The degree of microRNA-34b/c methylation in serum-circulating DNA is associated with malignant pleural mesothelioma

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Abstract

Objectives: Malignant pleural mesothelioma (MPM) is an aggressive tumor with a poor prognosis. microRNA-34b/c (miR-34b/c), which plays an important role in the pathogenesis of MPM, is frequently downregulated by DNA methylation in approximately 90% of MPM cases. In this study, we estimated the degree of miR-34b/c methylation in serum-circulating DNA using a digital methylation specific PCR assay (MSP).

Materials and Methods: A real-time MSP assay was performed using the SYBR Green method. The melting temperature (Tm) of each PCR product was examined using a melting curve analysis. For a digital MSP assay, 40 wells were analyzed per sample. A total of 110 serum samples from 48 MPM cases, 21 benign asbestos pleurisy (BAP) cases, and 41 healthy volunteers (HVs) were examined.

Results: Positive range of Tm value for miR-34b/c methylation was defined as 77.71 °C to 78.79 °C which was the mean ± 3 standard deviations of 40 wells of a positive control. The number of miR-34b/c methylated wells was counted per sample according to this criterion. The number of miR-34b/c methylated wells in MPM cases was significantly higher than that in BAP cases ($P = 0.03$) or HVs ($P < 0.001$). Advanced MPM cases tended to have higher number of miR-34b/c methylated wells

than early MPM cases. Receiver–operating characteristic (ROC) curve analysis revealed that three number of miR-34b/c methylated wells per sample was the best cut-off of positivity of MPM with a 67% of sensitivity and a 77% specificity for prediction. The area under the ROC curve was 0.77.

Conclusions: Our digital MSP assay can quantify miR-34b/c methylation in serum-circulating DNA. The degree of miR-34b/c methylation in serum-circulating DNA is associated with MPM, suggesting that this approach might be useful for the establishment of a new detection system for MPM.

1. Introduction

Asbestos exposure has been reported to cause asbestos-related diseases such as malignant pleural mesothelioma (MPM), primary lung cancer, and benign asbestos pleurisy (BAP) [1]. Although the use of asbestos has been strictly restricted, the number of MPM patients who had been expoed to asbestos is still increasing [2, 3]. MPM is an aggressive tumor with a dismal prognosis, with a median overall survival period of 12 months [2]. Approximately 85% – 90% of patients with MPM present with unresectable disease at the time of diagnosis [4]. Additionally, both MPM and BAP cases suffer from common symptoms caused by pleural effusion. These conditions are difficult to distinguish using not only radiological imaging tests such as chest X-ray and computed tomography, but also cytological examinations of pleural effusion [5, 6]. Therefore, pathological validation by means of an invasive pleural biopsy with a full-layer resection of the parietal pleura is strongly recommended [7], although the possibility of a sampling error at the time of biopsy is significant; whether a few pieces of the parietal pleura are actually representative of the entire pleural lesion is unclear [8]. Considering the difficulty associated with the pathological diagnosis of MPM, a definitive diagnosis based on pathological finding alone is occasionally challenging [9, 10]. Since misguided diagnoses lead to delays in treatment and early

diagnosis and subsequent treatment are thought to improve the clinical outcome of patients with MPM, a critical need exists for the development of a reliable and non-invasive test for the detection of MPM.

Reportedly, the downregulation of several tumor suppressive genes, such as *BMP3b* [11], *BMP6* [11], *IGFBP* [12], and *RASSF1A* [13], frequently occurrs as a result of DNA methylation in MPM cases. Similar to protein coding genes, microRNAs (miRs), which are a group of non-coding small RNAs that mostly regulate their target messenger-RNAs through posttranscriptional repression [14], are downregulated through the methylation of their promoter regions [15]. In fact, we have recently revealed that miR-34b/c, which plays an important role in the pathogenesis of MPM, is downregulated by promoter methylation in approximately 90% of MPM cases [16].

Blood examinations are less-invasive diagnostic methods and several serum biomarkers such as mesothelin, osteopontin, CYFRA21-1, and Fibulin-3 have been reported for the diagnosis of MPM [10, 17-19]. Among them, mesothelin has been well-studied and is currently considered to be the best serum biomarker of MPM available, although a recent systematic review of medical literature revealed a limited sensitivity [20]. The presence of nucleic acids in the blood was recognized more than 30 years ago [21]. Solid malignant tumors are known to release a significant amount

of genomic DNA into the systemic circulation probably through cellular necrosis and apoptosis [21, 22]. Therefore, cell-free circulating DNA in the serum or plasma is considered to be a source of useful biomarker during carcinogenesis [23, 24], although tumor-derived circulating DNAs are fragmented and present in the blood flow amidst a high background of normal cell-derived DNAs [22, 25]. Highly sensitive assays are required to detect tumor-specific genetic alterations in serum-circulating DNAs in patients with malignant tumors [23].

Digital PCR assays have been developed as a highly sensitive assay for the detection of rare genetic abnormalities amidst a high normal background. Digital PCR was originally developed as a tool for the amplification of individual molecules for purposes of identifying and counting individual DNA molecule sequence alterations[26], and now is applied to determine coding mutations, loss of heterozygosity, allelic imbalance and SNP polymorphisms [27, 28]. This principle has been also applied to DNA methylation analyses [29]. One of advantages of digital PCR is the sequestration of competing background molecules into negative wells that do not participate in the PCR amplification, leading to improve the ratio of template-to-background in the positive wells [29]. Particularly, competition for primer annealing by background DNA is a major problem in the detection of low-abundance methylation variants by MSP,

because sequence redundancy is increased in bisulfite converted DNA, which contains only three bases outside of sites of DNA methylation [29]. To the best of our knowledge, the digital PCR assay for the detection of methylation of miR genes has never been applied as a blood detection test for MPM.

To establish a new detection system for MPM, we developed a digital MSP assay to evaluate the degree of miR-34b/c methylation in serum-circulating DNAs in patients with MPM, comparing those in patients with BAP, and healthy volunteers (HVs).

2. Material and methods

2.1. Sample collection

We obtained more than 2 mL of peripheral blood samples from 48 MPM cases, 21 BAP cases, and 41 HVs at Okayama University Hospital, Okayama Rosai Hospital, and the National Hospital Organization Yamaguchi Ube Medical Center between August 2006 and August 2011. The characteristics of all 110 cases are shown in Table 1. The blood samples were centrifuged at 3500 rpm for 5 min within one hour after the collection, and the sera were collected and stored in aliquots at -80 °C at each institute until further experiments. As a positive control (POC), the supernatant of a culture medium for NCI-H290 (H290), an MPM cell line harboring heavy methylation

of miR-34b/c [16], was collected and stored at -80 °C. We also collected the supernatant of culture medium for LP9, a non-malignant peritoneal mesothelial cell line, as a negative control. H290 was a kind gift from Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). We purchased LP9 from the Coriell Cell Repository (Camden, NJ). Informed consent was obtained from each case at each institute. The study was approved by the ethics committee of Okayama University (approval number for the genome study, 173).

2.2. DNA extraction and bisulfite conversion

We extracted DNA from 1 mL of serum sample or supernatant of cell culture medium using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Carlsbad, CA), according to the manufacturer's recommendations, and eluted the DNA in 120 μL of the kit's elution buffer. The DNA concentration was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and the mean dosage of the extracted serum DNA was 4.8 ± 1.8 μg (40 ± 15 ng/μL). Among the 120 μL of extracted serum DNA, 20 μL of DNA (0.8 ± 0.3 μg) was used for bisulfite conversion using the Epitect Bisulfite Kit (Qiagen) and the DNA was eluted in 40 μL of the kit's elution buffer and

used as the templates for the assays described below. As for the H290 and LP9 cell lines, the concentrations of extracted DNA from the supernatant of cell culture medium were adjusted to 40 ng/μL. Twenty microliters (0.8 μg) were applied for bisulfite conversion using Epitect Bisulfite Kits (Qiagen) with 40 μL of the final elution.

2.3. Real-time methylation specific PCR (MSP) assay

We designed three sets of MSP primers for the predicted bisulfite-modified sequences based on the nucleotide sequence submitted to GenBank (gene accession numbers, NR_029839 for miR-34b and NR_035765 for miR-34c) and our previous report [16]. Among them, we decided to use the following primer set because of its high sensitivity (data not shown): forward primer, CGTACGGGGTCGAGAGAGT; reverse primer, CTCGACCCGAACTCCCACT. The length of the PCR product was 83 bp. A real-time MSP assay was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in a final volume of 20 μL per well containing 1 μL of bisulfited DNA (20 ± 7.5 ng/well for serum and 20 ng/well for cell supernatant), 10 μL of 2 x Power SYBR® Green PCR Master Mix (Applied Biosystems), and 0.3 μL of both 10 μM forward and reverse primers. The PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min, followed by 50 cycles of 94 °C for 15

seconds and 60 °C for 60 seconds. After PCR amplification, the melting temperature (Tm) of each PCR product was examined using a melting curve analysis.

2.4. Statistical analysis

Differences in the number of miR-34b/c methylated wells were compared between two categorized groups using the Mann-Whitney test. *P* values less than 0.05 were considered statistically significant. The receiver–operating characteristic (ROC) curve analysis was performed to determine the cut-off point for the number of miR-34b/c methylated wells. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Detection of positive wells containing miR-34b/c methylated DNA using a melting curve analysis

The fluorescent signal after PCR amplification was detected in all the wells, including those containing a water blank, because of nonspecific PCR reactions. To detect the positive wells containing miR-34b/c methylated DNA, we defined the positive range of the Tm value for miR-34b/c methylation. We investigated the Tm values of 40 wells of the POC samples, 40 wells of LP9 supernatant samples (negative

control), and 40 wells containing a water blank. The supernatants of the culture medium for the cell lines contained cell-derived DNA from apoptotic cells and were used as models of the serum samples. The range of the Tm values differed between the 40 POC wells (mean ± standard deviation [SD], 78.25 °C ± 0.18 °C) and the 40 water blank wells (75.01 °C ± 0.47 °C) (Figure 1). In addition, the length of the PCR product of the water blank was confirmed to be shorter than that of POC using gel electrophoresis (Supplemental Figure 1). Furthermore, the Tm values of 39 out of 40 of the wells of the LP9 supernatant sample (negative control) were within the range of the WB samples (Figure 1). We confirmed that none of the CpG sites that our MSP assay could detect were methylated in the LP9 cell lines using bisulfite sequencing (data not shown). According to this result, we defined the positive range of the Tm values for miR-34b/c methylated wells as 77.71 °C to 78.79 °C, which was within the mean Tm values ± 3 SDs of 40 wells of the POC samples.

3.2. Digital MSP assay for miR-34b/c methylation

In the preliminary study, we examined miR-34b/c methylation in 1 μL of bisulfited DNA from serum-circulating DNA using a real-time MSP assay (one well per sample). miR-34b/c methylation was not present in 1 μL of bisulfited DNA from

serum-circulating DNA from the MPM cases, even though the primary tumor harbored heavy miR-34b/c methylation. Considering the dilution effect of tumor-derived DNA in serum-circulating DNA, we repeated the real-time MSP assay for the same serum-circulating DNA and found that miR-34b/c methylation could occasionally be detected. Based on these findings, we decided to perform a real-time MSP assay for 40 PCR wells per serum-circulating DNA sample using the whole elution of bisulfited DNA (40 μL). The quantification of miR-34b/c methylation was performed using a digital MSP assay by counting the number of miR-34b/c methylated wells per sample. For this purpose, a total of 800 μL of PCR mixture containing 40 μL bisulfited DNA templates were first made, and we then distributed them in 20-μL aliquots per well for a total of 40 wells. After PCR amplification, the Tm value of each PCR product was calculated using a melting curve analysis, and the miR-34b/c methylation status of each PCR well was classified according to the positive range of the Tm values for miR-34b/c methylation. In every experiment, a POC sample was placed into a 96-well polypropylene PCR plate to confirm that the Tm value of the POC sample fell within the positive range for miR-34b/c methylation.

3.3. Quantification of miR-34b/c methylation using a digital PCR assay

A distribution map showing the Tm values for all the wells in all the cases was shown in Figure 1. Each group showed a characteristic distribution of Tm values. Biphasic peaks of Tm values were seen for the MPM and BAP cases, in which the low- and high-grade Tm values were comparable to those of the water blank (low-grade) and the POC of miR-34b/c methylation (high-grade), respectively. The Tm values of the HVs were mainly around that of the water blank.

The numbers of miR-34b/c methylated wells in the MPM cases was significantly higher than those in the BAP cases ($P = 0.03$) or the HVs ($P < 0.001$) (Figure 2). The BAP cases also had significantly higher numbers of miR-34b/c methylated wells than the HVs ($P = 0.01$).

3.4. Association between the numbers of miR-34b/c methylated wells and patient characteristics

We evaluated the association between the numbers of miR-34b/c methylated wells per sample and clinicopathological factors. No significant differences in the numbers of miR-34b/c methylated wells were seen when compared according to age, sex, smoking status, and histological subtype. MPM cases with an advanced clinical stage tended to exhibit higher numbers of miR-34b/c methylated wells than those with

an early clinical stage except in two cases (Supplemental Figure 2). These two MPM cases with clinical stage I had over 20 miR-34b/c methylated wells; one of the patients had 34 miR-34b/c methylated wells, while the other had 22 miR-34b/c methylated wells and he suffered from a rapid increase in the thickness of the pleura immediately after the initial diagnosis and collection of the serum sample, although the patient was subsequently lost to follow-up.

3.5. Optimal cut-off point for miR-34b/c methylation by ROC curve analysis

In order to determine the cut-off number of miR-34b/c methylated wells for MPM cases, we carried out ROC curve analysis comparing MPM cases versus other non-malignant cases (Figure 3 and Supplemental table 1). According the ROC curve for all cases, three number of methylated wells was the best cut-off of positivity of MPM with a 67% of sensitivity and a 77% specificity for prediction. The area under the ROC curve (AUC) was 0.77.

4. Discussion

In this study, we established a highly sensitive assay for the quantification of miR-34b/c methylation in serum-circulating DNA to distinguish MPM cases from BAP cases or HVs. Our assay showed that the degree of miR-34b/c methylation was

significantly higher in serum-circulating DNA from MPM cases than from BAP cases or HVs. MPM cases with an advanced clinical stage tend to have more miR-34b/c methylated wells than those with early stage disease, although two early-stage MPM cases did show heavy miR-34b/c methylation of their serum-circulating DNAs.

The dosage of DNA in the blood circulation itself is associated with tumor progression in patients with malignant tumors [30]. Needless to say, the degree of tumor-specific alterations in serum-circulating DNA can be considered a more specific marker for the detection of malignant tumors than the amount of total serum-circulating DNA. PCR reactions for the detection of these tumor-specific alterations in serum-circulating DNA can be interrupted not only by the fragmentation of DNA derived from tumor cells, but also by a high background of DNA derived from non-malignant cells. To overcome these difficulties, several sensitive assays have been developed [29, 31, 32] . Among them, digital PCR has been established as a highly sensitive assay for the detection of minor genetic alterations among a vast number of normal alleles [27, 29]. Digital PCR can also calculate the dosage of the genetic alteration in a sample by determining the percentage of PCR wells with a positive reaction [29], with more precise quantification enabled by analysis of more PCR wells per sample. Of note, although we used TaqMan-based real-time PCR assays in our

preliminary experiment, its sensitivity was low and we finally selected the present method (data not shown).

Our results showed that more than three miR-34b/c methylated wells yielded the highest discriminative ability with a 67% sensitivity and a 77% specificity for predicting the presence of MPM. The AUC was 0.77, indicating that the established assay had a moderate diagnostic accuracy for predicting the occurrence of MPM [33, 34] . As a positive test, a high specific threshold is typically required, and if we opt for a specificity of 95% (ie, a false-positive rate of one out of 20), a sensitivity of our assay results in 38%. For a negative test result to aid in excluding diagnosis, a high sensitive threshold is generally required. At a selected sensitivity of 95%, the specificity of this assay was 24%. These results suggested that the sensitivity and specificity of our assay is almost similar to those of serum mesothelin level in an individual patient data meta-analysis (AUC = 0.77, a sensitivity of 32% at 95% specificity) [20]. With regard to our sensitive assay, false positive cases are present in 43% of BAP cases and 12% of HVs using a cut-off value of three methylated wells. Further investigation is warranted for improvement of both sensitivity and specificity by combining with other biomarkers.

Two early-stage MPM cases exhibited heavy miR-34b/c methylation, indicating that our sensitive assay might detect miR-34b/c methylation during the early stage of MPM pathogenesis. Obviously, the limitation of clinical staging based on conventional radiological examinations should be considered, since one case experienced the rapid progression of the MPM soon after the initial diagnosis. The serum level of miR-34b/c methylation might reflect biological malignancy much more accurately than clinical staging. Further investigations of large-scaled studies are needed to clarify this issue. The sequential occurrence of other malignant tumors is another consideration, since miR34-b/c methylation can be observed in patients with other malignant tumors, such as lung [35, 36], colorectal [15], and gastric cancers [37]. Regarding this issue, these malignant tumors were not obviously coincidental in any case of this study.

In conclusion, our digital MSP assay can quantify miR-34b/c methylation in serum-circulating DNA, revealing that miR-34b/c methylation is more heavily and frequently present in serum-circulating DNA from MPM cases than from BAP cases or HVs. This approach might be useful for the establishment of a new detection system for MPM.

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Conflict of interest statement

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