Methylation Profiling of Mesothelioma Using Real-Time Methylation-Specific PCR:

A Pilot Study

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We tested whether methylation profiles generated by real-time methylation-specific PCR (MSP) can be useful in differentiating benign, reactive mesothelial cell proliferation (RM) from malignant mesothelioma (MM). Forty-two of the 63 cases (67%) yielded informative results for RARβ2, GPC3, CDKN2A *(p16),* TERT, *and* CCND2 *(cyclinD2) gene methylation. DNA methylation of any gene was observed in much higher frequency in MM cases than RM cases (63% vs. 33%,* $P < 0.05$*). Individual gene methylation was higher in the MM than the RM cases for most of the genes; however, this was not statistically significant (*RARβ2: *58% vs. 33%,* $P > 0.05$*;* GPC3: *36% vs. 27%,* $P > 0.05$*;* CDKN2A: *4% vs. 0%;* TERT: *4% vs. 0%), while* CCND2 *methylation was not detected in any case. Although preliminary, we demonstrate that real-time MSP can be applied to archival specimens and gene methylation profiling may have potential to be a useful ancillary tool to help distinguish MM from RM.* Diagn. Cytopathol. 2007;35:498–502. © 2007 Wiley-Liss, Inc.

Key Words: mesothelioma; gene methylatio profiling; real-time MSP

It is often difficult to differentiate malignant mesothelioma (MM) from reactive mesothelial cell proliferation (RM) in cytological specimens. Ancillary techniques, including immunohistochemical staining, have failed to completely eliminate diagnostic uncertainty. Methylation of tumor suppressor genes has been shown to be critical in cancer development, and methylation profiling has shown potential for clinical utility.[1,2] Mesotheliomas, like other malignancies, have been demonstrated to have a number of genes methylated, including *RASSF1A* and *CDKN2A* (p16).[3,4] In one study, *RASSF1A* was found methylated in 32% mesotheliomas,[4] and its methylation was correlated with loss of *RASSF1A* expression and the presence of SV40 DNA.[4,5] *CDKN2A* gene alterations are relatively common in MM, most often inactivated by homozygous deletion and less common by point mutation.[3] Inactivation of *CDKN2A* gene product expression by DNA methylation has also been found.[3] Another gene that has been found to be methylated in MM is *TERT*. The protein product of *TERT* gene is the catalytic subunit of telomerase, which is involved in maintaining telomere length. Surprisingly, the activation of *TERT* is related to DNA methylation of the promoter region, in contrast to most known tumor suppressor genes' down-regulation by DNA promoter methylation.[6–8] Reports regarding *TERT* activation and MM development have been mixed.[9,10] In contrast, in ovarian and cervical cancer, *TERT* methylation increases with age and is associated with a poor prognosis, regardless of *TERT* expression.[11] DNA sequence analysis of the *TERT* promoter region (−500 to +1, relative to ATG translational site) correlated DNA methylation with *TERT* expression among tumors and *TERT* positive cell lines.[7] Glypican 3 (*GPC3*) gene methylation has also been implicated in mesothelioma.[12] Allelic loss at *GPC3*, whose human homolog is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, was infrequent (6.9%) in MM cell lines, and no mutations were found. However, in majority of MM tumors and MM cell lines, *GPC3* transcript levels were markedly decreased, and the *GPC3* promoter region was shown to have aberrant methylation.[12] Similarly, methylation of *RARβ2* and *CCND2* (CyclinD2) gene has been associated with various types of cancers from breast, stomach, lung,

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to head and neck.[1,2,13–16] Clinical feasibility of methylation-specific PCR using cytology samples such as breast FNA biopsies and ductal-lavage specimens has been demonstrated.[1,17] Real-time MSP, which offers faster turn around time than conventional MSP and also has potential for semi-quantitative analysis, has been used in clinical research.[16,18]

In this article, we present results of a pilot study in which we investigate the use of methylation profiles of a panel of genes including *RARβ2, GPC3, CDKN2A, TERT,* and *CCND2* derived from real-time MSP to distinguish MM from RM using archival surgical and cytological specimens.

Materials and Methods

Tissues

Sixty-three cases were retrieved from the AFIP and University of Michigan archives with IRB approval. Those cases had either unstained slides or paraffin blocks prepared from biopsy or effusion available. Forty-one cases were MM, diagnosed by unequivocal morphological features and most also confirmed by immunohistochemical studies and clinical follow up information, while 22 cases were RM with different underlying diseases. Unstained slides were soaked with extraction buffer and scraped into Eppendorf tubes for DNA extraction.[1] Sections from paraffin blocks were cut at 4-μm thickness for Hemotoxylin/Eosin staining and morphologic examination, and 4–6 sections of 10-μm thickness sections were collected into an Eppendorf tube for genomic DNA extraction following previous established protocol.[1,19] Purified genomic DNA was treated with sodium bisulfite according to established protocols[1,19] and analyzed using a real-time MSP assay.

Real-Time Methylation-Specific PCR

Sodium bisulfite-treated genomic DNA was amplified using fluorescence-based real-time methylation-specific PCR. Methylation of *CDKN2A, RARβ2, CCND2, TERT,* and *GPC3* genes was examined using *β-Actin* and/or *Her-2* as the internal control for DNA quantification. Control of sodium bisulfite treatment was also ensured by including genomic DNA from the MDA-MB-231 cell line that generates positive result for *RARβ2* amplification reaction, since *RARβ2* is known to be methylated in this cell line.[1] Briefly, sodium bisulfite-converted genomic DNA was amplified with locus-specific PCR primers and dual labeled fluorogenic probes. During the extension phase of PCR, the 5′ to 3′ nuclease activity of Taq-polymerase cleaves the probe and releases the reporter, whose fluorescence can be detected by the laser detector of the ABI Prism 7700 Sequence Detection System. After crossing a fluorescence detection threshold, the PCR amplification results in a fluorescent signal proportional to the amount of PCR product generated. Primers and probes, specifically for sodium bisulfite-converted DNA sequences for *CDKN2A, RARβ2,* and *CCND2* genes, were based on published data.[16] For *HERT* and *GPC3,* the primers and probes were designed based on sequence analysis. All primer and probe sequences are listed in Table I. For DNA quantity control, *Her-2* primers and probes were used. Another control used was selected from a region of *β-Actin* that contains no CpG di-nucleotides thus not affected by DNA methylation status and sodium bisulfite-treatment (Table I). Real-time PCR was set up in a total volume of 50 μl containing 1X Taqman universal PCR master mix, 400 n*M* of each primer, 100 n*M* of probe and up to 2 μl sodium bisulfite treated-DNA samples. After an initial denature step at 50°C for 2 min and 95°C for 10 min, 45 or 50 cycles of 15 sec at 95°C and 1 min at 60°C were followed. Amplification data, collected by the 7700 sequence detector was analyzed using software developed by PE Applied Biosystems (Foster City, CA). Samples with reactions that show exponential increase of signal over cycle numbers were considered positive while reactions had no such increase was considered negative (Fig. 1).

Table I. List of Primers and Probes for the Real-Time MSP Assay

Actin	
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 AAC ACA-TAMRA
RARβ2	
Forward	TTT GAG GAT TGG GAT GTC GAG
Reverse	CGA ATC CTA CCC CGA CGA TA
Probe	FAM-CGC GAG CGA TTC GAG TAG GGT TTG TTT-TAMRA
Her-2	
Forward	ATG CAG ATT GCC AAG GTA TGC
Reverse	GGA AGC ACC CAT GTA GAC CTT CT
Probe	Vic-CCG GAG CAA ACC CCT ATG TCC ACA A-TARMA
CDKN2A(P16)	
Forward	GGG GAG AGT AGA TAG CGG GGC
Reverse	AAC CAA TCA ACC GAA AAT TCC ATA
Probe	FAM-TAC TCC CCG CCG CCG ACT CCA T-TAMRA
GPC3	
Forward	GGT CGG GAT CGT GCG TAT
Reverse	CCT ATC CCG AAA AAT CCA AAC TAA
Probe	FAM-CGT GTT TGG TGG TGG CGA TGT TG-TAMRA
TERT	
Forward	AAG CGC GGT TTA GAT TTT CG
Reverse	GAA TCC ACT AAA AAC CCG ACC TAA C
Probe	FAM-TTC GTT CGG AGT AGT TGC GTT GTC GG-TAMRA
CCND2	
Forward	TTT GAT TTAAGG ATG CGT TAG AGT ACG
Reverse	ACT TTC TCC CTA AAA ACC GAC TAC G
Probe	FAM-AAT CCG CCA ACA CGA TCG ACC CTA-TAMRA

Statistical Analysis

This was performed using Fisher Exact test; a two-sided P value of less than 0.05 was considered significant.

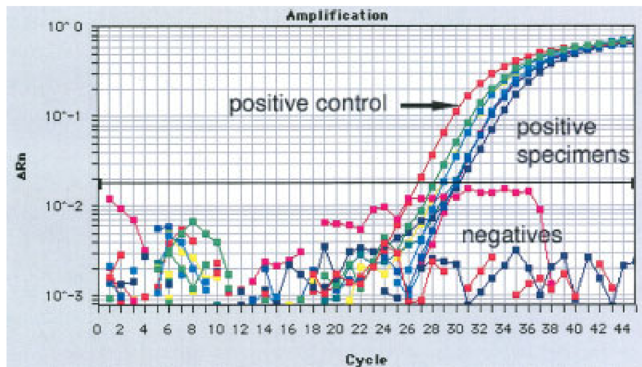

Fig. 1. Example of real time-PCR of the *β-Actin* gene amplification plot of one set of reactions. Each color represents one sample. On the graph of reaction (ΔRn) vs. Cycle number, the threshold cycle occurs where the sequence detection application begins to detect the increase in signal associated with exponential growth of PCR product (positive specimens). At any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies. With negative specimens, product lines do not show exponential increase or cross over the threshold. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table II. Demographic Data of the 25 Patients in the Cohort Show Similar Age Between Methylation Positive (+ Methyl) and Negative (− Methyl) Cases in MM or RM Groups

	Gender		*Age (mean, yr)*		
Diagnosis	*Male*	*Female*	*All cases*	*+ Methyl*	*− Methyl*
MM	7	5	63.2	63.4	62.8
RM	2	11	47.0	45.3	48.4

Results

Of the 63 cases, 42 (67%) yielded informative results (generated PCR products on either the *β-Actin* and/or *Her-2* amplification reaction, Fig. 1). Of these 42 cases, 27 were MM cases and 15 were from cases with diagnosis of RM. MM cases consisted of mostly the epithelial type (25 cases) with two cases of the sarcomatoid type. The demographic information was available from only 12 MM patients with similar gender distribution (7 male and 5 female) and a mean age of 63.2 yr and from 13 RM patients who were mostly female and slightly younger (11 female and 2 male, mean age: 47.0, Table II). In the MM group, 17 of the 27 cases demonstrated methylation of at least one of the genes tested (Fig. 2). Eight of them had two or more genes methylated. In contrast, in the RM group, methylation was found in only 5 of 15 cases, significantly lower than the MM group ($P < 0.05$, Fig. 2). Individually, the frequency of *RARβ2* gene methylation was higher in the MM cases than in the RM cases, although the difference was not statistically significant (15 of 27 cases vs. 4 of 15 cases, $P > 0.05$, Fig. 2). *GPC3* gene methylation frequency was also found to be higher in the MM group than the RM group (9 of 25 cases vs. 4 of 15 cases, $P > 0.05$, Fig. 2). Methylation of

	Genes					
MM cases	*RAR-B2*	*TERT*	*CDKN2A*	*GPC3*	*CCND2*	*Any gene*
1	■			■		■
2	■	■		■		■
3						
4	■					■
5	■			■		■
6	■			■		■
7	■					■
8						
9						
10						
11	■			■		■
12	■			■		■
13	■					■
14	■			■		■
15						
16			/	/	/	
17				■		■
18						
19				■		■
20			/	/	/	
21						
22	■					■
23	■		■			■
24	■					■
25						
26	■					■
27	■					■
Methylated	56%	4%	4%	38%	0	63%
RM cases						
1						
2						
3						
4						
5						
6	■			■		■
7	■			■		■
8						
9						
10	■					■
11	■			■		■
12						
13						
14	■			■		■
15						
Methylated	33%	0	0	27%	0	33%

Fig. 2. Gene methylation is more frequently observed in MM than RM cases. Solid-black block indicates methylation while empty block indicates no methylation observed and "/" indicates not performed.

TERT and *CDKN2A* was each found in one case of the MM group (4%), respectively, but none of the RM group. *CCND2* methylation was not detected in any of the cases examined (Fig. 2). *RASSF1A* was not methylated in any cases, although few cases were examined (data not shown).

There was no significant age difference of the patients with respect to their methylation status among the MM and RM groups (Table II). Although the MM group patient were older (mean age: 63.2) than the RM group, within each group the methylation positive or negative patients have similar age. In MM patient group, methylation positive patients had an average age of 63.4 while methylation negative patients with an average age of 62.8. In RM group, methylation positive patients had an average age of 45.3 while methylation negative patients with an average age of 48.4.

Discussion

We were able to use real-time MSP to acquire methylation profiles of MM and RM from archival paraffin sections in surgical biopsy and cytology effusion specimens. Real-time PCR offers rapid turnaround time and has the potential for semi-quantitative analysis. The rate of informative results ($\sim$67%) was similar to the conventional MSP method (R. Pu, data not shown) and the real-time MSP results from other investigators (*vide infra*). For example, one study showed 29 of 45 paraffin block specimens were informative for real-time PCR amplification.[20] The inability to obtain methylation profiles in 1/3 of the cases was most likely due to less than optimal DNA quality and low quantitative yields of DNA from the archival material. The informative rate can be enhanced by using fresh material if real-time MSP proved to be useful ancillary tool by more comprehensive studies in the future. Although our study did not show significant differences in individual gene methylation frequency, the overall methylation in MM was significantly higher than that of RM (63% vs. 33%, $P < 0.05$). In our study, MM patients were older than the RM patients, a potential problem in interpretation of the result since some DNA methylation events have been shown to be age-related phenomena.[11,21,22] However, in both MM and RM groups, the patient age was similar in methylation positive and negative patients, arguing against the possibility that aging contributes to the higher observed methylation percentage in MM group.

It is known that certain tumor suppressor gene methylation events may occur earlier than the other genes during tumorigenesis. *RARβ2* is noted to have moderate levels of methylation in benign and pre-cancer lesions, e.g. fibroadenoma and papilloma in breast.[1] We found methylation of *RARβ2* in 56% of MM cases, highest among all the genes examined, but also in 33% of RM cases. Our result suggests that methylation of *RARβ2* may occur in benign condition and, if involved in MM development, likely an early event, similar to its role in other types of neoplasia.[1,13] Similarly, *GPC3* gene methylation may be an early event during MM development as we found moderate level of methylation in MM (36%) as well as RM (27%) cases. Although alterations of *CDKN2A* are very common in MM, we found methylation of *CDKN2A* in only a small percentage of MM cases (4%), similar to 8.8% reported by others.[3] This suggests that methylation of *CDKN2A* does not play a major role in its down regulation in MM, in contrast to a high prevalence of *CDKN2A* methylation in other type of cancers, such as squamous cell carcinoma of the lung (90%).[13]

Methylation of *TERT* promoter, resulting in activating telomerase activity, was considered idiosyncratic in comparison to the usual function of methylation in down-regulation of gene expression.[6] Whether any of the CpG di-nucleotides in the *TERT* promoter region is important for its function in MM has not been analyzed in detail, although most of the promoter region has been shown to be methylated in other types of *TERT*-positive tumors and cell lines.[7] We found only one case of MM having DNA methylation in the promoter region that we interrogated while none in the benign cases. The sequences of our primers and probe correspond to the DNA sequence from -273 to -346 relative to ATG translation start site and contain 9 CpG di-nucleotides. Although *TERT* methylation was less common than we anticipated, it is possible that in MM, those 9 CpGs are not as frequently methylated as in other *TERT* positive tumors or cell lines studied by sequencing analysis.[7] Similarly, *CCND2* methylation was not found in any MM/RM cases examined. The *CCND2* sequences examined in this case, however, have been shown to be methylated in other malignancies.[1,16] This suggests that in MM tumorigenesis, *CCND2* methylation may not play as an important role as it does in other carcinomas.[1,16]

In summary, we found DNA methylation is more frequent in MM (epithelial type) than RM in most of the genes examined, although no individual gene showed statistically significant differences in methylation. The potential clinical utility of methylation profiling using real-time MSP to differentiate MM from RM and from other malignancy is supported by our results and other publication.[23] Increasing the number of samples and genes examined, and utilizing freshly obtained specimens may provide a more complete evaluation of whether methylation profiling can be used as an ancillary tool in differentiating mesothelioma from benign mesothelial proliferation.

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