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CD146 and insulin-like growth factor 2 mRNA-binding protein 3 predict prognosis of asbestos-induced rat mesothelioma

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Malignant mesothelioma (MM), which is associated with asbestos exposure, is one of the most deadly tumors in humans. Early MM is concealed in the serosal cavities and lacks specific clinical symptoms. For better treatment, early detection and prognostic markers are necessary. Recently, CD146 and insulin-like growth factor 2 mRNA-binding protein 3 (IMP3) were reported as possible positive markers of MM to distinguish from reactive mesothelia in humans. However, their application on MM of different species and its impact on survival remain to be elucidated. To disclose the utility of these molecules as early detection and prognostic markers of MM, we injected chrysotile or crocidolite intraperitoneally to rats, thus obtaining 26 peritoneal MM and establishing 11 cell lines. We immunostained CD146 and IMP3 using paraffin-embedded tissues and cell blocks and found CD146 and IMP3 expression in 58% (15/26) and 65% (17/26) of MM, respectively, but not in reactive mesothelia. There was no significant difference in both immunostainings for overexpression among the three histological subtypes of MM and the expression of CD146 and IMP3 was proportionally associated. Furthermore, the overexpression of CD146 and/or IMP3 was proportionally correlated with shortened survival. These results suggest that CD146 and IMP3 are useful diagnostic and prognostic markers of MM. (*Cancer Sci* 2013; 104: 989–995)

Malignant mesothelioma (MM) is one of the most lethal tumors in humans. In Japan[1] and the US,[2] approximately 1000 and 3000 patients, respectively, are diagnosed annually with MM. The characteristics of MM include the following: the majority of cases are associated with repeated asbestos exposure;[1] once diagnosed, the prognosis is overwhelmingly poor, with an average survival of 7.7 months after diagnosis; and it takes 30–40 years after the initial asbestos exposure for MM to occur.[3,4] Currently, four hypotheses have been proposed for the pathogenesis of asbestos-induced MM, namely, the oxidative stress theory, the chromosome tangling theory, the adsorption theory and the chronic inflammation theory.[5]

Epidemiologically, there is concrete evidence that respiratory exposure to asbestos is associated with MM. Based on the epidemiological data, the risk of carcinogenesis in commercially used asbestos has been calculated as the ratio of 1:100:500 for chrysotile [$Mg_6Si_4O_{10}(OH)_8$], amosite [Fe^{2+}, $Mg]_7Si_8O_{22}(OH)_2$] and crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$], respectively.[6,7] Although asbestos has been banned in most developed countries, MM is expected to occur for decades because of the long latency period. The peak of MM incidence in Japan is predicted to occur in the year 2025, with a cumulative 100 000 deaths estimated to be related to this neoplasm in the next 40 years.[2]

The early detection and subsequent effective therapy might improve the prognosis of MM. However, a lack of specific symptoms might conceal the existence of MM in the serosal cavities, enabling its rapid and progressive expansion to occur undetected. In addition to difficulties in including MM as a differential diagnosis, the pathological diagnosis of MM is sometimes difficult. Morphologically and immunohistochemically, MM presents in a heterogeneous manner. Furthermore, non-neoplastic mesothelia might demonstrate atypical features, mimicking invasion below the lining epithelium.[8,9] These difficulties need to be overcome to allow the early detection and subsequent treatment required to improve the prognosis of MM.

Recently, CD146, also known as melanoma cell adhesion molecule (MCAM or MUC18), was reported to be a useful positive marker of MM of both epithelioid and sarcomatoid types,[10] distinguishing them from reactive mesothelia.[11] CD146 is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. The overexpression of CD146 is associated with poor prognosis in several cancers, including melanoma, prostate cancer and triple-negative breast cancer, suggesting that it plays biological roles in cancer progression *in vivo*.[12,13] In addition to cancer, CD146 was detected in endothelial cells and mesenchymal stem cells, also suggesting the pivotal role of CD146 in angiogenesis, cardiovascular diseases and implantation.[12] Recently, it was shown that translation of CD146 mRNA is regulated by insulin-like growth factor 2 mRNA-binding protein 3 (IMP3).[14] The IMP are oncofetal proteins that have been implicated in mRNA turnover and translational control and have been found to be increased in some forms of invadopodia.[14] IMP3 has also been reported to be a poor prognostic marker of various neoplasms[15,16] and a positive marker for the detection of MM.[17]

Animal models can provide valuable information for understanding the asbestos-induced carcinogenic process in mesothelial cells.[18] In the present study, we describe the utility of CD146 and IMP3 as positive diagnostic markers of asbestos-induced MM in a rat model. For the first time, we report that in rats the overexpression of CD146 and IMP3 is associated with the poor prognosis of MM.

Materials and Methods

Materials. The primary antibodies used for immunohistochemistry are summarized in Table S1. Histofine Simple Stain rat Max-PO (multi) was obtained from Nichirei (Tokyo, Japan). Anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody

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(#7074) and anti-mouse IgG HRP-linked antibody (#7076) were from Cell Signaling (Danvers, MA, USA). Liquid DAB+ (K3468), BCIP/NBT substrate system (K0598) and anti-rabbit immunoglobulins/HRP antibody (P0448) were from DAKO (Carpinteria, CA, USA). Anti-rabbit immunoglobulins/alkaline phosphatase antibody (A2306) was from Sigma-Aldrich (St Louis, MO, USA). TACS-Blue label and nuclear fast red were from Trevigen (Gaithersburg, MD, USA). Immunosaver was from NisshinEM (Tokyo, Japan). Protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN, USA). Immobilon-P transfer membrane was from Millipore (Billerica, MA, USA). Can Get Signal immunoreaction enhancer solution was from Toyobo (Osaka, Japan). Blocking reagent N101 was from Nichiyu Life Sciences (Tokyo, Japan). ChemilumiOne Super and Protein Assay Bicinchoninate kit were from Nakalai Tesque (Kyoto, Japan). All other chemicals were of the highest quality available from Wako (Osaka, Japan).

Animal experiments and cell lines. The Animal Care Committee of Nagoya University Graduate School of Medicine approved this experiment. The care and handling of animals were in accordance with the National Institutes of Health Guidelines. During the experiment, the rats were housed in a temperature-controlled environment kept at 23°C with alternating 12-h light and 12-h dark cycles and were allowed free access to distilled water and basal chow diet (Funabashi F-1, Chiba, Japan). The experiment was carried out according to a previously described protocol of mesotheliomagenesis[18] with a minor modification. In brief, F_1 hybrid rats were bred in-house by crossing female Fischer344 and male Brown-Norway (BN/CIL) strains (Charles River, Yokohama, Japan). These F_1 hybrid rats received two intraperitoneal injections of 1 mL of asbestos fiber (5 mg/mL; chrysotile and crocidolite, Union for International Cancer Control grade) when they were 6 and 7 weeks old and were maintained under close observation for 600 days after asbestos administration. The rats were killed when abdominal distention due to ascites or marked weight loss was observed. A total of 26 rats (15 males and 11 females) were used for these studies (Table S2). After being killed, the organs (peritoneum, heart, lungs, liver, spleen, kidneys and testes/ovaries) were immediately removed. A portion of each tissue was fixed in 10% neutral formalin for histological examination while the rest of the tissue was frozen and preserved at −80°C for subsequent western blot analysis. The induced tumors were diagnosed as MM using hematoxylin and eosin staining and immunohistochemistry and the tumors were classified as either epithelioid mesothelioma (EM), biphasic mesothelioma (BM) or sarcomatoid mesothelioma (SM), as previously described.[18] Tumors from male rats were classified as SM in seven cases, BM in five cases and EM in three cases. Tumors from female rats were classified as SM in six cases, BM in four cases and EM in one case. When possible, mesothelioma cell lines were established from ascites, as previously described.[19] Some mesothelioma cell lines previously established in another project were also used.[18] Normal mesothelial cells on the surface of intraperitoneal organs were carefully collected, as previously described.[20]

Immunohistochemical analyses. Briefly, 4-μm sections were dewaxed in xylene and ethanol. The slides were subjected to high-temperature antigen retrieval. The methods of antigen retrieval and the dilution of each antibody used are summarized in Table S1. After antigen retrieval, the slides were dipped in methanol containing H_2O_2 [0.3% (v/v)] for 30 min to quench endogenous peroxidase activities. After washing with 10 mM phosphate-buffered saline (PBS) at pH 7.4, the slides were incubated with primary antibodies with microwave irradiation. The slides were then washed with PBS three times for 5 min each time[21] and SimpleStain Rat multi was applied to the slides. After washing with PBS three times, the protein expression was visualized as brown precipitates with liquid DAB+. After visualization, we performed Perls' iron staining, which changes brown hemosiderin deposits to blue, and nuclear counterstaining was performed with hematoxylin. We classified the signal intensity of CD146 as follows: 3+, strong complete membrane staining in more than 10% of the tumor cells; 2+, weak to moderate complete membrane staining in more than 10% of the tumor cells; 1+, faint/barely perceptible membrane staining in more than 10% of the tumor cells; or 0, no staining was observed or membrane staining was observed in <10% of the tumor cells. This classification was based on the scoring system previously reported for HER2/*neu* (c-*erb*B-2), which encodes a transmembrane glycoprotein.[22] As CD146 was detected in endothelia in tumor-associated microvessels, we used endothelia as an internal positive control. The IMP3 expression was classified as follows: 3+, positive staining was observed in more than 25% of the tumor cells; 2+, positive staining was observed in 10–25% of the tumor cells; 1+, positive staining was observed in 5–10% of the tumor cells; or 0, positive staining was observed in <5% of the tumor cells. The expression of hypoxia-inducible factor (HIF)-1α was evaluated according to the Allred scoring system from 0 to 8[23] and the intensity score was classified as follows: 3+, strong; 2+, intermediate; 1+, weak; or 0, negative. The proportion score was classified as follows: 5+, more than 67%; 4+, from 34 to 66%; 3+, from 10 to 33%; 2+, from 1 to 10%; 1+, <1%; or 0, negative. The total score was calculated by summing the intensity score (3–0) and the proportion score (5–0). To evaluate the correlation between HIF-1α expression and survival, we categorized 26 MM cases as follows: high-expression group, total score 6–8; moderate-expression group, total score 3–5; and low-expression group, total score 0 and 2. The expression of cyclin D1 was evaluated as positive or negative. The positivity of Ki-67 in tumor cells was counted and validated as the Ki-67 labeling index. For the double immunostaining of alpha-smooth muscle actin (α-SMA), desmin and AE1/3 (pan-cytokeratins) antibodies, which were raised in mice, we used DAB+ for color visualization. After high-temperature treatment for simultaneous antigen retrieval and inactivation of immune-complexes, the sections were incubated with CD146, followed by treatment with anti-rabbit immunoglobulins/HRP. The localization of CD146 was visualized using the TACS-Blue label. For the double immunostaining of mesothelin and podoplanin antibodies, which were raised in rabbits, we applied anti-rabbit immunoglobulins/alkaline phosphatase antibody after antigen retrieval and incubation with the primary antibody. We used BCIP/NBT substrate to develop the blue color for localization. After high temperature treatment, the sections were incubated with CD146 antibody, followed by visualization with DAB+.

Western blot analysis. This was performed using a standard procedure as previously described.[21] Mesothelioma cell lines and rat mesothelial tissue were lysed in radioimmunoprecipitation buffer (10 mM phosphate buffer pH 7.2, 2 mM EDTA, 150 mM NaCl, 0.1% SDS [w/v], 1% sodium deoxycholate [w/v], 1% Triton X-100 [v/v] and 50 mM sodium fluoride with protease inhibitor cocktail). The membranes were incubated with the primary antibodies for CD146 (1:2000), IMP3 (1:500) or β-actin (1:2000). The signal intensities of the bands were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis. For survival analyses, Kaplan–Meier curves were developed and log-rank tests were performed. The correlation between CD146 and IMP3 expression was analyzed using Fisher's exact test. The results are representative of three independent experiments and the error bars represent the standard error of means (SEM). The signal intensities were analyzed using unpaired *t*-test and the difference was considered to be significance when $P < 0.05$. These analyses were performed using the GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA).

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Results

Evaluation of CD146 expression in peritoneal MM using immunohistochemistry. In the 26 cases, we observed the peritoneal invasion of white nodular tumors of 1–5 mm diameter with massive bloody ascites (Table S2). To confirm the cellular origin of tumors, paraffin-embedded tissues were immunostained with the mesothelial markers, mesothelin, podoplanin, AE1/3 (pancytokeratin) and Wilms tumor 1 (WT1). The tumors were not always positive for all of the mesothelial markers, but some markers were positive in at least part of the tissue section. These immunohistochemical results are consistent with those of human MM, confirming that asbestos fibers can induce peritoneal MM. We show representative results of immunohistochemistry for both epithelioid and sarcomatoid mesotheliomas in Figure S1. In some of the MM sections, prominent membranous localization of CD146 was observed (Fig. 1a). Low-magnification images of immunostaining are also shown to visualize the overall distribution of positivity in the tissue (Fig. S2). The ratio of positivity was dependent on histological subtype. All EM cases (4/4) were positive, while 4/9 BM and 7/13 SM cases were positive (Table 1). While CD146 positivity was 100% in the EM cases, CD146 overexpression classified as 3+ or 2+ was observed in only 25% (1/4) of the EM cases. In the histologically high-grade subtypes (BM and SM), CD146 was positive in 50% of cases and its overexpression was observed in 23% (5/22) of cases. These results suggest that histological subtypes show an association with positivity but not with the overexpression of CD146.

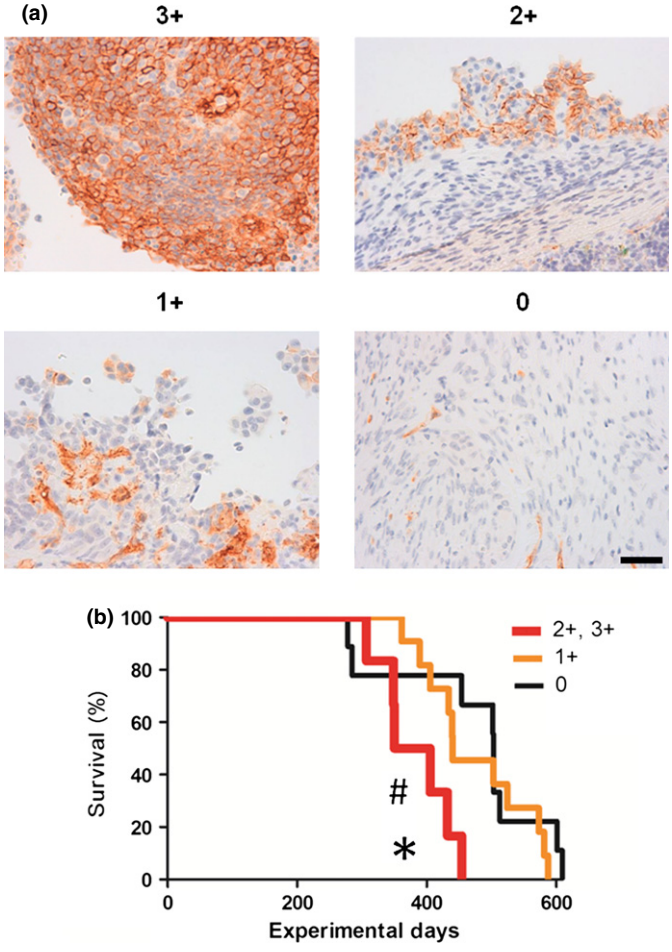

Fig. 1. Evaluation of CD146 expression in peritoneal malignant mesothelioma (MM) induced by asbestos in rats and its impact on survival. (a) CD146 expression was scored as 3+, 2+, 1+ or 0, as described in the materials and methods section (bar, 50 μm). A positive signal was observed in the endothelia. (b) Survival analyses showed that CD146 overexpression (2+, 3+) was associated with statistically significant shortened survival compared with either 1+ or 0 (#$P < 0.05$ *vs* 1+; *$P < 0.05$ *vs* 0). There was no statistically significant difference between 1+ and 0 ($P = 0.44$).

Occasionally, we observed non-tumorous CD146-positive cells proliferating beneath the normal mesothelial layer (Fig. 2). These CD146-positive cells extended to and eventually replaced the surface mesothelial layer. These CD146-positive cells were negative for two mesothelial markers, mesothelin and podoplanin. Focally, they co-immunostained for the myofibroblast markers desmin and α-SMA, indicating that CD146-positive cells share an immunophenotype with myofibroblasts (Fig. 2).

Evaluation of IMP3 expression in peritoneal MM using immunohistochemistry. The IMP3 expression was scored as 3+, 2+, 1+ or 0, as described in the materials and methods section (Fig. 3a). As summarized in Table 1, 65% (17/26) of cases were positive for IMP3. We analyzed the association between CD146 and IMP3 protein levels, as determined using immunohistochemistry. We observed that areas positive for IMP3 sometimes corresponded to those of CD146. However, this was not always the case. To analyze the correlation, we performed the Fisher's exact test and found that there was a significant association between the protein levels of CD146 and IMP3 ($P = 0.018$) (Table 2).

Survival analyses based on CD146 and IMP3 expression in peritoneal MM assessed using immunohistochemistry. Survival analysis using the log-rank test showed that overexpression of CD146 (2+, 3+) was correlated with shortened survival ($P < 0.05$) compared with either CD146 (1+) or CD146 (negative) cases (Fig. 1b). The IMP3 overexpression (2+, 3+) was also correlated with shortened survival compared with IMP3 (0, 1+) (*$P < 0.05$) (Fig. 3b). Survival analysis showed that the dual overexpression of CD146 and IMP3, corresponding to the $CD146^{high(2+,3+)}IMP3^{high(2+,3+)}$ phenotype, correlated with a statistically shortened survival time compared with cases with the $CD146^{low(0,1+)}IMP3^{low(0,1+)}$ phenotype (**$P < 0.01$) (Fig. 3c).

Evaluation of CD146 and IMP3 expression in peritoneal MM using western blot. CD146 was detected in the majority of mesothelioma cell lines that were established from ascites. There were differences in signal intensity between the lines (Fig. 4a). In 4/4 EM lines, the expression of CD146 was

Table 1. Immunohistochemical evaluation of CD146 and IMP3 expression

Histology subtype	CD146†				Positive ratio	IMP3‡				Positive ratio
	0	1+	2+	3+		0	1+	2+	3+	
EM	0	3	1	0	100% (4/4)	1	2	1	0	75.0% (3/4)
BM	5	2	1	1	44.4% (4/9)	4	2	2	1	55.6% (5/9)
SM	6	4	1	2	53.8% (7/13)	4	3	2	4	69.2% (9/13)

BM, biphasic mesothelioma; EM, epithelioid mesothelioma; IMP3, insulin-like growth factor 2 mRNA-binding protein 3; SM, sarcomatoid mesothelioma. †3+, Strong complete membrane staining in more than 10% of the tumor cells; 2+, weak to moderate complete membrane staining in more than 10% of the tumor cells; 1+, faint/barely perceptible membrane staining in more than 10% of the tumor cells; or 0, no staining was observed or membrane staining was observed in <10% of the tumor cells. ‡3+, Positive staining was observed in more than 25% of the tumor cells; 2+, positive staining was observed in 10–25% of the tumor cells; 1+, positive staining was observed in 5–10% of the tumor cells; or 0, positive staining was observed in <5% of the tumor cells.

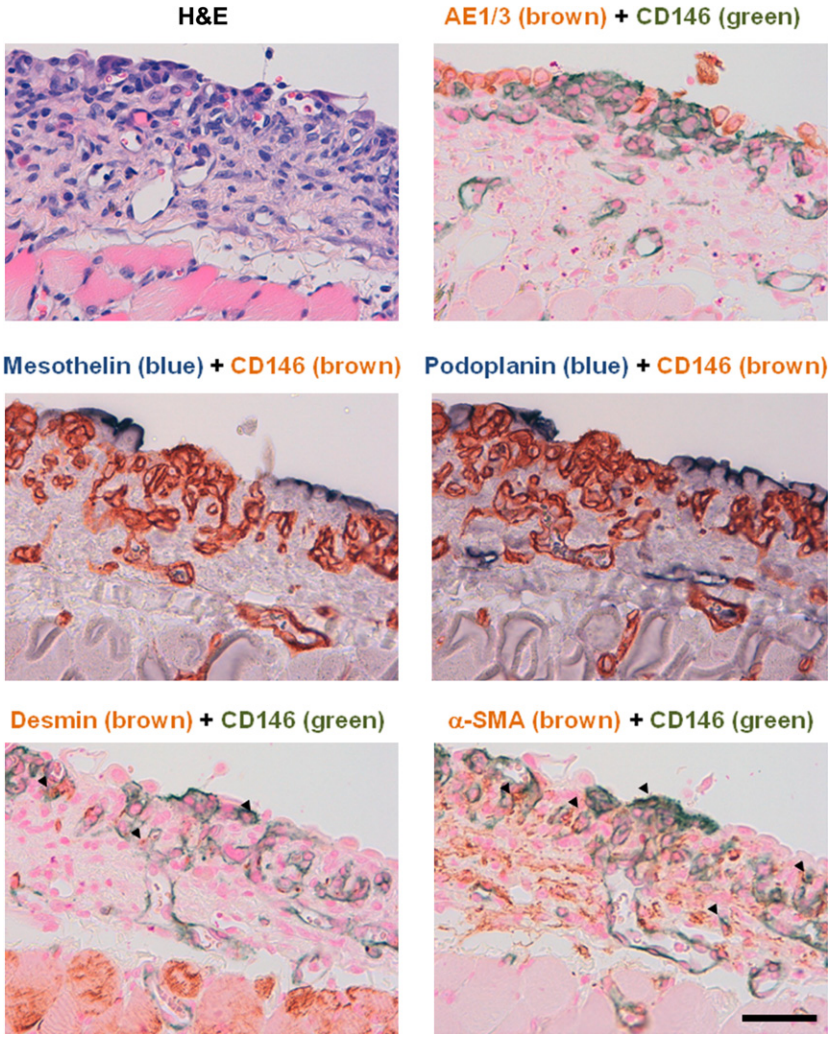

Fig. 2. Localization of CD146-positive cells during early carcinogenesis and their immunohistochemical phenotype. Representative results of immunohistochemical staining are shown. The CD146-positive cells proliferated to form a handshake-like luminal structure under the mesothelial layer. These CD146-positive cells extended to and replaced the superficial mesothelial layer. They were negative for mesothelin and podoplanin, which are mesothelial markers, and were focally positive for desmin and alpha-smooth muscle actin (α-SMA), which are myofibroblast markers (arrowheads; bar, 50 μm).

elevated. However, the overexpression of CD146 was low in SM and only occurred in 1/6 lines (Fig. 4b). The overexpression of IMP3 was also observed in EM (4/4 lines) and SM (2/6 lines) (Fig. 4c). Immunohistochemical staining demonstrated that the membranous signal of CD146 was in agreement with the western blot results (Fig. 4d).

Survival analyses based on cyclin D1, HIF-1α and Ki-67 expression in peritoneal MM assessed using immunohistochemistry. The stained specimens were evaluated as described in the materials and methods section. There was no significant difference between survival and the overexpression of HIF-1α, cyclin D1 or Ki-67 labeling as assessed using the log-rank test (Figs S3–S5). We observed the presence of Ki-67-positive lymphocytes in the red pulp of the spleen (Fig. S6a). To determine the effect of splenic lymphocytes on MM, we evaluated the association between Ki-67-positive lymphocytes and the Ki-67 labeling index of MM cells, as well as rat survival (Fig. S6b,c). There was no statistically significant association.

Discussion

Here we show for the first time that CD146 and IMP3 are expressed in peritoneal MM induced by asbestos in rats. Notably, their overexpression was correlated with a poor prognosis.

CD146, a transmembrane glycoprotein belonging to the immunoglobulin superfamily, functions as a calcium-independent adhesion molecule.[12] A recent human study involving the cytology of pleural effusions showed that CD146 is expressed in MM but not in reactive mesothelia, suggesting that CD146 might be a useful marker for differential diagnosis.[11]

We assessed CD146 expression in paraffin-embedded MM specimens and found that it was expressed in 100% of EM cases, 44% of BM cases and 58% of SM cases. To further confirm CD146 overexpression in MM, we evaluated CD146 protein expression in established MM cell lines and found that CD146 was reproducibly expressed in a variety of MM cell lines (Fig. 4a,b). The EM cell lines showed a high frequency of CD146 high immunopositivity (100%, 4/4), whereas the SM cell lines showed a low frequency of CD146 high immunopositivity (17%, 1/6). These results suggest that the histological subtype affects the protein levels of CD146. In comparison to the previous report in humans, which showed that 94% of MM cases were positive for CD146,[11] the present study in rats indicated that the CD146-positive ratio (58%, 15/26) was not as high. We believe that this difference is mainly due to the differences in histological subtype. In the former study, all cytology samples contained EM components (21 EM and 1 BM), whereas the present study contained 13 (50%) pure SM cases. Additionally, as the former study used alcohol as a fixative, the surface antigens might have been better preserved in the tissues. There were also differences in the histological complexity of tissues used in the two studies. As shown in Figure 1(a), CD146 immu-

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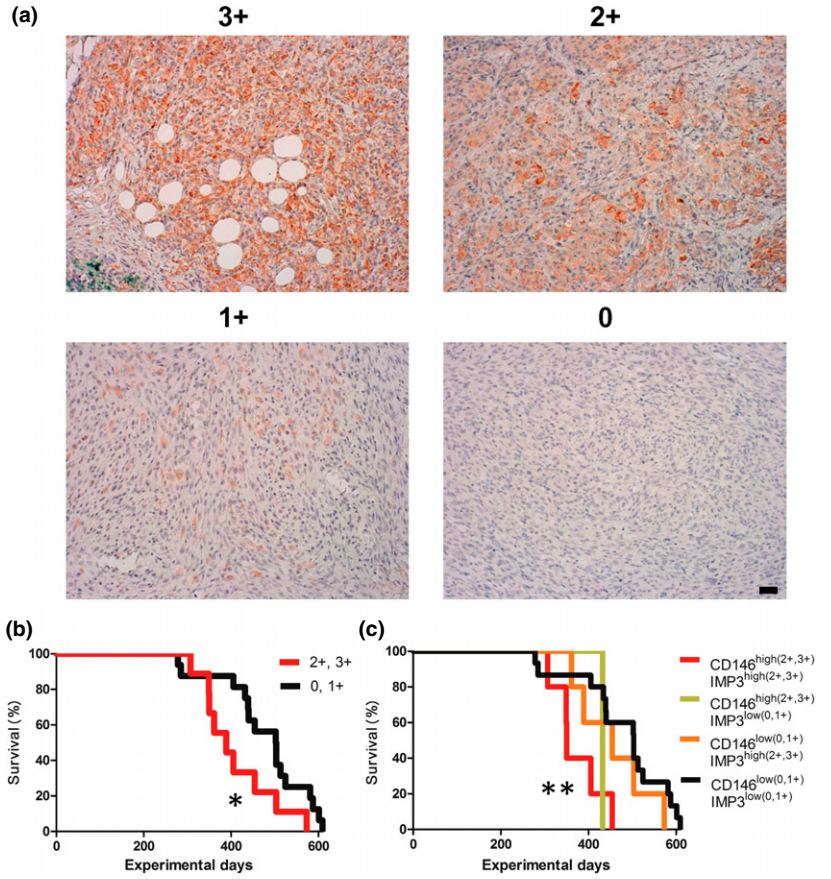

Fig. 3. Evaluation of insulin-like growth factor 2 mRNA-binding protein 3 (IMP3) expression in peritoneal malignant mesothelioma (MM) tissue sections and its impact on survival. (a) IMP3 was scored as 3+, 2+, 1+ or 0, as described in the material and methods section. Perls' iron staining was performed to show iron deposition (bar, 50 μm). (b) Survival analysis demonstrated that IMP3 overexpression (2+, 3+) was associated with statistically significant decreased survival compared with IMP3 (0, 1+) (*$P < 0.05$). (c) Survival analysis showed that the CD146$^{high(2+,3+)}$IMP3$^{high(2+,3+)}$ phenotype was associated with a statistically significant decrease in survival compared with the CD146$^{low(0,1+)}$IMP3$^{low(0,1+)}$ phenotype (**$P < 0.01$).

Table 2. Correlation of CD146 and IMP3 with immunohistochemical evaluation

	CD146†	
	3+, 2+	1+, 0
IMP3‡		
3+, 2+	5	5
1+, 0	1	15

Fisher's exact test for correlation of CD146 and IMP3, $P = 0.018$. IMP3, insulin-like growth factor 2 mRNA-binding protein 3. †3+, Strong complete membrane staining in more than 10% of the tumor cells; 2+, weak to moderate complete membrane staining in more than 10% of the tumor cells; 1+, faint/barely perceptible membrane staining in more than 10% of the tumor cells; or 0, no staining was observed or membrane staining was observed in <10% of the tumor cells. ‡3+, Positive staining was observed in more than 25% of the tumor cells; 2+, positive staining was observed in 10–25% of the tumor cells; 1+, positive staining was observed in 5–10% of the tumor cells; or 0, positive staining was observed in <5% of the tumor cells.

nopositivity was observed in the endothelia and pericytes,[24] thus it is difficult to distinguish whether the cells are stromal cells or MM cells. Here, we adopted a scoring system, which is similar to that used for HER2/*neu*, and a threshold of 10% was used to avoid inter-observer inconsistencies.[22] This strategy might help pathologists to standardize the validation of CD146-positive tumors.

Recently, CD146 was also recognized as a mesenchymal and stem cell marker for bone marrow-derived stem cells and amniochorionic membrane-derived cells, but not for adipose tissue.[25,26] CD146-positive non-tumorous cells, located beneath the normal mesothelial cells, were negative for mesothelial markers and focally positive for myofibroblast markers (Fig. 2). We hypothesize that the functional role of CD146-positive mesenchymal cells is to act as stomata of lymphatic vessels, which drain short and thin fibrous materials or small particles from the pleural and abdominal cavity. However, long and thin fibrous materials, including asbestos, are trapped and not cleared.[27] Once the asbestos fibers are stuck in the stoma, macrophages are activated and this is followed by persistent inflammation. Mesothelial cells, or presumptive mesothelial progenitor cells, are damaged and acquire mutations. This step is followed by the promotion phase of carcinogenesis. In the present study, it appears that CD146-positive myofibroblast-lineage mesenchymal cells emerged and proliferated, which is consistent with an emerging concept that myofibroblasts are a source of cells for the cancer stem cell niche.[28] In this microenvironment, CD146-positive myofibroblast-lineage mesenchymal cells presumably maintain the damaged mesothelial progenitor cells and promote their transformation into CD146-positive MM. Accordingly, in some foci, we observed the expansion and replacement of the mesothelial layer by CD146-positive cells, suggesting that the CD146-positive mesenchymal-lineage MM was induced (Fig. 2).

The IMP3, which has been reported to be a marker that differentiates MM from reactive mesothelia in humans,[17] was also a useful positive marker for MM in rats. The frequency of IMP3 immunopositivity was slightly higher than that of CD146 but CD146 seemed to mark MM cells more selectively than IMP3. It was previously reported that IMP3 regulated the translation of CD146 in HeLa cells.[14] In MM cases in which CD146 was overexpressed (+2 or +3), 83% (5/6) also showed IMP3 overexpression. Although the immunohistochemical staining did not fully demonstrate the colocalization of CD146 and IMP3, this pattern was clearly observed in cultured cell

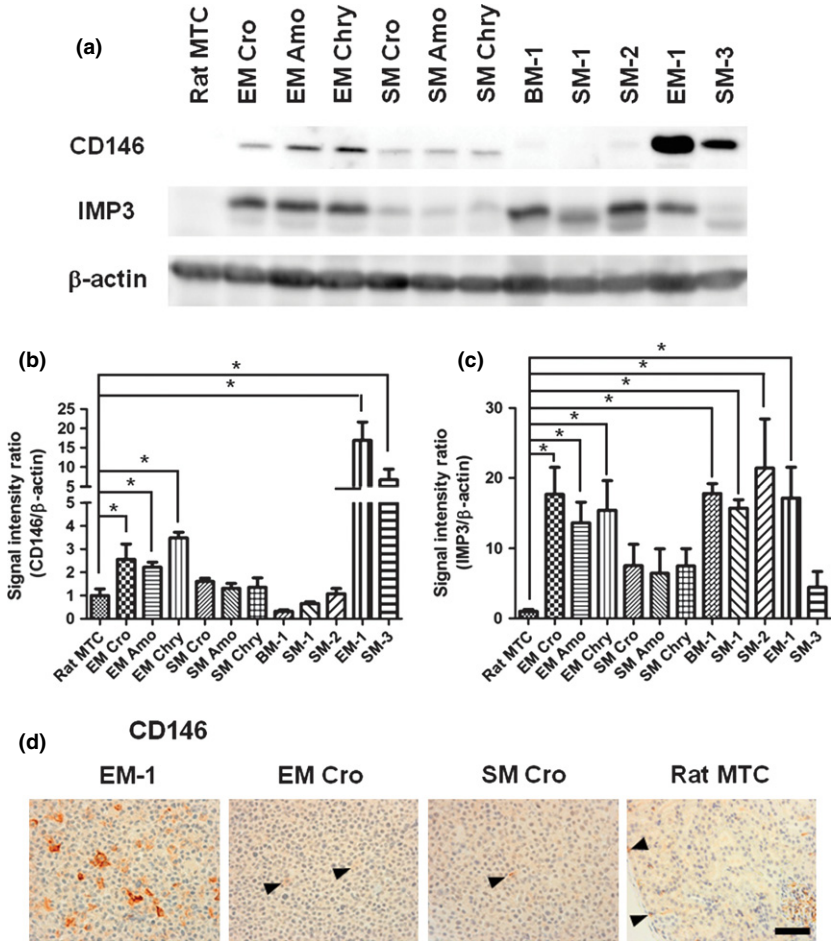

Fig. 4. Expression of CD146 and IMP3 in MM cell lines. Rat mesothelial cells (MTC) were used as the control mesothelial cells. They were scraped from the surface of peritoneal solid organs, as described in the materials and methods section. Epithelioid mesothelioma (EM) Cro, EM Amo and EM Chry are EM cell lines that were established via the intraperitoneal administration of crocidolite (Cro), amosite (Amo) and chrysotile (Chry), respectively, to rats. Sarcomatoid mesothelioma (SM) Cro, SM Amo and SM Chry are SM lines that were established via the intraperitoneal administration of crocidolite, amosite and chrysotile, respectively, to rats. BM-1 was established from case no. 26, whose histology was representative of biphasic mesothelioma. SM-1, SM-2, EM-1 and SM-3 were established from mesothelioma induced by chrysotile, amosite, crocidolite and amosite, respectively. (a) Three independent experiments were performed and the representative results of CD146, IMP3 and β-actin are shown. (b) The signal intensities of CD146 were analyzed using ImageJ software (*$P < 0.05$ *vs* rat MTC). (c) The signal intensities of IMP3 were analyzed using ImageJ software (*$P < 0.05$ *vs* rat MTC). (d) Representative results of CD146 immunohistochemical staining of paraffin-embedded cell blocks from cell lines. EM-1 demonstrated strong membranous staining. Some positive mesothelioma cells (arrowheads) were observed in EM Cro and a few positive mesothelioma cells (arrowheads) were observed in SM Cro. There were CD146-positive capillary vessels (arrowheads) beneath the thin capsule of the kidney and its glomerulus. Mesothelial cells were obtained from this rat kidney by scraping (bar, 50 μm).

lines (Fig. 4a–c; EM, 4/4 lines; SM, 0/1 line). While we have not shown the co-immunoprecipitation of IMP3 protein and CD146 mRNA, these results suggest that IMP3 might regulate the translation of CD146 also in rat MM.

The overexpression of CD146 or IMP3 has been associated with a poor prognosis in various cancers.[12,17] To elucidate the functional role of CD146 and IMP3 expression in MM *in vivo*, we analyzed the effect of their overexpression on survival. We found that CD146 and IMP3 overexpression was associated with significantly decreased survival (Figs 1b,3b). Notably, CD146 and IMP3 double-positive MM was most significantly associated with decreased survival, more so than any of the other immunophenotypes (Fig. 3c).

To evaluate the value of other candidate markers as prognostic markers for rat MM, we also analyzed HIF-1α,[29] cyclin D1[30] and Ki-67[31] expression using immunohistochemistry. Although each high-expression group showed decreased survival, none of the differences were statistically significant (Figs S3,S4,S5). These results suggest that CD146 and IMP3 are superior prognostic markers.

In the present study, we observed the presence of Ki-67-positive lymphocytes in the red pulp of the spleen, suggesting the involvement of the immune system in MM progression (Fig. S6a). Carcinogenic fibrous materials cause increased phagocytosis, leading to the secretion of inflammatory cytokines IL-1β and IL-6 by macrophages.[5,27] These inflammatory cytokines might modulate the responses of macrophages from inhibition to stimulation, promoting the tumor microenvironment.[32] A dysfunctional immune system might have enabled MM cells to escape from the fine immunological defense systems and expand into lethal tumors. To assess the effect of splenic lymphocytes on MM, we evaluated the association between Ki-67-positive lymphocytes and the Ki-67 labeling index of MM cells, as well as rat survival (Fig. S6b,c). These associations were not statistically significant in our model.

In conclusion, we observed for the first time that the overexpression of CD146 and IMP3 was associated with decreased survival in asbestos-induced peritoneal MM in rats. These results provide evidence that CD146 and IMP3 are good diagnostic and prognostic markers in MM and suggest that this rat model is ideal for studying preventive and therapeutic strategies for this currently fatal neoplasm.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunohistochemical staining of mesothelial markers.

Fig. S2. Immunohistochemical staining of CD146 in low magnification.

Fig. S3. Evaluation of HIF-1α expression in tissue sections of peritoneal malignant mesothelioma and its impact on survival.

Fig. S4. Evaluation of cyclin D1 expression in tissue sections of peritoneal malignant mesothelioma and its impact on survival.

Fig. S5. Evaluation of Ki-67 expression in tissue sections of peritoneal malignant mesothelioma and its impact on survival.

Fig. S6. Evaluation of Ki-67 expression in lymphocytes present in the splenic red pulp and its impact on survival.

Table S1. Antibodies used in the present study.

Table S2. Treatment, life span and histological subtype of rats used in the present study.