

Title Page

**Original research article**

**Title:** Highly expressed EZH2 in combination with BAP1 and MTAP loss, as detected by immunohistochemistry, is useful for differentiating malignant pleural mesothelioma from reactive mesothelial hyperplasia

**Running Title:** EZH2 in malignant pleural mesothelioma

**Authors**

Masayo Yoshimura<sup>a</sup>, Yoshiaki Kinoshita<sup>a</sup>, Makoto Hamasaki<sup>a</sup>, Shinji Matsumoto<sup>a</sup>, Tomoyuki Hida<sup>b</sup>, Yoshinao Oda<sup>b</sup>, Akinori Iwasaki<sup>c</sup>, and Kazuki Nabeshima<sup>a</sup>

**Affiliations**

<sup>a</sup>Department of Pathology, Fukuoka University School of Medicine and Hospital, Fukuoka, Japan

<sup>b</sup>Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>c</sup>Department of Thoracic Surgery, Fukuoka University Hospital and School of Medicine, Fukuoka, Japan

**Corresponding author**

Kazuki Nabeshima, M.D, Ph.D.

Department of Pathology, Fukuoka University Hospital and School of Medicine, 7-45-1

Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Phone: +81-92-801-1011, Fax: +81-92-863-8383

E-mail: [kaznabes@fukuoka-u.ac.jp](mailto:kaznabes@fukuoka-u.ac.jp)

## **Abstract**

**Objective:** Malignant pleural mesothelioma (MPM) is an aggressive neoplasm with poor prognosis. Loss of BRCA-associated protein 1 (BAP1) protein expression as detected by immunohistochemistry (IHC) and homozygous deletion (HD) of the 9p21 locus as detected by fluorescence *in situ* hybridization (FISH) permits differentiation of MPM from reactive mesothelial hyperplasia (RMH). We have previously reported that detecting the loss of methylthioadenosine phosphorylase (MTAP) using IHC is a surrogate assay for 9p21 FISH. Furthermore, enhancer of zeste homolog 2 (EZH2), which encodes a component of polycomb repressor complex 2 (PRC-2), has been overexpressed in various tumors as well as MPM. In the current study, we investigated whether EZH2 IHC assay, alone or in combination with BAP1 and MTAP IHC, is useful for distinguishing MPM from RMH.

**Materials and Methods:** We examined IHC expression of EZH2, BAP1, and MTAP, and 9p21 FISH in MPM (n = 38) and RMH (n = 29) and analyzed the sensitivity and specificity of each detection assay for distinguishing MPM from RMH.

**Results and Conclusion:** EZH2, BAP1, and MTAP IHC, and 9p21 FISH were characterized by a 100% specificity each and 44.7%, 52.6%, 47.4%, and 65.8%

sensitivities, respectively. A combination of EZH2 and BAP1 IHC, and 9p21 FISH showed the greatest sensitivity (89.5%). Using IHC alone (EZH2, BAP1, and MTAP IHC) also yielded a good sensitivity of 86.9%; this level is high enough for routine diagnostics. There were no statistically significant associations between expression of EZH2 and that of other markers (BAP1 and MTAP IHC) or 9p21 HD. However, a high expression level of EZH2 was significantly associated with short survival ( $P = 0.025$ ). In conclusion, adding a high expression level of EZH2 to a combination of BAP1 and MTAP loss, all detected by IHC, demonstrated useful for discriminating MPM from RMH.

### **Keywords**

Malignant pleural mesothelioma; reactive mesothelial hyperplasia; enhancer of zeste homolog 2; BRCA1-associated protein 1; methylthioadenosine phosphorylase; 9p21 fluorescence *in situ* hybridization.

### **Abbreviations**

9p21 FISH: 9p21 as detected by fluorescence *in situ* hybridization; BAP1: BRCA1-associated protein 1; BAP1 IHC: BAP1 expression as detected by immunohistochemistry; FISH: fluorescence *in situ* hybridization; HD: homozygous

deletion; IHC: immunohistochemistry; MPM: malignant pleural mesothelioma; MTPA

IHC: methylthioadenosine phosphorylase as detected by immunohistochemistry;

MTAP: methylthioadenosine phosphorylase; RMH: reactive mesothelial hyperplasia;

RT: room temperature; SSC: saline-sodium citrate; EZH2: enhancer of zeste homolog 2;

PRC2: polycomb repressor complex 2; IMP3: insulin-like growth factor mRNA binding protein 3; GLUT-1: glucose transporter-1; TTF-1: thyroid transcription factor-1; WT-1:

Wilms' tumor-1; CEA: carcinoembryonic antigen; EPP: extrapleural pneumonectomy;

PD: pleurectomy and decortication; PcG: Polycomb group; PRC1: polycomb repressive

complex 1; PH1: polyhomeotic 1; BMI1: B cell-specific Moloney murine leukemia

virus integration site 1; CBX: chromobox; RING1A/1B: Ring finger protein 1A/1B;

EED: embryonic ectoderm development; SUZ12: suppressor of zeste 12; RbAp48:

retinoblastoma (Rb)-associated proteins 48; H3K27me3: trimethylation of histone 3

lysine 27

## 1. Introduction

Malignant pleural mesothelioma (MPM) is the most common primary pleural tumor and aggressive neoplasm that arises from the mesothelium of the pleura. The prognosis is poor with a median survival of 9.2 to 11.2 months [1-3], although a better prognosis with a median survival of 5 years is reported for MPM with germline BAP1 mutations [4, 5]. In the diagnosis of MPM, it is important to differentiate it from reactive mesothelial hyperplasia (RMH). RMH is a benign process, but sometimes resembles MPM histologically and cytologically [6-8]. Therefore, certain immunohistochemical markers, such as desmin, epithelial membrane antigen (EMA) [9, 10], insulin-like growth factor mRNA binding protein 3 (IMP3) [10-12], glucose transporter-1 (GLUT-1) [12-15], and CD146 [12, 16, 17], have been used to distinguish RMH from MPM but these are not useful in an individual case [18].

Recently, two new markers: homozygous deletion (HD) of the 9p21 locus as detected by fluorescence *in situ* hybridization (FISH; 9p21 FISH) [18-21] and BRCA-1 associated protein 1 (BAP1) [18, 19, 22-27] loss as detected by immunohistochemistry (IHC; BAP1 IHC) have been reported to be useful for distinguishing MPM from RMH with 100% specificity. The sensitivity of these markers individually is insufficient; however, their combination yields a high sensitivity [18-20, 27].

*BAP1* gene is located on the short arm of chromosome 3 (3p21.1), and has a role in cell proliferation and growth inhibition. *BAP1* gene frequently shows inactivating mutations in 23-63.6% of MPM [22, 23, 28]. BAP1 nuclear staining is reliable evidence of BAP1 wild type, while cytoplasmic staining alone, no nuclear staining is reliable evidence of BAP1 mutation, since 99% of BAP1 mutations result in truncated BAP1 proteins with loss of the carboxyterminus that contains the nuclear localization signal (NLS), or are mutations in the catalytic domain with consequent loss of the deubiquitylase activity of BAP1 required for nuclear translocation [23, 29, 30].

9p21 FISH cannot be performed by all laboratories because it requires highly skilled staff and is expensive. We have previously reported that methylthioadenosine phosphorylase (MTAP) as detected by IHC (MTAP IHC) is a good surrogate assay to 9p21 FISH [31, 32]. In our previous study with epithelioid and biphasic MPM, MTAP loss was detected by IHC in 65%–75% of MPM with 9p21 HD. Furthermore, MTAP IHC distinguished malignant mesothelial proliferations from benign proliferations with a specificity of 100% and a sensitivity of 45.1% in tissue specimens and 43.2% in cell blocks from pleural effusions [31-33]. MTAP IHC showed 80% sensitivity and 100% specificity in differentiating sarcomatoid MPM from fibrous pleuritis [33]. Furthermore,

a combination of MTAP IHC and BAP1 IHC showed greater sensitivity (76.5–90%) in differentiating MPM from benign proliferations [31-33].

Enhancer of zeste homolog 2 (EZH2), which encodes a component of polycomb repressor complex 2 (PRC-2), has been overexpressed in MPM [34, 35]. EZH2 IHC showed high specificity in differentiating MPM from benign proliferations [35]. Thus, in the current study, we investigated whether a combination of three IHC methods (BAP1, MTAP, and EZH2) was effective for distinguishing MPM from RMH, compared with combinations of BAP1 IHC, EZH2 IHC, and 9p21 FISH.

## **2. Materials and Methods**

### *2.1 Case Selection*

This study included 38 MPM and 29 RMH cases. All cases were obtained from the pleural lesion file (including consultation cases) of the Department of Pathology, Fukuoka University Hospital, Fukuoka, Japan, between 2001 and 2015. Histological mesothelioma diagnosis and classification were performed following the World Health Organization guidelines and classification for 2015. The mesothelial nature of each tumor was confirmed using IHC assays. Calretinin, podoplanin (D2-40), cytokeratin 5/6, and Wilms' tumor-1 (WT-1) were used as positive mesothelial markers. While



thyroid transcription factor-1 (TTF-1), Ber-EP4, and carcinoembryonic antigen (CEA) were used as negative markers.

The study protocol was approved by the Ethics Committee of Fukuoka University Hospital (#11-7-11, 7/27/2011). Anonymous use of redundant tissues is part of the standard patient treatment agreement.

## *2.2 IHC*

For IHC, tissue was initially formalin-fixed, paraffin-embedded, and sliced into 4- $\mu$ m-thick sections. Endogenous peroxidase activity was then blocked using the blocking reagent included in the Dako EnVision Kit (Dako, California, USA) for 5 min at room temperature (RT) followed by epitope retrieval using pH 9.0 Tris-EDTA buffer for 20 or 40 min at 95 °C. IHC was then performed using the following antibodies: anti-EZH2 rabbit monoclonal antibody (clone D2C9; Cell Signaling Technology, Danvers, MA, USA; 1:100 dilution; 4 °C overnight), anti-BAP1 mouse monoclonal antibody (clone C-4; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 dilution; RT, 1 h), anti-MTAP mouse monoclonal (clone 2G4; Abnova, Taipei, Taiwan; 1:100 dilution; RT 30 min). Sections were then washed, incubated with ChemMate EnVision (Dako), and counterstained with hematoxylin. Specimens from breast tumors (for EZH2) and liver

tissue (for MTAP) served as positive controls. The surrounding stromal cells and lymphocytes were used as internal positive control cells for BAP1.

Nuclear staining in mesothelial cells with the same as, or higher intensity than, positive controls or internal positive controls was regarded as positive in EZH2 or BAP1 IHC. In MTAP IHC, cytoplasmic expression with nuclear staining in mesothelial cells at the same as, or higher intensity than, the positive control was interpreted as positive. Cytoplasmic positivity was considered a non-specific reaction for BAP1 staining. In each case, >500 tumor cells in five high power fields ( $\times 400$ ) were evaluated, and the proportion of cells positive for staining was calculated. We set the cut-off value at 50% for MTAP and BAP1 IHC as described previously [31, 32]. For EZH2 IHC, staining was divided into two groups, low expression (proportion of cells <50%) or high expression (proportion of cells >50%) according to the previous report [35].

### *2.3 FISH*

The FISH studies were carried out on 4- $\mu\text{m}$ -thick tissue sections as previously described [20, 31-33]. Sections were deparaffinized and rehydrated using descending alcohol dilutions, then washed with  $2\times$  saline-sodium citrate (SSC), exposed to pretreatment solution at 80 °C for 30 min (PathVysion HER2 DNA probe kit; Vysis, Downers Grove, IL, USA), and digested with pepsin solution (Sigma-Aldrich, Tokyo,

Japan) at 37 °C for 90 min. After refixation in 10% buffered formalin at RT for 10 min, the sections were treated with 2× SSC, dehydrated in ethanol, dried, and exposed to either of the two probes: 190 kb Vysis LSI *p16/CEP 9* (Abbott Japan, Tokyo, Japan) or 57 kb SureFISH 9p21.3 CDKN2A (Agilent technologies, TX, USA). Both the probes and tissue sections were denatured at 80 °C for 10 min in the probe solution provided (Abbott Japan), followed by hybridization at 37 °C for 20 h in ThermoBrite (Abbott Japan). Finally, the tissue sections were washed with 2× SSC containing 0.3% Tween 20 (Sigma, St. Louis, MO, USA) at 72 °C for 2 min and then with 2× SSC containing 0.1% Tween 20 at RT for 5 min. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in the antifade reagent (Vector Laboratories, Burlingame, CA, USA). Analyses were performed using a fluorescence microscope (Axio Imager Z1; Carl Zeiss MicroImaging, Jena, Germany) and Isis analysis system (MetaSystems, Altussheim, Germany) equipped with filter sets with single and dual-band exciters for spectrum green, spectrum orange, and DAPI (UV 360 nm).

HD was defined as the lack of both 9p21 signals. Heterozygous deletion was assumed when only one 9p21 signal was present or when the total number of 9p21 signals did not exceed one-half the total number of centromeric signals. At least 100 cells were scored for each case. Lymphocytes present in each preparation were used as internal negative controls and required to emit two signals per FISH probe. This result indicated that loss of the 9p21 signal was not due to pre-analytical factors (e.g., fixation or processing). We set the cut-off value at 11% for 9p21 HD as described previously [20, 31].

## *2.4 Statistical analysis*

Fisher's exact test was performed to investigate the association between EZH2 and other markers (BAP1 IHC, 9p21 FISH, and MTAP IHC). The Kaplan Meier method was performed for survival analyses, and the differences between survival curves were analyzed using the log-rank test. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics v24 (IBM, Armonk, NY, USA).

## **3. Results**

### *3.1 Clinicopathological characteristics*

Thirty-one of the 38 MPM cases were male and eight were female (M:F ratio: 4.8:1). The mean age at diagnosis was 65.3 y (range 33–86 y). One of the 38 MPM cases was obtained via autopsy, 13 were obtained via biopsy, and the remainder were via surgery including extrapleural pneumonectomy (EPP) and pleurectomy and decortication (PD). Histology showed 27 epithelioid, 6 biphasic, and 5 sarcomatoid

MPM. Follow-up period ranged from 20 to 4248 d (mean 995 d). All of the 29 RMH cases were obtained from patients with bullae formation (all male; mean age 32.5 y, range: 18–78 y).

### *3.2 IHCs and 9p21 FISH results*

Representative results of IHC and 9p21 FISH in RMH and MPM are shown in Figure 1 and 2, respectively. EZH2 expression was visible in the nucleus. MTAP IHC showed positive reactivity in the cytoplasm as well as nucleus where MTAP expression is retained. MTAP loss was defined as MTAP reactivity in the cytoplasm and nucleus at an intensity lower than that in the positive control. BAP1 expression was observed in the nucleus, and BAP1 loss was defined as nuclear reactivity at an intensity lower than that in the internal positive control. BAP1 nuclear, together with a weaker cytoplasmic reactivity, reactivity is reliable evidence of BAP1 wild type, while cytoplasmic reactivity alone, no nuclear reactivity is reliable evidence of BAP1 mutation as described [23].

The proportion of positivity for EZH2 in MPM cases showed a wide distribution from 4.5 to 93.9%, while that for EZH2 in RMH cases was confined to a range <40%

(Figure 3). According to the cut off value set at 50%, 44.7% of MPM cases were assessed as “high expression” while all RMH cases belonged to the “low expression” group.

### *3.3 Sensitivity and specificity of detection assays for discriminating MPM from RMH*

The sensitivity and specificity of each detection assay for discriminating MPM from RMH cases are summarized in Table 1. All of EZH2 IHC, BAP1 IHC, MTAP IHC, and 9p21 FISH were characterized by a 100% specificity and their sensitivities were 44.7%, 52.6%, 47.4%, and 65.8%, respectively. The specificity was 100% for all combinations of IHC (EZH2, BAP1, and MTAP) and 9p21 FISH. Sensitivity was low with EZH2 IHC alone, but this increased when combined with other markers. Although a combination of EZH2 IHC, BAP1 IHC, and 9p21 FISH gave the highest sensitivity (89.5%), that of EZH2, BAP1, and MTAP, which was only a combination of immunostaining, was as high as 86.8%.

### *3.4 Association between high EZH2 expression and other markers*

Table 2 shows the association of highly expressed EZH2 with other markers [BAP1 loss (IHC), 9p21 HD (FISH), and MTAP loss (IHC)]. Although highly expressed EZH2 and 9p21 HD showed a tendency to correlate, it was not statistically significant ( $P = 0.053$ ).

### 3.5 Survival analysis

Survival analysis was performed in 37 MPM patients (27 epithelioid MPM, 5 biphasic MPM, and 4 sarcomatoid MPM) available for follow-up (Figure 4). Overall survival (OS) was significantly longer in epithelioid MPM than sarcomatoid MPM ( $P = 0.041$ , Figure 4A). In all MPM cases the high EZH2 expression group had a significantly shorter OS than the low EZH2 expression group ( $P = 0.025$ , Figure 4B). However, when analyzed according to histological subtype, their prognostic effect was seen only in epithelioid MPM ( $P = 0.008$ , Figure 4C), and not in biphasic and sarcomatoid MPM ( $P = 0.831$ , Figure 4D).

## 4. Discussion

This study demonstrated the diagnostic application of EZH2 IHC for differentiating MPM from RMH. Several recent studies describe the utility of 9p21 FISH or BAP1 IHC for differentiating MPM from RMH [18-20, 36]. In the current study, sensitivity was low with EZH2 IHC alone, but when EZH2 IHC was combined with other markers, overall sensitivity increased. A combination of BAP1 IHC and 9p21 FISH yielded a sensitivity of approximate 80%, whereas adding EZH2 IHC to this combination increased the overall sensitivity to about 90% (Table 1). Moreover, adding EZH2 IHC to a combination of BAP1 and MTAP IHC yielded a high sensitivity of 86.8% and 100% specificity. Since 9p21 FISH cannot be performed in every facility, it is useful to have a diagnostic combination of IHC that can discriminate MPM from RMH.

Based on the proportion of positively stained cells in each case of MPM and RMH (Figure 3), an actual clinical cut-off value in discrimination between MPM and RMH of 50% was considered reasonable also by our team as reported previously [35]. The distribution of the proportion of positivity stained cells is different from those of BAP1 and MTAP [31]. BAP1 and MTAP exhibited a bimodal distribution of the proportion of positivity stained cells in MPM cases. However, for EZH2 there are some cases of positive cells among MPM patients showing around the cut-off value of 50%.



Thus, care should be taken in these cases.

Polycomb group (PcG) proteins are critical epigenetic mediators of stem cell pluripotency. The two main families of PcG protein complexes are polycomb repressive complex 1 (PRC1) and PRC2. PRC1 consists of four core subunits: polyhomeotic 1 (PH1), B-lymphoma Moloney murine leukemia virus insertion region-1 (BMI1), chromobox (CBX), and Ring finger protein 1A/1B (RING1A/1B). PRC2 consists of four core subunits: EZH2, embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and retinoblastoma (Rb)-associated protein 48 (RbAp48). EZH2 has histone methyltransferase activity with H3K27 as its substrate, which catalyses to H3K27me2 or H3K27me3. H3K27me3 has been reported to recruit PRC1 via proteins of the CBX family. RING1A/1B induces the monoubiquitination of histone 2A lysin 119 (H2AK119) [37]. Monoubiquitination of H2A is a critical mechanism in the control of initiation, elongation, and silencing of transcription and DNA repair [38]. On the other hand, the BAP1-ASXL1 complex has H2AK119ub deubiquitinase activity [39]. For this reason, BAP1 loss is considered to be related to a high expression of EZH2. A recent study described how BAP1 loss enhanced EZH2 expression and promoted proliferation of mesothelioma cell lines. Furthermore, it demonstrated that mesothelioma cells with BAP1 loss are sensitive to EZH2 pharmacologic inhibition

[40]. On the other hand, Sinozaki-Ushiku et al. showed no statistically significant association between BAP1 and EZH2 expression in MPM, suggesting that mechanisms underlying EZH2 overexpression and BAP1 loss may be distinct [35]. In this study, there was also no statistical association between high EZH2 expression and BAP1 loss (Table 2.  $P = 0.973$ ). These findings suggest that EZH2 high expression in MPM does not necessarily coincide with BAP1 loss, possibility due to other mechanisms or tumor environment.

This is the first report to demonstrate a relationship between highly expressed EZH2 and 9p21 homozygous deletion in MPM. This association showed a tendency to correlate, but was not statistically significant (Table 2.  $P = 0.053$ ). There was no correlation between BAP1 IHC or MTAP IHC and highly expressed EZH2 ( $P = 0.973$ ,  $P = 0.284$ ). Therefore, this lack of correlation among these three IHCs (BAP1, MTAP, EZH2) enabled the combination to offer an increased sensitivity in differential diagnosis between MPM and RMH. A major limitation of our study was the relatively small sample size. Further studies are required to assess the usefulness of EZH2 IHC.

High EZH2 overexpression has been reported in various cancers and particularly with the aggressiveness of tumors [41]. In this study, high EZH2 expression resulted in a significantly worse OS ( $P = 0.025$ ). Pharmacological inhibition of EZH2/PRC2 by 3-

Deazanplanocin A (DZNep), an *S*-adenosylhomocysteine (SAH) hydrolase inhibitor, has been shown to effectively inhibit growth of MPM cells [34]. Phase II clinical trials of tazemetostat, an EZH2 inhibitor, have been initiated (ClinicalTrials.gov, NCT02860286).

In conclusion, adding EZH2 to the diagnostic IHC panel for differentiating between MPM and RMH increased diagnostic sensitivity. Furthermore, highly expressed EZH2 was associated with poor prognosis.

### **Conflict of Interest**

All authors report that no potential conflicts of interest exist with any companies/organizations whose products or services have been referred to in this article.

### **Acknowledgement**

The authors thank Ms. K. Yano and Ms. M. Onitsuka, Department of Pathology, Fukuoka University School of Medicine and Hospital, for their skilled assistance with the 9p21 FISH and IHC assays. This work was supported in part by Grants-in-Aid for Scientific Research (#24590495) from the Japanese Society for the Promotion of Science; grants from the Research Center for Advanced Molecular Medicine, Fukuoka

University; and funds (#171032) from the Central Research Institute of Fukuoka

University.

## References

- [1] P. Beckett, J. Edwards, D. Fennell, et al., Demographics, management and survival of patients with malignant pleural mesothelioma in the National Lung Cancer Audit in England and Wales, *Lung Cancer* 88(3) (2015) 344-348.
- [2] R.A. Damhuis, A. Khakwani, H. De Schutter, et al., Treatment patterns and survival analysis in 9014 patients with malignant pleural mesothelioma from Belgium, the Netherlands and England, *Lung Cancer* 89(2) (2015) 212-217.
- [3] M. Farzin, C.W. Toon, A. Clarkson, et al., Loss of expression of BAP1 predicts longer survival in mesothelioma, *Pathology* 47(4) (2015) 302-307.
- [4] F. Baumann, E. Flores, A. Napolitano, et al., Mesothelioma patients with germline BAP1 mutations have 7-fold improved long-term survival, *Carcinogenesis*. 36(1) (2015) 76-81.
- [5] S. Pastorino, Y. Yoshikawa, H.I. Pass, et al., A Subset of Mesotheliomas With Improved Survival Occurring in Carriers of BAP1 and Other Germline Mutations, *J Clin Oncol.* 36(35) (2018) 3485-3494.
- [6] A. Hjerpe, V. Ascoli, C.W. Bedrossian, et al., Guidelines for the cytopathologic diagnosis of epithelioid and mixed-type malignant mesothelioma. Complementary statement from the International Mesothelioma Interest Group, also endorsed by the International Academy of Cytology and the Papanicolaou Society of Cytopathology, *Acta Cytol.* 59(1) (2015) 2-16.
- [7] A.N. Husain, T. Colby, N. Ordonez, et al., Guidelines for pathologic diagnosis of malignant mesothelioma: 2012 update of the consensus statement from the International Mesothelioma Interest Group, *Arch. Pathol. Lab. Med.* 137(5) (2013) 647-667.
- [8] S. Matsumoto, K. Nabeshima, T. Kamei, et al., Morphology of 9p21 homozygous deletion-positive pleural mesothelioma cells analyzed using fluorescence in situ hybridization and virtual microscope system in effusion cytology, *Cancer Cytopathol.* 121(8) (2013) 415-422.
- [9] R.L. Attanoos, A. Griffin, A.R. Gibbs, The use of immunohistochemistry in distinguishing reactive from neoplastic mesothelium. A novel use for desmin and comparative evaluation with epithelial membrane antigen, p53, platelet-derived growth factor-receptor, P-glycoprotein and Bcl-2, *Histopathology* 43(3) (2003) 231-238.
- [10] J. King, N. Thatcher, C. Pickering, et al., Sensitivity and specificity of immunohistochemical antibodies used to distinguish between benign and malignant pleural disease: a systematic review of published reports, *Histopathology* 49(6) (2006) 561-568.
- [11] A.F. Lee, A.M. Gown, A. Churg, IMP3 and GLUT-1 immunohistochemistry for distinguishing benign from malignant mesothelial proliferations, *Am. J. Surg. Pathol.* 37(3) (2013) 421-426.
- [12] H. Minato, N. Kurose, M. Fukushima, et al., Comparative immunohistochemical analysis of

IMP3, GLUT1, EMA, CD146, and desmin for distinguishing malignant mesothelioma from reactive mesothelial cells, *Am. J. Clin. Pathol.* 141(1) (2014) 85-93.

[13] A.N. Husain, M.K. Mirza, A. Gibbs, et al., How useful is GLUT-1 in differentiating mesothelial hyperplasia and fibrosing pleuritis from epithelioid and sarcomatoid mesotheliomas? An international collaborative study, *Lung Cancer* 83(3) (2014) 324-328.

[14] Y. Kato, K. Tsuta, K. Seki, et al., Immunohistochemical detection of GLUT-1 can discriminate between reactive mesothelium and malignant mesothelioma, *Mod. Pathol.* 20(2) (2007) 215-220.

[15] S.E. Monaco, Y. Shuai, M. Bansal, et al., The diagnostic utility of pre FISH and GLUT-1 immunohistochemical analysis in mesothelial proliferations, *Am. J. Clin. Pathol.* 135(4) (2011) 619-627.

[16] S. Bidlingmaier, J. He, Y. Wang, et al., Identification of MCAM/CD146 as the target antigen of a human monoclonal antibody that recognizes both epithelioid and sarcomatoid types of mesothelioma, *Cancer Res.* 69(4) (2009) 1570-1577.

[17] A. Sato, I. Torii, Y. Okamura, et al., Immunocytochemistry of CD146 is useful to discriminate between malignant pleural mesothelioma and reactive mesothelium, *Mod. Pathol.* 23(11) (2010) 1458-1466.

[18] A.N. Husain, T.V. Colby, N.G. Ordonez, et al., Guidelines for pathologic diagnosis of malignant mesothelioma 2017 update of the consensus statement from the International Mesothelioma Interest Group, *Arch. Pathol. Lab. Med.* 142(1) (2018) 89-108.

[19] A. Churg, B.S. Sheffield, F. Galateau-Salle, New markers for separating benign from malignant mesothelial proliferations: are we there yet?, *Arch. Pathol. Lab. Med.* 140(4) (2016) 318-321.

[20] T. Hida, M. Hamasaki, S. Matsumoto, et al., BAP1 immunohistochemistry and p16 FISH results in combination provide higher confidence in malignant pleural mesothelioma diagnosis: ROC analysis of the two tests, *Pathol. Int.* 66(10) (2016) 563-570.

[21] S. Chiosea, A. Krasinskas, P.T. Cagle, et al., Diagnostic importance of 9p21 homozygous deletion in malignant mesotheliomas, *Mod. Pathol.* 21(2008) 742-747.

[22] M. Bott, M. Brevet, B.S. Taylor, et al., The nuclear deubiquitinase BAP1 is commonly inactivated by somatic mutations and 3p21.1 losses in malignant pleural mesothelioma, *Nat Genet.* 43(7)(2011) 668-672.

[23] M. Nasu, M. Emi, S. Pastorino, et al., High incidence of Somatic BAP1 alterations is sporadic malignant mesothelioma, *J Thorac Oncol.* 10(4)(2015) 565-576.

[24] J.R. Testa, M. Cheung, J. Pei, et al., Germline BAP1 mutations predispose to malignant mesothelioma, *Nat Genet.* 43(10)(2011) 1022-1025.

[25] M. Cigognetti, S. Lonardi, S. Fisogni, et al., BAP1 (BRCA1-associated protein 1) is a highly

specific marker for differentiating mesothelioma from reactive mesothelial proliferations, *Mod. Pathol.* 28(8) (2015) 1043-1057.

[26] B.S. Sheffield, H.C. Hwang, A.F. Lee, et al., BAP1 immunohistochemistry and p16 FISH to separate benign from malignant mesothelial proliferations, *Am. J. Surg. Pathol.* 39(7) (2015) 977-982.

[27] L.M. Wang, Z.W. Shi, J.L. Wang, et al., Diagnostic accuracy of BRCA1-associated protein 1 in malignant mesothelioma: a meta-analysis, *Oncotarget* 8(40) (2017) 68863-68872.

[28] Y. Yoshikawa, A. Sato, T. Tsujimura, et al., Frequent inactivation of the *bap1* gene in epithelioid-type malignant mesothelioma, *Cancer Sci.* 103 (2012) 868-874

[29] A. Bononi, A. Napolitano, H.I. Pass, et al., Latest developments in our understanding of the pathogenesis of mesothelioma and the design of targeted therapies, *Expert Rev Respir Med.* 9(5) (2015) 633-654.

[30] N. Mashtalir, S. Daou, H. Barbour, et al., Autodeubiquitination protects the tumor suppressor BAP1 from cytoplasmic sequestration mediated by the atypical ubiquitin ligase UBE2O, *Mol Cell.* 54(3) (2014) 392-406.

[31] T. Hida, M. Hamasaki, S. Matsumoto, et al., Immunohistochemical detection of MTAP and BAP1 protein loss for mesothelioma diagnosis: Comparison with 9p21 FISH and BAP1 immunohistochemistry, *Lung Cancer* 104 (2017) 98-105.

[32] Y. Kinoshita, T. Hida, M. Hamasaki, et al., A combination of MTAP and BAP1 immunohistochemistry in pleural effusion cytology for the diagnosis of mesothelioma, *Cancer Cytopathol.* 126(1) (2018) 54-63.

[33] Y. Kinoshita, M. Hamasaki, M. Yoshimura, et al., A combination of MTAP and BAP1 immunohistochemistry is effective for distinguishing sarcomatoid mesothelioma from fibrous pleuritic, *Lung Cancer.* (October) (2018) Epub ahead of print. <https://doi.org/10.1016/j.lungcan.2018.09.019>

[34] C.D. Kemp, M. Rao, S. Xi, et al., Polycomb repressor complex-2 is a novel target for mesothelioma therapy, *Clin. Cancer Res.* 18(1) (2012) 77-90.

[35] A. Shinozaki-Ushiku, T. Ushiku, S. Morita, et al., Diagnostic utility of BAP1 and EZH2 expression in malignant mesothelioma, *Histopathology* 70(5) (2017) 722-733.

[36] S.M. McGregor, J. McElherne, A. Minor, et al., BAP1 immunohistochemistry has limited prognostic utility as a complement of CDKN2A (p16) fluorescence in situ hybridization in malignant pleural mesothelioma, *Hum. Pathol.* 60 (2017) 86-94.

[37] Y. Kondo, Targeting histone methyltransferase EZH2 as cancer treatment, *J. Biochem.* 156(5) (2014) 249-257.

[38] L. Gutierrez, K. Oktaba, J.C. Scheuermann, et al., The role of the histone H2A ubiquitinase Sce in Polycomb repression, *Development* 139(1) (2012) 117-127.

- [39] M. Carbone, H. Yang, H.I. Pass, et al., BAP1 and cancer, *Nat. Rev. Cancer* 13(3) (2013) 153-159.
- [40] L.M. LaFave, W. Beguelin, R. Koche, et al., Loss of BAP1 function leads to EZH2-dependent transformation, *Nat. Med.* 21(11) (2015) 1344-1349.
- [41] K.H. Kim, C.W. Roberts, Targeting EZH2 in cancer, *Nat. Med.* 22(2) (2016) 128-134.



## Figure Legends

### Figure 1.

Representative examples of H&E staining (A) and EZH2 (B), MTAP (C), and BAP1 (D) immunostaining and 9p21 FISH (E) in RMH. Original magnifications  $\times 400$  (A–D). (A): hyperplastic reactive mesothelial cells on the pleural surface, (B): low EZH2 expression, (C): retained MTAP IHC in mesothelial cells, (D) retained BAP1 IHC in mesothelial cells, (E): Normal pattern, with two CEP-9 green centromeric signals and two 9p21 red signals in a single nucleus (white arrow).

### Figure 2.

Representative examples of H&E staining (A) and EZH2 (B), MTAP (C), and BAP1 (D) immunostaining, and 9p21 FISH (E) in MPM. Original magnifications  $\times 400$  (A–D). (A): epithelioid MPM, (B): high EZH2 expression (nuclear staining), (C): loss of MTAP IHC in mesothelial cells (cytoplasmic/nuclear staining), (D) loss of BAP1 IHC in mesothelial cells (nuclear staining), (E): homozygous deletion, with two CEP-9 green centromeric signals and no 9p21 red signals in a single nucleus (white arrow).

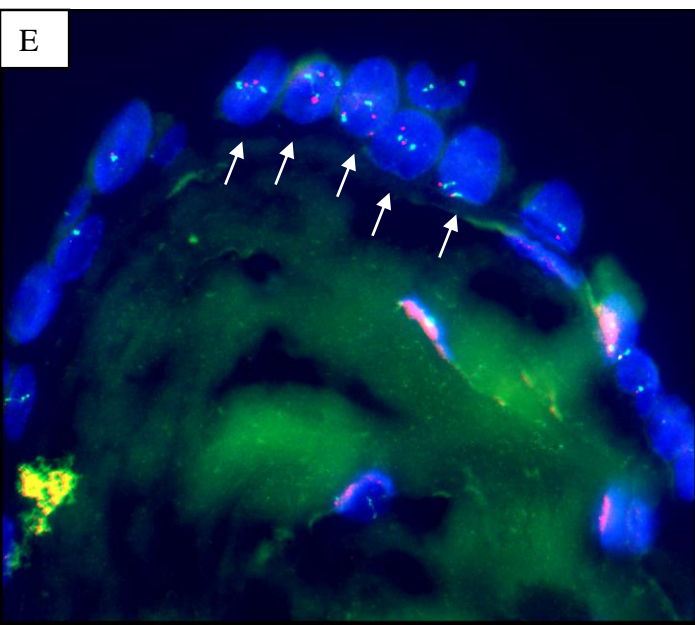
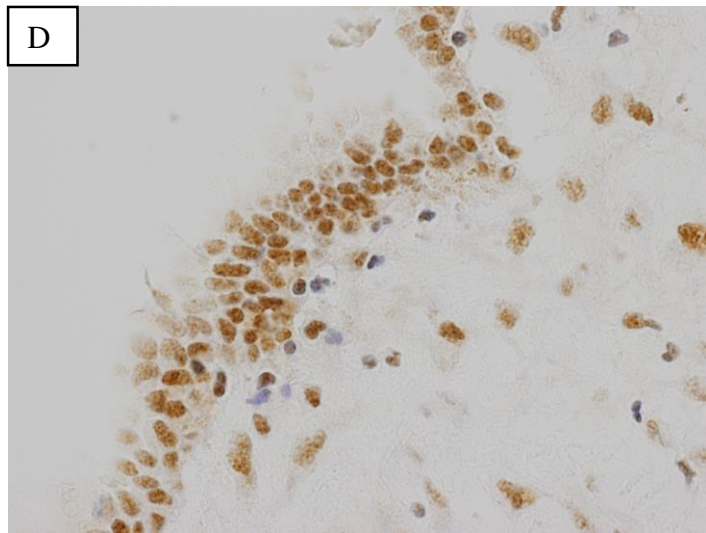
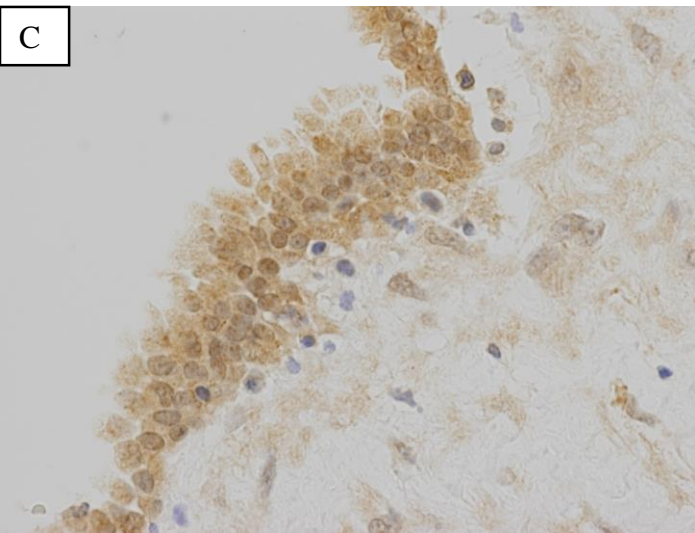
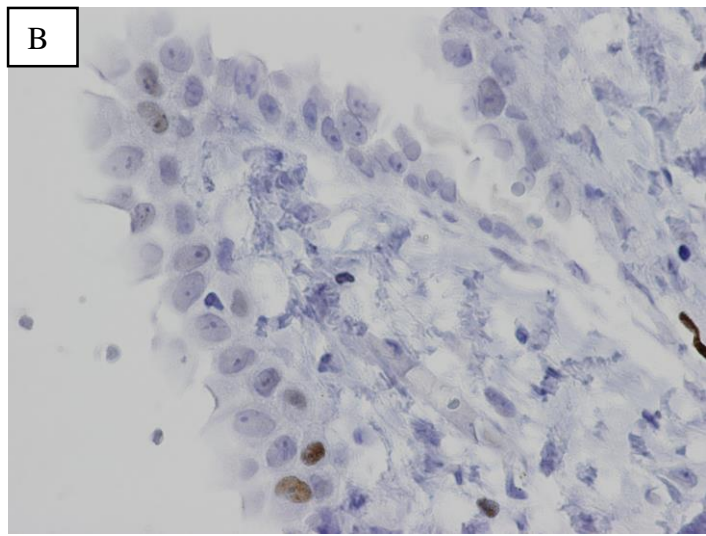
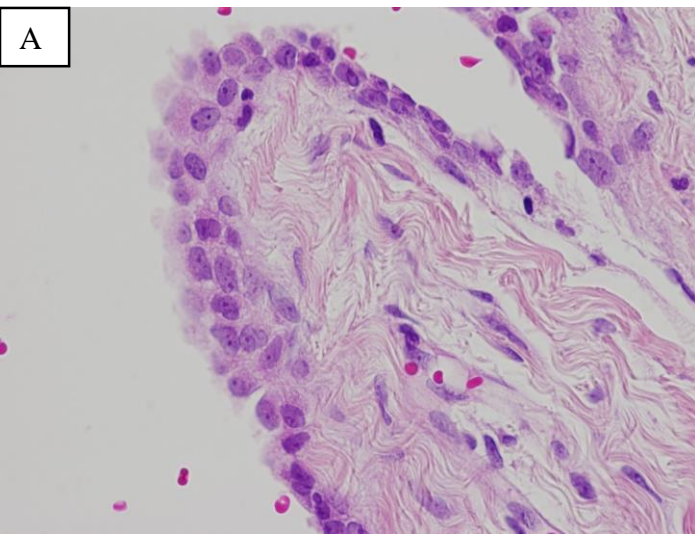
### Figure 3.

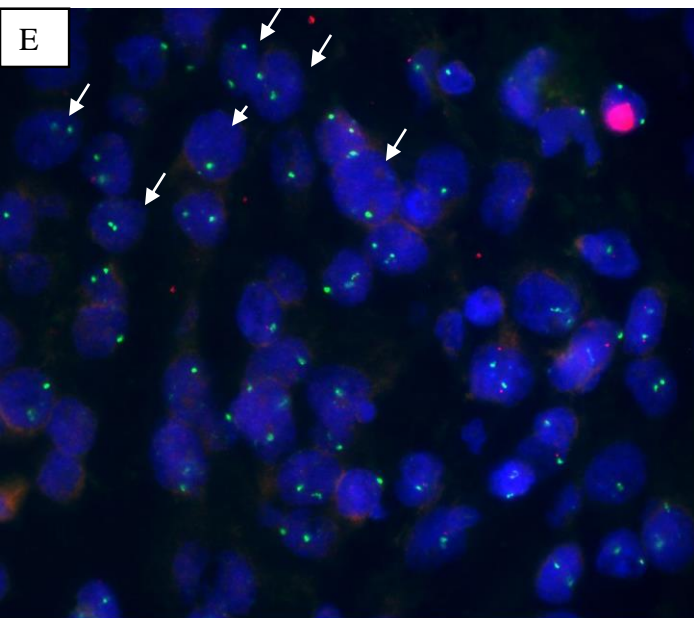
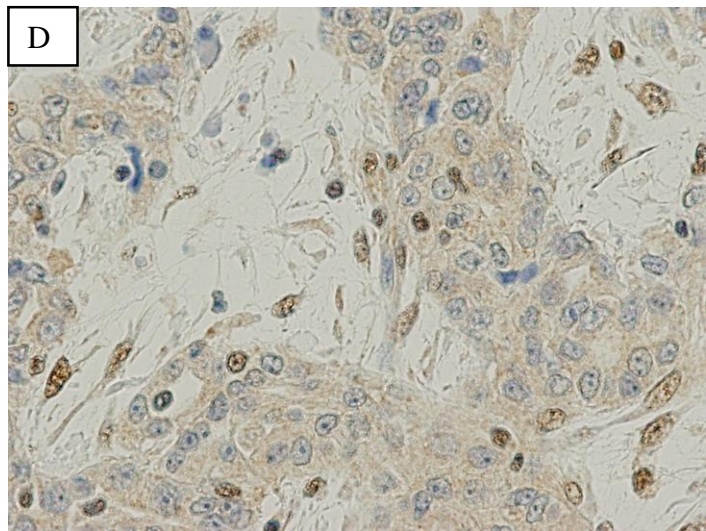
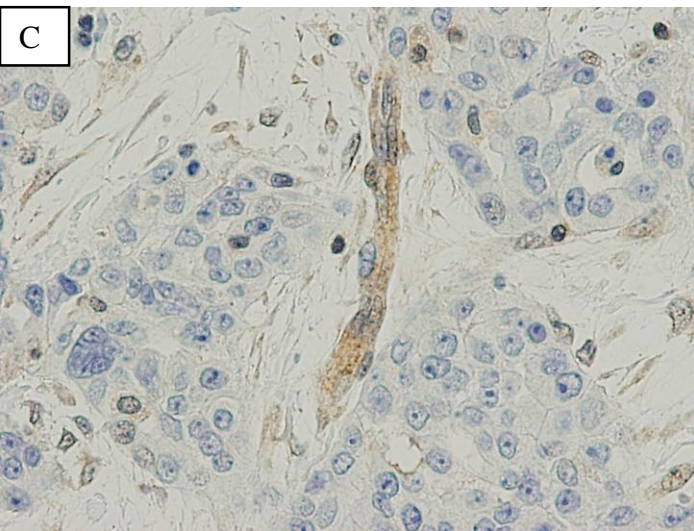
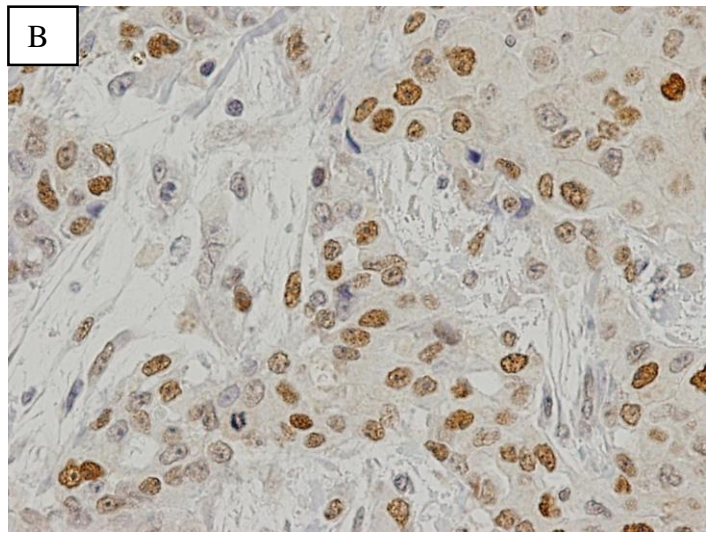
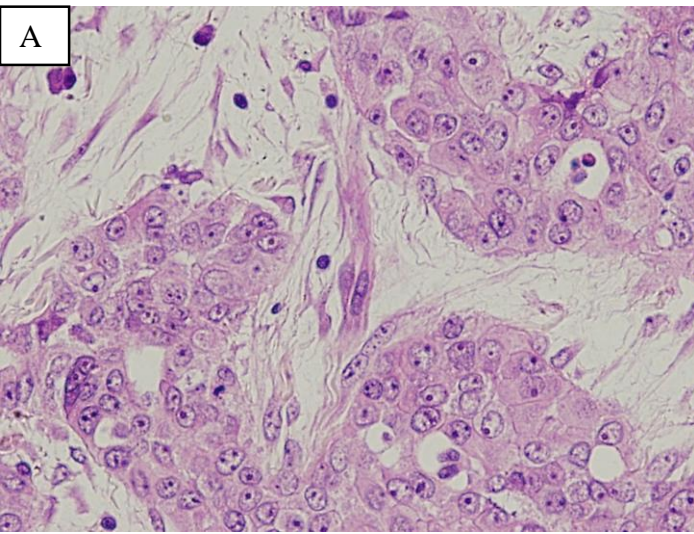
Proportion of cells expressing EZH2 in their nuclei in malignant pleural mesothelioma

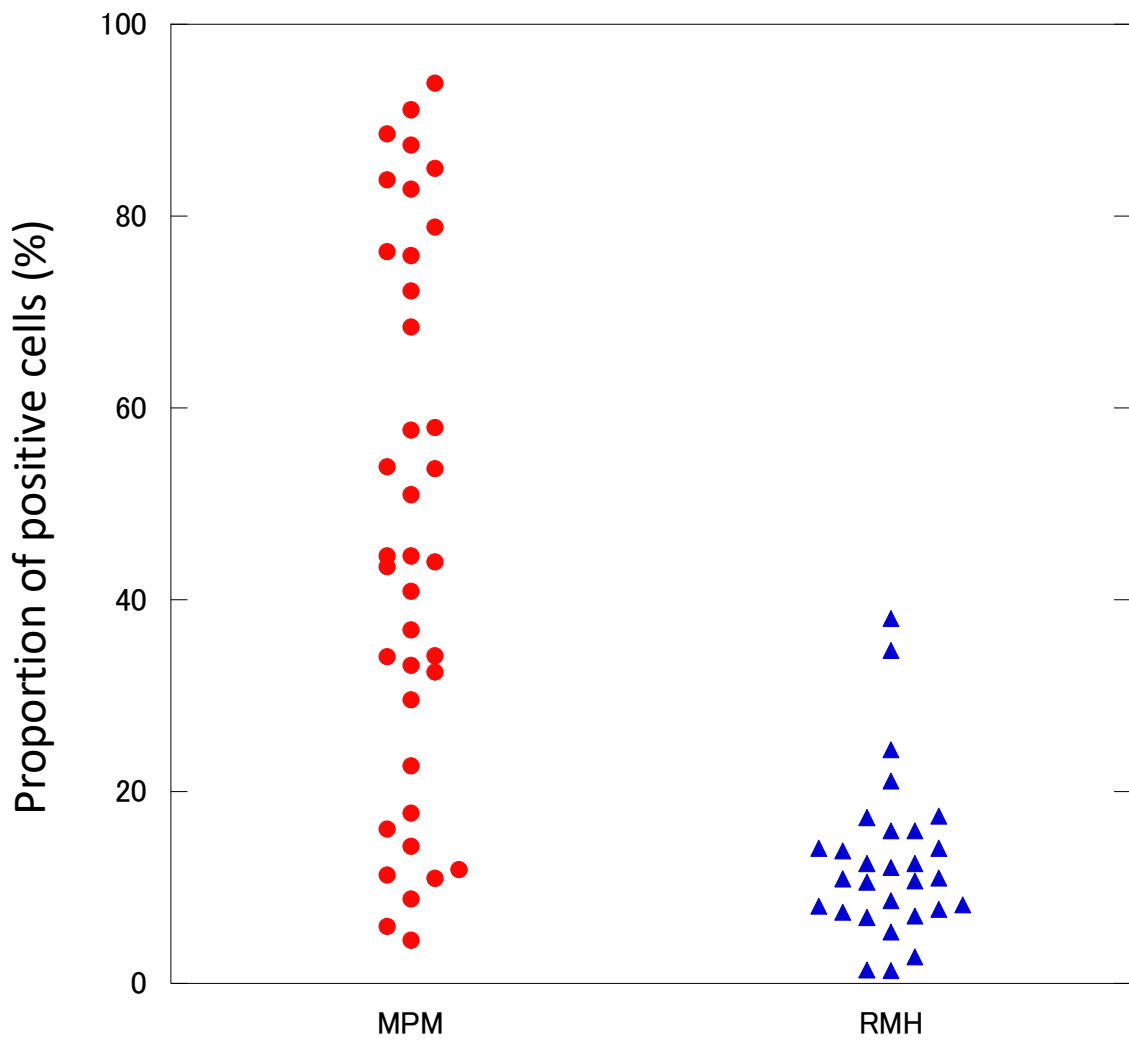
(MPM) and reactive mesothelial hyperplasia (RMH).

**Figure 4.**

Overall survival (OS) curves for patients with MPM. (A) Histology (E-MPM, epithelioid malignant pleural mesothelioma; B-MPM, biphasic MPM; S-MPM, sarcomatoid MPM). (B) EZH2 status, (C) EZH2 status in only E-MPM, (D) EZH2 status in B-MPM and S-MPM.







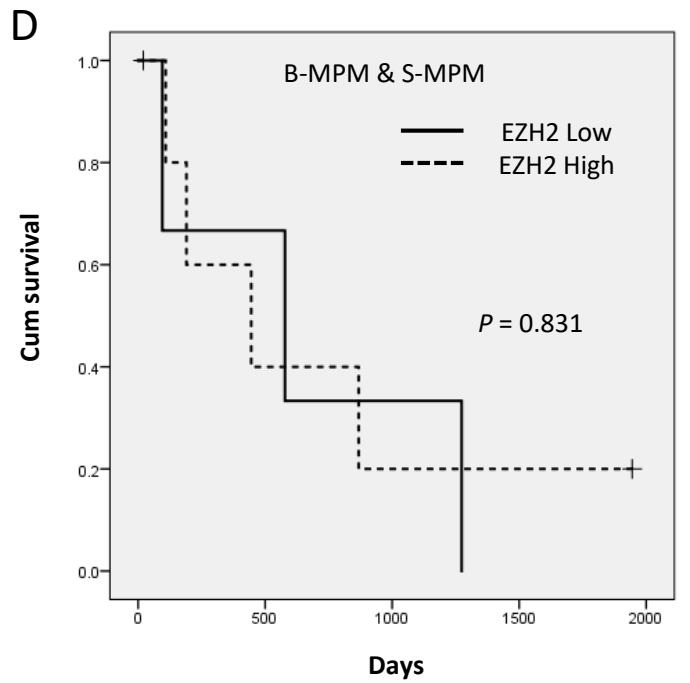
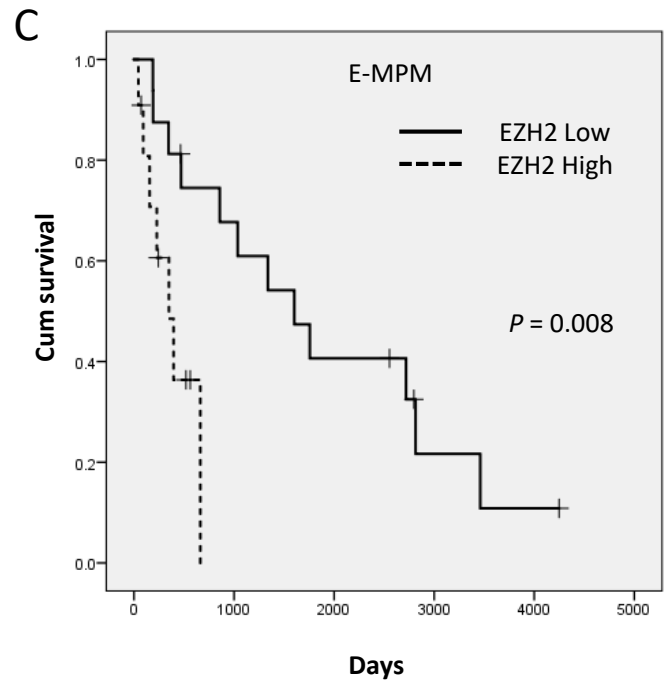
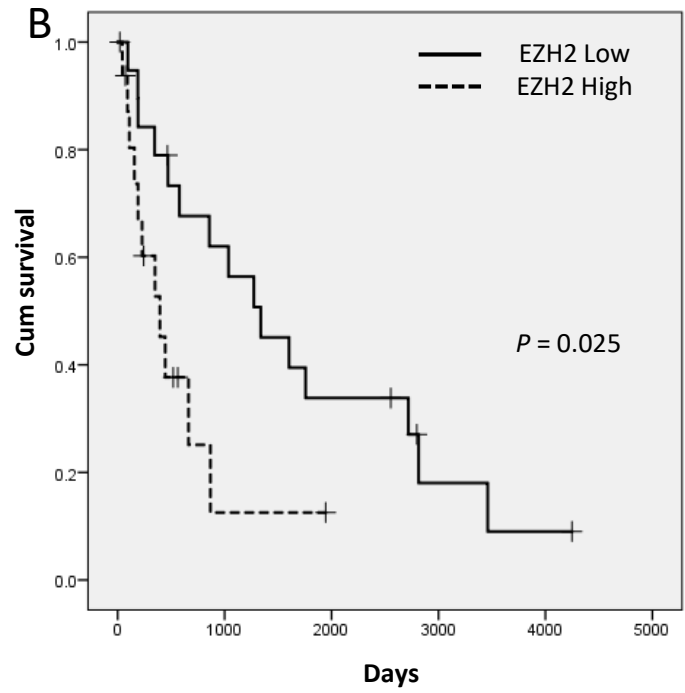
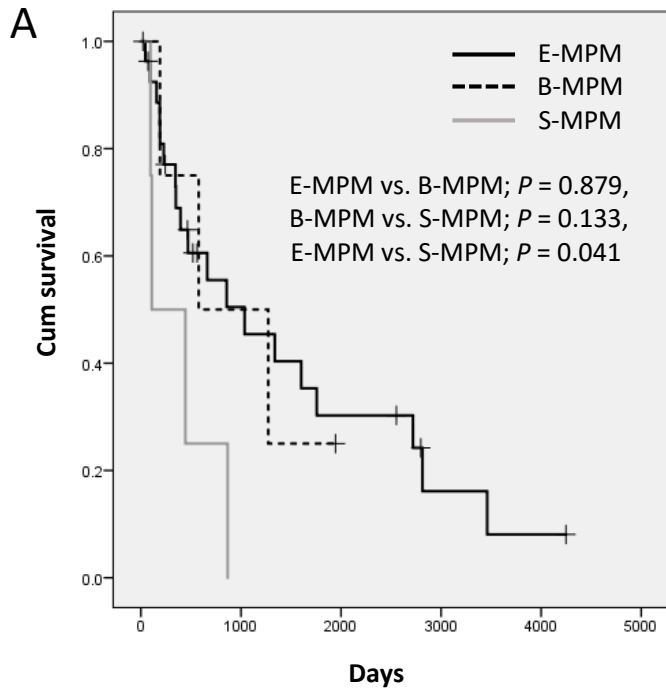


Table 1. Yoshimura M, et al.

Table 1. Diagnostic application of detection assays to differentiate malignant pleural mesothelioma from reactive mesothelial hyperplasia

		MPM (n = 38)	RMH (n = 29)	Sensitivity (%)	Specificity (%)
EZH2 IHC	High	17	0	44.7	100
	Low	21	29		
BAP1 IHC	Retained	20	0	52.6	100
	Loss	18	29		
MTAP IHC	Retained	18	0	47.9	100
	Loss	20	29		
9p21 FISH	HD	25	0	65.8	100
	Normal	13	29		
EZH2 / 9p21	High / HD	28	0	73.7	100
	Low / Normal	10	29		
EZH2 / BAP1	High / Loss	28	0	73.7	100
	Low / Retained	10	29		
EZH2 / MTAP	High / Loss	25	0	65.8	100
	Low / Retained	13	29		
BAP1 / 9p21	Loss / HD	31	0	81.6	100
	Retained / Normal	7	29		
BAP1 / MTAP	Retained / Retained	28	0	73.7	100
	Loss / Loss	10	29		
EZH2 / BAP1 / 9p21	High / Loss / HD	34	0	89.5	100
	Low / Retained / Normal	4	29		
EZH2 / BAP1 / MTAP	High / Loss / Loss	33	0	86.8	100
	Low / Retained / Retained	5	29		

Table 2. Association with high EZH2 expression and other markers (BAP1 IHC, 9p21 FISH, and MTAP IHC)

	EZH2 low expression	EZH2 high expression	<i>P</i> -value
BAP1 loss (-)	10	8	0.973
BAP1 loss (+)	11	9	
9p21 homozygous deletion (-)	10	3	0.053
9p21 homozygous deletion (+)	11	14	
MTAP loss (-)	6	7	0.284
MTAP loss (+)	13	7	