

Overexpression of CD26/DPPIV in mesothelioma tissue and mesothelioma cell lines

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Abstract. Mesothelioma, a highly aggressive cancer with poor prognosis and refractory to currently available therapies show increasing trends of its incidence in Japan and other developing countries. Although surgery is a gold standard for patients with early mesothelioma, most patients with advanced disease are not suitable for surgical resection and have option of palliative chemotherapy alone. One of the new treatment strategies for mesothelioma, the humanized anti-CD26 monoclonal antibody therapy is under development. CD26, a 110-kDa transmembrane glycoprotein with known dipeptidyl peptidase IV activity, plays a role in tumor development and its expression was reported in various human malignancies. This study determined the preliminary selection criteria for humanized monoclonal anti-CD26 antibody therapy. Eighty-one epithelioid (49 differentiated and 32 less differentiated), 34 sarcomatoid, 19 biphasic mesothelioma and 8 mesothelioma cell lines were immunohistochemically examined using 8 different commercially available anti-CD26 antibodies for membranous and cytoplasmic expression. The cytoplasmic expression of CD26 was observed in all histological types of mesothelioma, while the membranous expression of CD26 was found in 88% of differentiated and 69% of less differentiated epithelioid mesothelioma, and none of sarcomatoid mesothelioma with anti-CD26 antibodies with rabbit polyclonal anti-DPP4 antibody and similar results were also obtained with goat polyclonal anti-DPP4/CD26 antibody. These antibodies absorbed with soluble human CD26 proteins do not show CD26 expression in mesothelioma tissue, suggesting these two antibodies localize true CD26 protein. Seven mesothelioma cell lines, including sarcomatoid types, also showed membranous expression of CD26 in cellblock preparation.

CD26 vector transfection to CD26-negative MSTO-211H cells showed membranous expression of CD26 by flow cytometry, but not in tumor developed in NOD/SCID mice with inoculation of CD26 vector transfected MSTO-211H cells. We found that both rabbit and goat polyclonal antibodies are suitable for immunohistochemical evaluation of membranous expression of CD26 in mesothelioma.

Introduction

Mesothelioma, primarily associated with exposure to asbestos, causes about 2,500 deaths per year in the United States alone (1). Although the incidence of mesothelioma has been reported as declining in the USA and Canada, a recent study still show a slight increase in death due to mesothelioma in USA (2) and its incidence is rising in Europe, Japan and other developing countries, causing more deaths. The data obtained from the 'Vital Statistics' published by Ministry of Health, Labor and Welfare, Japan indicated that there is an increasing trend in the death due to mesothelioma, from 500 cases in 1995 to 710 cases in 2000, and 911 cases in 2005 (3). It is a highly aggressive cancer with poor prognosis and refractory to currently available therapies (4,5). The conventional modalities in management of mesothelioma by either surgery or radiotherapy and chemotherapy are not very effective, though some of the recent clinical trials of trimodality therapy with neoadjuvant chemotherapy followed by extra-pleural pneumonectomy and radiotherapy have shown few months survival advantage over conventional therapy (6-8). In population-based studies, survival times ranged from 5 months to 13.2 months (9). Although surgery is an option for patients with early-stage mesothelioma, most patients present with advanced locally invasive disease, not amenable to surgical resection. For these patients, the current best treatment is palliative combination chemotherapy with cisplatin and pemetrexed. While this treatment regimen relieves symptoms and has been shown to confer a modest survival benefit (10), median survival is still only 9-12 months from diagnosis.

Therefore, the new treatment strategies for management of mesothelioma are an urgent need in the coming years. Various therapeutic monoclonal antibodies have emerged as new treatment modality in the treatment of cancers. Trastuzumab

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Table I. Source of antibodies.

Source/manufacture	Catalogue no.	Host/clone	Primary choice of application
Santa Cruz	SC-9153	Rabbit/polyclonal	WB, IP, IF, ELISA
MBL International	DO68-1	Mouse (Clone 44-4)	FC, IHC
Abcam	Ab61825	Rabbit/polyclonal	IHC-Paraffin
Lifespan Biosciences (MBL International)	LS-A8060	Rabbit/polyclonal	IHC-Paraffin
Acris Antibodies GmbH	AP07044PU-N	Rabbit/polyclonal	IHC-Paraffin
Imgenex	IMG72095	Rabbit/polyclonal	IHC-Paraffin
Novus Biological	NB100-59021	Rabbit/polyclonal	IHC-Paraffin
R&D Systems	AF1180	Goat/polyclonal	WB, ELISA, IHC

WB, Western blotting; IP, immunoprecipitation; IF, immunofluorescence; ELISA, enzyme linked immunosorbent assay; FC, flow cytometry; IHC, immunohistochemistry.

targeting ErbB2 in breast cancer, cetuximab targeting EGFR or bevacizumab targeting VEGF in colorectal or lung cancers have already entered into standard clinical practice (reviewed in ref. 11). Humanized anti-CD26 monoclonal antibody having inhibitory effect on malignant mesothelioma cell growth *in vitro* and *in vivo* experiments has been reported (12), one of the a promising new developments for the antibody-based treatment of mesothelioma (12,13).

CD26 is a widely distributed 110-kDa transmembrane glycoprotein with known dipeptidyl peptidase IV activity in its extracellular domain (14,15). It is discovered as a lymphocyte cell surface antigen. It increases during T-cell activation as an essential role in the immune regulation and functions as a regulator of chemokine function. It has been reported to be expressed in various other tissues, placenta, kidney, lung, brain, liver, endothelium, and intestines (16-19). CD26 also plays a role in tumor development and its expression was reported in various human malignancies, mainly cancers of thyroid, breast, prostate, and ovary (13,20). We immunohistochemically evaluated the expression of CD26 in mesothelioma in determining the proper selection criteria of the mesothelioma patients for humanized monoclonal anti-CD26 antibody treatment.

Materials and methods

Tissue samples and mesothelioma cell lines. The formalin-fixed paraffin-embedded (FFPE) tissue materials from mesothelioma cases were obtained from the tissue archives of the Department of Pathology, Hiroshima University. They were derived from patients who had undergone surgical resection or autopsy. The clinicoradiological data were retrieved from the hospital records. The microscopic slides were reviewed and reclassified using the current histological classification of tumors of lung and pleura, World Health Organization 2004. Epithelioid mesothelioma was further sub-divided into two subtypes, i.e., 'differentiated' type (EM-D) and 'less-differentiated' type (EM-LD) based on the morphology of 'papillo-tubular structures' as an indicator of differentiation. Thus, EM-D were epithelioid mesothelioma showing papillo-tubular pattern, micropapillary pattern, and/or microcystic pattern and EM-LD showing solid nest, trabecular pattern,

signet-ring cell-like appearance and/or single cell infiltration pattern. One representative tissue block was used for immunohistochemical confirmation of mesothelioma using positive and negative antibody panels mentioned in our previous publications (21-25). This study consisted of 81 cases of epithelioid mesothelioma (EM) (61%), 34 cases of sarcomatoid mesothelioma (SM) (25%) and 19 cases of biphasic mesothelioma (BM) (14%). The mean age of patients was 61.9 years (23-87 years), and male/female ratio was 6:1. Forty-nine cases of epithelioid mesothelioma were differentiated type (EM-D) showing histological patterns of tubulopapillary, microcystic, or myxoid growth and 32 cases were less differentiated type (EM-LD) showing histological patterns of solid-sheet with polygonal, pleomorphic or bizarre giant cells. The study was carried out in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Six epithelial type (epithelioid) and 2 fibroblastic type (sarcomatoid) mesothelioma cell lines were purchased either from Riken, Tokyo, Japan (ACC-MESO1, ACC-MESO4, HMMME) or American Type Culture Collection, Manassas, VA, USA (NCI-H226, NCI-H2452, MSTO-211H, NCI-H28, NCI-H2052). All mesothelioma cell lines were maintained in RPMI-1640 media with 10% fetal bovine serum, and 1% kanamycin, and 1% fungizone (all purchased from Gibco) at 37°C with 5% CO₂.

Each of mesothelioma cell lines was cultured for 6-10 days on laminin-coated wells of Lab-Tek II chamber slide system (Thermo Fisher Scientific, Tokyo, Japan) and 100 mm CellBind culture dish (Corning Life Sciences, Corning, NY). Cells in chamber slides were directly fixed with 10% buffered formalin for 10 min. Cells in culture dish were subjected to cell-block preparation using glucomannan (HoldGel 110, EBIS1 cell-block construction kit, Asia Kizai, Tokyo, Japan) according to the manufacturer's protocol.

CD 26 immunohistochemistry. CD 26 immunohistochemistry was performed in 3-µm-tissue sections prepared from formalin-fixed paraffin-embedded tissue blocks in APS-coated slides. Sections were deparaffinized by four changes of xylene, and rehydrated with graded series of ethanol. Endogenous peroxidase was inactivated by treatment with 0.3% H₂O₂ in PBS for 30 min. The tissue sections were then incubated

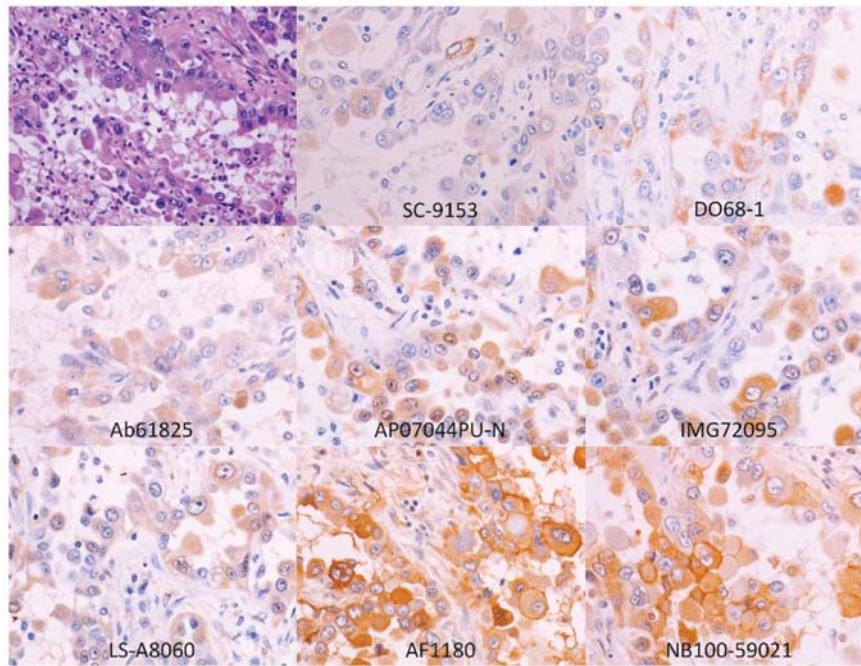


Figure 1. CD26 expression in epithelioid mesothelioma with various antibodies. Cytoplasmic expression of CD26 is observed with all the 8 anti-CD26/DPP4 antibodies studied. The membranous expression of CD26 is detected with only CD26-NB100/59021 and CD26-AF1180 antibodies.

with commercially available anti-CD26/DPPIV antibodies purchased from various sources (Table I) at 4°C overnight in a humidified chamber. The reaction was visualized using the Histofine Simple Stain kits (Nichirei Biosciences, Tokyo, Japan) and diaminobenzidine (Dojindo Laboratories, Tokyo, Japan) as the chromogen. The tissue sections were counterstained for nucleus with Mayer's haematoxylin. Similar immunohistochemical procedure was carried out with the omission of the primary antibody as a negative control. CD26 immunohistochemical procedure was also performed in mesothelioma cells on chamber slides and sections prepared from cell line blocks.

The membranous and cytoplasmic expression of CD26 was semi-quantitatively analyzed. The scoring system was based mainly on membranous staining: 0, no membranous staining of tumor cells; 1+, membrane staining in up to 25% of tumor cells; +2, membrane staining observed in 26-50% of tumor cells; +3, membrane staining in >50% of tumor cells.

Absorption study using soluble human CD26 proteins. Absorption study by soluble CD26 antigen was conducted to validate whether signal by immunohistochemistry using rabbit anti-human DPP4 polyclonal antibody (NB100-59021, Novus Biologicals, Littleton, CO, USA, hereafter we call as CD26-NB100/59021) or goat anti-human DPPIV/CD26 polyclonal antibody (AF1180, R&D Systems Inc., Minneapolis, MN, USA, hereafter we call as CD26-AF1180) corresponds to true CD26 localization or not. Two μg of rabbit anti-human CD26-NB100/59021 or goat anti-human polyclonal antibody (CD26-AF1180) was incubated with 5 μg of recombinant soluble human CD26 proteins (produced by CHO cells, provided by Dr Kei Ohnuma, Tokyo University) overnight at 4°C. The solution was then centrifuged at 5,000 \times g for 10 min and the pellet was discarded. The immunohistochemistry

using this absorbed primary antibody as primary antibody was carried out as described above.

CD26 transfection into CD26-negative MSTO-211H mesothelioma cells. MSTO-211H cells, which do not express CD26, were cultured as described above. MSTO-211H cells adhered to culture dish were transfected with a full-length CD26 subcloned retroviral shuttle plasmid pLNCX2 vector (Clontech Inc., Mountain View, CA, USA) using the Lipofectin reagent (Invitrogen). Fresh media with 10% FBS was replenished 18 h after transfection and cells were harvested after 48 h.

The cells were stained with anti-human CD26 monoclonal antibody (BA5, Phycoerythrin-conjugated, diluted 1:100, Dako) for analysis with a flow cytometer (EPICS XL-MCL, Beckman Coulter, Hialeah, USA). Normal mouse IgG2a (Sigma-Aldrich) was used as a control.

Inoculation of MSTO-211H cells into NOD/SCID mice. All *in vivo* studies were approved by the Animal Care and Use Committee of Keio University. NOD/SCID mice were bred and maintained in Department of Pathology, Keio University. The human CD26 vector transfected-MSTO-211H cells were inoculated subcutaneously into the left flank of 6-week old female NOD/SCID mice. The resected subcutaneous tumor was fixed with 10% formalin and embedded in paraffin for immunohistochemical study.

Results

CD26 expression in mesothelioma tissue. The immunohistochemical staining of CD26 expression was carried out with 8 different commercially available antibodies against CD26/DPP4. Using any of these antibodies, cytoplasmic expression of CD26 (cCD26) was observed in all mesothelioma cases (Fig. 1).

Table II. CD26 immunohistochemical score in mesothelioma.^a

	CD26-NB100/59021 expression				CD26-AF1180 expression			
	Score 0	Score 1	Score 2	Score 3	Score 0	Score 1	Score 2	Score 3
EM-D								
Cytoplasmic	0	3	12	34	0	2	12	35
Membranous	6	12	16	15	6	16	19	8
EM-LD								
Cytoplasmic	0	2	11	19	0	0	15	17
Membranous	10	7	11	4	10	7	12	3
BM-epithelioid component								
Cytoplasmic	0	1	8	9	0	1	6	11
Membranous	1	4	8	5	4	6	6	2
BM-sarcomatoid component								
Cytoplasmic	0	3	15	0	0	4	13	1
Membranous	18	0	0	0	18	0	0	0
Sarcomatoid mesothelioma								
Cytoplasmic	0	9	21	5	0	12	7	6
Membranous	35	0	0	0	35	0	0	0

^aDetailed data available online (<http://home.hiroshima-u.ac.jp/byouri2/JP/Supple.html>). EM-D, epithelioid mesothelioma-differentiated type; EM-LD, epithelioid mesothelioma-less differentiated type; BM, biphasic mesothelioma.

Table III. Comparison of CD26 immunohistochemical score of CD26 expression with two antibodies.

		NB100-59021			
		0	1+	2+	3+
AF1180	0	13	5	2	0
	1+	4	14	10	1
	2+	0	4	21	12
	3+	0	0	2	11

p-value <0.0001.

Table IV. Immunohistochemical score of membranous CD26 expression.

	Total	Immunohistochemical score (%)			
		0	1+	2+	3+
EM-D	49	6 (12.2)	12 (24.5)	16 (32.7)	15 (30.6)
EM-LD	32	10 (31.2)	7 (21.9)	11 (34.4)	4 (12.5)
BM (E)	18	1 (5.5)	4 (22.2)	8 (44.5)	5 (27.8)

EM-D, epithelioid mesothelioma differentiated type; EM-LD, epithelioid mesothelioma-less differentiated type. BM (E), epithelioid component of biphasic mesothelioma.

However, the membranous expression of CD26 (mCD26) could not be detected with SC-9153, DO-68, Ab61825, LS-A8060, AP01046PU-N, and IMG72095 antibodies. The membranous expression of CD26 in mesothelioma was only detected using either CD26-NB100/59021 or CD26-AF1180 antibodies.

CD26 expression was found in almost all cases of mesothelioma with membranous and/or cytoplasmic pattern using CD26-NB100/59021 or CD26-AF1180 antibody (Table II). The membranous expression patterns between these two antibodies were very similar (Fig. 1 and Table III). Using CD26-NB100/59021 antibody, the membranous expression of CD26 was found in 65 of 81 (80%) epithelioid mesotheliomas (Fig. 2, Table II), and 17 of 18 (89%) epithelioid component of biphasic mesothelioma. The membranous expression of CD26 was not found in sarcomatoid mesothelioma or sarcomatoid

component of biphasic mesothelioma (Fig. 2, Table II). Fifteen of 49 cases (31%) of EM-D showed mCD26 expression in >50% of tumor cells, 16 cases (33%) in 26-50% of tumor cells, and only 12 cases (25%) showed <25% of tumor cells (Table IV). Four of 32 cases (12%) of EM-LD showed mCD26 expression in >50% of tumor cells, 11 cases (34%) in 26-50% of tumor cells, and only 7 cases (22%) showed <25% of tumor cells (Table IV). Five of 18 cases (27%) of epithelioid component of BM showed mCD26 expression in >50% of tumor cells, 8 cases (44%) in 26-50% of tumor cells, and only 4 cases (22%) showed <25% of tumor cells (Table IV).

CD26 expression in mesothelioma cell lines. Seven out of 8 mesothelioma cell lines showed CD26 expression. MSTO-211H mesothelioma cell line had no expression of CD26. The

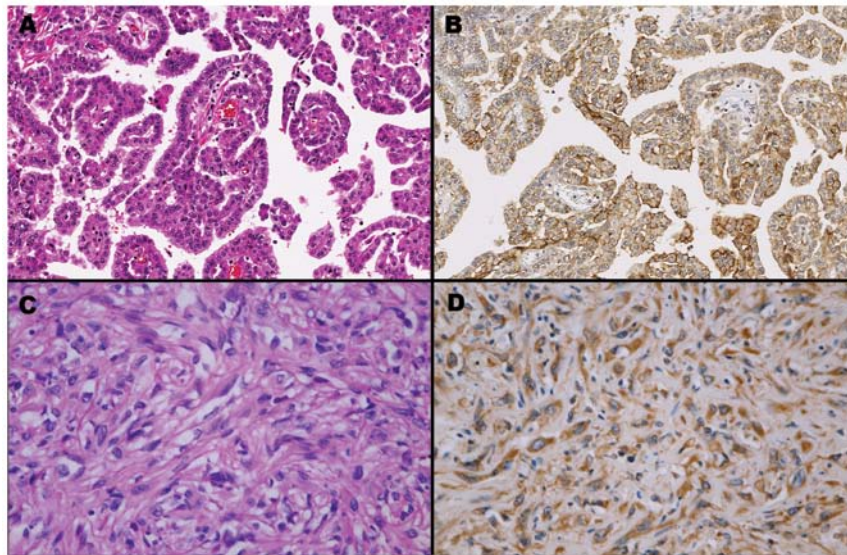


Figure 2. CD26 expression in mesothelioma using CD26-NB100/59021 antibody. Epithelioid mesothelioma differentiated type (A and B), shows membranous expression of CD26 and sarcomatoid mesothelioma (C and D) shows only cytoplasmic expression with inconspicuous membranous expression of CD26. (A and C) Hematoxylin eosin stain; (B and D) CD26 immunohistochemistry.

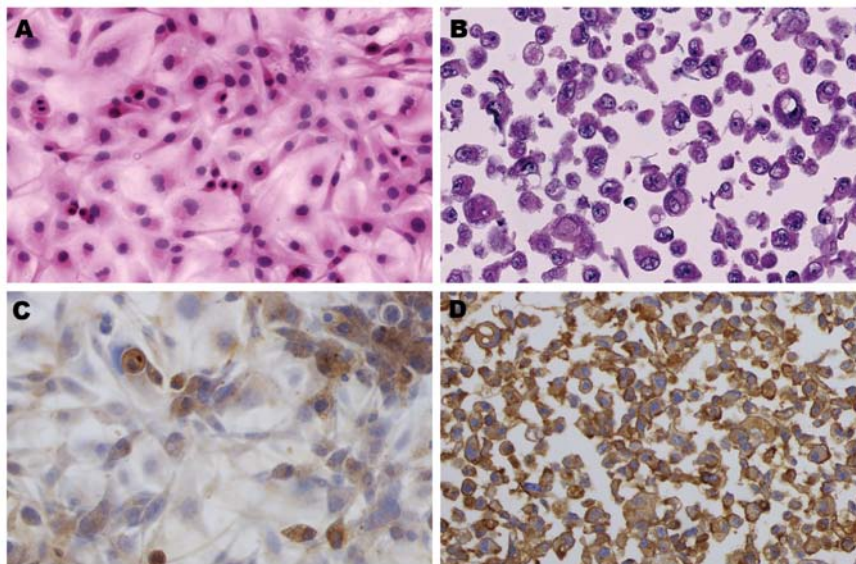


Figure 3. CD 26 expression in mesothelioma cell line. Mesothelioma cell line ACC-MESO-1, shows spindle cell growth in culture dish (A) and no membranous expression pattern of CD26 is detected (C). However, the cellblock preparation (B) from the same cell line shows membranous expression pattern of CD26 in tumor cells (D).

tumor cells derived from sarcomatoid mesothelioma when grown in chamber slides showed morphologically spindle-shaped tumor cells (Fig. 3A), and had primarily cytoplasmic CD26 expression pattern with very few cells showing weak membranous expression (Fig. 3B). The cellblock preparations from the same mesothelioma cells, which transformed to round to oval cells due to trypsinization, showed prominent cell membrane (Fig. 3C). In these preparations, mCD26 expression was clearly evident (Fig. 3D).

Validation of polyclonal antibodies for true localization of CD26. Immunohistochemistry using either absorbed primary antibody of rabbit anti-human CD26 polyclonal antibody

(CD26-NB100/59021) or goat anti-human CD26 polyclonal antibody (R&D, AF1180) showed disappearance of CD26 expression in mesothelioma tissue (Fig. 4), suggesting these two antibodies localizes true CD26 protein.

CD26 expression after CD26 vector transfection into mesothelioma cells and tumor development in NOD/SCID mice. MSTO-211H cells, which primarily do not express CD26, showed membranous expression of CD26 after transfected with a full-length CD26 subcloned retroviral shuttle plasmid pLNCX2 vector by flow cytometric analysis (Fig. 5A).

NOD/SCID mice after inoculation of CD26 vector transfected MSTO-211H cells developed mesothelioma. This tumor

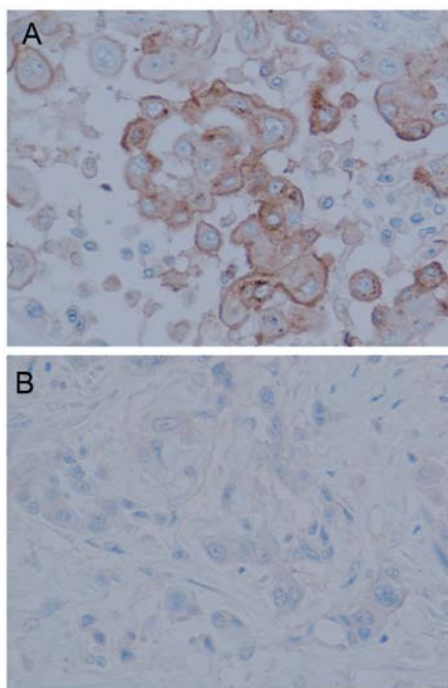


Figure 4. Absorbed CD26 reactions after soluble CD26 antigens in immunohistochemistry. The absorbed CD26 antibody is rabbit anti-human CD26 polyclonal antibody (CD26-NB100/59021) was incubated with recombinant soluble human CD26 proteins produced by CHO cells. CD26 immunoreactivity observed with anti-CD26 antibody (A) is not detected with absorbed CD26 antibody (B).

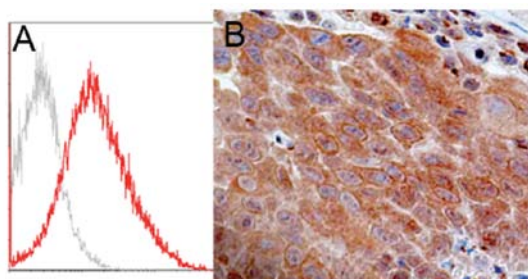


Figure 5. (A) CD26 vector-transfected MSTO-211H cells without fixation showing membranous expression of CD26 by flow cytometric analysis. Red curve, anti-CD26 antibody (phycoerythrin-conjugated monoclonal antibody, BA5); gray curve, control mouse IgG2a. (B) CD26 expression in human CD26 expression vector-transferred MSTO-211H cells. CD26 expression is mainly in the cytoplasm of mesothelioma developed after human CD26 vector-transferred MSTO-211H cells were inoculated into NOD/SCID mice. (Immunohistochemistry with goat anti-human CD26 polyclonal antibody (CD26-AF1180).

showed cytoplasmic expression of CD26. No membranous expression of CD26 could be observed (Fig. 5B).

Discussion

Various unconjugated or immuno-conjugated monoclonal antibody-based therapies have been developed and already established as a new treatment modality in lymphomas, breast, lung, or colorectal cancers. Anti-CD20 antibody (rituximab) in lymphomas, anti-HER2 antibody (trastuzumab) in breast cancer, anti-EGFR antibody (cetuximab) or anti-VEGF (beva-

cizumab) in colorectal or lung cancers have entered standard clinical practice (reviewed in ref. 11). Other antibodies like MDX-010 humanized mAb (anti-CTLA-4 antibody), which inhibit T-cell activation, are also supposed to be promising (26). The possibility of humanized anti-CD26 monoclonal antibody treatment for its inhibitory effect has been reported on malignant mesothelioma cell lines *in vitro* and *in vivo* experiments (12). This is the new promising strategy in antibody-based treatment of mesothelioma (12,13).

The success of all antibody-based treatments of cancer largely depends on the proper selection of the cases. One such selection criterion is by the immunohistochemical expression in the tumor cells. For example, the selection of breast cancer patients for anti-HER2 antibody therapy depends upon the HER-2 expression in the tumor cells of those patients using immunohistochemical study. All breast cancers do not express HER-2 when analyzed by immunohistochemical staining alone and many cases do need further analysis of HER-2 expression by fluorescence *in situ* hybridization (FISH). The interpretation manual has been developed for the management of such treatment (27). The same rule should be applied to humanized monoclonal CD26 antibody therapy to mesothelioma and proper guidelines for selection of mesothelioma cases are required. We have, in this study, analyzed various commercially available antibodies to detect an antibody with reproducibility in correct interpretation of CD26 expression in the archived mesothelioma tissue and/or mesothelioma cell lines. Out of more than 50 different commercially available antibodies, we have selected the antibodies available in the past and newly developed antibodies available recently for immunohistochemical staining in FFPE sections. We found CD26-NB100/59021 antibody from Novus Biologicals or AF1180 from R&D systems are equally good for interpretation of membranous CD26 expression. We also validated these two antibodies for true localization of CD26 protein by absorption study using soluble human CD26 proteins.

The morphology of mesothelioma is generally found to be an important prognostic factor, epithelioid mesothelioma cases show better survival than sarcomatoid mesothelioma cases (28,29). In the present study, none of sarcomatoid mesothelioma expressed membranous CD26 in the tumor cells. The reason for this may be due to the morphological characteristics of sarcomatoid mesothelioma, primarily spindled shape. This can be better explained by our findings of CD26 expression in mesothelioma cell lines. The mesothelioma cells grow as spindled cells in culture medium with inconspicuous cell membrane. This can be even observed in some of the mesothelioma cell lines derived from epithelioid mesothelioma cases. However, the same cells, when treated with trypsin, showed round to oval cells with distinct cell membrane. In this study, we found membranous CD26 expression in these mesothelioma cells. In addition, the mesothelioma cells, when analyzed by flow cytometry to detect expression of cell surface antigen, clearly showed CD26 expression. On the other hand, it can also be postulated that the development of sarcomatoid mesothelioma is by epithelial-mesenchymal transition from epithelioid mesothelioma. The concept of epithelial-mesenchymal transition was recognized at first as a central differentiation process in early embryogenic morphogenesis (30). The coordinated molecular and cellular changes defined as the reductions in

cell-cell adhesion, apical-basolateral polarity, and epithelial markers, as well as the acquisition of motility, spindle-cell shape, and mesenchymal markers. So, CD26 expression, which is prominent in epithelioid mesothelioma, more in differentiated type than less-differentiated type may have been lost after transition into the sarcomatoid mesothelioma, thus showing only cytoplasmic CD26 expression. Our CD26 vector transfected MSTO-211H cells, when inoculated to NOD/SCID mice, show only cytoplasmic expression though surface antigen is clearly expressed in flow cytometric analysis. The detection of surface CD26 by flow cytometry can be another method of analyzing mCD26 expression in pleural fluid from mesothelioma patients.

In conclusion, we found overexpression of CD26 in mesothelioma tissue and cell lines. By using NB100-59021 or AF1180 antibody, immunohistochemical evaluation of membranous CD26 expression in human mesothelioma tissues is possible for selecting mesothelioma cases for humanized monoclonal anti-CD26 antibody treatment. Sarcomatoid mesothelioma in FFPE which do not show membranous CD26 expression, though existence of membranous expression in mesothelioma cell blocks suggest other method to evaluate CD26 expression such as flow cytometry is needed for selecting these cases for humanized monoclonal anti-CD26 antibody therapy.

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