CD26 overexpression is associated with prolonged survival and enhanced chemosensitivity in malignant pleural mesothelioma

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Running title: CD26 is good predictor in chemosensitivity of MPM

The numbers of the text without references is 3,918 words, and this manuscripts contains 2 figures and 3 tables.
Translational relevance

Malignant pleural mesothelioma (MPM) is an aggressive cancer arising from the mesothelial cells lining the pleura. Due to the lack of efficacy of conventional treatments, novel therapeutic strategies are urgently needed to improve outcomes. In this study, we found that the CD26 molecule is expressed on the cell membrane of the epithelial and biphasic, but not the sarcomatoid, type of mesothelioma. Treatment outcome prediction study showed that CD26 membrane expression on MPM was closely correlated with disease responsiveness to chemotherapy. Mesothelioma cells expressing high level of CD26 displayed high proliferative activity, and microarray analysis of CD26-knockdown and CD26-transfected mesothelioma cells showed that CD26 expression was closely linked to expression of genes contributing to cell proliferation, cell cycle regulation, drug-induced apoptotic action, and chemotherapy resistance. Our results therefore suggest that CD26 is a clinically significant biomarker for the prediction of response to chemotherapy for MPM.
Abstract

Purpose: Malignant pleural mesothelioma (MPM) is an aggressive and therapy-resistant neoplasm arising from the pleural mesothelial cells, without established indicators to predict responsiveness to chemotherapy.

Experimental design: Our present study involving 79 MPM patients demonstrated that 73.4% of MPM expressed CD26 on cell membrane.

Results: The majority of epithelioid and biphasic type of MPM expressed CD26 on the cell membrane, whereas the sarcomatoid type demonstrated a lack of CD26 surface expression. Although the sarcomatoid type was associated with poor prognosis ($p<0.0001$), no significant relationship between CD26 expression and survival was observed. On the other hand, there was a trend for an association between response rate to chemotherapy and CD26 expression ($p=0.053$), with higher level of CD26 expression more likely to be linked to better response to chemotherapy. Moreover, CD26 expression was a significant factor associated with improved survival in patients who received chemotherapy (MST, 18.6 vs 10.7 months, $p=0.0083$). Furthermore, CD26 expression was significantly associated with better prognosis in patients receiving non-pemetrexed (PEM)-containing regimens (MST, 14.2 vs 7.4 months, $p=0.0042$), while there was no significant association between CD26 expression and survival time for patients receiving PEM-containing regimens. Our in vitro and microarray studies showed that mesothelioma cells expressing high CD26 displayed high proliferative activity, and CD26 expression was closely linked to cell cycle regulation, apoptosis, and chemotherapy resistance.

Conclusions: Our results strongly suggest that CD26 is a clinically significant biomarker for predicting response to chemotherapy for MPM.
Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from the mesothelial cells lining the pleura (1). It is generally associated with a history of asbestos exposure and has a very poor prognosis (2). In fact, the median survival is less than 12 months, with most patients dying within 10-17 months of their first symptoms. Once rare, the incident of MPM has increased in industrialized nations as a result of past widespread exposure to asbestos (3). The incident is predicted to increase further in the next decades, especially in developing countries where asbestos has not yet been prohibited (1-3). Due to the lack of efficacy of conventional treatments, novel therapeutic strategies are urgently needed to improve outcomes.

The identification of potential biomarkers would be helpful in managing three clinical aspects of MPM: early diagnosis, prognosis and response to therapy. However, there are presently no established indicators that have clinical significance for MPM. Given the fact that MPM remains a rare disease with several subtypes, the lack of accessibility to uniform tumor populations renders the search for biomarkers very difficult (4).

CD26 is a 110-kDa cell surface glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain (5) and is capable of cleaving N-terminal dipeptides with either l-proline or l-alanine at the penultimate position (5). CD26 activity is dependent on cell type and the microenvironment, factors that can influence its multiple biological roles (5-8). Although CD26 expression is enhanced following activation of resting T-cells, CD4+ CD26\textsuperscript{high} T-cells respond maximally to recall antigens such as tetanus toxoid (5). Cross-linking of CD26 and CD3 with solid-phase immobilized monoclonal antibodies (mAbs) can induce T-cell costimulation and IL-2 production by CD26+ T-cells (5). In addition, anti-CD26 antibody treatment of T-cells enhances tyrosine phosphorylation of...
signaling molecules such as CD3ζ and p56\textsuperscript{ck} (7). Moreover, DPPIV activity is required for CD26-mediated T-cell costimulation (5). CD26 may therefore have an important role in T-cell biology and overall immune function. Association with various proteins, including fibroblast-activation protein-α (FAP α), plasminogen, adenosine deaminase, CD45 and collagen, influences its activity (5). As a result, CD26 has an important, but complex, function in tumor behavior, with its biological effect dependent on the tumor type and the microenvironment. Likely as a result of this multifunctional characteristic, CD26 is associated with a high level of clinical aggressiveness in some tumors but a lower level in others (9, 10). For example, it is a marker of aggressive disease for certain subsets of T-cell non-Hodgkin's lymphomas/leukemias, with expression of CD26 on T-lymphoblastic lymphomas/acute lymphoblastic leukemia cells being associated with a worse outcome compared with CD26-negative tumors (12). CD26 is also expressed at high levels on renal carcinoma cells (13-15). Two recent studies also correlated CD26 expression with tumorigenesis. In an immunohistochemical analysis of 152 patients with gastric gastrointestinal stromal tumors, CD26 was found to be associated with a poorer overall survival (16). In addition, CD26 can serve as a prognostic marker in B-cell chronic lymphocytic leukemia (17). Furthermore, CD26 itself may be a novel therapeutic target. Anti-CD26 monoclonal antibody (mAb) treatment resulted in both in vitro and in vivo antitumor activity against several tumor types, including lymphoma and renal cell carcinoma (15,18).

More recently, we showed that CD26 is preferentially expressed on malignant mesothelioma cells but not on normal mesothelial cells, and suggested that membranous expression of CD26 indicates an importance in treatment of patients with MPM (19). More importantly, humanized anti-CD26 antibody inhibited growth of malignant mesothelioma cells and induced long term survival of tumor-transplanted SCID mice (20). Although we
recently showed that cells from certain CD26-positive mesothelioma cell lines appeared to include the cancer stem cell characteristics for malignant mesothelioma in addition to CD24 and CD9-positive cells (21), the role of CD26 for MPM in the clinical setting as a prognostic and therapeutic biomarker has not yet been clarified.

In the present study, we showed that the CD26 molecule is expressed on the cell membrane of the epithelial and biphasic, but not the sarcomatoid, type of mesothelioma. Importantly, treatment outcome prediction study showed that CD26 membrane expression on MPM was closely correlated with disease responsiveness to chemotherapy. Meanwhile, our in vitro studies showed that mesothelioma cells expressing high level of CD26 displayed high proliferative activity, and microarray analysis of CD26 knockdown and CD26-transfected mesothelioma cells showed that CD26 expression was closely linked to expression of genes contributing to cell proliferation, cell cycle regulation, drug-induced apoptotic action, and chemotherapy resistance. These data further argue for the potential clinical significance of CD26 in MPM. Our results therefore strongly suggest that the CD26 molecule is a clinically significant biomarker for the prediction of response to chemotherapy for MPM.
Materials and methods

Patients and tissue samples

Seventy-nine malignant pleural mesothelioma tissues were obtained from patients who had undergone biopsy or surgery at Okayama Rosai Hospital and National Hospital Organization (NHO) Yamaguchi-Ube Medical Center between 1998 and 2009. All patients were confirmed by histology. Histological sections from the mesothelioma patients were previously examined and classified into epithelioid, sarcomatoid and biphasic subtypes, according to the WHO histological classification by three independent pathologists (YT, VJA and KI), who were blinded to the results of the studies discussed in this paper. Sufficient specimen was collected from each patient to allow for evaluation by immunohistochemistry at Hiroshima University (22). The clinical stage and performance status (PS) were determined according to the criteria of the International Mesothelioma Interest Group (IMIG) TNM staging system for malignant pleural mesothelioma (23), and Eastern Cooperative Oncology Group (ECOG) PS scale. Survival terms were calculated from the date of diagnosis of mesothelioma. Radiologic response rate was assessed by modified Response Evaluation Criteria in Solid Tumors (RECIST) criteria, which have been validated in mesothelioma (24, 25).

Immunohistochemical staining

Immunohistochemical staining for CD26 was performed in 3-μm-tissue sections prepared from formalin-fixed paraffin-embedded tissue blocks. Endogenous peroxidase inactivation by treatment with 0.3% H₂O₂ in PBS for 30 min of deparaffinized sections was performed, followed by incubation with anti-CD26/DPPIV antibody (NB100-59021, Novus Biologicals, Littleton, CO, USA) 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 hematoxylin. Similar immunohistochemical procedure was carried out with the omission of the primary antibody as a negative control. CD26 reactivity on lymphocytes and/or endothelial cells in and around the tumor tissue was considered as internal positive control.

Membranous and cytoplasmic expression of CD26 was semi-quantitatively analyzed. CD26 expression was evaluated and verified independently by two pathologists (VJA and YT). Though cytoplasmic expression of CD26 was observed in many of the tumor cells in most of the cases, CD26 expression in mesothelioma was based on its membranous expression (19). In detail, it was scored as 0, absence of membranous expression on tumor cells; 1+, membranous expression on up to 25% of tumor cells; +2, membranous expression on 26-50% of tumor cells; +3, membranous expression on more than 50% of tumor cells. The representative figures of score 3, 2, 0 were shown in Figure S1.

Separation of mesothelioma cell line MESO-1 cells into CD26<sup>high</sup> and CD26<sup>negative</sup> cells and <em>in vitro</em> cell growth assay

Naturally CD26 positive MESO-1 cells were sorted by flow cytometric cell sorter (BD FACS Aria, Franklin Lakes, NJ) into CD26<sup>high</sup> and CD26<sup>negative</sup> cells following staining with anti-CD26-FITC. These cells were subsequently grown in 10% FCS-RPMI medium (containing penicilin and streptomycin) for the indicated days and living cells were then measured by MTT assay. MESO-1 cells sorted by a cell sorter were seeded (5 x 10<sup>4</sup>/well) in 96-well titer plates and were incubated for the indicated days in medium. Cell proliferation was assayed using TetraColor ONE according to the manufacturer’s instructions (Seikagaku...
Biobuisiness Corp.). Living cells were measured by the absorbance value at 450 nm. Data represent mean values ± standard errors calculated from at least three independent experiments in triplicate.

**In vitro cell proliferation assay of CD26-knockdown mesothelioma cell lines**

To further analyze the effect of CD26 expression on cell proliferation, in vitro cell proliferation assay was performed using CD26-knockdown MM cells (CD26-KD). For this purpose, we generated CD26-knockdown MM cells (CD26-KD) using naturally CD26-expressing MESO-11, JMN, and H28 cells transfected with siRNA against human CD26 (Sense strand, 5’-ACUCUAACUGAUUACUUA ATT-3’ was selected and purchased from QIAGEN). Decreased expression of CD26-KD MM cells were confirmed through flow cytometry (Figure S2). These cells were subsequently grown in 10% FCS-RPMI medium (containing penicillin and streptomycin) for the indicated days and living cells were then measured by MTT assay, as described above.

**Microarray data generation**

To analyze potential mechanisms involved in the association between CD26 expression and anti-cancer drug sensitivity on MM cells, we conducted a comprehensive analysis of mRNA expression profiles of MM cells differing in the level of CD26 expression. For this purpose, we used CD26-KD cells as generated above. Moreover, we used CD26-overexpressed MM cells (CD26-Trf) generated with retrovirally transfection of human CD26 into CD26-negative MSTO-211H (MSTO) cells (19). Total RNA was extracted from these cells using standardized protocols of conventional published methodology (RNeasy; QIAGEN). cRNA was then hybridized on TORAY 3D-Gene Human 25k chip (TORAY).
The amount of transcription product was measured, and a profile of mRNA in a sample was obtained by TORAY manufacturer’s protocol. Expression level was shown in log₂ ratio with the heatmap.

Statistical analysis

The chi-squared test or trend test was used to analyze the correlation between CD26 expression and clinicopathologic parameters. The probabilities of survival were estimated using the Kaplan-Meier method (26) and differences between patient groups were determined by the log-rank test. The multivariate analysis of prognostic factors in patients who received chemotherapy was performed using the Cox proportional hazard model. All reported P values are two-sided. A level of $p < 0.05$ was accepted as being statistically significant.
Results

Patient characteristics

Key patient characteristics in the present study are summarized in Table S1. The mean age of the MPM patients was 65.4 years. Most patients were men (89.9%) and had good performance status (PS 0, 1) (84.8%). Forty-nine patients (62.0%) had epithelioid histology. Fifty-three patients (67.1%) had stage I/II disease. Thirty-two patients (40.5%) underwent extrapleural pneumonectomy (EPP). Of the 56 patients who received chemotherapy, 31 patients were treated with pemetrexed (PEM) + cisplatin (CDDP), 4 with PEM + carboplatin (CBDCA), 2 with PEM only, 11 with gemcitabine (GEM) + vinorelbine (VNR), 4 with GEM + CDDP, 2 with GEM only, 1 with doxorubicin + CDDP, and 1 with mitomycin C + CDDP. 15 patients were treated with adjuvant chemotherapy. Thirty-seven patients (66.1%) received PEM-containing regimen. Seventeen patients (21.5%) received best supportive care (BSC) only.

CD26 expression in mesothelioma tissues

As shown in Table S1, 58 patients with mesothelioma (73.4%) expressed CD26 on the mesothelioma cell membrane. The majority of patients with epithelioid and biphasic type of mesothelioma expressed CD26 on the mesothelioma cell membrane, whereas none of the patients with the sarcomatoid type did. It should be noted that diffuse staining for CD26 in the cytoplasm of the mesothelioma cells was observed in all patient samples, even in patients with the sarcomatoid type (Figure S1, panel C).

The association between CD26 expression and key clinicopathologic factors is summarized in Table S1. A statistically significant correlation was observed between membranous CD26 expression on the malignant mesotheliomas and histologic type
However no statistically significant correlation between CD26 expression and gender ($p=0.901$), age ($p=0.552$), clinical stage ($p=0.555$), PS ($p=0.565$), EPP ($p=0.069$), CT ($p=0.236$), or BSC ($p=0.748$) was observed.

**CD26 expression and survival of mesothelioma patients**

The prognostic significance of CD26 membrane expression and other clinicopathological factors in patients with malignant pleural mesothelioma was evaluated by univariate analysis. As shown in Table 1, the median survival time (MST) for all mesothelioma patients in the current study was 13.9 months, with the 1-year survival rate being 58.9%. Older age, sarcomatoid histology, advanced stage, absence of EPP, absence of chemotherapy, and BSC were factors associated with poor prognosis. However, as shown in Fig 1A, no significant relationship between membranous CD26 expression in malignant mesothelioma and survival was observed (MST, 15.0 vs. 10.8 months, $p=0.1384$).

**CD26 expression and chemotherapy response and survival**

Of the 56 patients treated with chemotherapy, 15 patients had adjuvant chemotherapy, and one patient died prior to evaluation of response to chemotherapy. Therefore, we evaluated the relationship between membranous CD26 expression and response to chemotherapy for 40 patients. As shown in Table 2, the response rate to chemotherapy was marginally associated with membranous CD26 expression ($p=0.053$). There was a trend toward an association between higher level of CD26 membrane expression and better response to chemotherapy. Univariate analysis of survival time for patients who received chemotherapy is shown in Table 2. As shown in Table 2 and Figure 1A, membranous CD26 expression was a significant factor associated with better survival in patients who received
chemotherapy (MST, 18.6 vs. 10.7 months, \( p=0.0083 \)). Other clinicopathological factors, histology type, stage, EPP or PEM were also observed to be associated with overall survival (Table2). Multivariate analysis for the chemotherapy response showed histology (\( p=0.016 \)) and EPP (\( p=0.005 \)) had independent prognostic significance (Table S2). Membranous CD26 expression showed a hazard ratio of 1.947, but without statistically significant value (\( p=0.067 \)). (Table S2).

Since membranous CD26 expression appears to predict for treatment outcome in patients undergoing chemotherapy, patients who received chemotherapy were subdivided into two groups, patients with PEM-containing regimen and patients without PEM-containing regimen. As shown in Figure 1B, for patients treated with non-PEM-containing regimen, membranous CD26 expression was significantly associated with better survival (MST, 14.2 vs. 7.4 months, \( p=0.0042 \)). On the other hand, for those treated with PEM-containing regimen, CD26 expression was not significantly associated with survival (\( p=0.1225 \)). However, in the PEM-treated cohort, there was a trend toward better cumulative survival for patients with CD26-positive disease as compared to those with CD26-negative disease in 30+ months of follow-up (MST, 19.5 vs. 12.8 months, 2-year survival rate 37.9% vs. 20.0%). These results therefore suggest that membranous CD26 expression can potentially be a marker of response to chemotherapy in malignant mesothelioma.

**CD26 expression and proliferative activity of mesothelioma cells**

Since CD26 membrane expression on mesothelioma cells appears to be a predictive marker of response to chemotherapy, we next attempted to characterize in details the CD26-positive cells of mesothelioma cell lines. For this purpose we sorted naturally occurring CD26-positive MESO-1 mesothelioma cells and subdivided them into CD26-positive and
CD26-negative cells. Such cells were subsequently cultured for the indicated days. As shown in Figures 2A and 2B, CD26$^{\text{high}}$ cells always exhibited higher level of proliferative activity than CD26$^{\text{negative}}$ cells. Similarly, CD26$^{\text{high}}$ cells from other naturally occurring CD26-positive mesothelioma cell lines such as H226 and H28 cell lines consistently had higher level of proliferation than CD26$^{\text{negative}}$ cells from the same cell lines (data not shown). Moreover, CD26-knockdown of MESO-1, JMN and H28 cells resulted in reduced proliferation as compared to wild type CD26-positive MESO-1, JMN and H28 cells, respectively (Figs 2C (a)-(c)). These results hence suggest that CD26-positive mesothelioma cells have generally robust growth activity. Since highly proliferative cells are typically sensitive to chemotherapeutic agents, our present data support the notion that mesothelioma patients with tumors expressing high level of CD26 generally have chemosensitive disease.

**Microarray analysis for gene profiles in CD26-transfected or CD26-knockdown mesothelioma cells**

To understand the mechanism involved with the relatively greater level of chemosensitivity of CD26$^{\text{high}}$ mesothelioma cells, we analyzed the gene expression profiles of mesothelioma cells differing in CD26 expression level. For this purpose, we used the naturally CD26-positive mesothelioma cell line MESO-1, and the naturally CD26-negative mesothelioma cell line MSTO. As described in the materials and methods section, expression of CD26 protein in MESO-1 was reduced by siRNA against human CD26 (CD26-KD cells), and overexpression of CD26 was achieved in MSTO by human CD26 transfection (CD26-Trf cells). The expression profiles of genes in CD26-KD or CD26-Trf cells were analyzed using cDNA chips mounted with approximately 25,000 human oligo genes. Table 3 lists the five genes that differ in expression levels and that play a role in
tumor proliferation, cell cycle or anticancer drug sensitivity. Expression of the CD26 gene was found to validate CD26-knockdown or CD26 overexpression in CD26-KD MESO-1 or CD26-Trf MSTO, respectively (top of lists in Table 3). IHPK2, inositol hexakiphosphate kinase 2, is a member of a family of enzymes generating inositol pyrophosphate and mediates cell apoptosis (27). It had been shown that increased IHPK2 activity sensitizes cancer cells to anticancer drugs, whereas its depletion blocks cell death (28). The IHPK2 mRNA level was decreased in CD26-KD cells and was increased in CD26-Trf cells (Table 3), which may partly explain the enhanced chemosensitivity of CD26<sup>high</sup> mesothelioma cells.

Cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> mRNA was increased in CD26-KD cells and was reduced in CD26-Trf cells. The p21<sup>Cip1</sup> protein binds to and inhibits the activity of cyclin-CDK2 or CDK4 complexes, and thus functions as a cell cycle stopper at the G1 phase (29). The reduced level of p21<sup>Cip1</sup> expression in CD26<sup>high</sup> mesothelioma cells would allow the cells to progress through the cell cycle, resulting in greater chemosensitivity. DUSP1, dual specificity protein phosphatase 1, is MAP kinase phosphatase-1 and is a mediator of cancer chemoresistance (30). As shown in Table 3, DUSP1 level in CD26-Trf cells is lower than that in CD26-KD cells, data which support our findings of greater chemoresistance of CD26<sup>negative</sup> mesothelioma cells. PTTG1, pituitary tumor-transforming 1, or securin, is a protein involved in regulating the metaphase-anaphase transition and anaphase onset. Increased securin level is associated with favorable outcomes in invasive breast cancer (31). Securin (PTTG1) level was increased in CD26-Trf mesothelioma cells and decreased in CD26-KD mesothelioma cells (Table 3), hence suggesting that this protein may be marker of favorable outcome for malignant mesothelioma. Our data also indicated a difference in the expression of TRIM7 (tripartite motif-containing protein 7). While the role of TRIM7 in cancer has not yet been elucidated, this ubiquitin E3 ligase protein might function as a
regulator of cell cycle and proliferation, as had been shown with other E3 ligases (32).

Taken together, our results described above suggest that CD26 expression on mesothelioma cell membrane is a reliable biomarker for predicting potential chemosensitivity and clinical outcome for malignant mesothelioma.
Discussion

In this study, we showed that the majority of the epitheliod and biphasic type of mesothelioma expressed CD26 on the cell membrane while there was no CD26 surface expression for the sarcomatoid type. We also demonstrated that CD26 membrane expression was associated with improved survival in MPM patients who had received chemotherapy. Moreover, our *in vitro* studies of CD26 positive mesothelioma cell lines and microarray analysis of CD26 knockdown and CD26-transfected mesothelioma cells support the conclusion that CD26 surface expression is associated with and potentially contributes to mesothelioma chemosensitivity. Therefore, our present work strongly suggests that the CD26 molecule is a reliable biomarker for predicting potential therapeutic outcome following chemotherapy.

MPM is a fatal malignancy (1, 2). The overall survival of patients with MPM varies among different treatments and is also dependent on patients’ overall condition. Various patients’ characteristics have been studied for their potential effects on the survival of patients with MPM. Patients’ age, histology, stage, performance status, and therapeutic modality (e.g. EPP and/or chemotherapy) were identified as important predictors of prognosis in our study, consistent with previous reports by other investigators (33, 34).

Mesothelioma tumor growth is generally insidious but the usual clinical signs/symptoms of mesothelioma (dyspnea, cough and chest pain) are nonspecific, leading to late diagnosis for curative treatment. It is therefore important to search for disease markers that can help with early diagnosis and potentially improve prognosis. In this regard, serum osteopontin and mesothelin levels have been proposed to be early diagnostic markers for MPM (35, 36), although there have been doubts relating to their specificity and sensitivity. Mesothelin is directly produced by the tumor itself, and its level could mirror tumor burden,
as suggested by Robinson et al. (37), who found higher serum mesothelin levels in patients having larger tumors. Furthermore, elevated osteopontin level could stimulate tumor growth and spread, and thus high level of osteopontin may be associated with shorter survival (38). Similar correlation between high osteopontin expression and a shorter survival have been described in other tumors (39, 40). However, mesothelin and osteopontin have not been found to be predictors of treatment outcome, and more work is therefore needed to identify indicators of responsiveness to therapy that can be used to optimize treatment in MPM.

Few studies have identified potential predictors of responsiveness to chemotherapeutic agents as pemetrexed and/or cisplatin/carboplatin in patients with MPM (4). It is hypothesized that low expression of excision repair cross-complementation group 1 (ERCC1) might predict for increased sensitivity to platinum-based chemotherapy, possibly due to the saturation of the enzyme complex; conversely, high levels of ERCC1 may predict for resistance to platinum-based chemotherapy (4). Regarding pemetrexed treatment, thymidylate synthase (TS) expression level has been used to predict pemetrexed effectiveness (41, 42). Righi et al investigated the correlation between baseline expression levels of TS and ERCC1 genes evaluated by real-time PCR and immunohistochemistry and clinical outcomes in MPM patients treated with pemetrexed-based chemotherapy (42). They observed that low TS protein and mRNA levels are predictive of improved time to progression and overall survival.

Our present work showed that the response rate to chemotherapy was marginally associated with CD26 membrane expression, with higher expression level tending to be associated with better response to chemotherapy. This is the first report demonstrating that CD26 membrane expression level in mesothelioma is a marker for predicting treatment outcome for MPM patients receiving chemotherapy.
Previous studies on CD26 have yielded various results in different cancers. Preclinical studies showed that increased CD26 expression inhibited metastasis in ovarian cancer (10), while suppression of CD26 promoted metastasis in prostate cancer (43). On the other hand, inhibition of CD26 in renal cell carcinoma decreased tumor growth and reduced binding of the cancer cells to fibronectin and collagen (15). Moreover, clinical studies in thyroid cancer, gastrointestinal stromal tumors and T cell non-Hodgkin’s lymphoma /leukemias suggested that CD26 expression was associated with distant metastasis, recurrence after resection or poor survival (16,18,44). The multifunctional roles of CD26 may account for its varied roles in different cancers (11). Our recent studies also showed that humanized anti-CD26 mAb treatment of mouse xeno-graft models of human malignant mesothelioma cells drastically inhibited tumor growth in tumor-bearing mice, resulting in enhanced survival (20).

Our current in vitro studies showed that CD26 expression on mesothelioma cells was associated with enhanced proliferative activity. More importantly, microarray analyses of gene expression profiles in CD26-overexpressed or CD26-knockdown mesothelioma cells showed that CD26-expressing mesothelioma cells exhibited the upregulation of several genes which play a role in sensitizing cancer cells to anticancer drugs and which are associated with favorable outcome in invasive cancers. On the other hand, expression of CD26 in these mesothelioma cells was linked with the down-regulation of genes that have a role in cell-cycle progression and tumor sensitivity to chemotherapy.

In conclusion, our present work suggests that CD26 is an important biomarker for predicting sensitivity to chemotherapy, supported by our in vitro studies and microarray analyses. Moreover, since membranous CD26 expression can potentially predict tumor sensitivity to chemotherapy, knowledge of membranous CD26 expression in MPM may affect
clinical care of patients and treatment decision.
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Figure Legend

Figure 1

A. Kaplan-Meier survival curves of patients with mesothelioma with and without membranous CD26 expression. (a) survival curve of all MPM cases and (b) survival curve of MPM patients who received chemotherapy,

B. Kaplan-Meier survival curves of patients with MPM patients who received chemotherapy with PEM (a) and without PEM (b).

Figure 2

CD26 expression level in malignant mesothelioma (MM) cell lines correlates with cellular proliferation activity.

A. CD26-FITC labeled MESO-1 cells were sorted by flow cytometric cell sorter. Cells expressing high level of CD26 (CD26$^{high}$) were gated in more than 10$^2$ of FITC intensity, and CD26 negative cells (CD26$^{negative}$) were gated in less than 10$^0$ of FITC intensity.

B. CD26$^{high}$ or CD26$^{neg}$ MESO-1 cells sorted in (A) were grown in culture medium for the indicated days, and viable cells were measured by MTT assay. Higher proliferative activity was observed for CD26$^{high}$ cells compared to CD26$^{negative}$ cells (*, ** $p<0.01$).

C. CD26-knockdown (CD26-KD) MM cells were generated using siRNA against human CD26, which is naturally expressed in MESO-1 (a), JMN (b) and H28 MM cells (c). Cells were grown in culture medium for two days, and viable cells were measured by MTT assay. Decreased proliferative activity was observed for CD26-KD cells compared to CD26 naturally expressed wild type cells (contl. siRNA).
Figure 1

A

(a) All MPM cases

(b) MPM cases who received chemotherapy

CD26 positive
CD26 negative

Cumulative Survival Rate

Survival Time (months)

$p = 0.1384$

$p = 0.0083$

B

(a) non-PEM

(b) PEM

CD26 positive
CD26 negative

Cumulative Survival Rate

Survival Time (months)

$p = 0.1225$

$p = 0.0042$
Figure 2

A

CD26-FITC

CD26 high

CD26 negative

Contl. Ig

B

O.D. 450nm

Day 0
Day 1
Day 2
Day 3

CD26 high

CD26 negative

C

(a)

O.D. 450nm

p < 0.001

contl.

CD26-KD

MESO-1

(b)

O.D. 450nm

p < 0.005

contl.

CD26-KD

JMN

(c)

O.D. 450nm

p < 0.001

contl.

CD26-KD

H28
Table 1: Univariate analysis of survival for MPM cases

| Clinicopathological parameters | All MPM cases | | | | Cases with chemotherapy | | | |
|-------------------------------|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                               | MST (months) | 1-yr survival (%) | P value | MST (months) | 1-yr survival (%) | P value | MST (months) | 1-yr survival (%) | P value |
| Membranous CD26 expression    | Positive      | 15               | 64.4       | 0.1384        | 18.6            | 82.1           | 0.0083         |               | |
|                               | Negative      | 10.8             | 42.9       |               | 10.7            | 41.2           |               | 0.2345         | 10.7            | 41.2           | 0.0083         | |
| Sex                           | Male          | 14.1             | 57.9       |               | 16.8            | 70.6           |               |               | 16.8            | 70.6           |               | |
|                               | Female        | 12.8             | 66.7       | 0.2345        | 10              | 60             | 0.9618         |               | 10              | 60             | 0.9618         | |
| Age                           | < or = 65     | 16.3             | 68.4       |               | 16.7            | 72.7           |               |               | 16.7            | 72.7           |               | |
|                               | >65           | 11.7             | 47.3       | 0.0054        | 15.7            | 65.2           | 0.2309         |               | 15.7            | 65.2           | 0.2309         | |
| Histology                     | Epithelioid   | 15.2             | 64.6       |               | 18.6            | 81.8           |               |               | 18.6            | 81.8           |               | |
|                               | Biphasic      | 15               | 66.9       |               | 15              | 73.3           |               |               | 15              | 73.3           |               | |
|                               | Sarcomatoid   | 6.2              | 18.2       | 0.0001        | 6.2             | 12.5           | < 0.0001       |               | 6.2             | 12.5           | < 0.0001       | |
| Stage                         | I / II        | 15.2             | 62.8       |               | 18.6            | 70.3           |               |               | 18.6            | 70.3           |               | |
|                               | III / IV      | 10               | 48.9       | 0.0064        | 14.4            | 66.7           | 0.0242         |               | 14.4            | 66.7           | 0.0242         | |
| PS                            | 0 / 1         | 14.4             | 63.7       |               | 18.5            | 73.5           |               |               | 18.5            | 73.5           |               | |
|                               | 2 / 3         | 9.7              | 28.6       | 0.0055        | 11.6            | 42.9           | 0.057          |               | 11.6            | 42.9           | 0.057          | |
| EPP                           | EPP           | 19.5             | 71.9       |               | 23.7            | 83.3           |               |               | 23.7            | 83.3           |               | |
|                               | Non-EPP       | 11.7             | 49.3       | 0.0006        | 14.2            | 59.4           | 0.0018         |               | 14.2            | 59.4           | 0.0018         | |
| CT                            | CT            | 16.3             | 69.6       |               |                 |                |               |               |                 |                |                |               |
|                               | Non-CT        | 6.8              | 29.5       | 0.0315        |                 |                |               |               |                 |                |                |               |
| PEM                           | PEM           | 18.5             |           |               | 81.1            |               |               |               | 81.1            |               |               | |
|                               | Non-PEM       | 11.6             | 47.4       | 0.0379        |                 |                |               |               | 11.6            | 47.4           | 0.0379         | |
| BSC                           | EPP or CT     | 15.2             | 66.1       |               |                 |                |               |               |                 |                |                |               |
|                               | BSC           | 9.4              | 28.8       | 0.0006        |                 |                |               |               |                 |                |                |               |

PS, ECOG performance status; EPP, extrapleural pneumonectomy; CT, chemotherapy; BSC, best supportive care; PEM, pemetrexed regimen.
Table 2: Membrane CD26 expression and chemotherapy response

<table>
<thead>
<tr>
<th>CD26 expression</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>22</td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

*p = 0.053

PR, partial remission; SD, stable disease; PD, progressive disease.
Table 3
Heatmap view showing the results of gene expression analysis of CD26-knockdown (CD26-KD) MESO1 or CD26-transfected (CD26-Trf) MSTO cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log₂ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SYMBOL</strong></td>
<td><strong>NAME</strong></td>
</tr>
<tr>
<td>DPP4</td>
<td>Dipeptidyl peptidase VI</td>
</tr>
<tr>
<td>IHPK2</td>
<td>Inositol hexakisphosphate kinase 2</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor (p21)</td>
</tr>
<tr>
<td>DUSP1</td>
<td>Dual specificity protein phosphatase 1</td>
</tr>
<tr>
<td>PTTG1</td>
<td>Securin (Pituitary tumor-transforming protein</td>
</tr>
<tr>
<td>TRIM7</td>
<td>Tripartite motif-containing protein 7</td>
</tr>
</tbody>
</table>

Color Key

Expression value

-3.0 -2.0 -1.0 0 1.0 2.0 3.0
CD26 overexpression is associated with prolonged survival and enhanced chemosensitivity in malignant pleural mesothelioma


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