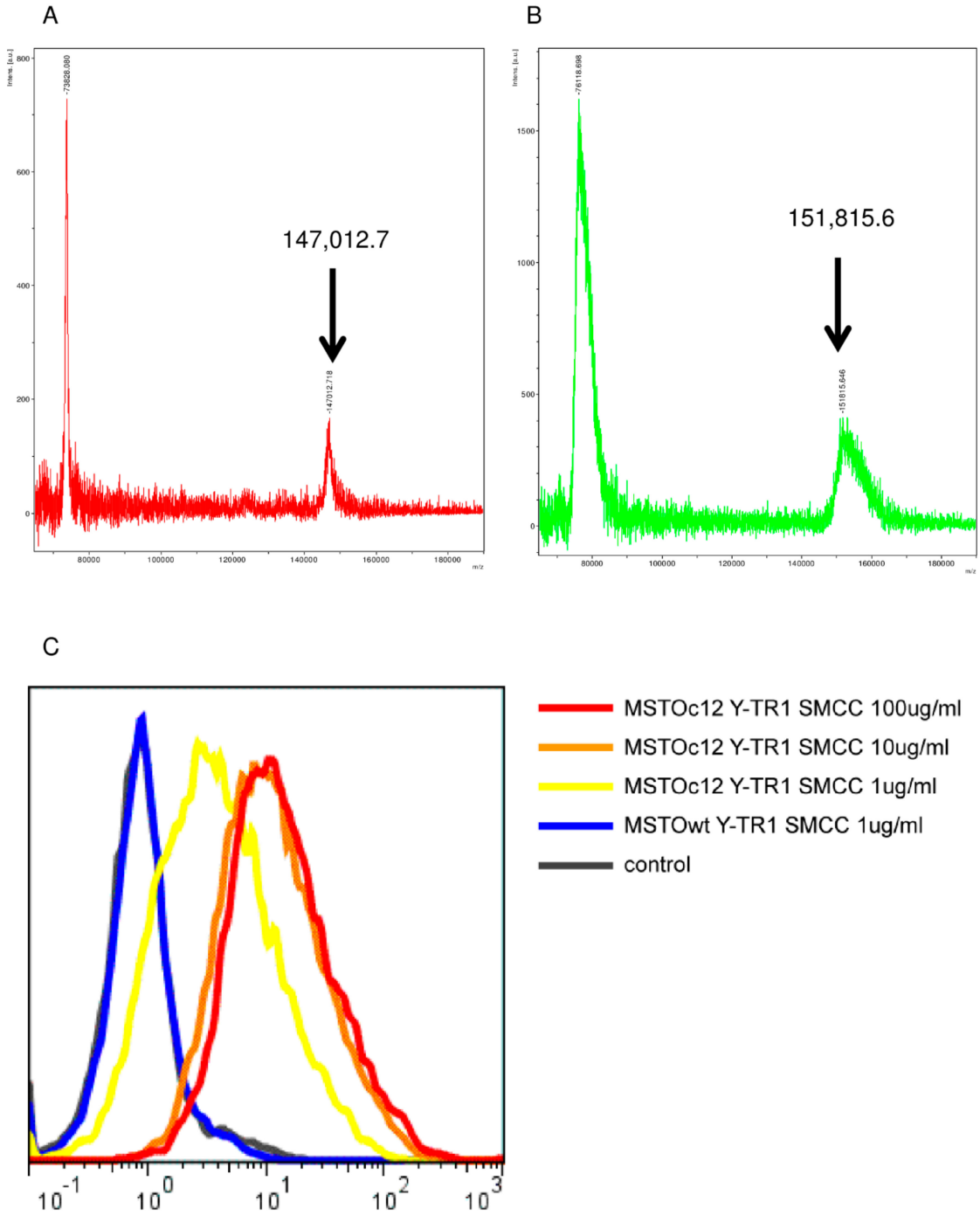

Figure 3. Biochemical analysis of Y-TR1. (**A**,**B**): The intact mass of unconjugated YS110 and Y-TR1 (SMCC) measured by the MALDI-TOF mass analysis. (**A**) Unconjugated YS110: 147,012.7; (**B**) Y-TR1 (SMCC): 151,815.6; (**C**) binding of Y-TR1 to the multiple myeloma (MM) cell line MSTO clone12 (CD26 positive). First antibody: Y-TR1 second antibody: Anti-human rabbit IgG FITC conjugate. Y-TR1 over 10 μg/mL showed intact binding to CD26-positive cells.

2.3. In Vitro Cytotoxicity of Y-TR1 against MM and Leukemia Cell Lines

The Y-TR1 showed dose-dependent cytotoxicity against CD26-positive MM and leukemia cell lines (Figure 4A,C–E). We compared the Y-TR1 cytotoxicity against the CD26-positive MM cell line MSTO clone12 and JMN between three linkers, SPDP, GMBS, and SMCC (Figure 4A,B). Comparison between three heterobifunctional linkers was performed, and IC50 against the MSTO clone12 of

Y-TR1 using SPDP, GMBS, and SMCC were 38 μg/mL, 18 μg/mL, and 15 μg/mL, respectively (Table 2). Since Y-TR1 conjugated by SMCC showed the best cytotoxicity in this experiment, we adopted Y-TR1 (SMCC) for further experiments. The cytotoxicity of Y-TR1 against various CD26-positive or -negative MM and leukemia cell lines was compared with unconjugated YS110 (Figure 4B). The IC50 values of Y-TR1 (SMCC) against various cell lines calculated from WST-1 assays are shown in Table 1. Against CD26-positive cells (MSTO clone12, JMN, Jurkat CD26 (+)), Y-TR1 showed remarkably higher cytotoxicity than the unconjugated YS110. Compared with the CD26 negative counterpart (MSTO wt, Jurkat CD26 (−)), CD26 positive cell lines (MSTO clone12, Jurkat CD26(+)) are more susceptible to the Y-TR1 cytotoxicity at the concentration of 20 μg/mL (Figure 4C,D). The in vitro influence of nonspecific binding of antibodies via the Fc receptor were evaluated by the cytotoxic assay of Y-TR1 with or without the Fc receptor blocking reagent. The Fc receptor blocking reagent had no significant effect on the in vitro cytotoxicity of Y-TR1 against MSTO clone12 cells (Supplementary Figure S1). In vitro cytotoxicity against CD26-positive non-cancer cells (primary dermal human microvascular endothelial cells, dHMVECs) was also tested, and Y-TR1 showed less cytotoxicity than against CD26-positive malignant cell lines significantly (Figure 4E). 40% reduction in the viability of dHMVECs was observed at the maximum concentration of Y-TR1 (60 μg/mL).

Table 2. Comparison of IC50 of Y-TR1 using various linkers (SPDP, GMBS, SMCC).

Conjugate	IC50 (μg/mL)
Y-TR1 SPDP	35
Y-TR1 GMBS	18
Y-TR1 SMCC	15

Y-TR1's equivalent of free TR-1 was calculated by IC50 of Y-TR1 (approximately 15 μg/mL = 99 nM) and unconjugated TR-1 (250 nM) against the MSTO clone12 cell line. The calculated value was approximately 2.5 Y-TR1 molecules equivalent (Figure 4F).

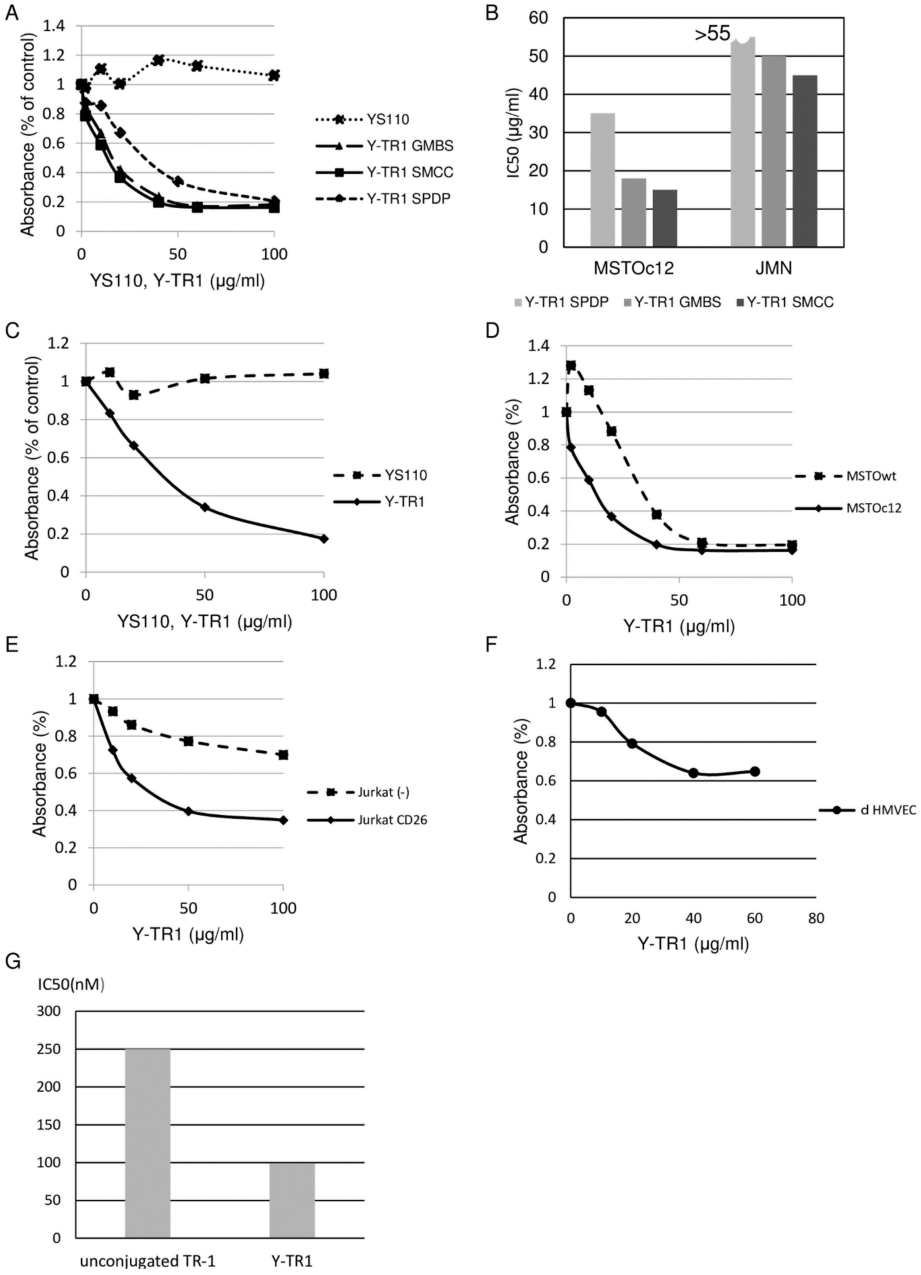

Figure 4. In vitro cytotoxicity of Y-TR1 against MM and leukemia cell lines. The representative results of at least three independent experiments are shown. (**A**) Comparison of cytotoxicity of Y-TR1 using various linkers against the CD26-positive MM cell line MSTO clone12. Y-TR1 using SMCC showed the highest cytotoxicity. The horizontal axis shows the concentration of the compounds in μg/mL. The vertical axis shows the percent of control of the absorbance value in the WST-1 assay; (**B**) comparison of IC50 of Y-TR1 using three linkers, SPDP, GMBS, and SMCC against CD26-positive MM cell lines MSTO clone12 and JMN; (**C**) in vitro cytotoxicity of TR-1 against the CD26-positive MM cell line JMN compared to unconjugated YS110. The horizontal and vertical axes show the same as indicated in (**A**); (**D** and **E**) higher in vitro cytotoxicity of Y-TR1 against the CD26-positive MM cell line MSTO clone12 compared to the CD26-negative counterpart MSTO wt (**D**) and CD26-positive leukemia cell line Jurkat CD26 (+) compared to the CD26-negative counterpart Jurkat (−) (**E**) at Y-TR1 concentration of 20 μg/mL. The vertical axis of the graph shows the percent of control in the WST-1 assay; (**F**) in vitro cytotoxicity of Y-TR1 against CD26-positive non-cancer adult dermal human microvascular endothelial cells (dHMVECs). Horizontal and vertical axes show the same as indicated in (**A**); (**G**) IC50 of unconjugated TR1 and Y-TR1 compared in molar concentration.

2.4. Nucler Translocation of Y-TR1

To confirm that Y-TR1 molecules are internalized and are transported into the nucleus in CD26 positive cancer cell lines as with YS110, the Western blot analysis of nuclear fraction using the anti-human IgG antibody and immunofluorescence staining using were conducted. In the western blot analysis of the CD26 positive JMN cell line, Y-TR1 molecules were detected by the Western blot analysis using the anti-human IgG antibody in both the cytoplasm and nuclear fraction after 30 min and 60 min of the Y-TR1 treatment (Figure 5A). Immunofluorescence staining using the Alexa Fluor 488 labeled anti-human IgG antibody observed under confocal laser microscope showed several dots colocalized with nuclear staining (Hoechst 33342) in Y-TR1 treated (60 min) JMN cells (Figure 5B).

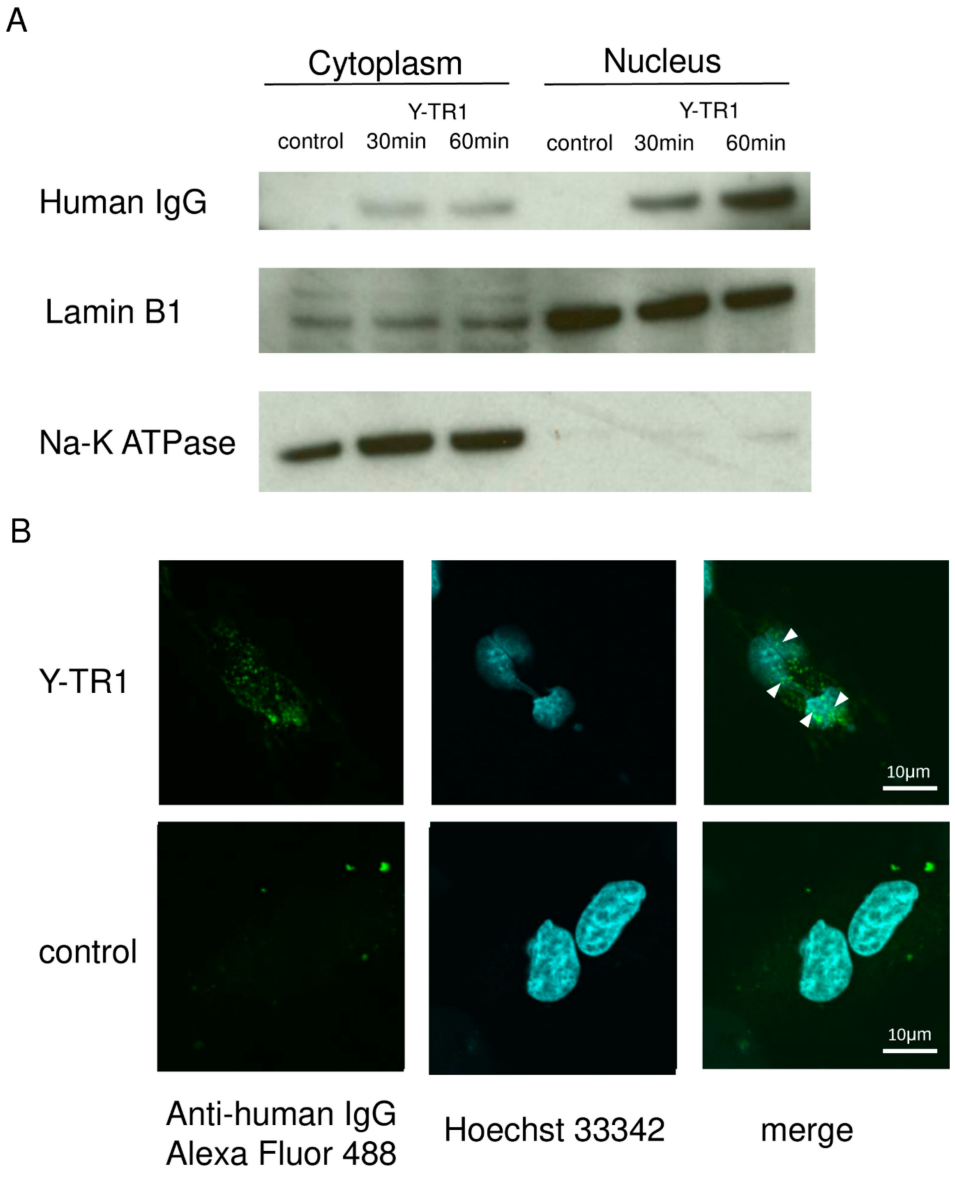

Figure 5. Nuclear translocation of Y-TR1. (**A**) Western blot analysis using anti-human IgG antibody detected Y-TR1 in the cytoplasm and the nuclear fraction of Y-TR1 treated CD26 positive JMN cells after 30 min and 60 min. Lamin B1 and Na-K ATPase were used as loading controls of the nuclear and cytoplasm fraction; (**B**) immunofluorescence staining observed under confocal laser microscopy of fixed JMN cells following 1 h of Y-TR1 treatment with the Alexa Fluor 488 labeled anti-human IgG antibody. Nuclear staining was done with Hoechst 33342. Localization of Y-TR1 (green) was observed in the nucleus (blue) as indicated by the white arrows. Scale bar: 10μm.

2.5. Apoptosis Assay

The induction of apoptosis was examined after the Y-TR1 treatment because triptolide has been reported to cause apoptosis in cancer cells [17]. After 48 h of the Y-TR1 treatment, the caspase 3/7 activity of triptolide-, TR1-, and Y-TR1-treated MSTO clone12 cells was elevated six to nine times against YS110-treated cells (Figure 5A). These results support the estimation that the cytotoxic effects of Y-TR1 are caused by internalized TR1.

2.6. Effects of Triptolide and Y-TR1 on Heat Induction of HSP70 in CD26-Positive MM Cells

To confirm that the cytotoxic effect of Y-TR1 is caused by RNA polymerase II repression as with triptolide, heat shock induction of HSP70, which is dependent on the RNA polymerase activity, was evaluated [17]. In both CD26-positive MM cell lines MSTO clone12 and JMN, the mRNA level of HSP70 after heat shock (45 °C, 2 h) in Y-TR1-treated cells was significantly lower than in YS110-treated cells significantly (Figure 5B,C).

2.7. In Vivo Anti-Tumor Effect of Y-TR1

The in vivo efficacy of Y-TR1 compared with the unconjugated YS110 in the NOD/SCID mouse xenograft model using the CD26-positive MM cell line JMN. The mean tumor volumes on day 55 estimated by the ellipsoid volume formula ($\pi/6 \times L \times W \times H$) [18] were compared between three groups (control, YS110, Y-TR1, $n = 10$) with Fisher's protected least protected difference multiple comparison test. The mean tumor volume of the Y-TR1 group (4 mg/kg weight, total 36 mg/kg Y-TR1 intraperitoneally) was significantly lower ($p < 0.05$) than the control or the YS110 group (Figure 6A). The mean tumor volume of the YS110 group (4 mg/kg weight, total 36 mg/kg Y-TR1 intraperitoneally) was not significantly altered compared with the control (Figure 6A). Two of the nine Y-TR1 group mice showed complete tumor growth prevention macroscopically at the time of sacrifice (day 55). One representative experiment out of two with similar results is shown. There were no clinical manifestations in mice treated with YS110 or Y-TR1. The mean body weight of the mice of each group at sacrifice was not significantly different in mice treated with YS110 or Y-TR1 (Supplementary Figure S2A). No pathological alterations were observed in the brain, heart, lung, liver, spleen, kidney, pancreas, digestive organs or adrenal glands of mice (Supplementary Figure S2B). On the other hand, the mean tumor weight of the YS110 only group and the Y-TR1 group (8 mg/kg weight, total 72 mg/kg of YS110 or Y-TR1 intraperitoneally) was significantly lower ($p < 0.05$ or $p < 0.025$) than that of the control group (Figure 6B). Furthermore, the mean tumor weight of the Y-TR1 group was significantly lower ($p < 0.05$) than that of the YS110 only group (Figure 6B). The statistical analyses were done using Fisher's protected least protected difference multiple comparison test ($n = 10$). In these tumors, the cell growth analysis was performed using MIB-1 (Ki67) staining. As a result, a decreased number of MIB-1-positive cells in Y-TR1-treated tumors was shown compared to IgG1- or YS110-treated tumors (Figure 6C).

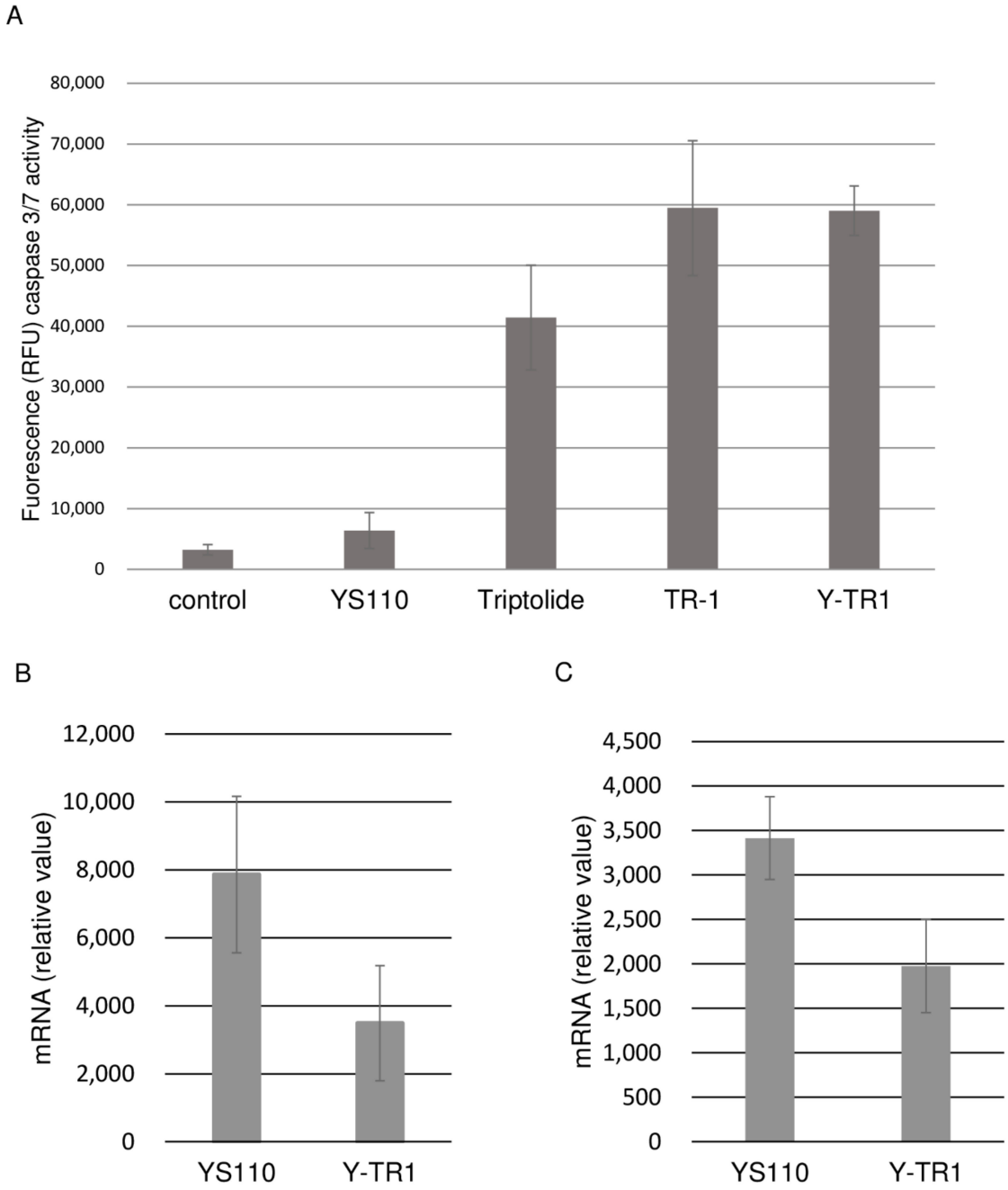

Figure 6. Investigation into the cytotoxic effect of TR-1. (**A**) Caspase 3/7 activity (represented in fluorescence) after 48 h of treatment with YS110, triptolide, TR1, and Y-TR1 in the CD26-positive MM cell line MSTO clone12. The activity is elevated in triptolide-, TR1-, and Y-TR1-treated cells. The vertical axis shows the intensity value measured by the fluorometer; (**B**, **C**) effects of Y-TR1 on the mRNA levels of HSP70 after heat induction. Relative mRNA levels of HSP70 after heat induction (45 °C, 2 h) were significantly lower in Y-TR1-treated cells compared to unconjugated YS110-treated cells in CD26-positive MM cell lines. A *t*-test at the $p = 0.05$ level was carried out as statistical analysis ($n = 10$). The error bar indicates one standard deviation; (**B**) MSTO clone12; (**C**) JMN.

3. Discussion

RNA polymerase II is indispensable for the transcription of almost all protein-coding genes, including those related to cell proliferation [19]. It was previously reported that the functional blockade of one of the subunits of RNA polymerase II, POLR2A, by RNAi strategies and treatment with

chemical compounds such as α-amanitin resulted in growth inhibition of cancer cells [20–22]. We have shown that nuclear accumulation of CD26 promoted POLR2A suppression, leading to a reduction in cell growth [13]. Therefore, we examined whether the novel Antibody-drug conjugate (ADC), Y-TR1 (YS110-TR1 conjugate), restrained POLR2A expression more strongly in the nucleus of tumor cells than YS110 alone. Triptolide has been demonstrated to possess a unique bioactive spectrum of anti-cancer activity and immunosuppressive efficacy; however, due to its poor water solubility and severe toxicity, triptolide cannot be used systemically in the clinic [14]. Therefore, we attempted to develop a new ADC, Y-TR1, which has a higher efficiency of anti-tumor action because triptolide was conjugated with humanized anti-human CD26 monoclonal antibody, YS110, with anti-tumor effects via the suppression of POLR2A transcription, retarded G2/M cell cycling, and antibody-dependent cell-mediated cytotoxicity (ADCC) / complement-dependent cytotoxicity (CDC) [13,23].

Recently, Liu Y et al. reported that cancer cells with hemizygous TP53 deletion were vulnerable to further suppression of such genes. POLR2A was identified as a gene that is almost always co-deleted with TP53 in human cancers [20]. Suppression of POLR2A with α-amanitin or small interfering RNAs selectively inhibits the proliferation, survival and tumorigenic potential of colorectal cancer cells with hemizygous TP53 loss in a p53-independent manner [24]. However, some previous clinical applications of POLR2A inhibitors, such as α-amanitin, have been limited due to their liver toxicity [25]. Therefore, they suggested that α-amanitin-based antibody–drug conjugates were highly effective therapeutic agents with reduced toxicity [26]. It was shown that low doses of the α-amanitin-conjugated anti-epithelial cell adhesion molecule (EpCAM) antibody lead to complete tumor regression in mouse models of human colorectal cancer with hemizygous deletion of POLR2A [24]. TP53 is frequently inactivated in mesothelioma, but mutations are rare. MDM2 and P14/ARF are upstream regulators of TP53 that may contribute to TP53 inactivation [27]. These results suggest that POLR2A may be a certain therapeutic target molecule for malignancies.

Most therapeutic mAbs are thought to disturb signal transduction within tumor cells or get rid of critical cell-surface antigens [28]. As a consequence, these effects may lead to the clearance of cancer cells. ErbB2 is known to be associated with a specific locus on the cyclooxygenase (COX) 2 promoter, activate the gene expressions, thereby inducing cell growth [29]. The humanized ErbB2 mAb trastuzumab inhibits the translocation of ErbB2 into the nucleus. Herein it revealed that in contrast to this ErbB2-Herceptin line, the YS110 treatment abundantly induces nuclear localization of CD26 and in consequentially suppresses POLR2A expression, leading to inhibition of tumor cell growth. These findings reveal that disturbance of nuclear transport of cell-surface antigens by mAbs may be effective targets for mAb therapy against malignancies. Recently it has be shown that some mAbs conjugated to payloads (e.g., radioisotopes, drugs, or toxins) may be targeted to direct inductions of tumor cell death [30–33]. The 90Y-radiolabeled anti-CD20 IgG1, Ibritumomab tiuxetan, has been examined to have substantial anti-tumor effects and is available for standard clinical practice as a therapy for lymphoma [34]. However, the potent cytotoxicity of these payloads may delay the development of novel conjugated antibodies. We have revealed the nuclear localization of anti-CD26 mAbs YS110 in a cell-surface CD26-dependent manner. This phenomenon implies that YS110 may be a target for specific intra-nuclear components, such as genomic DNA sequences and transcription factors. There have been previous reports on the nuclear localization of mAbs against cell-surface antigens, such as ME425 (against EGF receptor) and Br 15-6A (against carbohydrate Y determinant) [35,36].

As the clinical application of ADCs advances, difficulties in the drug-antibody ratio (DAR) control has been discussed recently. FDA approved ADC Kadcyla (T-DM1) is conjugated with the SMCC linker using the amino side chains of lysine residue of the antibody as with Y-TR1. As the limit of the procedure, the ADCs are a heterogenous mixture of ADCs with several DARs [37]. Though some of the efforts to develop methodologies to obtain homogenous ADCs using site specific conjugation have been proposed, many of these technologies require additional bio-engineering or chemical work and not fully established [37]. At the moment, we concluded that the conventional method using the SMCC linker is the best option considering accumulated data of Kadcyla and Adcetris (Brentuximab vedotin).

The overview of anti-cancer effects of antibody drug conjugate Y-TR1 was shown in Figures 7 and 8. It is expected that Y-TR1 bound to CD26 on the cell surface introduces cell death via immunological cytotoxicity such as ADCC and/or CDC [4,11]. On the other hand, CD26 and Y-TR1 are internalized into cytoplasm and then transported to the nucleus within 1 h as confirmed in this study by the immunofluorescence study and Western blot study. In the nucleus, the suppression of POLR2A transcription by the increased amount of intra-nuclear CD26 and the inhibition of TFIIH by TR1 impairs mRNA synthesis [12,13,16], as indicated in this study. Furthermore, YS110 retards directly cell cycling of cancer cells at both G1/s and G2/M [9,10,23].

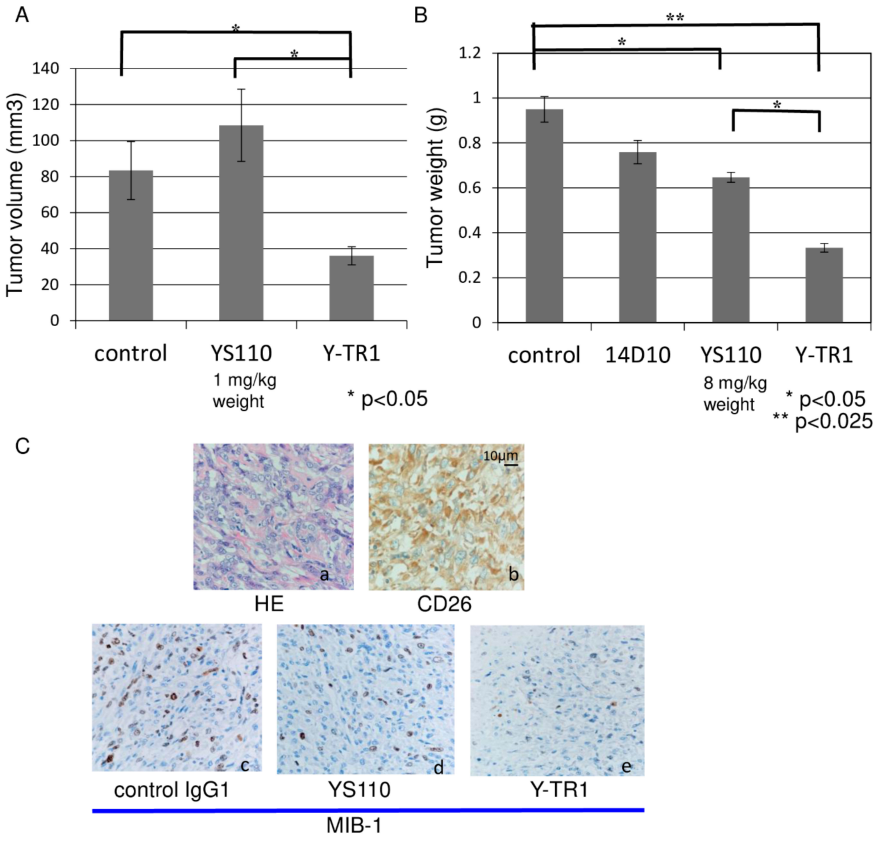

Figure 7. In vivo anti-tumor effect of Y-TR1 in the NOD/SCID mouse xenograft model using the CD26 positive MM cell line JMN. (**A**) Y-TR1 was administered intraperitoneally 4 mg/kg/dose, three times per week, for a total of nine doses from day zero of subcutaneous inoculation of 1 × 107 JMN cells. The average estimated tumor volume on day 55 was compared among three groups (control, YS110, Y-TR1, $n = 10$) with Fisher's protected least protected difference multiple comparison test. The mean tumor volume of the Y-TR1 group was significantly lower (* $p < 0.05$) than that of the control or YS110 group. The mean tumor volume of the YS110 group was not significantly altered compared with the control. An experiment out of two with similar results is shown; (**B**) Y-TR1 was administered intraperitoneally 8 mg/kg/dose, three times per week, for a total of nine doses. The average estimated tumor weight on day 42 was compared among three groups (control, 14D10, YS110, Y-TR1, $n = 10$) with Fisher's protected least protected difference multiple comparison test. The mean tumor weight of the YS110 or Y-TR1 groups was significantly lower (* $p < 0.05$ or ** $p < 0.025$, respectively) than that of the control group. The mean tumor weight of the Y-TR1 group was significantly lower (* $p < 0.05$) than that of the YS110 group. An experiment out of two with similar results is shown; (**C**) histological analysis of xenograft tumors of JMN cells. JMN-derived tumors show histopathology of sarcomatoid mesothelioma. (×20). a: Hematoxylin and eosin staining. b: Immunohistochemical staining with anti-human CD26 antibody revealed CD26 expression in tumor cells. c–e: MIB-1 (Ki67) staining showed a decreased number of MIB-1-positive cells in Y-TR1-treated tumors compared to IgG1- or YS110-treated tumors. Scale bar: 10 μm.

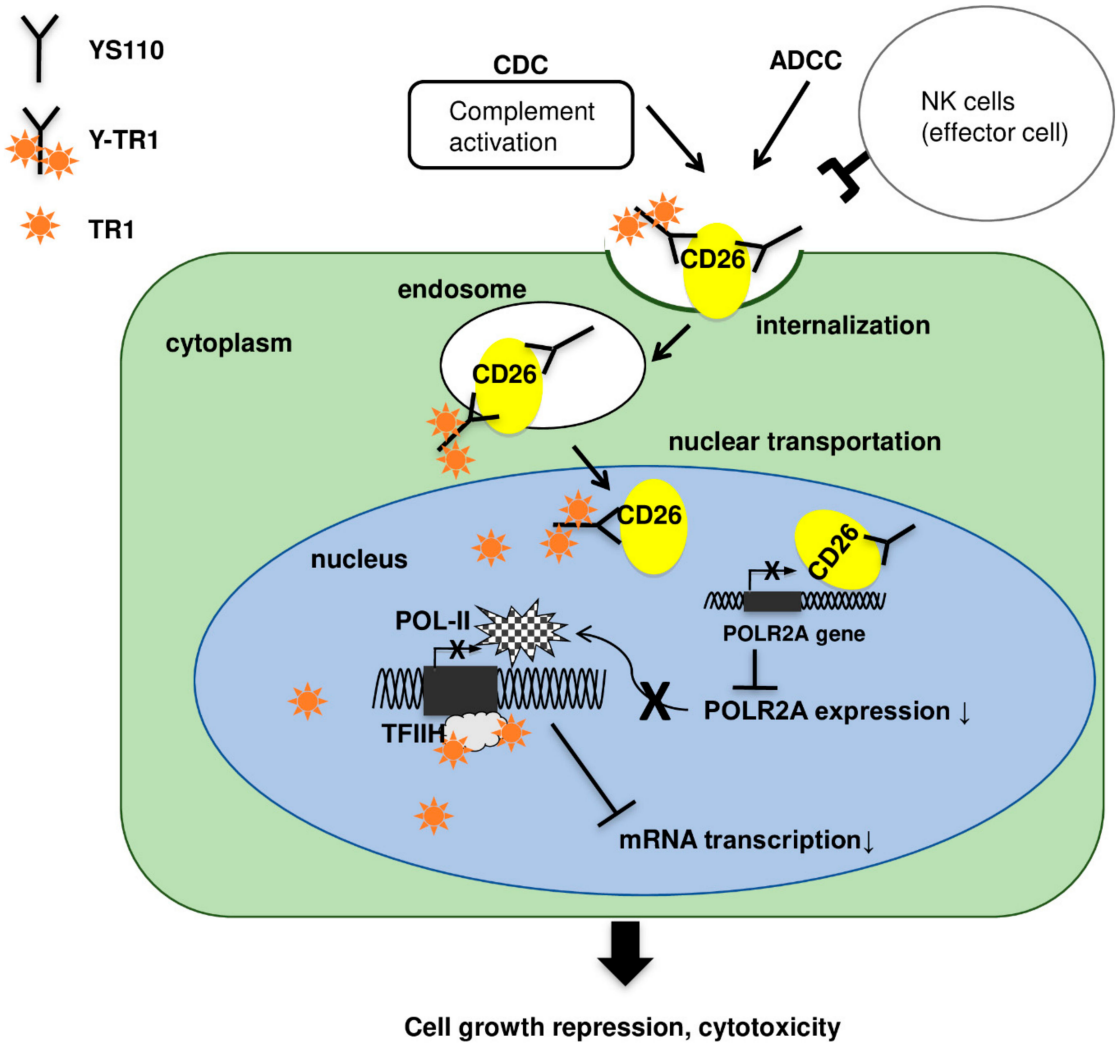

Figure 8. Y-TR1 has multiple anti-tumor effects as follows; (1) introduction of cell death via immunological cytotoxicity such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), (2) retarded cell cycling of both G1/S and G2/M, (3) suppression of POLR2A transcription by increased amount of intranuclear CD26, (4) inhibition of TFIIH by TR1 carried into the nucleus using conjugation of TR1 to YS110.

The LD50 of triptolide was reported as 0.83 mg/kg body weight in mice [38]. The calculated LD50 of TR-1, triptolide-derivative for conjugation to YS110, is 20.8 mg/kg body weight because the TR-1 has reduced anti-tumor activity to 1/25 of triptolide IC50. So, the LD50 of Y-TR1 may be calculated as 932.5 mg/kg body weight in the condition that all TR1 conjugated with YS110 was released from ADC, Y-TR1, because Y-TR1 has 6–7 molecules of TR1 on one molecule of Y-TR1. As a result, the clinical application of Y-TR1 may be expected at 6 mg/kg body weight in accord with the concentration of YS110 in the phase I clinical trial without toxicity of TR1 because the LD50 of Y-TR1 is estranged from the calculated LD50 of TR-1.

In consequence, the present data show certain evidences that induced that the nuclear localization of CD26 by the humanized anti-CD26 mAb YS110 promotes transcriptional repression of the POLR2A gene, and then, the internalization of YS110-TR-1 compound into the nucleus may inhibit TFIIH, resulting in growth suppression of cancer cells. Given that Y-TR-1 has a direct anti-proliferative effect on cancer cells, including malignant mesothelioma cells, these findings highlight the potential of rational therapy against CD26-positive cancers, not only through immunological ADCC and complementary activation effects but also by direct inhibition of cancer cell growth.

4. Materials and Methods

4.1. Reagents and Antibodies

The humanized anti-CD26 antibody YS110 was constructed from the anti-CD26 mouse monoclonal antibody 14D10 coding sequence as described previously [4]. Triptolide (Figure 1) was purchased from Shaanxi Taiji Huaqing Technology (Shaanxi, China), and an SH group was introduced by ChemGenesis Inc. (Tokyo, Japan). The triptolide derivative was designated TR1. TR-1 was provided as an S-S dimer for chemical stability (Figure 1).

4.2. Conjugation Protocols

Heterobifunctional linkers, SPDP (N-Succinimidyl 3-(2-pyridyldithio)-propionate) (Cat No. 21857, Thermo Scientific Inc.), GMBS (N-[γ-maleimidobutyryloxy]succinimide ester) (Cat No. 22309, Thermo Scientific Inc.), SMCC (succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) (Cat No. 22360, Thermo Scientific Inc.) were solved in dimethyl sulfoxide (DMSO) just before use. YS110 was modified with heterobifunctional linkers SPDP (15 times molar excess), GMBS (30 times molar excess), or SMCC (20 times molar excess) in PBS-EDTA pH 7.5 at room temperature for 30 min. The unreacted excess linkers were removed by the HiTrap Desalting Column (Cat No. Cat No. 21857, GE Healthcare Inc., Buckinghamshire, UK). The triptolide derivative TR1 S-S dimer was resolved in 100% ethanol and reduced using the immobilized TCEP reducing gel (Cat No. 77712, Thermo Scientific Inc.) for 2 h. The concentration of the SH group of reduced TR1-SH was measured by the DTNB ((5,5-dithio-bis-(2-nitrobenzoic acid)) assay. Linker-modified YS110 and TR-1-SH, at a ratio of 1:5.68, were reacted in PBS-EDTA pH 7.5 at room temperature overnight. Unreacted TR-1-SH was removed by the PD-10 Column (Cat No. 17085101, GE Healthcare Inc.). The product was sterilized by filtration with the Millex-GV Filter Unit 0.22 μm (Cat No. SLGV 013SL, EMD Millipore, Billerica, MA, USA), and the final concentration was measured using a BCA protein assay reagent kit (Cat No. 23225, Thermo Scientific Inc.). The outline figure of the conjugation protocol is indicated in Figure 1. The remaining unconjugated TR1-SH in the product was measured by the DTNB assay.

4.3. Cell Culture

MSTO-211H (MSTO) (American Type Cell Culture Collection, Manassas, VA, USA), a CD26-negative malignant mesothelioma cell line, was transfected with the CD26 gene and designated MSTO-clone12 [13]. Jurkat (American Type Cell Culture Collection), CD26 negative T-cell leukemia cell line, was transfected with the CD26 gene and designated Jurkat CD26(+) [39]. JMN, a CD26-positive cell line established from malignant mesothelioma, was provided by the Clinical Research Center, Institute of Medical Science, University of Tokyo. All the cell lines were grown in the RPMI medium (Cat No. 11875-093, Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies), ABPC (100 μg/mL), Streptomycin (100 μg/mL), 37 °C, 5% CO_2. dHMVEC (American Type Cell Culture Collection), primary dermal human microvascular endothelial cells were grown in the EGM-2MV Bullet Kit medium (Lonza, Basel, Switzerland) at 37 °C, 5% CO_2.

4.4. Mass Spectrometry Assay

The drug-antibody ratio of Y-TR1 was analyzed by the Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF mass) using Autoflex III (Bruker Corporation, Billerica, MA, USA) after ultrafiltration.

4.5. Binding Assay

To assess the binding of Y-TR1 to the CD26-positive MM cells, cultured MSTO-wt (CD26 negative) and MSTO-clone12 (CD26 positive) cells were collected, and 1 × 106 cells were incubated with 1 μg/mL,

10 μg/mL, and 100 μg/mL of Y-TR1 at 4 °C for 30 min. Cells were washed three times and incubated with FITC-conjugated rabbit anti-human IgG (Cat. No. 6140-02, Southern Biotech, Birmingham, AL, USA) at a 1:100 dilution at 4 °C for 30 min. After washing three times, the FACS analysis was carried out on Epics XL-MCL (Beckman Coulter, Brea, CA, USA).

4.6. Cytotoxicity Assay

The cytotoxic effects of triptolide, TR-1, YS110 and Y-TR1 against MM and T-cell leukemia cell lines were measured using the colorimetric cell proliferation kit WST-1 (Cat No. 11644807001, Roche Applied Science, Rotkreuz, Switzerland) based on the colorimetric detection of a formazan salt. In brief, 5 × 103 MSTO-wt, MSTO-clone12, JMN, Jurkat CD26(−), and Jurkat CD26(+) cultured in 96-well plates in the RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), ABPC(100 μg/mL), Streptomycin(100 μg/mL) with Triptolide (ranging from 0 nM to 100 nM), TR-1 (ranging from 0 nM to 1000 nM), YS110 (ranging from 0 μg/mL to 100 μg/mL), or Y-TR1 (ranging from 0 μg/mL to 100 μg/mL) for 48 h at 37 °C, 5% CO_2. To assess the influence of nonspecific binding of Y-TR1 to Fc receptors, the cytotoxicity assay of Y-TR1 against MSTO-clone12 cells carried out as above with 2 μg/mL of the Fc receptor blocking reagent, Human BD Fc Block (Cat.No. 564219, BD Life Sciences, Franklin Lakes, NJ). CD26-positive dHMVECs cultured in the EGM-2MV Bullet Kit medium (Lonza) underwent the same procedure as above. The WST-1 assay was carried out according to the manufacturer's instructions. The background absorbance of each sample at 630 nm was subtracted from the readings at 450 nm. The experiment was performed in triplicate, and the representative experiment is shown.

4.7. Western Blotting

Cultured CD26 positive MM cell line JMN cells were treated for 30 min or 60 min with Y-TR1 (2 μg/mL). Nuclear and cytoplasmic protein fractions were obtained using the NE-PER Nuclear and cytoplasmic extraction reagents (Cat No. 78833, Thermo Fischer Scientific Inc.) following the manufacturer's instructions. For the Western blot analysis, 20 μg of cytoplasmic fraction and 5 μg of nuclear protein were separated on an SDS-polyacrylamide gel and transferred to a PVDF membrane by the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). After blocking for 1 h in the Bullet Blocking One for Western Blotting reagent (Cat No. 13779-01, nacalai tesque, Kyoto, Japan), the membrane was incubated with Rabbit F(ab')2 Anti-Human IgG (H+L)-HRP antibody (Cat No. 6000-05, Southern Biotech) diluted 1:1000 in Can Get Signal Solution 2 (Cat. No. NKB-101T, TOYOBO, Osaka, Japan) for 30 min at room temperature and developed using the ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). The anti-Lamin B1 antibody (Cat. No. sc-6216, Santa Cruz Biotechnology, Dallas, TX, USA) and Anti-Na-K ATPase α1 antibody (Cat. No. sc-21712, Santa Cruz Biotechnology) were used as loading controls for the nuclear and membrane/cytoplasm fraction, respectively. The experiment was performed in triplicate, and the representative experiment is shown.

4.8. Immunofluorescence Staining

CD26 positive MM cell line JMN cells cultured on chamber slides were treated with Y-TR1 (2 μg/mL) for 60 min. PBS (phosphate buffered saline) were added to the control cells. The cells were fixed in 4% paraformaldehyde for 15 min and permeabilized by 0.1% TritonX-100 for 10 min at room temperature. The cells were incubated with Rabbit F(ab')2 anti-human IgG (H+L)-Alexa Fluor 488 (Cat. No. 6000-05, Southern Biotech) diluted 1:100 in Can Get Signal Solution 1 (Cat. No. NKB-101T, TOYOBO) for 60 min at room temperature. Nuclear staining was done with Hoechst 33342 (Cat. No. H3570, Thermo Scientific Inc.) diluted 1:2000 in 1% BSA for 10 min at room temperature. Stained cells were examined by the confocal laser microscopy FV10i (Olympus, Tokyo, Japan).

4.9. Apoptosis Assay

The CD26-positive MM cell line MSTO-clone12 was treated with YS110 (40 μg/mL), triptolide (20 nM), TR-1 (400 nM), and Y-TR1 (40 μg/mL) for 48 h. After 48 h of treatment, the groups of cells underwent apoptosis assays using the Apo-ONE Homogenous Caspase 3/7 assay (Cat. No. G7792, Promega Corporation, Madison, WI, USA). The assays were performed according to the manufacturer's instructions. In short, active caspase 3/7 in the lysed cells catalyzes profluorescent substrate tofluorescent product. The intensity of the fluorescence was measured using a fluorometer (Glomax Multi Detection System, Promega Corporation). The experiment was performed in triplicate, and the representative experiment is shown.

4.10. Heat Induction of HSP70 and Real Time PCR Assay

CD26-positive MM cell lines MSTO-clone12 and JMN were treated with unconjugated YS110 (40 μg/mL) or Y-TR1 (40 μg/mL) for 1 h before heat shock (45 °C, 2 h). After heat shock, the cells were lysed immediately to isolate the total RNA using an RNeasy mini kit (Cat. No. 74104, Qiagen, Hilden, Germany). Total RNA was reverse transcribed using Prime Script RT enzyme (Takara Bio Inc., Shiga, Japan), and cDNA was used for real-time PCR with the following primers. HSP70 (forward): CAC CAC CTA CTC CGA CAA CCA, HSP70 (reverse): GCG CCT AAT CTA CCT CCT CAA TG, (Invitrogen, Carlsbad, CA) beta actin (forward): TGG CAC CCA GCA CAA TGA A, beta actin (reverse): CTA AGT CAT AGT CCG CCT AGA AGC A (Takara Bio inc., Shiga, Japan). Real-time PCR reactions were performed using Thermal Cycler Dice TP800 (Takara Bio Inc.). The experiment was performed in triplicate, and the representative experiment is shown.

4.11. In Vivo Efficacy Assay and Toxicity Study

NOD/SCID (NOD/LtSz-scid) mice were maintained in a specific pathogen-free facility in micro-isolator cages and were provided with sterile food and water ad libitum. The animal protocol was approved by the Keio University Institutional Animal Care and Use Committee (approval number: 9184). A total of 1×107 cultured JMN cells were subcutaneously transplanted into female 6- to 8-week-old NOD/SCID mice. All 30 animals were randomly assigned into four treatment groups, i.e., control, YS110, 14D10 and Y-TR1 groups. From the day of transplantation, the YS110, 14D10 and Y-TR1 groups received 4 or 8 mg/kg/dose of YS110, 14D10 or Y-TR1 intraperitoneally three times a week, for a total of nine doses. The control group received an equivalent volume of human IgG1 (Sigma-Aldrich, Tokyo, Japan). When the tumor became apparently visible, the tumor was excised and measured by caliper and weighed. The estimated tumor volume was calculated by the formula of $\pi/6 \times L \times W \times W$ [18]. One representative experiment out of two with similar results is shown. Tumor tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. For histology, sections were stained with hematoxylin and eosin. For immunohistochemistry, sections were washed with PBS and subjected to antigen retrieval by heating at 100 °C in 0.01 M sodium citrate (pH 6.0) for 10 min and then treated with 3% H_2O_2 before incubation with the following primary antibodies: Goat anti-CD26 pAb (Cat. No. AF1180, R&D Systems, Minneapolis, MN, USA) (1:100) and mouse anti-Ki 67 mAb (MIB-1, NB600-1252, Novus Biologicals, Littleton, CO, USA) (1:100). As the toxicity assay, the mean body weight of the mice of each group at sacrifice was measured and histological observation of hematoxylin and eosin stained samples of organs (the brain, heart, lung, liver, spleen, kidney, pancreas, digestive organs or adrenal glands) was done.

4.12. Statistics

In the analysis of the real-time PCR assay, the *T*-test at the $p = 0.05$ level was carried out using the SPSS software (IBM, Armonk, NY, USA). Statistical significance between the mean tumor volumes or weights of the groups in the in vivo xenograft assay was assessed by Fisher's protected least-square

differences (PLSD) multiple comparison test. Statistical analyses were carried out using the SPSS software (IBM).

4.13. Study Approval

All experiments were approved by the Animal Care and Use Committee of Keio University and were performed in accordance with the institute guidelines (approval number: 9184).

5. Conclusions

We developed an antibody-drug conjugate (ADC, designated Y-TR1) with YS110 and an inhibitor, triptolide, for one of general transcription factors for Pol II, TFIIH, using cross-linking method. Y-TR1 revealed anti-tumor property against CD26 positive cancer cells both in vitro and in vivo. Induction of nuclear localization of CD26 by Y-TR1 promotes transcriptional repression of the POLR2A gene, furthermore the internalization of YS110-TR1 compound into the nucleus may inhibit TFIIH, resulting in impaired cancer cell growth.

6. Patents

Patent No. PCT/JP2016/076542 cancer treatment composition combining the anti-CD26 antibody and other anticancer agent.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/8/1138/s1, Figure S1: Influence of Fc blocking reagent (2 μg/mL) on the cytotoxicity of Y-TR1 against CD26 positive MM cell line MSTO clone12 (A) and CD26 negative counterpart MSTO wt (B) was not observed. Horizontal axis shows concentration of Y-TR1 in μg/mL. Vertical axis shows percent of control of absorbance value in WST-1 assay. Figure S2: (A) The mean body weight (g) of the mice of each group at sacrifice. The error bar indicates one standard deviation. There was no significant difference between each group. (B) Histological images (hematoxylin and eosin staining) of heart, lung, liver, and kidney of Y-TR1 treated mice and control (IgG1) mice. No pathological alterations were observed.

Author Contributions: M.H. designed the research, performed the experiments, analyzed the data and wrote the manuscript; M.H., K.Y., H.N. and T.Y. analyzed the data including in vitro and in vivo experiments; H.M. performed the cell culture and immunohistochemistry; H.Y. designed the chemical structure of the ADC; C.M., and M.S. contributed to the coordination of the research; T.Y. analyzed the data, wrote the manuscript and directed the project.

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Conflicts of Interest: The authors declare no conflict of interest.

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Phase I study of YS110, a recombinant humanized monoclonal antibody to CD26, in Japanese patients with advanced malignant pleural mesothelioma

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ABSTRACT

Objectives: CD26 is a transmembrane glycoprotein with dipeptidyl peptidase IV activity that is overexpressed in malignant pleural mesothelioma (MPM). We performed a phase I study to determine the maximum tolerated dose, pharmacokinetics, and antitumor activity of YS110, a monoclonal antibody to CD26, in Japanese patients with MPM intolerant of or refractory to prior standard therapies.

Material and methods: The study was designed as an open-label, 3 + 3 dose-escalation, phase I trial. Patients were sequentially assigned to three dosing cohorts (2, 4, or 6 mg/kg). Each 6-week treatment cycle consisted of YS110 administration weekly for 5 weeks followed by a 1-week rest period. Treatment was continued until disease progression, death, or intolerable toxicity. Corticosteroid, antihistamine, and acetaminophen administration before each infusion was adopted to limit infusion-related reactions (IRRs).

Results: Nine Japanese patients (seven men and two women, mean age of 62.2 years), three in each dosing cohort, were enrolled in the study. No patient developed a dose-limiting toxicity. Adverse events of grade 3 or 4 developed in seven patients, with the most common such event being a decreased lymphocyte count. Two patients had mild or moderate IRRs. The serum concentration of YS110 increased in a dose-dependent manner. Among seven patients evaluable for tumor response, four showed stable disease and one achieved a partial response.

Conclusions: YS110 showed promising antitumor efficacy and was generally well tolerated in Japanese patients with advanced MPM at doses of up to 6 mg/kg. YS110 will be tested at 6 mg/kg in a subsequent phase II study.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy that arises from the mesothelial lining of the pleura and is generally associated with asbestos exposure. [1] Although the use of asbestos has now been banned in several industrialized countries, the peak incidence of asbestos-related diseases such as MPM will likely occur between 2015 and 2030 [1]. MPM tends to be associated with a poor prognosis [1,2]. A large study of patients with MPM (n > 16,000) in the United States found that overall survival (OS) at 2 years was 26.5% for women and 16.6% for men, with the respective values at 5 years being 9.4% and 4.2%. [2]. Deaths from MPM are also estimated to increase in Japan, with a predicted peak in 2030 [3], consistent with the estimated trend in Europe.

Therapeutic options for MPM include chemotherapy, radiation therapy, surgery, or combinations of these modalities. [4,5] The role of surgery in the management of MPM remains unclear, given that well-conducted trials have been difficult to undertake and there are apparent postoperative complications. Chemotherapy regimens for patients with unresectable tumors usually consist of the combination of pemetrexed

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with a platinum agent (typically, cisplatin), either with or without bevacizumab. [4–6] However, even with multimodal therapy, treatment outcome for patients with MPM is poor, with most individuals dying within 2–3 years of diagnosis [7].

CD26, a 110-kDa type II transmembrane glycoprotein with dipeptidyl peptidase IV (DPPIV) activity, plays an important role in immune regulation. [8] CD26 is co-stimulator and caveolin is its ligand which is expressed on antigen present cell [9]. These are involved in memory T-cell activation and proliferation [9]. CD26 was found to be overexpressed in MPM cells, but not in benign mesothelial tissue [10,11]. CD26 was found in 80% of epithelioid mesothelioma and 78% in epithelioid component of biphasic mesothelioma [10]. While, in sarcomatoid mesothelioma or sarcomatoid component of biphasic mesothelioma, CD26 was not found [10]. CD26 is also expressed in various tumors, and its expression is reported to be a marker of several cancer stem cells including colorectal cancer, chronic myeloid leukemia, gastric adenocarcinoma and MPM [12]. Moreover, preclinical research indicates that blocking CD26 inhibits tumor growth in xenograft models of several human tumor types including non-Hodgkin T cell lymphoma, malignant mesothelioma, and renal cell carcinoma [11,13,14].

YS110 is a recombinant humanized monoclonal antibody that binds with high affinity (dissociation constant, 0.244 nM) to human CD26. Extensive in vitro and in vivo studies have shown that YS110 possesses antitumor activity for malignant mesothelioma cell lines. [11,15] Single or repeated intravenous administration of YS110 has also been found to be safe in nonhuman primates. [16] The first phase I study of YS110 in humans was conducted in France and found that its administration at doses up to 6 mg/kg weekly was generally well tolerated and showed promising efficacy in 33 patients with advanced or refractory CD26-expressing tumors including malignant mesothelioma, renal cell carcinoma, and urothelial carcinoma [16]. The most common adverse events (≥25%) were asthenia, condition aggravated, constipation, dyspnea and hypersensitivity. We have now performed a phase I clinical trial to assess the tolerability, safety, and pharmacokinetics of YS110 in Japanese patients with MPM as well as to determine the recommended dose and preliminary antitumor effects of the antibody. There were no specific regulatory requirements to conduct this phase I trial.

2. Material and methods

2.1. Patients

Patients aged 20 to 74 years with histologically confirmed advanced MPM of any histological subtype were enrolled (ClinicalTrials.gov identifier: NCT03177668). Patients were included if they were intolerant to, or their tumors were refractory to, existing antineoplastic drugs and no standard therapy was suitable. Other key inclusion criteria were the presence of a measurable tumor lesion as defined by modified Response Evaluation Criteria in Solid Tumors (RECIST), an Eastern Cooperative Oncology Group (ECOG) performance status of ≤1, a life expectancy of ≥12 weeks, and generally good organ function. Patients were included only if their most recent major surgery, antitumor drug treatment, or radiation therapy was at least 4 weeks ago. Patients were excluded if they had not recovered from toxicity due to previous chemotherapy, had tumor lesions in the central nervous system, had accompanying interstitial pneumonia or pulmonary edema requiring treatment, or had poorly controlled hypertension.

2.2. Study design

This was an open-label, standard 3 + 3 dose-escalation, phase I part of a phase I-II study. Patients were enrolled into three successive cohorts (dose of 2, 4, or 6 mg/kg) and received a 6-week cycle of YS110 treatment consisting of once-weekly infusions for 5 weeks (days 1, 8, 15, 22, and 29) followed by 1 week of rest. Three patients were enrolled in the first cohort and received YS110 at a dose of 2 mg/kg. Each patient was assessed for dose-limiting toxicity (DLT) up to day 18 relative to the first dose (DLT evaluation period). The principal investigators, medical expert, and sponsor determined whether a DLT had developed with reference to the following criteria: febrile neutropenia of grade ≥3, neutrophil count decline of grade 4, platelet count decline of grade 4 or requiring platelet transfusion, or nonhematologic toxicity of grade ≥3 with the exception either of any such toxicity—such as nausea, vomiting, anorexia, diarrhea, pyrexia, or electrolyte abnormalities—that could be controlled by appropriate treatment or of any infusion-related reaction (IRR) of grade 3 that could be controlled by a reduction in the rate or interruption of the infusion or by appropriate treatment. If none of the patients in the first cohort developed a DLT, three patients were assigned to the next dose cohort (YS110 at 4 mg/kg) and the process repeated. The patients in the last cohort were to receive YS110 at a dose of 6 mg/kg. If at any time a patient developed a DLT during treatment, three additional patients were enrolled in that cohort before moving to the next dose level. If two or more patients developed a DLT at any dose level, treatment was maintained at that dose, and no patients were enrolled in the next higher-dose cohort. Treatment was continued until disease progression, the development of unacceptable toxicity including a DLT, or the occurrence of a protocol deviation, or at the request of the patient. The first dose of cycle 2 and any subsequent cycles was administered immediately after evaluation of the patient on day 43 (± 3 days) of the previous cycle. The maximum tolerated dose for the phase II part of the phase I-II study was considered to be the highest dose at which < 33% of evaluated patients developed a DLT.

To minimize IRRs, we administered prophylactic d-chlorpheniramine maleate, methylprednisolone, dexamethasone, acetaminophen, and ranitidine hydrochloride according to a predefined schedule before infusion of YS110. Methylprednisolone could be omitted prior to doses 2–5 of each cycle at the discretion of the investigator.

The study protocol was approved by the Institutional Review Board of the four participating hospitals, and the study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. All patients provided written informed consent to participation after being given detailed information about the study.

2.3. Safety assessment

The main safety end point was determination of the recommended dose based on the occurrence of DLT. Patients were monitored for adverse events (AEs) throughout the study. Vital signs were monitored and the electrocardiogram recorded regularly during each drug infusion, and blood samples were collected for hematologic and biochemical assessments. Blood samples were also assayed for antibodies to YS110 including neutralizing activities. Investigators evaluated AEs according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE v4.03), and these events were assessed for a causal relation to YS110. Investigators also evaluated whether or not each AE was an IRR.

2.4. Pharmacokinetics

To assess the pharmacokinetic profile of YS110, we collected serial blood samples during cycle 1. The blood samples were collected before administration of YS110, at 10 min before the end of the infusion, and at 2, 6, 12, 24, 36, 48, and 168 h after the end of drug administration on days 1, 15, and 29. Blood samples were also collected at 72, 96, 120, and 144 h after drug administration on day 1 and at 336 h after drug administration on day 29. The serum concentration of YS110 was measured with a validated electrochemiluminescence assay performed on the Meso QuickPlex SQ120 platform (Meso Scale Diagnostics, Rockville, MD). Pharmacokinetic parameters were calculated by noncompartmental analysis with the use of WinNonlin software v7.0 (Certara USA, Princeton, NJ).

2.5. Efficacy assessment

Tumor response was determined by a central assessment committee using the modified RECIST criteria for the evaluation of response in MPM. [17] RECIST version 1.1 was applied if tumor assessment could not be performed according to the modified RECIST criteria. For observation (imaging) of lesions, contrast medium was used unless there was a specific reason (such as hypersensitivity) not to, and the same imaging method (such as computed tomography) was used under the same conditions (including slice thickness and use of contrast medium) as at baseline. Tumor response was defined in terms of the disease control rate, which is the proportion of patients with a complete response, a partial response (PR), or stable disease for ≥24 weeks. Progression-free survival (PFS) and OS were also assessed, with the former being defined as the time from the first day of treatment until confirmed progressive disease or death and the latter as the time from the first day of treatment to death.

2.6. Pharmacodynamics

Blood samples were also collected before, at the end of, and 24 h after YS110 infusion on days 1, 15, and 29 of cycle 1 for measurement of DPPIV activity, soluble CD26 concentration, and absolute values for lymphocyte subsets including T cell subsets ($CD3^+$/CD56–, $CD3^+$/$CD4^+$, $CD3^+$/$CD4^+$/$CD26^+$, $CD3^+$/$CD8^+$/CD56–, and $CD8^+$/$CD26^+$/CD56–) and natural killer cell subsets (CD3–/$CD56^+$, CD3–/$CD16^+$/$CD56^+$, and CD3–/$CD26^+$/$CD56^+$) as previously described. [18]

2.7. Statistical analysis

An interim evaluation of the phase I data, including results for the last patient up to 6 months after the onset of YS110 administration, was performed. The safety analysis set included all patients who received at least one dose of the study drug, and the pharmacokinetic population included all patients of the safety analysis set who had evaluable drug concentration data. Descriptive statistics were applied to analyze the study results. OS and PFS were evaluated with the Kaplan-Meier method, with censoring at data cutoff. A post hoc analysis examined the number and proportion of patients whose best overall response as determined by central evaluation was a complete response, a PR, or stable disease at the time of data cutoff, with exact 95% confidence intervals (CIs) being calculated with the Clopper-Pearson method.

3. Results

3.1. Patient characteristics

The characteristics of the study patients are shown in Table 1. Nine Japanese individuals (seven men and two women, with a mean age ± SD of 62.2 ± 9.72 years), three in each dose cohort, were enrolled in the study. The histological subtype of MPM was epithelioid in seven patients and biphasic in the other two. MPM was stage III in two patients and stage IV in seven. Five patients had metastatic disease at baseline. All patients had previously received chemotherapy, and one each had also undergone radiotherapy or surgery. All patients discontinued the study (Supplementary Fig. 1), with the most common reason for discontinuation being disease progression (n = 7). In the 2 mg/kg cohort, one patient discontinued treatment during cycle 1, one patient completed cycle 1, and one patient entered cycle 2. In the 4 mg/kg cohort, one patient entered cycle 2, one entered cycle 3, and one entered cycle 4. In the 6 mg/kg cohort, one patient discontinued treatment during cycle 1, one entered cycle 2, and one entered cycle 4.

3.2. Safety

During the DLT evaluation period (days 1–18) for each dose, no patient developed febrile neutropenia of grade ≥3, a neutrophil count decline of grade 4, a platelet count decline of grade 4 or requiring platelet transfusion, or any nonhematologic toxicity of grade ≥3 meeting the criteria for a DLT. Given that no DLTs were observed, the maximum tolerated dose was considered to be 6 mg/kg. All nine patients experienced at least one AE (Table 2). Six patients had treatment-related AEs, the most common of which included fatigue, blood creatinine increase, proteinuria, and rash (each observed in two patients) (Supplementary Table 1). AEs of grade 3 developed in seven patients, and an AE of grade 4 (lymphocyte count decrease) occurred in one patient. The AEs of grade 3 comprised four cases of lymphocyte count decrease and one each of hyponatremia, proteinuria, and nephrotic syndrome. All cases of lymphocyte count decrease were considered by investigators to be unrelated to YS110 but rather related to steroid, and all patients recovered. Hyponatremia, proteinuria, and nephrotic syndrome were considered to be possibly related to YS110, but these events could not be followed up until recovery because of the death of the patients due to disease progression.

There were no deaths associated with AEs. One patient who received YS110 at a dose of 2 mg/kg discontinued treatment after being hospitalized with a serious AE (nephrotic syndrome of grade 3). This was the only AE-related treatment discontinuation. Two patients interrupted treatment because of AEs, including chest pain, malaise, pyrexia, decreased appetite, proteinuria, and rash. Three IRRs (one of grade 2 and two of grade 1) developed in two patients. One patient in the 2 mg/kg cohort developed a rash and pyrexia that were classified as IRRs, and one patient in the 6 mg/kg cohort also had an IRR. None of these IRRs was severe.

3.3. Pharmacokinetics

Pharmacokinetic parameters for YS110 in serum determined after its administration at 2, 4, or 6 mg/kg are shown in Table 3. The maximum serum concentration (C_{max}) and area under the concentration-versus-time curve over the dosing interval (AUC_τ) on days 1 and 29 (fifth and final dose of cycle 1) tended to be proportional to dose level. Exposure to YS110 increased with repeat administration at each dose. The C_{max} and AUC_τ after administration of YS110 at 6 mg/kg on day 29 were thus 1.6 and 2.7 times, respectively, as high as those for day 1.

3.4. Immunogenicity

Antibodies with neutralizing activity to YS110 were detected after treatment in two patients. One patient in the 2 mg/kg cohort had developed antibodies to YS110 by day 29 of cycle 1, and neutralizing activity became apparent on day 43 of cycle 1. Another patient, in the 4 mg/kg cohort, had developed antibodies to YS110 by day 43 of cycle 1 and neutralizing activity on day 50 of cycle 1.

3.5. Efficacy

Seven of the nine study patients were evaluable for tumor response. The best overall response was a PR in one patient and stable disease in four patients (Fig. 1). The patient who achieved a PR was a 70-year-old woman with a baseline ECOG performance status of 1. The total size of her target lesion had decreased relative to baseline (evaluated as a PR) after one treatment cycle (Fig. 2). The patient discontinued treatment after cycle 4 because of progressive disease. Post hoc analysis revealed that 55.6% (95% CI, 21.2%–86.3%) of patients had stable disease or a PR after the first cycle of treatment. The median PFS was 3 months (95% CI, 1.4 months to not evaluable), and the PFS rate at 3 months was 45% (Supplementary Table 2). Median OS was 9.5 months (95% CI, 2.2 months to not evaluable), and the OS rate at 3 months was 78%

Table 1
Baseline Demographics and Clinical Characteristics of the Study Patients.

Characteristic	YS110 Dose			Total (n = 9)
	2 mg/kg (n = 3)	4 mg/kg (n = 3)	6 mg/kg (n = 3)	
Age (years), mean ± SD	61.3 ± 11.59	58.0 ± 13.11	67.3 ± 2.08	62.2 ± 9.72
Sex, n				
Male	2	2	3	7
Female	1	1	0	2
Weight (kg), mean ± SD	59.4 ± 11.02	67.6 ± 20.66	68.0 ± 6.73	65.0 ± 12.89
Tumor stage (IMIG TNM), n				
III	0	1	1	2
IV	3	2	2	7
ECOG performance status, n				
0	2	2	1	5
1	1	1	2	4
Tumor histology, n				
Epithelioid	3	3	1	7
Biphasic	0	0	2	2

IMIG, International Mesothelioma Interest Group; TNM, tumor-node-metastasis; ECOG, Eastern Cooperative Oncology Group.

Table 2
Frequency of Adverse Events in Each Dose Cohort.

Adverse event, number of patients	2 mg/kg		4 mg/kg		6 mg/kg	
	Grade 3 or 4	All grades	Grade 3 or 4	All grades	Grade 3 or 4	All grades
Hematologic						
Lymphocyte count decreased	2	2	2	2	1	2
Neutrophil count increased	0	0	0	0	0	1
White blood cell count increased	0	0	0	0	0	1
Nonhematologic						
Palpitations	0	0	0	0	0	1
Nausea	0	1	0	0	0	1
Vomiting	0	0	0	0	0	1
Toothache	0	0	0	1	0	0
Chest pain	0	0	0	0	0	1
Malaise	0	0	0	0	0	1
Fatigue	0	2	0	0	0	0
Pyrexia	0	1	0	0	0	0
Nasopharyngitis	0	0	0	0	0	1
Lung infection	0	0	0	0	0	1
Upper respiratory tract infection	0	0	0	1	0	0
Infusion related reaction	0	0	0	0	0	1
Hyponatremia	0	0	0	0	1	1
Decreased appetite	0	0	0	0	0	1
Dizziness	0	1	0	0	0	0
Insomnia	0	1	0	0	0	0
Urinary retention	0	0	0	0	0	1
Nephrotic syndrome	1	1	0	0	0	0
Dyspnea	0	0	0	0	0	1
Hiccups	0	1	0	1	0	0
Rash	0	1	0	1	0	0
Hypotension	0	0	0	0	0	1
Electrocardiogram QT prolonged	0	1	0	0	0	0
Laboratory abnormalities						
ALT increased	0	1	0	0	0	1
AST increased	0	1	0	0	0	1
Blood creatinine increased	0	1	0	1	0	0
Blood bilirubin increased	0	0	0	1	0	0
γ-Glutamyltransferase increased	0	1	0	0	0	0
Hypoalbuminemia	0	1	0	2	0	0
Hyperglycemia	0	0	0	1	0	0
Hypophosphatemia	0	1	0	0	0	0
Proteinuria	1	1	0	1	0	0
Hematuria	0	1	0	0	0	0

All adverse events were coded according to the Medical Dictionary for Regulatory, Activities (MedDRA) central coding dictionary, version 19.1 or later.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 3
Pharmacokinetic Parameters of YS110 in Cycle 1 for Each Dose Cohort.

Parameter *2 mg/kg*	Day 1 (n = 3)	Day 15 (n = 3)	Day 29 (n = 2)
$t_{½}$ (h)	21.65 ± 7.64	35.22 ± 2.55	32.76 ± 5.23
C_{max} (μg/mL)	38.13 ± 7.14	40.77 ± 3.18	56.95 ± 13.08
$AUC_{τ}$ (h μg mL^{-1})	1793 ± 570	2307 ± 705	3065 ± 1188
CL (mL h^{-1} kg^{-1})	1.19 ± 0.40	0.92 ± 0.25	0.71 ± 0.27
4 mg/kg	(n = 3)	(n = 3)	(n = 3)
$t_{½}$ (h)	55.69 ± 13.48	94.49 ± 42.67	86.72 ± 32.45
C_{max} (μg/mL)	99.47 ± 31.09	129.33 ± 35.73	146.33 ± 36.83
$AUC_{τ}$ (h μg mL^{-1})	6335 ± 1657	10987 ± 3940	13303 ± 5335
CL (mL h^{-1} kg^{-1})	0.57 ± 0.14	0.40 ± 0.15	0.35 ± 0.17
6 mg/kg	(n = 3)	(n = 2)	(n = 2)
$t_{½}$ (h)	68.50 ± 1.79	129.72 ± 28.79	170.25 ± 31.54
C_{max} (μg/mL)	162.67 ± 19.76	241.00 ± 11.31	265.50 ± 48.79
$AUC_{τ}$ (h μg mL^{-1})	10,400 ± 2086	22833 ± 1327	28252 ± 2705
CL (mL h^{-1} kg^{-1})	0.49 ± 0.12	0.26 ± 0.02	0.21 ± 0.02

Data are means ± SD.
$t_{½}$, elimination half-life; C_{max}, maximum serum concentration; $AUC_{τ}$, area under the serum concentration–versus–time curve over the dosing interval; CL, total body clearance.

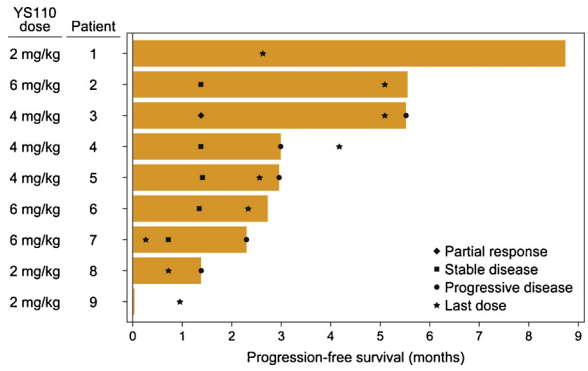

Fig. 1. Swimmer plot of YS110 efficacy at data cutoff. The length of each bar represents time to disease progression or death, whichever came first. Response symbols represent the best response. Patient 9 was censored at the date of first dose administration because of the absence of posttreatment lesion assessment.

(Supplementary Table 2).

3.6. Pharmacodynamics

Soluble CD26 concentration and DPPIV activity were measured in serum of all patients. The mean soluble CD26 concentration decreased from 734.6 μg/L at baseline to 333.4 μg/L after the first dose of YS110 and to 161.4 μg/L after the dose on day 15 and thereafter remained low (range, 122.7–236.3 μg/L) until the end of treatment (Fig. 3A). A similar reduction in DPPIV activity was also apparent (Fig. 3B). The number of $CD3^{+}/CD4^{+}$ T cells decreased from 520.9/μL (42.5% of

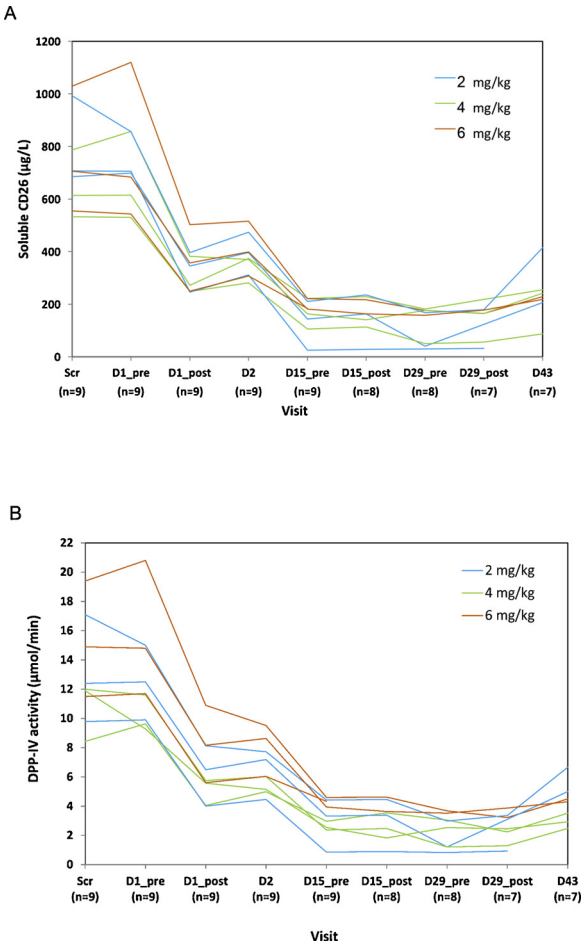

Fig. 3. Levels of soluble CD26 (*A*) and dipeptidyl peptidase IV (DDPIV) activity (*B*) in serum of the study patients at screening (Scr), before (pre) and after (post) YS110 administration on days (D) 1, 15, and 29, as well as on days 2 and 43 of cycle 1.

total lymphocytes) at baseline to 157.7/μL (38.8%) after the first treatment, whereas the number of $CD3^{+}/CD4^{+}/CD26^{+}$ cells decreased from 455.5/μL (37.3%) to 126.3/μL (31.8%). The number of CD3–/$CD56^{+}$ natural killer cells decreased from 243.3/μL (18.0%) to 32.7/μL (7.8%) after the first treatment, and the number of CD3–/$CD26^{+}$/$CD56^{+}$ cells decreased from 18.3/μL (1.9%) to 1.3/μL (0.40%).

4. Discussion

MPM is an aggressive thoracic tumor type with limited treatment options and a poor prognosis. Several novel therapeutic agents for MPM are under investigation, one of which is YS110, a humanized

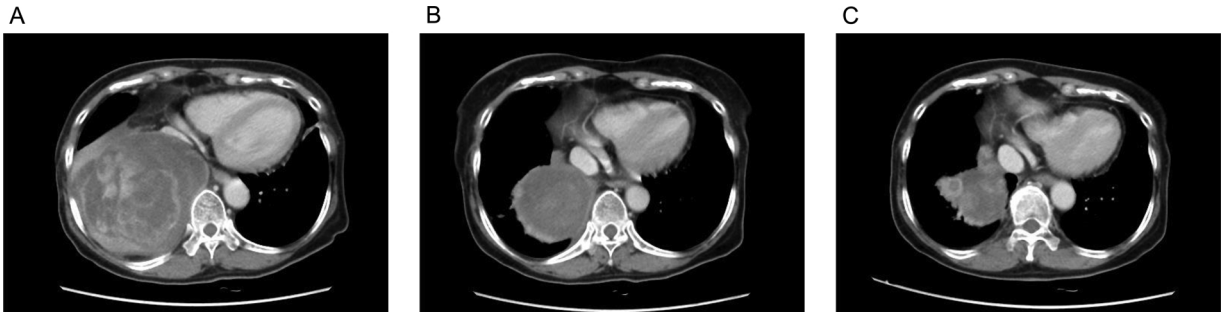

Fig. 2. Computed tomography scans of the lungs of the patient with a partial response performed at screening (*A*), on day 43 of cycle 1 (*B*), and on day 43 of cycle 2 (*C*).

monoclonal antibody that selectively binds with high affinity to the extracellular domain of CD26.

In the present study, YS110 treatment at doses of up to 6 mg/kg did not result in any DLTs in Japanese patients with advanced MPM who were intolerant of or whose tumors were refractory to current anticancer treatments. YS110 was also well tolerated in the first-in-human phase I trial performed with 33 heavily pretreated patients with advanced or refractory CD26-expressing tumors in France. [16] Together, these studies suggest that 6 mg/kg weekly is the recommended dose of YS110, and this dose is now under investigation in the ongoing phase II part of this study. In the French study, seven severe IRRs (six hypersensitivity reactions and one anaphylactic reaction) were observed in six patients (18.2%), with two of these IRRs being designated DLTs. In contrast, no severe IRRs occurred in the present study, and only three mild or moderate such reactions were apparent. This low rate of IRRs and their mild or moderate intensity in our study suggest that the prophylactic treatment to prevent them was efficacious. In the French trial, the first three cohorts of patients received the increasing doses of YS110 administered in the same volume of solution, with the result that patients in the later cohorts received YS110 at a higher concentration. [16] From cohort 4 onward, the volume of the YS110 solution was increased with each increase in dose. On the basis on this experience, the protocol of our trial was modified to include an increase in the volume of infused solution with increasing doses of YS110 for all cohorts, which may also have contributed to the low rate and intensity of IRRs.

Five of the nine patients treated with YS110 in the present study experienced a decrease in lymphocyte count of grade 3 or 4. This reduction in lymphocyte count may have been due to prophylactic corticosteroid administration to limit IRRs. Corticosteroids induce a transient decline in lymphocyte count as a result of the translocation of lymphocytes from blood to tissue. [19] The prompt recovery from the severe reduction in lymphocyte count apparent in the study subjects indicates that YS110 did not destroy lymphocytes. Moreover, a similar trend of a reduction in $CD3^+/CD4^+$ and $CD3^+/CD4^+/CD26^+$ T cell subsets was observed, suggesting that the lymphocyte count decrease was not restricted to cells expressing CD26. The changes in lymphocyte numbers were thus likely not entirely due to YS110 treatment.

The most severe AEs observed in the present study were hyponatremia, nephrotic syndrome, and proteinuria, which were each experienced by one patient and were considered to be related to treatment. One patient discontinued treatment because of serious nephrotic syndrome. The effects of YS110 on the kidneys have not been fully elucidated. [16] DPPIV/CD26 is highly expressed in the proximal tubules of the kidneys [20], and the circulating level of soluble CD26 may be a marker for impaired renal function [21]. We therefore cannot rule out the possibility that YS110 was responsible for the case of nephrotic syndrome. However, this patient had preexisting proteinuria that may have conferred a predisposition to the development of renal toxicity. In addition, no cases of renal toxicity were apparent in the French clinical study or in nonhuman primate toxicity tests [16]. The relation between CD26 inhibition and renal function requires further investigation.

One patient in the present study achieved a PR, which is the first such response reported for YS110, given that no PRs were manifest in the French phase I study. [16] The patient who achieved this response had an epithelioid tumor, and CD26 expression has been shown to be high in epithelioid cells [22]. Although the expression rate of CD26 in tumor tissue had not been determined at the time of data cutoff, it is possible that MPM tumors with an epithelioid histology are more sensitive to CD26 inhibition. We will investigate the relationship between CD26 expression or other biomarkers and clinical outcome of YS110. YS110 has been found to induce MPM cell lysis via antibody-dependent cytotoxicity [12,15,23]. It has also been shown to induce cell cycle arrest in $CD26^+$ malignant mesothelioma cells and to control the growth of MPM cells via up-regulation of the cyclin-dependent kinase inhibitors p21 or p27. [11,15] The antitumor effects of YS110 in vivo are therefore likely mediated by its binding to mesothelioma cells that express CD26.

The serum levels of both soluble CD26 and DPPIV activity decreased after treatment with YS110 according to a similar time course and then remained below baseline values for the duration of the study. Inhibition of DPPIV activity suppresses cleavage of the chemokine CXCL10, which is a ligand for the receptor CXCR3. By reducing the concentration of soluble CD26 and DPPIV activity, YS110 may enhance the migration of CXCR3-expressing effector T cells into the tumor parenchyma. [24,25] Inhibition of DPPIV activity was also recently shown to promote interleukin-33–dependent, eosinophil-mediated control of tumor growth by increasing the concentration of the chemokine CCL11 [23]. Inhibition of DPPIV activity may thus contribute to the antitumor action of YS110.

Our study has some limitations. The study design with the small number of patients in each dose cohort precluded examination of any racial differences in the pharmacokinetics of YS110. In addition, antibodies to YS110 with neutralizing activity were detected in two patients, but the effects of such neutralizing activity on pharmacokinetic parameters were not evaluated given that serial pharmacokinetic data were not obtained after cycle 1. Finally, we did not measure CD26 expression in tumor tissue and so were not able to examine the relation between CD26 expression and tumor response.

In conclusion, YS110 was generally well tolerated at doses up to 6 mg/kg in Japanese patients with advanced MPM. This dose is thus the recommended dose for evaluation in the phase II part of our phase I-II study. YS110 also showed promising antitumor efficacy in patients with MPM.

Author contributions

T.K., I.M., M.Y., Y.K., C.M., and K. Nakagawa contributed to the conception or design of the study. M.T., Y.O., H.H., T.H., J.S., T.S., K. Nosaki, and K. Nakagawa enrolled patients. M.Y. analyzed the study data. All authors contributed to data interpretation and writing of the manuscript and approved the final version of the manuscript.

Conflict of Interest Statement

M.T. received honoraria from Novartis Pharma, ONO Pharmaceutical, and Boehringer Ingelheim. Y.O. reports grants from Kissei during the conduct of the study; grants and personal fees from AstraZeneca, Chugai, Lilly, ONO, BMS, Pfizer, MSD, Kyorin, Takade, Novartis, Taiho, and Abbvie, personal fees from Celltrion, and grants from Amgen, Boehringer Ingelheim, ROXO, and Janssen, outside the submitted work. H.H. reports grants from Kissei Pharmaceutical Co. Ltd during the conduct of the study; grants and personal fees from Eli Lilly, Astra Zeneca, MSD, Ono, BMS, Novartis, Chugai, and Taiho, and grants from Daiichi-Sankyo, Genomic Health, Abbvie, and Meck Serono, outside the submitted work. T.H. reports grants and personal fees from Kissei during the conduct of the study; grants and personal fees from Ono Pharmaceutical Co., Ltd., Bristol-Meyers Squibb, Chugai Pharmaceutical Co., Ltd., AstraZeneca, Nippon Boehringer Ingelheim, Novartis, Eli Lilly, Taiho Pharmaceutical Co., Ltd., Pfizer, Clovis Oncology, MSD, and Ignyta, and grants from Merck Serono, Eisai, Takeda Pharmaceutical Co., Ltd., Dainippon Sumitomo Pharma, Abbvie, Kyowa Hakko Kirin, Daiichi Sankyo, Astellas, Servier, and Janssen Pharmaceutical, outside the submitted work. J.S. has nothing to disclose. T.S. reports grants from Kissei Pharmaceutical during the conduct of the study; grants and personal fees from Astellas Pharma, AstraZeneca, Chugai Pharmaceutical, Eli Lilly Japan, Kissei Pharmaceutical, MSD, Nippon Boehringer Ingelheim, Novartis Pharma, Pfizer Japan, and Takeda Pharmaceutical, personal fees from Bristol-Myers Squibb, Kyowa Hakko Kirin, Nippon Kayaku, Ono Pharmaceutical, Roche Singapore, Taiho Pharmaceutical, Thermo Fisher Scientific, and Yakult Honsha, and grants from Bayer Yakuhin,

Daiichi Sankyo, Eisai, LOXO Oncology, and Merck Serono, outside the submitted work. K. Nosaki has nothing to disclose. T.K. has nothing to disclose. I.M. and M.Y. are employee of Kissei Pharmaceutical. Y.K. is a members of the board, CEO of Y'sAC Co., Ltd.. Y'sAC Co., Ltd. owns the worldwide Patent of YS110, a humanized monoclonal antibody against CD26 molecule expressed on a surface membrane of cancer cells. C.M. is an inventor of the humanized anti-CD26 mAb YS110 (US Patent #7402698). Y's AC Co.,LTD (Tokyo,Japan) own this patent. C. Morimoto is a founding member and shareholder of this company. K. Nakagawa reports grants and personal fees from MSD K.K., Eli Lilly Japan K.K., Bristol Myers Squibb Company, Taiho Pharmaceutical Co.,Ltd., Ono Pharmaceutical Co.,Ltd., Chugai Pharmaceutical Co.,Ltd., AstraZeneca K.K., Astellas Pharma Inc., Novartis Pharma K.K., Nippon Boehringer Ingelheim Co.,Ltd., and Pfizer Japan Inc., grants from Merck Serono Co., Ltd., during the conduct of the study; grants and personal fees from Takeda Pharmaceutical Co.,Ltd., SymBio Pharmaceuticals Limited., and Daiichi Sankyo Co., Ltd., grants from ICON Japan K.K., PAREXEL International Corp., IQVIA Services JAPAN K.K., A2 Healthcare Corp., AbbVie Inc., EP-CRSU CO., LTD., Linical Co.,Ltd., Otsuka Pharmaceutical Co., Ltd., EPS International Co., Ltd., Quintiles Inc., CMIC Shift Zero K.K., Eisai Co., Ltd., Kissei Pharmaceutical Co.,Ltd., Kyowa Hakko Kirin Co.,Ltd, EPS Corporation., Bayer Yakuhin, Ltd, inVentiv Health Japan, GRITSONE ONCOLOGY.INC, GlaxoSmithKline K.K., Yakult Honsha Co., Ltd., and Covance Inc., and personal fees from KYORIN Pharmaceutical Co.,Ltd., CareNet,Inc, Nichi-Iko Pharmaceutical Co., Ltd., Hisamitsu Pharmaceutical Co.,Inc., YODOSHA CO., LTD., Clinical Trial Co., Ltd., MEDICUS SHUPPAN,Publishers Co., Ltd., AYUMI Pharmaceutical Corporation, Nikkei Business Publications, Inc., Thermo Fisher Scientific K.K., NANZANDO Co.,Ltd, Medical Review Co., Ltd., YOMIURI TELECASTING CORPORATION., and Reno. Medical K.K., outside the submitted work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.09.010.

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DPP8 is a novel therapeutic target for multiple myeloma

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Dipeptidyl peptidases (DPPs) are proteolytic enzymes that are ideal therapeutic targets in human diseases. Indeed, DPP4 inhibitors are widely used in clinical practice as anti-diabetic agents. In this paper, we show that DPP4 inhibitors also induced cell death in multiple human myeloma cells. Among five DPP4 inhibitors, only two of them, vildagliptin and saxagliptin, exhibited apparent cytotoxic effects on myeloma cell lines, without any difference in suppression of DPP4 activity. As these two DPP4 inhibitors are known to have off-target effects against DPP8/9, we employed the specific DPP8/9 inhibitor 1G244. 1G244 demonstrated anti-myeloma effects on several cell lines and CD138+ cells from patients as well as in murine xenograft model. Through siRNA silencing approach, we further confirmed that DPP8 but not DPP9 is a key molecule in inducing cell death induced by DPP8/9 inhibition. In fact, the expression of DPP8 in CD38+ cells from myeloma patients was higher than that of healthy volunteers. DPP8/9 inhibition induced apoptosis, as evidenced by activated form of PARP, caspases-3 and was suppressed by the pan-caspase inhibitor Z-VAD-FMK. Taken together, these results indicate that DPP8 is a novel therapeutic target for myeloma treatment.

Dipeptidyl peptidases (DPPs) are members of the serine protease subfamily S9B including DPP4, DPP8, DPP9 and fibroblast activation protein (FAP). DPPs selectively cleave N-terminal dipeptides (Xaa-Pro) from their substrates; therefore, they are considered to be ideal drug targets for the treatment of human diseases[1–6]. Notably, DPP4, also known as CD26, cleaves and inactivates the incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP)[7]. These proteins are secreted from enteroendocrine K and L cells and stimulate pancreatic beta cells so as to secrete insulin. An increased GLP-1 level is one of the major effects of DPP4-inhibitor treatment. DPP4 has thus become a molecular target for the management of diabetes mellitus (type 2)[8]. DPP4 inhibitors are now commonly used in clinical practice as anti-diabetic drugs to obtain satisfactory glycemic control in patients with type 2 diabetes mellitus[9].

Meanwhile, DPP4/CD26 is expressed on various cells and has a multitude of biological functions[10]. Numerous previous reports have demonstrated that CD26 is involved in T-cell function and regulation of the immune system[11–19]. Moreover, DPP4/CD26 is detectable on many types of cancer cells; examples include thyroid carcinoma, gastrointestinal stromal tumor, prostate carcinoma, lung carcinoma, hepatic cancer, colon carcinoma, renal cell cancer (RCC), and malignant pleural mesothelioma (MPM). Hematologic cancers such as T-acute lymphoblastic leukemia, T-anaplastic large cell lymphoma, and T-lymphoblastic lymphoma are also included[20–22].

We previously reported that DPP4/CD26 is not expressed on normal mesothelial cells but on MPM cells[23], therefore, CD26 is a potential therapeutic target in the management of MPM patients[24]. In addition, our *in vivo* experiments confirm the anti-tumor effects of anti-CD26 monoclonal antibody in murine xenograft systems of MPM[25–27] or RCC[28]. Expanding on our preclinical findings, we reported the promising results of the first-in-human phase 1 clinical study of YS110, an anti-CD26 recombinant DNA-derived humanized monoclonal antibody, regarding pharmacokinetics, pharmacodynamics, safety, and preliminary anti-tumor activities in patients with refractory MPM or RCC[29]. Furthermore, we also demonstrated that hematological cancers such as

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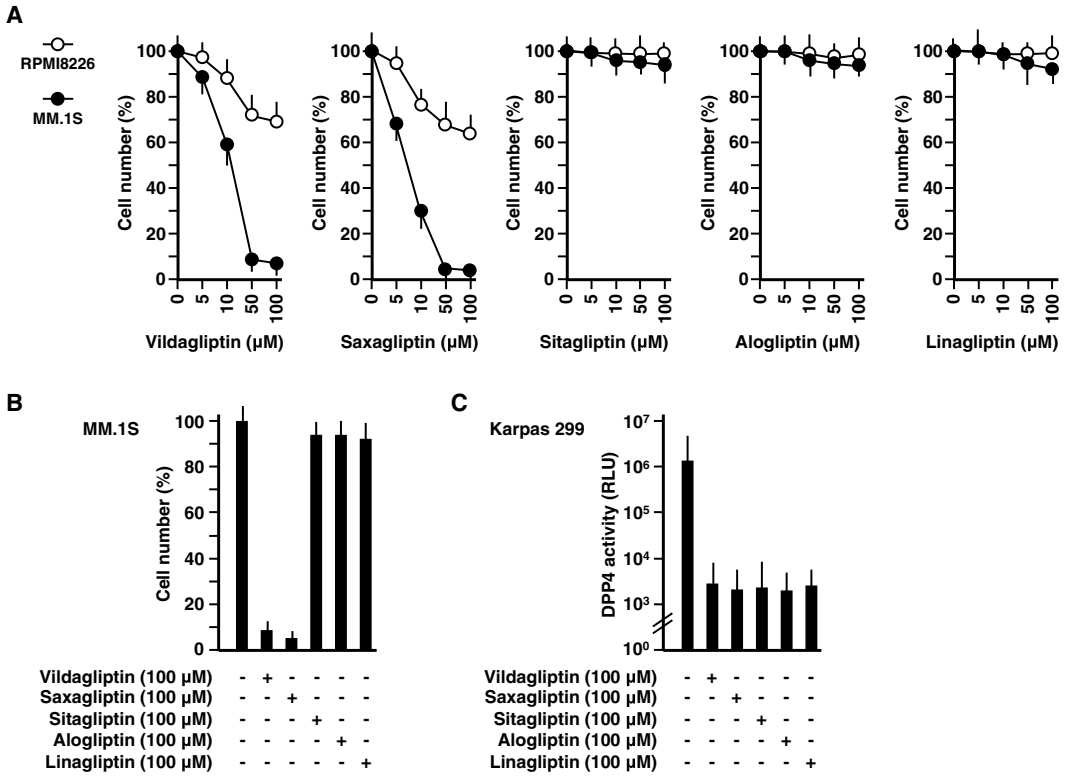

Figure 1. Cytotoxic effects of DPP4 inhibitors against multiple myeloma cell lines. (**A**) 1.0×10^5 MM.1 S (open circles) or RPMI8226 (closed circles) cells were cultured at doses of 0–100 μM DPP4 inhibitors (vildagliptin, saxagliptin, sitagliptin, alogliptin, or linagliptin) for 72 hours. Cell number was estimated by a colorimetric assay using WST-1 reagent (n = 6). (**B**) 1.0×10^5 MM.1 S cells were cultured with 100 μM DPP4 inhibitors (vildagliptin, saxagliptin, sitagliptin, alogliptin, or linagliptin) for 72 hours. Cell number was estimated by a colorimetric assay using WST-1 reagent (n = 6). (**C**) 1.0×10^5 Karpas 299 cells were cultured with 100 μM DPP4 inhibitors (vildagliptin, saxagliptin, sitagliptin, alogliptin, or linagliptin) for 24 hours, respectively. DPP4 activity was estimated using a luminogenic DPP4 substrate, Gly-Pro-aminoluciferin (n = 6). The data are representative of three separate experiments and presented as the mean ± SD.

T-anaplastic large cell lymphoma[30,31] and multiple myeloma[32] are also potential targets of CD26-directed therapies as well as MPM and RCC.

Therefore, herein we initially investigated the therapeutic efficacy of DPP4 inhibitors on multiple myeloma cells, work which subsequently led to the interesting findings indicating that DPP8 is a novel therapeutic target for multiple myeloma.

Results

Cytotoxic effects of DPP4 inhibitors against multiple myeloma cell lines. The cytotoxic effects of DPP4 inhibitors on multiple myeloma cell lines were examined using WST-1 cell proliferation assay system as shown in Fig. 1A. Vildagliptin treatment up to 100 μM led to decreased cell number of MM.1 S or RPMI8226 cells in a concentration-dependent manner till 7 and 70%, respectively. Nevertheless, 100 μM of vildagliptin is not achieved as a plasma concentration by the recommended oral daily dose (i.e. 100 mg) since oral administration of 200 mg of vildagliptin resulted in less than 5.0 μM of plasma concentration as demonstrated previously[33]. Similar cytotoxic effects were observed when cells were treated with saxagliptin; however, both cell lines were unaffected in the presence of sitagliptin, alogliptin, or linagliptin. As only vidagliptin and saxagliptin showed the marked cytotoxicity (Fig. 1B), it was assumed that the cytotoxicity of those two DPP4 inhibitors was due to stronger suppressive effects on DPP4 activity than the other three DPP4 inhibitors. However, surprisingly, the suppressive effects of these five DPP4 inhibitors on DPP4 activity were almost identical (Fig. 1C). In addition, the cytotoxic effects against the T-cell lymphoma cell line Karpas 299 was also observed only with vildagliptin and saxagliptin. (Supplementary Fig. 1A). These results indicated that vildagliptin and saxagliptin exerted their anti-myeloma activity by other mechanisms than DPP4-inhibition.

Anti-myeloma activity of DPP8/9 inhibitor. Based on previous work showing that vildagliptin and saxagliptin were classified into the same category (Class 1) of DPP4 inhibitors[34] and had non-negligible off-target effects on DPP8/9 activity[35], we hypothesized that vildagliptin and saxagliptin-induced inhibitory effects on DPP8/9 were the causal factor for their anti-myeloma activity. To further address this topic, we employed a specific DPP8/9 inhibitor, 1G244[36] to confirm whether DPP8/9 inhibition actually induced cell death in multiple myeloma cells. As shown in Fig. 2A, 1G244 dose-dependently decreased viable cell number of five multiple

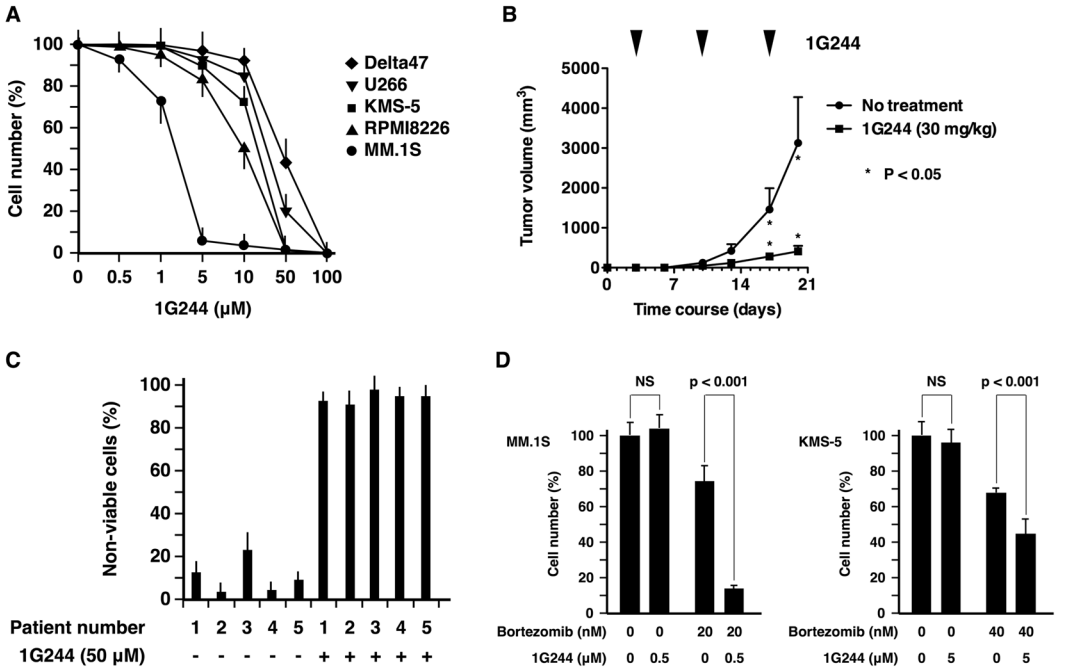

Figure 2. Anti-myeloma activity of DPP8/9 inhibitor. (**A**) 1.0×10^5 Delta47 (rhombuses), U266 (inverted triangles), KMS-5 (squares), RPMI8226 (triangles), or MM.1 S (circles) cells were cultured with 1G244 (0–100 μM) for 72 hours. Cell number was estimated by a colorimetric assay using WST-1 reagent (n = 6). (**B**) 0.5×10^5 MM.1 S cells were subcutaneously inoculated into NOG mice (n = 6). Three days after the inoculation, 1G244 (30 mg/kg) was administered subcutaneously once-a-week. The tumor volume was measured every third or fourth day. (**C**) $0.1–0.5 \times 10^5$ CD138 + myeloma cells from patients were cultured with 1G244 (50 μM) for 24 hr (patient number 2–5) or 48 hr (patient number 1) Non-viable cells were estimated by a flow cytometric analysis using 7-AAD reagent (n = 3). (**D**) 1.0×10^5 MM.1 S (left panel) or KMS-5 (right panel) cells were cultured with bortezomib (20 or 40 nM) with or without 1G244 (0.5 or 5 μM) for 72 hours, respectively. Cell number was estimated by a colorimetric assay using WST-1 reagent (n = 6). The data are presented as the mean ± SD.

myeloma cell lines as well as three T-cell lymphoma cell lines (Supplementary Fig. 1B). Almost complete cell death of all cell lines was observed at a dose of 100 μM. However, since it is known that 100 μM of 1G244 induced nonspecific cell death[37]; we therefore used 1G244 at levels below 50 μM in our subsequent experiments. Since, MM.1 S was the most susceptible among five cell lines, it was inoculated into mice to confirm the anti-myeloma effects of 1G244 *in vivo*. 1G244 was administered subcutaneously into mice at 30 mg/kg once a week, since daily intravenous injection of 1G244 at the same dosage has been linked to severe cyanosis in rats on day 4 or 5[36]. Once-a-week administration of 1G244 apparently suppressed the subcutaneous growth of MM.1 S cells with no other obvious clinical symptoms in mice (Fig. 2B). 1G244 effect on samples from patients with multiple myeloma was also examined. CD138-positive myeloma cells were efficiently isolated from bone marrow cells using magnetic beads (Supplementary Fig. 2). Myeloma cells of all five patients were regarded as non-viable (91–97%) following incubation with 50 μM 1G244 (Fig. 2C). It should be noted that myeloma cells of patient 2 had a deletion of chromosome 17p, being resistant to various chemotherapeutic and biologic agents such as bortezomib, lenalidomide, dexamethasone, cyclophosphamide, and doxorubicin. These results suggested that DPP8/9 inhibition induced cell death in myeloma cells via a distinctive signaling pathway which did not overlap with that of existing chemotherapeutic and biologic agents. Therefore, the effect of the combination of 1G244 and bortezomib was examined (Fig. 2D). 1G244 at a dose of 0.5 μM displayed no cytotoxic effects on MM.1 S cells; however, in combination with bortezomib (20 nM), the number of viable cells decreased significantly compared to the effect of bortezomib (20 nM) alone. Similar effects were observed on another myeloma cell line, KMS-5. These results suggested that DPP8/9 is a realistic potential therapeutic target for the management of multiple myeloma; however, it was reported that the cell death of THP-1 monocytes induced by 1G244 was not dependent on DPP8/9[38]. Therefore, we introduced small interfering RNAs (siRNAs) into our experiments to downregulate specifically the expression of either DPP8 or DPP9.

DPP8 as a target of myeloma therapy. In order to determine whether DPP8 or DPP9 is particularly responsible for the anti-myeloma activity of 1G244, we employed small interfering RNAs (siRNAs) specific to either DPP8 or DPP9, due to unavailability of specific chemical inhibitors to either DPP8 or DPP9 since these two DPPs closely resemble each other in structure[39,40]. As shown in Fig. 3A, treatment with siRNAs specific to DPP8 clearly decreased viable cell number; however, treatment with siRNA specific to DPP9 showed no such change. These results indicated that DDP8 is a novel target for myeloma therapy. Indeed, DPP8 was expressed at a higher

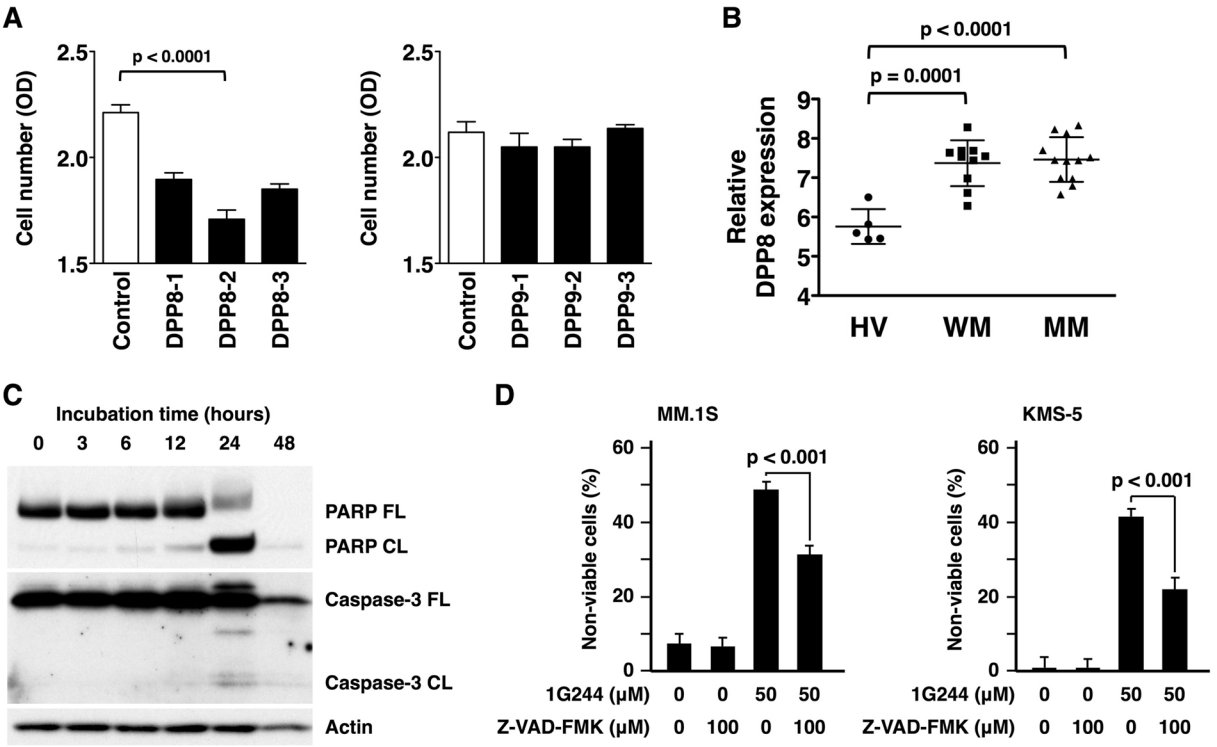

Figure 3. DPP8 as a target of myeloma therapy. (**A**) 1.0×10^5 MM.1 S cells were cultured with 20 nM DPP8 siRNAs (left) or DPP9 siRNAs (right) for 72 hours. Cell number was estimated by a colorimetric assay using WST-1 reagent (n = 6). (**B**) DPP8 gene expression of CD38 + bone marrow cells in healthy volunteers (HV) (n = 5) was compared to those in Waldenstrom's macroglobulinemia patients (WM) (n = 10) or in multiple myeloma (MM) patients (n = 12) based on a dataset record GDS2643. (**C**) 1×10^6 MM.1 S cells were cultured with 1G244 (50 μM) for 0–48 hours. The full length (FL) and cleaved form (CL) of PARP or caspase-3 were detected by Western blot analysis. β-Actin was used as a loading control. Full-length blots are presented in Supplementary Fig. 3. (**D**) 1.0×10^6 MM.1 S (left) or KMS-5 (right) cells were cultured with 1G244 (50 μM) with or without pan-caspase inhibitor, Z-VAD-FMK (100 μM) for 24 hours. Non-viable cells were estimated by a flow cytometric analysis using 7-AAD reagent (n = 6). The data are representative of three separate experiments except (**B**) and presented as the mean ± SD in (**A**,**B**,**D**).

level in CD38+ bone marrow cells of Waldenstrom's macroglobulinemia and multiple myeloma patients than those of healthy volunteers (Fig. 3B). Regarding the potential cellular mechanism involved in 1G244-induced cell death in multiple myeloma cells, apoptosis was the most likely, as cleaved forms of both caspase-3 and PARP were detected (Fig. 3C). Furthermore, 1G244-induced cell death was suppressed by the pan-caspase inhibitor Z-VAD-FMK (Fig. 3D). These findings led to our conclusion that the mechanism for cell death of multiple myeloma cells induced by DPP8-inhibition is apoptosis.

Discussion

Recently, inhibition of DPP8/9 has garnered attention as a new potential therapeutic strategy for acute myeloid leukemia (AML). Johnson DC *et al*. reported that Val-boroPro, a non-selective inhibitor of the post-proline cleaving serine proteases, as well as the specific DPP8/9 inbibitors 1G244 and L-allo-Ile-isoindoline induced cell death in many AML cell lines and primary AML samples[37]. Although it was demonstrated that Val-boroPro had no activity against any of the non-AML cell lines, it did not necessarily contradict our findings that DPP8/9 inhibition induced cell death in multiple myeloma cells. This is due to the fact that the twenty non-AML cell lines which were tested did not include multiple myeloma cell lines. Therefore, our present paper reports for the first time the anti-myeloma activity induced by the DPP8/9 inhibitor.

Meanwhile, Johnson DC *et al*. concluded that pyroptosis, an immunostimulatory form of programmed cell death, was the mechanism responsible for cell death induced by the DPP8/9 inhibition, being dependent on caspase-1 activation downstream of inflammasome formation[37]. In their report, caspase-1 expression was found as a key determinant of cell sensitivity to a DPP8/9 inhibitor and that treatment of sensitive cells with a DPP8/9 inhibitor induced the cleavage of pyroptotic substrate gasdermin D (GSDMD) but not the apoptotic substrate PARP. However, in contrast, our data showed that caspase-3 and PARP cleavage was clearly detected in 1G244-mediated cell death of multiple myeloma cells (Fig. 3C). We thus concluded that apoptotic cell death signaling was induced in multiple myeloma cells by DPP8/9 inhibition. Our conclusion does not completely oppose the possible involvement of pyroptotic cell death because cell death signaling involves a complex process. The inflammasome formation can activate caspase-8, which mediates the activation of downstream caspases such as

caspase-3, caspase-7, and caspase-9[41–43]. Besides, caspase-3 can be activated downstream of caspase-1 through inflammasome responses independent of caspase-8[44]. In the case of 1G244-dependent caspase-3 cleavage, additional detailed work is required to examine the potential involvement of inflammasome responses.

During the investigation of DPP8/9 inhibition-induced pyroptotic cell death signaling of AML cells, caspase recruitment domain-containing protein 8 (CARD8) was identified as an activator of pro-caspase-1[37]. Concerning the mechanism involved in DPP8/9 inhibition-mediated activation of CARD8, DPP9 was regarded as a novel interacting partner with CARD 8 as well as the NLR family member, pyrin domain containing 1 (NLRP1)[45]. NLRP1 is the human homolog of the mouse Nlrp1b, which is activated in murine macrophage cells treated with the DPP8/9 inhibitors[46]. DPP9 functions as an endogenous inhibitor of NLRP1; therefore, DPP8/9 inhibition activates NLRP1, leading to pyroptotic cell death. Interestingly, DPP9 contributes to the inhibition of NLRP1 not only by its catalytic function but also by physical interaction with NLRP1. These findings are based on the data showing that the catalytically inactive DPP9 point mutant S759A, which does not compromise the ability of DPP9 to bind NLRP1, led to significant but partial repression of NLRP1-dependent pyroptosis reaction, and DPP8/9 inhibitors caused the dissociation of DPP9 from NLRP1. While the inhibitory mechanism of DPP9 on NLRP1-dependent pyroptosis by its physical interaction with NLRP1 needs to be investigated further, it is important to note that some other biological function of DPP9 other than its catalytic activity may be revealed in the future.

While either CARD8 or NLRP1 may be involved in DPP9-dependent pyroptosis, a mechanism involved in DPP8-induced apoptotic cell death has not yet been elucidated. The catalytic activity of DPP8 is the most probable function that contributes to the induction of apoptotic cell death signaling; however, other possible involvement of its non-catalytic function such as protein interaction may also play a role.

In the present study, we speculate that its inhibitory effect on DPP8/9 is responsible for the anti-myeloma activity of vildagliptin; however, "off-target" effects should be taken into consideration when vildagliptin is used as an anti-cancer drug. Regarding this point, recent investigation demonstrated that vildagliptin reduced lung cancer growth exerted by surfactant-activated macrophages and NK cells via tumor necrosis-related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity[22].

In summary, our present work demonstrated that DPP8 is a novel target for multiple myeloma therapy inducing apoptotic cell death. Further development of specific inhibitors against DPP8 would provide promising therapeutic effects in human multiple myeloma.

Methods

Cell culture. Five human multiple myeloma cell lines, Delta47, U266, KMS-5, RPMI8226, and MM.1 S cells as well as three human T-cell lymphoma cell lines, Karpas 299, H9, and HUT102 cells were supplied by American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Gibco BRL, Tokyo, Japan) with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO).

Reagents. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) was purchased from Medical and Biological Laboratories (Nagoya, Japan). 1G244 was purchased from AK Scientific (Union city, CA). Alogliptin was purchased from ChemScene (Monmouth Junction, NJ). Linagliptin was purchased from BioVision (Milpitas, CA). Sitagliptin and Bortezomib were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Vildagliptin was purchased from LKT Laboratories (St. Paul, MN). Saxagliptin was purchased from Adooq Bioscience (Irvine, CA).

Cellular cytotoxicity. The number of viable cells seeded onto a 96-well culture plate was quantified using Premix WST-1 Cell Proliferation Assay System (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. Briefly, 10 μl of Premix WST-1 per 100 μl of culture medium was added to each well and the cells were incubated under the standard culture condition for 1 hour. WST reduction was determined with an automated ELISA plate reader, ImmunonMini NJ-2300 spectrophotometer (InterMed, Tokyo, Japan), at an optical density (OD) of 450–650 nm, as we described previously[47].

DPP4 activity. The DPP4 activity of the cell culture media was measured using DPPIV-Glo™ Protease Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 5 μl of the cell culture media was added to the mixture of a luminogenic substrate, Gly-Pro-aminoluciferin and a recombinant luciferase. After the release of aminoluciferin, substrate for luciferase by DPP4 cleavage and the following luciferase reaction, luminescence was recorded as relative light units (RLU) on a plate reader, Infinite M1000 Pro (Tecan, Männedorf, Switzerland).

***In vivo* studies.** NOD/Shi-scid IL-2Rγnull (NOG) female mice of age (6–7 weeks) and weight (19–21 g) were obtained from Central Institute for Experimental Animals (CIEA) (Kawasaki, Japan). The mice were kept under specific pathogen-free conditions with a 12 hour day and night cycle with free access to food and water, and received humane care in compliance with Institutional Guidelines. All experiments were approved by the Animal Care and Use Committee of Sapporo Medical University and were performed in accordance with the guidelines and regulations of the Animal Care and Use Committee of Sapporo Medical University. In order to examine the anti-myeloma activity of 1G244, 5×10^6 MM.1 S cells were inoculated subcutaneously on the left side at the back of NOG mice. Three days after the inoculation, 30 mg/kg of 1G244 was administered subcutaneously once-a-week. The growth of tumor was followed every third or fourth day by measurements with a caliper and its volume was calculated according to the following formula: $MD \times TL^2 \times 1/2$, where MD and TL are the maximum diameter and transverse length, respectively. The mice were sacrificed before the volume of the tumor mass reached 3,500 mm^3 for ethical reason, as we described previously[30].

Myeloma cells from patients. Multiple myeloma patients followed up between January 2000 and December 2015 in our hospital were retrospectively screened. Frozen bone marrow cells from five patients were obtained for analysis. Myeloma cells were positively selected using MACSprep™ Multiple Myeloma CD138 MicroBeads, human Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, bone marrow cells were suspended in MACS buffer and incubated with microbeads conjugated to monoclonal anti-human CD138 antibodies. The cells were then loaded onto MACS column. The magnetic labeled CD138+ cells were bound to the column and released from magnetic field using wash buffer. All patients were treated according to institutional review board-approved protocols and gave informed consent in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Sapporo Medical University.

Flow cytometry. The population of non-viable cells was estimated by a flow cytometric analysis using a standard flow cytometric viability probe, 7-Amino-Actinomycin (7-AAD) reagent (BD Biosciences, San Jose, CA) which permeates the membranes of both dead and damaged cells. Briefly, after the incubation with 7-AAD for 15 minutes at room temperature in the dark, cells were analyzed on the BD FACSCanto II (BD Biosciences) with FlowJo software 7.6.1 (Treestar, Ashland, OR).

Gene expression database. A dataset record GDS2643 was found in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) and used to compare DPP8 gene expression of CD38+bone marrow cells in healthy volunteers (HV) to those in Waldenstrom's macroglobulinemia patients (WM) or in multiple myeloma (MM) patients.

Preparation of siRNAs. Stealth siRNAs (Set of 3)™ (Invitrogen, Carlsbad, CA) targeting human DPP8 with the following sequences were used: HSS123433 (DPP8-1) (gga agg auc aua gau guc aua gau a); HSS123434 (DPP8-2) (gga ccu cau uca gac aga auc uau u); HSS123435 (DPP8-3): gcc ggu agu gga auu uau cac gua a. As a negative control, Low GC in Stealth RNAi Negative Control Kit™ (Invitrogen) was used. Also, Stealth siRNAs (Set of 3)™ targeting human DPP9 with the following sequences were used: HSS132085 (DPP9-1) (gac agg cag caa gaa ucc caa gau u); HSS132086 (DPP9-2) (gca agu acu cgg gcc uca uug uca a); HSS132087 (DPP9-3) (ccu gga agc aga ugc ugg auc auu u). As a negative control, Medium GC in Stealth RNAi Negative Control Kit™ was used.

Transfection of siRNAs. Multiple myeloma cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, MM.1 S cells were seeded at 1.0×10^5 cells/100 µL/well onto 96-well plates. The cells were then transfected with 2 pmol siRNA and 0.3 µL Lipofectamine RNAiMAX Reagent diluted with Opti-MEM Medium (Invitrogen) and cultured for 72 hours. This gives a final siRNA concentration of 20 nM.

Western blot analyses. Cells were lysed in a buffer containing 1% sodium dodecyl sulfate (SDS), 20 mM Tris-HCl pH 7.4, 5 µg/ml pepstatin A, 10 µg/ml leupeptin, 5 µg/ml aprotinin and 1 mM phenyl-methylsulfonyl fluoride and then heated for 5 minutes. After passage through a 20-gauge needle ten times and centrifugation at 15,000 rpm at 4 °C for 30 minutes, the aliquot was boiled in a standard reducing sample buffer for 3 minutes and subjected to SDS-polyacrylamide gel electrophoresis. It was followed by transfer to Immobilon-P membrane (Millipore, Bedford, MA) and hybridization with anti-poly (ADP-ribose) polymerase (PARP) antibody (#9542) (Cell Signaling, Danvers, MA), anti-caspsae-3 antibody (#9662) (Cell Signaling), and anti-actin antibody (sc-1615) (Santa Cruz Biotechnology). Proteins detected by these antibodies were visualized with horseradish peroxidase–conjugated anti-rabbit or goat antibody (Santa Cruz Biotechnology) followed by the use of enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden), as we described previously[47].

Statistical analysisn. The statistical significance of difference was evaluated by Student's t-test using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Statistical significance of $p < 0.05$ considered significant.

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Author contributions

T.S. designed and performed the experiments. A.T., K.T., S.I. and Y.K., helped in performing some experiments. K.N., T.K., R.H., N.I., K.O., S.K., K.A., A.W., J.M., M.A., I.Y. and C.M. provided scientific advice. T.S., P.J., M.U.R. and N.H.D. wrote the paper.

Competing interests
The authors declare no competing interests.

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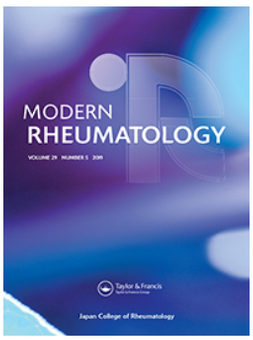

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REVIEW ARTICLE

Rheumatic diseases associated with immune checkpoint inhibitors in cancer immunotherapy

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ABSTRACT

Immune checkpoint inhibitors (ICIs) have drastically altered cancer treatment paradigms, with increasing numbers of novel ICIs being currently evaluated in numerous clinical trials for various cancers. ICIs release 'brakes' against tumor immunity to control cancer growth through T cell-dependent anti-tumor activity. Meanwhile, side effects associated with ICIs are directly related to their mechanism of action, as nonspecific immune activation targeting non-tumor organs results in undesirable off-target inflammation and autoimmunity. Accumulating data reveal that immune-related adverse events (irAEs) of ICIs in cancer patients can resemble various rheumatic diseases. Moreover, while patients with preexisting rheumatic diseases can theoretically experience irAEs and disease flares, observational studies have shown that ICIs can be used successfully in these patients. As ICIs continue to provide long-lasting disease control in cancer patients and their usage correspondingly increases, the rheumatologist will be managing new ICI-associated clinical entities mimicking common autoimmune diseases and will need to be prepared to rapidly diagnose and treat these irAEs. Early recognition and treatment of these rheumatic adverse events will allow for improved outcomes and quality of life for cancer patients faced with previously rapidly fatal disease.

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Introduction

Monoclonal antibodies (mAbs) against coinhibitory immune checkpoint molecules have demonstrated clinical activities in various malignancies [1]. Targets include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152), programmed cell-death protein 1 (PD-1 or CD279) and its ligand (PD-L1; B7-H1 or CD274), which negatively regulate T cell activation and T cell receptor (TCR) signaling, respectively. By disinhibiting these regulatory pathways, immune checkpoint inhibitors (ICIs) overcome self-tolerance and promote T cell-mediated expansion, leading to robust anti-tumor immunity [1]. Originally approved by the US Food and Drug Administration (FDA) for the treatment of advanced melanoma [2], ICIs have led to a paradigm shift in the field of cancer therapy, with the list of indications for ICI use in advanced cancers being now ever-expanding, to include non-small cell lung carcinoma, bladder cancer, head and neck squamous carcinoma, breast cancer, gastric cancer, colorectal carcinoma or solid tumors with high microsatellite instability or mismatch-repair deficiency, hepatocellular carcinoma, Merkel cell carcinoma, urothelial carcinoma, Hodgkin's lymphoma and leukemia [1].

As a consequence of their mechanism of action, ICI therapy can induce nonspecific immune activation, which can target non-tumor tissues. These side effects are collectively referred to as immune-related adverse events (irAEs) [3]. irAEs can resemble various rheumatic diseases, such as inflammatory arthritis (IA) [4], but also exhibit diverse manifestations throughout the body [5,6] (Figure 1). As indications for ICIs use expand and as these novel agents are combined with each other, it becomes increasingly important for rheumatologists to recognize irAEs and appropriate management. In this paper, we summarize the underlying immune mechanisms and the latest findings regarding the rheumatic manifestations and the general approach to management of ICI-associated irAEs in cancer patients treated with these novel agents. Reviewing many recently published work on rheumatic irAEs, this review will provide rheumatologists an updated understanding of these emerging cancer therapies, with particularly a focus on their associated immunopathologic mechanisms and rheumatic complications, and their management.

Normal immune response and immune homeostasis

The classical definition of immunity is protection from infectious pathogens, and the mechanisms of host defense fall into two broad categories, innate immunity and adaptive immunity [7]. During the innate response process, activation of antigen-presenting cells (APCs) leads to enhanced expression of costimulatory molecules. The principal T cell costimulatory molecule CD28 is recognized by the B7

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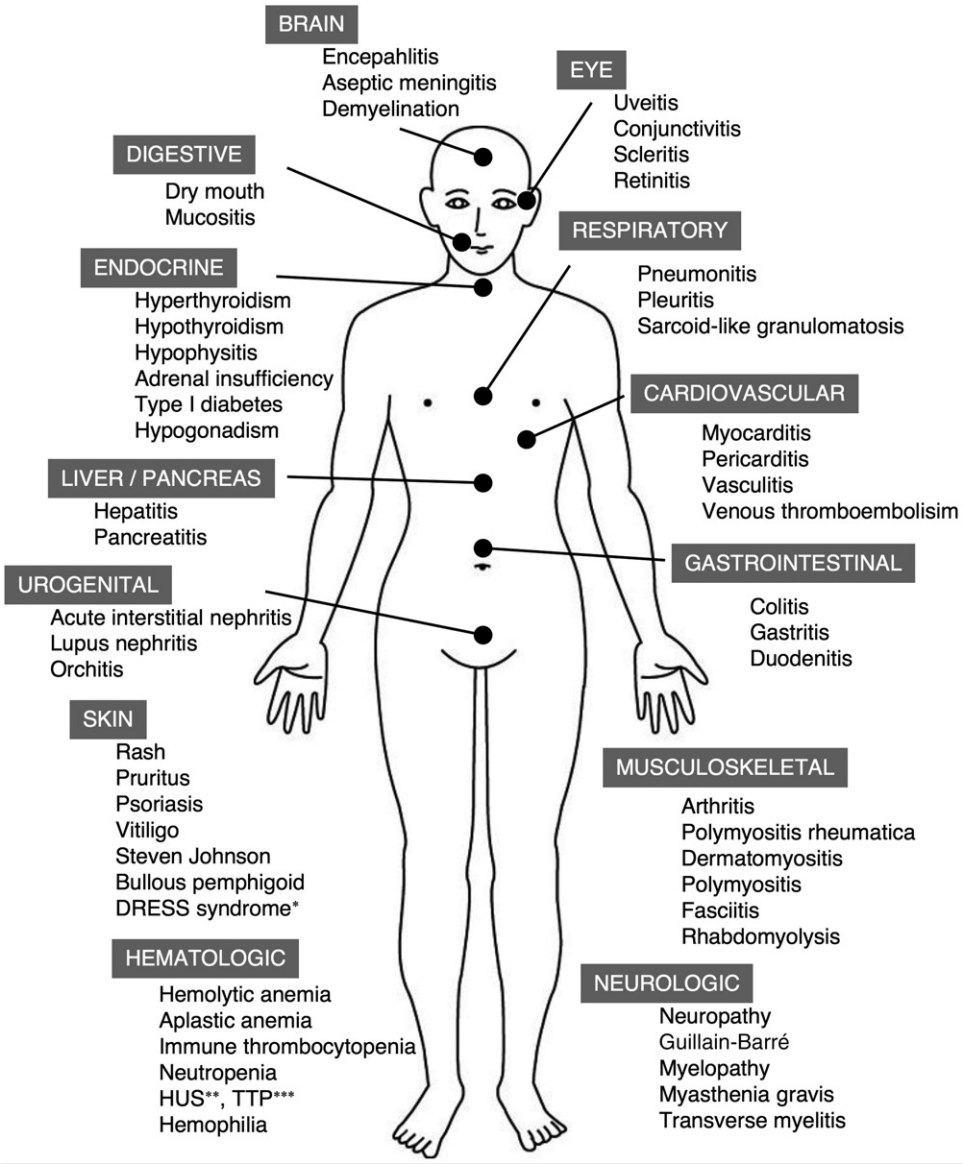

Figure 1. Spectrum of immune-related adverse events induced by immune checkpoint inhibitors. Details are described in the text. *DRESS: drug reaction with eosinophilia and systemic symptoms; **HUS: hemolytic uremic syndrome; ***TTP: thrombotic thrombocytopenic purpura.

molecules CD80 (B7-1) and CD86 (B7-2) that are expressed on APCs [8,9]. Antigen-specific T cells are activated by specific antigens ('signal 1') and the costimulatory molecules ('signal 2') [8,10] (Figure 2(A)). Once activated, proliferated and expanded clonally, antigen-specific T cells exhibit enhanced cell surface expression of immune inhibitory molecules (immune checkpoints) to prevent uncontrolled immune responses and inflammatory tissue damages and to maintain self-tolerance [8,9,11].

CTLA-4 is a transmembrane glycoprotein that is a homolog of the immune costimulatory protein CD28 [9,12] and plays a key role in the development of peripheral tolerance to self-proteins by neutralizing the function of CD28 [9,13]. CTLA-4 is a receptor that inhibits T cell activation by blocking CD28-CD80/CD86 engagement through its approximately 20 times greater affinity to CD80/CD86 on APCs [9,14–16] (Figure 2(B)). In addition, regulatory T cells (Treg), a CD4 subset involved in global regulation of the innate and adaptive immunity, constitutively express CTLA-4, which binds to CD80/CD86 on APCs to reduce their ability to activate T cells through CD28 [17] (Figure 2(C)). The significant role of CTLA-4 in immunity is clearly demonstrated in the CTLA-$4^{-/-}$ mouse model, with the animals being moribund at 3–4-week-old and exhibiting severe pancreatitis, myocarditis and T cell infiltration in the liver, heart, lung and pancreas [18,19].

PD-1 molecules are expressed on the T cell surface within 24 h of activation, and subsequently, disappear once the antigen is eradicated [8]. While CTLA-4 mainly affects naïve T cells, PD-1 is primarily expressed on mature T cells in peripheral tissues and the tumor microenvironment (TME) through downmodulation of TCR signaling [20], hence altering effector T cell survival, proliferation and biological function [21,22] (Figure 2(D)). There are two known ligands to PD-1: PD-L1 and PD-L2 (B7-DC or CD273) [21,22]. PD-L1 is widely expressed on hematopoietic and non-hematopoietic cells, including heart, endothelium, pancreatic islets, small bowel and placenta, while PD-L2 is

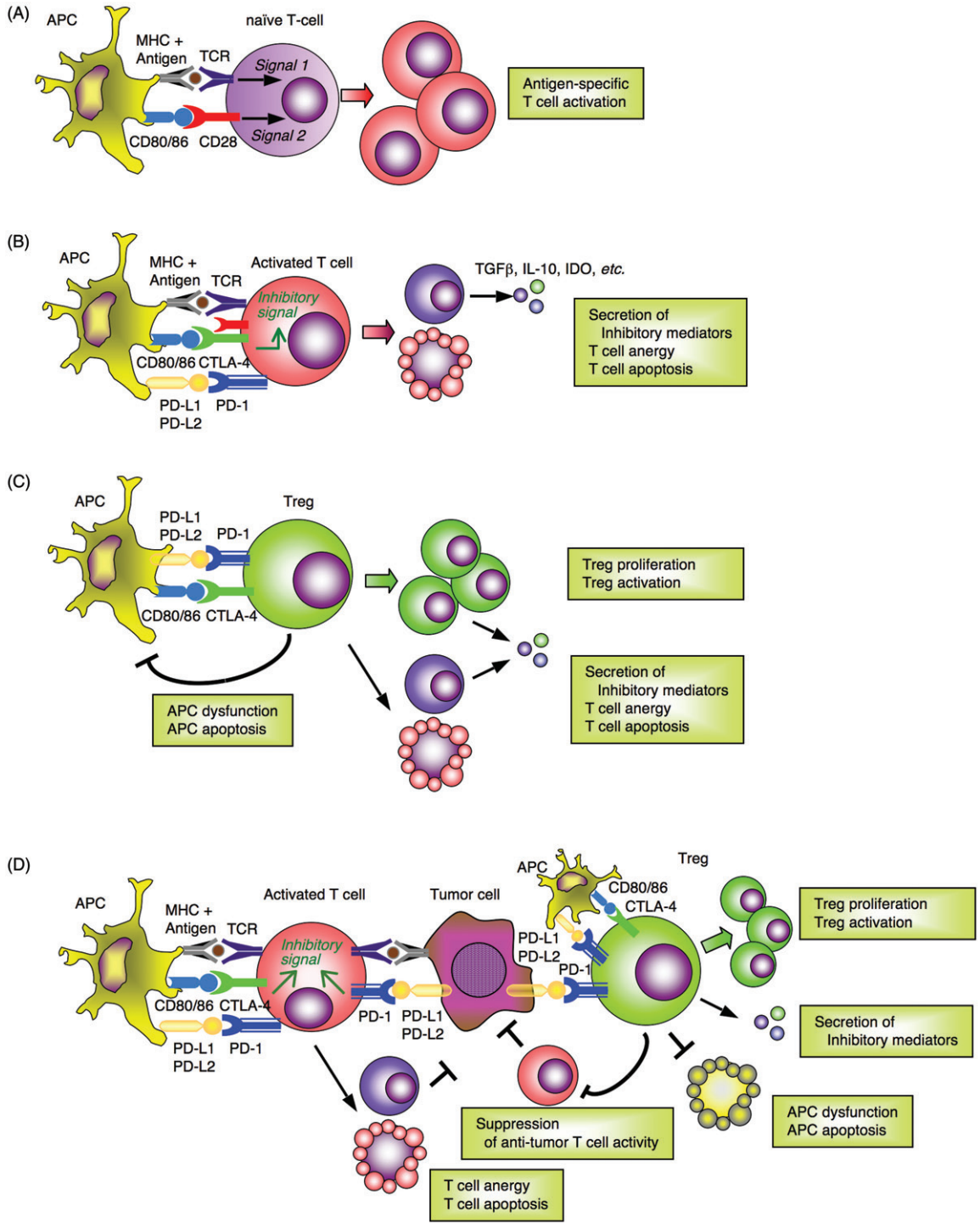

Figure 2. Mechanisms of T cell costimulation, coinhibition, and T cell dependent anti-tumor immunity. (**A**) Adaptive immune response develops in a stepwise fashion, consisting of initial antigen recognition, followed by activation of specific lymphocyte subsets that results in proliferation and differentiation into effector and memory cells, then elimination of the antigen, and decline of the response, with memory cells being the long-lived survivors of the process. In an activation step, T cells are primed by antigen-presenting cells (APCs) with antigen peptides loaded by major histocompatibility complex (MHC) molecule. TCR complex recognizes peptide antigens that are presented by MHC molecules (class I MHC for CD8+ and class II for CD4+ T cell) on the surface of APC, followed by engagement of CD28 on the surface of T cells by CD80 or CD86 expressed on APCs which provides a costimulatory second signal, cooperatively activating antigen-specific T cells. (**B**) After activation, T cells express PD-1 and CTLA-4 coinhibitory molecules that bind to PD-L1/PD-L2 and CD80/86 (with significantly higher affinity than CD28), respectively, leading to suppression of antigen-specific T cell activity through anergy and apoptosis, and secretion of inhibitory mediators including TGF-β, IL-10 and indoleamine 2,3-dioxygenase (IDO). If the antigen is presented to T cells without adequate levels of costimulatory signals, the cells become anergic to the antigen, a process which is mediated by coinhibitory molecules including CTLA-4 and PD-1. (**C**) Regulatory T cells (Treg) also constitutively express CTLA-4 and PD-1 as an inhibitory extrinsic mechanism leading to proliferation and activation of Treg, induction of T cell anergy and apoptosis, and secretion of inhibitory mediators. Meanwhile, a key role of Treg is to prevent immune reactions against self-antigens, a function mediated in part by the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, which inhibit lymphocyte activation and effector function. (**D**) Mechanisms described in panels of B and C on PD-1 and CTLA-4 immune checkpoint molecules lead to tumor escape in the tumor microenvironment. PD-L1 and PD-L2 are expressed on various tumor cells, which could partly explain the ability of tumor cells to evade the process of immune surveillance. Following continuous exposure of effector T cells to antigens, such as in the setting of the tumor microenvironment, T cells lose the ability to respond to the antigen, a process termed T cell exhaustion, with PD-1 signaling playing a critical role. PD-1 is also highly expressed on Treg, and enhances their proliferation and suppressive activity upon ligand binding, likely further helping tumor escape by suppressing effective immune response.

expressed mainly on dendritic cells and macrophages [22]. Induction of PD-L1 expression on tissue cells in the inflammatory regions may be a protective mechanism to downregulate effector T cell activity and reduce immune-mediated injury [23] (Figure 2(B)). PD-$1^{-/-}$ mice demonstrate evidence of autoimmunity, specifically, mild lupus-like autoimmunity and dilated cardiomyopathy [23,24]. The PD-1 knockout autoimmune effects appear to be less severe and display a later onset than those observed in CTLA-$4^{-/-}$ mice [22,25]. As is the case with CTLA-4, PD-1 is also highly expressed on Treg, and enhances their proliferation and suppressive activity upon ligand binding [26] (Figure 2(C)).

An important group of diseases which reflects the failure of the normal control mechanisms described above is autoimmune diseases, which result from the lack of tolerance to self-antigens. The mechanisms of self-tolerance can be broadly classified into two groups: central tolerance and peripheral tolerance [11]. In central tolerance, immature self-reactive T and B lymphocyte clones that recognize self-antigens during their maturation in the central lymphoid organs are eliminated or rendered harmless by negative selection [11]. Autoreactive lymphocytes which manage to escape from the central tolerance mechanisms are subsequently silenced in peripheral tolerance by anergy, Treg and apoptotic deletion [11] (Figure 2(B,C)).

Taken together, immune checkpoints such as CTLA-4 and PD-1 systems are regulatory inhibitory pathways that contribute to immune homeostasis, being essential in preventing autoimmunity, maintaining self-tolerance and avoiding tissue damage that could result from persistent immune activation.

Mechanism of action of immune checkpoint inhibitors

Multiple studies have demonstrated that many tumors use the same pathways involved in immune regulation to evade immune attack [1]. This realization has led to the development of mAbs that block CTLA-4 and PD-1 for tumor immunotherapy, by removing the brakes on the immune response and promoting responses against tumors [1]. The first approved ICI by FDA was ipilimumab, a fully human IgG_1 anti-CTLA-4 mAb, and subsequently, several agents including anti-PD-1 mAb and anti-PD-L1 mAb have been developed for clinical use as shown in Figure 3(A).

Anti-cytotoxic T-lymphocyte-associated antigen 4 inhibitors

Following the discovery of the CTLA-4 receptor in 1986, work involving a murine preclinical model revealed the anti-tumor activity of anti-CTLA-4 Ab [13]. Clinical studies subsequently demonstrated that ipilimumab extended survival time by nearly four months in patients with advanced melanoma [27,28]. Tremelimumab, a fully-human IgG_2 that also targets CTLA-4, is currently under development as monotherapy or combined therapy [29]. Treatment with CTLA-4 mAb results in persistent T cell activation by blocking the inhibitory pathway in the antigen priming phase (Figure 3(A,B)). Moreover, anti-CTLA-4 mAb-mediated inhibition increases the ratio of effector T cells to Treg in the TME, due to depletion of intratumoral Treg through complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) [30] (Figure 3(A)). Of note is that the therapeutic agent for rheumatoid arthritis (RA) abatacept, a fusion protein consisting of the extracellular domain of CTLA-4 and the Fc region of IgG_1, acts in an opposite manner as ICIs, by facilitating coinhibitory signaling of T cells through its binding affinity for CD80/CD86 [31,32].

Anti-programmed cell-death protein-1 inhibitors

Generation of tumor-reactive CD8+ T cells requires the successful processing and presentation of tumor-derived peptide antigens with class I major histocombatibility complex (MHC) molecules by APCs [10,33]. Once developed, tumor-specific CD8+ T cells subsequently differentiate into effector T cells, undergo clonal expansion, migrate to the TME, and ultimately eliminate tumor cells expressing tumor-specific antigens bound to class I MHC molecules through the release of cytotoxic granules [10]. The presence of enhanced PD-1 expression on CD8+ tumor infiltrating lymphocytes (TILs) may either reflect an anergic or exhausted state, consistent with the findings that cytokine production by PD-1+ TILs is decreased [34]. Initial studies showed that PD-1/PD-L1 blockade reversed the exhausted state of effector T cells in the TME, leading to the clinical development of anti-PD-1 inhibitors for cancer immunotherapy [20]. In addition, a large proportion of intratumoral CD4+ T cells are Treg with increased level of PD-1 expression. These findings thus provide an important scientific rationale for a therapeutic approach involving anti-tumor immunity through PD-1/PD-L1 blockade [35]. Currently, pembrolizumab, a humanized IgG_4 mAb, and nivolumab, a fully human IgG_4 mAb, are approved as anti-PD-1 mAbs for clinical use. Treatment with anti-PD-1 mAbs leads to persistent T cell activation by blocking the inhibitory pathway both in the antigen priming phase as well as the effector phase (Figure 3(A,B)).

Anti-programmed cell-death protein-ligand 1 inhibitors

Atezolizumab is a humanized IgG_1 anti-PD-L1 mAb, engineered to delete binding to the Fc receptor [36]. It upregulates T cell activation by blocking the interaction between PD-1 and PD-L1 or CD80 and PD-L1, with a safety profile similar to that of anti-PD-1 mAbs [37]. Other novel anti-PD-L1 mAbs being evaluated currently in various clinical trials are the fully human IgG_1 mAbs durvalumab and avelumab.

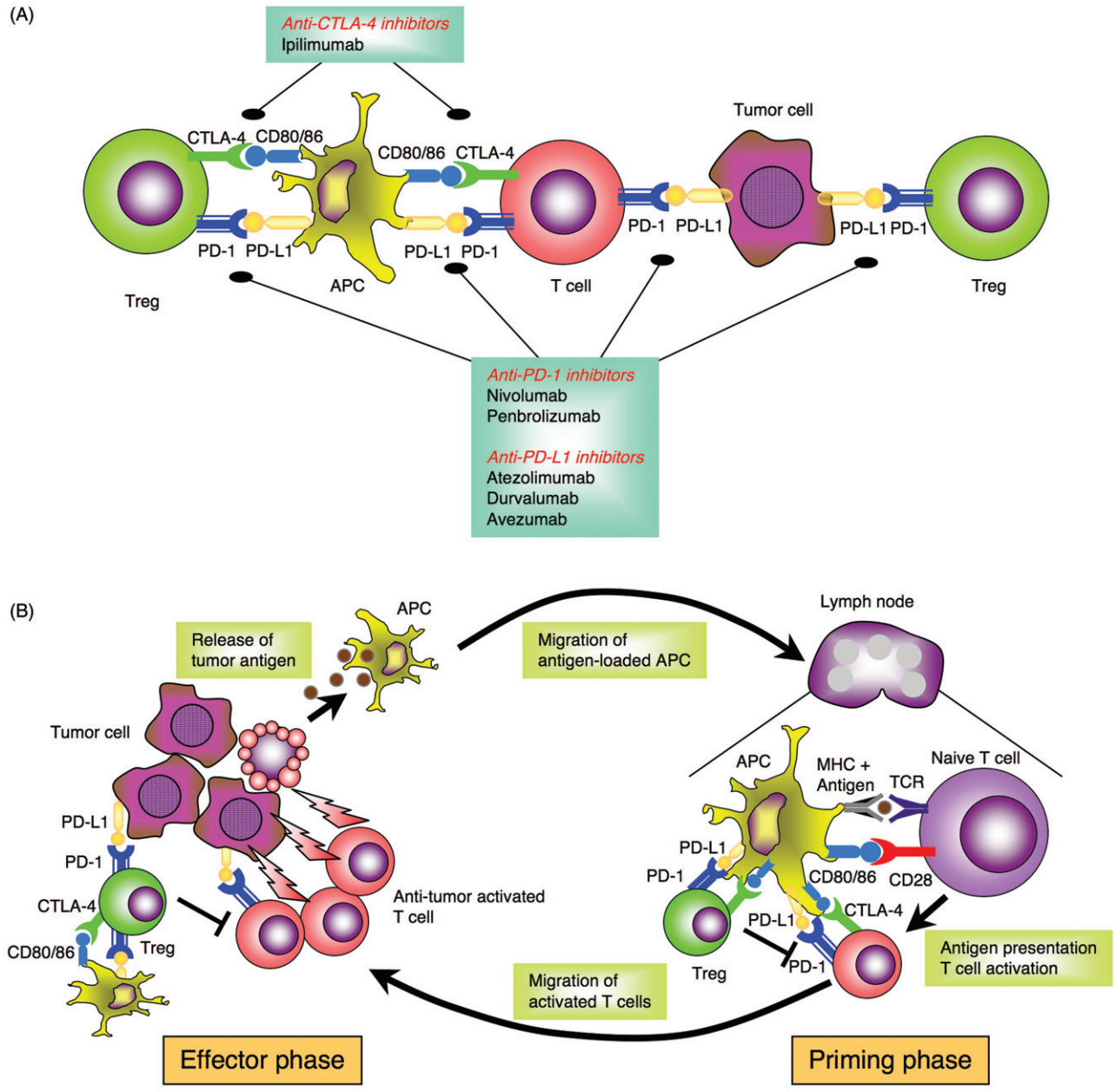

Figure 3. Points of action of anti-PD-1, anti-PD-L1 and anti-CTLA-4 inhibitors. **(A)** Anti-CTLA-4 inhibitor prevents CTLA-4 from binding to CD80/86, reinvigorating the inhibited T cell. Anti-PD-1/PD-L1 inhibitors restore down-modulated TCR signaling and reinvigorate the exhausted T cell. Anti-CTLA-4 and anti-PD-1/PD-L1 inhibitors also deplete regulatory T cells (Treg). **(B)** Cycle of tumor antigen loading to antigen-presenting cells (APCs), migration to lymph node of APC, tumor-specific T cell activation by antigen-loaded APC, accumulation of activated tumor-specific T cells in the tumor microenvironment and targeting of tumor cells. Activation of Treg concomitantly leads to tumor escape. Anti-CTLA-4 inhibitor results in persistent T cell activation by blockade of inhibitory pathway in antigen priming phase. Anti-PD-1/PD-L1 inhibitors result in persistent T cell activation by blockade of inhibitory pathway both in antigen priming phase and in effector phase. They also exert anti-tumor activity through depletion and suppression of Treg.

Combined therapy

The combination of ipilimumab and nivolumab has been approved for the treatment of metastatic melanoma by the US-FDA [38]. Other combined ICI therapy such as tremelimumab and durvalumab is under clinical trials for various cancers. Although these combinations may improve efficacy, they can result in significantly increased toxicity [3,6,39–43].

Immune-related adverse events

As discussed earlier, human immune system normally exists in a state of equilibrium in which lymphocyte activation for protection against pathogens is delicately balanced by the mechanisms of tolerance to prevent deleterious reactions against self-antigens, and the failure of tolerance allows for responses against self-antigens, leading to autoimmune diseases [8,11,44]. Consequently, ICI-mediated blocking of the inhibitory checkpoints can enhance immune activation to result in unwanted off-target effects, including immune-related and inflammatory events [3–6]. Involving any organ system (Figure 1), irAEs from ICIs are increasingly recognized as unique entities mimicking classical rheumatic diseases [4]. The accurate diagnosis and management of these side effects are of the utmost importance, given the fact that the use of ICIs in cancer patients with preexisting autoimmune disease

TABLE 1. Summary of relevant literature regarding arthro-musculoskeletal manifestations of rheumatic irAEs.

	No. of patients (N)	ICI drugs PD/CTLA/Combi[a] (N)	Treatment N/S/M/B[b] (N)	ICI therapy (N)	Outcome of irAEs (N)	Ref.
Inflammatory arthritis	20 (3.8%) 7, RA-pattern 2, PsA-pattern 11, PMR	6/1/0 2/0/0 11/0/0	0/7/1/0 2/0/1/0 2/9/0/0	19, Continued 1, Withheld	20, Remission	[48]
–	30	16/0/14	–/24/3/7	Cessation	3, Remission 18, Persistence	[46]
–	14 (3.5%)	12/1/1	11/14/8/0	3, Continued 3, Withheld 8, Cessation	3, Remission 10, Persistence 1, Unknown	[49]
Myositis	5 (0.8%)	5/0/0	0/5/0/0	Discontinued	3, Remission 2, Fatal	[55]
Myositis-fasciitis	2 (0.9%)	2/0/0	N/A	Discontinued	Remission after ICI cessation	[59]
Non-inflammatory musculoskeletal conditions	15 (2.8%)	14/0/1	2/3/0/0	Continued	Remission	[48]

irAEs: immune related adverse events; ICI: immune checkpoint inhibitor; N/A: not applicable; Ref: reference.
[a]PD, anti-PD-1 or anti-PD-L1 therapy; CLTA, anti-CTLA-4 therapy, Combi, anti-PD plus anti-CTLA-4 therapy.
[b]N, NSAIDS; S, Corticosteroids; M, Methotrexate; B, TNF inhibitor.

is expected to increase in the future as ICI therapy becomes more prevalent in a variety of human neoplasms [3].

Arthritis

While arthralgia and myalgia were by far the most commonly reported rheumatic irAEs in clinical trials [45,46], their exact prevalence may have been underestimated since only high-grade irAEs were noticed in some trials. On the other hand, case series and case reports have provided details on patients with IA including seropositive RA [47]. Large cohort studies on ICIs and rheumatic irAEs have been recently reported (Table 1). A single-center prospective study in France revealed that 35 patients (6.6%) among 524 patients receiving ICIs developed musculoskeletal symptoms [48]. All but two patients had no prior history of autoimmune disease – one with axial spondyloarthritis (AxSpA) and one with psoriasis (PSO). Among 20 patients (3.8%) who developed IA, 11 patients (1.9%) were diagnosed with polymyalgia rheumatica (PMR), exhibiting clinical findings that fulfilled the 2012 EULAR (European League Against Rheumatism)/ACR (American College of Rheumatology) criteria for PMR, and 1 patient was diagnosed with PMR based on the typical clinical presentation and complete disease resolution following treatment with 12.5 mg of prednisone. One patient with preexisting stable condition of AxSpA developed a PMR-like condition 20 days after commencement of ICI therapy. Seven patients (1.3%) developed bilateral and symmetric hand pain and stiffness, mimicking RA. One patient had a positive result for anti-cyclic citrullinated peptide (CCP) antibodies while testing negative for rheumatoid factor (RF). Two patients (0.4%) developed psoriatic arthritis (PsA), including one with pre-existing PSO. All of nine patients with clinical findings mimicking RA or PsA required prednisone treatment, which resulted in clinical improvement or remission. Two patients required methotrexate (MTX) to achieve remission of IA. All patients but one continued on ICI therapy. For the one exception, ICI therapy was temporally withheld as per the requirements of the study protocol in which this patient participated.

More recently, investigators at Johns Hopkins University reported a retrospective longitudinal cohort study on IA patients receiving ICI therapy with no prior history of autoimmune disease [45]. Thirty patients with ICI-induced IA were identified in longitudinal visits to Rheumatology from January 1, 2013 to July 1, 2017 (The incidence of IA in this study was not ascertained since the overall size of the patient population was not stated). Fourteen patients treated with combined CTLA-4/PD-1 therapy were more likely to present with knee arthritis, to have higher levels of C-reactive protein (CRP) and to have negative results for anti-CCP antibodies, RF and anti-nuclear antibodies (ANA). Sixteen patients treated with PD-1 or PD-L1 monotherapy were more likely to have initial small joint involvement and to have IA as their only irAEs. One patient had low levels of anti-CCP antibodies, one had a high titer of RF and one had low titer of ANA. Twenty four among 30 IA patients required systemic steroids for the management of IA. Ten patients had additional immunosuppressant including tumor necrosis factor-inhibitors (TNFi) and/or MTX with clinical improvement of their arthritis. Those receiving combined ICI therapy were more likely to require additional immunosuppressant. Tumor progression while on TNFi and/or MTX was not observed in those with initial tumor response to ICIs. Outcome regarding IA symptoms was evaluated in 21 patients with clinic visits at least 3 months following cessation of their ICI treatment. Eighteen patients still exhibited IA symptoms after ICI discontinuation.

A group from Israel has also reported 14 patients (3.5%) with rheumatic manifestations among 400 patients receiving ICI therapy between January 1, 2013 and April 30, 2017 [49]. Twelve patients were treated with anti-PD-1 mAb, one with anti-CTLA-4 mAb, and one with a combination of anti-PD-1 and anti-CTLA-4 mAbs. IA was identified in 12 patients (3.0%), including 4 patients with predisposing factors such as a personal or family history of PSO, a prior episode of uveitis or anti-CCP antibodies positivity. Other rheumatic diseases such as pulmonary sarcoidosis and biopsy-proven eosinophilic fasciitis were diagnosed in two patients (0.5%). Treatment of IA with non-steroidal anti-

inflammatory drugs (NSAIDs) was mostly unsuccessful while steroid therapy was beneficial in dose ≥20 mg/day. The addition of MTX allowed steroid tapering without an excess of adverse events or tumor progression in the short follow-up time available. There was no patient treated with TNFi in this cohort study. Among 14 patients with rheumatic manifestations, ICI therapy was discontinued in 8 patients, temporarily withheld in 3 patients and continued in 3 patients. Among the 8 patients who stopped ICI treatment, 3 patients experienced remission and had their anti-rheumatic medicine withdrawn, while 5 patients continued on anti-rheumatic medication with low disease activity. In the 6 patients with continued or temporarily withheld ICI therapy, all patients but one continued on anti-rheumatic medication with low or moderate disease activity (one patient with ICI therapy withheld was classified as 'unknown' for anti-rheumatic medication and rheumatic disease status).

Findings from recent large cohort studies indicated that IA appears to be the most common type of rheumatic irAEs, mimicking seronegative RA and PMR [50,51]. Most patients with IA have been reported to be seronegative for anti-CCP antibodies or RF. Meanwhile, in general, imaging studies including magnetic resonance imaging and ultrasonography have shown joint erosion, tenosynovitis, Doppler-positive synovitis and joint effusion [52–54]. It is therefore important for the rheumatologist to recognize IA as an irAEs related to ICI therapy and to understand the diagnosis and management of IA with atypical signs/symptoms of arthralgia and myalgia, given the expected increase use of ICIs in cancer patients in the future.

Inflammatory and non-inflammatory muscle disease

Myositis is less common than IA (Table 1). One retrospective study which included 654 patients receiving anti-PD-1 therapy showed that biopsy-proven myositis was diagnosed in five patients (0.8%) [55]. A severe case of dermatomyositis related to anti-CTLA-4 mAb administration for metastatic melanoma has been reported [56]. The signs/symptoms were initially resolved by treatment with steroids and discontinuation of ICI therapy. The patient was again treated with anti-CTLA-4 mAb on recurrence, followed by prompt flaring of dermatomyositis. Recently, three cases of ICI-related muscle disorder were reported in patients with pulmonary adenocarcinoma by French investigators [57]. These patients had initially moderate bilateral proximal weakness with elevated levels of serum creatine kinase. Two patients subsequently developed myastheniform symptoms while one patient's case was complicated by severe myocarditis. One case of ICI-related myo-fasciitis has also been reported [58]. The muscle symptoms were resolved by treatment with steroids and discontinuation of ICI therapy, while myocarditis was irreversible. A recent retrospective study of 220 patients with anti-PD-1 therapy showed that 2 patients (0.9%) developed symptomatic inflammatory myositis with fasciitis in lower extremities [59]. The French group above also reported that non-inflammatory musculoskeletal conditions developed in 15 patients of 35 rheumatic irAEs among 524 patients receiving ICIs (2.8%) [48]. The symptoms were characterized by arthralgia of proximal or distal joints, which worsened with physical activity and improved with rest, and the absence of joint stiffness. Elevated levels of CRP were observed in 4 patients, likely associated with their malignancies since increased CRP values had been present prior to the development of rheumatic symptoms. The patients were managed successfully with NSAIDs, analgesics and/or physiotherapy, and no modification of ICI therapy was necessary.

Other rheumatic immune-related adverse events

Sicca syndrome including dry mouth with or without dry eyes has been reported in patients receiving ICI therapy [46,54,60]. Johns Hopkins investigators described four patients who developed sicca syndrome associated with ICI therapy [54]. Three patients had positive results for ANA while one patient was positive for anti-La/SSB antibodies with low titer. Dry mouth tended to be more severe than dry eyes. Most patients with ICI-related siccas syndrome have reported not to have concomitant parotitis, in contrast to the typical form of sicca syndrome including Sjögren's syndrome.

irAEs involving blood vessels such as vasculitides are quite rare and appear to be at a reported rate of less than 1% [61]. Recent work elucidated the molecular mechanisms involved in immune checkpoint-mediated medium and large vessel vasculitis such as giant cell arteritis (GCA) [62], which may be the most commonly described vascular IRAE in patients undergoing ICI therapy. Two cases of GCA with PMR following anti-CTLA-4 mAb administration were reported, with high responsiveness to steroids [63]. One case of isolated lymphocytic uterine vasculitis and digital vasculitis was also reported [64]. More recently, a case of small vessel vasculitis during anti-CTLA-4 mAb therapy was reported [65]. After receiving anti-CTLA-4 mAb therapy for melanoma, this patient developed digital vasculitis with negative results for ANA, cytoplasmic and perinuclear anti-neutrophil cytoplasmic antibody (C- and P-ANCA), and cryoglobulin. Despite intensive treatment with high dose steroids, epoprostenol, botulinum toxin and rituximab, the patient had to undergo multiple distal digital amputations.

One patient with melanoma developed nephrotic syndrome after two doses of anti-CTLA-4 mAb [66], with results from a kidney biopsy suggestive of lupus nephritis. Glomerulonephritis resolved following treatment with anti-coagulation and steroids. Circulating anti-double-stranded deoxyribonucleic acid (dsDNA) antibodies appeared concomitantly and subsided following withdrawal of ipilimumab.

Cases of sarcoidosis or sarcoid-like reactions related to ICI therapy have also been reported [60,67,68]. Biopsy is the gold standard for evaluation of new lesions to guide management and to minimize the risk of premature discontinuation of ICI therapy with the potential to provide durable tumor response. Management of patients should be tailored

for each individual situation. In general, asymptomatic patients benefiting from ICI therapy with sufficient tumor response can be continued on therapy with appropriate monitoring, while symptomatic patients may need long courses of steroids or secondary immunosuppressants to control the inflammatory process and avoid organ dysfunction and fibrosis caused by sarcoidosis or sarcoid-like reactions.

Non-rheumatic immune-related adverse events

Skin manifestations are the most common irAEs in all ICIs [6,69], including rash, vitiligo, pruritus and bullous pemphigoid. A recent meta-analysis showed that development of a rash with ipilimumab is fairly common, with mild cases occurring in about 24% of patients and high-grade rashes occurring in 2% [70]. In patients with anti-PD-1, skin toxicities have been reported to occur in 30–40% [71–74]. On the other hand, severe cutaneous irAEs such as toxic epidermal necrolysis rarely developed [6,69].

Enterocolitis as gastrointestinal irAEs are manifested by diarrhea, obstruction, perforation and toxic megacolon [75]. Onset is usually 10–12 weeks following the commencement of treatment [75–77]. Diarrhea occurs in up to 30% of patients receiving anti-CTLA-4 mAb therapy and less frequently in patients undergoing anti-PD-1 therapy [6]. Enterocolitis is most pronounced in patients treated with combination therapy [6,78]. Colonoscopic and histologic findings resemble those observed in inflammatory bowel disease [79].

Several endocrinopathies have been reported in patients receiving ICI therapy, with thyroiditis being the most common, often presenting as hypothyroidism but occasionally as hyperthyroidism, occurring in 6–20% of patients with ICI therapy [80–82]. The pituitary gland can also be affected by ICI therapy, manifesting as hypophysitis, which can occur up to 1–16% of patients [2,39,40,83,84]. Other endocrinopathies include autoimmune diabetes mellitus (DM) or type 1 DM, pancreatitis, hypogonadism and primary adrenal insufficiency [80,81]. Although the acute inflammatory process can be treated, most patients with ICI-induced endocrinopathies develop long-term sequelae and require long-term hormone replacement therapy [81].

Neurologic irAEs are less frequently reported and include paresthesia, altered sensation, aseptic meningitis, encephalopathy, seizures, transverse myelitis, acute and chronic inflammatory demyelinating polyneuropathy, metabolic myopathy, Guillain-Barré syndrome and myasthenia gravis-like syndrome [85].

Pneumonitis is found in less than 5% of patients, ranging from dyspnea to hypoxic respiratory failure [86,87]. The median time to onset is 2.8 months [87]. High dose steroids therapy is required for moderate to severe pneumonitis. ICI-induced pneumonitis is reported with both anti-PD-1 and anti-CTLA-4 therapy and occurs more often with combination therapy [86].

Autoimmune hepatitis is manifested as elevated levels of hepatic enzymes and occurs in up to 5% of patients [2,39,40,72,84,88]. Liver biopsy reveals a pan lobular active hepatitis picture with a predominant CD8-positive inflammatory infiltrate [89]. More rarely, predominant injury to bile ducts can be seen with mild portal mononuclear infiltrate around proliferated bile ductules.

Myocarditis related to ICI therapy has been rarely reported to cause severe irAEs [90]. With the increased application of ICI therapy, incidence of ICI-induced myocarditis is seen to rise over time. A recent report indicated that there were 46 deaths among the 101 patients with severe myocarditis following ICI therapy [91]. Fatality rate was higher with combination therapy than with monotherapy. Myocarditis induced by ICIs tends to occur early after treatment initiation, has a generally fulminant course and responds to higher steroids doses [92].

Other reported ICI-mediated irAEs include uveitis, conjunctivitis, scleritis, retinitis, pericarditis, acute kidney injury, acute interstitial nephritis, rhabdomyolysis, hemolytic anemia, thrombocytopenia, neutropenia and hemophilia [5,60,93–96].

Immune-related adverse events with preexisting rheumatic diseases

While the underlying mechanisms involved in the development of irAEs are not completely understood, the nonspecific upregulation of T cell activation and the suppression of Treg activity resulting from ICI treatment could conceivably exacerbate inflammation and autoimmunity in patients with preexisting autoimmune diseases. It is important to understand whether irAE development in patients with preexisting rheumatic diseases represents flares of their disease or new autoimmune events following ICI therapy. Of note is the fact that patients with preexisting autoimmune or rheumatic disease were typically excluded from the original trials, resulting in a relative paucity of data to fully address this issue. Retrospective analyses have demonstrated that a flare of preexisting autoimmune disease was induced by ICI therapy in 6–43% of patients with preexisting autoimmune disease and that new irAEs developed in 16–33% of the cohorts [97–100]. In general, flares were mild, occurred more often in those with active autoimmune disease, did not lead to discontinuation of ICI therapy, and were readily manageable with standard therapies when intervened in a timely fashion [5]. While preexisting autoimmune diseases should not be an absolute contraindication to ICIs, a careful assessment of disease activity is important prior to starting ICI therapy because of the risk of potential flares.

Management of immune related adverse events in cancer treatment

No definitive prospective trial for the treatment of irAEs has been conducted, and therefore the best approaches and recommendations are based on expert consensus opinion [3]. Several recent publications proposed useful clinical recommendations for the management of irAEs [5,93,101]. The diagnosis of irAEs is primarily clinical, and most patients do

not express the more generic autoantibodies. Many of the initial symptoms, such as arthralgia and fatigue, are relatively nonspecific and can potentially arise from comorbidities or concomitant use of other medications. Approach to the diagnosis and management of irAEs always includes a thorough evaluation for infection. Most patients with irAEs are initially treated with steroids and supportive therapy. The initial steroid dose depends on the relative disease severity, the relative degree of end-organ damages and the presence of potentially life-threatening signs/symptoms [5,93,101].

irAEs are graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) [102], which were developed primarily to standardize reporting of adverse events for clinical trials, although they are included in toxicity management algorithms in recent irAEs guidelines [5,93,101]. As general recommendation guidelines, for grade 1 toxicities, ICI therapy may be continued with close monitoring, with the exception of some neurologic (such as aseptic meningitis, encephalitis and transverse myelitis), hematologic (such as aplastic anemia, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura and hemophilia), and cardiac toxicities (such as myocarditis, pericarditis and arrhythmia). For grade 2 toxicities, ICI therapy should be withheld, and generally lower doses of steroids may be administered. For grade 3 toxicities, ICI therapy should be withheld, and high doses of steroids may be administered with a gradual tapering course with resolution of signs/symptoms. Grade 4 toxicities warrant permanent discontinuation of ICIs, with the possible exception of endocrinopathies controlled by hormone or insulin replacement. Of note is that for the relative rate situations where steroids are not effective, other immunosuppressive agents would need to be used, taken into consideration the patients' overall performance status and end-organ functions. For non-life-threatening rheumatic events such as IA, while there are no clear guidelines, published reports suggest that most patients respond well to moderate doses of steroids [5,93,101]. Occasionally, MTX or TNFi might be necessary to allow for quicker tapering of steroids. Meanwhile, severe colitis will require discontinuation of ICIs and treatment with high dose steroids and possibly other immunosuppressive drugs such as TNFi. Recent large observational studies have demonstrated that treatment with TNFi is not associated with increased risks of tumor development, cancer progression, recurrence or survival when used to treat IA such as RA [103,104]. However, it should be noted that the risk for tumor progression or impaired cancer response is theoretically possible with TNFi [105].

The decision to recommence ICI therapy following resolution of high-grade irAEs represents a challenge for rheumatologists as well as oncologists. The safety of temporarily withholding ICI therapy in patients who developed high-grade irAEs with the combination of ipilimumab/nivolumab has been studied [106]. This retrospective analysis was to evaluate the safety and efficacy of re-challenging 80 patients with anti-PD-1 monotherapy who discontinued anti-CTLA-4/anti-PD-1 combination therapy for metastatic melanoma due to clinically significant irAEs (including colitis, hepatitis and pneumonitis). Fourteen patients (18%) had recurrent irAEs at a median of 14 days following resumption of prior ICI therapy (including 1 patient with grade 5 Steven-Johnson syndrome). Moreover, distinct toxicities occurred in an additional 17 (21%) patients. Of the 14 patients with recurrence of the same irAEs, 7 had grade 3–4 toxicities, and 10 discontinued treatment due to the recurrent irAEs. Colitis was less likely to recur than other irAEs, with only 2 of 33 (6%) patients experiencing recurrent colitis or diarrhea with anti-PD-1 resumption. With the exception of endocrine toxicities which can be treated with hormone replacement therapy, recent guidelines recommend permanent discontinuation of ICIs following a CTCAE grade 4 toxicity [5,93,101]. Due to the potential for morbidity and mortality, permanent discontinuation for grade 1 cardiac toxicities and grade 3 hepatitis, pneumonitis, neurologic, hematologic and ophthalmologic toxicities are recommended [5,93,101]. Prospective studies are needed to determine whether resumption of anti-PD-1 maintenance is beneficial for patients who cease combination ICI therapy due to toxicity.

Conclusions

Despite their proven efficacies in the treatment of various human neoplasms, ICIs can cause severe irAEs that limit their full therapeutic benefits and result in considerable morbidity and mortality. The role of the rheumatologist will be of increasing importance as ICI therapy becomes more established in cancer treatment, given its demonstrated benefits in many cancer patients, including those with advanced diseases refractory to other treatment modalities. As shown in recent large cohort studies, increased awareness of IA, as well as other rheumatic manifestations, as an adverse association with ICI therapy is required to make the correct diagnosis and determine the correct course of action. The CTCAE grading system has recently been noted to be insufficiently suitable for grading the severity of many rheumatic complications, and while rheumatology-specific modifications of the CTCAE have been proposed [107], these changes have not been applied to ICI trials to date. Rheumatic irAEs can be late adverse events occurring up to 2 years following initiation of ICI therapy [60,105], and occasionally even after the patient has stopped the therapy. Until larger, well-powered studies are available to help determine in a more precise way the potential risks of ICI therapy, careful evaluation of the risks and benefits and individual preferences need to be considered when making decisions regarding ICI therapy for patients with cancer and autoimmune disease.

Conflict of interest

None.

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Review Article

Monoclonal Antibody Therapies in Multiple Myeloma: A Challenge to Develop Novel Targets

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The treatment options in multiple myeloma (MM) has changed dramatically over the past decade with the development of novel agents such as proteasome inhibitors (PIs); bortezomib and immunomodulatory drugs (IMiDs); thalidomide, and lenalidomide which revealed high efficacy and improvement of overall survival (OS) in MM patients. However, despite these progresses, most patients relapse and become eventually refractory to these therapies. Thus, the development of novel, targeted immunotherapies has been pursued aggressively. Recently, next-generation PIs; carfilzomib and ixazomib, IMiD; pomalidomide, histone deacetylase inhibitor (HDADi); panobinostat and monoclonal antibodies (MoAbs); and elotuzumab and daratumumab have emerged, and especially, combination of mAbs plus novel agents has led to dramatic improvements in the outcome of MM patients. The field of immune therapies has been accelerating in the treatment of hematological malignancies and has also taken center stage in MM. This review focuses on an overview of current status of novel MoAb therapy including bispecific T-cell engager (BiTE) antibody (BsAb), antibody-drug conjugate (ADC), and chimeric antigen receptor (CAR) T cells, in relapsed or refractory MM (RRMM). Lastly, investigational novel MoAb-based therapy to overcome immunotherapy resistance in MM is shown.

1. Introduction

The treatment options in MM has changed dramatically over the past decade with the emergence of novel agents including proteasome inhibitors (PIs, bortezomib) and immunomodulatory drugs (IMiDs, thalidomide and lenalidomide) and exerts a remarkable impact on the outcome of MM patients [1–3]. However, most patients who achieve a prolonged response following initial therapy may ultimately relapse or become refractory. Thus, the development of novel, targeted immunotherapies has been pursued aggressively. Recently, next-generation PIs (carfilzomib and ixazomib) [4–9], IMiDs (pomalidomide) [10–12], histone deacetylase inhibitor (HDACi, panobinostat) [13–15], and the monoclonal antibodies (MoAbs, elotuzumab and daratumumab) have emerged and further improved the clinical outcome in MM patients who are refractory to prior treatments [12, 16–36]. Importantly, MM remains a chronic disease, so in order to overcome the disease relapse, ongoing challenges to pursue novel therapeutic strategies as well as predictive biomarkers for response or resistance to immunotherapies are required. Furthermore, these novel therapies are expected to be potentially useful in the treatment options for patients who are ineligible for autologous stem cell transplantation (SCT) followed by high-dose chemotherapy [37].

Monoclonal antibody (MoAb) therapies have been accelerating and shown to be able to improve the outcome of cancers [38]. In hematological malignancies, rituximab, a chimeric murine/human anti-CD20 monoclonal $IgG_{1\kappa}$ antibody or of atumumab, a humanized anti-CD20 monoclonal $IgG_{1\kappa}$ antibody, targeting CD20 on B cells, is currently indicated for the treatment of B-cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). It exerts significant activity in combination with cytotoxic anticancer drugs [38, 39].

Although these progresses in immune therapies and their application for the treatment of MM have not succeeded until recently, these therapeutic strategies have finally attained a breakthrough with the development of the MoAb therapies targeting surface molecules, expressed in MM cells, such as elotuzumab, a humanized anti-CS1/SLAMF7 monoclonal antibody, and daratumumab, a humanized anti-CD38 monoclonal antibody, both of which have been approved in the treatment of relapsed or refractory MM (RRMM) patients who received at least three prior therapies including PIs and iMiDs [40–43]. Herein, we review an overview of the current status of MoAb therapies in RRMM. In addition, we introduce investigational novel MoAb therapies in RRMM and show future direction toward immunotherapy resistance in MM.

2. Monoclonal Antibodies (MoAbs) in MM

Potential MoAbs target various kinds of antigens including growth factors, signaling molecules, cell surface proteins, and molecule of adhesion. Ideally, these MoAb-therapeutic targets should be predominantly expressed on a majority of MM cells, but not on normal hematopoietic cells or non-hematopoietic tissues. MoAb therapies involve several mechanisms including direct cytotoxic effects, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cellular cytotoxicity (CDC), and interference with cell-to-cell interactions [40–43]. Other mechanisms include the use of intracellular toxins or radioactive isotopes conjugated to MoAbs after its internalization into tumor cells, which reveal cytotoxicity against tumor cells beyond those bearing MoAb target antigens [40–43].

2.1. CD20 and Rituximab. CD20 is a transmembrane phosphoprotein expressed on committed B lymphoid cells through the all stages of their development, but its expression is reduced in plasma cells. Rituximab, a chimeric murine/human anti-CD20 monoclonal $IgG_{1\kappa}$ antibody targeting CD20 on B cells, is currently indicated for the treatment of B-cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) [39]. It exerts significant activity in combination with cytotoxic anticancer drugs. However, CD20 is present only in a few plasma cells and is absent in most of plasma cells in MM. Therefore, few selected MM patients achieved only minimal responses (MD) [44–46]. Moreover, MM cells express increased levels of complement-inhibitory proteins which result in the reduction of CDC via rituximab against tumor cells.

2.2. CS1/SLAMF7 and Elotuzumab. Elotuzumab is a humanized IgG_1 monoclonal antibody which targets SLAMF7, known as CS1, a glycoprotein, intensely expressed on MM cells and normal plasma cells as well as natural killer (NK) cells. It induces cytotoxicity against MM cells via NK cell-associated ADCC, NK cell activation, and inhibition of the interaction between MM cells and bone marrow stromal cells (BMSCs). Elotuzumab revealed intensive anti-MM efficacy and safety profiles when combined with IMiDs or PIs in previously treated RRMM [12, 16–21] (Table 1). The phase II results demonstrated that elotuzumab in combination with lenalidomide plus dexamethasone (Rd) in patients with RRMM showed safety and efficacy which was better than previously noted with Rd [17, 18]. Moreover, results of the phase III trial ELOQUENT-2 clearly proved the benefit of adding elotuzumab to Rd for the treatment of RRMM [18]. The overall response rates (ORRs) were 79% for the elotuzumab group and 66% for the control group; the PFS rate was 68 vs. 57% for the elotuzumab and control groups at 1 year and 41 vs. 27% at 2 years; the median PFS was 19.4 vs. 14.9 months for the elotuzumab and control groups [19]. Based on the results of these trials, elotuzumab attained food and drug administration (FDA) approval in 2015 in combination with Rd for the treatment of RRMM patients, who previously received two or three prior therapies. A phase III randomized study of Rd with or without elotuzumab in previously treated MM patients is currently ongoing. Phase II trials of elotuzumab plus pomalidomide and dexamethasone (EPd) vs Pd in 117 patients who received >2 prior therapies revealed that after a follow-up period of 9 months, EPd had a longer median PFS (10.3 vs 4.7 month) and a better ORR (53 vs 26%) [12]. Phase II trials of elotuzumab plus bortezomib and dexamethasone (EBd) vs Bd in 77 patients who had received one to three prior therapies showed that EBd had a longer median PFS (9.7 vs 6.9 months). However, there was no deference in ORR between EBd group and Bd group (66% vs 63%) [20, 21].

2.3. CD38 and Daratumumab. Daratumumab is a humanized IgG_1-kappa monoclonal antibody targeting CD38, which is 46-kDa type II transmembrane glycoprotein, broadly expressed on plasma cells as well as lymphoid cells, myeloid cells, and nonhematopoietic tissues. It is also expressed in OCs. CD38 retains multiple functions including ectoenzymatic activity, signal transduction, and receptor-mediated regulation of cell adhesion [22, 23]. In preclinical studies, daratumumab revealed anti-MM cytotoxicity through multiple mechanisms including ADCC, ADCP, CDC, and direct apoptosis via FcR-mediated cross linking of daratumumab *in vitro* [24–26] (Table 2). Of note, no difference was revealed in daratumumab-associated ADCC or CDC between newly diagnosed and RRMM patients. The level of CD38 expression in MM cells was reported to be related to daratumumab-associated ADCC and CDC [24–26]. Moreover, daratumumab has several effects on the immune system. It increases CD8+/CD4+ and CD8+ Treg ratios as well as memory T cells, while decreasing naïve T cells, which enhance the overall immune response to MM cells [27].

Daratumumab revealed anti-MM efficacy as monotherapy as well as in combination with novel agents in heavily pretreated RRMM patients, which resulted in FDA approval in 2015. The GEN501 and SIRIUS trials demonstrated that daratumumab is active as monotherapy in RRMM patients [28, 29]. It showed improved ORRs regardless of refractoriness to prior therapies including PIs and IMiDs (31%). [30]. Phase III Castor trials revealed that

Table 1: Summary of clinical trials in anti-CS1/SLAMF7 antibody in relapsed/refractory MM.

References		Phase	Regimen	ORR (%)	PFS (mo)	OS
Richardson et al. [17]		2	Elo + Rd	84.00%	NA	NA
Lonial et al. [18]	ELOAUENT2	3	Rd ± Elo	79% vs 66%	19.4 mo vs 14.9 mo	NA
Dimopoulos et al. [12]		2	Pd ± Elo	53% vs 26%	10.3 mo vs 4.7 mo	NA
Jakubowiak et al. [20]	Elo-Bd	2	Bd ± Elo	66% vs 63%	9.7 mo vs 6.9 mo	1 yr 85% vs 74%
Zonder et al. [16]	Phase1 Elo	1	Elo Dose Escalation	MTD not identified	NA	NA
Jakubowiak, et al. [21]	Elo-Bd	1	Elo + Bd	48.00%	9.5 mo	NA
Lonial, et al. [19]	Elo-Rd	1	Elo + Rd	82.00%	NA	NA

MM, multiple myeloma; Elo, elotuzumab; Rd, lenalidomide plus dexamethasone; Pd, pomalidomide plus dexamethasone; Bd, bortezomib plus dexamethasone, NA, not available; MTD, maximum tolerated dose.

Table 2: Summary of clinical trials in anti-CD38 antibody in relapse/refractory MM.

References		Phase	Regimen	ORR (%)	PFS (mo)	OS
Lokhorst et al. [28]	GEN501	1/2	Dara monotherapy	36%	5.6 mo	1 yr 77%
Lonial et al. [29]	SIRIUS	2	Dara monotherapy	17%	3.7 mo	1 yr 65%
Spencer et al. [32]	CASTOR	3	Bd ± Dara	83% vs 63%	1.5 yr 48% vs 8%	NA
Palumbo et al. [31]	CASTOR	3	Bd ± Dara	83% vs 63%	1 yr 61% vs 27%	NA
Dimopoulos et al. [33]	POLLUX	3	Rd ± Dara	93% vs 76%	1 yr 83% vs 60%	NA
Dimopoulos et al. [34]	POLLUX		Rd ± Dara	93% vs76%	2 yr 68% vs 41%	NA
Chari et al. [35]	EQULLEUS	1b	Pd ± Dara	60%	1 yr 42%	1 yr 89%

MM, multiple myeloma; Dara; daratumumab, Rd, lenalidomide plus dexamethasone; Bd, bortezomib plus dexamethasone; Pd, pomalidomide plus dexamethasone; NA, not available; MTD, maximum tolerated dose.

daratumumab significantly improved ORR, PFS, and time to progression (TTP) in combination with Bd, ORR (83% vs 63%), the 12-month rate of PFS (61% vs 27%), and TTP at 12 months (65% vs 29%) [31]. Another phase III Castor study also revealed a significant benefit of D-Bd over Bd regardless of treatment history or cytogenetic risk [32]. Phase III POLLUX trials demonstrated remarkable efficacy of daratumumab in combination with lenalidomide plus dexamethasone (DRd) in patients with RRMM [33, 34]. The ORR was 92.9% in DRd group versus 72.9% in Rd group. DRd improved PFS compared with Rd with 12-month PFS rates of 83.2% in DRd group versus 60.1% in Rd group and 24-month PFS rate of 68.0% versus 40.9%, restrictively [33, 34]. The EQUULEUS study led to the FDA approval of daratumumab in combination with Pd in 2017 for RRMM patients who have received 2 or more prior line of therapy including lenalidomide and a PI. The median PFS was 8.8 months, the 12-month PFS rate was 42%, the median OS was 17.5 months, and the median 12-month survival rate was 66% [35].

3. Novel Target Antigens in MoAb Therapies in MM

3.1. CD38 and Isatuximab. Isatuximab is a chimeric IgG_1-kappa anti-CD38 monoclonal antibody which selectively binds to a unique epitope on human CD38 receptor and elicits anti-MM activity by direct apoptosis, ADCC, and ADCP [47]. CDC was triggered in less than half of MM patients with high levels of CD38 in MM cells. A phase 1b open-label, dose escalation study showed that 57 patients who had received at least one prior line of therapy attained ORR of 52% by isatuximab plus Rd in 42 evaluable lenalidomide-refractory patients, and overall median PFS was 8.5 months [48]. Another phase 1b study of isatuximab plus Pd in patients with RRMM who had received more than 2 prior therapies also revealed that ORR was 62%; median duration of response was 18.7 months; and PFS was 17.6 months [49].

3.2. Interleukin-6 (IL6) and Siltuximab. Interleukin-6 is an important cytokine for the growth and survival of MM cells. It is chiefly produced by BMSCs and increased by several cytokines. A chimeric anti-IL-6 antibody, siltuximab, revealed cytotoxicity in MM patients who was refractory to dexamethasone [50]. In addition, it increased cytotoxicity with Bd in combination, whereas in a phase 2 randomized study of siltuximab plus bortezomib, the addition of siltuximab to bortezomib did not appear to improve PFS or OS in refractory MM patients [51]. The other study showed that there were no responses to siltuximab but combination therapy with dexamethasone yielded a partial or minimal response rate of 23%, in dexamethasone-refractory MM [51].

3.3. PD-1/PD-L1 Inhibitors. Programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway is a negative regulator of immune activation [52]. Recently, there are discrepancies concerning programmed death PD-L1 expression on plasma cells in MM. Several data demonstrated that PD-L1 is overexpressed on MM plasma cells but not on normal plasma cells [53–56]. It was reported that PD-L1 expression on plasma cells was associated with increased risk of progression from smoldering MM (SMM) into MM [57], whereas other reports showed that no difference was detected in PD-L1 expression on plasma cells between MM, SMM, monoclonal gammopathy of undetermined significance (MGUS), and healthy individuals

[58, 59]. Similarly, discordant results were reported regarding PD-1 expression on immune cells, including T cells and NK cells in MM. Paiva et al. showed that PD-1 was overexpressed on CD4+ and CD8+ T cells in MM patients [58]. Benson et al. demonstrated that PD-1 expression was increased on NK cells from MM patients, compared with normal NK cells, whereas Paiva et al. demonstrated there was no difference between these cells [58, 60].

Among hematological malignancies, antibody blockade of the PD-1/PD-L1 pathway is a highly effective therapeutic approach for patients with classical Hodgkin lymphoma, 97% of which typically exhibits an overexpression of PD-L1 due to the alteration in chromosome 9p24.1 (54). Therefore, the PD-1/PD-L1 axis is a good target for MoAbs, leading immune cells to kill tumor cells. The use of nivolumab, a human IgG4 MoAb which blocks the interaction with PD-L1 and PD-L2 by binding to the PD-1 receptor on activated immune cells, was approved by FDA in 2016 for the treatment of relapsed or progressed Hodgkin lymphoma [52]. However, the outcome of checkpoint blockade by monotherapy with PD-1/PD-L1 inhibitors is unsatisfactory in MM, compared with solid tumors due to the reduced immune dysfunction in MM [58, 59]. In contrast, lenalidomide enhances the effect of PD-1/PD-L1 blockade on both T cell- and NK cell-mediated cytotoxicity. The combination therapy of lenalidomide plus PD-1/PD-L1 inhibitors increased interferon γ by BM-derived effector cells in MM and was associated with increased apoptosis of MM cells, suggesting synergistic cytotoxic effects [56, 61, 62]. There are only limited data from clinical trials of PD1/PDL1 MoAbs in MM patients. The phase Ib trial of nivolumab monotherapy in 27 RRMM patients showed the stabilization of disease status in 17 patients, lasting a median of 11.4 weeks [63]. A phase I study of pembrolizumab with Rd in RRMM patients revealed a partial response rate of 50% [61, 62, 64, 65]. A phase 3 study of the combination of Rd with or without pembrolizumab was performed in transplant ineligible newly diagnosed MM patients (KEYNOTE-185 trial) [61, 62, 64]. A Phase 3 study of the combination of Pd with or without pembrolizumab was conducted in the KEYNOTE-183 trial, and it led FDA to discontinue the trial, due to increased risk of death of patients [61, 62, 65].

3.4. Bispecific T-Cell Engager (BiTE) Antibodies (BsAb). Bispecific T-cell engager (BiTE) antibodies (BsAbs) are constructs, composed of 2 linked MoAbs which target 2 epitopes. One arm of antibody, scFvs, binds to CD3 on tumor-specific T cells, while the other arm binds to tumor-specific antigen on tumor cells [66, 67]. Cross linkage of T cells to the tumor cells causes T cells to release cytotoxic molecules such as perforin, which creates transmembrane pores in tumor cells, and granzyme B, which initiates apoptosis toward tumor cells. In addition, cytokine production from T cells activates its proliferation to kill tumor cells. BsAbs are characterized by small size (5 kDa), which induces high efficacy toward tumor cells, but its serum half-life is short [66, 67]. B-cell maturation antigen (BCMA) belongs to tumor necrosis factor superfamily member 17, also named "TNFRSF17 or CD269," which is uniformly expressed in malignant plasma cells but not in normal essential non-hematopoietic tissues, and only restricted expression is detected in normal hematopoietic cells including normal plasma cells and mature B lymphocytes. Thus, it is a highly plasma cell specific antigen and has a central role in regulating B-cell maturation and differentiation into plasma cells by engaging a proliferation-inducing ligand (APRIL) cells. This expression pattern leads to the development of BCMA-specific mAbs, BsAbs, antibody-drug conjugates (ADCs), and chimeric T cell receptor (CAR) T cells [68–70]. BsAb, BI-836909 (AMG420), the first bispecific scFv, simultaneously binds to CD3+ T cells and BCMA + MM cells which make a cross linking between both cells to induce cytolytic synapse, activate T cells, and lyse BCMA + MM cells. In phase I study in RRMM patients, it exhibited potent and high efficacy by depleting BCMA + MM cells [68–70]. CD3xCD38 BsAb, engineered to direct T cells to CD38 on tumor cells, was also developed. The phase 1 multicenter study of GBR1342 is underway [71].

3.5. Antibody-Drug Conjugates (ADCs). Antibody-drug conjugate is composed of recombinant MoAbs, bound to cytotoxic chemical agents through synthetic chemical linkers. MoAbs bind to the cell surface antigen on tumor cells and are internalized with the chemicals. Thus, the cytotoxic chemicals are released and transported from lysosome into cytosol to kill tumor cells [72]. GSK2857916 is a humanized and IgG_1 MoAb with high affinity to BCMA with afucosylated Fc linked to auristatin F noncleavable linker, maleimidocaproyl. In preclinical study, it binds to BCMA + MM cells and induces G2/M arrest and apoptosis by the activation of caspase 3/7 and 8. The naked form of ADC augmented effector-mediated cytotoxicity including ADCC and ADCP against patient MM cells [72]. In MM xenograft models, GSK2857916 depletes MM cells but surrounding BCMA-BM accessory cells remain unharmed. Its cytotoxicity is further increased by GSK2857916 plus lenalidomide in combination. In phase 1 study of GSK2857916 in RRMM patients, GAK2857916 monotherapy revealed a 60% response rate and median PFS of 7.9 months [73, 74]. Anti-BCMA approaches, alone or in combination with iMIDs or immune checkpoint inhibitors, will be evaluated in clinical trials in MM [70].

3.6. Chimeric Antigen Receptor (CAR) T Cells. CARs are fusion proteins incorporating an antigen-recognition domain and T-cell signaling domain. T cells are genetically modified to express CARs, which specifically recognize target antigens on tumor cells [75–77]. CAR T-cell therapy has already approved by FDA and European Medicine Agency (EMA) for the treatment of relapsed of refractory B-acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL) [75–77]. CAR-expressing T cells targeting CD19 revealed efficacy in patients with acute lymphoblastic leukemia (ALL) or B-cell NHL. This success of CAR-T cells against leukemia or lymphoma has encouraged the development of CAR-T therapies for MM. In the first

Table 3: Investigational monoclonal antibodies in MM.

Target molecule	mAb	Type	Clinical trials
CD138	Indatuximab ravtansine	ADC	Inda ± Rena ORR 78% vs 4%
CD56	Lorvotuzumab	ADC	Lorv+/Rd ORR 56% vs 7%
CD40	Dacetuzumab, lucatumumab	Humanized	Luc; 4% attained prolonged PR
CD74	Milatuzumab	Humanized	No objective responses
BAFF	Tabalumab	Humanized	Bd + Taba; ORR 44%
BCMA	GSK2857916	ADC	MTD not determined
GRP78	PAT-SM6	Humanized	MTD not determined
IGF-1R	AVE1642	Humanized	No objective responses
ICAM-1	BI-505	Humanized	No objective responses
CD26	YS110 (huCD26mAb)	Humanized	Best responses 50%

ADC, antibody-drug conjugate; Lena, lenalidomide; Inda, indatuximab ravatansine, Rd, lenalidomide plus dexamethasone; Lorv, lorvotuzumab; Luc, lucatumumab; PR, partial response; Bd, bortezomib + dexamethasone; Taba, tabalumab; MTD, maximum tolerated doses.

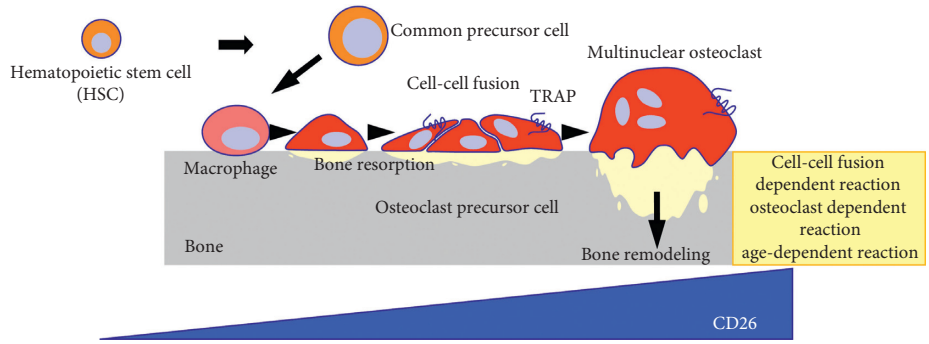

Figure 1: CD26 in human osteoclast development CD26 expression is increased during human osteoclast (OC) development.

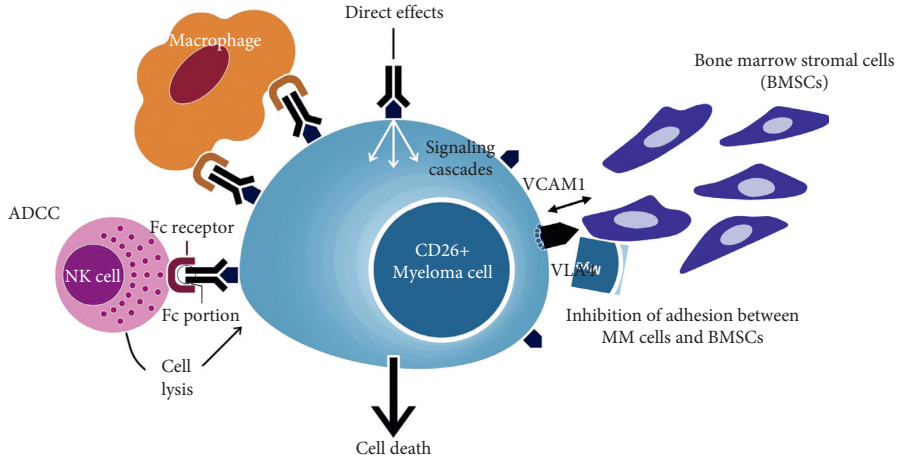

Figure 2: Humanized anti-CD26 monoclonal antibody (huCD26mAb): mechanisms of action huCD26mAb inhibits CD26 + MM cell growth chiefly via ADCC.

human clinical trials, Carpenter et al. designed the first novel CAR targeting BCMA in MM and demonstrated CAR-BCMA T cells had powerful activity against MM that was resistant to standard therapies [78, 79]. Moreover, bb2121 was produced by transducing autologous T cells with a lentiviral vector encoding a second-generation CAR incorporating an anti-BCMA single-chain variable fragment, CD137 costimulatory motif, and a CD3-zeta signaling domain [80]. A phase 1 clinical study of bb2121 in heavily pretreated RRMM patients revealed that 85% of the patients had a clinical response lasting a median of 10.9 months without any ongoing MM therapies [80]. Currently, CAR-T cell therapy for MM remains experimental. CAR-T cell therapy is a potentially life-threatening therapeutic approach, which needs to be administrated in experience hospitals. Now, phase 3 trials are just starting for RRMM in

2019. In addition, novel CARs targeting alternative plasma cell antigens including CD38, CD44v6, and SLAMF7(CS) are being developed [81, 82].

4. Experimental Research in Novel MoAb Therapy in RRMM

4.1. Investigational MoAbs. Target antigens for MoAb are either cell surface membrane proteins or soluble factors including cytokines or chemokines expressed or secreted in MM cells. Their functions include MM cell growth, cellular adhesion, angiogenesis, apoptosis, and cell-to-cell contact between MM cells microenvironmental cells. Investigational mAbs targeting CD138, CD56, CD40, CD74, BAFF, BCMA, GRP78, IGF-1R, and ICAM-1 are preclinically developed, and several of them are in clinical trials [83–92] (Table 3).

4.2. Humanized Anti-CD26 Monoclonal Antibody (huCD26mAb). CD26 is a 110 kDa transmembrane glycoprotein with dipeptidyl peptidase (DPPIV) activity, which is widely expressed in various normal cells such as T lymphocytes, natural killer (NK) cells, basophils, eosinophils, endothelial cells, and epithelial cells [93–96]. In addition, CD26 is expressed in several tumor cells including malignant lymphoma, mesothelioma, renal cell carcinoma, and hepatocellular carcinoma and is involved in T-cell activation and tumorigenesis [97, 98]. We have recently characterized CD26 as a potential therapeutic target for the treatment of MM [99]. We identified CD26 expression in human osteoclasts (OCs) in healthy individuals (Figure 1). Its expression is further increased in osteoclasts in osteolytic bone tumors including MM, adenocarcinoma, lung cancer, and osteosarcoma. huCD26mAb, a humanized IgG_1 monoclonal antibody that directly targets CD26, inhibits human OC differentiation *in vitro* and *in vivo* analysis [99]. In the bone marrow tissue of MM patients, we found that CD26 was present in plasma cells around OCs or endothelial cells. *In vitro* immunostaining or flow cytometry studies revealed that although CD26 expression was low or absent on MM cell lines cultured alone, it was intensely and uniformly expressed on MM cell lines cocultured with OCs [100]. The augmented CD26 expression in MM cells was exploited to enhance cytotoxicity of huCD26mAb chiefly via a substantial increase in antibody-dependent cytotoxicity (ADCC) against MM cells, direct effects or inhibition of the adhesion between MM cells and BM stromal cells (BMSCs) (Figure 2). Moreover, huCD26mAb in combination with the existing standards of care including bortezomib and lenalidomide synergistically enhanced huCD26mAb-induced ADCC activity against CD26 + MM cells compared with each agent alone [100]. Lastly, therapeutic effect of huCD26mAb against MM cell growth and its related osteolytic lesion was also validated *in vivo*, using a xenograft model: an intrabone tumor model of MM. Our preclinical results demonstrated that huCD26mAb elicited significant anti-MM efficacy by impairing both CD26 + MM cells and OCs *in vivo*, suggesting that CD26 could be an ideal therapeutic target of antibody-based therapy in RRMM [100].

5. Conclusion

During the last decades, therapeutic strategies in MM have dramatically changed. MoAbs act synergistically with backbone regimens including iMIDs, PIs, or HDACi and have benefits to overcome resistance to prior therapies. The future treatment options of MM to overcome resistance are promising by combination with MoAbs plus these novel agents, check point inhibitors or CAR T-cell therapy.

Conflicts of Interest

The authors declare they have no conflicts of interest.

Supplementary Materials

CD26 in human osteoclast development humanized anti-CD26 monoclonal antibody (huCD26mAb): mechanisms of action summary of clinical trials in anti-CS1/SLAMF7 antibody in relapsed/refractory MM. Summary of clinical trials in anti-CD38 antibody in relapsed/refractory MM. Investigational monoclonal antibodies in MM. (*Supplementary Materials*)

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CORRESPONDENCE

Reply to 'MUC4 staining in sarcomatoid carcinomas' by Berg et al.

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We appreciate Berg et al. for their study to replicate the findings of our study published in *Modern Pathology* [1]. They studied MUC4 expression in 6 sarcomatoid mesotheliomas and 13 sarcomatoid carcinomas of the lung and prematurely concluded its low applicability in differentiating sarcomatoid mesothelioma from sarcomatoid carcinoma of the lung. Their study also showed no MUC4 expression in all six cases of sarcomatoid mesothelioma, which is similar to our study. Regarding MUC4 expression in sarcomatoid carcinoma of the lung they found one case with diffuse expression, three cases showing focal expression, and nine cases with no expression. They have not given the detailed histology of their cases of sarcomatoid carcinoma. In our publication, we included and analyzed the MUC4 expression in 5 cases of spindle cell carcinoma, and 24 cases of pleomorphic carcinoma including the sarcomatoid (spindled cells) component. We found no expression of MUC4 in all five pure spindled cell types, but found most of the sarcomatoid component of pleomorphic carcinoma to have focal to diffuse expression. We agree with them to some extent about the staining of MUC4 in sarcomatoid carcinoma, provided if their cases were dominantly pure sarcomatoid type (spindle cell carcinoma). Further study of MUC4 expression in sarcomatoid carcinoma of the lung is needed with more cases of pleomorphic carcinoma with spindled cell components.

Recently, we also studied and published the MUC4 expression as a useful immunohistochemical marker in differentiating epithelioid mesothelioma from both lung adenocarcinoma and lung squamous cell carcinoma [2]. The negative MUC4 expression has 100% specificity and 86% sensitivity to differentiate epithelioid mesothelioma from lung adenocarcinoma and squamous cell carcinoma.

We still believe MUC4 expression has applicability in differentiating sarcomatoid mesothelioma from sarcomatoid carcinoma of the lung, because the negative MUC4 expression has 100% specificity (supported by 100% specificity in the study of Kyra KB also), although the sensitivity has a discrepancy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Droplet digital PCR as a novel system for the detection of microRNA-34b/c methylation in circulating DNA in malignant pleural mesothelioma

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Abstract. Malignant pleural mesothelioma (MPM) is a rare malignancy arising from the pleura that is difficult to diagnose, contributing to its dismal prognosis. Previously, we reported that the degree of microRNA (miR)-34b/c methylation in circulating DNA is associated with the development of MPM. Herein, we present a newly developed droplet digital PCR (ddPCR)-based assay for the detection of miR-34b/c methylation in circulating DNA in patients with MPM. We originally prepared two probes within a short amplicon of 60 bp, designing one from the positive strand and the other from the complementary strand. The two probes functioned cooperatively, and our established assay detected DNA methylation accurately in the preliminary validation. We subsequently verified this assay using clinical samples. Serum samples from 35 cases of MPM, 29 cases of pleural plaque and 10 healthy volunteers were collected from 3 different institutions and used in this study. We divided the samples into 2 groups (group A, n=33; group B, n=41). A receiver-operating characteristic curve analysis using the samples in group A determined the optimal cut-off value for the diagnosis of MPM, with a sensitivity of 76.9% and a specificity of 90%. On the other hand, the use of the same criterion yielded a sensitivity of 59.1% and a specificity of 100% in group B, and corresponding values of 65.7 and 94.9% for the entire cohort, indicating a moderate sensitivity and a high specificity. In addition, when the analysis was focused on stage II or more advanced MPM, the sensitivity improved to 81.8%, suggesting the possibility that the methylated allele frequency in MPM may be associated with the stage of disease progression. On the whole, the findings of this study indicate that miR-34b/c methylation in circulating DNA is a promising biomarker for the prediction of disease progression in patients with MPM.

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Abbreviations: MPM, malignant pleural mesothelioma; cfRNA, circulating cell-free RNA; cfDNA, circulating cell-free DNA; ctDNA, circulating cell-free tumor DNA; miR or miRNA, microRNA; ddPCR, droplet digital PCR; PP, pleural plaque; HV, healthy volunteers; LNA, locked nucleic acids; SNPs, single nucleotide polymorphisms

Key words: malignant pleural mesothelioma, droplet digital PCR, microRNA-34b/c, cfDNA, methylation

Introduction

Malignant pleural mesothelioma (MPM) is a rare and highly aggressive tumor arising from the pleura or other mesothelial surfaces and is most commonly associated with asbestos exposure, which is known as a major risk factor. Although asbestos use is now prohibited in Western countries, the incidence of MPM is not expected to decrease in the near future due to the long incubation period between asbestos exposure and the onset of MPM (1,2). Moreover, asbestos continues to be used in many developing and emerging economies, such as countries in Southeast Asia, suggesting the possibility of future epidemics of MPM. In the majority of cases, MPM is only diagnosed at an advanced disease stage; therefore, the development of a novel diagnostic approach is warranted (3,4).

Recently, the concept of a 'liquid biopsy' for the diagnosis and monitoring of diseases has attracted attention. Several

studies have suggested that the individual genetic profiles of cancers are highly heterogeneous and that these profiles can even change during the course of the disease, particularly in response to treatment (5,6). At present, the molecular profiles of patients with solid tumors are generally established using surgically resected or biopsy specimens. However, the use of tissue biopsies is limited by their invasiveness, making it difficult to grasp chronological alterations in molecular profiles and potentially missing some genomic changes. A liquid biopsy originally referred to an analysis of the genomic profiles of circulating tumor cells (7), and this method has attracted particular interest among experts in the field of clinical oncology. This definition has now been extended to include various tumor components, such as circulating cell-free RNA (cfRNA), circulating cell-free DNA (cfDNA), circulating cell-free tumor DNA (ctDNA) and exosomes, and this technique enables clinicians to repeatedly and non-invasively explore real-time changes in the genomic profiles of human cancers.

MicroRNAs (miRNAs or miRs) are a group of small noncoding, endogenous, single-stranded RNAs that play an essential role in the regulation of gene expression. A number of studies have reported that the aberrant hypermethylation of CpG islands in the promoter regions is closely related to the silencing of tumor-suppressive miRs in several types of cancer (8-11). We previously identified that among several miRs, the epigenetic silencing of miR-34b/c by aberrant methylation in the promoter region plays an important role in the tumorigenesis of MPM (12). miR-34s have been discovered to be direct transcriptional targets of p53, and to constitute a part of the p53 tumor suppressor network regulating cell cycle arrest, apoptosis and senescence (13,14). As regards the application of miRs as biomarkers, Suzuki *et al* reported that the aberrant methylation of miR-34b/c in biopsy specimens was a predictive marker of metachronous gastric cancer (15). Wu *et al* also reported that the detection of methylation in the promoter regions of miR-34s using stool DNA was useful as a screening biomarker for colorectal cancer (16). Additionally, we have previously revealed that the degree of miR-34b/c methylation in serum-circulating DNA is associated with the development of MPM (17). Although the origins of ctDNA differ, these previous studies suggest the possibility that the methylation of the miR-34b/c promoter is a promising biomarker.

In our previous study in 2011, we compared the degree of methylation using MPM cell lines, MPM tissues and nonmalignant mesothelial primary cultures that were established from pleural effusions of cancer-free patients, and we have shown that the promoter of miR-34b/c is highly methylated in MPM (12). Based on these findings, the aim of the present study was to apply the miR-34b/c methylation specifically observed in MPM to the diagnosis and prediction of the disease progression. For this purpose, in this study, we established a novel assay with which to detect DNA methylation in the blood using droplet digital PCR (ddPCR) technology, enabling the highly sensitive and quantitative detection of target genes (18). In ddPCR, the input DNA is distributed among approximately 20,000 droplets, and each droplet contains 1 or fewer copies of the target or background DNA; this makes it possible to detect 0.001% of the target gene from the background DNA (19-21). Our established assay was then verified using serum samples from patients with MPM, pleural plaque patients and healthy volunteers.

Materials and methods

Clinical samples and cell lines. We collected >1 ml peripheral blood sample from 35 cases of MPM, 29 cases of pleural plaque (PP) and 10 healthy volunteers (HV) at the Okayama University Hospital (Okayama, Japan), Okayama Rosai Hospital (Okayama, Japan), or the National Hospital Organization, Yamaguchi Ube Medical Center (Yamaguchi, Japan), between January, 2005 and January, 2015. The details are described in Table I. The age of all the healthy volunteers was >20 years and healthy individuals who were not any current medications were recruited. None of the participants had a medical history of cancer other than MPM, and all the blood samples were collected before any type of treatment. The blood samples were immediately centrifuged at 5,000 x g for 5 min, and the separated serum samples were stored at -80˚C at the respective institutions. In addition, 3 surgically resected MPM specimens obtained from the National Hospital Organization, Yamaguchi Ube Medical Center were also subjected to the methylation assay. All the tissues were frozen in liquid nitrogen immediately after surgery and stored at -80˚C. This study was conducted with the approval of the Institutional Review Board/Ethical Committee of Okayama University; each of the participants provided written informed consent for the sample collection. All the experiments were performed in accordance with the Declaration of Helsinki.

We also used two human MPM cell lines [NCI-H28 (H28), NCI-H2052 (H2052)] and one human normal mesothelial cell line (MeT-5A) as positive and negative controls, respectively. The H28 and H2052 cells were obtained as kind gifts 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, USA). The MeT-5A cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For the cell lines that had been stored long-term in liquid nitrogen, a DNA fingerprinting analysis by short tandem repeat profiling (the PowerPlex 1.2 System, Promega, Madison, WI, USA) was performed for cell authentication. The cells were maintained in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FBS and cultured in a humidified incubator under 5% CO_2 at 37˚C, and the samples were routinely tested for mycoplasma using the Venor GeM OneStep kit (Minerva Biolabs, Berlin, Germany).

DNA extraction, bisulfite conversion, and bisulfite DNA sequencing. We extracted DNA from the serum samples using the QIAamp Circulating Nucleic Acid kit (Qiagen, Venlo, The Netherlands). The DNA concentrations were quantified using the Qubit 2.0 Fluorometer and Qubit dsDNA HS or BR assay kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was also extracted from the MPM tissues using the phenol-chloroform method. DNA was extracted from the cell lines using the DNeasy Blood and Tissue kit (Qiagen). Genomic DNA was subjected to bisulfite conversion using the Epitect Bisulfite kit (Qiagen), and the methylation status of miR-34b/c

Table I. Patient characteristics.

A, Patients in group A

Characteristic	MPM (n=13)	PP (n=20)
Median (range), years	71 (51-90)	69.5 (65-72)
Sex, male/female	9/4	20/0
Smoking history		
Never	3	5
Former/current	10	15
Histological subtypes		
Epithelioid	6	N/A
Biphasic	4	N/A
Sarcomatoid	3	N/A
Clinical stage		
I	3	N/A
II	2	N/A
III	5	N/A
IV	1	N/A
Unknown	2	N/A

B, Patients in group B

Characteristic	MPM (n=22)	PP (n=9)	HV (n=10)
Median (range), years	61.5 (49-86)	77 (60-86)	31 (25-37)
Sex, male/female	19/3	9/0	10/0
Smoking history			
Never	6	3	8
Former/current	16	6	2
Histological subtypes			
Epithelioid	15	N/A	N/A
Biphasic	5	N/A	N/A
Sarcomatoid	2	N/A	N/A
Clinical stage			
I	8	N/A	N/A
II	5	N/A	N/A
III	5	N/A	N/A
IV	4	N/A	N/A

MPM, malignant pleural mesothelioma; PP, pleural plaque; HV, healthy volunteer.

was determined using bisulfite DNA sequencing as previously described (12,17). The raw sequence chromatograms were analyzed using Chromas Lite software version 2.6.5 (available at http://technelysium. com.au/wp/ chromas/). The degree of methylation was determined by comparing the intensity of the sequencing electropherogram of cytosine with that of thymine at each of the CpG sites. Based on the electropherograms, we quantified the relative ratios between the heights of each of the waves, as described previously (Fig. 1A) (22), and classified the degree of methylation into three groups, as follows: Low-methylated, degree of methylation <20%; moderately methylated, degree of methylation between 20 and 70%; and highly methylated, degree of methylation >70%.

Primers and TaqMan probes. The sequences of the primers and TaqMan probes used in this study were designed based on the nucleotide sequence submitted to GenBank (GenBank accession numbers NR 029839.1 for miR-34b and NR 035765.1 for miR-34c). The melting temperature (Tm) of each primer was calculated using the Oligo Calculator (http://mbcf149.dfci.harvard.edu/docs/oligocalc.html). The primers were synthesized by Invitrogen (Thermo Fisher Scientific, Yokohama, Japan). The primers, including the mixed-base and TaqMan probes containing the locked nucleic acids (LNAs) were designed using the IDT Biophysics software (https://www.idtdna.com/ pages/tools) and were synthesized by Integra ted DNA Technologies KK (Tokyo, Japan).

ddPCR assay for miR-34b/c methylation detection. ddPCR was performed using the QX200 Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA). EpiTect Control DNAs (methylated or unmethylated and bisulfite-converted human DNA, QIAGEN) were used for the assay validation. The total volume of the PCR mixture used for the assay was 22 μl, containing 10 μl of ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 1 μM of each primer, 0.25 μM of each probe and 200 μM of dNTP. As for the amount of DNA, 10 μl of cfDNA extracted from the serum was used, while a total of 5 ng of DNA (methylated and bisulfite-converted human control DNA) was applied for the validation of the assay. The PCR conditions were described in detail in our previous study (23). The annealing temperatures were optimized by gradient PCR. The PCR products were then subjected to analysis with the QX-200 droplet reader and QuantaSoft analysis software (Version 1.7.4.0917) (Bio-Rad). The former measures the fluorescence value of each droplet, and the latter measures the number of positive and negative droplets in each sample and calculates the fraction of positive droplets by a Poisson algorithm. QuantaSoft analysis software cannot display the fluorescence intensity of each droplet and standard deviation.

Statistical analysis. All *in vitro* experiments were performed at least 3 times. Data are represented as the means ± standard deviation. The concentrations of the target alleles were calculated using QuantaSoft software (Bio-Rad) based on Poisson's distribution. The receiver-operating characteristic (ROC) curve analysis was performed using JMP® 9.0.0 for Windows (SAS Institute, Inc., Cary, NC, USA). A one-way ANOVA followed by Bonferroni's multiple comparisons test was conducted using GraphPad Prism, version 7 (GraphPad Software, San Diego, CA, USA). Probability values (P-values)<0.05 was considered to indicate statistically significant differences.

Results

Appropriate sequences for primer design. First, we examined candidate sequences suitable for the primer and TaqMan probe design based on some key points, as follows: i) Multiple CpG sites were included in the target sequence to increase the sensitivity; ii) CpG sites were not included in the primer sequences; and iii) the frequency of single nucleotide polymorphisms (SNPs)

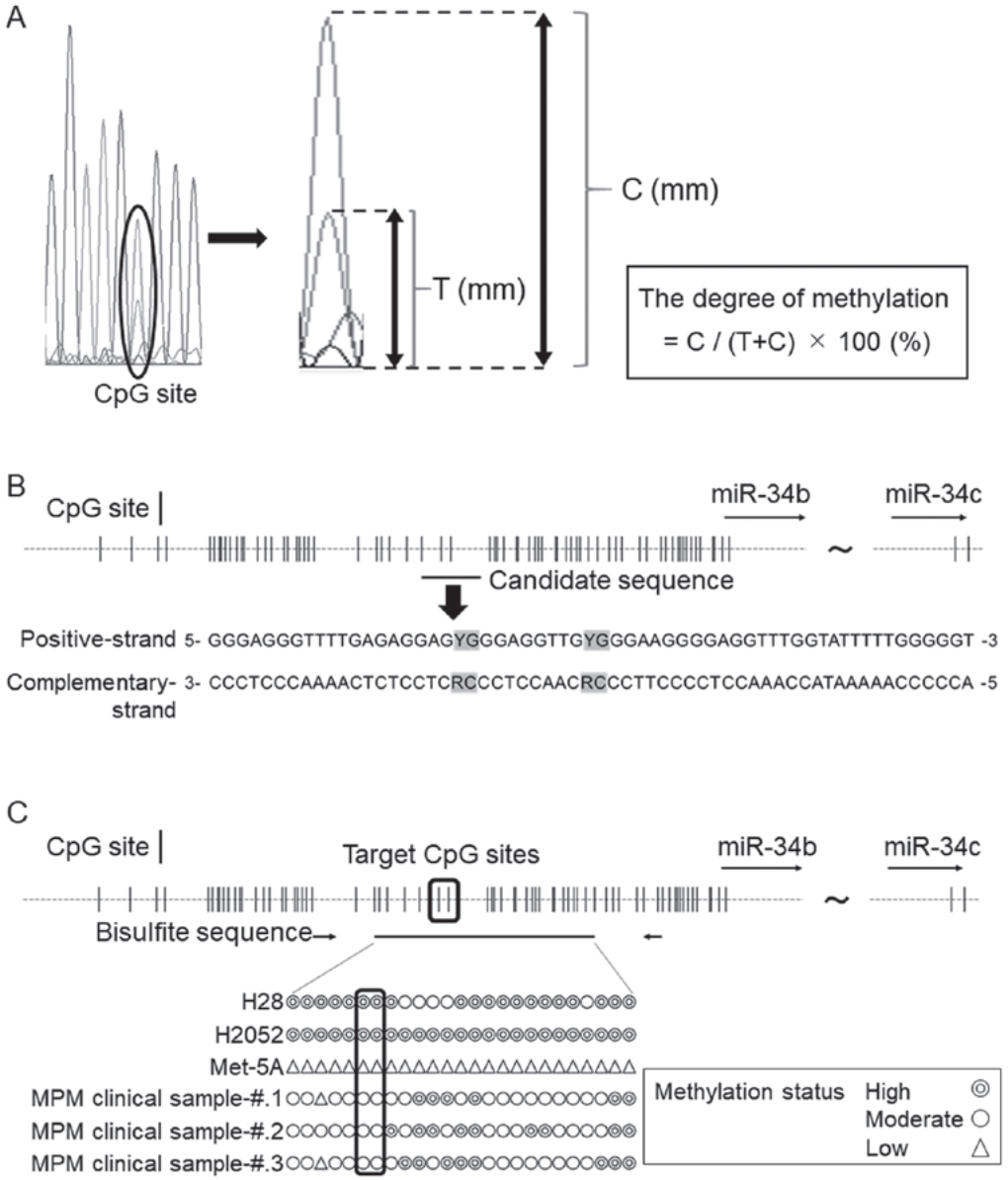

Figure 1. Optimal probe and primer design. (A) Method for calculating the degree of methylation from sequencing electropherograms. C, cytosine (methylated allele); T, thymine (unmethylated allele). (B) Schema of the miR-34b/c promoter region. CpG sites included in the selected sequence are highlighted in gray. G, guanine; A, adenine; T, thymine; C, cytosine; Y, pyrimidine; R, purine. (C) Methylation statuses of 2 MPM cell lines, 1 normal mesothelial cell line, and 3 MPM tissue specimens. Double circles represents a highly methylated status, a single circle represents a moderately methylated status, and a triangle represents a low methylation status. The target CpG sites and the methylation status are surrounded by the black line. MPM, malignant pleural mesothelioma.

was relatively low in the target sequences. In addition, we made the amplicon size as small as possible to increase the sensitivity of ctDNA detection, as described previously (23). One of the candidate sequences is shown in Fig. 1B, and the SNPs in this target region, as provided by the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/projects/SNP/), are listed in supplementary Table SI. The possible frequency of SNPs in this region was ≤0.02%, which reinforced the validity of this sequence. To confirm the methylation status of the two CpG sites included in this sequence, we performed bisulfite DNA sequencing. The results revealed that both CpG sites were moderately or highly methylated in both the MPM cell lines and the MPM clinical specimens, but not in the normal mesothelial cell line (Fig. 1C). Based on these results, we designed the primers as shown in Table II. Validation of the primer sets was performed to identify possible non-specific reactions, and we confirmed the specificity of the primers (data not shown).

Probe design and assay validation. Herein, we present a schema representing the principle on which our methylation detection assay was based (Fig. 2). The two CpG sites were detected separately by two TaqMan probes with the same fluorescent dye, and thus we examined the optimal probe design. As both CpG sites in this sequence were located close to each other, we designed one probe based on the sequence of the positive strand (Probe-P), and the other based on the sequence of the complementary strand (Probe-C). In addition, in order to obtain a sufficient match-mismatch Tm difference, the probes were fabricated using LNAs. Based on these concepts, we designed

Table II. Sequences of primers and probes.

	Oligo name	Oligo sequences 5' to 3'	Tm (˚C)	Product size (bp)	Match-mismatch Tm difference (˚C)
Primers	MPM-Fw	GGGAGGGTTTTGAGAGGAG	62.54	60	NA
	MPM-Rv	ACCCCCAAAAATACCAAACC	63.28		NA
	MSP-Fw	AGAGAGTTAGTTTTAGGGTTTGGG	61.5	358	NA
	MSP-Rv	CCTCRAACCCCATTTCAC	62.95		NA
Probes	Probe-P	FAM/AC+CT C+CC+GCT/IABLFQ	65.41	NA	21.03
	Probe-C1	FAM/TTG+CGGG+AAGGGG/IABLFQ	64.07	NA	14.75
	Probe-C2	FAM/TG+CGG+G+A+AGG/IABLFQ	63.23	NA	17.83
	Probe-C3	FAM/AGGTT+G+C+GGGAAG/IABLFQ	63.56	NA	11.85
	Probe-C4	FAM/TG+CGGGAAGGGGAG/IABLFQ	64.65	NA	13.29

ddPCR, droplet digital PCR; Tm, melting temperature; IABLFQ, Iowa Black FQ™ ; NA, not available; locked nucleic acid, +N; R, mixed base (A or G).

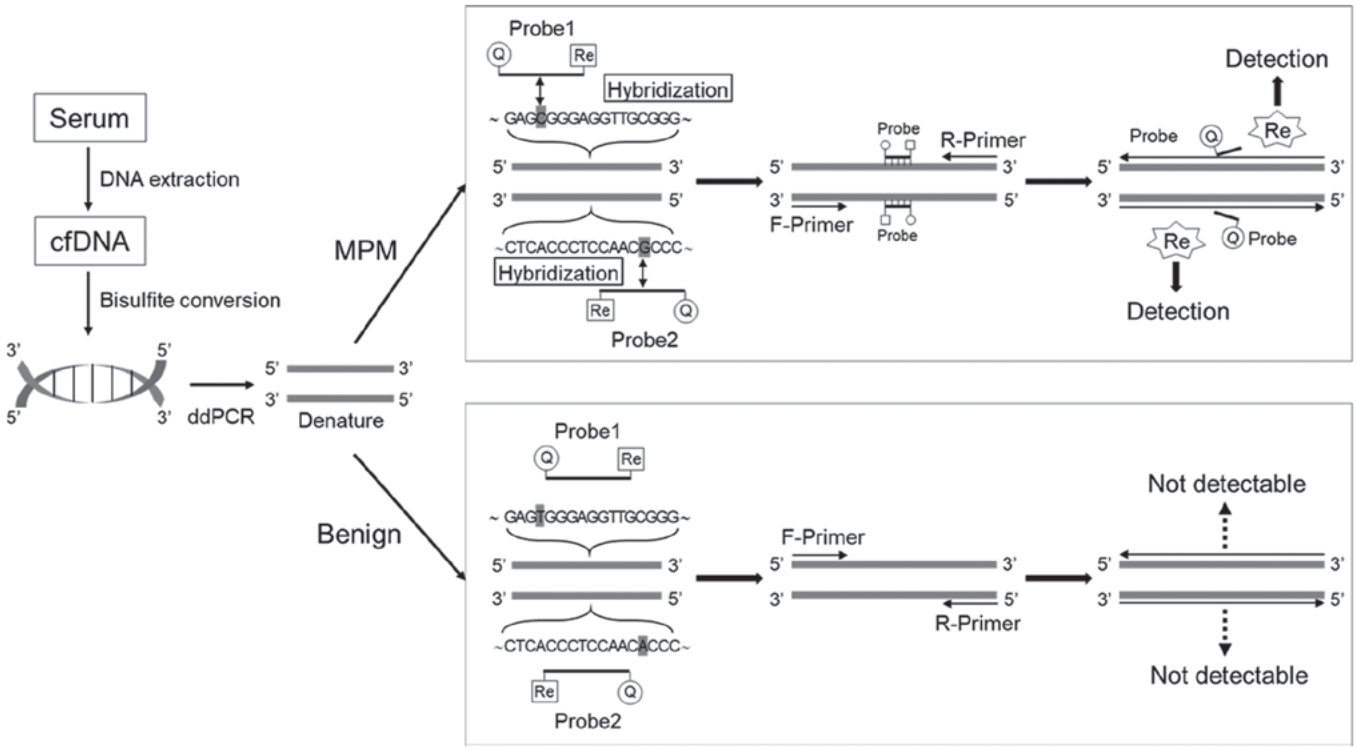

Figure 2. Schematic diagram of the assay established in this study. The target CpG sites of each probe are highlighted in gray. cfDNA, circulating cell-free DNA; MPM, malignant pleural mesothelioma.

several probe sets (Probe-P, Probe-C#1-4) (Table II). To verify the validity of these probes, and to consider the optimum annealing temperatures, gradient PCR was conducted within the range of 51˚C to 61˚C. As a result, Probe-P and Probe-C#1 had a higher fluorescence, compared with the other probes (Fig. 3A and B). Determining whether the two probes would function properly without competition was also important for successful methylation detection. Therefore, to test the interaction between Probe-P and Probe-C#1, we performed the same experiment using the two probes in combination. We found that the fluorescence intensity was enhanced when the probes were used in combination, suggesting that the probes functioned cooperatively (Fig. 3C and D), and the optimal annealing temperature was determined to be 53.1˚C. Lastly, we confirmed whether this assay could correctly distinguish between methylated and unmethylated DNA. As shown in Fig. 3E and F, the number of droplets with a fluorescence intensity >3,000 was noticeably larger in the methylated DNA group.

Clinical application of the established assay. We then evaluated the feasibility of the clinical application of this

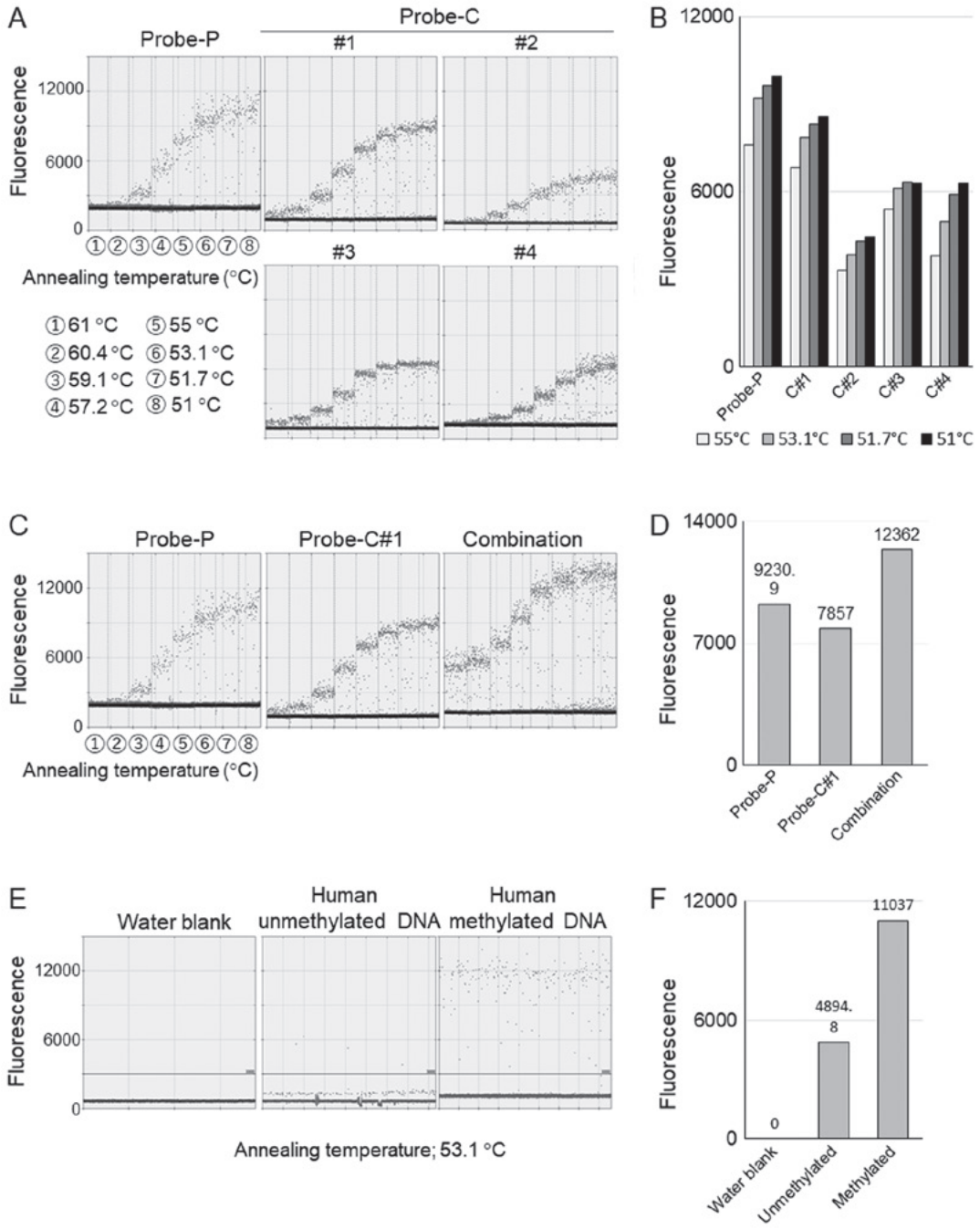

Figure 3. Validation of the established assay. (A) Validation of probes. Gradient PCR was conducted within an annealing temperature range of 51 to 61˚C; the numbered circles indicate the following temperatures: 1, 61˚C; 2, 60.4˚C; 3, 59.1˚C; 4, 57.2˚C; 5, 55˚C; 6, 53.1˚C; 7, 51.7˚C; and 8, 51˚C. Probe-P represents the probe designed based on the sequence of the positive strand, and Probe-C represents the probe designed based on the sequence of the complementary strand. (B) The mean fluorescence values of droplets with a fluorescence intensity of over 3,000. (C) Verification of combined use of the probes. The annealing temperature was ranged from 51 to 61˚C; the numbered circles indicate the following temperatures: 1, 61˚C; 2, 60.4˚C; 3, 59.1˚C; 4, 57.2˚C; 5, 55˚C; 6, 53.1˚C; 7, 51.7˚C; and 8, 51˚C. The use of Probe-P and Probe-C#1 in combination was associated with an enhanced fluorescence intensity, compared with that of each probe alone (right panel). (D) The mean fluorescence values of droplets with a fluorescence intensity of over 3,000 at annealing temperature of 53.1˚C. (E) Verification of established assay using methylated and non-methylated human DNA. (F) The mean fluorescence values of droplets with a fluorescence intensity of over 3,000 at annealing temperature of 53.1˚C.

assay. We divided the serum samples (35 cases of MPM, 29 cases of PP and 10 HVs) into group A (n=33) and group B (n=41) according to their collection site: Samples obtained from the Okayama Rosai Hospital were classified as group A, while those obtained from the other two institutions were classified as group B. The characteristics of the patients in the 2 groups are summarized in Table I. The median concentration of cfDNA extracted from the serum was 1.47 ng/μl for the MPM cases and 1.44 ng/μl for the others. Firstly, to determine the positive criterion, we conducted an ROC curve analysis comparing the MPM cases with other non-malignant cases using samples from group A (Fig. 4A and Table SII). The results indicated that the presence of at least 3 droplets with a fluorescence of over a threshold value of 7,000 was the optimal cut-off for the diagnosis of MPM, with a sensitivity of 76.9% and a specificity of 90%.

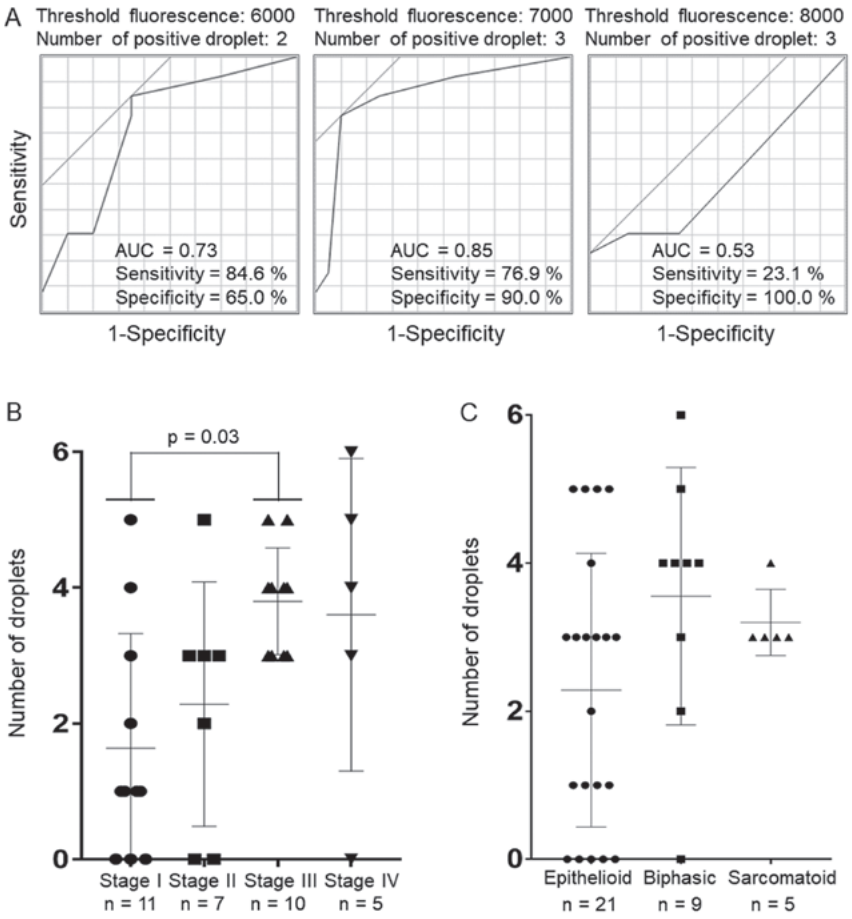

Figure 4. Validation for clinical application of the established assay. (A) Comparison of receiver-operating characteristic (ROC) curves among threshold fluorescence values of 6,000-8,000. ROC curves for the positive criterion of methylation detection are shown. AUC, area under the ROC curve. (B) Number of droplets with fluorescence over the threshold value of 7,000 according to the clinical stage of MPM. The bars represent the mean with standard deviation. MPM, malignant pleural mesothelioma. (C) Number of droplets with fluorescence over the threshold value of 7,000 according to the histological subtypes of MPM. The bars represent the means ± standard deviation.

Subsequently, we evaluated the validity of this criterion. The results are shown in Table III. The sensitivity and specificity for the diagnosis of MPM in the group B samples were 59.1 and 100%, respectively, while those for the entire cohort were 65.7 and 94.9%, respectively, indicating a moderate sensitivity and a high specificity. In addition, when we focused on the diagnosis of only stage II or more advanced MPM, the sensitivity increased to 81.8%. Actually, there were significant differences in the number of droplets with fluorescence values of at least 7,000 per case among stages (one-way ANOVA, P=0.02) (Bonferroni's post hoc test; stage I vs. stage II, P>0.99; stage I vs. stage III, P=0.03; stage I vs. stage IV, P>0.18; stage II vs. stage III, P=0.39; stage II vs. stage IV, P>0.99; stage III vs. stage IV, P>0.99), suggesting that the methylated allele frequency may be associated with the stage of MPM progression (Fig. 4B). On the other hand, methylation was not detected in one case with clinical stage IV MPM. We also assessed whether the histological subtypes were associated with the methylated allele frequency. However, no significant difference in the methylated allele frequency was observed among the histological subtypes (one-way ANOVA, P=0.16) (Bonferroni's post-hoc test; epithelioid vs. biphasic, P=0.21; epithelioid vs. sarcomatoid, P=0.87; biphasic vs. sarcomatoid, P>0.99) (Fig. 4C).

Table III. Assay sensitivity and specificity of each group.

	MPM	PP or HV
Group A (n=33)		
Positive	10	2
Negative	3	18
Sensitivity, 76.9%; specificity, 90.0%		
Group B (n=41)		
Positive	13	0
Negative	9	19
Sensitivity, 59.1%; specificity, 100%		
Entire cohort (n=74)		
Positive	23	2
Negative	12	37
Sensitivity, 65.7%; specificity, 94.9%		
Stage II or more advanced MPM (n=61)		
Positive	18	2
Negative	4	37
Sensitivity, 81.8%; specificity, 94.9%		

MPM, malignant pleural mesothelioma; PP, pleural plaque; HV, healthy volunteer.

Discussion

In this study, we established a TaqMan-based ddPCR assay for the detection of the methylation of the miR-34b/c promoter region in circulating DNA. The design of the two probes, one from the positive strand and the other from the complementary strand, allowed the successful detection of the methylation of the two CpG sites located close to each other, with an overall specificity of 94.9%. Although the sensitivity of our assay was limited to 65.7%, when the analysis was focused on the detection of stage II or more advanced cases of MPM, the sensitivity increased to 81.8%, and there was a tendency that the methylated allele frequency was higher in more advanced MPM. These findings suggest that the methylation status may be positively associated with the stage of MPM progression and that it may be useful for predicting tumor progression. As for the association between the methylation status of tumor suppressor genes and the disease progression, Jezkova *et al* also reported that the hypermethylation of RASSF1A and PITX2, which are known for the tumor suppressor gene in breast cancer, is significantly associated with tumor stage in breast cancer patients (24). On the other hand, Guo *et al* mentioned that there was no significant difference in the methylation status of HOXD10, which functions as a tumor suppressor in hepatocellular carcinoma (HCC), between the HCC patients with stage I and II and those with stage III and stage IV (25). Thus, whether the degree of the promoter methylation can predict the tumor progression may depend on the type of cancer and gene. In addition, in our series, methylation was not detected in one case despite the patient having clinical stage IV MPM; therefore, further studies of the tumor characteristics that may be particularly related to the degree of methylation are required.

It is well known that both chemotherapy and radiotherapy induce DNA methylation changes, and chemotherapy or radiation-induced alterations in DNA methylation result in changes in the biological response to the treatment. Recently, Flanagan *et al* reported that platinum-based chemotherapy induces DNA methylation changes in blood DNA, and the methylation levels in blood DNA at the time of relapse can reflect the clinical outcome of cancer patients (26). Sun *et al* also reported that the promoter methylation level of RASSF1A was affected by oxaliplatin-based chemotherapy, and the methylation status in blood DNA can be used to predict the outcome of patients with colorectal cancer (27). Thus, the influences of treatments on methylation statuses are a very important issue that should be examined in the future, and miR-34b/c is no exception. Therefore, the samples that were used in the present study were collected before any treatment was administered.

Several circulating biomarkers have been reported for the diagnosis of MPM, including the soluble mesothelin-related peptides, osteopontin, fibulin-3 and miRs (28,29). As for protein markers, while they exhibit excellent specificity, their poor sensitivity reduces their diagnostic usefulness (30-32). As regards circulating miRs, although some miRs exhibit diagnostic potential for MPM, there are problems, such as their origin (whether they are derived from tumor cells or hematopoietic cells is still controversial) that need to be resolved, and the majority of the analyses of cfRNA in the blood remain exploratory (21). On the other hand, few studies have reported the usefulness of a diagnostic method targeting the degree of methylation of DNA, not the miR or protein itself, for MPM. Several studies have reported the existence of a strong association between the methylation status in tumor tissue samples and that in ctDNA from blood samples; therefore, targeting ctDNA methylation is reasonable (33-36). As suggested by previous studies, a combination of various approaches could be useful to increase the sensitivity, and targeting circulating methylated DNA may be a worthwhile addition (28,29).

Whereas we used a SYBR-Green-based real-time MSP assay (48 wells/sample) in our previous study, we adopted a TaqMan-based ddPCR assay (20,000 droplets/well) in the present study to improve the specificity and accuracy of the detection of methylated DNA from amongst a large amount of background DNA. As a result, the specificity of the assay was improved to 94.9%, compared with that in our previous study. On the other hand, the sensitivity of the established assay was limited to 65.7%. The median dosage of cfDNA in this study was approximately 15 ng/sample, corresponding to 4,500 haploid genome equivalents. Considering the capability of ddPCR, it is possible to process larger amounts of cfDNA. Increasing the dosage of DNA may lead to an improvement in sensitivity. Recently, cfDNA in body fluids other than blood, such as urine or stool, has also attracted attention as useful biomarkers of cancer (37,38). The collection of these samples offers the advantage of being truly non-invasive and allowing large sample volumes to be collected, which may compensate for the disadvantage of the rather limited amount of cfDNA in the blood. In addition, the concentration of ctDNA is one of the key factors for successful cancer detection using a liquid biopsy, and it is well known that the proportion of ctDNA in cfDNA varies among patients depending on the tumor localization, size, vascularization, and clearance, ranging from <0.005 to 90% in several types of cancer (39-42). However, the association between ctDNA and total cfDNA in MPM remains unclear; therefore, further investigation of this issue using liquid biopsies in patients with MPM will be our next task.

This study had some limitations. First, the sample size was too small to enable a definitive conclusion, and the groups in this study were not matched for background characteristics, such as age and sex. Considering the rarity of MPM, large clinical trials would be preferable. Second, plasma samples are more suitable than serum samples for cfDNA analyses due to the lower background level of wild-type DNA in the former (21,43). Therefore, our established assay should be validated using plasma samples. These factors could have introduced some bias to our results.

In conclusion, in this study, we established a novel detection system for the promoter methylation of miR-34b/c using ddPCR. Our findings suggest the possibility that miR-34b/c methylation in ctDNA could be a promising circulating biomarker for the prediction of disease progression in MPM.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS, JS, HY, KS and SToyooka conceived and designed experiments. HS, STanaka, HTo and KN conducted the experiments. HS and STomida analyzed data and prepared the figures. KA, NF, HTa, KO and TK contributed to the sample collection. HS, JS and SToyooka wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted with the approval of the Institutional Review Board/Ethical Committee of Okayama University; each of the participants provided written informed consent for the sample collection. All the experiments were performed in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE Open Access

Physician requests by patients with malignant pleural mesothelioma in Japan

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Abstract

Background: Malignant pleural mesothelioma (MPM) is a fatal and rare disease that is caused by the inhalation of asbestos. Treatment and care requests made by MPM patients to their physicians were collected and analyzed.

Methods: This cross-sectional survey was part of a larger study ($N = 133$) regarding the quality of life of MPM patients. Specific responses to two open-ended questions related to patients' requests regarding treatment and care were quantified, analyzed and divided into categories based on content.

Results: Responses ($N = 217$) from MPM patients ($N = 73$) were categorized into 24 subcategories and then abstracted into 6 categories. The majority of requests were related to patient-physician communication. Patients wanted clear and understandable explanations about MPM and wanted their physician to deliver treatment based on the patient's perspective by accepting and empathizing with their anxiety and pain. Patients expected physicians to be dedicated to their care and establish an improved medical support system for MPM patients.

Conclusion: Patients with MPM had a variety of unmet needs from their physicians. Physicians who provide care to MPM patients should receive training in both communication skills and stress management. A multidisciplinary care system that includes respiratory and palliative care for MPM patients should be established.

Keywords: Asbestos, Communication, Mesothelioma, Patient-centered care, Support

Background

Globally, exposure to asbestos in the workplace is now considered one of the main causes of work-related deaths with one-half of these deaths attributable to cancers, including malignant pleural mesothelioma (MPM) [1]. The number of deaths from MPM in Japan was greater than 1400 in 2015 [2]. This number is expected to grow by 2040 [3]. MPM is fatal [4, 5] and causes debilitating physical symptoms, such as pain, dyspnea, fatigue, loss of appetite, and sweating [6]. Patients with MPM also experience emotional difficulties, including the shock of diagnosis [7], anxiety and depression [8], or guilt and shame [9]. In addition, patients have complained of a lack of information about the disease and a lack of compensation from their insurance providers [10]. Patients have also expressed anger toward their employers who did not alert them to the hazards of asbestos [8, 11], in response to their own ambivalence toward working in an unhealthy environment versus supporting their family [8], and as a result of the stress of dealing with asbestos-related lawsuits [8, 12, 13]. For patients with MPM, a multidisciplinary approach involving a psychologist specialized in taking care of cancer patients and their families is recommended [14]. In Japan, physicians are the major source of information and support for patients with MPM. Unfortunately, some patients with MPM have not been well informed, and physicians were unable to meet their needs. This lack of rapport and communication eventually led to dissatisfaction with their attending physician and had a negative impact on patients' quality of life (QOL) [10]. Given the importance of the physician-patient relationship, it is important to further investigate what MPM patients need from their physicians to address their current gap in knowledge of the disease. The current study is part of a larger study regarding the QOL of

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patients with MPM. The aim is to determine the needs of patients within the health services by quantifying the requests to their physicians and qualitatively analyzing their answers to two open-ended questions regarding these requests.

Methods

Study design

This study is a part of a major study about QOL and intention of care among MPM patients. This study is a cross-sectional descriptive study that used a mailed survey [15]. In brief, an invitation to participate in the study was sent to 422 cancer hospitals in Japan; 64 hospitals (15.2%) agreed to participate. In February 2016, the participating hospitals distributed 438 questionnaires to their patients with MPM. Additional questionnaires were mailed in March 2016 to 94 MPM patients who were identified through patient and family support groups, which have 15 branches in Japan. The completed questionnaires were mailed back to the researchers by the end of April 2016. Basic demographic and medical data of the participants were gathered using a separate researcher-constructed, patient self-administered questionnaire. The questionnaire contained 72 questions regarding the QOL of MPM patients and related factors. In total, 88 (20.1%) questionnaires were returned. Of the 94 questionnaires that were sent to the patients and family support groups, 45 (47.9%) were returned. In total, 133 questionnaires were collected, and 73 (54.9%) participants answered the two open-ended questions referred to as "requests to physicians." Table 1 describes the characteristics of the participants. In the current study, we evaluated the answers to open-ended questions: (1) "What do you request from your doctor about your diagnosis and treatment?" and (2) "Describe the attitude and words you want from your doctor (Additional file 1)."

Table 1 Demographic and Medical Characteristics of the Study Participants ($N = 73$)

Characteristic	Response	n	%
Gender	Male	61	83.6
	Female	12	16.4
Age in years (mean ± SD)		66.8 ± 11.3	
MPM Treatment Received			
Surgery	I did not have	43	58.9
	I had	30	41.1
Chemotherapy	I never had	13	17.8
	I had before	29	39.7
	I am having now	31	42.5
Radiotherapy	I never had	52	71.2
	I had before	19	26.0
	I am having now	2	2.7
Palliative care	I never had	39	53.4
	I had before	9	12.3
	I am having now	25	34.2
ECOG Performance Status	0	12	16.4
	1	40	54.8
	2	7	9.6
	3	13	17.8
	4	1	1.4
Relationship with Their Physician	Very good	30	41.1
	Good	31	42.5
	Moderate	9	12.3
	Not very good	2	2.7
	Poor	1	1.4

ECOG, Eastern Cooperative Oncology Group; SD, standard deviation

Data analysis

Basic medical and demographic information was tallied, and the percentages and mean values were calculated. The answers to the questions were analyzed using the qualitative content analysis procedures of Graneheim and Lundman [16]. Initial categories were created by grouping similar words and phrases. The authors discussed the definitions and examples that emerged through the content analysis to enhance the representation and add clarity to categories, definitions, and examples. Responses that were not easily ascribed to a specific category were discussed and assigned to an appropriate category when the research team achieved 100% consensus. This process was repeated until all the responses were coded [17]. Finally, two researchers verified all the answers and tallied the number of times each category and subcategory was mentioned. The prevalence was compared between patients who received palliative care and those who did not receive palliative care. Comparisons between independent groups were performed using the chi-square test.

Ethical considerations

Ethical approval for the study was obtained from the Okayama Rosai Hospital Ethics Review Board. Eligible MPM patients received written information about the study, including their right to confidentiality, to refuse participation, or to withdraw at any point in the study without penalty.

Results

Requests to the physician

The 217 requests by 73 respondents were categorized into 24 subcategories and were finally integrated into six

categories. Table 2 displays the categorized requests to physicians by MPM patients.

Understandable explanations to meet patient's needs

Among the 217 requests, 80 concerned explanations from their doctor. The most frequent requests were to tell the cause of the symptoms, explain the curability and prognosis of the disease, and provide a treatment plan ($n = 41$).

> *"A doctor told me 'You have 2 years to go.' However, I was so healthy and could not imagine how this could be happening. I was in a panic because I did not know what to do next. Later, another doctor said 'Live as you lived. When you have pain, I will introduce you to a doctor for pain.' This explanation gave me back my life."* (#18 Male)

The second most frequent request was to provide information about their disease in simple words ($n = 12$).

> *"'There is no change, the same as the last time.' [He] does not explain anything. How is it the same? Is it good or bad? Why does he think so? If he based his diagnosis upon data, show them to me."* (#47 Male)

Patients with MPM exhibited great concern regarding examinations. They wanted their physician to explain

Table 2 Requests to Physicians by MPM Patients (217 requests; $N = 73$)

Categories / Subcategories		Times mentioned	% of Sample
1. Understandable explanation to meet the patient's needs		80	
1.1	Explain the cause of the symptoms, curability and prognosis of the disease, and provide a treatment plan	41	56.2
1.2	Use simple words	12	16.4
1.3	Explain the purpose, benefits, risk and results of examinations	10	14.0
1.4	Inform about all treatment options	10	14.0
1.5	Give advice about daily activities	3	4.1
1.6	Spend enough time on explanations	2	2.7
1.7	Confirm patient's understanding and allow them to ask questions	2	2.7
2. Patient-centered treatment		39	
2.1	Minimize the physical impact of treatment	11	15.1
2.2	Do not give up on the treatment	10	14.0
2.3	Respect patient's intention	9	12.3
2.4	Careful clinical assessment to not miss clinical signs of progression	9	12.3
3. Improvement of treatment and support systems for MPM		35	
3.1	Develop country-wide specialized care system	16	21.9
3.2	Develop new drugs	10	14.0
3.3	Improve information systems	9	12.3
4. Emotional support		32	
4.1	Be kind and cheerful	11	15.1
4.2	Sympathize with patient's anxiety	10	14.0
4.3	Have a reliable attitude	6	8.2
4.4	Empathy for victims of asbestos	3	4.1
4.5	Visit patient as often as possible	2	2.7
5. Customize "breaking the bad news"		24	
5.1	Tell everything including bad news	17	23.3
5.2	Do not inform about bad news	5	6.8
5.3	Customize the contents and the way of informing	2	2.7
6. Dedication to the treatment of MPM		7	
6.1	Confront intractable disease	4	5.4
6.2	Learn about MPM	3	4.1

MPM, malignant pleural mesothelioma

the purpose, benefits and risks, and results of examinations ($n = 10$). *"Explain concretely why I need an examination and do not forget to tell me the results, including my data compared with normal levels. Being well-informed and knowing my results eases my anxiety and gives me a sense of control. I feel that I am not that bad yet."* (#72 Male)

"I want to know if the chemotherapy worked on my tumor." (#10 Male)

In addition, the respondents wanted to know all the treatment options (n = 10). *"I need to know the latest treatment."* (#81 Male)

"Does any treatment work for patients with MPM?" (#89 Male)

Furthermore, some respondents wanted advice about preparation. ($n = 3$) "*My doctor let me know the benefits of palliative care and advised me to introduce it at an early stage. It was helpful because I had time to prepare."* (#72 Male)

Patients with MPM wanted their physician to spend enough time on explanations ($n = 2$). *"I know doctors are very busy. However, please understand that each patient needs time to understand what you said. Please do give us information so that we can understand one thing and then go further with the explanation. If you only explain things one-by-one, we never understand and get confused."* (#2 Male)

Finally, patients with MPM wanted their physician to confirm their understanding of the explanation and allow them to ask questions (n = 2). "*My doctor always asks me 'Is there anything you want to ask me?' You will never know how greatly I appreciate him. It is the greatest gift for patients."* (#45 Male)

Patient-centered treatment

Eleven patients requested the minimization of the physical impact of the treatment.

"*I do not want to suffer from heavy treatment. Just relieve my pain and let me stay at home until the last day."* (#78 Male)

Other respondents wanted their physician to not give up on treatment ($n = 10$). *"My doctor said I cannot receive chemotherapy any more, but I really want to receive treatment. I hope my doctor never gives up on my treatment … .I feel safe as long as I receive treatment."* (#75 Male)

Nine respondents commented that their physician should respect patients' intentions because they were not treated in the way they wanted. "*My doctor came to me and said, 'Move to another hospital. The members of the medical conference decided not to treat you here anymore.' How can they say that? Patients are completely reliant on their doctors; at the very least, treatment must include the patient's perspective."* (#120 Male)

"I hope my doctor not only treats my tumor but also takes care of me. I am not a box with cancer, but a living person." (#123 Male)

Another 9 patients with MPM wanted their physician to perform a careful clinical assessment to not miss clinical signs of progression ($n = 9$). *"I want my doctor to check very carefully to identify progress as soon as possible because MPM has no effective treatment. However, he repeats the same examination in a mechanical way. This makes me uneasy."* (#99 Male)

Need for improvement of treatment and a support system for MPM

Some patients described specific suggestions to improve support systems. The participants wanted the development of country-wide specialized care systems ($n = 16$), development of new drugs ($n = 10$), and improvement of information systems ($n = 2$).

"Because MPM is a difficult disease, I want to be treated by a specialist. I am disappointed that there is no specialist in my area." (#36 Male)

"Develop a test for early disease detection and develop a medical care service as soon as possible." (#12 Male)

"We need a liaison to consult with about MPM. It is so hard to collect information about the disease and hospitals for individual patients and their family." (#113 Male)

Emotional support

The participants wanted their physicians to be kind and cheerful ($n = 11$), to sympathize with patients' anxiety (n = 10), to have a reliable attitude ($n = 9$), and to visit the patient as often as possible (n = 2).

"No one can cheer me up but the doctor. I want my doctor to say, 'it is alright.' I was so happy when he said, 'Let's work together'." (#8 Male)

"When I am very anxious, I ask my doctor the same question many times. He says, 'I explained that before,

didn't I?' He is angry, and it makes me more anxious. I hope he allows me to ask questions as many times as I want." (#102 Male)

"My doctor pays attention to the computer and does not look at me. I hope he looks me in the eye." (#113 Male)

"My doctor came to me and smiled at me. It was only for a minute, but it worked and made me feel so relieved. I want him to come as often as possible." (#45 Male)

Furthermore, patients with MPM wanted to be considered as a victim of the use of asbestos and expected their physician to have empathy with victims of asbestos ($n = 3$).*"If I were to die from another cancer, I would not suffer like this. I am so resentful that I will die from asbestos; this feeling prevents me from facing my problems. How dare my doctor say 'patients with MPM are not the only ones who are suffering?'"* (#106 Male)

Customize "breaking the bad news"

Some of the participants wanted their physicians to inform them about everything including bad news ($n = 17$). In contrast, some did not want to be informed about bad news ($n = 5$) or requested that doctors customize the content and way of presenting bad news ($n = 2$).

"*I want my doctor to tell me everything, including bad news."* (#64 Male)

"I was already shocked to learn that I have MPM; it was cruel to tell me the time I had left." (#112 Male)

"Don't tell me the bad news. Just let me know something good." (#75 Female)

Dedication to the treatment of MPM

Patients wanted their physicians to confront the intractable disease ($n = 4$) and to learn more about MPM ($n = 3$).

"I hope my doctor has enough ambition and passion to battle the difficult disease of MPM." (#127 Male)

"My doctor's priority is to make money from us. They do not have the spirit to take care of us on our deathbed." (#120 Male)

"Doctors are the only hope for patients. I beg them to learn more about MPM." (#65 Male)

We compared these categorized requests according to MPM patients with or without palliative care. MPM patients who did not receive palliative care described more requests concerning understandable explanations, need for improvement of treatment and support systems, and dedication to the treatment of MPM than those who received palliative care. Among these requests, there was statistical significance concerning communication regarding the cause of the symptoms, curability and prognosis of the disease, and treatment plan ($p = 0.030$) (Additional file 2: Table S1).

Discussion

This study was part of a larger study about the QOL of MPM patients and sought to reveal their healthcare-related needs, particularly regarding interactions with their physician. Patients with MPM wanted their physicians to provide supportive communication, patient-centered care, and an attitude of dedication and commitment to their treatment. Most requests to their physicians concerned the content and method of communication. Patients wanted precise information about their condition, even if it was raw data from examinations. Patients also wanted the doctor to explain in laymen's terms how the condition would affect their daily lives. A previous study of patients with MPM also identified the difficulty of physicians in establishing rapport and engaging in a fruitful two-way communication [18]. The style of communication requested by patients with MPM was similar to studies of other cancers: a two-way exchange of information [19, 20]; and communication to provide the patient with data [21, 22]. Additionally, patients wanted to be allowed to ask questions [22], to be treated by physicians with insightful and empathetic attitudes [23, 24], and to be assured of on-going support [24].

The requests for emotional support were clearly evident in this study. The need for physicians to provide emotional support was documented in previous studies [23, 24], including one in which physicians were considered the most important source of psychological support [25]. In particular, our study indicated that MPM patients had an extra need for empathy due to their perception of being victims of asbestos. Additionally, the diagnosis of MPM engendered deep resentment given the circumstances surrounding their exposure to asbestos [10, 12, 26], feelings of injustice [12], and feelings of being traumatized [27].

This study also indicated that many patients with MPM wished for clear and complete information about their disease and its prognosis, while a smaller number of patients wanted the information to be delivered in a more indirect and vague manner. Yanagihara reported that Japanese patients wanted bad news to be minimized and to be conservative [28]. Patients with MPM were reported to have high levels of uncertainty and feelings of a lack of control leading to psychosocial distress since receiving their diagnosis [29]. Physicians should take

these differences into account when they present the diagnosis and prognosis of MPM to their patients.

It is fundamental that any treatment is the result of mutual decision-making between the patient and the physician. Our study demonstrated the frustration of some patients with MPM who could not receive chemotherapy due to a safety issue, leaving them feeling not cared for or abandoned. In addition, the current study indicated that patients who did not receive palliative care described more requests than those who received palliative care. One possible explanation would be a difficulty of physicians to tell the curability and prognosis of the disease to the patients. Miyashita et al. evaluated end-of-life cancer care in designated cancer centers and palliative care units and reported that care evaluation score was lower in designated cancer centers than in palliative care units concerning physical care by physician, help with decision making, and knowing what to expect about future condition [30]. Unfortunately, Japan has a limited care system for patients with MPM [31]. An integrated care and support system is urgently needed with a multidisciplinary approach that includes physicians, nurses, psychologists, support groups, and medical social workers.

Patients with MPM also expect their physicians to have updated knowledge about MPM and continued interest in searching for new methods of treatment. Patients certainly did not want their doctor to be stymied or to give up on them. Budych et al. previously indicated that patients with rare diseases prefer that their physician make most of the decisions regarding their care [32].

Limitations of this study include a low participation rate from hospitals (approximately 20%), although approximately half of the questionnaires were returned from the support groups. This study is also biased toward patients in the early stages of MPM and those with a good relationship with their physicians. However, given that other studies support the findings of this research, the risk of this bias is less of a concern. Further research should include a longitudinal, mixed-methods study that utilizes standardized instruments in addition to interviews with patients and physicians to shed more light on the specific needs of both groups.

Conclusion

This study indicated that patients with MPM had a variety of needs unmet by their physicians, even if they were in the early stages of the disease, and most had good relationships with their physicians. In addition, the current study indicated that patients who did not receive palliative care described more requests than those who received palliative care. Physicians should consider introducing shared decision-making and empathic verbal and nonverbal communication with dedication to the treatment of MPM. Physicians who provide care to MPM patients should receive training in both communication skills and stress management. A multidisciplinary care system that includes respiratory and palliative nurse specialists should be established for patients with MPM.

Additional file

Additional file 1: Questionnaire about quality of life of people with malignant pleural mesothelioma. (DOCX 17 kb)

Additional file 2: Table S1. (DOCX 22 kb)

Abbreviations

MPM: Malignant pleural mesothelioma; QOL: Quality of life

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Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

YNIO and NF made substantial contributions to the conception and design. YN, KA, JN, and KHara made substantial contributions to data acquisition. YN, IO, KA, KHotta, KK, and TK made substantial contributions to data analysis and interpretation. YN and NF were involved in drafting the manuscript. NF provided the final approval of the version to be published.

Ethical approval and consent to participate

This study was approved by the institutional review board of Okayama Rosai Hospital (approval no. 2017–22). This study was also approved by the institutional review board of each hospital or institution that distributed the questionnaire to their patients, according to their policy. The study was conducted based on the ethical principles of avoiding harm, voluntary participation, anonymity, and protection of privacy and personal information. The purpose, procedures, and confidentiality of the study were explained in written format. The participants were informed that nonparticipation would not disadvantage them. Return of the answered questionnaire was considered to constitute the patient's consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Small intestinal intussusception in an adult

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CLINICAL QUESTION

A 74-year-old man with a history of occupational asbestos exposure in a shipyard was diagnosed with lymphohistiocytoid mesothelioma (LHM). Systemic chemotherapy consisted of cisplatin, and pemetrexed was administered. After six courses of chemotherapy, multiple tumours had regressed. Six months after chemotherapy, the tumours on the pleura exhibited regrowth and partly invaded the liver. Salvage chemotherapies, including irinotecan and vinorelbine, were administered and his disease was stable for several months. Then he suddenly vomited and complained of abdominal pain. Contrast CT showed small intestine intussusception. Partial resection of the small intestine was performed. We found a tumour in the resected small intestine.

Review the high-quality, interactive digital Aperio slide at http://virtualacp.com/JCPCases/jclinpath-2017-204973/ and consider your diagnosis.

WHAT IS YOUR DIAGNOSIS?

A. Gastrointestinal stromal tumour
B. Leiomyosarcoma
C. Lymphohistiocytoid mesothelioma
D. Malignant schwanoma sheath tumour
E. Sarcomatous mesothelioma

The correct answer is after the discussion.

DISCUSSION

Recently, we reported a case of LHM.[1] The current report is of his subsequent clinical course.

Autopsy revealed multiple tumours on the pleura, peritoneum and pericardium. Metastasis to both lungs, the liver, stomach, colon, rectum, thyroid, left kidney, spleen and lumbar spine was also found. Histological examination revealed that these tumours showed similar aspects as of the tumour in the small intestine.

Pathological examination of the tumour in the small intestine revealed spindle-shaped cell proliferation with few lymphocyte infiltrations (figure 1). Immunostaining was positive for calretinin, CAM5.2, WT-1 and AE1/3, and negative for CK20, CD34 and desmin. Based on these findings, the tumour was diagnosed as a metastasis of malignant pleural mesothelioma (MPM), sarcomatous subtype. He died 2 months after the surgery due to the progression of MPM.

LHM was first reported in 1988.[2] Histologically, it is characterised by histiocytic tumour cell proliferation with lymphocyte infiltration. There are diverging opinions whether it is epithelioid or sarcomatous. Galateau-Sallé *et al*[3] suggested that the survival in patients with LHM is more like that of the epithelioid or biphasic subtype of MPM. On the other hand, Kawai *et al*[4] reported a patient diagnosed with LHM whose autopsy indicated sarcomatous mesothelioma.

It is still controversial whether LHM should be categorised as an epithelioid or sarcomatous subtype of MPM. The current case suggests that it is similar to the sarcomatous subtype.

The principal histological differential diagnoses of a tumour in the small intestine include leiomyosarcoma, gastrointestinal stromal tumour and neurogenic tumours.

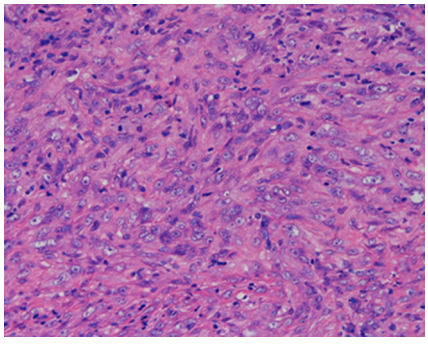

Figure 1 Pathological examination shows spindle-shaped cell proliferation with few lymphocyte infiltrations.

ANSWER

E. Sarcomatous mesothelioma

Take home messages

- Lymphohistiocytoid mesothelioma (LHM) is characterised by histiocytic tumour cell proliferation with lymphocyte infiltration.
- It is still controversial whether LHM should be categorised as an epithelioid or a sarcomatous subtype of malignant pleural mesothelioma.
- The current case suggests that it is similar to the sarcomatous subtype.

Handling editor Iskander Chaudhry.

Contributors KT, MA and NF treated the patient and drafted the paper. TO, JN, YM, YF, SW and SO contributed to data monitoring, and drafted and revised the paper. TI and HS contributed to pathological examination of the patient. TK approved the final work to be published.

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Clinical Trials: Immunotherapy

Clinical Cancer Research

Clinical Efficacy and Safety of Nivolumab: Results of a Multicenter, Open-label, Single-arm, Japanese Phase II study in Malignant Pleural Mesothelioma (MERIT)

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Abstract

Purpose: Malignant pleural mesothelioma (MPM) is a rare and aggressive malignancy with poor prognosis. Patients with MPM who do not respond to standard first-line chemotherapy have limited treatment options. We evaluated the efficacy and safety of nivolumab, an immune checkpoint inhibitor, for the treatment of advanced or metastatic MPM.

Patients and Methods: Japanese patients with unresectable, advanced, or metastatic MPM resistant or intolerant to ≤2 regimens of chemotherapy and ≥1 measurable lesion (s) were enrolled. Patients received nivolumab 240 mg intravenously every 2 weeks until progressive disease or unacceptable toxicity. The primary endpoint was objective response rate by central assessment according to the Modified Response Evaluation Criteria in Solid Tumors. Adverse events (AEs) and treatment-related AEs (TRAEs) were evaluated.

Results: Thirty-four patients were enrolled between July 2016 and October 2016. Median follow-up was 16.8 (range: 1.8–20.2) months. Ten (29%, 95% confidence interval, 16.8–46.2) patients showed a centrally assessed objective response. The objective response rates were 26% (7/27), 67% (2/3), and 25% (1/4) patients for epithelioid, sarcomatoid, and biphasic histologic subtypes, respectively. Median duration of response was 11.1 months with a 68% disease control rate. Median overall survival and progression-free survival were 17.3 and 6.1 months, respectively. The objective response rate was 40% with programmed death-ligand 1 expression ≥1% and 8% with <1%. Thirty-two patients (94%) experienced AEs and 26 (76%) experienced TRAEs.

Conclusions: Nivolumab met the primary endpoint as second- or third-line treatment for patients with MPM and showed promising efficacy with manageable toxicity.

See related commentary by Mansfield and Zauderer, p. 5438

Introduction

Malignant pleural mesothelioma (MPM) is a rare and aggressive malignancy, responsible for 1,550 malignancy-related deaths in Japan in 2016 (1). In Japan, MPM is more common in men than women given their increased likelihood of occupational exposure to asbestos, and MPM most commonly affects elderly people (median age, 68 years; ref. 2, 3), in part, because of the long latency of the effects of asbestos exposure, which typically occur 30–50 years postexposure (4).

The median survival for patients with MPM is 7.9 months based on studies of newly diagnosed patients in Japan (2, 5).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Malignant pleural mesothelioma (MPM) is a rare malignancy with poor prognosis, and patients who do not respond to first-line chemotherapy have limited treatment options. In this (multicenter, open-label, single-arm, Japanese phase II study in malignant pleural mesothelioma) study, we evaluated the efficacy and safety of nivolumab, an immune checkpoint inhibitor, for the treatment of advanced or metastatic MPM in patients intolerant or resistant to ≤2 regimens of chemotherapy. Nivolumab yielded an objective response rate of 29%, median overall survival of 17.3 months, and progression-free survival of 6.1 months. Its efficacy appeared promising in all histologic subtypes (epithelioid, sarcomatoid, and biphasic) and in PD-L1 ≥1% and <1% patients, although our sample size was small. Nivolumab showed manageable toxicity. While our study lacked a comparator, our findings reflect those of similar trials and suggest that nivolumab provides a clinical benefit and is a potential second- or third-line treatment option for MPM.

Most patients are diagnosed with advanced-stage MPM and receive first-line chemotherapy with pemetrexed and cisplatin (PC). This regimen provides a survival benefit over cisplatin alone (12.1 months and 9.3 months, respectively; ref. 6). Carboplatin is less toxic and more convenient than cisplatin, and combination therapy for MPM with carboplatin and pemetrexed has been evaluated, yielding an overall survival (OS) and progression-free survival (PFS) comparable with that of PC (7–9). Furthermore, adding bevacizumab to PC significantly improved survival benefit by 2.7 months in comparison with PC (10). However, patients with MPM who do not respond to first-line treatment with PC have no standard treatment. National Comprehensive Cancer Network (NCCN) guidelines recommend treatment with nivolumab with or without ipilimumab (11) and pembrolizumab is also a treatment option, but no drug had yet been approved for second-line treatment of MPM before starting this study.

Programmed death ligand 1 (PD-L1) is the ligand to the human programmed death-1 (PD-1) receptor. It is expressed in the tumors of patients with MPM (12–15): in 40% of patients with MPM according to one clinical investigation (12) and in 70% according to data from archived patient tissue (13). PD-L1 expression is correlated with a poor prognosis in MPM (12–15). Nivolumab is a human mAb to the PD-1 receptor that inhibits the interaction between PD-1 and its ligands, PD-L1 or PD-L2. Furthermore, nivolumab is approved for the treatment of various subtypes of malignancies (16).

We hypothesized that nivolumab would be a potential second- or third-line treatment option for MPM. Thus, the multicenter, open-label, single-arm, Japanese phase II study in MPM (MERIT) study evaluated the clinical efficacy and safety of nivolumab in Japanese patients with advanced or metastatic MPM resistant/intolerant to ≤2 regimens of platinum-based chemotherapy in combination with pemetrexed. This study started before the NCCN guideline recommended nivolumab for second-line treatment of MPM (11).

Patients and Methods

Study design and patients

This was a multicenter, open-label, single-arm phase II study conducted from June 16, 2016 to March 14, 2018 (data cut-off date), at 15 centers in Japan (Supplementary Table S1). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The study protocol was reviewed and approved by the institutional review board of each site before study initiation. This study is registered with clinicaltrials.jp (JapicCTI-163247). All patients provided written informed consent.

Selection and description of patients

Eligible patients were men and women ages ≥20 years with histologically confirmed MPM, unresectable advanced or metastatic MPM without surgery, or MPM resistant or intolerant to ≤2 regimens of chemotherapy including platinum-based combination therapy with pemetrexed; and had ≥1 measurable lesion(s) as defined in the Modified Response Evaluation Criteria in Solid Tumors (mRECIST) in MPM (17) and confirmed by imaging within 14 days before enrollment, available tumor tissue samples (fresh or archival) for analysis of PD-L1 expression, and an Eastern Cooperative Oncology Group performance status of 0 or 1. Main exclusion criteria were severe hypersensitivity reactions to any other drug, including antibody products; concurrent autoimmune disease or a history of chronic or recurrent autoimmune disease; multiple primary cancers; brain or meningeal metastases; current or history of interstitial lung disease or pulmonary fibrosis diagnosed on the basis of imaging or clinical findings; and previous treatment with nivolumab, anti-PD-1 antibody, anti-PD-L1, or PD-L2, or any other therapeutic antibodies or pharmacotherapies for T-cell regulation.

Procedures

Patients received 240-mg nivolumab via intravenous 30-min infusion every 2 weeks on day 1 of each cycle until any criterion for nivolumab discontinuation was met (Supplementary Table S2). Neither dose nor administration mode of nivolumab could be adjusted. Therapies prohibited during the study period included immunosuppressants, corticosteroids at doses exceeding 10 mg/day prednisone equivalent, antitumor therapies (e.g., chemotherapy, molecular-targeted therapy, and immunotherapy), concurrent radiotherapy, pleurodesis, and surgical therapies for malignant tumors.

Patients underwent tumor imaging by computed tomography or magnetic resonance imaging every three cycles. The target lesions in pleura were measured uni-dimensionally as the largest tumor thickness perpendicular to the chest wall or mediastinum according to modified RECIST (17); those in nonpleura were measured according to RECIST version 1.1.

PD-L1 expression analysis was performed in a central laboratory (Cancer Genetics, Inc.) using (fresh or archival) tumor tissue samples with 28-8 antibody (Dako). One or more formalin-fixed, paraffin-embedded (FFPE) blocks of tumor tissue samples collected by core needle biopsy, excisional biopsy, or incisional biopsy of ≥5 FFPE unstained slide samples (serial tissue sections) were analyzed for PD-L1 status. Each tumor tissue sample was required to contain ≥100 evaluable tumor cells. PD-L1–positive

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status was defined as membranous staining in ≥1% of tumor cells. Samples were classified as "not evaluable (NE)" if the biological conditions of the sample rendered the stained cell membranes difficult to assess, even if the samples otherwise met the evaluation criteria.

Outcomes

The primary endpoint was centrally assessed objective response according to mRECIST. The objective response rate was defined as the proportion of patients whose best overall response was complete response (CR) or partial response (PR). Secondary endpoints were investigator-assessed objective response rate and percent change in the sum of tumor sizes of target lesions; disease control rate, OS, PFS, duration of response, time to response, and best overall response assessed centrally. In addition, subgroup analyses of tumor response, PFS, OS by PD-L1 expression (<1% and ≥1%), and histologic subtype were performed.

OS was defined as the time from the first nivolumab dose to death from any cause. PFS was defined as the time from the first nivolumab dose to progressive disease (PD) or death from any cause. Disease control rate was the percentage of patients whose best overall response was CR, PR, or stable disease (SD).

Adverse events (AEs) and treatment-related AEs (TRAEs) were monitored throughout the study period and graded according to the Japanese translation (Japan Clinical Oncology Group edition) of the NCI Common Terminology Criteria for Adverse Events, version 4.0. AEs of special interest were prespecified as endocrine disorders, gastrointestinal toxicity, hepatotoxicity, pulmonary toxicity, nephrotoxicity, skin toxicity, and hypersensitivity/infusion reactions.

Statistical analysis

As there was no available standard treatment for the target population, the lower threshold for response was set at 5%, and an expected objective response rate of 19% was used for this study. We calculated that ≥29 patients would be required to detect a significant difference in the objective response rate with a power of 80% and a one-sided significance level of 0.025. To account for the estimated 10% dropout rate, we planned to recruit 32 patients. The full analysis set was used for the analysis of the efficacy endpoints, and the safety analysis set for the analysis of baseline demographic and clinical characteristics and safety endpoints. Frequency distribution and summary statistics were used for baseline characteristics. The objective response and disease control rates and their two-sided 95% confidence intervals (CI) were calculated. Medians and two-sided 95% CIs for OS, PFS, and duration of response were calculated using the Kaplan–Meier method. OS and PFS rates, and their two-sided 95% CIs, were calculated at 6 and 12 months depending on the duration of follow-up. The percentages of patients with best overall response of CR, PR, SD, PD, and NE were calculated. Statistical analyses were performed with SAS version 9.3 (SAS Institute Inc.).

Results

Most patients were male (29/34 patients, 85%), with a median age of 68.0 years; 27/34 patients (79%) had an epithelioid subtype (Table 1). Patients received a median of 12.5 (range, 1–42) doses; the median duration of treatment was 6.8 (range, 0.03–19.1) months. The median relative dose intensity was 96% (range, 62%–112%). Six patients (18%) were still on treatment, and 28 (82%) discontinued treatment at data cutoff. The reasons for discontinuation included PD (22 patients, 65%); unequivocal clinical progression attributable to PD (5 patients, 15%); development of grade ≥2 interstitial lung disease or pneumonitis (4 patients, 12%); lack of nivolumab administration for 6 weeks due to AE onset (2 patients, 6%); and continuation of treatment judged as inappropriate by the principal investigator (1 patient, 3%). Some patients had more than one reason for discontinuation. All 34 patients were included in both the full and safety analysis sets. Median follow-up was 16.8 (range, 1.8–20.2) months.

Table 1. Baseline demographic and clinical characteristics

	Nivolumab *N* = 34
Sex	
Male	29 (85)
Female	5 (15)
Age, years, median (range)	68.0 (43–78)
Body mass index, kg/m^2, median (range)	22.1 (15.8–29.0)
Number of prior treatment(s)	
1	24 (71)
2	10 (29)
Performance status	
0	13 (38)
1	21 (62)
Previous systemic therapy	
First line	
Pemetrexed + cisplatin/carboplatin	31 (91)
Pemetrexed + cisplatin + BBI608	2 (6)
Pemetrexed + cisplatin + bevacizumab	1 (3)
Second line	
Gemcitabine	3 (9)
Pemetrexed + cisplatin/carboplatin	3 (9)
Pemetrexed	2 (6)
Other	2 (6)
PD-L1 status	
≥1%	20 (59)
<1%	12 (35)
NE	2 (6)
Histological subtype	
Epithelioid	27 (79)
Biphasic	4 (12)
Sarcomatoid	3 (9)

NOTE: Data are *n* (%), unless otherwise stated.

Ten (29%; 95% CI, 16.8–46.2) of 34 patients had an objective response by central assessment (Table 2), and all were PR. The response rate by site according to mRECIST was identical. The disease control rate was 68% (95% CI, 50.8–80.9; Table 2). Regarding the best overall response, 10 (29%) patients had PR, 13 (38%) had SD, 9 (26%) had PD, and 2 (6%) were NE (Table 2). In addition, central review confirmed that 1 patient had no measurable lesions.

The Kaplan–Meier curves for OS and PFS are shown in Fig. 1A and B. Median OS was 17.3 months (95% CI, 11.5–not reached), with OS rates of 85% (95% CI, 68.2–93.6) and 59% (95% CI, 40.6–73.2) at 6 and 12 months, respectively. Median PFS was 6.1 months (95% CI, 2.9–9.9), with PFS rates of 52% (95% CI, 33.5–66.9) and 32% (95% CI, 16.4–47.9) at 6 and 12 months, respectively. At data cutoff, 3 of 10 patients (30%) had an ongoing response. The median duration of response was 11.1 months (95% CI, 3.5–16.2), with median time to response of 2.63 (range, 1.0–6.9) months. Among responders, the median reduction in target lesions from baseline (depth of response) was 61% (interquartile range, 48–72).

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Table 2. Efficacy of nivolumab

	N	Tumor response (95% CI)
Objective response rate ($n = 34$)	10	29% (16.8–46.2)
Epithelioid ($n = 27$)	7	26% (13.2–44.7)
Biphasic ($n = 4$)	1	25% (4.6–69.9)
Sarcomatoid ($n = 3$)	2	67% (20.8–93.9)
Disease control rate ($n = 34$)	23	68% (50.8–80.9)
Best overall response rate ($n = 34$)		
CR	0	0% (0.0–10.2)
PR	10	29% (16.8–46.2)
SD	13	38% (23.9–55.0)
PD	9	26%
NE	2	6%

NOTE: All results are from the central assessment according to mRECIST. 95% CIs were calculated using the Wilson method; 95% CIs were not calculated for the PD or NE categories.

Tumor shrinkage was observed in all histologic subtypes, especially in 6 of 7 patients with either sarcomatoid or biphasic histologic subtype, slight tumor growth was observed in 1 remaining patient. Therefore, the disease control rate in sarcomatoid/biphasic patients was 100% (Fig. 2A). Tumor shrinkage was observed, regardless of PD-L1 status. Among PD-L1 evaluable patients, tumor shrinkage occurred in 14 of 20 (70%) patients with PD-L1 expression ≥1% and 4 of 12 (33%) patients with PD-L1 expression <1% (Fig. 2A). A long duration of response was recorded with a median duration of 11.1 months (95% CI, 3.5–16.2; Fig. 2B). Patients with tumor shrinkage tended to maintain the tumor response (Fig. 3).

The objective response rate by histologic subtype is reported in Table 2. The objective response rates were 26%, 67%, and 25% for epithelioid, sarcomatoid, and biphasic histologic

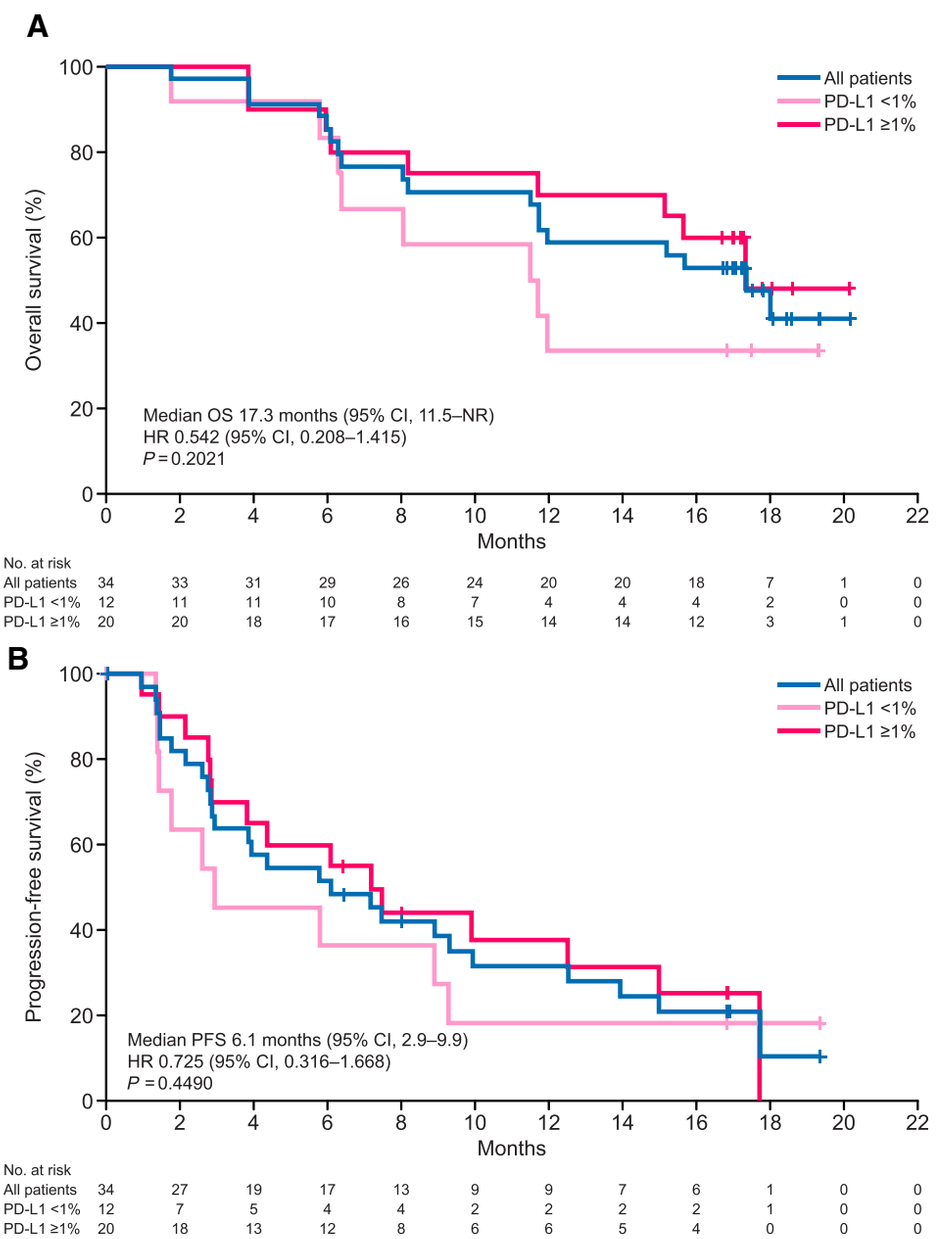

Figure 1.
Kaplan–Meier curves for OS (**A**) and PFS (**B**), for all patients and according to PD-L1 expression status. Median OS and PFS were calculated using values for all patients. HRs denote a comparison between the PD-L1 ≥1% and <1% groups. NR, not reached.

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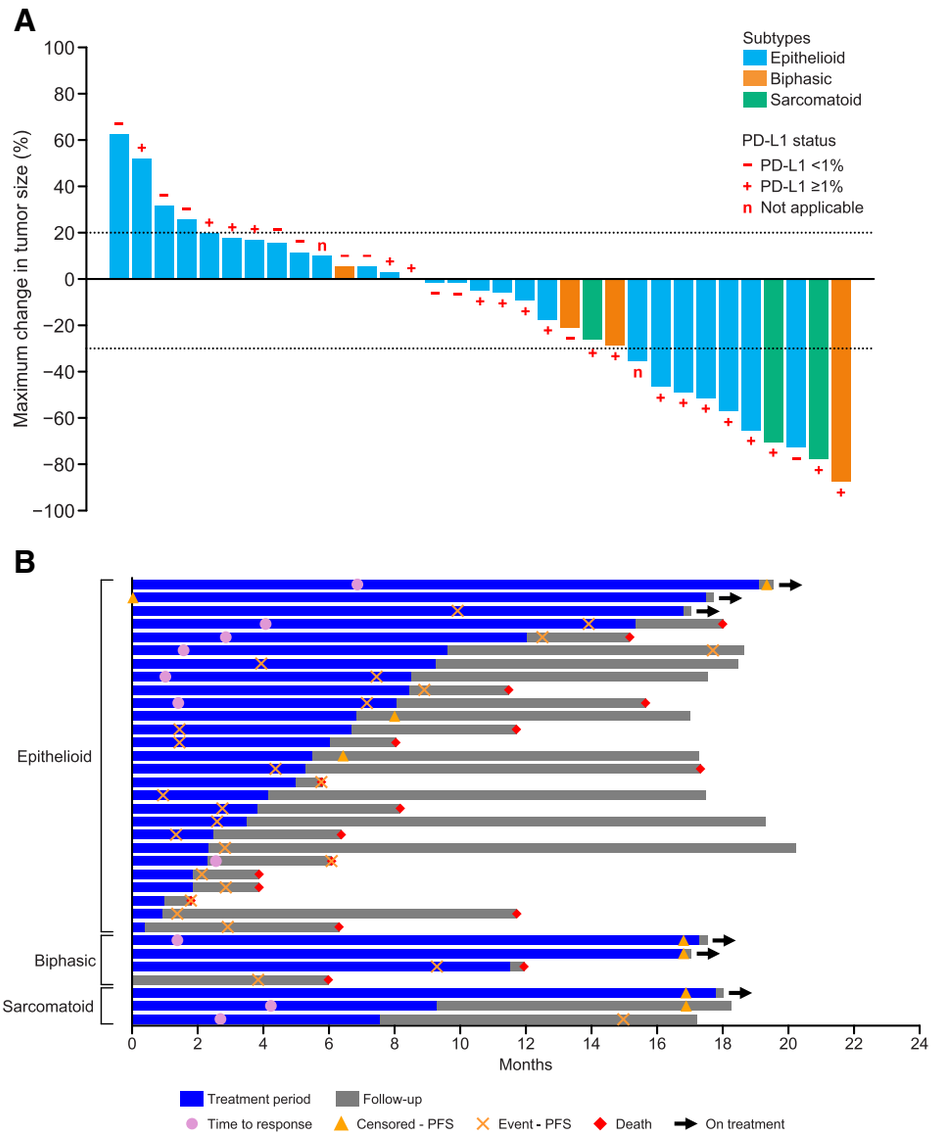

Figure 2.

Percent change in the sum of tumor size by histologic subtype and PD-L1 expression status. The waterfall plot shows the maximum percentage changes from baseline in target lesions by histologic subtype and PD-L1 expression status (**A**), with the length and direction of the bars indicating either an increase or decrease in the target lesion size of each patient. The swimmer's plot (**B**) shows treatment exposure and response duration, with bar length corresponding to the duration of treatment for each patient. Central assessment was performed with lesions defined as the largest tumor thickness perpendicular to the chest wall or mediastinum measured uni-dimensionally according to mRECIST.

subtypes, respectively. The subgroup analysis of OS and PFS by histologic subtype exhibited trends, with prolonged OS and PFS for patients with nonepithelioid subtype (Supplementary Fig. S1A and B). Results of tumor response analysis by PD-L1 expression are shown in Supplementary Table S3. The objective response rate differed by PD-L1 expression (40% for ≥1% vs. 8% for <1%, respectively). Similar trends were observed among patients with different PD-L1 expression levels (≥5% vs. <5% and ≥10% vs. <10%). The subgroup analysis of OS and PFS by PD-L1 status exhibited trends, with prolonged OS and PFS for patients with PD-L1 ≥1% versus <1% [hazard ratio (HR) for OS 0.542 (95% CI, 0.208–1.415; $P = 0.2021$); HR for PFS 0.725 (95% CI, 0.316–1.668; $P = 0.4490$); Fig. 1A and B].

All-cause AEs occurring in ≥5% of patients are shown in Table 3. Most patients (94%) experienced AEs and 16 (47%) patients experienced grade ≥3 AEs. A total of 26 patients (76%) experienced TRAEs, and 11 patients (32%) experienced Grade ≥3 TRAEs. Serious AEs occurred in 14 patients (41%), with 11 patients (32%) having serious TRAEs. Four patients (12%) experienced AEs leading to study treatment discontinuation [two events of interstitial pneumonia (1, grade 2; 1, grade 3) and two events of pneumonitis (both grade 3)]. No fatal AEs occurred between study start and either 28 days after the last nivolumab dose or the start of poststudy treatment. Regarding TRAEs with an incidence of ≥10%, rash occurred in 6 patients (18%); lipase increased, 5 (15%); and diarrhea and amylase increased, 4 each (12%).

The following AEs of special interest occurred: type 1 diabetes mellitus in 1 patient (3%), hypopituitarism in 1 patient (3%), hypothyroidism in 2 patients (6%); and blood thyroid stimulating hormone decreased, blood thyroid stimulating hormone increased, and thyroid function test abnormal in 1 patient (3%) each; diarrhea in 6 (18%) patients; gamma-glutamyltransferase increased in 2 patients (6%); alanine aminotransferase increased, aspartate aminotransferase increased, blood bilirubin increased, and blood alkaline phosphatase

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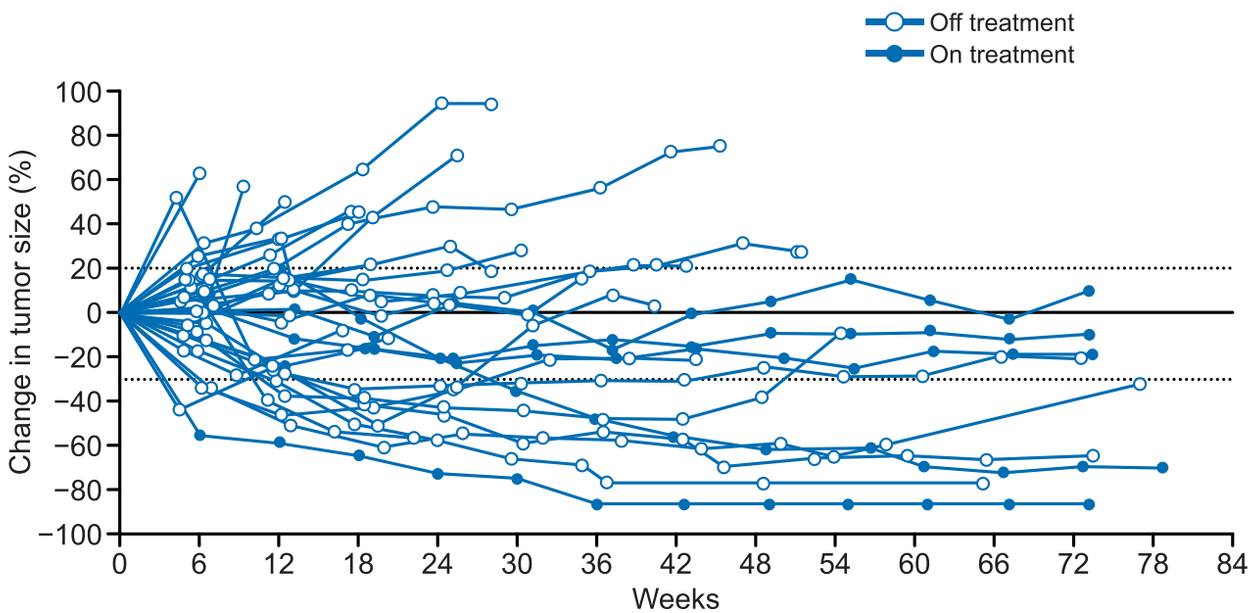

Figure 3.
Percent change in target tumor size over time. Central assessment was performed according to mRECIST.

increased in 1 patient (3%) each; interstitial lung disease and pneumonitis in 2 patients (6%) each; blood creatinine increased in 1 patient (3%); rash in 6 patients (18%), rash maculopapular in 2 patients (6%), and blister, eczema, rash pruritic, skin exfoliation, and urticaria in 1 patient (3%) each; and hypersensitivity in 1 patient (3%). Grade 3–4 AEs of special interest were diarrhea, gamma-glutamyltransferase increased, and pneumonitis in 2 patients (6%) each, and type 1 diabetes mellitus, hypopituitarism, alanine aminotransferase increased, aspartate aminotransferase increased, interstitial lung disease, and rash and hypersensitivity in 1 patient each (3%).

Discussion

MPM is a very aggressive malignancy with a poor prognosis. To develop better therapies for mesothelioma, recent research has focused on the role of immune cells within the tumor microenvironment. Treatment with immune checkpoint inhibitors, which reactivate immune responses that are silenced by immune checkpoints, has shown promising results (18).

The present results suggest that patients with advanced or metastatic MPM resistant or intolerant to the standard treatment may benefit from treatment with nivolumab. Previous studies of standard treatment in advanced or recurrent MPM reported response rates of 0%–2% with placebo or best supportive care and 0%–4.5% with investigational products (19–21). Efficacy of nivolumab for pretreated MPM was reported in previous studies (MAPS2 and NivoMes trials; ref. 22, 23). In addition, the KEYNOTE-028 study showed an objective response rate (investigator assessed according to RECIST guideline, version 1.1) of 20% (95% CI, 6.8–40.7) in previously treated patients with PD-L1–positive MPM receiving pembrolizumab 10 mg/kg every 2 weeks (24). In this study, an objective response rate of 29% was confirmed by central assessment according to mRECIST in patients with MPM and was concordant with the results of other similar studies (22–24). These results suggest that anti-PD-1 antibodies have a high potential for becoming a new treatment option for MPM.

Sarcomatoid or biphasic histologic subtypes are known predictors of poor prognosis (25), and PC therapy has little effect on these histologic subtypes (26). In this study, the objective response in patients with sarcomatoid and biphasic histologic subtypes was 2 of 3 and 1 of 4 patients, respectively. These results indicate that nivolumab had a beneficial effect in these histologic subtypes for which no previous treatment has been shown to be effective. This further supports the use of immune checkpoint inhibitors as potential treatment options to manage MPM. Interestingly, the PD-L1 expression rate was $\geq$50% in the three responders with sarcomatoid and biphasic histologic subtype (data not shown). However, these results should be interpreted with caution as there were only 7 patients with these subtypes. Further study in a larger number of patients with these histologic subtypes is warranted to confirm our findings.

Previous studies have shown that positive PD-L1 expression status has been associated with worse survival outcomes compared with negative PD-L1 expression status (14, 15). In this study, both PD-L1–positive and PD-L1–negative patients responded to nivolumab, and although not significant, differences in OS and PFS with PD-L1 expression status favored positive PD-L1 expression. While promising, these results must be considered in the context of the study design and size, and the fact that the PD-L1 analysis was exploratory. A greater number of patients showing PD-L1 expression responded to nivolumab, although some patients without PD-L1 expression also showed responses. This study was not powered to study differences in response or survival between categories of PD-L1 expression, but this is a critical area for future study in larger, comparative trials.

Patients who have PD after initial chemotherapy are generally expected to have a poor prognosis, advanced symptoms, and worsened condition compared with chemotherapy-naïve

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Table 3. AEs

	Nivolumab *N* = 34	
	Any grade	Grade 3–4
Any AEs	32 (94)	16 (47)
Most common AEs by preferred term (≥5% of patients)		
Viral upper respiratory tract infection	10 (29)	0 (0)
Weight decreased	7 (21)	0 (0)
Diarrhea	6 (18)	2 (6)
Rash	6 (18)	1 (3)
Pyrexia	6 (18)	0 (0)
Lipase increased	5 (15)	4 (12)
Stomatitis	5 (15)	1 (3)
Nausea	5 (15)	0 (0)
Amylase increased	4 (12)	2 (6)
Decreased appetite	4 (12)	2 (6)
Arthralgia	4 (12)	0 (0)
Vomiting	3 (9)	0 (0)
Fatigue	3 (9)	0 (0)
Malaise	3 (9)	0 (0)
Upper respiratory tract infection	3 (9)	0 (0)
Gamma-glutamyltransferase increased	2 (6)	2 (6)
Pneumonitis	2 (6)	2 (6)
Anemia	2 (6)	1 (3)
Hypophosphatemia	2 (6)	1 (3)
Interstitial lung disease	2 (6)	1 (3)
Hypothyroidism	2 (6)	0 (0)
Constipation	2 (6)	0 (0)
Dental caries	2 (6)	0 (0)
Mucosal inflammation	2 (6)	0 (0)
Edema peripheral	2 (6)	0 (0)
Lymphocyte count decreased	2 (6)	0 (0)
Hyperkalemia	2 (6)	0 (0)
Hypoalbuminemia	2 (6)	0 (0)
Myalgia	2 (6)	0 (0)
Dyspnea	2 (6)	0 (0)
Pneumothorax	2 (6)	0 (0)
Rash maculo-papular	2 (6)	0 (0)
AEs leading to discontinuation of study treatment	4 (12)	3 (9)
AEs leading to interruption of study treatment	15 (44)	10 (29)

NOTE: Data are presented as *n* (%).

patients. In fact, a PFS of 1.6–1.7 months and an OS of 5.4–4.9 months was reported in patients with MPM resistant/intolerant to standard treatment who received single-agent vinorelbine, single-agent gemcitabine, or both agents (27). Conversely, in this study, the median PFS and median OS were 6.1 months and 17.3 months, respectively, which were comparable with the results of previous studies in patients requiring second- and third-line treatment with nivolumab with or without ipilimumab (22, 23) and pembrolizumab (24). These findings suggest that nivolumab provides a clinical benefit and could be considered an option for second- or third-line treatment for MPM.

Regarding the safety profile, of the 34 patients receiving nivolumab, 32 (94%) and 26 (76%) patients experienced AEs and TRAEs, respectively. No deaths related to AEs were reported. Nivolumab is approved for the treatment of various cancer types and has been administered to many patients. In our opinion, the safety profile of nivolumab in this study did not differ greatly from that in other cancer types for which nivolumab has already been approved.

In conclusion, the primary endpoint was met in patients with advanced or metastatic MPM resistant or intolerant to maximally two regimens of chemotherapy including platinum-based combination therapy with pemetrexed who received nivolumab as second- or third-line treatment. Nivolumab showed a promising overall response rate of 29% and appeared to yield encouraging PFS and OS results across a range of histologic subtypes, and in patients with PD-L1 expression. Nivolumab had a manageable toxicity profile. Adequately powered, randomized, controlled trials are needed before definitive conclusions can be drawn regarding the survival benefits of nivolumab.

Disclosure of Potential Conflicts of Interest

M. Okada reports receiving commercial research grants from Ono Pharmaceutical and Bristol-Myers Squibb and speakers bureau honoraria from Ono Pharmaceutical and Bristol-Myers Squibb. T. Kijima reports receiving speakers bureau honoraria from Ono Pharmaceutical. K. Aoe reports receiving commercial research grants from Ono Pharmaceutical, Bristol-Myers Squibb, AstraZeneca, MSD, Novartis, and Eli Lilly. T. Kato reports receiving speakers bureau honoraria from AbbVie, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Eli Lilly, Kyowa Hakko Kirin, Merck Serono, MSD, Nitto Denko, Novartis, Ono Pharmaceutical, Pfizer, Sumitomo Dainippon Pharma, Taiho Pharmaceutical, Takeda Pharmaceutical, and F. Hoffman-La Roche, is a consultant/advisory board member for AstraZeneca, Eli Lilly, MSD, and Chugai Pharmaceutical, and reports that an immediate family member is an employee of Eli Lilly. N. Fujimoto reports receiving speakers bureau honoraria from Ono Pharmaceutical, Bristol-Myers Squibb, Nippon Boehringer Ingelheim, Chugai Pharmaceutical, Hisamitsu Pharmaceutical, Daiichi Sankyo, and Astellas Pharma, and is a consultant/advisory board member for Ono Pharmaceutical, Bristol-Myers Squibb, Nippon Boehringer Ingelheim, and Kyorin Pharmaceutical. K. Nakagawa reports receiving commercial research grants from MSD, Eli Lilly Japan, Bristol-Myers Squibb, Taiho Pharmaceutical, Ono Pharmaceutical, Chugai Pharmaceutical, Merck Serono, AstraZeneca, Astellas Pharma, Novartis Pharma, Pfizer Japan, and Nippon Boehringer Ingelheim, other commercial research support from ICON Japan, Takeda Pharmaceutical, PAREXEL International, IQVIA Services Japan, A2 Healthcare, AbbVie, SymBio Pharmaceuticals, EP-CRSU, Linical, Otsuka Pharmaceutical, EPS International, Quintiles, CMIC Shift Zero, Eisai, Kissei Pharmaceutical, Kyowa Hakko Kirin, EPS, Daiichi Sankyo, Bayer Yakuhin, inVentiv Health Japan, Gritstone Oncology, GlaxoSmithKline, Yakult Honsha, and Covance, and speakers bureau honoraria from MSD, Bristol-Myers Squibb, Eli Lilly Japan, Ono Pharmaceutical, Chugai Pharmaceutical, AstraZeneca, Astellas Pharma, Novartis Pharma, Nippon Boehringer Ingelheim, Pfizer Japan, Takeda Pharmaceutical, SymBio Pharmaceuticals, Daiichi Sankyo, Kyorin Pharmaceutical, CareNet, Nichi-Iko Pharmaceutical, Hisamitsu Pharmaceutical, Yodosha, Clinical Trial Co., MEDICUS SHUPPAN Publishers, AYUMI Pharmaceutical, Nikkei Business Publications, Thermo Fisher Scientific, Nanzando, Medical Review Co., Yomiuri Telecasting, and Reno. Medical. T. Hida reports receiving speakers bureau honoraria from Ono Pharmaceutical, Bristol-Meyers Squibb, Chugai Pharmaceutical, AstraZeneca, and MSD. S. Oizumi reports receiving other commercial research support from Bristol-Myers Squibb, Kyowa Hakko Kirin, Merck Serono, and Pfizer, and speakers bureau honoraria from AstraZeneca and Eli Lilly. F. Imamura reports receiving speakers bureau honoraria from AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceutical, Eli Lilly Japan, MSD, Ono Pharmaceutical, and Taiho Pharmaceutical. T. Takahashi reports receiving speakers bureau honoraria from Ono Pharmaceutical, MSD, and Chugai Pharmaceutical. M. Takenoyama reports receiving commercial research grants and speakers bureau honoraria from Bristol-Myers Squibb, AstraZeneca, Chugai Pharmaceutical, MSD, and Ono Pharmaceutical. H. Tanaka reports receiving speakers bureau honoraria from Ono Pharmaceutical and Bristol-Myers Squibb. Y. Ohe reports receiving commercial research grants from AstraZeneca, Chugai Pharmaceutical, Eli Lilly, Ono Pharmaceutical, Bristol-Myers Squibb, Kyorin Pharmaceutical, Dainippon Sumitomo Pharma, Pfizer, Taiho Pharmaceutical, Novartis, Kissei Pharmaceutical, Ignyta, Takeda Pharmaceutical, Daiichi Sankyo Pharmaceutical Co., Ltd, and Janssen, speakers bureau honoraria from AstraZeneca, Chugai Pharmaceutical, Eli Lilly, Ono Pharmaceutical, Bristol-Myers Squibb, Boehringer Ingelheim, Bayer, Pfizer, MSD, and Taiho Pharmaceutical, and is a consultant/advisory board member for AstraZeneca, Chugai Pharmaceutical, Ono Pharmaceutical, Bristol-Myers Squibb, Kyorin Pharmaceutical, Celltrion, and Amgen. No potential conflicts of interest were disclosed by the other authors.

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Clinical Cancer Research

Clinical Efficacy and Safety of Nivolumab: Results of a Multicenter, Open-label, Single-arm, Japanese Phase II study in Malignant Pleural Mesothelioma (MERIT)

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RESEARCH ARTICLE Open Access

Serum levels of the chemokine CCL2 are elevated in malignant pleural mesothelioma patients

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Abstract

Background: Malignant pleural mesothelioma (MPM) is a debilitating disease of the pleural cavity. It is primarily associated with previous inhalation of asbestos fibers. These fibers initiate an oxidant coupled inflammatory response. Repeated exposure to asbestos fibers results in a prolonged inflammatory response and cycles of tissue damage and repair. The inflammation-associated cycles of tissue damage and repair are intimately involved in the development of asbestos-associated cancers. Macrophages are a key component of asbestos-associated inflammation and play essential roles in the etiology of a variety of cancers. Macrophages are also a source of C-C motif chemokine ligand 2 (CCL2), and a variety of tumor-types express CCL2. High levels of CCL2 are present in the pleural effusions of mesothelioma patients, however, CCL2 has not been examined in the serum of mesothelioma patients.

Methods: The present study was carried out with 50 MPM patients and 356 subjects who were possibly exposed to asbestos but did not have disease symptoms and 41 healthy volunteers without a history of exposure to asbestos. The levels of CCL2 in the serum of the study participants was determined using ELISA.

Results: Levels of CCL2 were significantly elevated in the serum of patients with advanced MPM.

Conclusions: Our findings are consistent with the premise that the CCL2/CCR2 axis and myeloid-derived cells play an important role in MPM and disease progression. Therapies are being developed that target CCL2/CCR2 and tumor resident myeloid cells, and clinical trials are being pursued that use these therapies as part of the treatment regimen. The results of trials with patients with a similar serum CCL2 pattern as MPM patients will have important implications for the treatment of MPM.

Keywords: Asbestos, Cancer, Malignant pleural mesothelioma, CCL2

Background

A causal association between exposure to at least some types of asbestos and lung carcinomas and malignant pleural mesothelioma (MPM) has been long recognized [1], and in 2012 the WHO/International Agency for Research on Cancer (IARC, Lyon) classified all forms of asbestos (chrysotile, crocidolite, amosite, tremolite, actinolite, and anthophyllite) as carcinogenic to humans [2]. The 2014 updated Helsinki Criteria notes that while the use of asbestos is banned in many industrialized countries, the global production of asbestos remains at over two million metric tons a year, with an estimated 125 million people being exposed to asbestos in the workplace [3]. Furthermore, workers engaged in cleaning debris at sites of natural disasters and workers involved in demolition work may be exposed to asbestos. For example, asbestos-related disease is predicted to be significant in workers engaged in debris cleaning operations after the Great Hanshin Earthquake that occurred in Japan in 1995. Worldwide, asbestos exposure results in

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an estimated 255,000 deaths annually, with a significant fraction (over 30,000 in 2016) of these deaths due to mesothelioma [4]. In Japan, the number of patients that die of MPM is currently 1500 a year (Vital Statistics, Ministry of Health Labour and Welfare, Japan, 2015), and the incidence of MPM is predicted to remain relatively high in the coming years due to past exposure to asbestos.

Macrophages are considered to be essential constituents of many types of solid tumors [5, 6], and mesotheliomas are heavily infiltrated by macrophages [7–10]. The subtypes of macrophages within a tumor is heterogeneous [11]; in general however, tumor development is associated with the presence of macrophages with M2-like characteristics, particularly in patients with a poor prognosis [8, 12–14]. M2-like macrophages function in the resolution of inflammation and in protection and repair of damaged tissue [15–18]. One of the basic functions of M2-like macrophages that is associated with tissue protection and repair is immunosuppression [11], and tumors have generally been found to contain macrophages with immunosuppressive characteristics [5, 19–22].

Another important myeloid cell population that is associated with tumors are myeloid-derived suppressor cells, and there is almost universal agreement that accumulation of myeloid cells with MDSC-like phenotypes in the blood or tumor correlates with disease progression, poor prognosis, poor response to therapy, and decreased overall survival [23–29]. MDSCs are associated with tumor progression in mouse models of mesothelioma [30–32], and MDSCs are believed to be associated with mesotheliomas in human patients [33, 34].

C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), is expressed in most human cancers [35–37], and plays a key role in the recruitment of macrophages and MDSCs [35, 36, 38–40]. In general agreement with the findings that tumors accumulate macrophages and MDSCs that have pro-tumorigenic properties and express CCL2 and that CCL2 expression in tumor tissue is associated with advanced tumor stage and worse prognosis, there are several studies that report elevated levels of CCL2 in the serum of cancer patients and/or an association between elevated serum CCL2 and poor prognosis [41–51]. Other studies, however, found either no association between the serum CCL2 levels of cancer patients and clinical variables or that lower serum CCL2 levels were associated with poor prognosis or that higher serum CCL2 levels were associated with favorable prognosis [52–60].

Whether the disparate findings of the studies cited above are due to differences in tumor stage, CCL2 being associated with a tumorigenic response in some cases and to a tumoricidal response in others, differing immune suppression mechanisms in different tumor types or the patient cohorts studied, or to some other factor is not known. It is clear, however, that the role of CCL2 in tumorigenesis is likely to be affected by tumor-specific factors. The current study was undertaken to investigate serum CCL2 levels in mesothelioma patients. We found that serum CCL2 levels were increased in mesothelioma patients and that this increase was dependent on advancing mesothelioma stage.

Methods

Subjects

Healthy, unexposed volunteers (41 volunteers; 10 females and 31 males; age 56 ± 20.0 years; Range 23–91 years): Serum samples were collected from teaching and research staff at the Nagoya City University Graduate School of Medical Sciences and residents/patients at Nogoyashi Koseiin Medical Welfare Center Hospital (Koseiin Hospital). These subjects had no history of exposure to asbestos and were free from lung and pleural lesions on periodical (once or twice a year) institutional health examinations.

Healthy subjects possibly exposed to asbestos (356 subjects; 33 females and 323 males; age 68.7 ± 8.3 years; Range 35–96 years): Serum samples were collected from patients who visited or were hospitalized in the Japan Labour Health and Welfare Organization Asahi Rosai Hospital and the Saiseikai Chuwa Hospital. All of the enrolled subjects possibly exposed to asbestos had certified documents issued by the Japanese Ministry of Health, Labour and Welfare for the compensation of medical care. These subjects had no detectable asbestos-associated disease. Since the hospital records of patients not suffering from mesothelioma were not available to us, it is not known whether any of the subjects in this group had a health condition or treatment that would increase their serum CCL2 levels, for example see patient 356 (Additional file 1: Table S1). However, while there was a tendency for this group to have higher serum CCL2 levels compared to the healthy, unexposed volunteers, the difference between these groups was not statistically significant.

Mesothelioma patients (50 patients; 5 females and 45 males; age 72.5 ± 8.6 years; Range 57–99 years): Serum samples were collected from patients who were hospitalized in the Okayama Rosai Hospital, Asahi Rosai Hospital, Saiseikai Chuwa Hospital, Daido Hospital, and Nagoya City University Hospital. The diagnosis of MPM was made by biopsy examination combined with chest computed tomography examinations. Histological types of MPM were sarcomatoid, epithelioid, and biphasic.

All participants were provided written informed consent before inclusion in the study. Serum samples were then obtained, coded, and stored in aliquots at − 80 °C until use.

Assay method

Enzyme-linked immune-absorbent assay (ELISA) kits (CCL2: DCP00, R&D systems, Minneapolis, USA) were used for measuring CCL2, following the manufacturer's instructions. The minimum detectable level of human CCL2 ranged between 0.57 and 10.0 pg/ml for these ELISA kits. All samples had measured CCL2 levels above the minimum detectable levels.

Statistics

In Table 1, patient age and serum CCL2 levels are presented as mean ± SD. In Tables 2, 3, 4 and 5, Analysis of Variance (ANOVA) was used to calculate the estimated marginal means and standard errors. Fisher's exact test was used to test the significance of the differences of the nominal data (the data pertaining to gender). The Kruskal-Wallis (one-way ANOVA) test was used to test the significance of the differences in patient age. Analysis of covariance (ANCOVA) was used to compare the estimated marginal means of serum CCL2 levels adjusted for the covariates of age and gender. The homogeneity of the variance of the serum CCL2 levels was tested using Welch's test. The significance of the differences between the means was tested using the Bonferroni test when the variance was homogenous and Tamhane's T2 test when the variance was not homogenous. p-values were determined using pairwise comparison tests (pairwise comparisons are shown in Additional file 3: Tables S3 - S8). p-values < 0.05 were considered statistically significant. All statistical analyses were carried out with statistical software package SPSS 24.0 (SPSS, Chicago, IL, USA).

Results

A summary of the gender, age, and serum CCL2 levels of the study subjects is shown in Table 1. Individual CCL2 levels are shown in Additional file 1: Table S1. The pairwise comparisons of the groups is shown in Additional file 3: Tables S3 and S4. The mean CCL2 level in the serum of the mesothelioma patients is significantly elevated compared to the Possibly Exposed (no apparent disease) group, and this increase is dependent on the stage of the disease.

It is known that serum CCL2 levels increase with age [61–63], and as can be seen in Table 1 the mean CCL2 level in the serum of the Possibly Exposed (no apparent disease) group, age 68.7 ± 8.3 yrs., is higher than that of the Unexposed (no apparent disease) group, age 56.0 ± 20.0 yrs.: the age ranges of the study participants are shown in Additional file 2: Table S2. Analysis of the age of the patients using the Kruskal-Wallis (one-way ANOVA) test shows an age difference between the patients in the different groups ($p < 0.05$). Fisher's exact test also shows a gender difference between groups ($p < 0.05$): see Methods for the gender of the study participants. Therefore, the data was re-analyzed based on covariates of age (67.97) and gender (1.11). In Tables 2, 3, 4 and 5, Analysis of Variance (ANOVA) was used to calculate the estimated marginal means and standard error. Subsequently, Analysis of covariance (ANCOVA) was used to compare the estimated marginal means adjusted for covariates of age and gender.

Tables 2 and 3 show the unadjusted serum CCL2 means and 95% confidence intervals and the estimated CCL2 means and 95% confidence intervals when the data is adjusted based on the covariates of age and gender. In Table 2, the data was adjusted using the Unexposed (no apparent disease), Possibly Exposed (no apparent disease), and Mesothelioma (all patients) groups. The pairwise comparisons of these groups is shown in Additional file 3: Table S5. In Table 3, the data was adjusted using the Unexposed (no apparent disease), Possibly Exposed (no apparent disease), and Mesothelioma stages 1–4 groups. The pairwise comparisons of these groups is shown in Additional file 3: Table S6. After adjusting the data, the estimated mean CCL2 level

Table 1 Gender, age, and serum CCL2 levels of the study subjects. (Individual patient data is shown in Additional file 1: Table S1)

	Number of Patients	Gender		Age	Serum CCL2 (pg/ml)
		Women	Men		
Unexposed (no apparent disease)	41	10	31	56.0 ± 20.0	275.2 ± 98.2
Possibly Exposed (no apparent disease)	356	33	323	68.7 ± 8.3	307.5 ± 117.7
Mesothelioma (all patients)	50	5	45	72.5 ± 8.6	421.3 ± 295.1[a,b]
Mesothelioma (stage 1 patients)	12	0	12	72.8 ± 9.1	289.9 ± 115.4
Mesothelioma (stage 2 patients)	5	0	5	75.6 ± 7.1	281.0 ± 111.2
Mesothelioma (stage 3 patients)	14	1	13	74.3 ± 10.7	486.0 ± 333.4[c,d]
Mesothelioma (stage 4 patients)	19	4	15	70.2 ± 6.8	493.5 ± 346.7[c,d]

[a]Different from the Unexposed (no apparent disease) group at $p < 0.01$
[b]Different from the Possibly Exposed (no apparent disease) group at $p < 0.05$
[c]Different from the Unexposed (no apparent disease) and the Possibly Exposed (no apparent disease) groups at $p < 0.001$
[d]Different from the Mesothelioma (stage 1 patients) group at $p < 0.01$

Table 2 Serum CCL2 levels of the study subjects after adjusting the data for the covariates of gender and age

	Unadjusted Data (ANOVA)				Adjusted Data (ANCOVA)			
	Serum CCL2 (pg/ml)	Std Error	95% CI		Estimated Serum CCL2 (pg/ml)	Std Error	95% CI	
			Lower Limit	Upper Limit			Lower Limit	Upper Limit
Unexposed (no apparent disease)	275.2	22.9	230.2	320.3	303.5	24.2	256.0	351.1
Possibly Exposed (no apparent disease)	307.5	7.8	292.2	322.8	305.6	7.7	290.4	320.8
Mesothelioma (all patients)	421.3[a,c]	20.8	380.5	462.1	411.8[b,c]	20.8	370.9	452.6

[a]Different from the Unexposed (no apparent disease) group at $p < 0.001$
[b]Different from the Unexposed (no apparent disease) group at $p < 0.01$
[c]Different from the Possibly Exposed (no apparent disease) group at $p < 0.001$

in the serum of the mesothelioma patients is significantly elevated compared to the Possibly Exposed (no apparent disease) group, and this increase is dependent on the stage of the disease.

Two patients in the Mesothelioma group, patients 31 and 50 (Additional file 1: Table S1), had extraordinarily high levels of serum CCL2. Removal of these two patients reduces the serum CCL2 levels in the mesothelioma all patients, stage 3 patients, and stage 4 patients groups to 368.5 ± 138.1, 402.7 ± 123.2, and 420.5 ± 141.9, respectively. Tables 4 and 5 show the results when these two patients are removed from data analysis. Table 4 shows the unadjusted serum CCL2 means and 95% confidence intervals and the estimated CCL2 means and 95% confidence intervals when the data is adjusted based on the covariates of age and gender using the Unexposed (no apparent disease), Possibly Exposed (no apparent disease), and Mesothelioma (all patients) groups. The pairwise comparisons of these groups is shown in Additional file 3: Table S7. Table 5 shows the unadjusted serum CCL2 means and 95% confidence intervals and the estimated CCL2 means and 95% confidence intervals when the data is adjusted based on the covariates of age and gender using the Unexposed (no apparent disease), Possibly Exposed (no apparent disease), and Mesothelioma stages 1–4 groups. The pairwise comparisons of these groups is shown in Additional file 3: Table S8. After removal of patients 31 and 50 from the data analysis, CCL2 levels in the mesothelioma patients are still significantly higher than the CCL2 levels in the Unexposed (no apparent disease) and the Possibly Exposed (no apparent disease) groups, and this increase is dependent on the stage of the disease.

Discussion

In this study we measured the levels of CCL2 in the serum of 41 healthy volunteers who have not been exposed to asbestos, 356 healthy subjects who have possibly been exposed to asbestos, and 50 mesothelioma patients. The mean CCL2 level in the serum of the mesothelioma patients was significantly elevated compared to both the healthy volunteers who have not been exposed to asbestos and the healthy subjects who have possibly been exposed to asbestos (see Table 1). However, it is known that serum CCL2 levels increase with normal aging [61–63], and analysis of the age of the patients using the Kruskal-Wallis (one-way ANOVA) test showed an age difference between the patients in the Possibly Exposed (no apparent disease) and the mesothelioma groups. Fisher's exact test also showed a gender difference between these groups. Therefore, the data was re-analyzed based on covariates of age (67.97) and gender (1.11). Re-analysis of the data after adjusting for age and gender did not change the conclusions of the study:

Table 3 Serum CCL2 levels of the study subjects after adjusting the data for the covariates of gender and age

	Unadjusted Data (ANOVA)				Adjusted Data (ANCOVA)			
	Serum CCL2 (pg/ml)	Std Error	95% CI		Estimated Serum CCL2 (pg/ml)	Std Error	95% CI	
			Lower Limit	Upper Limit			Lower Limit	Upper Limit
Unexposed (no apparent disease)	275.2	22.9	230.2	320.3	305.5	24.2	256.0	351.1
Possibly Exposed (no apparent disease)	307.5	7.8	292.2	322.8	305.4	7.7	290.4	320.8
Mesothelioma (stage 1 patients)	289.9	41.5	208.4	371.4	275.7	41.1	195.0	356.5
Mesothelioma (stage 2 patients)	281.0	64.4	154.7	407.3	261.0	63.6	136.0	386.0
Mesothelioma (stage 3 patients)	486.0[a,c,d]	38.4	410.5	561.5	471.4[b,c,d]	38.1	396.4	546.3
Mesothelioma (stage 4 patients)	493.5[a,c,d]	33.0	428.7	558.3	492.5[a,c,d,e]	32.7	428.3	556.7

[a]Different from the Unexposed (no apparent disease) group at $p < 0.001$
[b]Different from the Unexposed (no apparent disease) group at $p < 0.01$
[c]Different from the Possibly Exposed (no apparent disease) group at $p < 0.001$
[d]Different from the Mesothelioma stage 1 patients group at $p < 0.01$
[e]Different from the Mesothelioma stage 2 patients group at $p < 0.05$

Table 4 Serum CCL2 levels of the study subjects after removing patients 31 and 50 and adjusting the data for the covariates of gender and age

	Unadjusted Data (ANOVA)				Adjusted Data (ANCOVA)			
	Serum CCL2 (pg/ml)	Std Error	95% CI		Estimated Serum CCL2 (pg/ml)	Std Error	95% CI	
			Lower Limit	Upper Limit			Lower Limit	Upper Limit
Unexposed (no apparent disease)	275.2	18.5	238.9	311.6	308.6	19.3	270.7	346.4
Possibly Exposed (no apparent disease)	307.5	6.3	295.2	319.9	305.4	6.1	293.3	317.4
Mesothelioma (all patients)	368.5[a]	17.1	334.9	402.1	356.0[b]	16.9	322.8	389.2

[a]Different from the Unexposed (no apparent disease) and the Possibly Exposed (no apparent disease) groups at $p < 0.01$
[b]Different from the Possibly Exposed (no apparent disease) groups at $p < 0.05$

serum CCL2 was elevated in mesothelioma patients (see Table 2). Mesothelioma patients 31 and 50 (see Additional file 1: Table S1) had exceptionally high levels of CCL2. After removal of these two patients' data from analysis, serum CCL2 was still elevated in mesothelioma patients (see Table 4). Therefore, our data indicate that serum CCL2 levels were increased in mesothelioma patients and this increase was not dependent on the age of the patients in the Mesothelioma group or on the presence of the two patients in the Mesothelioma group with exceptionally high levels of serum CCL2. Elevated CCL2 in the serum of mesothelioma patients is in agreement with the high levels of CCL2 present in the pleural effusions of mesothelioma patients reported by Gueugnon et al. [64].

The increase in the serum levels of CCL2 in the mesothelioma patients was dependent on the stage of the disease (see Table 1). Reanalysis of the data adjusting for age and gender also indicated elevated levels of serum CCL2 depended on mesothelioma stage (see Tables 2 and 3). The dependence on mesothelioma stage was still apparent after removal of the two mesothelioma patients with exceptionally high levels of serum CCL2 from data analysis (see Tables 4 and 5). Therefore, as with the increase in the levels of CCL2 in the serum of mesothelioma patients, the dependence of this increase on disease stage was not due to the age of the patients in the Mesothelioma group or on the presence of the two patients in the Mesothelioma group with exceptionally high levels of serum CCL2.

The mean CCL2 level in the serum of the healthy subjects who have possibly been exposed to asbestos was elevated compared to the healthy volunteers who have not been exposed to asbestos. However, as noted above, it is known that serum CCL2 levels increase during normal ageing [61–63]. Thus, the levels of CCL2 in the serum in these two groups followed the expected pattern, lower in the healthy unexposed group consisting of primarily younger patients and higher in the healthy possibly exposed group consisting of primarily older patients.

Several studies have reported that increased expression of CCL2 in tumor tissue is associated with advanced tumor stage and worse prognosis: These studies include patients with breast cancer [65–68], prostate cancer [69, 70], gastric cancer [71], colorectal cancer [72, 73], esophageal squamous cell carcinoma [74], head and neck squamous cell carcinoma [75], and glial tumors [47]. In agreement with these findings, a number of studies report elevated levels of CCL2 in the serum of cancer patients and/or an association between elevated serum CCL2 and poor prognosis: Moogooei et al. [47] and Pan et al. [48] report elevated levels of serum CCL2 in patients with glial tumors and lung cancer. Lu et al. [45] and Sharma et al. [49] report an association between

Table 5 Serum CCL2 levels of the study subjects after removing patients 31 and 50 and adjusting the data for the covariates of gender and age

	Unadjusted Data (ANOVA)				Adjusted Data (ANCOVA)			
	Serum CCL2 (pg/ml)	Std Error	95% CI		Estimated Serum CCL2 (pg/ml)	Std Error	95% CI	
			Lower Limit	Upper Limit			Lower Limit	Upper Limit
Unexposed (no apparent disease)	275.2	18.3	239.3	311.2	305.5	24.2	256.0	351.1
Possibly Exposed (no apparent disease)	307.5	6.2	295.3	319.7	305.4	7.7	290.4	320.8
Mesothelioma (stage 1 patients)	289.9	33.8	223.5	356.4	275.7	41.1	195.0	356.5
Mesothelioma (stage 2 patients)	281.0	52.4	178.1	383.9	261.0	63.6	136.0	386.0
Mesothelioma (stage 3 patients)	402.7[b]	32.5	338.9	466.5	471.4	38.1	396.4	546.3
Mesothelioma (stage 4 patients)	420.5[a,c,d]	27.6	366.3	474.8	492.5[b,c,d]	32.7	428.3	556.7

[a]Different from the Unexposed (no apparent disease) group at $p < 0.001$
[b]Different from the Unexposed (no apparent disease) group at $p < 0.05$
[c]Different from the Possibly Exposed (no apparent disease) group at $p < 0.01$
[d]Different from the Mesothelioma stage 1 patients group at $p < 0.05$

elevated serum CCL2 levels and poor prognosis in patients with prostate cancer, and Lu et al. [44] report an association between elevated serum CCL2 levels and poor prognosis in patients with nasopharyngeal cancer. Cai et al. [41], Wang et al. [50], Wu et al. [51], Lubowicka et al. [46], and Hefler et al. [42] report elevated levels of serum CCL2 in patients with lung, liver, gastric, breast, and ovarian cancer and that increased serum CCL2 was associated with poor prognosis. Lebrecht et al. [43] did not find a difference in serum CCL2 levels between breast cancer patients and normal donors, but they did find an association between serum CCL2 and poor prognosis.

However, there are also reports that increased expression of CCL2 in tumor tissue is associated with better prognosis: These studies include patients with gastric cancer [59], colorectal cancer [76], liver cancer [77], and non-small cell lung cancer [78]. There are also a number of studies, that report either that serum CCL2 levels in cancer patients are not related to clinical variables or that higher serum CCL2 levels are associated with a better prognosis and/or that lower serum CCL2 levels are associated with worse prognosis. Tas et al. [58], Tsaur et al. [60], and Monti et al. [56] found elevated serum CCL2 levels in patients with gastric, prostate, and pancreas cancer. However, Tas et al. report that while gastric cancer patients who responded to chemotherapy had lower serum CCL2 than non-responders, there was no association between serum CCL2 and any measured clinical variables; Tsuar et al. report that elevated serum CCL2 was negatively correlated with PSA value in prostate cancer patients; and Monti et al. report that elevated serum CCL2 was associated with increased survival in pancreas cancer patients. Farren et al. [54] also report that elevated serum CCL2 levels correlated with increased survival in pancreas cancer patients. Sullivan et al. [57] report that there was no difference in serum CCL2 levels between pancreas cancer patients and normal donors and that serum CCL2 did not correlate with any measured clinico-pathological parameters. Koper et al. [55], Ding et al. [53], and Tonouchi et al. [59] report that serum CCL2 levels were decreased in patients with astrocytic brain tumors, oral squamous cell carcinoma, and gastric cancer, and Tonouchi et al. report CCL2 levels tended to decrease in accordance with disease progression and that decreased serum CCL2 levels were associated with poor survival. Dehqanzada et al. [52] report that elevated serum CCL2 levels correlated with favorable prognostic variables in patients with breast cancer.

Thus, the association between serum CCL2 levels and different cancers appears to be variable. Since mesotheliomas are heavily infiltrated by macrophages [7–10] and likely to be infiltrated by MDSCs [33, 34], our finding that CCL2 is elevated in the serum of patients with advanced mesothelioma is consistent with a disease in which the CCL2/CCR2 axis and myeloid-derived cells play an important part. Consequently, therapies that prove effective against other cancers in which the CCL2/CCR2 axis and myeloid-derived cells are associated with disease progression may also prove effective with mesothelioma patients. There is considerable interest in developing therapies that target CCL2/CCR2 and tumor-resident myeloid cells [5, 22, 79–85]. Numerous clinical trials employing these therapies as part of the treatment regimen have been carried out or are currently being pursued [86–94]. The success or failure of these trials will have important implications for the treatment of mesothelioma. Another aspect of increased CCL2 in the serum of mesothelioma patients is that it may be possible to use serum CCL2 to monitor a patient's response to treatment [95].

Conclusions

CCL2 levels are elevated in mesothelioma patients and the increase is dependent on the stage of the disease. This is consistent with the premise that the CCL2/CCR2 axis and myeloid-derived cells play an important role in mesothelioma and disease progression. Other types of cancer also cause stage-dependent increases in serum CCL2. Therapies are being developed that target CCL2/CCR2 and tumor resident myeloid cells, and clinical trials are being pursued that use these therapies as part of the treatment regimen. The results of trials with patients with a similar pattern of CCL2 as mesothelioma patients will have important implications for the treatment of mesothelioma.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-019-6419-1.

Additional file 1: Table S1. Serum CCL2 levels: Individual patient data.

Additional file 2: Table S2. Age of the study participants.

Additional file 3: Table S3. Pairwise comparisons of the Unexposed_No apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Patients groups. **Table S4.** Pairwise comparisons of the Unexposed_no apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Stage 1–4 groups. **Table S5.** Pairwise comparisons of the Unexposed_no apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Patients groups. **Table S6.** Pairwise comparisons of the Unexposed_no apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Stages 1–4 groups. **Table S7.** Pairwise comparisons of the Unexposed_no apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Patients groups, with patients 31 and 50 removed from data analysis. **Table S8.** Pairwise comparisons of the Unexposed_no apparent disease, Possibly Exposed_no apparent disease, and Mesothelioma Stages 1–4 groups, with patients 31 and 50 removed from data analysis.

Abbreviations

CCL2: C-C motif chemokine ligand 2; MCP-1: Monocyte chemotactic protein-1; MDSC: Myeloid-derived suppressor cell; MPM: Malignant pleural mesothelioma

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None.

Authors' contributions
TK: Conception of the study, acquisition of patient samples, data analysis. NF: Acquisition of patient samples. TE: Data analysis and interpretation. TOmori Conception of the study. TOguri: Acquisition of patient samples. AN: Acquisition of patient samples. TY: Acquisition of patient samples. MK: Acquisition of patient samples. IU: Acquisition of patient samples. MN: Acquisition of patient samples. KY: Acquisition of patient samples. TT: Acquisition of patient samples. MT: Acquisition of patient samples. YY: Acquisition of patient samples. KT: Data analysis and interpretation. YM: Data analysis and interpretation. JX: Draft the manuscript. ST: Data interpretation, draft the manuscript. MA: Perform ELISAs, draft the manuscript. WTA: Perform ELISAs, draft the manuscript. DBA: Perform ELISAs, draft the manuscript. HT: Conception of the study, draft the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
All data is available in Addition file S1.

Ethics approval and consent to participate
All participants were provided written informed consent before inclusion in the study. This study was conducted under the approval of the ethics committees of the Nogoyashi Koseiin Medical Welfare Center Hospital, Okayama Rosai Hospital, Asahi Rosai Hospital, Saiseikai Chuwa Hospital, Daido Hospital, and Nagoya City University for the use of materials and analysis.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Chapter

Immunocheckpoint Blockade in Malignant Pleural Mesothelioma

Nobukazu Fujimoto

Abstract

Targeting immunocheckpoint with immunomodulatory monoclonal antibodies has proven to be an effective antitumor strategy across a variety of cancers. The immunosuppressive tumor microenvironment in malignant pleural mesothelioma (MPM) has suggested that MPM might benefit from this kind of immunotherapy. In recent years, immunocheckpoint inhibitors (ICIs) have shown encouraging results for patients with MPM. Antibodies against programmed death 1 (PD-1) and PD-ligand 1 (PD-L1) have demonstrated favorable response, progression-free survival, and overall survival. The toxicity profiles were similar to those observed with ICIs in other malignancies, like melanoma and non-small cell lung cancer, and they appeared to be manageable. Nivolumab, an anti-PD-1 antibody, was approved in Japan for advanced or metastatic MPM patients resistant or intolerant to other chemotherapies. Important future issues include developing a combination therapy, where ICIs are combined with other agents (including other ICIs), and developing biomarkers for determining which patients might respond well and which might experience unacceptable toxicities.

Keywords: durvalumab, immunocheckpoint, nivolumab, pembrolizumab, PD-1

1. Introduction

Malignant pleural mesothelioma (MPM) is a rare pleural malignancy that is associated with asbestos exposure. Gemba et al. reported that more than 70% of malignant mesothelioma cases in Japan were associated with occupational or environmental asbestos exposure [1]. MPM is a highly aggressive neoplasm with a poor prognosis; the median overall survival (OS) is only about 12 months. Systemic chemotherapy with platinum plus pemetrexed is the recommended first-line systemic therapy for advanced MPM [2]. Some clinical trials have examined the efficacy of new agents to improve the results of the platinum/pemetrexed combination; however, no new agent has demonstrated significant clinical efficacy. Thus, the pemetrexed/platinum combination remains the standard treatment.

Currently, there is no recommended treatment option for MPM after first-line platinum/pemetrexed chemotherapy. Re-treatment with pemetrexed-based chemotherapy is a reasonable option for patients that achieved durable disease control with the first-line chemotherapy [3]. Other treatment options of salvage chemotherapy include vinorelbine and gemcitabine; however, the median OS with these agents only ranges from 5 to 10 months [4, 5]. Other experimental agents, such as angiogenesis inhibitors [6] or tyrosine kinase inhibitors [7], have not demonstrated efficacy.

Targeting immunocheckpoint with immunomodulatory monoclonal antibodies was shown to be an effective antitumor strategy across a variety of cancers [8]. The immunosuppressive tumor microenvironment in MPM has suggested that MPM might benefit from this kind of immunotherapy [9, 10]. In fact, in recent years, immunocheckpoint inhibitors (ICIs) have shown some encouraging results for patients with MPM.

In this chapter, we review recent clinical findings on several ICIs, including anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibody, anti-programmed death 1 (PD-1) antibody, and anti-PD-ligand 1 (PD-L1) antibody, for treating patients with MPM.

2. Anti-CTLA-4 antibody

Anti-CTLA-4 antibody was the first ICI described for treating MPM. Phase II studies demonstrated that tremelimumab, a selective human monoclonal antibody against CTLA-4, showed favorable activity as a second-line treatment for MPM [11, 12]. However, a double-blind study that compared tremelimumab to placebo in subjects with previously treated, unresectable malignant mesothelioma (DETERMINE study) failed to demonstrate differences in OS or progression-free survival (PFS) between the treatment and placebo groups [13]. After that, anti-CTLA-4 antibodies were studied in combination with an anti-PD-1 or anti-PD-L1 antibody.

3. Anti-PD-L1 antibody

Avelumab is a human IgG1 monoclonal antibody that targets PD-L1 [14]. A phase 1b open-label study (JAVELIN solid tumor) was conducted in patients with unresectable mesothelioma that progressed after platinum/pemetrexed treatment; patients were enrolled at 25 sites in three countries [15]. Of 53 patients treated, the objective response rate (RR) was 9% (95% confidence interval [95%CI]: 3.1–20.7%); one patient experienced a complete response, and four patients experienced a partial response. Responses were durable (median, 15.2 months; 95%CI: 11.1 to non-estimable) and occurred in patients with PD-L1-positive tumors (RR: 19%; 95%CI: 4.0–45.6) and PD-L1-negative tumors (RR: 7%; 95%CI: 0.9–24.3), based on a 5% or greater cutoff for PD-L1 expression. The median PFS was 4.1 months (95%CI: 1.4–6.2), and the 12-month PFS rate was 17.4% (95%CI: 7.7–30.4). The median OS was 10.7 months (95%CI: 6.4–20.2).

4. Anti-PD-1 antibody

4.1 Pembrolizumab

A nonrandomized, phase Ib trial was conducted to test pembrolizumab in patients with PD-1-positive MPM that had been treated previously. In the preliminary report, 20% of patients experienced an objective response, 72% experienced disease control, and the median OS was 18 months (95%CI: 9.4 to non-estimable) [16]. Then, a phase II trial assessed pembrolizumab activity in 65 unselected patients with MPM [17]. The objective RR was 19% and the disease control rate was 66%. The median PFS was 4.5 months (95%CI: 2.3–6.2), and the median OS was 11.5 months (95%CI: 7.6–14).

After those promising results, pembrolizumab was used off-label in Switzerland and Australia [18]. A total of 93 patients (48 from Switzerland and 45 from Australia) were treated. In those cohorts, the overall RR was 18%, the median PFS was 3.1 months, and the median OS was 7.2 months. Among patients with the non-epithelioid histological subtype, pembrolizumab treatment improved the objective RR (24% vs. 16%; $p = 0.54$) and the median PFS (5.6 vs. 2.8 months; $p = 0.02$).

4.2 Nivolumab

Another anti-PD-1 antibody, nivolumab, was first tested in recurrent MPM in the Netherlands [19]. In that single-center trial, patients with MPM received 3 mg/kg intravenous nivolumab every 2 weeks. Of the 34 patients included, eight patients (24%) displayed a partial response and another eight displayed stable disease, which resulted in a disease control rate of 47%. Japanese investigators also evaluated the efficacy and safety of nivolumab for advanced MPM in patients that were resistant or intolerant to prior chemotherapy [20]. Thirty-four patients were enrolled, and 10 patients (29.4%, 95%CI: 16.8–46.2) showed an objective response in a central assessment. Objective RRs were 25.9, 66.7, and 25.0% for epithelioid, sarcomatous, and biphasic histological subtypes, respectively (**Figure 1**). The median OS and PFS were 17.3 and 6.1 months, respectively (**Figure 2a** and **b**). Based on these findings,

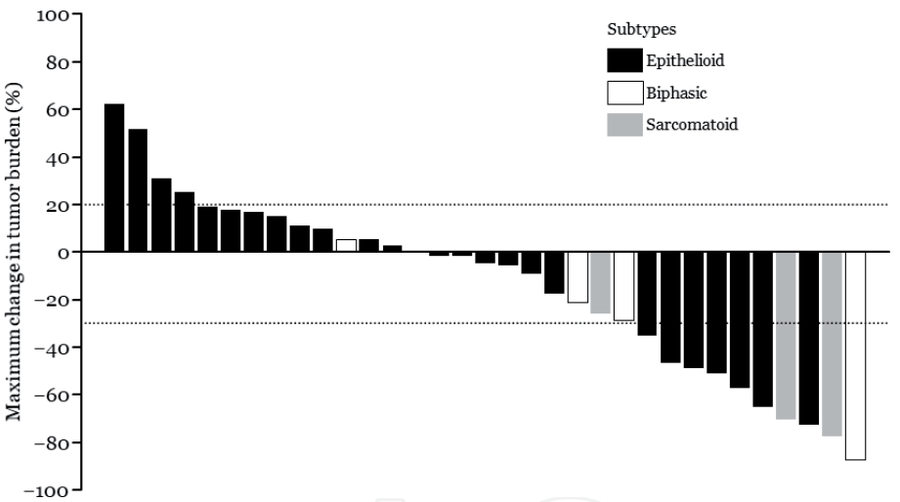

Figure 1.
A waterfall plot of the MERIT study results, which demonstrates the maximum percentage changes compared to baseline in target lesions of each patient, according to histological subtype (Ref. [20]).

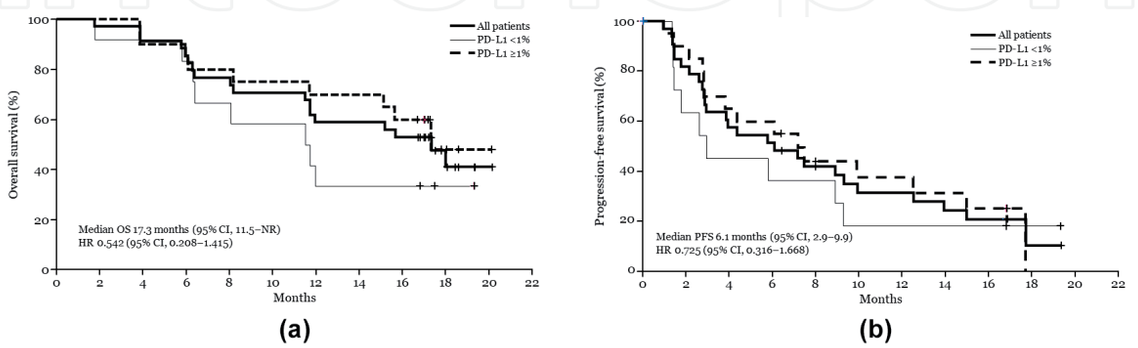

Figure 2.
Kaplan-Meier curves show survival for all patients and for patients grouped according to programmed death-ligand 1 (PD-L1) expression in the MERIT study (Ref. [20]). (a) Overall survival (OS); (b) progression-free survival (PFS). HRs compare the PD-L1 ≥ 1% group to the <1% group. CI, confidence interval; HR, hazard ratio; NR, not reached.

nivolumab was approved in Japan for patients with advanced or metastatic MPM that are resistant or intolerant to previous chemotherapy.

Although the effect requires confirmation in larger clinical trials, nivolumab and pembrolizumab might offer hope for patients with MPM.

5. Toxicity

The toxicity of these ICIs was acceptable in MPM. A study on pembrolizumab toxicity found grade 3 and 4 events, including adrenal insufficiency (3%), pneumonitis (3%), skin rash (3%), colitis (1.6%), confusion (1.6%), hepatitis (1.6%), and hyperglycemia (1.6%), and one grade 5 event of hepatitis (1.6%) [17]. In a study on nivolumab, adverse events of any grade occurred in 26 patients (76%), including fatigue (29%) and pruritus (15%) [19]. In that study, treatment-related grade 3 and 4 adverse events were reported in nine patients (26%); most events were pneumonitis, gastrointestinal disorders, and laboratory disorders. One treatment-related death was due to pneumonitis, but it was probably initiated by concurrent amiodarone therapy. These toxicity profiles were similar to those observed in other malignancies, including melanoma and non-small cell lung cancer (NSCLC), and they appeared to be manageable.

6. Future perspectives

Based on the promising results described above, ICIs could play a primary role in the treatment of MPM. An important issue for the future is whether ICIs can be combined with other agents, including other ICIs. For example, given the synergy between the PD-1/PD-L1 and CTLA-4 pathways in T-cell activation, a combination treatment with antibodies that target PD-1 or PD-L1 and CTLA-4 warrants investigation [22].

NIBIT-MESO-1 was an open-label, nonrandomized, phase II study that investigated the efficacy and safety of first- or second-line tremelimumab, a monoclonal antibody against CTLA-4, combined with durvalumab, a monoclonal antibody against PD-L1 [23]. In that study, patients with unresectable pleural or peritoneal mesothelioma received one dose of intravenous tremelimumab and durvalumab delivered every 4 weeks, for a total of four doses. This was followed by maintenance treatment with intravenous durvalumab. Of 40 patients, 11 (28%) displayed an objective response. The median PFS was 5.7 months (95%CI: 1.7–9.7), and the median OS was 16.6 months (95%CI: 13.1–20.1). Toxicity related to treatment was generally manageable and reversible.

Another multicenter, randomized, phase II study was conducted in France [24]. In that study, patients were randomly allocated to nivolumab or nivolumab plus ipilimumab. In the intention-to-treat population, the primary endpoint, 12-week disease control, was achieved by 25 (40%; 95%CI: 28–52) of 63 patients in the nivolumab group and by 32 (52%; 95%CI: 39–64) of 62 patients in the combination group. The most frequent grade 3 adverse events were asthenia (N = one [2%] with nivolumab vs. three [5%] with the combination), an asymptomatic increase in aspartate aminotransferase or alanine aminotransferase (N = none with nivolumab vs. four [7%] of each with the combination), and an asymptomatic increase in lipase (N = two [3%] with nivolumab vs. one [2%] with the combination). These findings indicated that the combination of anti-CTLA-4 and anti-PD1/PD-L1 antibodies appeared to be active and had a good safety profile in patients with MPM. Currently, there is an ongoing phase III, randomized, open-label trial for testing nivolumab in combination with ipilimumab vs. pemetrexed with cisplatin or carboplatin as a first-line therapy in unresectable MPM. The primary endpoint of the study, OS, will be reported in the near future.

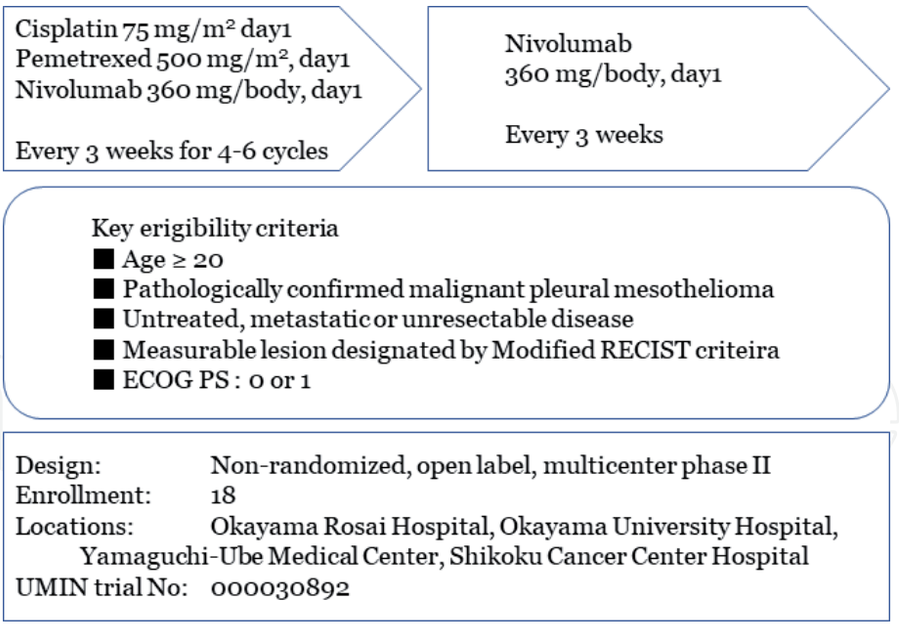

Figure 3.
Overview of a phase II trial for testing a first-line combination chemotherapy with cisplatin/pemetrexed and nivolumab for treating unresectable malignant pleural mesothelioma (Ref. [21]). RECIST, response evaluation criteria in solid tumors; ECOG, eastern cooperative oncology group; PS, performance status.

The combination of an anti-PD-1/PD-L1 antibody and conventional chemotherapy is also under investigation. Nowak et al. presented results from a phase II trial that tested durvalumab combined with cisplatin/pemetrexed in MPM [25]. The primary endpoint, PFS at 6 months, was 57% (N = 31/54; 95%CI: 45–68), the median PFS time was 6.9 months (95%CI: 5.5–9.0), and the objective RR was 48% (95%CI: 35–61). Grade 3–5 adverse events occurred in 36 patients, including neutropenia in 13%, nausea in 11%, anemia in 7%, fatigue in 6%, and any grade of peripheral neuropathy in 35%. The authors have conducted another phase II study to test the combination of nivolumab and cisplatin/pemetrexed, which is currently in progress (**Figure 3**) [21]. A large-scale randomized study for testing the combination of pembrolizumab and cisplatin/pemetrexed is also in progress. Based on whether these combination regimens, which include anti-PD1/PD-L1 antibodies, demonstrate sufficient activity, safety, and tolerability as first-line treatments, the standard regimen of cisplatin/pemetrexed might be replaced.

Another important issue is whether biomarkers can be developed to determine which patients might expect a response and which might expect unacceptable toxicity. Previous studies in patients with MPM have shown that tumors with positive PD-L1 expression were associated with worse survival outcomes compared to those with negative PD-L1 expression [26]. Although an optimal PD-L1 expression threshold could not be identified, a trend was observed, where a higher RR and more durable PFS were associated with increasing PD-L1 expression, in studies on pembrolizumab [17, 18] and nivolumab [20]. In some neoplasms, the tumor mutation burden or the tumor microenvironment was associated with the response to ICIs; however, those associations have not been established as biomarkers in MPM.

7. Conclusion

The prognosis of MPM remains poor. Recent encouraging results have suggested that a PD-1/PD-L1 blockade might be an effective treatment option

for MPM. Although the effect requires confirmation in larger clinical trials, nivolumab and pembrolizumab might offer hope for patients with MPM. Further study is warranted to develop more effective treatment strategies, such as combining ICI with other ICIs or with conventional chemotherapy, and to establish biomarkers for distinguishing patients that might respond to treatment from those likely to develop unacceptable toxicities.

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Conflict of interest

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GVHD の発症と慢性炎症

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Key words：移植片対宿主病（GVHD），慢性炎症，HMGB1，DAMPs，インフラマソーム

Abstract

移植片対宿主病（GVHD）の発症には移植前処置後の慢性炎症が関与し，その本態は「免疫学的自己と非自己の識別の破綻」と考えられる。前処置後の損傷組織から放出される DAMPs（特に HMGB1）は GVHD 発症のトリガーであり，好中球の活性化や自己 DNA の TLR への提示など，自然免疫の活性化に作用する。炎症性サイトカインを制御するインフラマソームは DAMPs と相互に作用するので，GVHD の病態の本質的な解明のためには HMGB1 だけではなく，インフラマソームも含めた慢性炎症反応の機序を明らかにすることが必要である。

はじめに

移植片対宿主病（graft-versus-host disease: GVHD）は同種造血幹細胞移植における重要な合併症である。レシピエントのアロ抗原を認識して活性化されたドナー由来の T 細胞がレシピエントの臓器を傷害する病態と考えられていて，GVHD を発症した臓器の病理組織像では T 細胞の浸潤が認められる。しかし，これは GVHD の病態形成が完成した，いわば終末期の病理組織像であるので，この所見のみでは病像の進展経過はわからない。GVHD の本態を理解するためには，移植の前処置から GVHD の発症に至るまでの一連の造血幹細胞移植の経過の中での免疫学的な応答を解明する必要がある。また，臨床現場での同種造血幹細胞移植では通常，主要組織適合抗原である HLA の適合したドナーから採取された造血細胞が移植されているにもかかわらず，GVHD が発症するということは，幹細胞移植後に「免疫学的寛容」が誘導されていないだけでなく，「自己」が免疫細胞によって正しく認識されていない可能性が考えられる。そこで，本稿では GVHD の病態を「免疫学的自己と非自己の識別の破綻」と考えて，同種造血幹細胞移植における初期免疫応答と GVHD の発症との関連について概説する。

1．HMGB1 に誘導される炎症が GVHD 発症のトリガーとなる

GVHD の発症経過では Initiation phase とも言うべき段階がある[1)]。同種造血幹細胞移植では，ドナー由来の造血幹細胞を生着させるためにレシピエントの免疫を抑制する目的で移植前処置が行われるわけであるが，放射線照射や抗癌剤投与による移植前処置でレシピエントの組織が傷害されると danger signal が細胞外に放出されて，その炎症を誘導する作用によりレシピエントの抗原

Chronic inflammation affects GVHD

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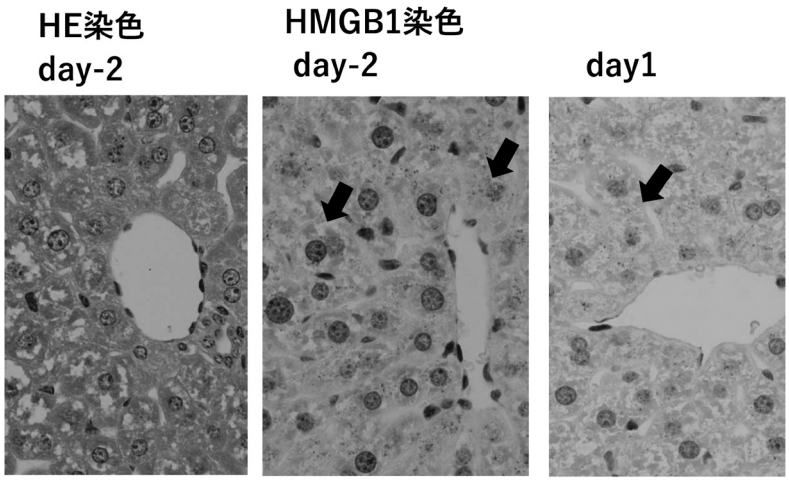

図1　核内から報酬された HMGB1（抗 HMGB1 抗体による免疫染色）
移植前処置を受けたマウスの肝細胞の核から細胞質へ HMGB 1が放出されている。

提示細胞が活性化されると考えられ，活性化されたレシピエントの抗原提示細胞がドナーの T 細胞を活性化するという概念が提唱されている。しかし，一方で樹状細胞による抗原提示は GVHD の誘導に必要ではなく，移植後の炎症反応によって MHC Class Ⅱを発現するようになった非造血系細胞による抗原提示が GVHD に関与しているとする報告[2] もあり，GVHD を引き起こす同種抗原提示の機序については未だ解明されていない点も多い。

danger signal は病原体由来で外因性因子の PAMPs（pathogen-associated molecular patterns）と，生体が侵襲を受けて傷害された組織・細胞から放出される内因性因子の DAMPs（damage-associated molecular patterns）に大別される。DAMPs は病原体によらない無菌性の炎症の誘因となり免疫細胞を活性化することから，移植前処置に伴う組織傷害のために細胞外に放出された DAMPs によって誘導される炎症反応が GVHD を引き起こすと考えられている。移植前処置でレシピエントの組織が傷害されて急性期の炎症反応が起こった時に大量の DAMPs が細胞外に放出されることで免疫反応の過剰な亢進が起こると急性の炎症反応が収束して消退することなく慢性炎症に進展することにより，慢性炎症が GVHD 発症の誘因となる可能性がある。

DAMPs には ATP，尿酸，HMGB1，S100 蛋白，熱ショック蛋白，IL-33 などの様々な種類があることが知られているが，筆者らは遅発性の炎症性メディエーターとして見出された HMGB1（high mobility group box 1）に着目した[3]。HMGB 1 は定常状態では細胞の核内に存在して DNA の安定化に関わっているが，細胞が損傷を受けて HMGB1 が細胞外に放出されると炎症性サイトカインとしての機能を示すことが知られている。そこで，移植前処置によって生じる炎症反応が GVHD を引き起こすという観点から移植後早期の免疫応答のトリガーである HMGB1 の関与について検討を行った。

急性 GVHD マウスモデルを用いて移植前処置後に HMGB1 の血中濃度を測定すると同時に，肝臓を摘出して抗 HMGB1 抗体で免疫組織染色を行ったところ day -2 の時点で移植前処置によって傷害された肝臓の細胞核内から HMGB1 が放出されていることが確認できた（**図1**）。しかし，HMGB1 の血中濃度の経時変化を調べたところ

day-3 から day 0 の間で血中濃度の上昇は認められず，day14 に血中濃度のピークに達した。

LPS 敗血症誘導マウスモデルにおいても IL-1 や TNF- α の血中濃度がピークに達する時期より遅れて HMGB1 の血中濃度がピークに達することが報告されており[3]，急性 GVHD マウスモデルにおいても同様に炎症反応の急性期の時期より遅れて HMGB1 の血中濃度が上昇する経時変化が認められた。**図1**に示されるように細胞が傷害を受けてから短期間で HMGB1 が核外に放出されているが，まだ細胞質内にとどまっているためにすぐには血中濃度の上昇として反映されない可能性がある。

2．GVHD の発症と好中球の関連

筆者らが急性 GVHD マウスモデルでの HMGB1 の血中動態を調べると同時に標的臓器である肝臓の組織所見を調べたところ，HMGB1 の血中濃度がピークとなる時期にほぼ一致して好中球の浸潤所見が認められた（**図2**）。また，リンパ球の浸潤は認められなかった。これは，HMGB1 が自然免疫細胞である好中球を活性化し，好中球が GVHD の初期病態に関与している可能性を示唆する所見と考えられる。他にも好中球が GVHD の病態に関与しているとする報告があり，Socié らは消化管 GVHD が疑われた同種幹細胞移植 95 症例に対して病理組織学的な検討を行い，1視野あたりに 20 個以上の好中球の浸潤があれば，90 日以内の移植関連死亡との相関が認められたことを報告している[4]。また，Qiao らは Busulfan と Cyclophosphamide の前処置後に幹細胞移植を行ったマウスモデルで肝臓に好中球が浸潤していることを報告している[5]。

HMGB1 の受容体は，パターン認識受容体である終末糖化産物受容体（receptor for advanced glycation end products: RAGE）や Toll 様受容体（Toll-like receptor）の TLR2，TLR4，TLR9

day 20 肝組織　HE染色

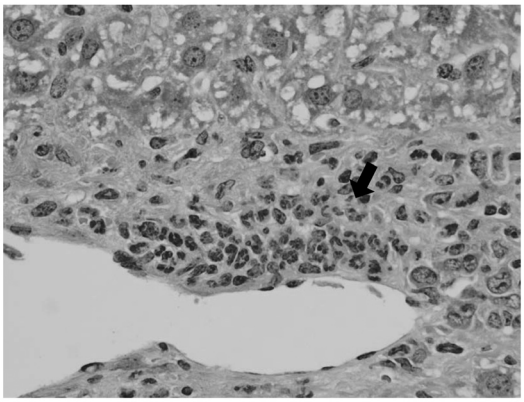

図2　肝組織内の好中球浸潤像
HMGB1 の血中濃度がピークとなった時期に一致して肝臓内に多数の好中球浸潤が認められる。

であり，HMGB1 はこれらの受容体を介して炎症を引き起こすが，GVHD に関与する受容体が何かはまだ明確になっていない。HMGB1 が TLR4 を介してマクロファージ細胞株である RAW264.7細胞を活性化することと，TLR4 を発現させた HEK293 細胞が HMGB1 で活性化されたことの報告や，パラコート中毒による急性肺傷害のマウスモデルで TLR4 を介して HMGB1 により活性化された好中球の肺浸潤が認められたことの報告などがある。しかし，これらの報告は内因性の danger signal で引き起こされた炎症反応によるマクロファージや好中球の活性化とは異なる機序を示したものと考えられる。一方では LPS 敗血症マウスモデルにおいて HMGB1 は好中球の遊走を動員するが，マクロファージは動員しないことや，HMGB1 は TLR4 ではなく RAGE を介して好中球を活性化させることの報告があり，HMGB1 は好中球に直接作用するのではなく，ケモカインを介して好中球を活性化して遊走させる可能性も考えられている。いずれにしても，これまで GVHD の病態形成にはあまりかかわっていないと考えられていた自然免疫系の好中球が GVHD に関与している可能性が報告されるようになってきたこと

は興味深い。好中球は病原体を処理する時に貪食以外にNETs（neutrophil extracellular traps）と言われる抗菌作用物質（ミエロペルオキシダーゼやエラスターゼなど）を放出する機能を有している。NETsは臓器への損傷を及ぼすことがあり，マウスの肝臓の虚血/再灌流モデルでは好中球がNETsを放出して肝臓を傷害する。HMGB1は好中球に対してNETs放出させる作用を示すことが知られており，好中球のHMGB1を欠損させたマウスでは好中球NETsの放出が減弱し，肝臓へのダメージが軽減されたことが報告されていることからNETsがGVHDの組織傷害に作用している可能性がある。

3.GVHDの発症における非自己の認識

GVHDの発症において移植前処置で生じた炎症反応により活性化されたレシピエントの抗原提示細胞がドナーT細胞を活性化させる，という考え方が通説となっているが，GVHDのエフェクター細胞であるT細胞がどのようにして活性化されるのか，その機序はまだ不明な点が多い。また，GVHDの本態を「免疫学的自己と非自己の識別の破綻」と考えた時に，移植前処置による炎症が生じた状況下では，どのような機序で「自己」が「非自己」として認識されてしまうのかについてもまだ十分には明らかにされていない。

HMGB1はIL-1αやIL-1βと複合体を形成することが知られている以外に，細菌のDNAやウイルスのRNA，エンドトキシンと結合して複合体を形成するが，自己免疫の発症においてHMGB1は自己のDNAと結合して複合体を形成し，TLR9を介して樹状細胞を活性化することが報告されている[6]。HMGB1は核内に存在している時は自己のDNAと結合して安定化させる機能を有するが，組織が損傷を受けて細胞外に放出されたHMGB1がDAMPsとして作用する時は病原体成分のDNAやRNAと結合して複合体を形成し，外因性の病原体成分がパターン認識されるように提示して自然免疫系を活性化すると考えられる。一方，HMGB1が自己組織由来のDNAと結合することは自己の組織が損傷を受けたことを警告するのが目的で，自然免疫系による損傷組織の除去を促し組織を修復させるために生体にとって必要な反応のはずである。しかし，炎症反応が収束せずに慢性炎症化して自然免疫の反応が過剰となってしまうと，HMGB1と複合体を形成していた自己DNAを免疫系が除去すべき「非自己」と誤認識して傷害する反応が起こるのかもしれない。自己DNAと結合したHMGB1がGVHDの発症においてどのような役割を果たすのか，今後の解明が待たれる。

活性化された好中球がGVHDの標的臓器へ浸潤し，GVHDの発症に関わっていると想定されることから，好中球が「自己と非自己の識別」にどのように関わるのか，抗原提示細胞として機能しうるのかについても考える必要がある。好中球はケモカインを産生してT細胞の遊走に作用する一方で，T細胞が産生するIFN-γにより好中球はMHC-classⅡや共刺激分子であるCD80やCD86，CD40などを発現して抗原提示細胞としての機能を有し，CD4陽性T細胞と好中球が相互作用することが報告されている[7]ので，GVHDの初期の病態形成からエフェクター細胞であるT細胞の誘導に至るまでの過程における好中球の役割について今後さらに解析が進むことが望まれる。

4. 炎症反応の制御によるGVHD発症抑制

GVHDの本態は「自己と非自己の識別の破綻」であり，その「破綻」を誘導する原因となるのが移植前処置の後に続く慢性的な炎症反応だとすれば，「慢性炎症」がGVHDの病態を形成する本質な問題であり，移植前処置で生じた炎症反

応を収束させることが GVHD の本質的な治療方法になり得るのではないかと考えられる。したがって筆者らは GVHD 発症のトリガーである HMGB1 の作用を阻害することによって移植前処置後の炎症を阻止することが GVHD の発症抑制につながる可能性を検討している。

HMGB1 の作用を阻害する分子として DPP Ⅳ（dipeptidyl peptidase Ⅳ /CD26）が報告されている。しかし，筆者らの検討では直接的な阻害作用ではなく，何らかの分子を介した間接的な阻害効果の可能性があるため追加検討を進めている。また，抗 HMGB1 抗体による GVHD の発症抑制を検討するために，急性 GVHD マウスモデルで抗 HMGB1 抗体を day-4 から day0 までに投与したところ，抗 HMGB1 抗体投与群では非投与群に比べて GVHD に伴う体重減少が起こりにくい傾向が認められた。しかし，前述のように移植前処置後の HMGB1 の血中濃度は Day14 頃にピークとなることから，移植後のどの時点で抗 HMGB1 抗体を投与すれば HMGB1 の炎症惹起作用に対する適切な阻害効果を得ることができるのかはまだ不明であり，今後の検討を要する。

炎症反応の制御を考える上で，HMGB1 の作用を制御する因子や自然免疫系の制御機構についても検討を加える必要がある。HMGB1 は IL-1 と結合するが，炎症性サイトカインである IL-1 の産生を制御するのがインフラマソーム（inflammasomes）である。インフラマソーム は NLR（Nucleotide binding oligomerization domain-like receptor），ASC（apoptosis-associated speck-like protein containing a caspase recruitment domain），caspase-1 から成る，IL-1β の産生を制御する蛋白の複合体であり，炎症反応の制御に関わっている。代表的なインフラソマームである NLRP3（nucleotide-binding domain and leucin-rich repeat-containing family, pyrin domains-containing protein 3）インフラマソームは DAMPs や PAMPs によって活性化されることが知られている。したがって，移植前処置により損傷組織から DAMPs が放出されると，インフラソマームが活性化されて IL-1 が産生されると考えられる。一方で NLRP3 インフラマソームはマクロファージなど免疫細胞からの HMGB1 放出に作用していることが報告されている[8]。したがって，移植前処置で炎症反応が生じた後，HMGB1 と NLRP3 インフラソマームが相互作用することで炎症反応が遷延して慢性化する要因になっている可能性がある。さらに，抗原特異的 CD 8陽性 T 細胞の活性化には抗原提示細胞が産生する NLRP3 インフラマソームが作用している可能性を指摘した報告[9]がある。

このように炎症の制御機構を見ていくと，GVHD の発症を抑制するためには発症のトリガーである HMGB 1 の作用を阻害するだけでなく，HMGB1 の細胞外放出を制御すると同時に T 細胞の活性化にも作用する NLRP3 インフラマソームの作用を抑制することも GVHD の発症や増悪を防ぐために検討する必要があるかもしれない（**図3**）。

おわりに

「自己と非自己の識別」は免疫学の根源的な命題である。GVHD の本態が慢性的な炎症反応によって誘導される「自己と非自己の識別の破綻」であると考えれば，従来のように樹状細胞による抗原提示やエフェクター T 細胞について論じるだけではなく，GVHD 発症の初期免疫応答に関わる自然免疫系の作用を明らかにしていくことが重要である。特に慢性炎症による自然免疫の過剰反応という状況下で提示された「自己」がどのようなプロセスを経て「非自己」と認識されて GVHD のエフェクター T 細胞が誘導されるのか，など未解明の点が多い。GVHD の本質的な理解に基づく

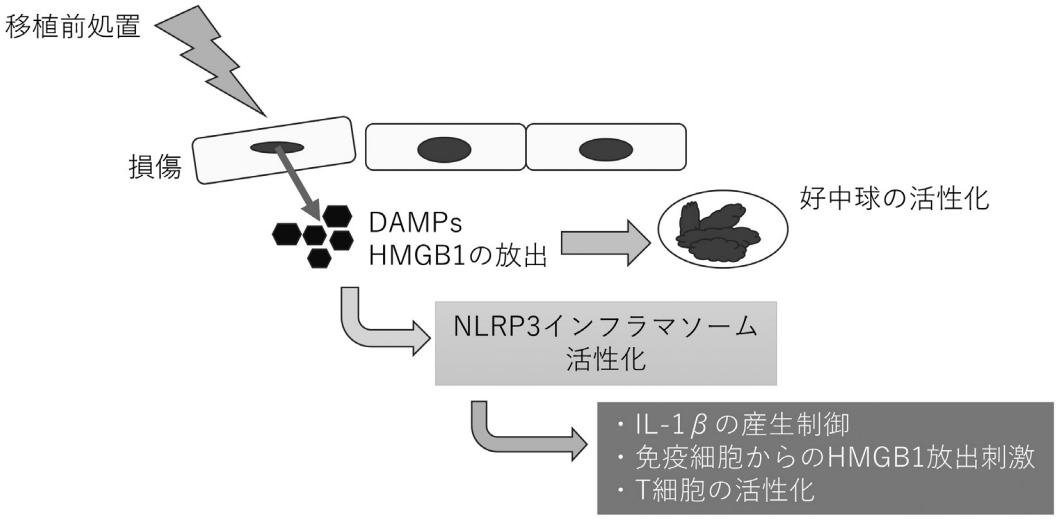

図3　移植前処置後の炎症に伴う免疫応答
移植前処置後に DAMP s、HMGB1 が放出されて誘導された炎症反応によって，自然免疫の活性が亢進し、GVHD の発症を引き起こすと考えられる。

治療のためには，これらの検討課題が今後解明されていくことが期待される。

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石綿(アスベスト)が人体に与える健康影響

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きしもと たくみ ● 1978年岡山大学医学部卒業。呼吸器内科医で職業性呼吸器疾患及び産業保健に関わり約35年になる。専門はじん肺及び石綿関連疾患の診断。日本職業・災害医学会理事、日本産業衛生学会代議員、日本呼吸器学会専門医・指導医、環境省中央環境審議会委員及び石綿健康被害判定小委員会委員長。

石綿繊維は気道を経由して吸入することにより細気管支・肺胞に到達する。また、肺に入った石綿繊維はリンパを介して胸膜腔に達するため、肺あるいは胸膜(臓側胸膜あるいは壁側胸膜)に病変を形成する(**図1**)。石綿繊維を体内に吸入したことを医学的に証明する所見として石綿小体・繊維と胸膜プラークがある。

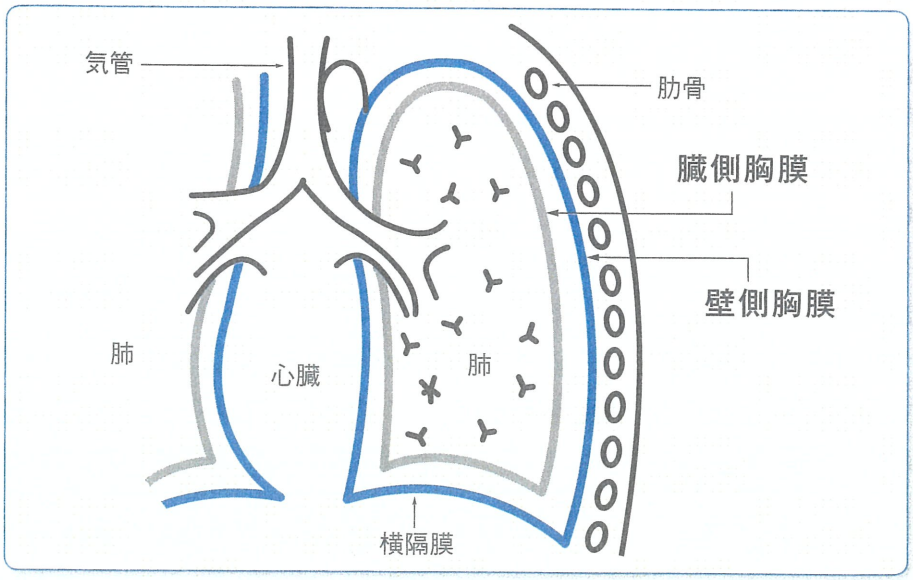
1. 石綿ばく露の医学的所見

(1)石綿小体・繊維

気道を介して吸入した石綿繊維は生体内で肺胞マクロファージ等の白血球が処理するが、処理しきれず、タンパク質などが繊維に付着したものが石綿小体である。石綿小体は光学顕微鏡で肺組織内あるいは気管支肺胞洗浄液にて確認できるが、その色は鉄タンパクであるフェリチンあるいはヘモジデリンに由来する(**図2**)。

石綿繊維については電子顕微鏡による確認が必要となる(**図3**)。石綿繊維とその他の繊維状物質を鑑別するにはX線回折装置を用いて繊維を構成する金属の成分分析が必要である。

図1. 臓側胸膜と壁側胸膜(イラスト画)



(2)胸膜プラーク

胸膜プラークは壁側胸膜にできる線維性の硬い組織である。通常、胸部単純写真、CTによって確認することができるが、薄い胸膜プラークは肉眼でしか確認できないため、手術や剖検時に壁側胸膜を丁寧に観察する必要がある。石綿低濃度ばく露によっても発生するが、病的なものではなく、石綿ばく露があったとする医学的証拠となる。

現在では胸膜プラークが石綿肺という用語としばしば混同されているため、適正な用語使用が望まれる。

図2. 石綿小体（光学顕微鏡像）

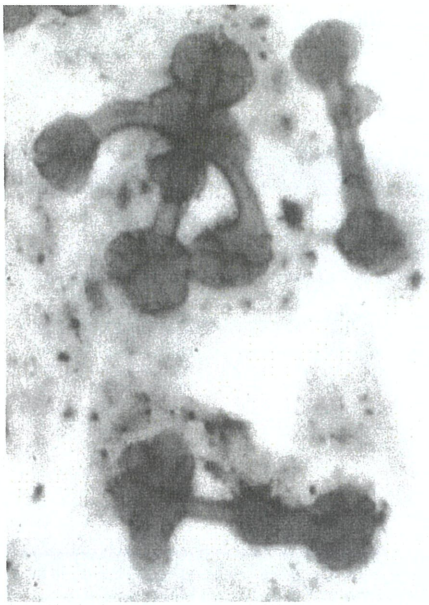
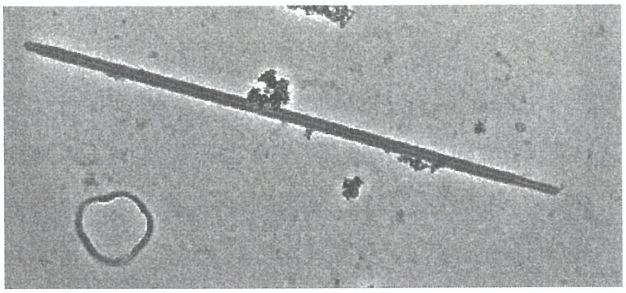


図3. 石綿繊維のⅠ種（クリソタイル）（透過型電子顕微鏡像）



2. 石綿関連疾患

良性病変として肺に石綿肺、胸膜には良性石綿胸水・びまん性胸膜肥厚を生じる。一方、悪性腫瘍として、肺には肺がんを、胸膜をはじめ、腹膜、心膜、精巣鞘膜に中皮腫を発症させることが医学的に明らかにされている**（表1）**。

これら疾患のうち、悪性腫瘍の場合には吸入する石綿繊維の種類に関係が深く、石綿吹付作業や水道管製造に使用され環境ばく露が社会問題となったクロシドライト（青石綿）の発がん性が最も高い。しかし、日本で過去に最も多く使用されたクリソタイル（白石綿）についても肺がんおよび中皮腫の発がん性が確認されている。

石綿がこれら疾患を招来して健康に影響を与えることが明らかになったのは産業革命後の19世紀末からである。また、医学的に最初に明らかになった疾患は石綿肺である。

表1. 石綿による呼吸器疾患の分類

	炎症・線維化	悪性腫瘍
肺実質	石綿肺	肺がん
胸　膜	良性石綿胸水 びまん性胸膜肥厚	胸膜中皮腫

（1）石綿肺

石綿肺は石綿高濃度ばく露によって発生するじん肺の1種であり、珪肺（けいはい）とは異なり胸部単純写真上不整形陰影（線維化）を呈する。発症までの潜伏期間は大阪泉南の石綿紡績作業ではわずか5年程度であったと報告されているが、通常は10年以上を要する。

しかし、日本では作業環境改善がなされて以降、石綿高濃度ばく露がなくなったため、ほとんど認められなくなった。中国では現在でも石綿紡績作業等高濃度石綿ばく露作業が行われており、石綿肺と診断されている作業者は少なくない。

（2）石綿肺がん

石綿による肺がん発生には、石綿高濃度ばく露が必要であると考えられているが、石綿単独ばく露での肺がん発生頻度はそれほど高くなく、喫煙が相乗あるいは相加作用として重要であり、石綿肺がん患者の大半が喫煙者である。

石綿ばく露によって発生する肺がんには組織学的な特徴はなく、一般人肺がんと同様である。石綿初回ばく露から肺がん発症までの潜伏期間は40年以上と長いため、今後も増加が予想されている。日本では石綿ばく露による肺がんの労災あるいは救済法による認定においては、胸膜プラークの存在や肺内石綿小体・繊維の定量が重要視されている。

石綿吹付作業、石綿紡績作業、石綿セメント製造作業者に発生した石綿肺がんでは医証は求められず、作業期間が5年以上であれば労災認定されることになっている。

（3）中皮腫

中皮腫は胸膜、腹膜、心膜、精巣鞘膜に発生する悪性腫瘍であるが、中皮細胞ががんにも肉腫にも分化する性質を持つため、中皮腫と呼ばれる。

中皮腫の約80％が石綿ばく露に起因するが、石綿ばく露単独で発症するため喫煙との関連性はない。石綿肺がんと同様、石綿初回ばく露から40年以上の長い潜伏期間を要することから、今後日本でも増加すると予想されている。事実、中皮腫による死亡者数は1995年には１年間で500人であったが、2017年には1,555人と３倍以上に増加している。増加の著しい中皮腫は男性の胸膜中皮腫であり、過去の石綿ばく露との関連性が明らかである。

診断は腫瘍組織によるが、顕微鏡下で観察してもがんに類似した上皮型、肉腫に類似の肉腫型とこれら二者が混じりあう二相型の３種類のタイプに分類される。中皮腫に特徴的な免疫抗体がないため、複数の抗体やその他の手法を用いて確定診断をする必要がある。現在でもなおその確定診断は難しく、そのため誤った診断がなされていることも少なくない。特に肺がんや卵巣がんあるいは良性疾患である線維性胸膜炎との鑑別診断が必要となる。

一方、治療は限られており、早期病変を診断した場合には壁側胸膜切除・臓側胸膜剝離術という手術療法が行われる。化学療法としては唯一シスプラチン＋ペメトレキセド併用療法が有効で、この治療が無効になった際には免疫チェックポイント阻害剤であるニボルマブの使用が承認された。しかし、これ以外に治療方法がなく予後不良な疾患である。

（４）良性石綿胸水

石綿ばく露により臓側胸膜に炎症が生じるために胸水が貯留する疾患を良性石綿胸水という。良性とは非悪性という意味であり、臨床経過が良好であるという意味ではない。胸水を穿刺すると滲出液で、悪性腫瘍細胞を認めないことが大原則であるが、本疾患の診断基準は今のところ定められていない。

また、本疾患は発生機序が不明であるため治療方法がないのが現状である。ほとんどの症例では自然に胸水は減少するが、完全に消失することは少なく、あとに器質化胸水あるいはびまん性胸膜肥厚を残すことが多い。早期の胸膜中皮腫との鑑別が重要であるため、胸腔鏡による検査と胸膜の生検が必要になることもある。労災では補償の対象疾病となっているが救済法では対象外である。

（５）びまん性胸膜肥厚

広範囲で肺の一葉以上を巻き込むような胸膜の線維化をびまん性胸膜肥厚という。臓側胸膜の線維化で通常壁側胸膜との癒着を来している。そのため、肺の膨張が妨げられることによって、肺活量が低下する疾患である。石綿ばく露以外でも発生するため、石綿ばく露歴が明確であることを必要とし、その他のこのような病態を来し得る疾患、たとえば心臓手術後やリウマチ胸膜炎、がん性胸膜炎等を鑑別する必要がある。石綿ばく露による場合には良性石綿胸水後に発生することが多いと報告されている。

労災・救済の対象はその病変の範囲が胸部単純写真正面像で、片側の場合は片側胸郭全体の1/2以上、両側の場合は両側胸郭全体の1/4を超えるものである。

潜伏期間は石綿肺がんや中皮腫と同様40年以上である。著しい呼吸機能障害を伴う本疾患は比較的予後が不良であることが明らかになりつつあるため的確な診断、適切な治療が望まれる。

3. 今後の対策

石綿関連疾患は石綿吸入後の潜伏期間が長い疾患が多いことから、吸入してもすぐに症状が出ない。しかし、石綿肺がんや中皮腫のみならずびまん性胸膜肥厚は一度発症すると予後不良であることが多い。過去に使用された石綿が負の遺産として沢山残っている日本では、建物の解体作業がこれからピークを迎えるため、新たな石綿吸入機会も少なくない。石綿関連疾患防止のために最も重要なことは石綿吸入防止対策である。そのためには、電動ファン付き防じんマスク等を使用した適切な石綿吸入防止策が望まれる。

また、過去の石綿ばく露者については、石綿関連疾患発症の可能性があるため早期診断・早期治療が必要である。医師をはじめとする医療従事者及び石綿ばく露者はこれら疾患についての知識を持っておくことが必要である。

石綿関連疾患の診断と治療

Diagnosis and treatment of asbestos related diseases

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＜要　約＞

2006年に兵庫県尼崎市の旧クボタ神崎工場周辺で中皮腫多発事案が報じられて13年が経過した。石綿関連疾患の潜伏期間はおおむね40年以上と長い。戦後の経済発展とともに石綿を汎用してきた日本において今後もこれら疾患の増加が予想されている。石綿関連疾患は産業保健関係者から臨床医まで幅広い対応が必要である。診断および治療に大きな進歩を見せている中皮腫を中心に最近15年に投稿された論文のレビューを行った。

キーワード：石綿肺、石綿肺がん、中皮腫、良性石綿胸水、びまん性胸膜肥厚

Ⅰ．石綿肺

1．疫　学

石綿肺とは、石綿（アスベスト）へのばく露による肺の線維化であり、石綿へのばく露による疾患としては、最も古くから知られている疾患である。欧米では1890年代から、石綿紡織工場の従業員の事例が報告されてきた。石綿のばく露量が減少した1968年以降に初めて石綿にばく露した労働者の石綿肺による死亡のリスクは低下している[1)]。最近では、造船労働者における石綿肺の頻度は高くないが、建設労働者で多数報告[2)]されている。日本では、石綿肺による死亡数は1968年には５人とされていたが、その後の推移は、厚生労働省人口動態統計によると**表１**のようになる。その多くは石綿紡織業の従業者と思われ、女性の割合は20％程度であるこ

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表1　石綿肺での死亡者数

年　代	石綿肺での死亡者数		
	男	女	計
1971～1975	18	10	28
1976～1980	24	8	32
1981～1985	34	11	45
1986～1990	59	17	76
1991～1995	66	22	88
1996～2000	117	24	141
2001～2005	109	16	125

（厚生労働省人口動態統計による）

とが特徴といえる。中国では今でも紡績作業は行われており、石綿肺の発生頻度は少なくない。

環境省と厚生労働省の主導により制定された石綿による健康被害の救済に関する法律（以下、石綿健康被害救済法）における石綿肺の患者の認定数をみると、年間数十名である。判定委員会では、石綿肺分科会を設けて認定の可否を判断しているが、最も患者数の多い特発性間質性肺炎（肺線維症）や他の原因によるじん肺症、あるいは喫煙による肺気腫に随伴する線維化などとの鑑別が必要とされている。一方、クロシドライト鉱山でのばく露があった南アフリカでは石綿肺の症例が減少しているが、石綿肺には肺がんや中皮腫の合併が多くなっており、石綿肺自体で死亡する人は高齢化している[3)]。

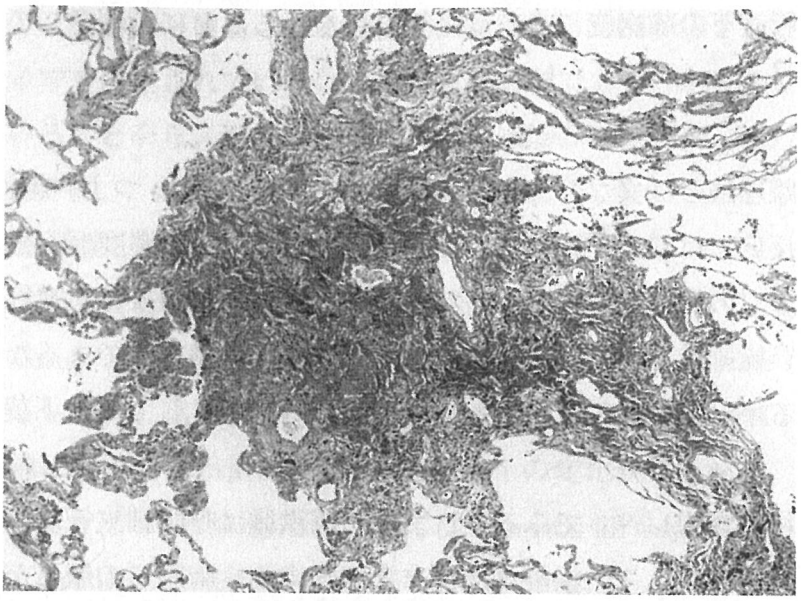
診断における要点は職業性石綿ばく露歴により高濃度石綿ばく露が存在することを詳細に聴取することと、石綿による胸膜プラークやびまん性胸膜肥厚の存在や肺組織内あるいは喀痰や気管支肺胞洗浄液中の石綿小体の証明が鑑別上必要である。1型のような軽度の石綿肺は進展が遅く、慢性間質性肺炎のように進行が速くないこと、また急性増悪しないことなどが特徴である[4)]。

2．画像診断

石綿高濃度ばく露によって発生するじん肺で、胸部単純写真上、両側下肺野外側を中心とした不整形陰影を呈す。また、不整形陰影が出現する前から、胸部聴診上両側肺底部に捻髪音を聴取することが多い。じん肺法では、じん肺標準フィルムの1型以上の所見を認める場合にのみ石綿肺と診断する。石綿肺の進行とともに微細な線状・網状影が両側下肺野外側から内側、上方に向かって進展し、下肺野が縮小する。さらに病変が進展するに従って線状・網状影は粗大になっていく。それとともに心陰影の境界は不鮮明（shaggy heart）となり、中・下肺野に輪状の蜂窩肺による輪状影を呈する場合もある。特発性間質性肺炎（Idiopathic pulmonary fibrosis/Usual interstitial pneumonia, IPF/UIP）に比し進行は緩徐である。

胸部画像上、IPF/UIPとの鑑別には注意が必要である。high resolution CT（HRCT）におけるsubpleural dotsやcurvilinear linesというような石綿肺に特徴的とされる所見は、病理所

(A)



(B)

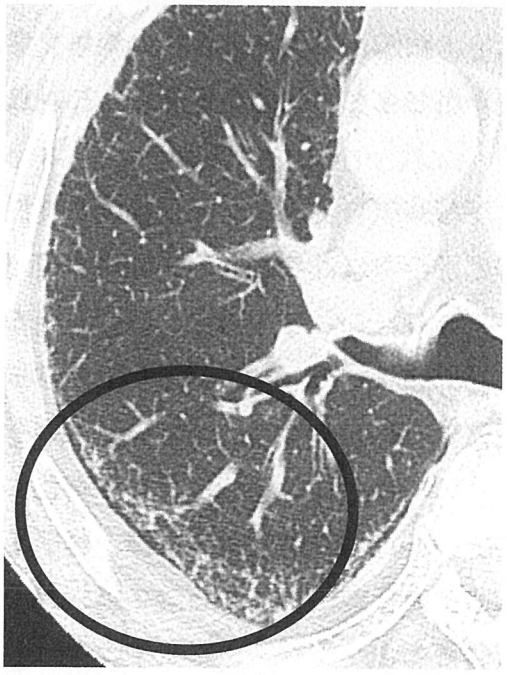


図１　石綿肺の初期像：（A）呼吸細気管支周囲の線維化をみる（HE 染色所見）。
（B）Subpleural curvilinear line とよばれる（CT 画像所見）。

見における細気管支周囲から末梢へと進展する線維化と対応しており、小葉辺縁から生じるIPF/UIPとの鑑別診断に有用である[5,6]（**図１**）。また、石綿肺においては、石綿ばく露により生じる胸膜病変である胸膜プラークやびまん性胸膜肥厚を伴うことが多いことも石綿肺と診断する根拠となりうるが、これら胸膜病変を伴う肺線維症が石綿肺であるとは限らない。石綿肺に胸膜プラークを伴わない頻度が20％という報告もあり[7]、胸膜病変を必ず伴うというわけではない。かつて石綿肺は大量に石綿繊維を吸入する作業場で発生しており、胸膜プラークの潜伏期間である15年以内に石綿肺が発症していたことが胸膜病変を伴わない典型的な石綿肺であったことも考慮しておくべきである。特に肺病変が進行した症例では軽微な石灰化を伴わない

胸膜プラークは胸膜直下の肺病変により同定しづらくなることにも留意しておく必要がある。

3．病理診断

珪肺を生じる遊離珪酸とは異なり、繊維状珪酸塩である石綿は、つよい線維化を引き起こし、ばく露量と線維化反応には量-反応関係があるといわれる[8]。高濃度ばく露者では短期間で石綿肺を生じるが、多くの例では、10年から20年の経過で緩徐に線維化が進行する。石綿繊維の種類からみると長い繊維ほど線維化を起こしやすい。石綿は繊維状であるために、一旦末梢肺に入ると排除される率が低い。したがって線維化の肺内分布としては、下葉下部にみることが多い。

線維化の進展を組織学的レベルでみると、まず石綿繊維は呼吸細気管支壁へ侵入し、その壁に線維化による肥厚を来す。この線維化は終末細気管支から肺胞道の壁にかけてその周囲の間質に広がり、さらに肺胞壁におよぶと肺胞腔の狭小化を生じて、呼吸細気管支を中心とした線維化巣を形成する[9]。線維化が進行すると、胸膜と接して、帯状の線維化像を形成し、無気肺硬化像とよばれる。残存する気腔の拡張が生じると、線維化巣とともに、蜂窩肺の形態を示すこともある。

こうした線維化の過程の中では、まず呼吸細気管支すなわち小葉中心性に生じる線維化であることが石綿肺の特徴で、他の疾患との鑑別の際にはこの点の認識が重要となる。石綿へのばく露の指標として石綿小体はよく知られており、これを組織標本で認めることも重要ではあるが、その存在部位に留意する必要がある。肺胞腔内のマクロファージや線維化をみない肺胞壁に認めた場合は、石綿が線維化を来したとする根拠にはならず、石綿肺と診断することは難しい。早期病変であれば、呼吸細気管支壁あるいはこれを中心とした線維化巣内に石綿小体をみることは石綿肺の診断にとって有用である。進行期の病変としては、胸膜下の帯状線維化巣内に石綿小体をみることが石綿肺の根拠となる。この場合は、石綿小体以外でも肺内には多くの含鉄小体を含むことがあり、石綿小体とするか否かの判断が重要である。

1986年のアメリカ胸部疾患学会は石綿肺の診断基準として、細気管支から末梢に進展する線維化と細気管支周囲への石綿小体あるいは石綿繊維の沈着を強調していた[10]。しかし、1997年のヘルシンキクライテリア[11]および2004年のアメリカ胸部疾患学会[12]の定義では、よく膨らませた肺においてびまん性肺線維症があり組織切片上1 cm^2の領域に2個以上の石綿小体あるいは被覆されていない石綿繊維を認める場合に石綿肺と定義すると変更した。この定義であれば、量-反応関係のないUIP型の慢性間質性肺炎であっても石綿肺と診断すべき症例が存在することになる。慢性間質性肺炎が増加する現在、石綿肺の定義の見直しとして、2010年のアメリカ呼吸器病理医会の石綿肺レポートでは石綿肺の特徴として、細気管支周囲からの線維化が末梢へと進展するとした1986年の定義が復活し、①炎症をほとんど欠く、②ゆっくりとした進展でfibroblastic fociを認めない、③臓側胸膜の弱い線維化を伴う病態とともに石綿小体の存在が重要であるとしている[13]。しかし、1997年のヘルシンキクライテリア[11]が述べているびまん性肺

線維症で一定以上の石綿小体が検出される慢性間質性肺炎と鑑別ができないようなUIP型石綿肺というようなタイプも否定されていないので注意が必要である。特に日本ではIPF/UIPによる死亡が増加していることから、石綿肺とIPF/UIPの鑑別には慎重であることが求められる。

4．石綿肺のGrade分類

1982年、CAP-NIOSHから提唱された線維化の強さと拡がりをもとにした分類を改訂して、2004年に示されたGrade分類[14]がよく使われている（**表2**）。

しかし、このGrade分類にもさまざまな問題点があることが指摘されている。Grade 1の線維化ははたして石綿によるものか否か、また、Grade 1の変化がGrade 3、4と進行していくものなのかなどである。そこでGrade 1についてはasbestos airway diseaseという名称も提案されている。また、Grade 4の蜂窩肺を形成する高度な線維化を石綿肺とする際に、特発性肺線維症などの疾患との鑑別が困難なことが多い。この場合、Grade 4の所見以外に高度な線維化のないGrade 1～3の呼吸細気管支周囲の線維化を認めることが重要である。通常の病理診断時に、蜂窩肺を伴う高度な線維化巣に少数の石綿小体を認めて、安易に石綿肺の診断を下さないことが大切である[15]。

5．石綿肺の鑑別診断

(1) 特発性間質性肺炎との鑑別

間質の線維化は、薬剤や放射線など医療性要因、膠原病やサルコイドーシスなどの全身性疾患との合併、過敏性肺炎や感染症肺炎などの局所の炎症によるものなど、その原因は多岐にわたる。

なかでも原因不明なものを、特発性間質性肺炎（idiopathic interstitial pneumonia, IIP）とよぶが、これは多彩な像を示し、6つの主な組織型と2つのまれな組織型および分類不能型に分けられている。この中で患者数が多くかつ難治性であるのが、IPFであり、病理組織学的には、通常型の間質性肺炎（UIP）パターンを示し、固有の肺胞構造は破壊され、胸膜直下に蜂窩肺を形成する。IPFは慢性に進行し、予後不良である[15]。

表2　石綿肺のGrade分類

Grade 1	呼吸細気管支壁とそれに接した肺胞壁の第一列までに限局する線維化で、観察標本上、その像が、呼吸細気管支数の半分以上にみられる場合。それに満たない場合は、Grade 0とする。
Grade 2	呼吸細気管支壁とそれに接した肺胞壁の第二列以上や肺胞道壁におよぶ線維化で、隣接した病変との間に健常な肺胞組織があり、接合を示さない場合。
Grade 3	線維化の進展によって、隣接する同様の病変との接合を示す場合。線維化が高度であっても蜂窩肺所見を認めない場合。
Grade 4	蜂窩肺を伴う高度な線維化を示す場合。

非特異性間質性肺炎（nonspecific interstitial pneumonia, NSIP）は細胞性胞隔炎を主体とするcellular NSIP（c-NSIP）と、肺胞構造の破壊と線維化を示すfibrosing NSIP（f-NSIP）がある。石綿肺との鑑別には、上記のIPFとf-NSIPが対象となる。石綿肺が呼吸細気管支を中心とする小葉の病変であるのに対し、IPFは小葉の辺縁に病変が生じ、f-NSIPは汎小葉性に病変をみる、という病変の局在に留意すべきである。これらの点から、進行した症例や検索しうる範囲が狭い場合は鑑別が困難となる。

(2) その他

喫煙にもとづく所見は、石綿ばく露者にはよく合併している。喫煙は気道中心に小葉中心性病変をつくるため鑑別が必要となる。また、喫煙による肺気腫が高度である場合、小葉中心から肺胞隔壁に拡がる線維化をみることがあり、鑑別が難しくなる。石綿小体の存在などを手がかりに鑑別をすすめるしかない。

慢性過敏性肺炎（Chronic hypersensitivity pneumonia, CHP）も石綿肺との鑑別を要する疾患である。この場合、細気管支の腔内および壁内に、結合性の緩い小型の肉芽腫がみられたり、異物型巨細胞の出現をみることがある。組織像は多彩で、小葉中心部の線維化病変相互、あるいは小葉中心部と小葉辺縁部の線維化病変をつなぐ線維化がみられ、石綿肺にも類似した所見を示すことがある[16]。

6．石綿肺の現状

石綿肺が発生するためには最低でも25石綿繊維/mL×年の石綿繊維の吸入が必要となる。欧米各国では石綿禁止あるいは使用量の低下から1968年以降石綿肺による死亡が激減していると報じられているが、中国、ロシアなど現在でも石綿生産あるいは使用している国々では減少していない[3]。剖検による病理学的な検討を行うとfalse negativeの診断が多いことも事実である。石綿肺の病理診断と画像診断には矛盾が生じることも指摘されており、病理診断で石綿肺と診断された症例の画像を後方視的にレビューしてもじん肺診断の１型であると診断される例が少なくないことも事実である[2]。

治療法は喀痰、咳や呼吸困難などの自覚症状に対する対症療法のみであるため、新たな石綿粉じん吸入を防止することが必要である。

Ⅱ．石綿肺がん

1．石綿ばく露と肺がん発生

石綿に関連する悪性腫瘍には、中皮腫と肺がんがある。中皮腫が石綿以外の要因がほとんど知られていないのに対し、肺がんは喫煙、粉じん、大気汚染などさまざまな原因により発生する。

石綿による肺がん発生には、石綿高濃度ばく露が必要であると考えられている。石綿高濃度ばく露による石綿肺に合併する肺がんを過去には石綿肺がんと称していたが、最近では石綿肺を伴わない肺がんの存在も明らかとなった[17]。

石綿による肺がんの発生には量 - 反応関係があり、ばく露量が多いほど肺がん発生頻度は高い。また、肺がん発生の最大の要因は喫煙である。1979年には、Hammondらが北アメリカの断熱作業者を1966年時の喫煙有無別に肺がん死亡率を調査して以下の結果を報告した。石綿単独ばく露での肺がん発生頻度はそうでない場合の5.2倍であるが、喫煙により53.2倍になると報告している[18]。しかし、同じ対象の断熱作業者を1981年から2008年まで追跡したところ、石綿ばく露のある喫煙者の肺がん発生頻度は28.4倍であった。同じ石綿ばく露者でも石綿肺のない作業者では14.4倍であり、石綿肺合併者においては36.8倍であったと報告している[19]。石綿ばく露量が多ければ喫煙が相乗効果となるが、そうでなければ相加作用のみであり、石綿ばく露量によって、喫煙の効果は異なることが明らかとなった。

2．石綿肺がんの臨床

日常の臨床で遭遇する「石綿肺がん」に、明らかな臨床的な特徴はない。組織学的にも、腺癌、扁平上皮癌、大細胞癌、および小細胞癌のいずれもがばく露のない人と同様の頻度で発生する。大気中の石綿濃度が１石綿繊維 /mL の職場に25年（週40時間）働いた場合のばく露量（25-100石綿繊維 /mL ×年）に相当する累積ばく露量がある場合に肺がんの危険度が２倍に増加するとされており[11]、石綿ばく露作業歴や、胸部画像所見あるいは肉眼的な胸膜プラークの存在、肺内の石綿小体などの医学的所見をもとに石綿ばく露の程度が推定され、労災補償あるいは石綿健康被害救済法による救済給付の対象となる。

ただし、過去の日本において石綿肺を合併させるほどの石綿高濃度ばく露が明らかであった石綿吹付作業者、石綿紡績作業者、石綿セメント製造作業者に発生した石綿肺がんでは石綿ばく露による医証は求められず、作業期間が５年以上であれば労災認定されることになっている。

全国労災病院でまとめられた調査結果によれば、石綿肺がん152例の診断時年齢中央値は72歳で、喫煙者が90％を占めていた。また、その98％が職業性石綿ばく露で、造船作業、建設作業、石綿製品製造作業の頻度が高かった。職業性石綿ばく露期間中央値は31年で、潜伏期間は47年であった[20]。

フランスの2003年から2013年までの石綿肺がんで労災補償された男性146例を後方視的に検討したところ、診断時年齢は63.2歳で、そのうち69.9％が造船労働者で、7.5％がビル建設業者であった。喫煙者が日本の報告と同様90％で、１日の喫煙本数は30.4本と重喫煙者が多数を占めた。組織型では腺癌が45.9％で最も多く、扁平上皮癌が38.4％であった[21]。

現在は肺線維症を示す石綿肺に合併した肺がん症例が減少しているので、通常人の肺がんと同様の治療方法が適応となる。特に腺癌の占める比率が高いため、分子標的治療の対象が多いので多彩な治療方法が選択可能である。また、過去の石綿ばく露者では国の指定機関により年

２回の胸部単純写真による定期健康診断を受けることが可能であるため、肺がんの早期診断頻度も高くなっていることから手術適応となる症例も増加している。

３．石綿肺がんマーカー

最近、石綿による肺がんの遺伝子レベルでのマーカーが報告されるようになってきた。2p16[22]は石綿繊維量と関連が深く、この部分のDNA欠損が石綿ばく露の無い肺がん例では９％であるのに対して500万本以上石綿繊維が認められた例では22%の欠損があったと報告されている。9p21.3に存在するp16/CDKN2Aは中皮腫でホモ接合性欠失（homozygous deletion）が知られているが、石綿肺がんでも同様の変異が50％あることが報告されている[23]。また、9q33.1の変異はすべての組織型の石綿肺がんで石綿ばく露との関連性が観察されているため、石綿によるDNA障害の潜在的な部位となっている可能性がある[24]。さらに19p13の欠失も腺癌を除く肺がんで石綿ばく露との関連性が示されている[25]。19p13、9q33.1、2p16の欠失や変異は、石綿繊維500万本以上が検出された肺がん患者においてそれ以下の症例と比べて有意に高いことから、石綿肺がんのマーカーになると報告されている[26]。

４．石綿による肺がんの労災認定基準

石綿による肺がんの労災認定基準は以下のとおりである。(1)から(6)のいずれかに相当する場合、労災認定の対象となる。

(1) 第１型以上の石綿肺

(2) 胸膜プラーク＋10年以上の石綿ばく露作業従事期間

(3) 以下のいずれか＋１年以上の石綿ばく露作業従事期間

ア 乾燥肺重量１g当たり5,000本以上の石綿小体

イ 乾燥肺重量１g当たり200万本以上の石綿繊維（５μm超）

ウ 乾燥肺重量１g当たり500万本以上の石綿繊維（１μm超）

エ 気管支鏡肺胞洗浄液１mL中５本以上の石綿小体

オ 肺組織切片中の石綿小体または石綿繊維

(4) 以下のいずれか＋１年以上の石綿ばく露作業従事期間

ア 胸部正面エックス線写真により胸膜プラークと判断できる明らかな陰影が認められ、かつ、胸部CT画像により当該陰影が胸膜プラークとして確認されるもの

イ 胸部CT画像で胸膜プラークを認め、左右いずれか一側の胸部CT画像上、胸膜プラークが最も広範囲に描出されたスライスで、その広がりが胸壁内側の４分の１以上のもの

(5) 特定の石綿ばく露作業への従事期間が５年以上あること

- 石綿製品製造（石綿紡織製品・石綿セメント製品）
- 石綿吹付作業

(6) 著しい呼吸機能障害を伴い、職業性石綿ばく露期間が３年以上あるびまん性胸膜肥厚

5．石綿肺がんの石綿健康被害救済法による認定基準

1997年のヘルシンキクライテリア[11),27)]は石綿ばく露による肺がん発生を２倍にする基準として、25石綿繊維/mL×年を提唱している。この基準を満たす医学的所見は以下の(1)から(6)の如くである。

(1) 乾燥肺重量１g当たり5,000～15,000本以上の石綿小体
(2) 乾燥肺重量１g当たり200万本以上の石綿繊維（５μm超）
(3) 乾燥肺重量１g当たり500万本以上の石綿繊維（１μm超）
(4) 気管支鏡肺胞洗浄液１mL中５本以上の石綿小体
(5) 病理組織標本上に石綿小体あるいは石綿繊維を確認できる
(6) 胸部エックス線検査またはCT検査上、胸膜プラーク所見があること＋胸部エックス線検査でじん肺法に定める第１型以上と同様の肺線維化所見があり、胸部CT検査においても肺線維化所見が認められること

以上を満たす肺がん症例は、日本の石綿健康被害救済法およびイギリス以外のヨーロッパ諸国の石綿肺がんの認定基準となっている[22)]。しかし、アジアの国々では石綿肺がんの基準は石綿肺というじん肺に合併した肺がんあるいは著しい呼吸機能障害を伴う石綿肺に合併した肺がんに限って労災補償されているのが現状である。以上のように日本における石綿肺がんの基準は寛容であることから、労災および救済法における基準を満たすような症例に対しては積極的な申請が望まれる。

Ⅲ．中皮腫

1．疫　学

石綿関連疾患における報告や統計は石綿使用を中止したか、大幅に減少した国々からのものである。世界で現在石綿を使用している量は400万t／年であるが、そのほとんどがアジア諸国と旧ソ連圏の国々での消費である。特に世界で人口が多い10番目までの国で石綿を中止している国は日本のみである。発展途上国では、石綿関連疾患の認知度は低く、適切な診断、登録等が行われておらず、今後同疾患が増加する懸念がある[28)]。日本での中皮腫の発生は1980年代まではまれであり、なじみの薄い腫瘍のひとつであったが、1990年代半ばから急激にその発生数の増加がみられるようになった。これは、1960年代からの高度経済成長期に石綿の使用が急激に増えたことによって、それから30～40年の潜伏期を経て、中皮腫の発生頻度の増加が生じたと考えられる。この事実は、中皮腫の発生が石綿へのばく露と密接に関連し、初回ばく露から中皮腫の発生までに長い時間経過が必要であることを示している。さらに、1995年からは、死亡統計において胸膜・腹膜の腫瘍は明確に区別され、男女別、部位別の死亡者数を見ることが可能となったが、これを各年毎にみてみると、男性の胸膜中皮腫が増加の大半の部分を占めることが明らかである（**図２**）。この結果は、主として職業性の石綿ばく露によって中皮腫を発症

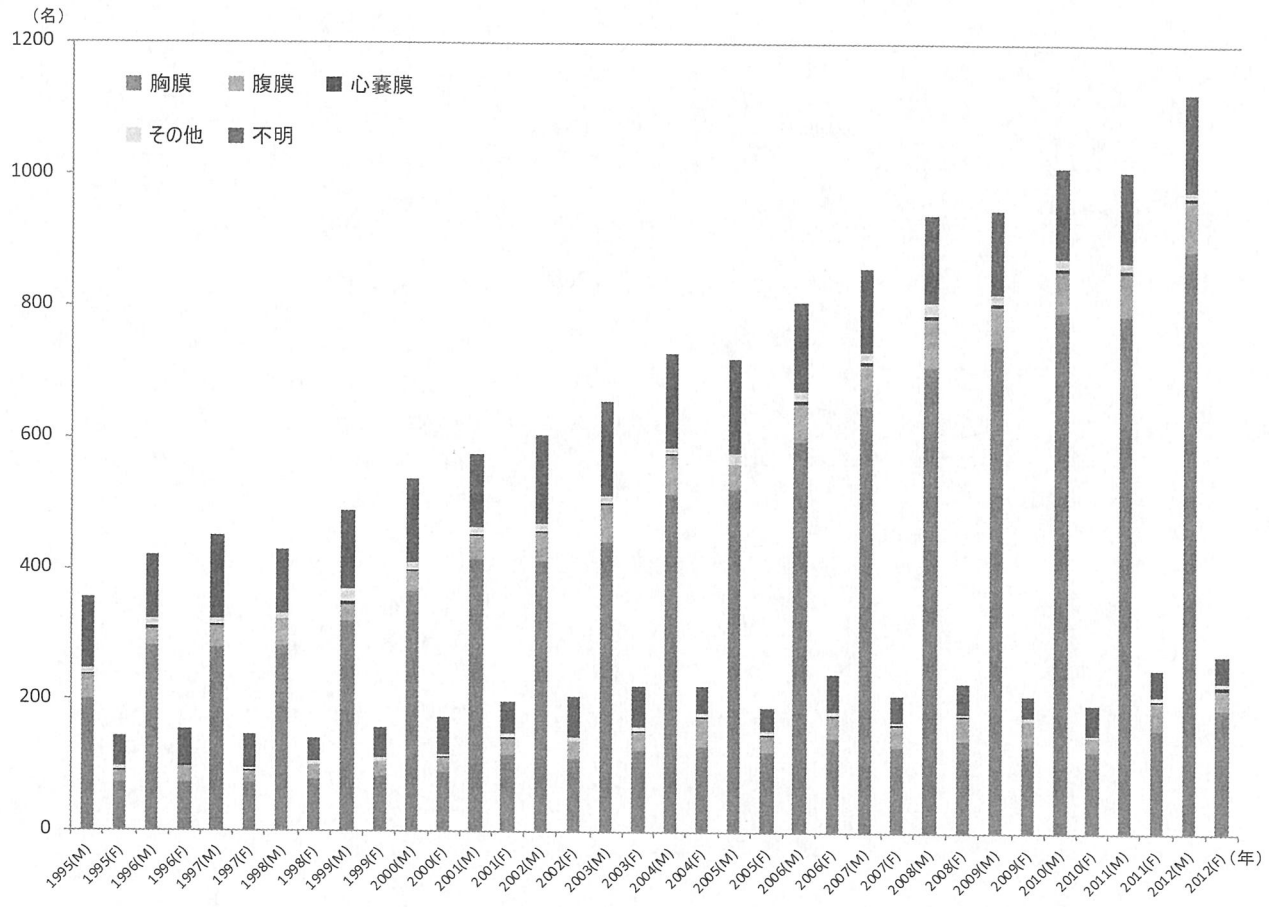


図２　中皮腫の発生の経年的推移（1995-2012）（部位別、男女別）

した人が急増したことを裏付けている。

一方、ひとつの国における石綿の使用量と中皮腫の発生との関連を検討した報告によると、年間約200tの使用について１例の中皮腫が発生するという。これに従うと、日本における石綿の使用量のピークは1974年のおよそ35.2万ｔであり、40年後（2014年）の中皮腫の発生数はおよそ1,700例となる。実際にこの年の中皮腫の死亡者数は1,376例であり、推測値に近い値を示している。2006年まで続いた石綿の使用を考えると、2040年頃までは同程度の中皮腫の発生が続くと予想される。

日本における中皮腫の疫学的な調査は少ない。日本全国で2003～2008年に中皮腫で死亡したのは死亡統計上6,030例であった。そのうち画像、病理学的な調査を行った結果929例は医学的に中皮腫であることが確認された。そのうち85.5％が胸膜、13.2％が腹膜、0.8％が心膜、0.5％が精巣鞘膜原発であった。診断時年齢の中央値は68歳で職業性石綿ばく露は73.7％、環境ばく露は2.2％、家庭内ばく露は0.9％で全体の76.8％が石綿ばく露によることが証明された。ばく露期間中央値は30年で、潜伏期間の中央値は43年であった。職業性石綿ばく露の職種別では建設業が131例、造船業が91例、配管作業が46例、電気工事が44例と多かった[29]。尼崎市における職業性石綿ばく露による男性中皮腫の SMR（standardized mortality ratio）は6.75、非職業性石綿ばく露による女性中皮腫の SMR は14.88でいずれも予想より有意に増加していた[30]。この事

実は子供の頃の石綿セメント工場周囲での環境ばく露が後に中皮腫の発生に影響するとする報告にも一致する[31]。

ジェノヴァ（イタリア）での1960～1980年までの造船所の労働者（3,984例）を2014年12月までfollow-upしたところ、胸膜中皮腫のSMRは5.75であった。399例のうち22.6％の90例は石綿肺がんであり、石綿ばく露によるattributable fractionは労働者の49.6％であった[32]。イタリアのクリソタイル鉱山労働者での中皮腫死亡はSMRが5.54であったが、肺がん死亡の有意な増加は確認できなかった[33]。

バーリ（イタリア）での石綿セメント工場従業員の胸膜中皮腫のSMRは、最後のばく露後20～29年でピークに達するが、腹膜中皮腫では20年後にプラトーに達する。肺がんはそのどちらよりも過剰な死亡者数であった[34]。石綿中止後の中皮腫のリスクを調査した６つの研究結果から、中皮腫は中止後10年でrelative risk（RR）は1.02で石綿肺がんは0.91であり中止後も10年間は両疾患とも減少しないことが判った[35]。スウェーデンでは1982年に石綿禁止としたが、1961～2009年までの癌登録をみれば24種の職業で中皮腫のリスクが増加していた。そのうち男性では配管工のSMRが4.99で最も高かった[36]。オーストラリアは2003年に石綿禁止をして2016年まで経過を見ているが、ようやくピークを迎えようとしている。中皮腫は1982～2016年までに16,679人が診断されたが、そのうち男性が84％であった[37]。

ブローニ（イタリア）の石綿セメント工場[38]では職業性石綿ばく露のみならず環境ばく露、家庭内ばく露が確認されており、中皮腫は低濃度でも発症していることが確認された。環境ばく露や家庭内ばく露で中皮腫になった症例では、職業性ばく露による中皮腫に比較して生命予後が悪かった。中皮腫は若いうちの石綿ばく露が発症に関連深く、ばく露量が多ければ多いほど潜伏期間が短いと報告されている[39]。

フランスにおける胸膜中皮腫と石綿ばく露との関係による調査では女性の場合は非職業性石綿ばく露、すなわち家庭内ばく露や環境ばく露が大きく影響するとされており、男女によってそのばく露形態が異なると考えられる[40]。同じフランスからの報告によれば、石綿単独ばく露での中皮腫のodds ratio（OR）は4.3であるのに対して、石綿＋鉱物繊維では17.6、また石綿＋シリカでは9.8と鉱物繊維やシリカと石綿の混合は、ばく露により中皮腫の発生率が高まると報告されている[41]。

石綿コホートスタディー55例を基に中皮腫発生から計算された石綿繊維の種類別の肺がん発生頻度は、中皮腫１に対してクロシドライトが0.5、クリソタイルが6.1、アモサイトが4.0、混合石綿が1.9であった。クロシドライト以外の石綿による肺がん発生は少なくとも２倍以上である[42]。

２．画像診断

胸部単純写真の役割は限定的であるが、胸水を伴う片側胸郭のびまん性の不整胸膜肥厚を呈し、進行すると胸郭容量低下を生じることはよく知られている[43]。ただ、このような所見は癌

性胸膜炎をはじめとする悪性胸膜病変で同じように認められ、非特異的である。

CTは中皮腫の存在診断、病期診断、治療効果判定に主たる役割を果たしている。その画像所見として、胸膜に沿った進展傾向があることにより、環状胸膜肥厚（“pleural rind”）や葉間胸膜浸潤の所見を呈する[44]。また悪性病変としての局所浸潤を示す所見として、胸壁浸潤による胸膜外脂肪織や肋間筋、肋骨への浸潤所見を呈する[45]。また、ドレナージチューブ挿入部や生検のルート沿いに腫瘍浸潤を認めることもよく知られている[46]。早期病変としては縦隔側胸膜肥厚所見が強調されており[47]、葉間胸膜肥厚とともに注意すべき所見である。

MRIはルーチン検査としては施行されないが、手術適応例で胸壁浸潤や横隔膜浸潤が問題となる際に用いられることがある[43]。通常筋肉に比しＴ１軽度高信号、Ｔ２高信号を呈し、Gd造影剤にて強く造影される。拡散強調像が良悪の鑑別に有用との報告もある[48]。

FDG-PET/CTは良悪の鑑別やリンパ節転移、遠隔転移の精査の時に用いられており、リンパ節転移など転移診断には一定の評価が得られているが[49]、CTにて良悪の判断が難しいような症例での有用性はまだ確立されていない[50]。

３．病理診断

⑴　中皮腫の医学的診断の重要性

2005年８月におこったクボタショック（クボタ尼崎工場の周辺住民に中皮腫が集団的に発生した）以降、一般生活環境下での石綿へのばく露による中皮腫の発生が注目され、ばく露の形態として、①職業性、②家族間、③生活環境下、という分類がなされるようになった。クボタショックは一種の公害であるとの認識から、石綿健康被害救済法が制定され、被害者の救済事業が始まった。これによって中皮腫と診断された人は、石綿へのばく露状況の如何を問わず救済されることになり、中皮腫の診断の確実性が求められ、CT検査などによる画像診断と生検などによる病理組織学診断の重要性が増すこととなった。殊に病理診断は最終診断として扱われ、統一された基準による適確な診断が必要となった。

2005年以前の中皮腫の病理診断は、HE染色による所見を基盤とし、ヒアルロン酸を主体とする酸性粘液多糖類の有無をみるアルシャン青染色や、腫瘍の局在が肺内か肺外かを判断するためのEVG染色に加え、ケラチンなどの抗体を用いた少数の免疫組織化学的染色にもとづいた診断であった。限られた染色であることに加え、その所見の判断基準も曖昧であったことから、誤った診断をつけられた例の割合が大きい状況が生じた。例えば、Takeshimaらによる日本での検討[51]では約17％が誤診と判定されている。

中皮腫の病理診断は、前述の救済法にもとづく判定小委員会と、その後に発足した労災補償制度にもとづく被害者の判定を行う石綿確定診断委員会において、複数の病理医の討議にもとづく診断と、さらに放射線科医による画像診断との摺り合わせを行うことによって、その診断精度は大きく上昇した。これはヨーロッパ、特にフランスやベルギーなどにおける総合討論による診断の確定という仕組みを参考としたものであった。さらにその後、中皮腫に

特異的な抗原に対する抗体を用いた免疫組織化学的染色が次々と開発され、加えて近年、遺伝子・分子レベルでの異常を検索することが可能となり、中皮腫の病理診断は飛躍的に進歩することとなった。

(2) 中皮腫の病理診断

中皮腫は、胸膜、腹膜、心膜、精巣鞘膜から生じる腫瘍で、これら漿膜に存在する中皮細胞に由来する。この中皮細胞は、漿膜腔に面して一層に並ぶ立方状ないし扁平な細胞であるが、胸腔内あるいは腹腔内の病変たとえば胸水や腹水の貯留や胸膜炎や腹膜炎などで、反応性に個々の細胞が腫大し異型性を示し、また、細胞の増殖によって過形成性病変を形成する。一層の中皮細胞の下には紡錘形細胞が少量存在するが、この紡錘形細胞も上皮様細胞に由来し、そのphenotypeを共有する。特にこの紡錘形細胞は、cytokeratinを細胞質内骨格フィラメントとしてもち、この点が他の間葉系の紡錘形細胞である線維芽細胞（fibroblast）や筋線維芽細胞（myofibroblast）とは異なる。胸膜や腹膜に炎症を生じた場合は、中皮細胞下に紡錘形細胞の増殖がみられ、cytokeratinとdesminをもつ細胞が認められる。腫瘍化した中皮細胞では、cytokeratinは保持されるが、desminは発現しない。この所見が後述する鑑別診断に有用となる[52]。

中皮腫の組織型は**表３**のように分類される。上皮性悪性腫瘍（癌腫）の形態をとる上皮型中皮腫、非上皮性悪性腫瘍（肉腫）の形態をとる肉腫型中皮腫、両者の所見が混在する（いずれかが少なくとも10％以上を占める）二相型中皮腫に３大別され、それぞれの占める割合は**表３**に示すとおりである。二相型中皮腫の報告をみると、上皮型中皮腫の間質成分である紡錘形細胞の増殖を腫瘍成分と見誤られた例、あるいは上皮型中皮腫の分化度が低くなり肉腫型と判断した例がみられ、日本では二相型の割合が多くなる傾向があるが、標本を再検す

表３　中皮腫の組織型分類と各々の占める割合

組織型		占める割合
びまん性中皮腫	Diffuse malignant mesothelioma	90%
上皮型中皮腫	Epithelioid mesothelioma	60%
肉腫型中皮腫	Sarcomatoid mesothelioma	20%
線維形成型中皮腫	Desmoplastic mesothelioma	
二相型中皮腫	Biphasic mesothelioma	20%
限局性中皮腫	Localized malignant mesothelioma	10%
上皮型中皮腫	Epithelioid mesothelioma	
肉腫型中皮腫	Sarcomatoid mesothelioma	
二相型中皮腫	Biphasic mesothelioma	
高分化乳頭状中皮腫	Well-differentiated papillary mesothelioma	a few
アデノマトイド腫瘍	Adenomatoid tumor	a few
特殊型中皮腫		a few

（中皮腫瘍取扱い規約、2018年より改変）

ると二相型中皮腫の割合は約20％程度と判断され、欧米との間に大きな差異はない。これに加えて、特殊型として、さまざまな分化像を示す腫瘍もあることが知られている。このような多彩な形態を示す腫瘍であるが故に、多くの腫瘍あるいは非腫瘍性病変との鑑別診断が重要となる。

従来は、上皮型中皮腫と肺原発の腺癌をはじめとする癌腫、肉腫型中皮腫と胸腔内に生じる他の肉腫との鑑別が重要であったが、近年、早期の上皮型中皮腫が多く見出されるようになって、過形成性病変などの非腫瘍性病変との鑑別が必要となり、また、肉腫型あるいは線維形成型中皮腫と線維性胸膜炎の鑑別に苦慮する例が増加してきた。これらについて、それぞれにその鑑別の要点を述べてみたい。

ア．上皮型中皮腫と肺原発の癌腫との鑑別

胸膜に生じた中皮腫は、基本的には胸膜にそって肺を囲繞するように進展する。一方、肺原発の癌腫は肺内に腫瘤を形成するので、EVG染色によって臓側胸膜の位置をみることで、病変が肺外なのか肺内なのかを確認することが必要となる。中皮腫でもびまん性の拡がりを示さず、胸膜に結節性病変を形成する場合があるし、肺原発の癌腫でもまれに、臓側胸膜直下に発生して胸膜へ進展し、胸膜に沿った拡がりを示して、偽中皮腫様肺癌（pseudomesotheliomatous carcinoma）とよばれることがある[53]。この場合、組織型は腺癌でも扁平上皮癌でもありうる。

組織学的レベルで鑑別が必要な場合は、免疫組織化学的染色が有用である。中皮腫にとって陽性マーカーと陰性マーカー（多くは肺がんにとって陽性マーカーとなる）を**表４**に示す。中皮腫では、乳頭腺管状構造を分化度の指標とすると、高分化型の場合は、中皮細胞としての陽性マーカーはいずれも陽性となるが、分化度が下がるにつれて、陽性の頻度も低くなり、判断に苦慮することとなる。

イ．肉腫型中皮腫と胸腔内に生じる肉腫との鑑別

肉腫型中皮腫では、増殖する紡錘形細胞はcytokeratin（CAM5.2あるいはAE1/AE3）の陽性を示すが、前述した中皮細胞マーカーの陽性率は低く、まったく染色されないことも多い。D2-40とα-SMAは陽性であることが多いが、細胞密度の高い胸膜炎でも陽性となることがあり、鑑別には使えない。

胸腔内に生じる肉腫としては、滑膜肉腫（synovial sarcoma）、上皮様血管内皮腫（epithelioid hemangioendothelioma）あるいは血管肉腫（angiosarcoma）などがあげられる。滑膜肉腫は単相型（monophasic type）と二相型（biphasic type）とがあるが、前者は肉腫型中皮腫と、後者は二相型中皮腫との鑑別が必要となる。免疫組織化学的染色では、滑膜肉腫ではcytokeratinが陽性となり、中皮腫との鑑別には使えないが、transducin-like enhancer of split 1（TLE1）の陽性は特異度が高いとされる。しかし現在、滑膜肉腫の診

表４　中皮腫の免疫組織化学的染色

	胸　膜	腹　膜
中皮腫マーカー	Calretinin WT-1 D2-40（podoplanin） CAM5.2, AE1/AE3 EMA（細胞膜に陽性）	Calretinin WT-1 D2-40（podoplanin） CAM5.2, AE1/AE3 EMA（細胞膜に陽性）
肺がんマーカー （胸膜中皮腫の陰性マーカー）	CEA TTF-1 NapsinA Claudin 4 p40/p63 MOC31 BerEP4	
女性生殖器腫瘍マーカー （腹膜中皮腫の陰性マーカー）		CEA MOC31 BerEP-4 Claudin 4 ER PgR
反応性中皮細胞マーカー （上皮型中皮腫、肉腫型中皮腫の陰性マーカー）	Desmin	

断では融合遺伝子（SYT-SSX）の検出が決め手とされており、遺伝子検索が必須である[54]。

血管性腫瘍は欧米では報告例が多いが、日本ではまれである。分化度の高い場合は、CD34、CD31、Factor Ⅷ related antigen（第Ⅷ因子関連抗原）が陽性であるが、分化度が低くなるとこれらは陰性であるため鑑別が難しくなる。

ウ．早期の上皮型中皮腫と反応性中皮細胞過形成との鑑別（図３）

上皮型中皮腫では、細胞異型性はそれほど目立たない例が多いので、上皮様中皮細胞の増生（過形成）部分の細胞異型性のみでは良悪性の判断は困難である。したがって、上皮下への浸潤性増殖の有無をみることが必要となる。胸膜の肥厚が軽度で、胸壁の脂肪組織や横紋筋組織が含まれている場合は、それらへの浸潤を判断することは容易であるが、炎症が持続すると上皮様中皮細胞が膠原線維増生部分に取りこまれ、中皮腫の浸潤に類似した形態を示すことになる。直線状あるいは整った腺管様の配列をとる場合は、取り込まれた非腫瘍性中皮細胞であり、単離した細胞をみる場合や、腺管様構造が複雑な形態を示す場合は中皮腫の浸潤とみなされる。

免疫組織化学的染色では、EMAが細胞膜に陽性であり、desminが細胞質に陰性である場合は中皮腫であると判断できる。反応性の中皮細胞の増殖では、その逆となる。近年、分子・

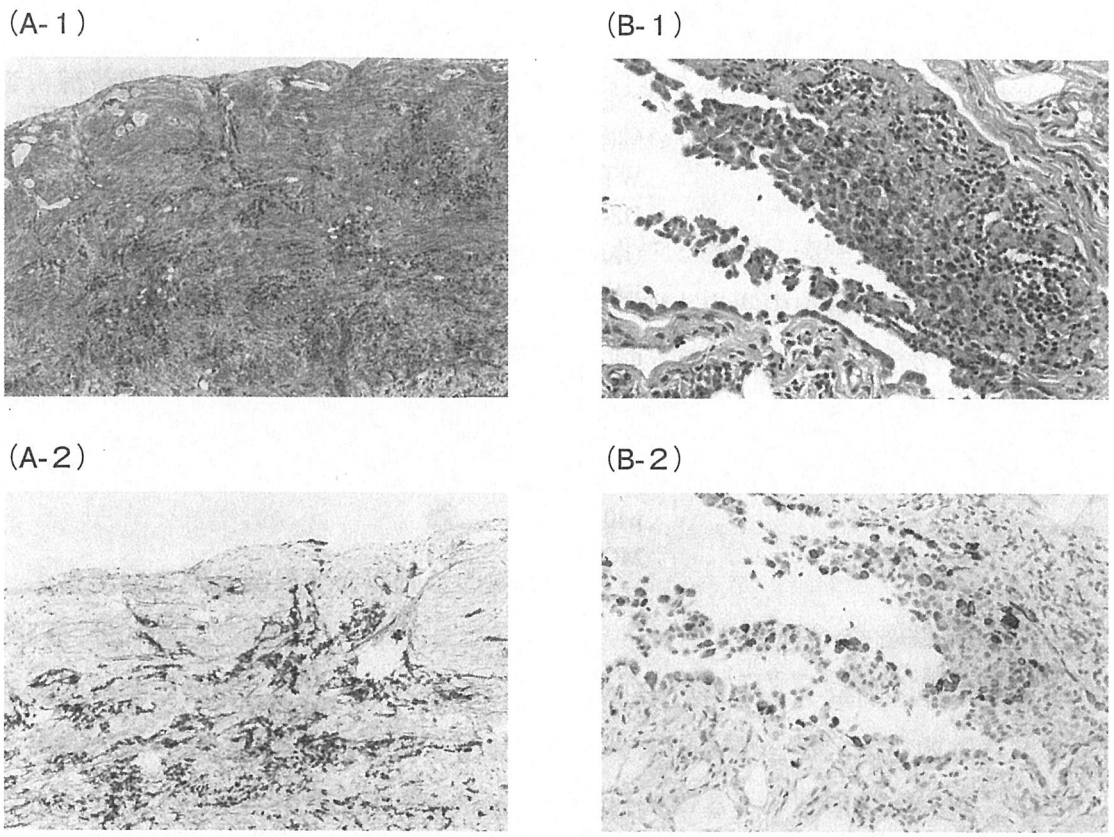


図３　上皮型中皮腫（A）と反応性中皮細胞過形成（B）：A-１は HE 染色、A-２は EMA の免疫染色で陽性である。B-１は HE 染色、B-２は EMA の免疫染色で陰性である。

図４　中皮腫における FISH 法による p16遺伝子の欠失：赤色のシグナルの脱落をみる。

遺伝子レベルの研究の進展により、BRCA1-associated protein 1（BAP1）遺伝子の欠失によるBAP1蛋白およびmethylthioadenosine phosphorylase（MTAP）蛋白の陰性化が中皮腫の指標となることが示されているが、免疫組織化学的染色では50％程度の症例にしか陰性化はみられない[55]。p16遺伝子の欠失によるp16蛋白の陰性化も中皮腫の指標となるが、p16蛋白の免疫組織化学的染色は信頼性が低く、FISH 法による観察が必要である[56]（図４）。

こうした組織標本による観察以外の手法による良悪性の判断ができるようになり、上皮

下への浸潤所見が明確でないために従来“atypical mesothelial proliferation”としか診断できなかった例の中に、非浸潤性中皮腫“mesothelioma in situ”が含まれることが明らかとなった[57]。また、胸水の細胞診のみによって中皮腫の診断を下さなければならない場合も、セルブロックの免疫組織化学的染色やFISH法によって、EMA、desminの所見のみならず、BAP1の欠失やp16の欠失をみることで、良悪性の判断が可能となっている。

表５　線維性胸膜炎と肉腫型（線維形成型）中皮腫との鑑別点

線維性胸膜炎	肉腫型（線維形成型）中皮腫
• 細胞密度の段階的変化 （“zonation”あり） （表層側→高い、深部→低い）	• “zonation”を認めない
• 膠原線維の走行は概ね胸膜表面に並行である	• 膠原線維の走行は方向が一定でなく、ときに“storiform pattern”様の配列をみる
• 胸壁（深部）脂肪組織などへの結節状進展はない	• 胸壁（深部）脂肪組織への結節状進展をみる
• 表層側では細胞異型性が目立つ	• 細胞異型性は低い
• 胸膜表面に対し、垂直な毛細血管をみる	• 毛細血管は目立たない
• 壊死巣はない	• 壊死巣をみることがある
• 肉腫様所見はない	• 細胞密度の高い肉腫様所見を示す部位がある

（AFIP Atlas, 2006, を改変）

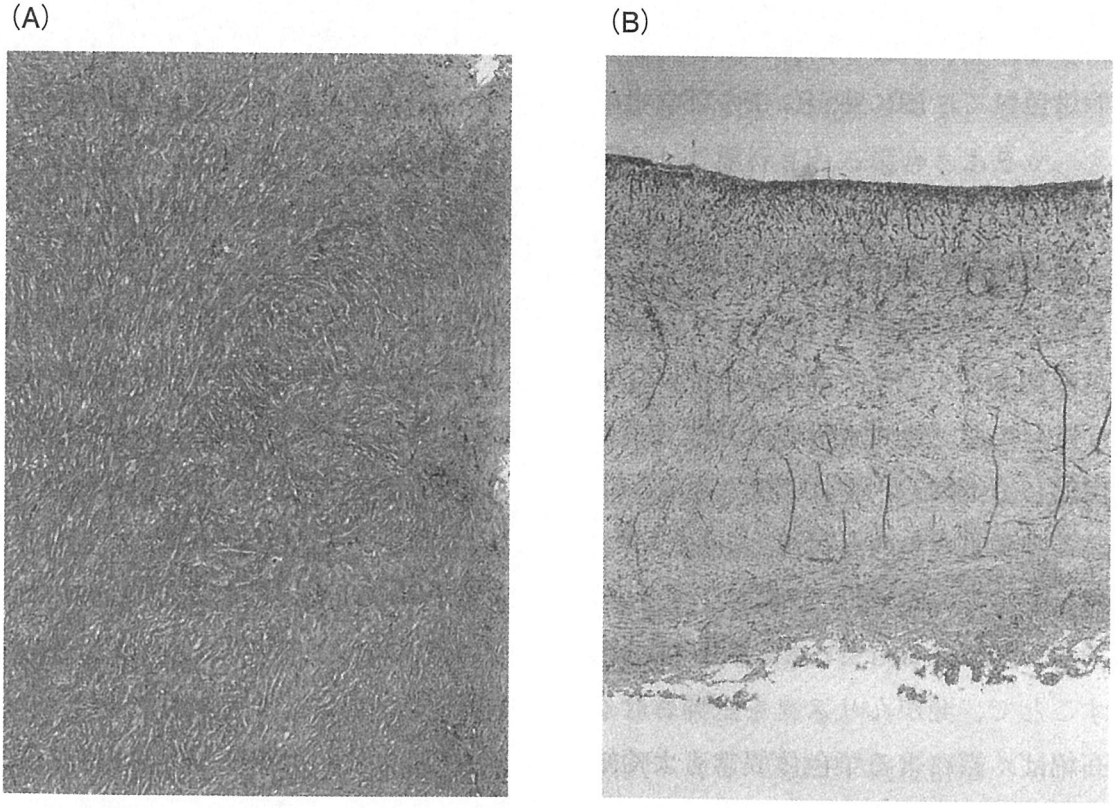


図５　線維形成型（肉腫型）中皮腫（A）と線維性胸膜炎（B）：中皮腫では膠原線維の走行の乱れがつよい。線維性胸膜炎では“zonation”を認め、膠原線維の走行はほぼ並行である。

エ．線維形成型（肉腫型）中皮腫と線維性胸膜炎との鑑別

肉腫型中皮腫の亜型として、腫瘍細胞の細胞密度が低く、間質の膠原線維量の多い例を線維形成型中皮腫（desmoplastic mesothelioma）とよぶ。こうした例は胸膜炎との鑑別が必要となるが、両者の鑑別点は**表５**のようにまとめられる[58]。判断を適切に行うためには、生検材料の場合は特に、胸膜表面から胸壁まで垂直方向に深く組織を採取することが肝要である。胸腔鏡検査の場合、胸膜の表面のみを“piece by piece”に採取した材料では、**表５**にある“zonation”の有無の判断や胸壁組織への進展の有無が判断できない（**図５**）。

オ．腹膜中皮腫の鑑別診断

腹膜中皮腫は、中皮腫全体の10％程度を占めるにすぎない。その成因として、従来は胸膜例に比して大量の石綿へのばく露があるとされてきたが、近年の例では必ずしもそうした例のみではないことに気付く。胸膜例は男性例が圧倒的に多いが、腹膜例の男女比はほぼ均等である。また、組織学的には大半が上皮型中皮腫であり、低分化な上皮型中皮腫を肉腫型と見誤らないことが大切である。

鑑別すべき腫瘍としては、腹腔内臓器（胃、大腸、膵臓、胆管など）原発の癌腫の播種性転移があげられる。消化管ではCEA、大腸ではCOX2、膵臓や胆管ではCA19-9などの免疫組織化学的染色が陽性となり、腺癌ならばclaudin4、扁平上皮癌ならばp40が陽性となることも鑑別には有用である。女性の場合は、卵巣をはじめとする女性生殖器原発の癌腫、特に漿液性癌（serous carcinoma）、淡明細胞癌（clear cell carcinoma）との鑑別が重要である。この場合は、細胞学的および組織学的特徴を注意深くみることと、免疫組織化学的染色にて、ER、PgR、PAX8などの陽性所見が中皮腫ではないことを示唆する[59]。

(3) 中皮腫の発生病理と遺伝子異常

ア．石綿繊維の発がん機序

石綿は、活性酸素種（reactive oxygen species, ROS）を発生させる。鉄イオンを多く含むクロシドライトやアモサイトでは、フェントン反応によって過酸化水素（H_2O_2）からヒドロキシルラジカル（OH）を産生することがよく知られている。また、炎症細胞によって二次的にもROSが産生され、特に石綿を貪食したマクロファージからの産生が重視されている。産生されたROSによってDNA損傷が引き起こされる。例えば、グアニンが酸化修飾を受けて、8-ヒドロキシデオキシグアノシン（8-OHdG）となると、これはDNA酸化損傷マーカーとなる。8-OHdGは、DNA複製時にG→Tトランスバージョンを引き起こすことで、発がんリスクを上昇させることになる[60]。

石綿ばく露による染色体異常もよく知られるが、これは、細胞内に貪食された石綿繊維が細胞分裂期の染色体分離に必要な分子に吸着して物理的な干渉を与えることによる。その結果、DNAの２重鎖切断や染色体内欠失などが引き起こされる。中皮腫にみられる染色

体異常はLOH解析やCGH解析によって検討されてきたが、染色体増幅（gain）は、5p、7p、7q、8q、17qなどにみられ、欠失（loss）は、1p、3p、6q、9q、13q、14q、15q、22qなどにみられると報告されている[61]。

遺伝子異常については、cyclin-dependent kinase inhibitor 2A（CDKN2A）遺伝子、neurofibromatosis type 2（NF2）遺伝子およびBAP1遺伝子の異常についての検討がすすんでいる。CDKN2A遺伝子は、$p16^{INK4a}$と$p14^{ARF}$をコードし、いずれもその不活性化が細胞のがん化を促進することになる。NF2遺伝子は神経線維腫症２型の原因遺伝子として同定されたが、中皮腫の40～50％で不活性化が認められる。BAP1遺伝子は、腫瘍抑制遺伝子であるが、酵素活性領域や核内局在シグナル領域の不活性化変異によってその腫瘍抑制機能が失われる。また、BAP1遺伝子の生殖系列細胞変異（germline mutation）をもつ家系が発見され、中皮腫やブドウ膜黒色腫を含む多様ながんが家系内で発生することが報告されている。NF2遺伝子はマーリン（Merlin）とよばれる蛋白をコードし、マーリンは細胞内接着に関与し、また下流シグナル伝達系としてmTOR系やHippoシグナル伝達系を活性化して細胞増殖の停止機能をもつ[60]。

いずれの遺伝子についても100％の中皮腫で異常がみられるわけではないので、中皮腫発生に関わる特定の遺伝子であるとはいえない。

４．治　療

悪性胸膜中皮腫の治療は、IMIG（International Mesothelioma Interest Group）分類による病期分類とWHO分類による組織分類にもとづき決定される。一般に、切除可能症例では手術療法が、切除不可能症例や術後再発症例では全身化学療法が治療の主体となる。放射線療法は手術療法や化学療法と組み合わせた集学的治療の一環として施行される場合もあるが、多くは疼痛コントロール目的の緩和療法として施行される。

(1)　手術療法

手術方法には、胸膜外肺全摘術（Extrapleural Pneumonectomy, EPP）と胸膜切除剝皮術（Pleurectomy/Decortication, P/D）の２種類がある。EPPは、壁側胸膜、臓側胸膜と肺、横隔膜、心膜を切除し、人工の膜で横隔膜と心膜を再建する。P/Dは、壁側胸膜と臓側胸膜を切除し、必要な場合に横隔膜や心膜を切除再建する。両術式の最大の違いは、肺の摘出であり、EPPは肺を摘出するが、P/Dでは肺は摘出しない。腫瘍の減量効果は、EPPのほうが高いとされ、またEPPでは術後の放射線治療が可能となる。P/DはEPPに比べ、心肺機能や全身状態がやや低下している症例でも適応となる可能性がある。

システマティックレビューによると、上皮型症例に対して、EPP施行群とベストサポーティブケア群における生存期間の中央値はそれぞれ19か月、７か月であり、手術の長期生存への寄与が報告された[62]。また1,365人の中皮腫患者に対して、手術に加え化学療法を施行した

群と化学療法単独群では手術＋化学療法群で有意な延長が認められた[63]。また5,937人の中皮腫患者において、背景因子を補正しても手術を受けた患者群は非手術患者群に比べ有意に良好な予後が得られたとの報告がある[64]。我が国における中皮腫患者の後方視的研究においても、手術施行例の予後は比較的良好であり[65]、十分に選択された症例群において、外科治療は予後の改善に寄与するものと推定される。

EPP と P/D を比較する別のメタアナリシスで、術後短期死亡率が EPP で有意に高いが(4.4% vs 1.7%, p<0.05)[66]、長期予後は同等あるいは手術死亡率に差はなく、生存期間の中央値は同等と報告されている（16か月 vs 19か月、統計学的有意差なし)[67]。EPP と P/D を直接比較した前方視的試験はないためどちらが優れているのかの結論は出ないが、いずれにしても手術単独での治療成績は充分でなく、治療戦略を構築する上では呼吸器外科医、腫瘍内科医を含む治療チームによる集学的治療が望ましい。メタアナリシスにおいて、手術を含む集学的治療が生存率に寄与する可能性が指摘されている。また日本における EPP を含む集学的治療に関する第Ⅱ相臨床試験において42例の登録患者におけるMSTは19.9か月、治療関連死亡は9.5％であった[68]。手術を行う場合には術後に肉眼的完全切除を得られることが重要と考えられ、耐術能があるかどうか呼吸機能・心機能などを十分に評価する必要がある。

(2) 放射線療法

根治目的の放射線治療は、集学的治療のひとつとして EPP 後の片側胸郭照射として用いられてきた。EPP後の局所制御に片側胸郭照射が有効であるとされながらも、完遂率の低さや、生存率向上への寄与が低いことが指摘されてきた。いずれの報告も後方視的研究もしくは第Ⅱ相試験であり、片側胸郭照射の有用性を検証する第Ⅲ相試験は現在のところ存在しない。

P/D 後の照射については、P/D 後に通常照射での片側胸郭照射（median 42.5Gy）を施行した123例の報告があり、２年全生存率23%、１年局所制御割合42% で、Grade３以上放射線肺臓炎が10.6%（Grade ５：１例）であり、P/D 後の片側胸郭照射は有効な治療選択肢ではないとされている[69]。一方、P/D 施行後および非切除例に対して IMRT を用いた片側胸郭照射を行った36症例について２年生存率がそれぞれ53%、28% であったが、Grade ３以上の放射線肺臓炎が20%（Grade５：１例）と報告されている[70]。MD Anderson Cancer Center（MDACC）におけるマッチング比較では、P/D 施行後、IMRT にて45Gy の片側胸郭照射を行った24症例（P/D-IMRT 群）と EPP 施行後に IMRT を行った24例（EPP-IMRT 群）における生存期間の中央値は28.4か月と14.2か月（p=0.04）でP/D-IMRT群でやや良好で、Grade４-５の有害事象に有意差は認めなかった（０% vs 12.5%, p=0.23)[71]。現時点では海外のガイドラインにおいても毒性の観点から P/D 後の放射線治療は推奨されていない。P/D 術後または手術非適応症例に対して放射線療法を行う場合には、臨床試験として行われるべきである。

(3) 化学療法

進行例および術後の再発例は内科的治療の対象となり、化学療法の適応となる。胸膜中皮腫に対する化学療法では、未治療の切除不能症例に対し、シスプラチン、ペメトレキセドの併用療法とシスプラチン単剤とを比較した臨床第Ⅲ相試験で、全生存期間、無増悪生存期間、奏効率いずれにおいても併用群で有意に良好であり（生存期間の中央値12.3か月 vs 9.3か月、無増悪生存期間の中央値5.7か月 vs 3.9か月、奏効率41.3% vs 16.7%）、現時点での標準治療とされている[72]。高齢や合併症のためシスプラチンの併用がためらわれる場合、カルボプラチンの投与も考慮される。カルボプラチンとペメトレキセドの併用療法については２つの第Ⅱ相試験があり、上述のシスプラチンとの併用療法に比べ奏効率はやや劣るが（18.6%、25%）、増悪までの期間（6.5M、8.0M）および全生存期間（12.7M、14M）はほぼ同等の結果であり、毒性も骨髄抑制以外は軽度であった[73,74]。ただし、カルボプラチンは悪性胸膜中皮腫に対し承認されていない点は注意が必要である。初回化学療法としてのこれらの併用療法の至適投与回数は４-６コースとされており、その後のペメトレキセドによる維持療法については明確なエビデンスはない。

プラチナ製剤とペメトレキセドに対し不応となった場合、治療法の選択肢は限られる。二次治療におけるビノレルビンあるいはゲムシタビンの成績は、いずれも第Ⅱ相試験において奏効率は７-24%、全生存期間の中央値は8.0-10.6か月と報告されている[75-77]。ビノレルビンとゲムシタビンの併用療法については、第Ⅱ相試験においてそれぞれの単剤療法を上回る成績が報告されておらず、一方で血液毒性が増加するとされている。それ以外にもこれまでにいくつかの新規製剤について臨床試験が行われてきたものの、有用性は示されなかった。近年行われた既治療の日本人の胸膜中皮腫患者（一次治療後24人、二次治療後10人）を対象としたニボルマブの臨床第Ⅱ相試験（MERIT 試験）において、組織型に関わらず奏効率29.4％、無増悪生存期間の中央値2.6か月、６か月の生存率74％、全生存期間の中央値17.4か月との成績が報告された[78]。この試験の結果に基づきニボルマブは2018年８月に、「がん化学療法後に増悪した切除不能な進行・再発の悪性胸膜中皮腫」に対して承認された。ニボルマブを含む免疫チェックポイント阻害剤については今後、既存の化学療法との併用、あるいは初回治療としての有用性への期待が高まっており、現在も臨床試験が進められている。

その他の治療法としては、血管新生阻害剤であるベバシズマブやニンテダニブを用いた臨床試験において一部で有望な結果が報告されたものの、実用には至っていない。遺伝子治療や抗体療法についても臨床試験が進められているが、これらについてもこれまでのところ臨床的な有用性は確認されていない。さらなる治療成績の改善のため、今後も引き続き有効な治療法の開発が切望される。

(4) 集学的治療

手術可能なⅠ－Ⅲ期の悪性胸膜中皮腫症例には、術前または術後のどちらかに化学療法、

また術後に放射線療法を加える集学的治療が推奨される。Sugarbakerらは、手術、化学療法、放射線療法の三者併用療法において長期生存が得られる可能性があることを報告している[79]。これらの三者併用療法の安全性および忍容性については、十分に選択された症例に対して一定の評価が得られていると思われるが、周術期化学療法を術前または術後のどちらに行うべきかについては前方視的比較試験の報告はなく、各施設の方針に委ねられている。三者併用療法の付加的療法として術中胸腔内温熱化学療法が有効との報告もある。

(5) 緩和医療

胸膜中皮腫では、胸膜刺激や胸壁浸潤により病初期より疼痛を来すことが多い。疼痛緩和目的で放射線治療を施行した報告では、約60％の症例に疼痛緩和が得られたと報告されている[80,81]。これらの報告では、主として40Gy/20回または36Gy/13回が用いられている。また、胸膜中皮腫では多くの場合胸水貯留により労作時呼吸困難、胸部圧迫感などが生じる。胸水制御と症状軽減を目的とした胸腔ドレナージおよび胸膜癒着術は、外科治療や化学療法などの積極的な抗癌治療の適応の有無にかかわらず考慮される。硬化剤には通常タルク（含水珪酸マグネシウム）またはOK432が用いられる。タルクを用いた172人の前方視的観察研究では、胸水コントロール率は３か月時点で49％（85/172例）、１年生存者においては93％（79/85例）と報告されている[82]。

Ⅳ．良性石綿胸水

１．定　義

良性石綿胸水とは、1964年にEisenstadtが初めて報告した石綿繊維による臓側胸膜の炎症によって生じる胸膜炎である[83]。発生機序としては石綿繊維の臓側胸膜への機械刺激あるいはadjuvant効果による自己免疫機序、胸膜線維化による壁側胸膜のリンパ排出孔の閉塞が考えられているが、現在でも不明のままである。Eplerら[84]の基準を用いれば、①石綿ばく露歴がある、②胸水の存在を確認できる、③胸水を来すその他の疾患を除外できる、④３年以内に悪性腫瘍の発生がない、である。

良性とは臨床経過が良好であるという意味では無く、悪性ではないということである。

２．診断と治療

職業性石綿ばく露歴があり、胸部画像上悪性腫瘍を示唆する所見、たとえば縦隔側胸膜肥厚等の胸膜中皮腫を示唆する所見がないことが前提になる。胸水の性状はほとんどが血性で、胸水中には主にリンパ球が増加する滲出液である。他疾患を除外するためには、胸水細胞診が陰性で、胸水中のヒアルロン酸、CEA、ADA、Cyfra21-1等の値が概ね正常範囲内であることを満たす。以上が臨床上の判断基準である[85]。

石綿ばく露との関連については量 - 反応関係があると報告されている[86]。石綿初回ばく露からの潜伏期間は石綿関連疾患では最も短いとされており、10年以内に発生する唯一の疾患である。しかし、実際には50.5年と長い潜伏期間があったという報告もある[87]。

我が国における良性石綿胸水の疫学調査はわずかであり、臨床報告も少ないが[88, 89]、海外においても疫学調査や原因追及のための研究はほとんど行われていない。そのため、参考とすべき診断基準は日本あるいは海外の論文等にも記載されていない。

現在では早期の胸膜中皮腫との鑑別が必要であるため、胸膜生検が行われる頻度が増加している。中皮腫細胞と反応性中皮細胞あるいは病理組織における早期中皮腫と良性石綿胸水（線維性胸膜炎）の鑑別にはBAP1、MTAPおよびp16遺伝子の9p21のhomozygous deletionをFISH法で確認することが有用と報告されている[90-92]。

発生機序が明らかでないため、治療方法はなく、対症的に胸水を抜いてコントロールする程度である。

3．労災補償対象と予後

本疾患を労災対象としている国は日本とフランスのみである。我が国では2003年から労災対象となっているが、石綿健康被害救済法の対象とはなっていない。労災対象となる場合でも、すべてが厚生労働省での協議によって決定されている。胸水が消退あるいは残存して器質化胸水を残すと後述のびまん性胸膜肥厚化して拘束性の呼吸機能障害を来すこともあるので経過観察が必要となる。

環境省委託研究で行った全国調査では、石綿ばく露による著しい呼吸機能障害を伴うびまん性胸膜肥厚症例259例のうち45.6％に相当する118例は、良性石綿胸水の既往があったと報告されている[93]。近年、良性石綿胸水からびまん性胸膜肥厚へと移行し３年以内に拘束性呼吸機能障害によって死亡に至ったという症例報告も行われるようになった[88, 89]。

上述の調査結果から、著しい呼吸機能障害を伴うびまん性胸膜肥厚と診断されてからの生存期間中央値は34.5か月と報告[93]されていることから、良性石綿胸水の予後は必ずしも楽観できるわけではないと考えて、胸水が消失あるいは器質化した後も慎重な経過観察が必要である。

Ⅴ．びまん性胸膜肥厚

びまん性胸膜肥厚（diffuse pleural thickening, DPT）は石綿による非腫瘍性胸膜疾患の１つであり、両側または片側の広範な胸膜肥厚を特徴とする。病理学的には臓側胸膜の慢性線維性胸膜炎であるが、その病変がしばしば壁側胸膜にもおよび、両者が癒着するため拘束性の呼吸機能障害による著しい呼吸機能障害を来す場合がある。石綿ばく露によるびまん性胸膜肥厚は、2003年に労災補償の対象疾患として加えられ、2006年以降は、業務上と認定されるための基準が示されている。また、石綿健康被害救済法が2010年７月１日に改正された際、石綿肺とともに石綿による健康被害疾病のひとつに加えられている。

1．石綿ばく露によるびまん性胸膜肥厚の診断

日本のびまん性胸膜肥厚の画像上の労災あるいは救済の認定診断基準は、両側性の場合はそれぞれ胸郭の４分の１以上、片側性の場合は胸郭の２分の１以上に拡がる連続した胸膜肥厚である。明らかな職業性石綿ばく露歴があり、かつ石綿ばく露以外の原因による胸膜肥厚ではないことが前提となる。びまん性胸膜肥厚の広がりの程度は、胸部単純写真における側胸壁の頭尾方向の長さで決められる。ただ、胸部単純写真だけでは胸膜プラークや癒着を伴わない胸膜肥厚との鑑別[94]が困難なこともあるため、胸部CTでの確認が必要である。胸部CT肺野条件におけるCrow's feet signの所見は臓側胸膜の線維化を示しており、CTにおけるこのsignの範囲を胸部単純写真と照らし合わせれば、びまん性胸膜肥厚における胸膜肥厚の範囲を確認できる。一方、円形無気肺も臓側胸膜の線維化によって肺実質が無気肺化する所見であり、びまん性胸膜肥厚の際によく認められる所見[95]である。

びまん性胸膜肥厚における画像上のcriteriaとしてイギリスにおいて労災認定基準として用いられている所見に肋骨横隔膜角の消失がある。この所見があると横隔膜との癒着によりFVC、TLCが減少することはよく知られた事実であるが、現在の日本におけるびまん性胸膜肥厚の認定診断基準では肋骨横隔膜角の消失の有無は問わない。以前の診断基準では胸部単純写真にて５㎜以上の肥厚を認めることを必要としていたが、胸膜肥厚の厚さを胸部単純写真で評価することは極めて困難で再現性が悪いため、現在は認定診断基準に含まれていない。

2．びまん性胸膜肥厚の呼吸機能障害

Fujimotoら[96]はびまん性胸膜肥厚の胸膜肥厚の厚さは%VCに影響を与えないが、Costophrenic Angle（CPA）の鈍化は%VCを有意に減少させると報告している。一方、de Fonsekaら[97]はCPAの消失がなくともFVCを低下させると報告している。正常群のFVCは98.9%で、CPAの消失がないびまん性胸膜肥厚では83.5%に低下する。しかし、片側性のCPAの消失は79.5%に、両側性になると66.7%へと低下することから、呼吸機能、特にFVC、TLCの低下にCPAの消失が大きくかかわることは明らかである[97]。

びまん性胸膜肥厚には低濃度石綿ばく露によっても発生する胸膜プラークの合併頻度が高く、加藤[95]も88.1%であったと報告している。胸膜プラークの存在により呼吸機能に影響を与えるかどうかについては議論が多いところであるが、近年の報告ではわずかながらのFVC、$FEV_{1.0}$の減少をもたらすことが事実[98]のようで、プラークの範囲の程度によりFVC、TLCが減少するようである[99]。びまん性胸膜肥厚ではプラークを含む壁側胸膜と線維化を来した臓側胸膜の癒着があることから、びまん性胸膜肥厚の呼吸機能障害にプラークの影響も考慮する必要がある。

石綿ばく露者の呼吸機能について掲載された論文のメタアナリシスでは、胸部画像上に異常所見がなくても有意にVC、$FEV_{1.0}$が低下していることが判った[100]。また、びまん性胸膜肥厚では石綿肺に比して$FEV_{1.0}$、FVC、peak flowはともに低下率が高く、肺実質障害がなくとも

呼吸機能低下が著しいことが判ると報告されている[101]。

3．びまん性胸膜肥厚の労災認定および救済法の基準

石綿によるびまん性胸膜肥厚により著しい呼吸機能障害を呈する場合、労災認定の対象となるが、その基準は以下のごとくである。

(1) まず拘束性換気障害を判定するためにパーセント肺活量（%VC）を測定し、この値が60%未満である場合に著しい呼吸機能障害があると判定される。

(2) (1)において%VCが著しい呼吸機能障害があると判定される値に満たない場合、ある程度の減少がみられるときには閉塞性換気障害の程度を評価する。具体的には、%VCが60%以上80%未満である場合に、1秒率が70%未満、かつパーセント1秒量が50%未満であるときに著しい呼吸機能障害ありと判定される。

(3) さらに(2)において著しい呼吸機能障害ありと判定されない場合でも、血液ガス分析により動脈圧酸素分圧が60Torr以下である場合、または肺胞気動脈血酸素分圧較差（$AaDO_2$）が一定の限界値を超えている場合には著しい呼吸機能障害があると判定される。これら呼吸機能検査結果に3年以上の職業性石綿ばく露歴と上述の胸部単純写真上の範囲を満たせば労災認定となる。

一方、救済の認定では胸部画像上の基準は労災認定に同じであるが、呼吸機能検査結果では%VCが60%未満である場合のみで、閉塞性呼吸器障害の基準の適応は無く、%VCが60%以上80%未満である場合には血液ガス分析結果が上述の(3)に合致すれば救済されることになっている。また、職業性石綿ばく露歴も3年以上という縛りも厳しくない。

4．びまん性胸膜肥厚の臨床像

岸本ら[102]は、日本における石綿ばく露によって発生したびまん性胸膜肥厚で労災認定基準の画像条件を満たす106例について臨床上の特徴や石綿ばく露との関連について報告している。その報告によると、70歳以上の男性が大半で、喫煙歴が調査可能であった104例のうち88例（84.6%）が喫煙者で、その過半数が重喫煙者であった。何らかの自覚症状を主訴として診断された症例が56例（52.8%）で、石綿健康管理手帳等の健康診断をきっかけに診断された症例が45例（42.5%）であった。職業歴では石綿製品製造作業、造船所内作業、建設作業、断熱・保温作業など、いわゆる中等度以上の石綿ばく露と思われる職種が大半を占めた。石綿ばく露期間の中央値は25年、石綿初回ばく露からびまん性胸膜肥厚診断までの期間の中央値は46.5年であった。胸部画像所見では、胸膜プラークは86例（81.1%）と高率にみられたが、石綿肺の合併は7例（6.6%）のみであった。また、良性石綿胸水の既往歴がある症例が53例（50.0%）あった。これらの症例のうち、著しい呼吸機能障害を来していた67例においては、自覚症状により診断された症例が45例（67.2%）と多く、そのうち呼吸困難を主訴とした症例が36例（80.0%）を占めた。また、呼吸困難度も高度呼吸機能障害を来した症例が多かった。診断時からの生存期間の

中央値は23.5か月と比較的予後不良であった。肺がんを合併した症例はわずか２例であり、その２例はいずれも石綿肺を合併していた。その他の大半は慢性呼吸不全あるいは急性肺炎の合併により死亡していた。

５．良性石綿胸水とびまん性胸膜肥厚

上述のように、びまん性胸膜肥厚症例の約半数は良性石綿胸水の既往がある。その場合、「良性石綿胸水」がどの時点で「びまん性胸膜肥厚化」したのかは、特に労災あるいは救済の認定の上で問題となる。加藤、岸本らは[103]、良性石綿胸水から胸水が器質化し、びまん性胸膜肥厚に至った症例について胸部CT画像を検討し、胸水の器質化のポイントとして、①胸水内部の不均一性（胸水の高吸収化）、②胸郭容量低下、③胸水貯留部位におけるCrow's feet signの存在、④胸水量の固定化、⑤胸水内エアーの存在、を提唱している。そして５項目中３項目以上を満たせば、びまん性胸膜肥厚化して肺の再膨張が不可逆となると報告している（ただし②を含んで３点となる場合には胸水量が３か月以上変わらない固定化の確認が必要）。ある程度以上の胸水が長期的に貯留し器質化して高度呼吸機能障害を来す症例について、胸水の器質化にどの程度の期間を要するのか、どのような画像所見をもって非可逆的な器質化胸水と判断するのか等について、さらなる検討が望まれる。

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103) 加藤勝也, 芦澤和人, 酒井文和, 栗原泰之, 岸本卓巳. 石綿ばく露によるびまん性胸膜肥厚のうち胸水の器質化過程を観察できた症例に関する検討. 平成28年度環境省請負業務　石綿関連疾患に係る医学的所見の解析調査業務（石綿肺等の鑑別診断の在り方に関する調査編）報告書. 2017; 38-51.

石綿（アスベスト）関連肺・胸膜疾患の的確な診断と新規治療法の導入

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欧米では以前から、中皮腫の約80％が石綿ばく露により発生しその大半が職業性石綿ばく露によると報告されていたが、本邦では石綿ばく露と中皮腫の発生に関する全国的な調査・研究はなされていなかった。平成17年のいわゆる「クボタ・ショック」を受け、当機構では厚生労働科学特別研究として「職業性石綿ばく露と中皮腫発生に関する研究」（研究代表者 岸本卓巳）を開始した。人口動態統計で把握し得た、平成15年から20年の６年間に中皮腫で死亡したとされる約6,000例について、遺族及び死亡診断書作成病院の了解を得て診療録、画像、病理標本の収集及び遺族に対するアンケート調査結果を検討し、わが国でも中皮腫の約80％が職業性ばく露を主体とした石綿ばく露が原因となって発生していたことを明らかにした。

その後の厚生労働科学研究「職業性石綿ばく露による肺・胸膜病変の経過観察と肺がん・中皮腫発生に関する研究」「胸膜中皮腫の的確な診断方法に関する研究－鑑別診断と症例収集－」（いずれも研究代表者岸本卓巳）では、胸膜中皮腫の診断精度の向上を図るため、中皮腫の画像パターンの解析、胸水の分子診断マーカーの診断意義についての検討、病理学的鑑別診断のための新規免疫組織化学マーカーの探索を行ったほか、石綿健康管理手帳取得者を対象とした低線量胸部CT検査を行い、肺がんや早期の中皮腫を診断するための有用性について報告した。また、石綿肺や胸膜中皮腫の鑑別疾患として重要な良性石綿胸水及びびまん性胸膜肥厚にも着目し、症例収集を行い臨床的特徴の解明に取り組んでいる。

さらに、労災疾病臨床研究補助金事業「胸膜中皮腫に対する新規治療法の臨床導入に関する研究」（研究代表者 藤本伸一）では、悪性胸膜中皮腫に対する新たな治療法として抗PD-１抗体の有用性を検討するため「切除不能悪性胸膜中皮腫に対する初回化学療法としてのシスプラチン、ペメトレキセドおよびニボルマブ併用化学療法の第II相試験」を医師主導治験として企画、立案し、治験を実施中である。

また、中皮腫患者におけるQuality of lifeの実態を明らかにするため、全国規模での横断的な調査を行った。これらの調査を通じて、中皮腫患者は様々な困難や要望を抱えていることが明らかとなり、これらの要望に応える具体的な方策として中皮腫患者や家族に適切な情報を提供する「患者さんとご家族のための胸膜中皮腫ハンドブック」（**図**）を開発した。現在は引き続き、労災疾病臨床研究補助金事業「石綿関連胸膜疾患における個別化治療とケアの確立」（研究代表者藤本伸一）において、胸膜中皮腫の早期診断や治療に応用可能なバイオマーカーの探索や、石綿ばく露によるびまん性胸膜肥厚における著しい呼吸機能障害を客観的に評価する指標の確立に取り組んでいる。

石綿関連疾患を早期に発見・診断し治療につなげ、また、労災・救済認定の迅速・適正化等に寄与することが我々の継続的な研究テーマである。

図. 適切な情報提供のために開発されたハンドブック

胸膜・腹膜の病理

胸膜・腹膜疾患への臨床的アプローチ

—治療を中心として—

藤本伸一

病理と臨床・別刷
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特集　胸膜・腹膜の病理

胸膜・腹膜疾患への臨床的アプローチ
―治療を中心として―

藤本伸一*

はじめに

石綿に関連する胸膜疾患には悪性疾患である胸膜中皮腫と，良性疾患としての良性石綿胸水およびびまん性胸膜肥厚が含まれる．腹膜疾患としては腹膜中皮腫があり，これまでのところ石綿に関連する良性の腹膜疾患の概念はない．本稿ではこのうち胸膜中皮腫と腹膜中皮腫を中心に，治療を中心とした臨床的アプローチについて概説する．

Ⅰ．胸膜中皮腫の治療

胸膜中皮腫の治療は，病期分類[1]と組織分類[2]に基づき決定される．切除可能な症例では手術療法が，切除不能例や術後再発例では全身化学療法が治療の主体となる．放射線療法は手術療法や化学療法と組み合わせた集学的治療の一環として施行される場合もあるが，多くは疼痛コントロール目的の緩和療法の一部として施行される．

1．胸膜中皮腫の手術

胸膜中皮腫の手術には，主に胸膜外肺全摘術 extrapleural pneumonectomy（EPP）と胸膜切除剝皮術 pleurectomy/decortication（P/D）の2種類がある．EPPは壁側胸膜，臓側胸膜と肺，横隔膜，心膜を切除し，人工の膜で横隔膜と心膜を再建する．P/Dは壁側胸膜と臓側胸膜を切除し，必要な場合に横隔膜や心膜を切除再建する．両術式の最大の違いは肺の摘出であり，EPPは肺を摘出するがP/Dでは肺は温存される．腫瘍の減量効果はEPPのほうが高く，またEPPでは術後の放射線治療が可能となる．P/DはEPPに比べると患者の負担が軽いと考えられ，高齢者や，心肺機能や全身状態がやや低下している症例でも適応となる可能性がある．

システマティックレビューによると，上皮型中皮腫症例において，EPP施行群とベストサポーティブケア群における生存期間の中央値はそれぞれ19ヵ月，7ヵ月であり，手術の長期生存への寄与が報告されている[3]．また手術に加え化学療法を施行した群と化学療法単独群では手術＋化学療法群で有意な生存期間の延長が認められたとの報告がある[4]．同様に，背景因子を補正しても手術を受けた患者群は非手術患者群に比べ有意に良好な予後が得られたとの報告がある[5]．我が国における胸膜中皮腫患者の後ろ向き研究においても，手術施行例の予後は比較的良好であり[6]（図1），十分に選択された症例においては，外科手術は予後の改善に寄与するものと推定される．

EPPとP/Dを直接比較した前向き試験はないためどちらが優れているのかの結論はなかなか得られない．EPPとP/Dを比較したメタアナリシスでは，術後短期死亡率がEPPで有意に高いが（4.4% vs 1.7%，$p<0.05$）[7]，長期予後は同等，あるいは手術死亡率に差はなく生存期間の中央値は同等と報告されている（16ヵ月 vs 19ヵ月，統計学的有意差なし）[8]．長期予後に差がないのであれば肺が温存され負担の軽いP/Dを，というのが最近の術式選択の傾向と思われる．また肉腫型中皮腫では，それ自体でさらに手術適応が限られる．いずれにしても手術単独での治療成績は十分でないため，治療戦略を構築する上では呼吸器外科医，腫瘍内科医を含む治療チームによる集学的治療が望ましい．

2．胸膜中皮腫における根治目的の放射線療法

胸膜中皮腫における根治目的の放射線治療は，集学的治療の一つとしてEPP後の片側胸郭照射として用いられてきた．EPP後の局所制御に片側胸郭照射が有効であるとされながらも，完遂率の低さや，生存率向上への寄与が低いことが指摘されてきた．ただいずれの報告も後方視的研究もしくは第Ⅱ相試験であり，

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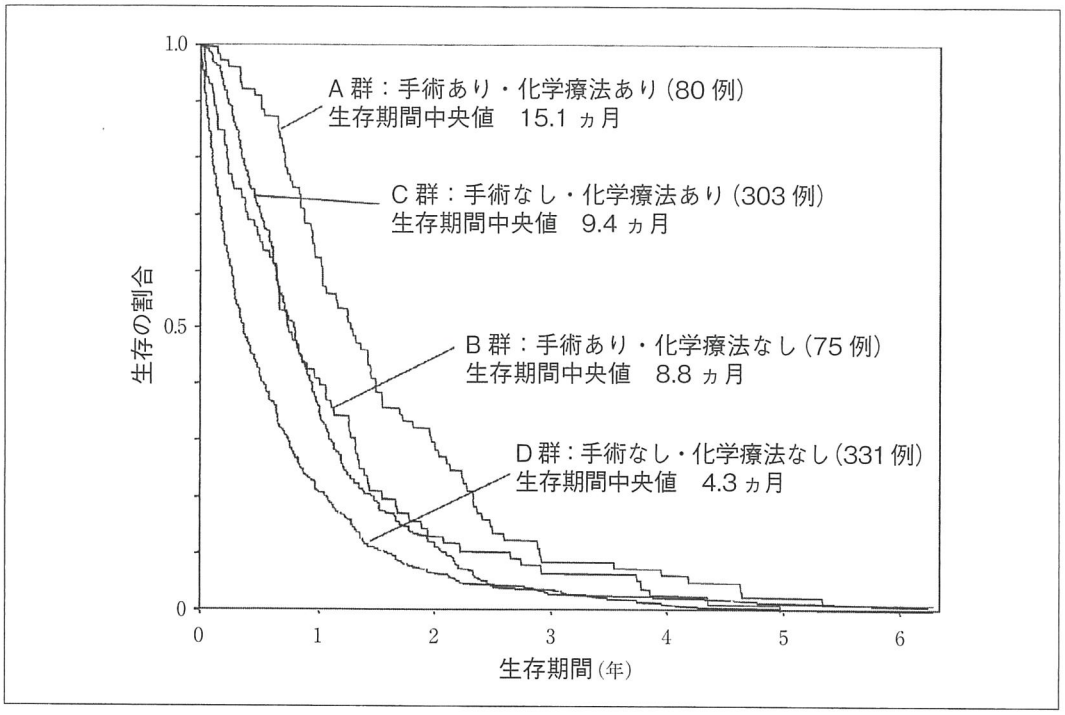


図1　治療法別の胸膜中皮腫の生存期間（文献6より一部改変）

片側胸郭照射の有用性を検証する第Ⅲ相試験は現在のところ存在しない．

P/D後の照射についても検討されているが，P/D後に通常照射での片側胸郭照射を施行した報告では，2年全生存率23％，1年局所制御割合42％で，Grade 3以上の放射線肺臓炎が10.6％（Grade 5：1例）と高頻度であるため，P/D後の片側胸郭照射は有効な治療選択肢ではないとされている[9]．一方，P/D施行後および非切除例に対して強度変調放射線治療 intensity modulated radiation therapy（IMRT）を用いた片側胸郭照射を行った症例において，2年生存率がそれぞれ53％，28％であったが，Grade 3以上の放射線肺臓炎が20％に生じている[10]．現時点では毒性の観点からP/D後の放射線治療は推奨されていない．P/D施行後または手術非適応症例に根治目的にて放射線療法を行う場合は，臨床試験として行われるべきである．

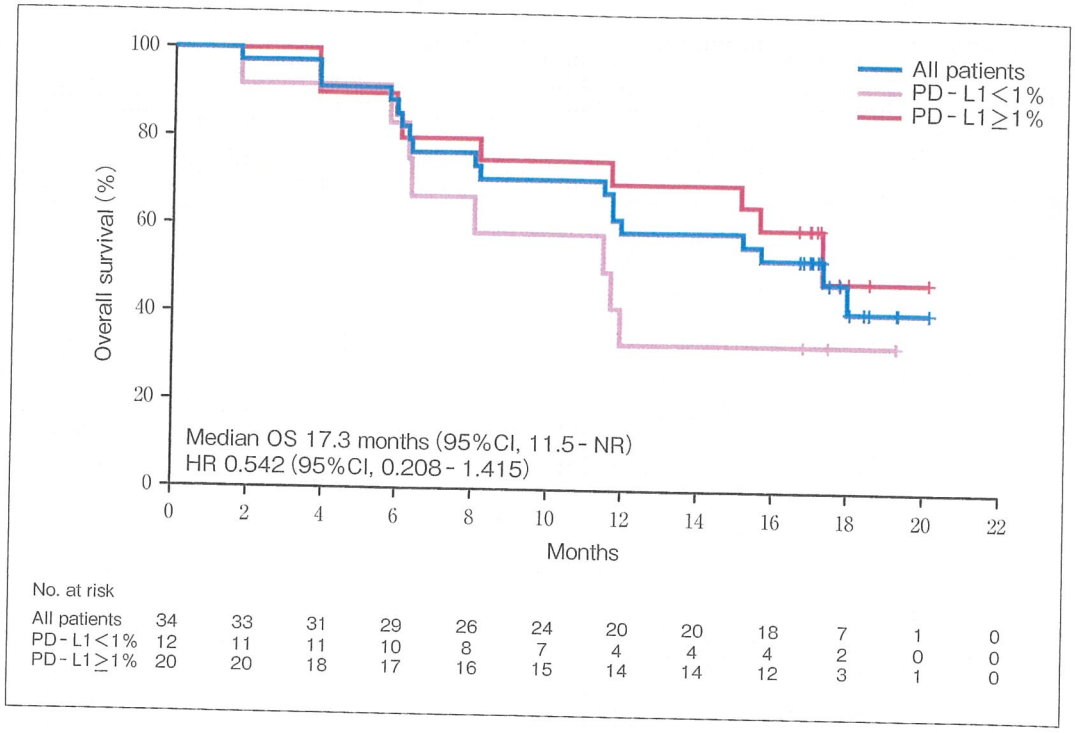
3．胸膜中皮腫における化学療法

進行例および術後の再発例は化学療法の対象となる．未治療の切除不能症例に対し，シスプラチン，ペメトレキセドの併用療法とシスプラチン単剤とを比較した臨床第Ⅲ相試験で全生存期間，無増悪生存期間，奏効率いずれにおいても併用群で有意に良好であり（生存期間の中央値12.3ヵ月 vs 9.3ヵ月，無増悪生存期間の中央値5.7ヵ月 vs 3.9ヵ月，奏効率41.3％ vs 16.7％）現時点での標準治療とされている[11]．高齢や合併症のためシスプラチンの投与がためらわれる場合，カルボプラチンの投与が考慮される．カルボプラチンとペメトレキセドの併用療法については2つの第Ⅱ相試験があり，上述のシスプラチンとの併用療法に比べ奏効率はやや劣るが（18.6％，25％），増悪までの期間（6.5ヵ月，8.0ヵ月）および全生存期間（12.7ヵ月，14ヵ月）はほぼ同等の結果であり，毒性も骨髄抑制以外は軽度であった[12,13]．ただし，カルボプラチンは悪性胸膜中皮腫に対し承認されていない点は注意が必要である．初回化学療法としてのこれらの併用療法の至適投与回数は4〜6コースとされており，その後のペメトレキセドによる維持療法については明確なエビデンスはない．

プラチナ製剤とペメトレキセドに対し不応となった場合，治療法の選択肢は限られる．二次治療におけるビノレルビンあるいはゲムシタビンの成績は，いずれも第Ⅱ相試験において奏効率は7〜24％，全生存期間の中央値は8.0〜10.6ヵ月と報告されている[14-16]．ビノレルビンとゲムシタビンの併用療法については，第Ⅱ相試験においてそれぞれの単剤療法を上回る成績が報告されておらず，一方で血液毒性が増加するとされている．それ以外にもこれまでに幾つかの新規製剤について臨床試験が行われてきたものの，有用性は示されなかった．

ここ数年，悪性黒色腫や非小細胞肺癌を中心に複数の癌腫に対し臨床導入が相次いでいる免疫チェックポイント阻害薬は，胸膜中皮腫患者にとっても福音とな

図2　既治療胸膜中皮腫に対するニボルマブ投与例の生存率（文献17より一部改変）



りつつある．既治療の日本人の胸膜中皮腫患者（一次治療後24人，二次治療後10人）を対象としたニボルマブの臨床第Ⅱ相試験（MERIT試験）において，組織型にかかわらず奏効率29.4%，無増悪生存期間の中央値2.6ヵ月，6ヵ月の生存率74%，全生存期間の中央値17.4ヵ月との成績が報告された[17]（**図2**）．この試験の結果に基づきニボルマブは2018年8月に，「がん化学療法後に増悪した切除不能な進行・再発の悪性胸膜中皮腫」に対して承認された．ニボルマブを含む免疫チェックポイント阻害薬については今後，既存の化学療法との併用，あるいは初回治療としての有用性への期待が高まっており，現在も臨床試験が進められている．筆者らは，切除不能例に対する初回化学療法としてのシスプラチン，ペメトレキセドおよびニボルマブの併用化学療法の有用性と安全性を評価する臨床第Ⅱ相試験を医師主導治験として施行しており[18]，既に症例集積を完了している．

その他の治療法としては，血管新生阻害薬であるベバシズマブやニンテダニブを用いた臨床試験において一部で有望な結果が報告されたものの，実用には至っていない．遺伝子治療や抗体療法についても臨床試験が進められているが，これらについてもこれまでのところ臨床的な有用性は確認されていない．さらなる治療成績の改善のため，今後も引き続き有効な治療法の開発が切望される．

4. 集学的治療

上述のように，外科手術単独での治療成績は良好とはいえず，集学的治療が生存率に寄与する可能性が指摘されている．手術可能なⅠ～Ⅲ期の悪性胸膜中皮腫症例には，術前または術後のどちらかに化学療法，またEPP施行例においては術後に放射線療法を加える集学的治療が検討される．Sugarbakerらは，手術，化学療法，放射線療法の三者併用療法において長期生存が得られる可能性があることを報告している[19]．また本邦におけるEPPを含む集学的治療に関する第Ⅱ相臨床試験において42例の登録患者におけるMSTは19.9ヵ月，治療関連死亡率は9.5%であった[20]．手術を行う場合には肉眼的完全切除を得られることが重要であるが，耐術能に加え術後の化学療法に耐えうるかも含めて呼吸機能・心機能などを十分に評価する必要がある．

5. 緩和医療

胸膜中皮腫は，多くの場合石綿ばく露から30～40年の潜伏期を経て発症するため，高齢で発症するケースが多い．また様々な合併症を有するケースも多く，上述の外科手術や化学療法の適応とならず緩和医療の対象となるケースが少なくない．また胸膜中皮腫では，病初期より胸水貯留による息切れや胸膜刺激や胸壁浸潤による疼痛を訴えることが多く，緩和医療の果たす役割が非常に大きい．疼痛緩和目的で放射線治療を施行した報告では，約60%の症例に疼痛緩和が得

られたと報告されている[21,22]．胸水制御と症状軽減を目的とした胸腔ドレナージおよび胸膜癒着術は，外科治療や化学療法などの積極的な抗癌治療の適応の有無にかかわらず考慮される．癒着には通常タルクまたはOK432（ピシバニール®）が用いられる．

また中皮腫患者においては，後述の腹膜例も含め，疼痛や呼吸困難などの苦痛症状を呈する頻度が高いこと，有効な治療法が限られること，また職業歴に起因する場合が多いことなどから，身体面のみならず精神的あるいは社会的なサポートを要するケースが多い[23]．緩和ケア医や医療ソーシャルワーカーなども含めた多職種によるサポートが望ましい．

Ⅱ．腹膜中皮腫

腹膜中皮腫は中皮腫全体の10～20％を占める．胸膜中皮腫に比べ特に若年の女性患者の割合が多い[24]．肺，胸膜に比べ吸入された石綿繊維の腹膜腔への到達はイメージされにくいが，腹膜中皮腫はむしろ胸膜中皮腫よりも高濃度の石綿ばく露後に発生することが示唆されている[25]．石綿ばく露以外の要因としては，胸膜同様鉱物由来の線維，*BAP1*遺伝子変異，放射線ばく露のほか，腹膜炎，繰り返す憩室炎，脳室腹膜シャントなどの報告があるが，原因が特定できない症例も少なくない[24,26]．自覚症状としては腹痛，腹部膨満感，食欲不振，体重減少を訴えることが多い．画像上は腹水のみのものから，腹膜の単発ないし多発の結節，腫瘤を呈する場合がある．

根治的治療法は存在せず，標準的治療法（外科治療，内科治療）も確立されていない．限局性で切除可能と判断される場合は確定診断も兼ねて切除の対象となる場合がある．切除不能な腹膜中皮腫に対し，我が国で承認されている薬剤はない．胸膜中皮腫に準じてシスプラチンあるいはカルボプラチンとペメトレキセドの併用療法が施行されているものと思われるが，その治療成績の報告は多くない[27]．

Ⅲ．良性石綿胸水

良性石綿胸水は，石綿ばく露によって生じる非悪性の胸水貯留をきたす疾患であり，石綿（アスベスト）胸膜炎とも呼ばれる．Eplerらによる診断基準は，①石綿ばく露歴がある，②胸部X線あるいは胸水穿刺で胸水の存在が確認される，③石綿ばく露以外に胸水貯留の原因がない，④胸水確認後3年以内に悪性腫瘍を認めないという4点である[28]．Hillerdalらは，胸部CTなどの画像診断で詳細な臨床経過を観察した場合には，発症後1年の経過観察でよいとしているが[29]，未だに診断基準が確立されているとは言い難い疾患である．石綿肺，原発性肺癌，中皮腫，びまん性胸膜肥厚とともに労災補償の対象疾患となっているが，その認定基準も明記されておらず，全例厚生労働省における協議によって決定されている．

良性石綿胸水の診断は，つまり結核性胸膜炎や細菌性胸膜炎，膠原病に伴う胸膜炎，また慢性腎不全や慢性心不全など多種多様な胸水貯留をきたす疾患を除外することである．中でも特に石綿ばく露歴がある場合，胸膜中皮腫をいかに除外するかが最も重要であり，胸水細胞診やCEA（carcinoembryonic antigen），アデノシンデアミナーゼ，ヒアルロン酸など胸水中の各種マーカーの検索はもちろん，可能な限り胸腔鏡検査を行い胸膜生検にて組織診断を得ることが重要である[30]．

良性石綿胸水に対する治療法は確立されていない．一定量以上胸水が貯留し息切れ，呼吸困難をきたす場合，胸腔穿刺，胸腔ドレナージにより胸水の排液が行われる．胸水貯留を繰り返しコントロールが困難な場合，ステロイド療法の報告があるが効果は限定的である．また良性石綿胸水の約半数は，後述するびまん性胸膜肥厚へ移行する．

Ⅳ．びまん性胸膜肥厚

びまん性胸膜肥厚は石綿による非腫瘍性胸膜疾患の一つであり，両側または一側の広範な胸膜肥厚を特徴とする．病理学的には臓側胸膜の慢性線維性胸膜炎であるが，その病変がしばしば壁側胸膜にも及び，両者が癒着するため拘束性の呼吸機能障害をきたす[31]．著しい呼吸機能障害を呈する場合，労災認定あるいは「石綿による健康被害の救済に関する法律」における救済の対象となる．

岸本らは，石綿ばく露によって発生したびまん性胸膜肥厚106例について臨床上の特徴や石綿ばく露との関連について報告している[32]．それによると，70歳以上の男性が大半で，多くが重喫煙者であり，石綿製品製造作業，造船所内作業，建設作業，断熱・保温作業など，いわゆる中等度以上の石綿ばく露と思われる職種が大半を占めた．石綿ばく露期間の中央値は25年，石綿初回ばく露からびまん性胸膜肥厚診断までの期間の中央値は中央値46.5年であった．高度の呼吸

機能障害をきたした症例が多く，診断時からの生存期間の中央値は23.5ヵ月と予後不良であった．肺癌を合併した症例が2例あるが，その他の大半は慢性呼吸不全あるいは急性肺炎の合併により死亡していた．

びまん性胸膜肥厚に対する治療法も確立していない．びまん性胸膜肥厚の進行を抑える薬剤はなく，対症的に気管支拡張薬，鎮咳薬，去痰薬などが用いられる．呼吸機能障害の程度によっては酸素療法が導入されるが，上述のように予後は不良である．

V．「病理医へ，ここを伝えてほしい」

胸膜，腹膜中皮腫を中心に治療法および鑑別すべき診断として良性石綿胸水，びまん性胸膜肥厚について述べた．臨床医が必要とするのは「速く，確実な病理診断」に尽きるが，そのためには臨床医と病理医のきめ細かい情報共有が必要である．稿の最後に，「臨床医が，病理医から伝えてほしいこと」を述べる．

1．検体は十分か

臨床的に胸膜中皮腫が疑われるのは，胸水貯留ないし胸膜の不整な肥厚，あるいは腫瘤を呈する場合である．まず臨床医は，画像および臨床所見から「明らかに悪性腫瘍であるかどうか」を判断する．具体的な所見としては，明らかな腫瘤の形成，周囲の肋骨や胸壁への浸潤，リンパ節や他臓器への転移などである．また不整な胸膜肥厚が短期間に増悪している場合も十分に悪性腫瘍を疑う．この場合臨床医は何らかの手段で胸水あるいは可能な限り胸膜組織，腫瘍組織を採取し検体として提出するが，病理医にはまず「診断するに十分な検体が得られているか」を判断し伝えていただきたいと思う．ここで臨床医が要求する診断は，「胸膜中皮腫か，そうでなければ他の何らかの悪性腫瘍」である．この場合「良悪性の判断がつきかねる」ような検体はそもそも不適であり，その場合臨床医は早急に追加の検体を得る手段を講じる必要がある．胸膜中皮腫の病理診断には複数の免疫組織化学染色が必要であるし，また結果的にたとえば肺癌である場合，その治療方針の決定には昨今種々のドライバー遺伝子の検索が必要であり，そこでも一定量以上の検体を必要とする．まず臨床医と病理医は，「明らかに悪性腫瘍であるかどうか」の情報を共有し，その観点から十分な検体であるかどうかを共有する必要がある．

2．診断確定までの時間

中皮腫の診断には様々な鑑別疾患があり，複数の免疫組織化学染色や遺伝子検索を要する．またこれらの検索は外注や他施設へのコンサルテーションを要することも少なくない．臨床医と病理医は，「診断までにどのくらいの時間を要するか」を共有する必要がある．例えば画像上は胸水貯留とわずかな胸膜肥厚のみ，のような場合，ある程度時間を要してでも抜かりのない診断を得る必要があろう．また上述のような「明らかに悪性腫瘍」の場合，特に病変の進展が速いケースでは一日も早い診断確定が要求される．臨床医は，時間的猶予がある状況なのかを病理医に伝え，その上で両者は「診断にはどれくらいの時間がかかりそうか」の情報を共有する必要がある．

3．労災あるいは救済認定

中皮腫は胸膜発生にせよ腹膜発生にせよ，診断が確定した場合我が国では労災補償を受けるか石綿健康被害救済法にて救済されることとなる．その認定に際し病理診断は極めて重要である．通常のヘマトキシリン・エオジン染色に加え，免疫組織化学染色における複数の陽性マーカー，陰性マーカーが必要であり，その結果は医証として提出される．病理データが不足する場合，追加の検体提出や専門機関での追加検索を要し，結果的に認定されるまでに余分な時間を要するか場合によっては認定に至らない場合もある．中皮腫の病理診断にあたっては中皮腫特有の，石綿に関わる社会的な側面を考慮し，その病理診断が労災あるいは救済認定にかなうものであるかどうかも念頭に置いた上で免疫組織化学染色も含めた所見を整理する必要がある．

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