Quantitative detection of *RASSF1A* DNA promoter methylation in tumors and serum of patients with serous epithelial ovarian cancer

Amy E. BonDurant [a], Zhiqing Huang [a], Regina S. Whitaker [a], Lauren R. Simel [a], Andrew Berchuck [a], Susan K. Murphy [a,b,]*

[a] *Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC, 27708, USA*
[b] *Department of Pathology, Duke University Medical Center, Durham, NC 27710, USA*

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ABSTRACT

Objective. Detection of cell free tumor-specific DNA methylation has been proposed as a potentially useful noninvasive mechanism to detect malignancies, including ovarian cancer, and to monitor response to treatment. However, there are few easily implemented quantitative approaches available for DNA methylation analysis. Our objectives were to develop an absolute quantitative method for detection of DNA methylation using *RASSF1A*, a known target of promoter methylation in ovarian cancer, and test the ability to detect *RASSF1A* methylation in tumors and serum specimens of women with ovarian cancer.

Methods. Bisulfite modified DNAs were subjected to real time PCR using nondiscriminatory PCR primers and a probe with sequence containing a single CpG site, theoretically able to capture the methylation status of that CpG for every allele within a given specimen. Input DNA was normalized to *ACTB* levels detected simultaneously by assay multiplexing. Methylation levels were established by comparison to results obtained from universally methylated DNA.

Results. The assay was able to detect one methylated *RASSF1A* allele in 100,000 unmethylated alleles. *RASSF1A* was methylated in 54 of 106 (51%) invasive serous ovarian cancers analyzed and methylation status was concordant in 20/20 matched preoperative serum–tumor pairs. Serial serum specimens taken over the course of treatment for 8 of 9 patients showed fluctuations in *RASSF1A* methylation concomitant with disease status.

Conclusions. This novel assay provides a real-time PCR-based method for absolute quantitation of DNA methylation. Our results support feasibility of monitoring *RASSF1A* methylation from serum samples taken over the course of treatment from women with ovarian cancer.

Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States and its molecular pathogenesis remains poorly understood. There is evidence that both genetic and epigenetic events play a role [1–4], with epigenetic changes occurring early in the carcinogenic process [5]. Understanding how these changes give rise to cancer, and how such alterations might be exploited to develop new diagnostic and therapeutic opportunities is a critical component of improving patient survival. It has become increasingly clear that epigenetic alterations in cancer are more frequent than genetic alterations, and these changes encompass both DNA methylation and histone modifications [4]. Many genes are indeed silenced in ovarian malignancies by DNA methylation [6,7], including *RASSF1*, which is methylated in 10–50% of ovarian cancers [7–11]. *RASSF1* encodes seven known alternatively spliced transcripts, with *RASSF1A* and *RASSF1C* constituting the major isoforms that are transcribed from different promoters located within distinct CpG islands. Expression of the *RASSF1A* isoform is selectively affected by DNA methylation, and is also uniquely responsible among the *RASSF1* isoforms for the reported tumor suppressor effects of RASSF1 [12,13].

The ease with which DNA methylation can be detected and the stability of this epigenetic mark have made it an attractive target for development of assays designed to detect malignancy from specimens obtained through noninvasive means. PCR-based methods have allowed for development of highly sensitive assays that are able to detect aberrant methylation of tumor DNA in serum or plasma [8,14–16]. For example, methylation-specific PCR is able to provide a positive signal for one methylated allele among one thousand that are

* Corresponding author at: B226 Levine Science Research Center, Research Drive, Box 91012 Durham, NC 27708, USA. Fax: +1 919 684 5336.
E-mail addresses: amyebondurant@gmail.com (A.E. BonDurant), huang028@mc.duke.edu (Z. Huang), whita004@mc.duke.edu (R.S. Whitaker), lrs3@duke.edu (L.R. Simel), berch001@mc.duke.edu (A. Berchuck), murph035@mc.duke.edu (S.K. Murphy).

unmethylated [17]. Similar assays have been developed that use real time detection of PCR products generated from methylated and unmethylated templates. In this technique, two primers and a probe are designed to anneal to sequence that contains multiple potentially methylated CpG dinucleotides in DNA that has undergone modification by sodium bisulfite. This chemical modification results in conversion of unmethylated cytosines to uracils leaving methylated cytosines unaffected [18]. Amplification and detection of methylation using real time PCR occur as the primers are extended during polymerization and the reporter fluorophore is released from the quencher fluorophore at the 3′ end of the probe through the 5′ nuclease activity of the Taq DNA polymerase. Fluorescence intensity in the reaction is measured at each cycling step, and is directly proportional to the amplicons synthesized. Unless multiple sets of primers and probes are designed to account for heterogeneous patterns of methylation (wherein some CpGs within the primer/probe target sequences on a given allele are methylated while others within the target sequences are unmethylated), the measurements obtained represent only those from DNA with a homogeneous pattern of methylation [19].

Herein we describe a real time PCR assay for quantifying *RASSF1A* promoter methylation in which the primers anneal to sequence devoid of CpGs while the probe anneals to a region containing a single CpG dinucleotide. Following validation of this assay, we analyzed ovarian cancer tumor tissue and serum specimens to determine if the assay is able to detect *RASSF1A* methylation. We also used this assay to evaluate serial serum specimens collected prior to and post surgery and chemotherapy to assess how *RASSF1A* methylation status might change over the course of treatment.

Materials and methods

Specimens

Normal and malignant specimens used for these studies were derived from the Duke University Gynecologic Oncology Tissue Bank, which collects specimens for banking and research purposes following provision of written informed consent by each participant under a Duke University Institutional Review Board approved protocol, IRB #0566-08-2R9ER. Use of de-identified specimens from the tissue bank for the present study was approved by the Duke University Institutional Review Board (IRB #6534-07EX). Affymetrix U133A gene expression microarray data was derived from our previously published data [20] for 45 serous epithelial ovarian cancer specimens (3 stage I/II, 42 stage III/IV). A complete clinical response for these patients was defined by the presence of: 1) normalization of CA125 levels, 2) a normal CT scan and 3) an office examination with no evidence of disease. An incomplete clinical response was defined as failure to meet any one of these three criteria.

DNA extraction

DNA from frozen tumors was purified using Gentra Systems reagents according to the manufacturer's protocol (Qiagen; Valencia, CA). For DNA isolation from serum samples, 500 μl serum was incubated with an equal volume of a 1% solution of SDS and 20 mg/ml proteinase K with occasional mixing at 50 °C for 48 h. 25 μl of a 20% solution of proteinase K was added three times daily. Samples underwent phenol:chloroform extraction twice followed by addition of 500 μl of 7.5 M NH₄OAc and 2 μl of glycogen solution (Qiagen). 4 ml of absolute ethanol was added followed by gentle inversion and incubation at −80 °C for ≥30 min. The samples were centrifuged for 35 min at 4 °C (5220 rcf), washed with 5 ml 70% ethanol and centrifuged for 15 min at 4 °C. The pellet was air dried for 10 min at ambient temperature and resuspended using 20 μl Hydrating Solution (Qiagen, Valencia, CA) overnight at 4 °C. DNA yields ranged from 25 ng/μl to 215 ng/μl.

Bisulfite modification

Genomic DNA (250–500 ng) was treated with sodium bisulfite as previously described [21]. DNA was recovered in 25–50 μl nuclease-free water and stored at 4 °C.

Radiolabeled bisulfite sequencing

Amplicons (698 bp) were produced from 30 ng of bisulfite modified DNA using Platinum Taq DNA Polymerase (Invitrogen; Carlsbad, Ca), 3 mM MgCl₂, 0.2 mM dNTPs and 0.4 μM each of the forward primer (listed 5′ to 3′) GGA GGG AAG GAA GGG TAA G and reverse primer AAC AAC CAC CTC TAC TCA TCT ATA ACC. PCR conditions were as follows: 94 °C × 3 min; then 35 cycles of 94 °C × 30 s, 64 °C × 30 s, 72 °C × 30 s; this was followed by a 5 min extension at 72 °C. Amplicons were resolved on 2% agarose gels, purified, and sequenced using the forward primer listed above with the ThermoSequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation; Cleveland, OH). The region sequenced includes the CpG site queried using real time PCR, described below. The dried gel was imaged using the Molecular Dynamics Storm Phosphorimaging System and ImageQuant software (GE Healthcare; Piscataway, NJ). The percent methylation for individual CpG dinucleotides was determined using the formula:% methylation = [volume T / (volume T + volume C)] × 100.

Quantitative real time PCR methylation assay

Multiplexed real time PCR reactions were performed on an Applied Biosystems 7900HT instrument. Controls were run on each plate and included defined mixtures of unmethylated and universally methylated DNAs (Millipore; Billerica, MA) in addition to reactions with all components except for the DNA template. Twenty five microliter reaction volumes were used and contained 40 ng of template DNA, 2X master mix (Applied Biosystems; Foster City, CA), 900 nM final concentration of all four primers, and 250 nM final concentration each of the *RASSF1A* and *ACTB* probes. *ACTB* primers and probe sequence were based on a prior report [22] and were as follows (listed 5′ to 3′): forward, TGG TGA TGG AGG AGG TTT AGT AAG T; reverse, AAC CAA TAA AAC CTA CTC CTC CCT TAA; probe, FAM-ACC ACC ACC CAA CAC ACA ATA ACA-TAMRA. *RASSF1A* primers and probe sequences were: forward, AAG GAG GGA AGG AAG GGT AAG G; reverse, CCT CCC CCA AAA TCC AAA CTA A and the probe specific for methylated *RASSF1A* DNA: VIC-ACT ATA AAA CCC GAA AAC-(MGB). Thermocycler conditions were as follows: 50 °C × 2 min; 95 °C × 10 min; 50 cycles of: 95 °C × 15 s, 55 °C × 30 s, 68 °C × 30 s. Assay sensitivity was determined using only the *RASSF1A* probe and primers for defined copy numbers of bisulfite modified plasmid DNA containing the methylated version of the *RASSF1A* sequence and Qiagen's Epitect unmethylated control DNA. For measurements of *RASSF1A* methylation from genomic DNA, we calculated the average ratio of methylated DNA in each sample relative to the methylation present in the undiluted universally methylated DNA from triplicate measures. The amount of input DNA for all specimens, including the universally methylated DNA, was normalized using the Ct values for *ACTB*.

Results

We used a modified version of the MethyLight assay [19] to quantify DNA methylation. We used primers that amplify bisulfite modified genomic DNA regardless of methylation status and combined this with a probe that anneals to sequence containing a single CpG dinucleotide. Radiolabeled bisulfite sequencing was performed first to examine the promoter region in ovarian malignancies and from these results we selected one CpG within this region for assay design. This CpG is located 41 bp upstream of the annotated *RASSF1A* transcription start site (Fig. 1A) and exhibits a similar level of methylation to that of eight other adjacent CpGs measured in 30 ovarian cancers (mean, 11.8%, sd = 16.1% for target CpG versus a range of means
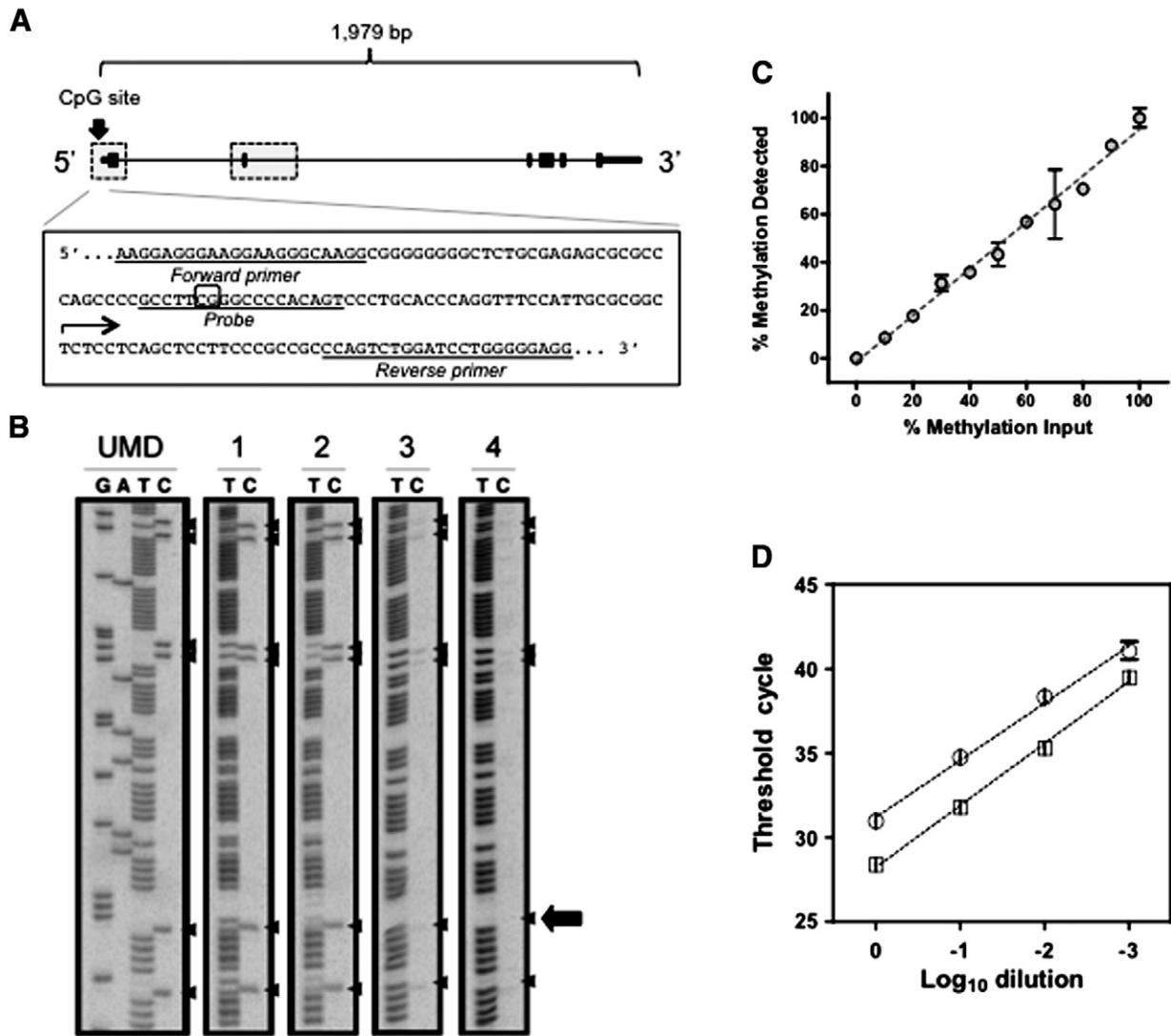
Fig. 1. *RASSF1A* locus and promoter methylation. (A) Schematic of the *RASSF1* locus at chromosome 3p21.31 (GRCh37/hg19; UCSC Genome Browser [34]), showing the exons (tall rectangles) and noncoding regions (short rectangles) of the *RASSF1A* isoform. Gray boxes with dashed outlines denote CpG islands. The relative position of the CpG site queried by the real time PCR methylation assay is indicated by the arrow. The original (non-bisulfite modified) nucleotide sequence of the region assayed is shown below, with positions of the PCR primers and CG-containing probe indicated. Right facing arrow, *RASSF1A* transcription start site. (B) Representative bisulfite sequencing data showing methylation of five specimens at the *RASSF1A* promoter region, spanning the CpG site contained within the probe used for real time PCR (black arrow). UMD, universally methylated DNA. Samples 1–4 are representative ovarian cancer specimens with higher (1 and 2) and lower (3 and 4) levels of *RASSF1A* methylation. All four nucleotides are shown for the UMD specimen for sequence context while only T and C nucleotide lanes are shown for the ovarian cancer specimens. Bands in the C lane are indicative of the presence of methylated cytosines while bands at the corresponding position in the T lanes are indicative of unmethylated cytosines. Arrowheads denote cytosines in CpG context. (C) Performance of the methylation-specific probe in real time PCR reactions using defined mixtures of bisulfite modified universally methylated and unmethylated DNAs. Real-time PCR reactions were run in triplicate and were analyzed relative to the UMD specimen. Average methylation +/− SEM is shown. R squared = 0.99. (D) Performance of the *RASSF1A* methylation-specific probe (squares) and the *ACTB* probe (circles) on a dilution series of bisulfite modified UMD. The *ACTB* primers and probe are non-discriminatory, as they do not anneal to sequence containing CG dinucleotides. Error bars, SEM.

from 6.5%, sd = 7.6% to 18.9%, sd = 21.7% for eight other adjacent ÇpG sites; representative data shown in Fig. 1B). In addition, the sequence context of this CpG allowed for design of a PCR assay using primers that do not anneal to sequence containing CpG dinucleotides.

The primers and single CpG-containing probe performed well in accurately detecting 10% incremental increases in levels of methylation using defined mixtures of bisulfite modified methylated and unmethylated DNAs (r squared = 0.99, p<0.0001; Fig. 1C). We also tested the sensitivity of the assay by using defined copy numbers of bisulfite modified methylated and unmethylated DNAs. Cycle threshold values showed a linear, progressive increase as the amount of methylated copies decreased, from methylated:unmethylated copy number ratios of 10,000:1 to 1:100,000 (r squared = 0.96, p<0.0001; Fig. S1). These results indicate a high degree of sensitivity in that one methylated copy can be detected in a background of 100,000 unmethylated copies.

An *ACTB* assay was used as a control for DNA input. The primers and probe for *ACTB* were designed to the bisulfite modified sequence but do not encompass any CpG dinucleotides [22]. We performed independent experiments in which a 10-fold dilution series of the bisulfite modified universally methylated DNA was used to assess performance for both *RASSF1A* and *ACTB* probes, which were labeled with different fluorophores to allow for multiplexed reactions. The results from all experiments (*RASSF1A*, N = 24; *ACTB*, N = 18) were averaged and plotted against the log of the dilution (Fig. 1D). The reactions produced linear trend lines over a four-log range with r squared values of 0.995 for *ACTB* (p = 0.003) and 0.997 for *RASSF1A* (p = 0.001).

Due to the prominent variability in the normal and malignant content of ovarian tumor tissues, we used the quantitative methylation data to classify tumors as exhibiting an unmethylated or methylated profile. Prior studies have used a threshold of >0% methylation with

respect to a fully methylated reference control to assign a methylated status to *RASSF1A* [23–25] since this particular promoter region is known to be unmethylated in normal tissues. However, we wished to define the background level above which *RASSF1A* could be considered to exhibit methylation using our methodology. To this end, peripheral blood lymphocytes (PBLs) are known to be unmethylated at *RASSF1A* [26,27]. We therefore measured methylation of PBLs from women without disease and showed an average level of methylation of 1.6% (SD = 1.3%, N = 17; data not shown). We used a threshold of >4.1% methylation to classify a tumor as being methylated (average plus two SDs; data shown in Fig. S2).

We used the real time PCR methylation assay to examine *RASSF1* methylation in 106 serous epithelial ovarian cancers (Fig. 2, Fig. S2). Fifty four of the 106 tumors exhibited hypermethylation. Analysis of clinical data did not reveal any significant associations between methylation status and race, stage, debulking status of the tumors, clinical response or survival (Table 1, Patient characteristics). However, *RASSF1A* was more likely to be methylated in women who were over 60 years of age at diagnosis, consistent with the idea that alterations in methylation status can arise in relation to the aging process [28]. Comparison of the real time PCR results for a subset of the ovarian tumors to those obtained by quantitative bisulfite sequencing (e.g., Fig. 1B) for the single CpG dinucleotide queried by the real time PCR assay showed that methylation values obtained by the two methods were significantly positively correlated (N = 53; Spearman r = 0.79, 95% CI, 0.66 to 0.87; p<0.0001; Fig. 3).

Tumor DNA is present in serum or plasma in addition to other body fluids, and methylation status can be detected following bisulfite modification using sensitive PCR-based assays [29]. We therefore examined the ability of the *RASSF1A* real time PCR assay to detect methylation in serum specimens from women with ovarian malignancies. To define a threshold for classifying a serum sample as hypermethylated, we analyzed serum specimens taken from seven women without evident malignancy. The average level of *RASSF1A* methylation in the unaffected women, normalized to *ACTB*, was 2.2% (SD = 1.8%). We therefore defined hypermethylation in serum as that exceeding 5.8% (average plus two SDs). The actual methylation values measured in serum from 21 patients with serous epithelial ovarian cancer are shown in Fig. 2. Analysis of the available 20 matched tumor specimens showed 100% concordance in methylation status with the serum (Fig. S3), indicating that detection of the malignancy in serum is highly sensitive. These results validate an earlier study showing *RASSF1A* methylation present in tumors was detectable in serum specimens of ovarian cancer patients [8]. Statistical analysis of the methylation status in the serum showed no significant association with race, stage, debulking status or age at diagnosis (Table 1).

We also analyzed serum and matched tumors for nine patients with stage III/IV serous epithelial ovarian cancer over the course of

Table 1
Patient characteristics.

	Tumor methylated/total (%)[a]	P value	Serum methylated/total (%)[a]	P value
Race				
Caucasian	42/82 (51.2%)	0.91[b]	17/20 (85.0%)	1.0[c]
Black	8/15 (53.3%)		1/1 (100%)	
Other	4/9 (44.4%)		NA	
Stage (invasive)				
I/II	1/4 (25.0%)	0.72[b]	0/1 (0%)	0.08[b]
III	45/88 (51.1%)		13/15 (86.7%)	
IV	7/12 (58.3%)		3/3 (100%)	
Unknown	1/2 (50.0%)		2/2 (100%)	
Debulking status (stages III–IV invasive)				
Optimal	25/44 (56.8%)	0.31[c]	5/5 (100%)	1.0[c]
Suboptimal	24/52 (46.2%)		9/11 (81.8%)	
Age at diagnosis				
≤60 years	21/52 (40.4%)	0.03[c]	7/8 (87.5%)	1.0[c]
>60 years	32/51 (62.7%)		11/13 (84.6%)	
Clinical response[d]				
Complete	11/27 (40.7%)	0.34[c]	ND	–
Incomplete	4/16 (25.0%)		ND	
Survival				
≤3 years	8/25 (32.0%)	0.74[c]	ND	–
≥7 years	7/17 (41.2%)		ND	

NA, Not applicable.
ND, Not determined.
[a] Methylated specimens exhibited >4.1% methylation for tumors and >5.8% for serum.
[b] Chi square test.
[c] Fisher's exact test.
[d] Complete clinical response is defined as normalization of CA125 values, normal CT scan and normal office examination for at least one month following primary therapy; incomplete clinical response is failure to meet any one or more of the three criteria.

treatment. Methylation of the tumor and serum at the time of surgery was measured along with serum taken at various time points (minimum, four months) following completion of primary treatment (Fig. 4). Tumors from all nine patients exhibited *RASSF1A* hypermethylation. For four of five patients with disease in remission, *RASSF1A* methylation levels decreased in serum specimens taken after completion of primary therapy relative to the methylation detected in serum at the time of surgery (compare S2 to S1 specimens, Fig. 4). Patient 2 had a normal CA125 value in spite of the elevation of *RASSF1A* methylation in the post-surgical serum specimen. For three of four patients with persistent or relapsed disease, *RASSF1A* methylation increased in the serum
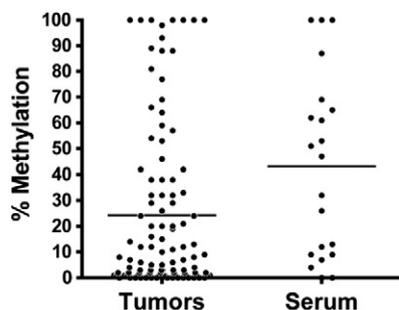

Fig. 2. *RASSF1A* methylation in cancers and serum specimens from women with serous epithelial ovarian cancer. Y-axis, distribution in percent methylation as detected in triplicate using *RASSF1A* quantitative real time methylation PCR relative to universally methylated DNA and normalized to the *ACTB* values obtained in parallel for each specimen. The horizontal lines represent the means for each group.
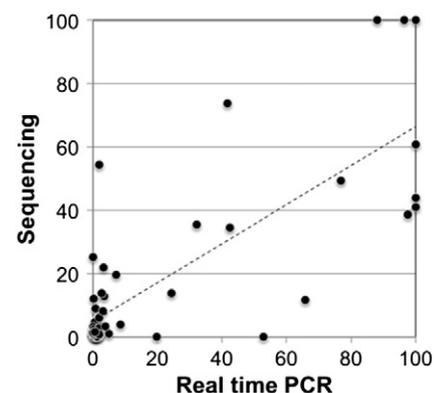

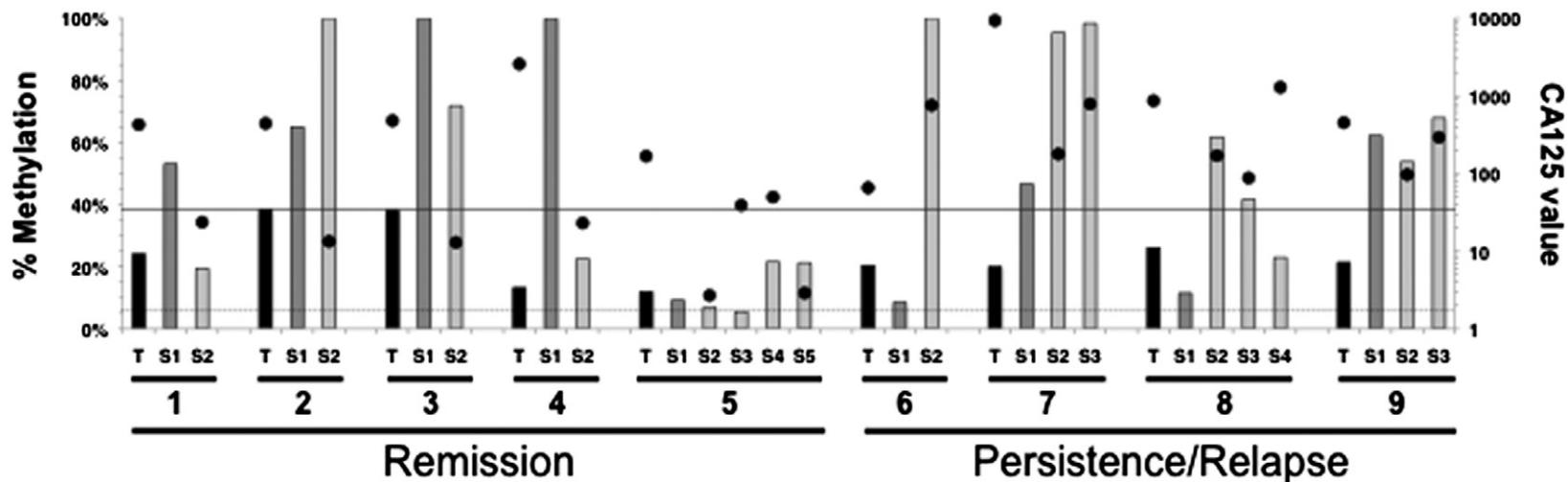Fig. 3. *RASSF1A* methylation in ovarian cancer and lymphocyte specimens using real time PCR versus bisulfite sequencing. Percent methylation is shown on both axes, as detected by real time PCR using the single CpG-containing fluorescent probe (x axis) versus methylation detected by radioactive manual sequencing followed by phosphorimager analysis (y axis) for the single CpG site queried in the real time PCR methylation assay.

Fig. 4. *RASSF1A* methylation in tumor, serum, and serial postoperative serum specimens over time. Real time quantitative PCR methylation analysis of specimens from nine individual patients with serous epithelial ovarian cancer. The primary y axis shows percent methylation. The secondary y axis indicates CA125 values, which are shown by the black dots on the bar graph. The solid line across the graph indicates the threshold for a normal CA125 value. T, tumor (black bars); S1, serum specimen taken at the time of surgery (dark gray bars); S2–S5, serial serum specimens obtained during patient follow up (range, four months to three years; light gray bars); dashed line, threshold level above which a serum specimen is defined as hypermethylated.

specimens taken after completion of primary therapy. The S2 specimen for patient 6 was obtained three years post-surgery, at which point serum *RASSF1A* was highly methylated and disease relapse had occurred. For patient 9, *RASSF1A* methylation decreased at five months post-surgery, but then increased above the level detected at the time of surgery less than two months later. The change of methylation status following surgery and chemotherapy do not significantly differ between these two groups, perhaps owing to small sample size (two-tailed Fisher's Exact test, $p = 0.21$). However, these results indicate that *RASSF1A* methylation status can be readily monitored in serum specimens using our real time assay, and suggest that quantitative assessment of *RASSF1A* methylation may provide information relevant to disease progression. Additional studies will be required to confirm these findings.

Discussion

The data presented here support that our novel real time PCR methylation assay is a sensitive method to quantify *RASSF1A* methylation. We observed 100% concordance in *RASSF1A* methylation status between serum and matched tumor specimens using this approach. Other studies have reported 75%–91% concordance [8,14,30]. This high level of specificity enables easy detection of *RASSF1A* methylation from a blood specimen and the ability to quantitatively monitor methylation and perhaps even disease status over the course of treatment.

Real time PCR methylation assays of the kind we have described here are not appropriate for discovery, but rather for high throughput analysis of genes or other genomic regions already defined as targets of hypermethylation. A strength of our assay design is that it is theoretically capable of accounting for the methylation status of every allele in a given population, since only a single CpG is used for discrimination. However, detection of a single CpG site is also a potential limitation, since CpG site-specific methylation is possible as is functional methylation of localized regions [31,32]. It is therefore imperative to identify a CpG site for which the methylation status will provide information with respect to the experimental question addressed and whose sequence context allows for placement of nondiscriminatory PCR primers flanking the region of interest.

A previous report showed that the presence or absence of methylated *RASSF1A* DNA in serum specimens of breast cancer patients during the course of treatment with tamoxifen was indicative of response, whereby the presence or emergence of methylated *RASSF1A* in serum reflected resistance and poor prognosis [33]. Our quantitative assay detected hypermethylation of *RASSF1A* in patient serum, demonstrating that the assay performs well in detecting methylation changes in specimens with limited DNA. We were also able to determine that *RASSF1A* methylation in serum DNA changes over time. We note that although there were fluctuations in serum *RASSF1A* methylation in these serial specimens, the methylation levels never returned to normal. It is presently unclear if this reflects the stability of tumor DNA in the peripheral circulation, or if this can be attributed to release of DNA from presumed small numbers of residual tumor cells during remission. This observation underscores the importance of accurately quantifying methylation in this setting.

In conclusion, we have developed a real time PCR methylation assay for quantifying DNA methylation through use of non-discriminatory PCR primers and a probe that queries the methylation status of a single CpG dinucleotide, theoretically capturing the methylation status of every allele in a given specimen. This assay can be used to analyze DNA methylation using cell free DNA as we have shown through analysis of serum specimens that were collected over the course of treatment from women with ovarian cancer. These types of assays may augment clinical assessment of patient status and treatment response in light of the decreased availability of tumor specimens from ovarian cancer patients with progressive or relapsed disease.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ygyno.2011.08.029.

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