated using chi-square tests. PMR values were calculated using Microsoft Excel 2007. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant. GraphPad Prism V5.0 (GraphPad Software, San Diego, CA) was used to draw all the graphs.

RESULTS

MethyLight assay development The methylation levels of DAPK1 and SOX1 in tumor tissue samples were quantified by the MethyLight assay. The linearity and accuracy of the MethyLight method were examined by preparing a serial dilution of a positive standard. The total amounts of input DNA in each dilution were determined. The standard curves of COL2A1, SOX1 and DAPK1 were generated with MSs1-treated normal tissue sample in a multiplex PCR (Fig. 2, A and C). The R^2 values of the three standard curves were 0.962, 0.989 and 0.998 for COL2A1, SOX1 and DAPK1, respectively. These data indicated good reproducibility in standards of different samples.

Evaluation of the multiplex MethyLight assay The normalized ratios of the singleplex MethyLight assay strongly correlated with those of the multiplex assay for both the markers (Fig. 3, A and B). After optimization, the results of the multiplex MethyLight assay were compared with results from the singleplex MethyLight assay. We found a maximum difference in threshold cycle (Ct) of 1.2 between singleplex and multiplex MethyLight PCR (mean, 0.5; range, 0.0–0.9). Correlation coefficients were as follows: $R^2 = 0.964$ and 0.943 for DAPK1 and SOX1, respectively. Of note, the same Ct ratios between singleplex and multiplex assay could be obtained when the threshold settings were adjusted for both the markers and COL2A1. These results indicate that the developed multiplex MethyLight assay shows an equal performance with the singleplex assay in detecting methylation in these samples.

Quantitative analysis of methylation in ovarian tissues The methylation level of DAPK1 and SOX1 in the 42 ovarian cancer tissue samples and seven normal tissue samples was compared. The distribution of PMR values

Fig. 2. Standard curves of (A) COL2A1, (B) SOX1 and (C) DAPK1 generated with MSs1-treated normal tissue sample in a multiplex PCR. The R^2 values of the three standard curves were 0.962, 0.989 and 0.998 for COL2A1, SOX1 and DAPK1, respectively. These data indicate good reproducibility in standards of different samples.

is illustrated in Fig. 4, A and B. The methylation level of the majority of the methylated ovarian cancer samples ranged from 4 to 96%. Various parameters reflecting the methylation status of DAPK1 and SOX1 gene promoters in ovarian cancer and normal tissue samples are summarized in Table 2. A chi-square test demonstrated that

Fig. 3. Correlation between the ΔCq values obtained with singleplex and multiplex MethyLight assays. The R^2 values for the two markers were 0.964 and 0.943 for (A) DAPK1 and (B) SOX1, respectively. The ΔCq values between singleplex and multiplex quantitative real-time PCR differ due to differences in set thresholds.

Fig. 4. Distribution of PMR values of DAPK1 and SOX1 genes in ovarian cancer and corresponding noncancerous tissue samples.

Table 2. Sensitivity and Methylation Index of singleplex and multiplex MethyLight assays in epithelial ovarian cancer (EOC) patients

	Singleplex MethyLight assay		Multiplex MethyLight assay
	DAPK1 methylation status	SOX1 methylation status	Combination of both genes (at least one gene is methylated)
Sensitivity (%)	61.90	83.30	90.40
<i>P</i> value	0.180	0.011	0.0004
Methylation Index	0.62	0.83	0.90

SOX1 promoter methylation was significantly higher in cancer samples than in normal samples ($P = 0.011$), whereas this difference between cancer and normal samples was not significant for DAPK1 promoter methylation ($P = 0.18$), when analyzed separately in a singleplex assay, whereas the detection frequency and significance level increased several-fold when these genes were analyzed together in a multiplex assay ($P = 0.0004$). P values calculated through the chi-square test determine the sta-

tistical significance of the trend of methylation positivity in the tumor samples in singleplex and multiplex assays. The Methylation Index is calculated as the proportion of methylated samples in the total number of samples. To test the potential of methylated DNA as a screening marker, we analyzed the sensitivity of these genes together in the MethyLight assay. The sensitivity was found to be 62% (26 of 42) and 83% (35 of 42) for DAPK1 and SOX1, respectively, when analyzed separately in the

singleplex assay, but increased to 90% in the multiplex assay when either or both of the SOX1 and the DAPK1 gene promoters showed methylation.

DISCUSSION

Development of ovarian cancer is a multistep process associated with genetic and epigenetic alterations. Methylation of promoter CpG islands and suppression of multiple tumor suppressor genes is frequently observed in the development of cancer and may occur at different stages of ovarian cancer. Assays to measure DNA methylation are potentially very useful in clinical practice, because many genes have been shown to be methylated and functionally silenced in a variety of human neoplasias.

In this study, we evaluated precision and performance characteristics of MethyLight, a real-time PCR assay, for two tumor suppressor genes. The MethyLight method has major advantages: it is a quantitative and relatively simple assay procedure; moreover, a minute amount and modest quality of DNA template are required, making the method compatible with plasma and serum samples and with small biopsies. The multiplex assay is thus useful clinically, combining measurement of multiple methylation biomarkers with high throughput in a single test, and thereby provides more useful data for diagnosis (Zhang et al., 2010).

A large number of genes have been identified as hypermethylated in EOC, such as p16, RASSF1A, OPCML, DAPK, SOX1, PAX1 and SPARC (Bammidi et al., 2012; Ibanez de Caceres et al., 2004; Sellar et al., 2003; Collins et al., 2006; Su et al., 2009; Socha et al., 2009), with the reported frequency of methylation varying widely among independent studies. To improve the specificity, we chose two tumor suppressor genes to construct the MethyLight assay, based on the role and function of genes in ovarian cancer, their relatively high hypermethylation rate in epithelial ovarian carcinoma and their consequent inactivation.

DAPK1 and SOX1 were selected for this study because their promoter regions are aberrantly methylated in ovarian cancer tissues and peripheral blood. DAPK1 is a tumor suppressor gene and has been reported to be suppressed or silenced in different cancers. Häfner et al. (2011) reported 50% and 35.3% DAPK1 methylation in primary tissue and 56% and 23.8% in serum samples from ovarian cancer and leiomyoma patients, respectively, whereas no samples from healthy women showed methylation of DAPK. Another study indicated 67% methylation in the DAPK1 promoter region of EOC tumors; again, no methylation was observed in healthy control samples (Collins et al., 2006). SOX1 is involved in the regulation of embryonic neural development. SOX1 methylation was analyzed, within the set of genes

SFRP1, SFRP2, SOX1 and LMX1A, by methylation-specific PCR (MSP) and was correlated with recurrence and overall survival of ovarian cancer patients. Alone, SOX1 methylation was found to be 58.7%, whereas combining the data for SFRP1, SOX1 and LMX1A increased the sensitivity and specificity rates to 73.08 and 75% (Su et al., 2009).

A MethyLight assay was performed to analyze DNA methylation of a panel of genes including DAPK1 for the detection of bladder cancer, where the promoter regions of TERT, RASSF1A, DAPK1, BCL2 and TNFRSF25 genes showed significant increases in methylation levels when compared with levels in nonmalignant adjacent tissue ($P < 0.01$) (Friedrich et al., 2004). A quantitative MSP analysis has confirmed that promoter hypermethylation of SOX1 is significantly more frequent in hepatocellular carcinomas than in control livers ($P < 0.0001$) (Shih et al., 2013). No study has analyzed the methylation status of the DAPK1 and SOX1 genes in combination, using the MethyLight assay as diagnostic marker, or for screening individuals at risk. With the multiplex MethyLight assay as developed in the present study, methylation of the promoter regions of these genes was detected successfully in both cancerous and normal ovarian tissue samples. We evaluated the reliability and the sensitivity of this multiplex MethyLight assay. The results obtained from this assay were stable and reproducible. On the basis of the multiplex MethyLight assay, we found that in some tissue samples, only one of the two genes (DAPK1 and SOX1) exhibited aberrant methylation. Combining the two genes for the assay, the sensitivity for detection of ovarian cancer in tissues reached 90%. Further analysis such as pyrosequencing should be undertaken to identify consistently methylated CpG sites; similarly, a MethyLight assay involving more genes needs to be developed to find the best set of biomarkers for the development of an early blood/serum-based diagnostic assay. Methylation studies in colorectal cancer have led to the development of a blood-based Septin 9 biomarker test kit called Epi proColon 2.0 by Epigenomics, which is a commercial success in both diagnostic and prognostic analysis. Similar work needs to be done in the case of ovarian cancer, as there is no reliable methylation-based biomarker available so far.

Ovarian cancer often goes undetected until it has spread beyond the pelvis and abdomen; it is more difficult to treat and is frequently fatal at this later stage. A CA-125 blood-based biomarker test, which detects the level of CA-125 in blood serum, has already been developed to check the progression and recurrence of ovarian cancer. CA-125 is a protein produced in more than 90% of advanced epithelial ovarian cancers. The major limitations with this biomarker are its lack of sensitivity and specificity for detecting early stages of ovarian cancer, especially in premenopausal women, which leads to false-

positive and false-negative (Stephen et al., 2013). Accumulated data indicate that the aberrant methylation of specific genes in the plasma or serum of patients with different carcinomas may be a useful epigenetic marker for early-stage disease. Therefore, for effective screening, there is a need to develop a blood-based marker with high sensitivity for early detection and sufficient specificity to protect patients against false-positive results and low survival rates (when diagnosis is made at late stage of disease). Since the combined sensitivity for DAPK1 and SOX1 reaches 90%, these genes appear to have great potential to be evaluated for their methylation level in cell-free DNA or serum DNA.

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