that, by controlling the expression level of these proteins, CHFR modulates the cell cycle regulation (1). Collectively, CHFR plays an important role in the prophase checkpoint by ubiquitinating and degrading several target genes, including PARP-1, Aurora A, and Plk-1. In dead, knocking down of *PARP1*and *AURKA* showed arithmetic effect on repressing cells to progress into mitosis induced by mitotic stress (supplemental Fig. 5*S*), supporting the idea that CHFR might be a master regulator of the prophase checkpoint and a tumor suppressor. In human cancers, increased expression levels of PARP-1 have been reported (32), indicating that abnormal PARP-1 expression could be associated with malignancies (33). Our present data show that a negative correlation between the expression of CHFR and PARP-1 implies that silencing in *CHFR* could cause overexpression of PARP-1 and result in the checkpoint deregulation and tumorigenesis.

As we reported previously, expression of CHFR correlated with resistance to microtubule inhibitors such as docetaxel, and abolishing CHFR caused mitotic catastrophe and sensitized cancer cells to the drugs (see Fig. 6, *left* and *center panels*) (21, 26). On the basis of the novel idea that the PARylation activity of PARP-1 regulated CHFR-dependent checkpoint function, in the next set of experiments we demonstrated that PARP inhibitors inhibited autoPARylation of PARP-1 and CHFR-dependent polyubiquitination/degradation of PARP-1 induced by mitotic inhibitors and attenuated the mitotic checkpoint, resulting in enhanced response to docetaxel in *CHFR*-expressing cells (Fig. 6, *right panel*). Small molecule inhibitors of PARP have been developed as sensitizers to DNA-damaging chemotherapy or ionizing radiation, and six potent and specific PARP inhibitors are currently undergoing clinical development for various cancers (16, 33). Recently, clinical phase II trials using PARP inhibitors olaparib or iniparib, in combination with a microtubule inhibitor, paclitaxel, have started in gastric cancers and breast cancers. As the results show in Fig. 5, PARP inhibitors sensitized cancer cells with CHFR-dependent resistance to microtubule inhibitors, which sheds new light on a potential strategy for the combined usage of PARP inhibitors with microtubule inhibitors.

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RESEARCH ARTICLE

Characterization of DNA hypermethylation in two cases of peritoneal mesothelioma

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Abstract Malignant mesothelioma (MM) is a rare disease with a poor prognosis. Pleural mesothelioma, which is the most common type of MM, is considered to be caused by asbestos exposure and is increasing in incidence, with about 15,000 new cases diagnosed worldwide annually. On the other hand, peritoneal mesothelioma is a very rare type of MM; thus, its pathogenesis is even less understood than pleural mesothelioma. Recent research on the pathogenesis of malignant pleural mesothelioma has indicated that both epigenetic and genetic alterations contribute to tumorigenesis. Here, we hypothesize that peritoneal mesothelioma also has an epigenetic alteration in the same genes (Kazal-type serine peptidase inhibitor domain 1 (*KAZALD1*), transmembrane protein 30B (*TMEM30B*), and mitogen-activated protein kinase 13 (*MAPK13*)). Our goal is to identify DNA methylation of these three candidate genes in two peritoneal mesothelioma cases. Laser capture microdissection was used to separate diseased sections of formalin-fixed paraffin-embedded samples from one surgically resected tissue (epithelial type) and one autopsy tissue (sarcomatous type). Genomic DNA was subsequently extracted by the standard phenol chloroform method. The DNA was then treated with sodium bisulphite, and pyrosequencing analysis was used to quantitatively analyze the methylation of candidate genes reported to be hypermethylated in malignant pleural mesothelioma (*KAZALD1*, *TMEM30B*, and *MAPK13*). *TMEM30B* and *MAPK13* were not methylated in either case. However, *KAZALD1* was highly methylated in sarcomatoid-type peritoneal mesothelioma. We first report that the *KAZALD1* gene was hypermethylated in sarcomatoid-type malignant peritoneal mesothelioma.

Keywords Mesothelioma · DNA methylation · Peritoneal

Abbreviations

MM	Malignant mesothelioma
MPM	Malignant pleural mesothelioma
MPEM	Malignant peritoneal mesothelioma

Ryota Hama and Yoshiyuki Watanabe contributed equally to this work.

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NF2	Neurofibromatosis type 2
KAZALD1	Kazal-type serine peptidase inhibitor domain 1
TMEM30B	Transmembrane protein 30B
MAPK13	Mitogen-activated protein kinase 13
PCR	Polymerase chain reaction
CT	Computed tomography
US	Ultrasound

Introduction

Malignant mesothelioma (MM) is a rare tumor arising from serous membranes covering cells of mesodermal origin, and it frequently has unpredictable and poor prognosis with very few curatively resected cases. Identification of the primary lesion, diagnosis, and treatment has been difficult. As a result, the disease mechanism remains poorly understood. A consensus has not been reached on the source; some believe that MM originates from mesothelial cells since they differentiate into both epithelial cells and fibroblasts, while others believe that mesenchymal cells are the source [1, 2]. MM is clinically categorized as localized or diffuse. Localized mesothelioma is often considered a benign tumor, while diffuse mesothelioma, the more common of the two, is mostly malignant. There are three basic histological types of mesothelioma: epithelial, biphasic (mixed) and sarcomatoid (fibrous). Malignant pleural mesothelioma (MPM) is most commonly the epithelial type. Malignant peritoneal mesothelioma (MPEM) is most commonly epithelial, followed by biphasic and sarcomatoid type, both of which are extremely rare [3].

MM occurs in the pleura, peritonea, and, although uncommon, in the pericardium, epididymis, and other sites. As a percentage, the occurrence of mesothelioma is 65.6–85.7 % in the pleura, 12.5–32.7 % in the peritonea, and 1.6–14.3 % in the pericardium, indicating that mesothelioma originating in the peritonea occur with a quarter to a third of the frequency of those originating in the pleura [4–6].

Asbestos exposure has been associated with MM ever since numerous patients with diffuse MM were identified among people who had worked in South African asbestos mines [7–10]. As there is typically a 20- to 50-year gap between exposure and the onset of symptoms, vigilance remains necessary in 2012. More cases are expected to arise in persons born in or before the 1960s, when asbestos restrictions were initiated. Those at risk are the asbestos workers themselves and their family members.

Inhaled asbestos is thought to arrive in the peritoneal cavity via the lymph nodes or enterally in sputum or drinking water. Asbestos-related substances (crocidolite, amosite, anthophyllite, and chrysotile), as well as silica, thorotrast, tritium, and other substances, have been implicated in MPEM, which appears in about 50 % of all malignant MPM cases [11–13]. Recurrent peritonitis, which is attributable to diverticulitis, has also been labeled a cause of MPEM [14]. Nevertheless, details of its pathogenesis remain unclear. It is urgently necessary to better characterize this disease, which is minimally sensitive to chemotherapy and radiotherapy, and carries a very poor prognosis.

We clinically encountered two cases of rare MPEM, one localized and the other diffuse. The patient with localized MPEM was surgically cured and survived; however, the patient with diffuse MPEM was ineligible for surgical treatment. The malignancy was minimally sensitive to chemotherapy, and as a result, the patient died. Neither patient had a background suggesting asbestos exposure.

It is necessary to identify the molecular mechanisms and key genes in MPEM development with the aim of using this information to develop new diagnostic tools and identify target molecules for new therapy. The most frequently inactivated tumor suppressor genes in human malignancies are *p53* and $p16^{INK4a}/p14^{ARF}$. MPM shows frequent genetic inactivation (deletions and point mutations) of $p16^{INK4a}$ *(CDKN2A)*/$p14^{ARF}$ and neurofibromatosis type 2 (NF2), and infrequent mutation of *p53* and *KRAS* [15–18]. MPEM also shows frequent genetic inactivation of $p16^{INK4a}$ *(CDKN2A)*/$p14^{ARF}$ [19]. However, there have not been any reports of mutations of NF2, p53, and KRAS in MPEM.

DNA methylation is one of the epigenetic modifications that are altered in neoplasia [20]. Hypermethylation of CpG-rich sequence regions, called CpG islands, located in the promoter of genes have been shown to be commonly implicated in silencing tumor suppressor genes in neoplasia [21] and have also been thought to be important in aging, inflammation, and in its association with increased risk of tumor formation [22, 23]. The relationship between promoter DNA hypermethylation and inflammation has been documented in inflammatory bowel diseases, many types of cancer, and MPM [24–27]. One example is Goto et al.'s discovery of three candidate genes (Kazal-type serine peptidase inhibitor domain 1 (*KAZALD1*), transmembrane protein 30B (*TMEM30B*), and mitogen-activated protein kinase 13 (*MAPK13*)) that were highly methylated in MPM [27]. The association between MPM and MPEM, including mechanisms and epidemiology, is not clearly understood. We hypothesized that genes where hypermethylation is linked to MPM (TMEM30B, KAZALD1, and MAPK13) may also contribute to MPEM onset due to the presence of long-term inflammation [14].

To investigate and diagnose our two MPEM cases, we analyze and compare the results using various immunohistological staining techniques. To clarify the mechanism of MPEM, we next confirm genetic alteration in genes p53, KRAS, and BRAF, as well as epigenetic alteration in genes TMEM30B, KAZALD1, and MAPK13, in our two MPEM cases.

Methods

Clinical samples

Subjects were two patients hospitalized at St. Marianna University School of Medicine Hospital with peritoneal mesothelioma over a 9-year period from 2003 to 2012. One patient with localized peritoneal mesothelioma, who survived following surgical resection, the other with diffuse peritoneal mesothelioma, who died and was autopsied. We thoroughly informed the patient or a family member about our research prior to analyzing a portion of the affected tissue.

Laser microdissection and DNA extraction

Samples were embedded in paraffin. Ten-micrometer sections were fixed to microscope slides and stained with malachite green. Next, the PALM MicroBeam (Carl Zeiss Microscopy, Peabody, MA), a laser microdissection system, was used to remove and isolate the tumor portions under a microscope. DNA was extracted from the collected tumor tissue using the QIAamp DNA Formalin-Fixed Paraffin-Embedded Tissue kit (QIAGEN, Valencia, CA).

Pyrosequencing analysis

The extracted DNA was bisulfite-processed with an EpiTect Bisulfite kit (QIAGEN, Valencia, CA). Biotinylated polymerase chain reaction (PCR) was then conducted with primers designed using a sequence specific for the CpG islands found in the promoter regions of the three genes *KAZALD1*, *TMEM30B*, and *MAPK13* (Supplementary Table 1). For each PCR, the denaturation step was carried out at 95 °C for 30 s, the annealing step at 60 °C for 30 s, and the extension step at 72 °C for 30 s. Biotinylated PCR products were captured with streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and were then incubated with sequencing primers. Next, a pyrosequencer (PyroMark; QIAGEN, Valencia, CA) was used to quantitatively analyze the methylation levels of the promoter region CpG sites of the genes. Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation at detected sites was used as a representative value for each gene promoter. Comparative validation was performed using as controls a mixture of DNA extracted from the blood

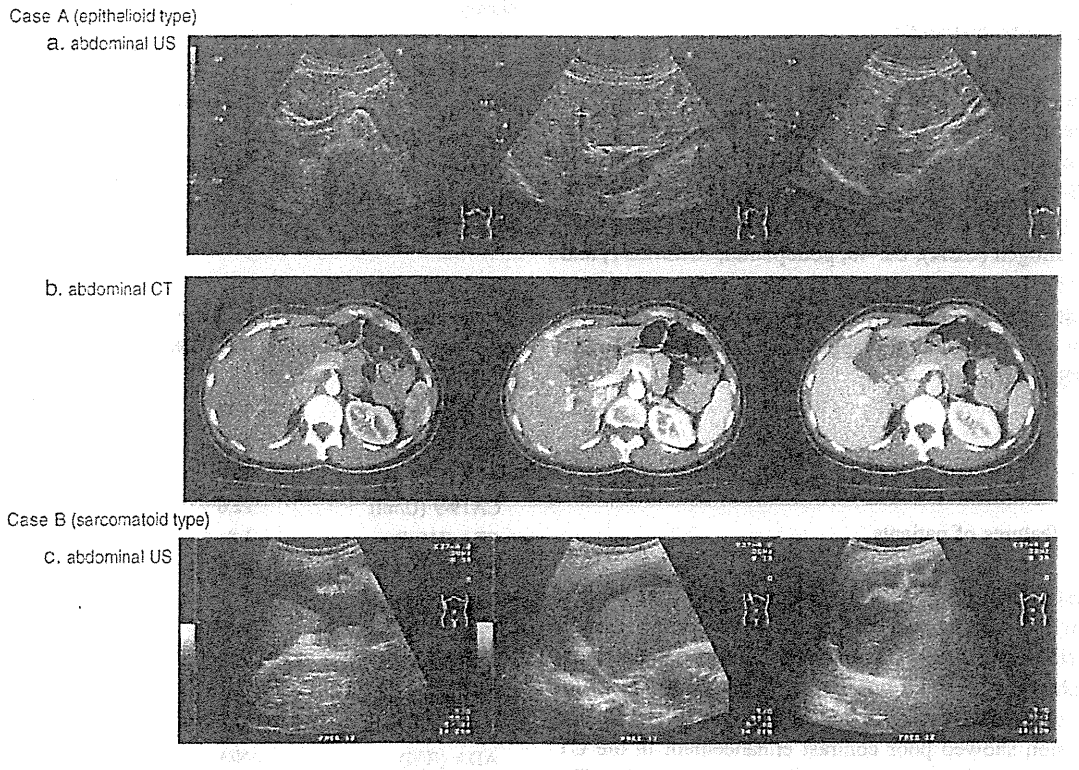

Fig. 1 Abdominal imaging in *cases A and B*. a 43-year-old woman with epithelial-type malignant peritoneal mesothelioma (*localized MPEM*). Abdominal ultrasound shows a round-shaped mass located near the hilar of the liver (*case A*). b Axial view of abdominal CT scan shows a 4-cm mass near the hilar liver (*case A*); c 75-year-old man with sarcomatoid-type malignant peritoneal mesothelioma (*diffuse MPEM*). Abdominal ultrasound shows massive ascites and irregular, nodular thickening of the parietal peritoneum and bowel wall (*case B*)

of multiple healthy persons (negative control) and the SssI-processed version of this DNA (positive control). All primer sequence information is shown in Supplementary Table 1.

p53, *KRAS*, and *BRAF* mutation analysis

PCR of the extracted DNA was conducted with primers designed for exons 2 to 11 of the *p53* gene. For the *KRAS* gene, PCR was conducted using primers designed for codons 12, 13, and 61, which are frequently mutated in cancer. For the *BRAF* gene, PCR was conducted using a primer designed for V600E, which is also frequently mutated in cancer. For each PCR, the denaturation step was carried out at 94 °C for 30 s, the annealing step at 55 °C for 30 s, and the extension step at 64 °C for 60 s. Platinum® PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA) was used as the PCR mix. PCR products were purified with Centri-Sep™ Columns (Applied Biosystems, Foster City, CA) and then analyzed in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) with BigDye® Terminator v3.1 (Applied Biosystems). Mutations of the determined sequences were assessed using Geneious Pro 5.5 (Biomatters, Auckland, New Zealand). Primer sequences are shown in Supplementary Table 1.

Immunohistological staining

Samples were embedded in paraffin and fixed to microscope slides in 2-μm-thick sections. Sections were stained with hematoxylin and eosin (HE) and several immunostains (HBME-1, mesothelial cell, calretinin, epithelial membrane antigen (EMA), AE/AE3, cytokeratin, vimentin, carcinoembryonic antigen (CEA), D2-40, podoplanin, *KAZALD1*, and *MAPK13*). Two pathologists specializing in the gastrointestinal system interpreted the resulting images to assess staining. We could not find any appropriate specific antibodies for TMEM30B.

Results

Clinical features of patients

The first patient (case A) learned from a doctor at another hospital that she had an abdominal mass lesion, though diagnosis was difficult. Ultrasound (US) and computed tomography (CT) showed one large, round-shaped mass lesion (4 cm) near the hilar region of the liver (Fig. 1a). The mass lesion showed poor contrast enhancement in the CT scan and was constructed from small, separate nodules. The mass was surgically resected and was later assigned a histological diagnosis of localized MPEM (Fig. 1b).

The second patient (case B) came to the outpatient clinic with persisting symptoms of chronic diarrhea. Colonoscopy failed to reveal any abnormalities. Abdominal US showed ascites and irregular, nodular thickening of the parietal peritoneum and bowel wall (Fig. 1c). MPEM was therefore suspected. An explorative laparotomy biopsy was performed, and a diagnosis of diffuse MPEM was made from the peritoneal biopsy tissue (Fig. 1d).

The two cases of MPEM were clinically classifiable as localized and diffuse MPEM. The patient histories were not highly suggestive of asbestos exposure. The clinical features of the two patients are summarized in Table 1.

Immunohistological staining

In both cases, HE staining, as well as additional immunohistochemistry (HBME-1, mesothelial cell, calretinin, EMA,

Table 1 Clinical features of the two patients

	Case A Localized type	Case B Diffuse type
Age	43	75
Gender	Female	Male
History		
Surgical treatment	(−)	(+) appendicitis
Diverticulums	(−)	(+) multiple
Smoking status	Unknown	Unknown
Asbestos exposure	(−)	(−)
Alcohol	Minimal	Minimal
Performance status	1	1
Pleural effusion (ml)	(−)	Rt (800 ml) and Lt (1,000 ml)
Abdominal dropsy (ml)	(−)	3,800 ml
Pathological subtype	Epithelial type	Sarcomatous type
Stage	II (T3N0M0)	III (T4N0M0)
Blood test		
CRP (mg/dl)	0.03	10.5
LDH (IU/l)	142	170
ALP (IU/l)	230	187
CA19-9 (U/ml)	22.6	60
CEA (U/ml)	1.8	1.4
CA125 (U/ml)	93.5	546
Abdominal dropsy		
Color	N/A	Yellowish brown
Rivalta reaction	N/A	(+)
Cell count (μl)	N/A	12,288
Hyaluronic acid (ng/ml)	N/A	38,400
ADA (IU/l)	N/A	13
CEA (U/ml)	N/A	<0.5
CA19-9 (U/ml)	N/A	<5

epithelial membrane antigen, AE/AE3, cytokeratin, vimentin, CEA, carcinoembryonic antigen, D2-40, podoplanin, *KAZALD1*, and *MAPK13*) were examined. In case A, large, round, or polygonal cells formed epithelioid clusters and showed papillary growth, which led to a diagnosis of epithelioid type MPEM (Fig. 2a). In case B, atypical spindle or ovoid cells were scattered in dense, collagenous fibers, leading to a diagnosis of sarcomatoid (desmoplastic variant) type MPEM (Fig. 2a).

We next examined additional immunohistochemistry (HBME-1, Calretinin, EMA, AE/AE3, vimentin, CEA, and D2-40) in both cases. HBME-1 and calretinin were used to determine whether the tissue is mesothelial. EMA, AE/AE3 can be diagnosed as epithelial. Vimentin is a type III intermediate filament protein that is expressed in mesenchymal cells. CEA and D2-40 are useful as a differential diagnosis between MM and adenocarcinoma. We successfully diagnosed the two cases of MPEM as epithelioid type and sarcomatous type (Fig. 2b–h).

Moreover, we examined the immunohistochemistry of two candidate genes (*KAZALD1* and *MAPK13*) using specific antibodies. Both cases A and B were similarly stained with *KAZALD1* and *MAPK13* (Fig. 3a, b).

p53, *KRAS*, and *BRAF* mutation analysis

No *p53*, *KRAS*, or *BRAF* mutations were noted in either case (Fig. 4a–c).

DNA methylation profiling in MPEM

We analyzed DNA methylation of three candidate genes, *KAZALD1*, *MAPK13*, and *TMEM30B* in two MPEM samples by means of quantitative bisulfite pyrosequencing. A methylation level of >15 % was considered methylation positive [28]. These genes have a CpG island in its promoter region and we made specific primers for use in bisulfite pyrosequencing analysis (Fig. 5). *TMEM30B* (case A, 1.0 %

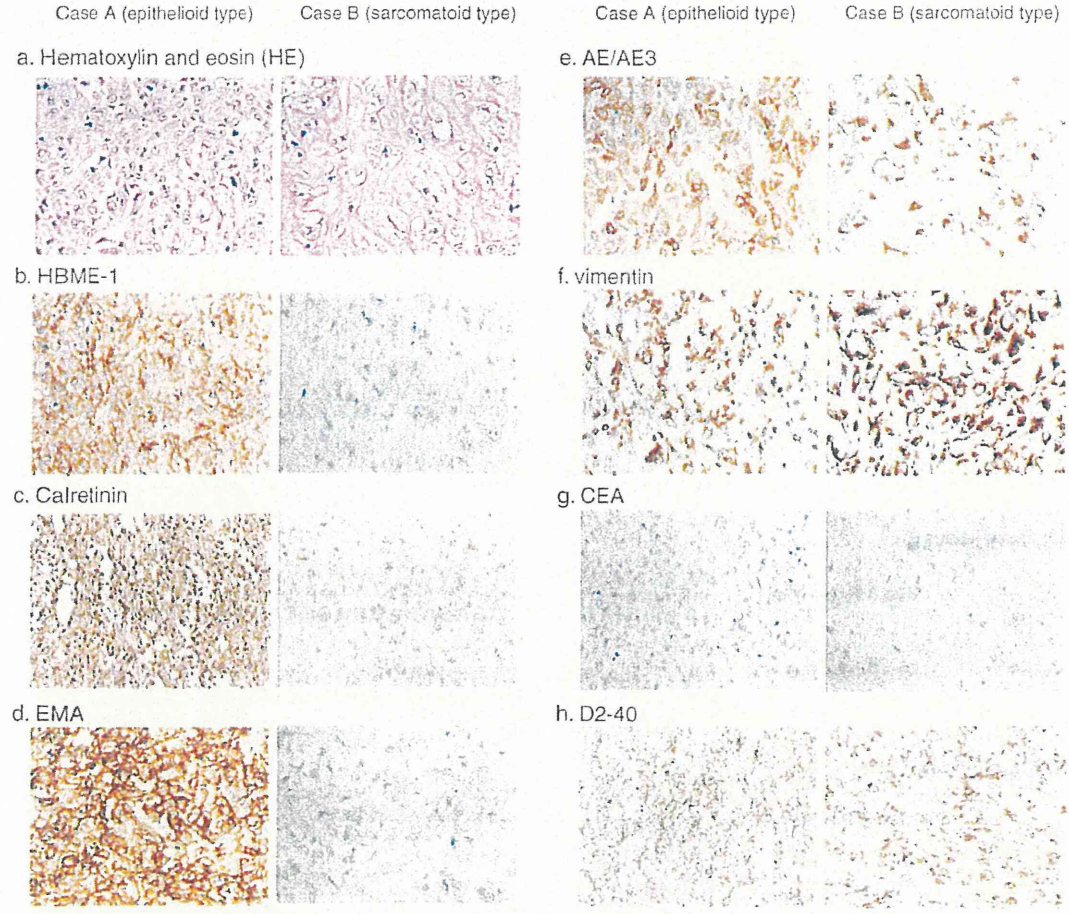

Fig. 2 Immunohistochemistry. **a** Hematoxylin and eosin (*HE*) staining. Large, round, or polygonal cells formed epithelioid clusters and showed papillary growth in *case A*. In *case B*, atypical spindle or ovoid cells were scattered in dense, collagenous fibers. Immunostaining supported the diagnosis of mesothelioma; both tumors were positive for cytokeratin (*AE1/AE3*), EMA, vimentin, calretinin, and D2-40 but negative for CEA (**b** *HBME-1*, **c** *Calretinin*, **d** *EMA*, **e** *AE/AE3*, **f** *vimentin*, **g** *CEA*, and **h** *D2-40*)

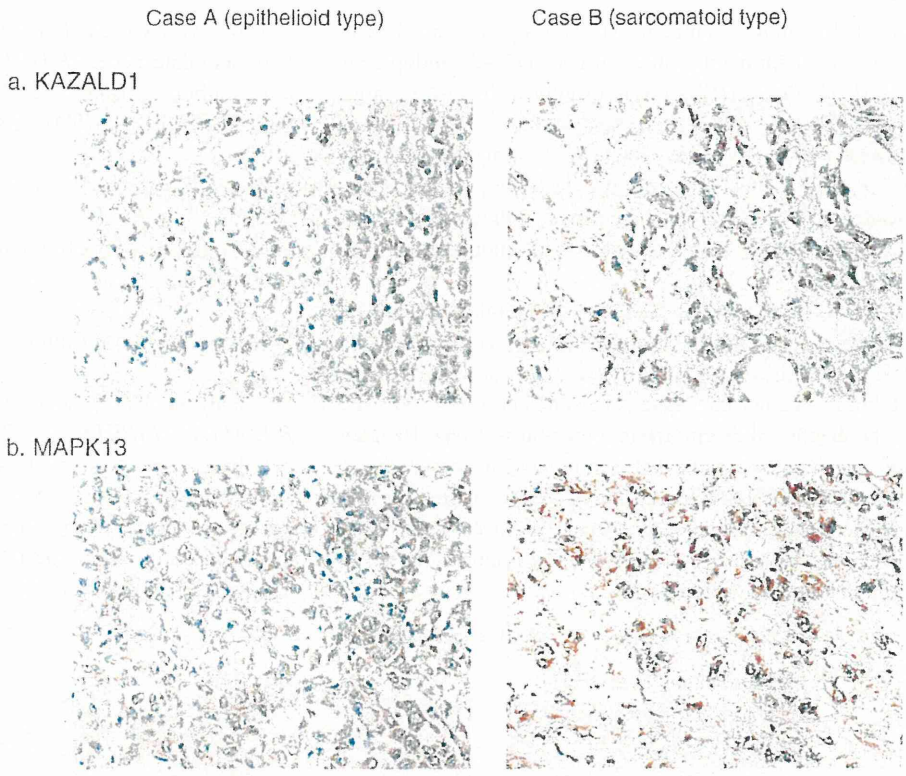

Fig. 3 Immunostaining shows a similar staining in both *cases A and B* in *KAZALD1* (**a**) and *MAPK13* (**b**)

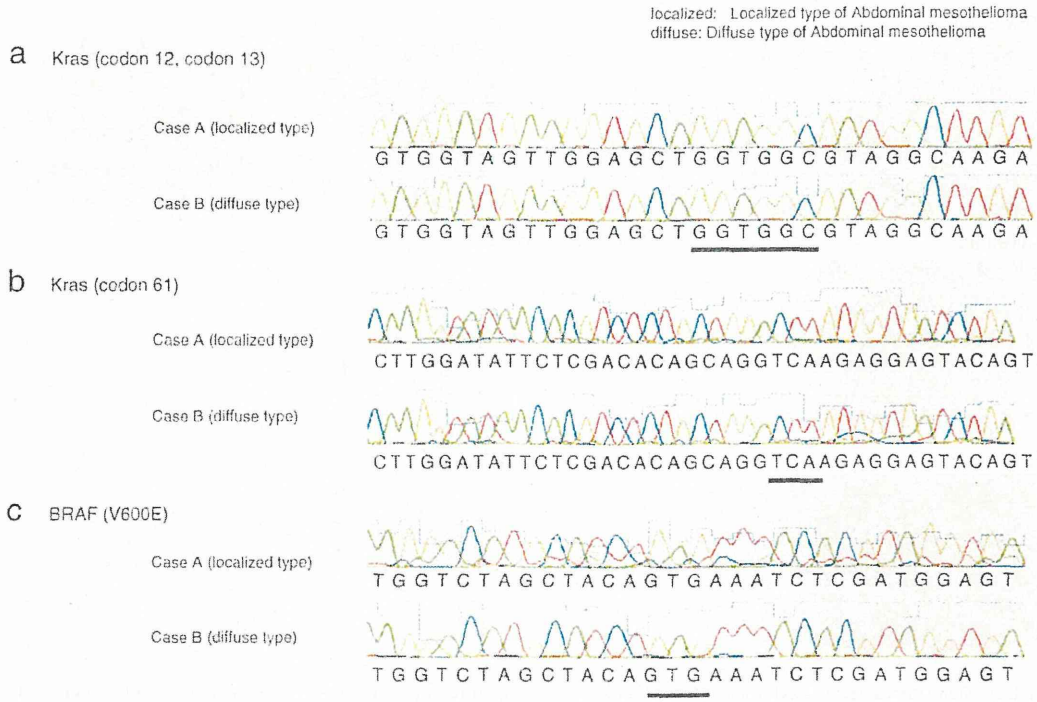

Fig. 4 Mutation analysis of *KRAS* and *BRAF*. a Somatic mutations in codons 12, 13, and 61 of *KRAS* were not seen in either of *case A or B*. b Somatic mutations in V600E of *BRAF* were not seen in either of *case A* or *B* by direct sequencing analysis

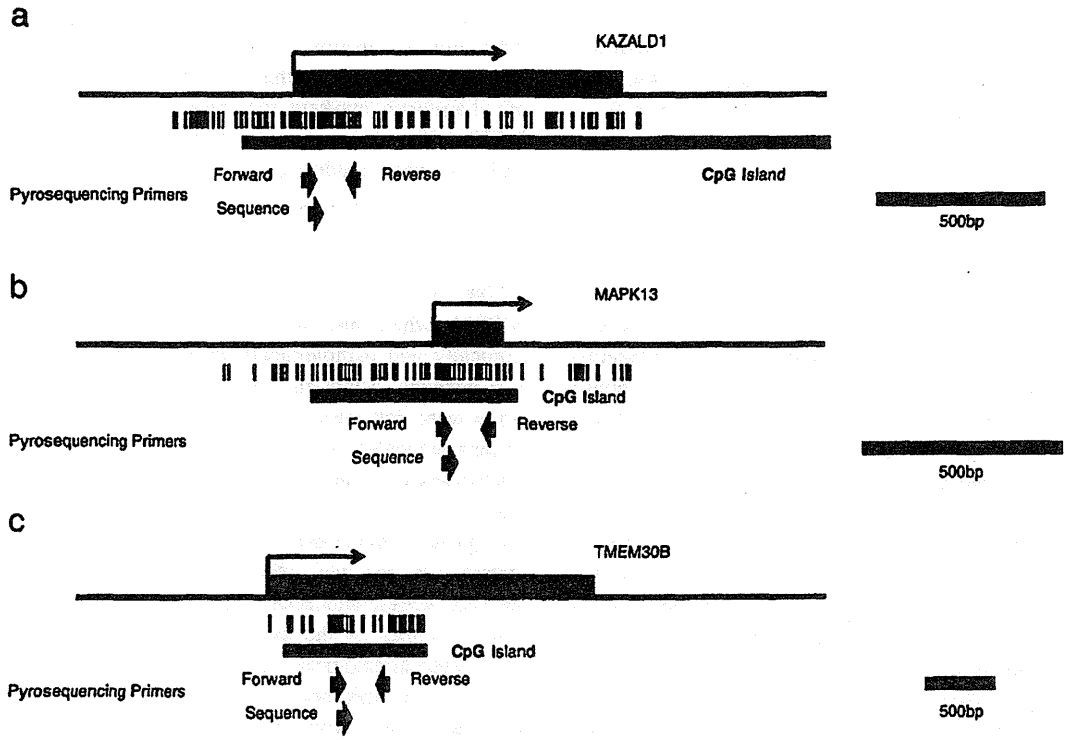

Fig. 5 Genomic structures of promoter region. a *KAZALD1*, b *MAPK13*, and c *TMEM30B* have a CpG island in its promoter region. Bisulfite pyrosequencing was performed using primers that cover these promoter regions for the genes

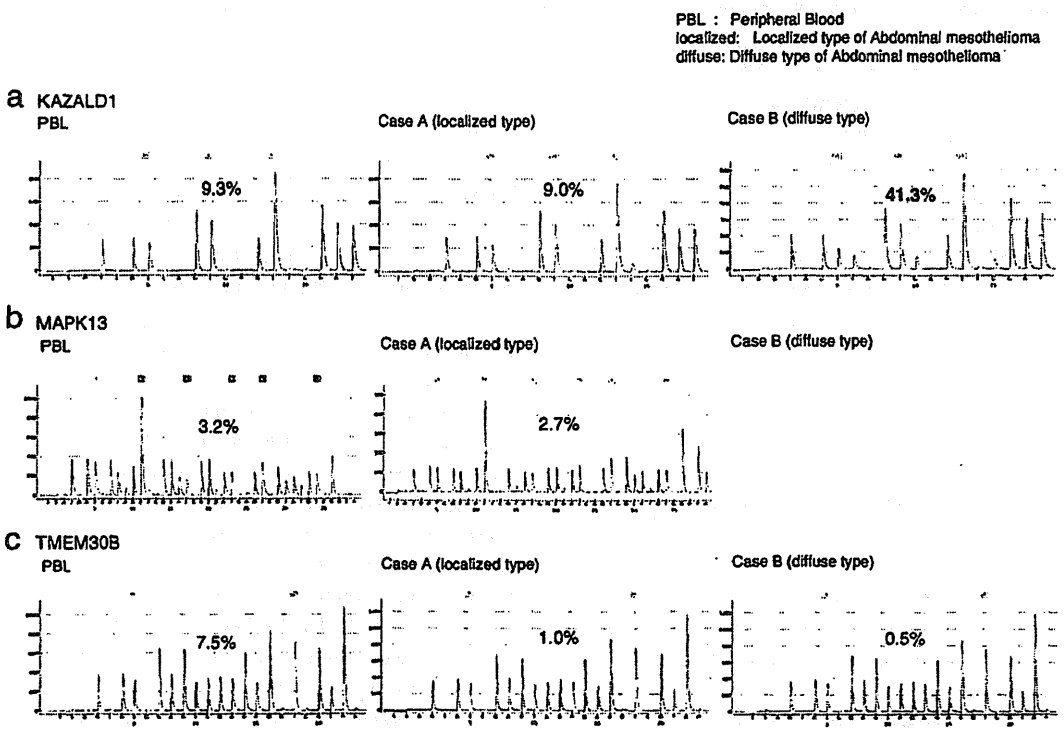

Fig. 6 Pyrogram of three genes. Methylation level of *KAZALD1* (a), *MAPK13* (b), and *TMEM30B* (c) were measured by quantitative bisulfite pyrosequencing. Only the *KAZALD1* methylation was seen in case B

and case B, 0.5 %) and *MAPK13* (case A, 2.7 % and case B, not amplified) were not methylated in either case. However, *KAZALD1* was highly methylated in sarcomatoid type MPEM (case A, 9.0 % and case B, 41.3 %) (Fig. 6a–c).

Discussion

Epigenetics, in addition to genetics, is widely recognized for its key role in tumorigenesis. Epigenetic changes collectively refer to changes that contribute as modifying elements without changing actual gene sequences. DNA hypermethylation of gene promoter regions is recognized as the primary mechanism of tumor suppressor gene silencing. Epigenetic abnormalities outnumber genetic abnormalities in gastrointestinal cancer, and epigenetic abnormalities are known to contribute to oncogenesis by deactivating a variety of tumor suppressor genes [21, 29–41]. *Helicobacter pylori* infection and related hypermethylation have recently been implicated in gastric cancer [42–47]. Recent research is beginning to show that identifying which tumor suppressor genes are methylated could assist in both disease screening and determining drug sensitivity, while also identifying the potential for lymph node and distant metastasis and prognosis. These genes show great potential as biomarkers [21, 48–52].

Epigenetic abnormalities have also been implicated in MPM pathogenesis. An investigation conducted with a cell line and clinical samples from MPM showed frequent gene inactivation by microRNA 34b/c methylation in both the cell line and clinical samples. MicroRNA 34b/c has the potential to suppress tumor growth after these genes were transfected [53]. Genome-wide gene methylation analysis of MPM showed that DNA methylation patterns were distinct from those of lung adenocarcinoma. *TMEM30B*, *KAZALD1*, and *MAPK13* were identified as methylated genes specific to MPM, highlighting the potential clinical application of these genes as molecular biomarkers [27]. Unfortunately, however, studies that evaluate the relevance of DNA methylation of MPEM are non-existent.

Mutation analyses, however, have been performed, identifying a loss of *BAP1 (BRCA1-associated protein 1)* expression due to high-frequency gene mutation in MPM [54]. Mutations in *p53*, *KRAS* and *EGFR*, which are frequent in malignancies, are rare in MM. Gene alterations are useful not only in understanding the mechanism of MM but also in applying molecular markers for screening, diagnosis, and incidences of tailor-made therapy. However, these reports show that only epithelioid and a few biphasic types of MPM have a genetic alteration (deletion, missense mutation, nonsense mutation, and frameshift mutation) of *BAP1*. *BAP1* is known as a DNA damage-response gene (homologous recombination) and could be connected to cytotoxic drugs and platinum-based chemotherapy (e.g., cisplatin+ and pemetrexed) response in epithelioid type MM [55, 56]. Although sarcomatoid type MPEM has a more severe and poor prognosis than epithelioid type, we have no appropriate diagnostic markers for therapy of sarcomatoid type MPEM. The chromosomal instabilities in MPM that have been reported include 1p, 1q, 3p, 4p15.1–p15.3, 4q25–q26, 4q33–q34, 5p, 6p, 6q, 7p, 8p, 8q, 9p, 10p13-pter, 13q, 14q11.1–q12, 14q23–q24, 15q, 17p12-pter, 17q and 22q [57–60].

We first investigated epigenetic abnormalities in MPEM, which had not been previously studied, in two clinically and pathologically differing cases of MPEM at our hospital. Although *TMEM30B* and *MAPK13* methylation were not present in either case, *KAZALD1* was only hypermethylated (41.3 %) in case B (clinically diffuse; pathologically sarcomatoid type MPEM) (Fig. 6a–c). *KAZALD1* gene encodes a secreted member of the insulin growth factor-binding protein superfamily, but its details are not well identified. Gene mutation analysis of *p53*, *KRAS*, and *BRAF* revealed no distinct abnormalities in either of the cases (Fig. 4a–c). Immunostaining and other histological evaluations showed evidence consistent with peritoneal mesothelioma (case A compatible with epithelioid type, and case B compatible with sarcomatoid type MPEM) in both cases (Fig. 2a). The staining results from immunohistochemistry of KAZALD1 and MAPK13 were similar in both cases A and B. Analyzing quantitative gene expression of KAZALD1 using RNA from both cases may help us better understand the mechanism of gene silencing caused by DNA methylation.

Aberrant DNA methylation plays a crucial role in the development and progression of human cancers, and is also frequently observed in chronic inflammation and precancerous lesions [22, 61]. Chronic pleuritis caused by asbestos exposure is a well-known key mechanism of MPM. Similarly, some reports suggested that recurring chronic diverticulitis may trigger the development of MPEM [62–64]. Our patient in case B also had an episode of recurring chronic diverticulitis.

Ou study is the possibly first attempt to analyze epigenetic alterations by limited numbers of the cases (only one localized and one diffuse type of MPEM). Thus, it is difficult to identify the epigenetic mechanisms and relationship between DNA methylation and MPEM. We should confirm these epigenetic phenotypes using a large number of validation sets of MPEM samples and clarify these mechanisms in the next step. Based on our results, we suspect that case B developed MPEM by *KAZALD1* metyhlation and might be correlated with chronic diverticulitis.

Our findings suggest that characterizing *KAZALD1* hypermethylation in sarcomatoid type-MPEM provides clinically useful information in the form of molecular, diagnostic, and therapeutic markers.

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Conflicts of interest None

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