

Aminopeptidase N/CD13 as a Potential Therapeutic Target in Malignant Pleural Mesothelioma

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Take-home: Aminopeptidase N is a potential therapeutic target in mesothelioma
exhibiting high Aminopeptidase N expression.

Abstract

Angiogenesis is a crucial factor in the progression of malignant pleural mesothelioma (MPM), and antiangiogenic strategies might be effective against MPM. Aminopeptidase N/CD13 (APN/CD13) promotes tumour angiogenesis and is associated with poor prognosis; however, its clinical significance in MPM remains unclear.

In 37 consecutive patients with surgically resected MPM, we evaluated the association between immunohistochemical APN/CD13 expression in resected tumours and survival.

Additionally, the antitumour and antiangiogenic effects of MT95-4, a fully humanized anti-APN/CD13 monoclonal antibody, were evaluated in mice orthotopically implanted with EHMES-10 (abundantly expressing APN/CD13) and MSTO-211H (scarcely expressing APN/CD13) MPM cells.

High tumour APN/CD13 expression was associated with poor prognosis in MPM patients ($P = 0.04$), and MT95-4 treatment reduced tumour growth and angiogenesis in mice harbouring EHMES-10, but not MSTO-211H, cells. Furthermore, in mice harbouring EHMES-10 cells, MT95-4 combined with cisplatin more effectively suppressed tumour progression than cisplatin alone.

Taken together these results suggested that APN/CD13 is implicated in the aggressiveness of MPM. Here, MT95-4 treatment reduced tumour progression likely by

inhibiting angiogenesis, suggesting APN/CD13 as a potential molecular target for MPM treatment. Additionally, combination treatment with MT95-4 and cisplatin could represent a promising approach to treating MPM exhibiting high APN/CD13 expression.

Introduction

Malignant pleural mesothelioma (MPM) is a rare, but aggressive malignant tumour with poor prognosis, with the number of MPM-related deaths continuing to increase worldwide [1, 2]. Only a minority of patients with MPM is eligible for multimodality therapy, including surgery and radiotherapy, and effective therapeutic strategies for patients with inoperable MPM are limited; therefore, novel systemic therapies are needed.

Angiogenesis is a crucial factor in the progression of solid tumours, and microvessel density is an independent prognostic factor of MPM [3–6], indicating that antiangiogenic strategies might be beneficial for MPM treatment. This is supported by a previous report showing that addition of bevacizumab to pemetrexed + cisplatin treatment improved survival in patients with MPM [7]

Aminopeptidase N (APN/CD13) is a Zn^{2+} -dependent, membrane-bound ectopeptidase that catalyses the cleavage of N-terminal amino acids from small peptides. APN/CD13 consists of 967 amino acids and a short N-terminal cytoplasmic domain, a single transmembrane domain, and a large extracellular domain, which contains the active site. APN/CD13 is widely expressed in mammalian cells, such as renal proximal tubular epithelial cells and myeloid progenitor cells, as well as in the small intestine

epithelium and central nervous system synaptic membranes. Additionally, APN/CD13 is a multifunctional enzyme that is also involved in viral receptor function, cellular differentiation, and antigen presentation [8–12]. Moreover, APN/CD13 is associated with tumour progression through the promotion of both tumour cell invasion and angiogenesis [13–18]. The expression of APN/CD13 in various types of tumours, including lung, pancreatic, and colon cancers as well as acute lymphoblastic leukaemia, has been reported [19–22]; however, the clinical significance of its expression in MPM remains to be elucidated. On the basis of these findings, we hypothesized that APN/CD13 is associated with MPM progression and is an attractive molecular target for the treatment of MPM. To determine the significance of APN/CD13 in MPM, we investigated correlations between APN/CD13 expression and patient survival by performing immunohistochemical staining of resected tumours in patients with surgically resected MPM. Additionally, to determine the efficacy of APN/CD13 as a molecular target in MPM treatment, we examined the antitumour and antiangiogenic effects of MT95-4, a fully humanized anti-APN/CD13 monoclonal antibody (mAb), in an orthotopic implantation mouse model of MPM. Using this animal model, we also examined whether MT95-4 affected the antitumour effect of the cytotoxic agent cisplatin.

Methods

Clinical and Pathological Characteristics of Patients

We examined 37 consecutive patients with MPM who underwent preoperative chemotherapy (cisplatin + pemetrexed) and radical surgery at the Department of Thoracic Surgery of Hiroshima University (Hiroshima, Japan) from February 2008 to August 2015. Major eligibility criteria for radical surgery was histologically confirmed diagnosis of MPM, including clinical subtypes T0-3, N0-2, M0 disease; no prior treatment for the disease; age 20-75 years; Eastern Cooperative Oncology Group performance status 0 or 1; predicted postoperative forced expiratory volume >1000 mL in 1 s. By immunohistochemical staining, we investigated APN/CD13 expression in resected tumours obtained from patients with MPM. Six patients who died of MPM-unrelated causes were excluded. This study was approved by the Ethics Committees of Hiroshima University Hospital and conducted in accordance with the ethical standards established by the Helsinki Declaration of 1975. All patients gave informed consent in writing and permission to use their samples.

APN/CD13 Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens were incubated with a mouse mAb against human APN/CD13 (Leica Biosystems, San Jose, CA, USA) at 4°C

overnight, washed in phosphate-buffered saline, and incubated with a biotinylated rabbit anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA). The immunoreaction was amplified using a VECTASTAIN ABC kit (Vector Laboratories), and antibody binding was visualized with 3,3-diaminobenzidine solution acting as a chromogen. The sections were then counterstained with haematoxylin and dehydrated. Some sections were incubated with normal murine IgG as negative controls.

Specimen Classification Based on Immunohistochemical Staining

All immunostained sections were reviewed by two pathologists who were blind to the clinical status of the patients. The proportion of tumour cells showing high or low staining was determined in five random microscopic fields by counting individual tumour cells at high magnification. The APN/CD13 expression level was evaluated in at least 200 tumour cells at a magnification of 400×. The mean proportion of APN/CD13-positive tumour cells in the tumours was 17%; therefore, we set 17% as a cut-off value to categorize tumours with high or low APN/CD13 expression.

Cells and Culture

Two human MPM cell lines, EHMES-10 and MSTO-211H, were used in this study. EHMES-10 cells were established from the pleural effusion of a patient with MPM at Ehime University [23,24], and MSTO-211H cells were purchased from the

American Type Culture Collection (Manassas, VA, USA). BALB/c Mouse Primary Pulmonary Artery Endothelial Cells were purchased from Cell biologic (Chicago, IL, USA). MPM cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Mouse endothelial cells were cultured in mouse endothelial cell medium/w kit (Cell biologic). All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. MSTO-211H and mouse artery endothelial cells were used within 6 months after thawing, and EHMES-10 cells were authenticated by short-tandem-repeat analyses (Promega, Tokyo, Japan).

Reagents and Animals

MT-95-4 was developed in our laboratory as previously reported [25], and cisplatin was purchased from Nippon Kayaku (Tokyo, Japan). Male severe combined immunodeficient (SCID) mice (6-weeks old) were purchased from CLEA Japan (Osaka, Japan). Animals were maintained according to guidelines for the ethical use of animals in research at Hiroshima University.

Flow-cytometric Analysis

Flow cytometric analyses of MPM cell lines were performed as previously described [25]

Quantitative Real-time PCR

RNA extraction, reverse transcription to cDNA, and real-time quantitative PCR were performed as previously described [25]. The primers used were as follows: APN/CD13 (Hs00174265_m1), vascular endothelial growth factor (Hs00900055_m1), and β -actin (4352935E).

Orthotopic Implantation Model

EHMES-10 (3×10^6) or MSTO-211H (1×10^6) cells were injected into the thoracic cavity of SCID mice as previously described [23], and the mice were randomly assigned to control or drug-treatment groups. MT95-4 (0.3 or 1 mg/kg) and control human IgG (Sigma-Aldrich, St. Louis, MO, USA) were injected intraperitoneally twice weekly, and cisplatin (3 mg/kg) was injected intraperitoneally once weekly. All mice were killed on day 28 (EHMES-10) or day 21 (MSTO-211H) after tumour cell inoculation, the thoracic tumours were carefully removed and weighed, and pleural effusions were harvested using a 1-mL syringe, followed by volumetric measurement.

Evaluation of Microvessel Density in Thoracic Tumours

Microvessel density was evaluated following immunohistochemical staining for CD31 in thoracic tumours as previously described [26].

Proliferation Assay

EHMES-10 or MSTO-211H (1×10^4 /100 mL) cells were seeded in 96-well plates and incubated with MT95-4 at various concentrations for 36 hours. Cell proliferation was assessed by measuring the absorbance of the medium in each well using the Cell Counting kit-8 (DOJINDO, Kumamoto, Japan).

***In Vivo* Analyses of Apoptosis**

Frozen tissue sections were used for *in situ* apoptosis detection by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) using the *In Situ* Apoptosis Detection Kit (Takara Biomedicals, Shiga, Japan). Images were captured using a microscope at a magnification of 200 \times (model BZ-9000; Keyence), and the positive cells were determined by counting 1000 cells per slide.

Angiogenesis Antibody Array Analysis

Angiogenesis antibody array analysis was performed according to the manufacturer's protocol. Additional detail is provided in an online data supplement.

Quantification of Vascular Endothelial Growth Factor (VEGF) Protein

EHMES-10 (1×10^5) cells were seeded in 24-well plates and cultured in the presence of MT95-4 (40 μ g/mL) or human control IgG for 12 hours or 48 hours. The VEGF concentration in the culture supernatant was measured using an ELISA kit according to the manufacturer's instructions (R&D Systems).

Matrix Degradation Assay

A matrix Degradation assay was performed using the Cell Invasion Assay Kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. In brief, cells were seeded on the upper surface of the insert membrane pre-coated with matrix proteins. Degradation of the matrix proteins was necessary for seeded cells to pass through the pores of the membrane. Finally, the invaded cells were stained and quantified. For mouse primary pulmonary artery endothelial cells, a suspension of 2×10^5 cells in various concentrations of recombinant human APN/CD13 was seeded on the upper surface of the insert membrane pre-coated with basement membrane matrix solution. For EHMES-10 and MSTO-211H cells, a suspension of 2×10^5 cells containing MT95-4 (20 $\mu\text{g}/\text{mL}$) or human control IgG were seeded on the upper surface of the insert membrane pre-coated with matrix proteins. Invasive cells were counted in three random microscopic fields per well after 48 hours.

Statistical Analysis

Fisher's exact test and the Mann-Whitney *U* test were performed to determine significant differences between APN/CD13 expression and clinicopathological parameters. Overall survival was recorded from the date of surgery to the date of death due to MPM. The Kaplan-Meier method was used to estimate the probability of survival,

and significance was assessed by the log-rank test. Univariate and multivariate analyses of predictive factors for prognosis were performed using the Cox proportional hazard regression model to assess APN/CD13 expression as independent prognostic marker for MPM. Independent predictors were identified from among the parameters whose *P*-values were < 0.1 in a univariate analysis. For *in vitro* experiments, data are expressed as the mean \pm SEM, and differences between groups were analysed by Student's *t* test or one-way ANOVA with Dunnett's *post hoc* test. For comparisons between groups *in vivo*, one-way ANOVA with Dunnett's *post hoc* test or Bonferroni's multiple-comparison test was performed. Statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA) and JMP software package (version 13.0; SAS Institute Inc., Cary, NC, USA). All *P*-values were recorded as 2-tailed, and $P < 0.05$ was considered statistically significant.

Results

APN/CD13 expression is associated with shorter overall survival in MPM patients

To investigate the clinical significance of APN/CD13 in MPM, we immunohistochemically evaluated APN/CD13 expression in resected tumours and analysed its association with survival in MPM patients. Representative images of positive and negative immunohistochemical staining of APN/CD13 in resected tumours are shown in Figure 1A and 1B, respectively. We observed APN/CD13 staining throughout the cell membrane and cytoplasm of tumour cells, and 15 and 16 tumours were categorized as exhibiting high and low APN/CD13 expression, respectively. The relationships between APN/CD13 expression and clinicopathological variables are shown in Table 1. The median age at surgery was 64 years (range: 48–72 years), and 19 patients died during the follow-up period. No significant relationships between APN/CD13 expression and age, smoking history, the effect of neoadjuvant chemotherapy, or histology were observed; however, we could not investigate the relationship between APN/CD13 expression and sex because female patients were not included. The median survival time of patients with high levels of APN/CD13 expression was significantly shorter than that of patients with low levels of APN/CD13 expression (17.3 vs. 54.4 months, $P = 0.04$; Figure 1C). Further, to investigate

APN/CD13 as an independent prognostic marker, univariate and multivariate analyses were performed using Cox proportional hazard regression model. A multivariate analysis demonstrated that both high APN/CD13 expression and histology (non-epithelioid) were independently associated with poor prognosis among the factors with $P < 0.1$ in a univariate analysis (Table2).

MT95-4 suppresses tumour progression in an APN/CD13-expression-dependent manner

To determine the efficacy of APN/CD13 as a molecular target in MPM, we examined the antitumour effects of MT95-4, a fully humanized anti-APN/CD13 mAb, in an orthotopic implantation mouse model of MPM, using the MPM cell lines EHMES-10 and MSTO-211H. Both quantitative real-time PCR and flow-cytometric analysis showed that EHMES-10 cells abundantly expressed APN/CD13, whereas MSTO-211H cells scarcely expressed APN/CD13 (Figure 2A and 2B). *In vivo* administration of MT95-4 at doses of 0.3 mg/kg and 1.0 mg/kg significantly reduced tumour weight (ANOVA $P = 0.03$ and $P = 0.01$, respectively). Additionally, administration of MT95-4 at 1.0 mg/kg significantly reduced the amount of pleural effusion (ANOVA $P = 0.02$) in mice harbouring EHMES-10 cells (Figure 2C and 2D); however, in mice harbouring MSTO-211H cells, MT95-4 administration reduced

neither tumour weight nor the amount of pleural effusion (Figure 2E and 2F).

MT95-4 suppresses tumour angiogenesis in an APN/CD13-expression-dependent manner

To investigate the antiangiogenic effect of MT95-4 in intrapleural tumours, we evaluated the microvessel density of intrapleural tumours in mice harbouring EHEMS-10 or MSTO-211H cells by immunohistochemical staining for CD31. MT95-4 administration significantly decreased microvessel density [ANOVA (all) $P < 0.001$] in intrapleural tumours in mice harbouring EHEMS-10 cells (Figure 3A and 3B), but not in mice harbouring MSTO-211H cells (Figure 3C and 3D).

MT95-4 enhances the antitumour effect of cisplatin in mouse models of MPM

Because the antitumour effect of MT95-4 was demonstrated in mice harbouring MPM cells exhibiting high levels of APN/CD13 expression, we investigated whether MT95-4 administration affected the antitumour effect of the cytotoxic agent cisplatin. Mice harbouring EHEMS-10 cells were treated with vehicle, MT95-4 alone, cisplatin alone, or MT95-4 and cisplatin combination therapy. As shown in Figure 4A and 4B, MT95-4 and cisplatin combination therapy significantly reduced both tumour weight ($P = 0.009$, ANOVA) and the amount of pleural effusion ($P = 0.04$, ANOVA) as compared with cisplatin alone. Moreover, MT95-4 and cisplatin combination therapy

significantly prolonged the survival time of mice harbouring EHMES-10 cells (Figure 4C).

Effects of MT95-4 on the degrees of proliferation and apoptosis.

MT95-4 had an antitumour effect by inhibiting angiogenesis *in vivo*; we further investigated other mechanisms underlying the anti-tumour effect. A proliferation assay showed that MT95-4 did not suppress the proliferation rate of both EHMES-10 and MSTO-211H cells *in vitro* (Figure 5A and 5B). Next, we evaluated apoptosis *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) using frozen tissue sections, and found that MT95-4 alone did not significantly increase the number of apoptotic cells. However, MT95-4 in combination with cisplatin significantly increased the number of apoptotic cells (Figure 5C and 5D)

Antiangiogenic effect of MT95-4 depends on the inhibition of extracellular matrix degradation, but not the inhibition of angiogenic factors

Because the antiangiogenic activity of MT95-4 was demonstrated *in vivo*, we explored the mechanism underlying the antiangiogenic effects of MT95-4. We first investigated whether MT95-4 directly affected the expression of angiogenic factors produced by tumour cells *in vitro*. Using an angiogenesis antibody array, the expression levels of 43 angiogenic factors were compared between EHMES-10 cells treated with or

without MT95-4. As shown in Figure 6A, treatment of EHMES-10 cells with MT95-4 did not affect the expression of angiogenic factors. To verify these findings, we evaluated the protein and mRNA levels of the most important angiogenic factor, VEGF, in EHMES-10 cells treated with MT95-4. Our results showed that treatment of EHMES-10 cells with MT95-4 did not affect either VEGF protein (Figure 6B) or mRNA (Figure 6C) levels. Given that the angiogenic factor did not play a key role in the antiangiogenic effect of MT95-4, we hypothesized that the antiangiogenic effect of MT95-4 *in vivo* was achieved by the inhibition of extracellular matrix degradation, thereby inhibiting the invasion of vascular endothelial cells into the perivascular stroma. To evaluate this hypothesis, we performed a matrix degradation assay. First, to assess whether MT95-4 suppresses extracellular matrix degradation via the blockade of APN/CD13 of mesothelial cells, a matrix degradation assay was performed using a EHMES-10 (abundantly expressing APN/CD13) or MSTO-211H (scarcely expressing APN/CD13) cell suspension containing MT95-4 (20 µg/mL) or human control IgG. MT95-4 suppressed the invasion of EHMES-10, but not MSTO-211H (new Figure 6D and E). Second, we investigated whether APN/CD13 directly promotes the invasion of mouse vascular endothelial cells by the degradation of the matrix proteins. A Matrix degradation assay using mouse primary pulmonary artery endothelial cell suspensions

containing various concentrations of recombinant human APN/CD13 showed that recombinant human APN/CD13 promoted the invasion of endothelial cells in a dose-dependent manner (new Figure 6F).

Discussion

In this study, we found that high APN/CD13 expression was potentially associated with poor prognosis in MPM patients, and demonstrated that MT95-4, a fully humanized anti-APN/CD13 mAb, exerted antitumour and antiangiogenic effects in mice harbouring EHMES-10 cells (abundantly expressing APN/CD13), but not in those harbouring MSTO-211H cells (scarcely expressing APN/CD13). In mice harbouring EHMES-10 cells, administration of MT95-4 in combination with cisplatin suppressed tumour progression more effectively and prolonged survival as compared with treatment with cisplatin alone. Additionally, *in vitro* experiments demonstrated that the antiangiogenic effect of MT95-4 might be mediated by the inhibition of extracellular matrix degradation and thereby the inhibition of vascular endothelial cell invasion, but not by the suppression of angiogenic factors produced by tumour cells.

Our results clearly show that high APN/CD13 expression in tumour cells was associated with poor prognosis in MPM patients. Although APN/CD13 expression is associated with poor prognosis in various tumour types [19–22], possibly through its ability to promote tumour cell invasion and angiogenesis [13–18], its clinical significance in MPM remains unknown. To the best of our knowledge, this represents the first study reporting an association between APN/CD13 expression in tumour cells

and MPM patient prognosis. These results indicate that APN/CD13 is involved in tumour aggressiveness in MPM patients.

To examine the efficacy of APN/CD13 as a therapeutic target in MPM, we tested the antitumour effect of MT95-4, a fully humanized anti-APN/CD13 mAb established in our laboratory [25], in an orthotopic implantation mouse model of MPM, using MPM cells exhibiting high or low APN/CD13 expression. We found that MT95-4 administration suppressed tumour progression and angiogenesis only in tumours exhibiting high APN/CD13 expression. These results suggest that a therapeutic strategy targeting APN/CD13 might constitute a promising treatment option for MPM exhibiting high APN/CD13 expression. Because MT95-4 treatment alone did not affect the cell proliferation (Figure 5A and B), apoptosis (Figure 5C and D), or senescence (data not shown), we suggest that MT95-4 suppresses tumour progression mainly by inhibiting angiogenesis in MPM tumours exhibiting high APN/CD13 expression.

A previous study reported that addition of bevacizumab to pemetrexed + cisplatin treatment significantly improved overall survival in patients with unresectable MPM [7], thereby reaffirming the importance of antiangiogenic therapy in MPM. In this study, we demonstrated an antiangiogenic effect of neutralization of APN/CD13 expression in tumour cells in an MPM mouse model harbouring MPM cells exhibiting

high APN/CD13 expression. This result agreed with those of previous studies reporting that APN/CD13 neutralization or knockdown in tumour cells decreased intratumoural microvessel density in mice [25, 27]. Bevacizumab administration inhibits the activity of VEGF secreted by tumour cells; however, our results demonstrated that MT95-4 treatment did not suppress VEGF expression in MPM cells. Additionally, use of an angiogenic antibody array enabled us to observe that MT95-4 treatment did not affect the expression levels of 43 angiogenic factors, including VEGF, produced by MPM cells. These results suggest that the antiangiogenic effect associated with MT95-4 treatment was not mediated by the suppression of angiogenic factors. A matrix degradation assay showed that the invasiveness of vascular endothelial cells was enhanced in the presence of APN/CD13 and MT95-4 suppressed extracellular matrix degradation by tumour cells by inhibiting APN/CD13 activity. Consequently, we suggest that the antiangiogenic effect of MT95-4 treatment was achieved by the inhibition of APN/CD13 activity, which is involved in the degradation of extracellular matrix elements, such as entactin and type IV collagen [13, 28], which are components of the basement membrane, thereby inhibiting the release and migration of vascular endothelial cells into the perivascular stroma [29, 30].

APN/CD13 expressed in both tumour cells and non-malignant stromal cells promotes tumour progression and angiogenesis [27]. Because MT95-4 does not recognize murine APN/CD13 [25], the antitumour and antiangiogenic effects of MT95-4 described in this study might have been achieved by inhibiting the activity of APN/CD13 expressed only in tumour cells. When used for patients with MPM, MT95-4 may exert stronger antitumour effects through inhibition of APN/CD13 activity in both tumour and stromal cells. Although APN/CD13 expression is not limited to the tumour microenvironment, a therapeutic approach involving MT95-4-targeting APN/CD13 should be feasible in clinical use. Notably, APN/CD13 deficiency in mice does not result in physiological or histological abnormalities [31], and Ubenimex, an APN/CD13 inhibitor, have been used in clinical practice without severe adverse effects [32].

Additionally, we found that MT95-4 treatment in combination with cisplatin, a fundamental cytotoxic agent used for MPM treatment, exerted stronger antitumour effects as compared with MT95-4 treatment alone in a mouse model of MPM. It was previously reported that CD13-overexpressing xenografts exhibited a reduced sensitivity to cisplatin *in vivo* [33]. Moreover, an APN/CD13 inhibitor, Ubenimex, enhanced the effect of anticancer drugs, including cisplatin, by inducing cancer cell apoptosis [34]. In our study, MT95-4 in combination with cisplatin, but not MT95-4

alone, significantly increased the number of apoptotic cells, indicating that the tumour suppressing effect of the combination treatment might be explained by the induction of apoptosis mediated by MT95-4 by inhibiting APN/CD13 activity in tumour cells. These results indicate that MT95-4 treatment in combination with cisplatin could represent a promising therapeutic strategy for the treatment of MPM-specific tumours exhibiting high APN/CD13 expression.

There were some limitations in this study. First, the number of patients included in the prognostic analysis was small, and the significance of APN/CD13 expression in MPM needs to be validated in larger and prospective clinical studies. Second, all MPM patients in this study had undergone preoperative chemotherapy, and little is known regarding whether chemotherapy affects the levels of APN/CD13 expression in tumour cells. Third, we were unable to reveal the precise mechanisms underlying the antitumour and antiangiogenic effects associated with MT95-4 treatment. Therefore, further investigations to clarify this point are necessary.

In conclusion, we showed that APN/CD13 expression was potentially associated with poor prognosis in MPM patients. Moreover, our results revealed that MT95-4, a fully humanized anti-APN/CD13 mAb, suppressed tumour progression likely by inhibiting angiogenesis in an orthotopic implantation mouse model of MPM

exhibiting high levels of APN/CD13 expression. Additionally, we demonstrated that MT95-4 treatment enhanced the antitumour effect associated with cisplatin, a fundamental cytotoxic agent used for MPM treatment. These findings suggest that APN/CD13 represents a potential therapeutic target in MPM, and that MT95-4 in combination with cisplatin could be a promising therapeutic strategy for the treatment of MPM-specific tumours exhibiting high levels of APN/CD13 expression.

Author contributions: TO: Conception and design, collection of data, data analysis and interpretation, manuscript writing. TN: Conception and design, data analysis and interpretation, revision of manuscript. YT, SA: Conception and design, data analysis and interpretation. HH, TM, SM, YH, HI, KF, MM and NK: Data analysis and interpretation. YM and MO: Collection of data, provision of study material. NH: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Table 1. Relationships between APN/CD13 expression and various clinical and pathologic variables.

Variables	APN/CD13			<i>P</i>
	Total (<i>n</i>)	high (<i>n</i>)	low (<i>n</i>)	
Sex				
Female	0	0	0	>0.999*
Male	31	15	16	
Median age (range)	64 (48–72)	64 (55–72)	63 (48–70)	
Smoking status				
Never	5	3	2	0.654*
Smoker	26	12	14	
Effect of neoadjuvant chemotherapy				
PR	14	7	7	>0.999 [†]
SD	17	8	9	
Histology				
Epithelioid	23	10	13	0.591 [†]
Biphasic	7	5	2	
Sarcomatoid	1	0	1	
Total	31	15	16	

* Fisher's exact test.

[†] Mann-Whitney *U* test.

Table 2. Univariate and multivariate analyses of clinical and pathologic variables for MPM patients

Variables	Hazard Ratio	95% confidence interval		P-Value
		Lower	Upper	
Univariate analysis				
Age > 64	2.415	0.75281	8.34558	0.137
High APN/CD13 expression	3.943	1.05241	19.11679	0.0416
Effect of neoadjuvant chemotherapy	1.496	0.51525	4.58769	0.4572
smoker	0.361	0.07528	2.56382	0.2679
Histology (non-epithelioid)	6.299	1.03425	48.1547	0.0461
Multivariate analysis				
High APN/CD13 expression	4.088	1.08272	19.88195	0.0375
Histology (non-epithelioid)	6.748	1.08035	52.63304	0.0416

Figure Legends

Figure 1.

Immunohistochemical staining for APN/CD13 in tumour tissues operatively obtained from patients with MPM.

A) Representative image of negative APN/CD13 staining. B) Representative image of positive APN/CD13 staining. C) Overall survival of patients with MPM in relation to the status of APN/CD13 expression in tumours. *P*-values were determined by the log-rank test.

Figure 2.

***In vivo* effect of MT95-4 on thoracic tumour volume and pleural effusion in an orthotopic implantation model of MPM.**

A) Expression levels of *APN/CD13* mRNA in human MPM cells analysed by quantitative real-time PCR. Data represent the mean \pm SEM of triplicate samples. ****P* < 0.001. B) APN/CD13 expression on the surface of human MPM cells analysed by flow cytometry. C–F) Evaluation of tumour weight and pleural effusion produced by MPM cells in SCID mice using (C, D) EHMES-10 or (E, F) MSTO-211H cells. EHMES-10 (3×10^6) or MSTO-211H (1×10^6) cells were inoculated into the thoracic cavity of SCID mice, followed by treatment with MT95-4 (0.3 mg/kg or 1 mg/kg) or an

isotype-matched control mAb intraperitoneal injected twice weekly. Thoracic tumour weight and pleural effusion were evaluated at 4 weeks (EHMES-10) or 3 weeks (MSTO-211H) after tumour cell inoculation. Each bar represents the mean value associated with seven mice per group. Data were analysed by one-way ANOVA with Dunnett's *post hoc* test. * $P < 0.05$ vs. control group.

NS, not significant.

Figure 3.

Effect of MT95-4 treatment on angiogenesis in thoracic tumours.

A–D) Mice transplanted with (A, B) EHMES-10 or (C, D) MSTO-211H cells were treated with MT95-4 (0.3 mg/kg or 1 mg/kg) or an isotype-matched control mAb by intraperitoneal injection twice weekly. The area of CD31-positive vessels in mice transplanted with (A) EHMES-10 or (C) MSTO-211H cells was calculated as described in the Methods ($n = 7$ /group). Data were analysed by one-way ANOVA with Dunnett's *post hoc* test. *** $P < 0.001$ vs. control group.

B, D) Immunohistochemical staining for CD31 in thoracic tumours derived from (B) EHMES-10 or (D) MSTO-211H cells. Scale bar: 100 μm .

NS, not significant.

Figure 4.

***In vivo* effect of MT95-4 treatment in combination with cisplatin in an orthotopic implantation model of MPM using EHMES-10 cells.**

A, B) Effect of MT95-4 treatment in combination with cisplatin on (A) tumour weight and (B) pleural effusion in SCID mice harbouring EHMES-10 cells. EHMES-10 (3×10^6) cells were inoculated into the thoracic cavity of SCID mice, followed by treatment with MT95-4 (0.3 mg/kg intraperitoneally twice weekly), cisplatin (3 mg/kg intraperitoneally once weekly), or MT95-4 (0.3 mg/kg) in combination with cisplatin (3 mg/kg). Thoracic tumour weight and pleural effusion were evaluated 4 weeks after tumour cell inoculation. Each bar represents the mean value associated with eight mice per group. Data were analysed by one-way ANOVA and Bonferroni's multiple-comparison test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. C) Effect of MT95-4 treatment in combination with cisplatin on survival of mice harbouring EHMES-10 cells. P was determined by the log-rank test. $*P < 0.05$, compared with the control group; $***P < 0.001$, compared with the control group; $^\dagger P < 0.01$, compared with the cisplatin monotherapy group.

NS, not significant.

Figure 5.

Effects of MT95-4 on the degrees of proliferation and apoptosis.

Proliferation was quantified in (A) EHMES-10 cell and (B) MSTO-211H cells was quantified after 36 hours in the presence of various concentrations of MT95-4. Data represent the mean values (SEM) of triplicate samples. (C, D) *In vivo* analyses of apoptosis in thoracic tumours produced by EHMES-10 cells. Mice harbouring EHMES-10 cells were treated with MT95-4 (0.3 mg/kg intraperitoneally twice weekly), cisplatin (3 mg/kg intraperitoneally once weekly), or MT95-4 (0.3 mg/kg) in combination with cisplatin (3 mg/kg). Four weeks after tumour cell inoculation, the mice were sacrificed. (C) Thoracic tumours were evaluated for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). The positive cells were calculated as described in the Methods ($n = 3/\text{group}$). (D) Quantitative immunohistochemical analysis. Data were analysed by one-way ANOVA with Dunnett's *post-hoc* test. $**P < 0.01$ vs. control group. NS, not significant.

Figure 6

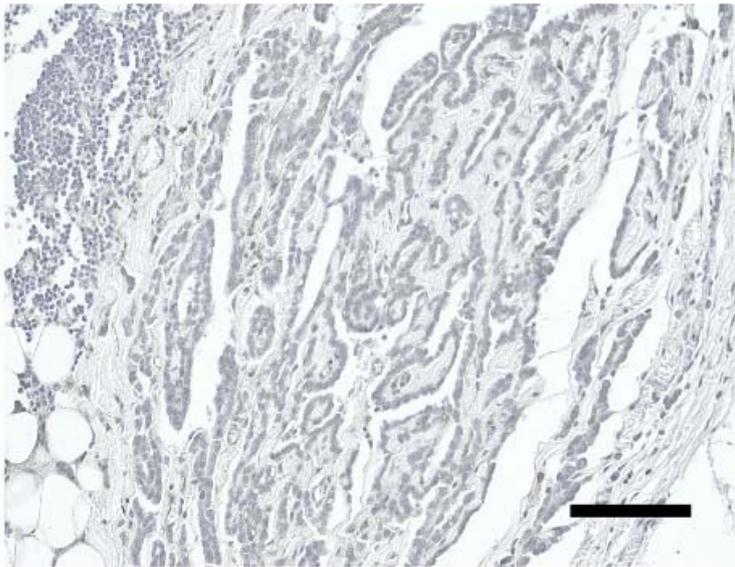
Antiangiogenic effect of MT95-4 depended on the inhibition of matrix degradation, but not the inhibition of angiogenic factors

A) Angiogenesis antibody array analysis of EHMES-10 cells treated with MT95-4 or human control IgG. (B, C) EHMES-10 cells (1×10^5) were seeded on 24-well plates and cultured with MT95-4 (40 $\mu\text{g}/\text{mL}$) or human control IgG for 24 hours. The densities

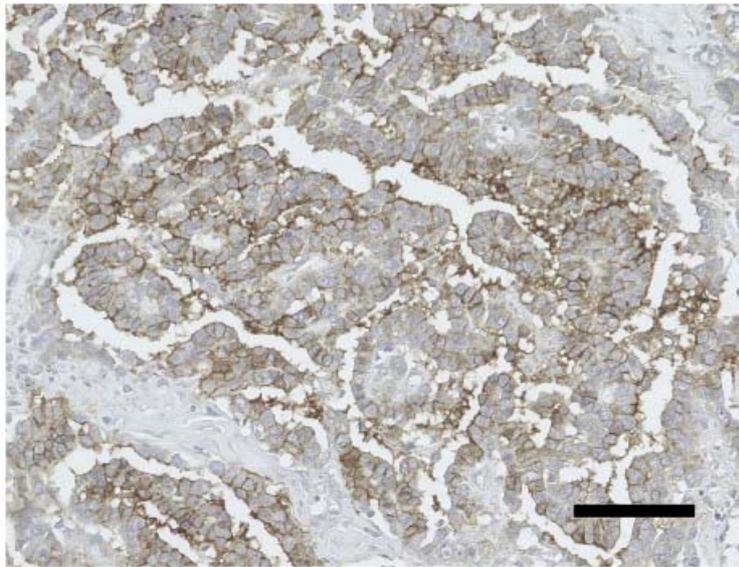
of individual spots were visualized using a chemiluminescence detection system. B) VEGF concentrations in culture media were measured by ELISA, and (C) *VEGF* mRNA levels were analysed by quantitative real-time PCR. (D, E) Effects of MT95-4 on the invasion of D) EHMES-10 cells and E) MSTO-211H cells in matrix degradation assay. (F) Effect of recombinant human APN/CD13 on the invasion of mouse primary pulmonary artery endothelial cells. The number of invasive cells was counted in three random microscopic fields per well. Data represent the mean \pm SEM of triplicate samples, and data were analysed by Student's *t* test or one-way ANOVA with Dunnett's *post-hoc* test. **P* < 0.05 vs. control group. NS, not significant.

Figure 1

A



B



C

