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Methionine Aminopeptidase 2 as a Potential Therapeutic Target for Human Non-Small-Cell Lung Cancers

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. Methionine aminopeptidase 2 (MetAP2) is a bi-functional protein that plays a critical role in the regulation of post-translational processing and protein synthesis.

Objectives. We studied whether MetAP2 is activated and expressed in human non-small-cell lung cancer (NSCLC) tissues and whether inactivation of MetAP2 activity, with its specific inhibitor fumagillin, potentially inhibits proliferation of NSCLC cells.

Material and Methods. The expression and function of MetAP2 were evaluated in NSCLC tissues, primary cell cultures and cell lines using immunohistochemistry, RT-PCR, Western blot, aminopeptidase activity assay and flow cytometry. MetAP2 expression was also studied in relation to clinicopathological factors.

Results. MetAP2 expression in NSCLC, including adenocarcinoma (ADC) and squamous cell carcinoma (SCC), showed a moderate to strong positive reaction while normal appearing bronchial epithelium showed weak staining and normal alveolar epithelial cells were widely negative. A high MetAP2 mRNA and protein expression was found in NSCLC tissues. The aminopeptidase activity in NSCLC was 2-fold higher than that in normal lung tissues. In a series of 41 ADC patients, MetAP2 expression was significantly correlated with patient's outcome or survival time. Inhibition of MetAP2 by fumagillin in SCC cell lines revealed a significant increase in caspase-3 activity as compared to the control ($p = 0.001$).

Conclusions. Our results indicate that MetAP2 is involved in NSCLC and is an important regulator of proliferative and apoptotic targets. Thus inhibition of MetAP2, such as by fumagillin, may be a potential therapeutic modality for prevention of tumor cell growth, development and progression in NSCLC patients (*Adv Clin Exp Med* 2016, 25, 1, 117–128).

Key words: apoptosis, methionine aminopeptidase 2 (MetAP2), myristoylation, fumagillin, non-small-cell lung cancer (NSCLC).

Non-small-cell lung cancer (NSCLC) is one of the most difficult malignancies to treat. Despite years of research, the prognosis for patients with lung cancer remains poor, with a five-year survival rate of 14%. Nevertheless, lung cancer may be curable in its early stages and most patients derive some benefit from treatment, such as longer survival or amelioration of symptoms [1]. DNA ploidy analysis may be efficient to estimate malignant potential in lung ADCs [2]. Recently, clinical

application of molecular targeted therapy such as epidermal growth factor receptor (EGFR) has improved prognosis. Accordingly, the overall survival of patients with metastatic NSCLC evidencing EGFR mutations has improved to 27–30.5 months when treated with EGFR [3]. Therefore, molecular targeted therapy for the appropriate population, using good predictive markers, is indispensable.

The gene expression profile analysis of human esophageal cell carcinoma cell lines after early

response to ionizing irradiation using cDNA microarray screening disclosed MetAP2 genes that were modulated in irradiation [4]. The MetAP2 gene may be useful in understanding the molecular basis of radiotherapy and in developing strategies to augment its effect or establish novel, less hazardous alternative adjuvant therapies. Overexpression of MetAP2 in immortalized bronchial epithelial cell line NL20 accelerated growth and was reversed using treatment with MetAP2 inhibitors. Thus MetAP2 plays an important role in tumor cell growth and may contribute to tumorigenesis [5, 6].

Protein synthesis starts with an initiator methionine in both prokaryotes and eukaryotes. The translational process on ribosomes starts with methionine. In order for the newly synthesized protein to be transported to its exact intracellular location, the methionine at the NH₂-terminal is removed. After the removal of methionine *via* MetAP, protein myristoylation takes place by the enzyme *N*-myristoyl-transferase (NMT). NMT is a cytosolic enzyme in eukaryotic cells [7]. The process is essential for further amino terminal modifications (e.g. acetylation by *N*- α acetyltransferase and myristoylation of glycine by *N*-myristoyl-transferase). The structural alterations from these modifications are essential in cell proliferation [8].

In eukaryotes, two isoforms of MetAP have been identified as MetAP1 and MetAP2 [9]. Both MetAP1 and MetAP2 are essential components of the cell growth machinery. In yeasts and humans, two proteins are known to possess MetAP2 activity and are known as MetAP1 and MetAP2. Downregulation of either MetAP1 or MetAP2 protein expression by small interfering RNA (siRNA) significantly inhibited the proliferation of human endothelial cells [10].

MetAP2 has attracted much more attention than MetAP1 due to the discovery of MetAP2 as a target molecule of the anti-angiogenic compounds, fumagillin and ovalicin [11]. Identification of MetAP2 as the cellular target of fumagillin class molecules, and the significant growth inhibition observed in cells sensitive to MetAP2 inhibition suggested the direct involvement of MetAP2 in the regulation of cell proliferation [12, 13].

Targeting the angiogenesis process has become an important strategy for inhibiting tumor growth. Fumagillin and its derivatives have been known to exert their inhibitory effects by specifically and covalently binding to MetAP2 [14, 15]. Inhibitors of angiogenesis can be classified into 2 groups, specific and nonspecific factors. Non-specific inhibitors, angiostatin, a tissue inhibitor of metalloproteinases-2 (TIMP-2), and endostatin have attracted

interest because of their strong antitumor effect. Recently, much research has focused on TNP-470, a synthetic analog of fumagillin, and its derivatives (IDR-803, IDR-804, IDR-805, CKD-732) [16].

High expression of MetAP2 has been demonstrated in breast, colorectal and cholangio-carcinoma [17–19]. Suppression of hepatoma growth and angiogenesis by fumagillin has also been reported [20]. As for NSCLC, moderate-to-high MetAP2 staining was identified only in lung carcinoma cell lines not lung cancer tissues [5]. Here we demonstrate the first description of increased MetAP expression in NSCLC tissues, primary cell cultures from NSCLC and cell lines. Our findings strongly suggest that the inhibition of MetAP2 by fumagillin or its analogs may be a potential target for tumor cell growth, development and progression in NSCLC patients.

Material and Methods

Material

We investigated 41 cases of NSCLC histologically classified as ADC [21], ranging in age from 45 to 80 years (mean, 67 years). The age, gender, tumor size, histological grading, nodal metastasis, staging and patient outcome were evaluated by reviewing the medical and pathologic records. Tumor size was evaluated using the greatest perpendicular diameter of each lung lesion. In addition, we studied surgical tissues from 8 cases of histologically classified primary NSCLC including 4 cases of ADC and 4 cases of SCC in order to evaluate MetAP2 aminopeptidase enzyme activity, mRNA expression, immunohistochemical localization and protein expression. Ten cases of normal lung tissues freshly obtained from resected benign lesions served as control. The other tissue samples were fixed in neutral-buffered formaldehyde and processed for histological and immunohistochemical evaluation. Furthermore, we studied cell cultures from RERF-LC-AI (well differentiated) and LC-1/sq (moderately differentiated) human SCC cell lines for MetAP2 inhibition assay by a novel inhibitor of MetAP2, fumagillin.

Methods

Cell Lines and Culture Condition

RERF-LC-AI (well differentiated) and LC-1/sq (moderately differentiated) human SCC cell lines were purchased from Riken BioResource Center (Tsukuba, Japan). The tumor cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 IU/mL

penicillin, 100 ug/mL streptomycin, and 0.25% amphotericin B at 37°C in a humidified atmosphere in a 5% CO₂ incubator. The cells were treated with trypsin (0.25%), harvested and processed for secondary cultures in the same culture medium until they reached 80% confluence.

In this study, tumor cells obtained at the low-passage cultures (passages at 4–5) were used.

Preparation of Tissues

The tissue samples (mean weight, 300 mg) were homogenized in 10 volumes of 0.01 mol/L phosphate-buffered saline (PBS) pH 7.4 and sonicated at 40 amplitudes for 2 min to obtain the soluble fraction. After centrifugation at 4°C, the supernatants were used to measure MetAP2 aminopeptidase activity and proteins in triplicate. For caspase-3 activity assay, primary cell cultures from NSCLC and normal lung tissues were harvested by centrifugation at 10,000 rpm for 5 min at 4°C and counted. For each case, 1×10^6 cells re-suspended in ice cold cell lysis buffer, and sonicated at 40 amplitudes for 30 s.

The cell lysates were centrifuged at 10,000 rpm for 3 min, and the supernatants were transferred to a microcentrifuge tube for caspase-3 activity assay.

MetAP2 Aminopeptidase

Activity Assay

MetAP2 aminopeptidase activity was determined by hydrolysis of methionine L-Leu-*p*-nitroanilide as a substrate, described elsewhere [22]. For MetAP2 activity, an assay mixture (500 µL) containing 50 mM Tris-HCl at pH 7.5 and 0.25 mM methionine L-Leu-*p*-nitroanilide as a substrate with an appropriate concentration of the enzyme was used. The reaction mixtures were incubated at 37°C for 30 min, left for 15 min on ice, and followed by spectrophotometric determination at 405 nm. The amount of aminopeptidase activity that released one micromole of L-Leu-*p*-nitroanilide per minute under assay conditions was defined as one unit.

Semi-Quantitative RT-PCR Analysis of MetAP2 mRNA Expression

The total RNA was extracted using an RNeasy mini kit (Quiagen, Hilden, Germany), according to the manufacturer's instructions. Before cDNA synthesis, the RNA was incubated with DNase and then precipitation was done using 95% ethanol. The precipitated RNA was then electrophoresed on 2.0% agarose-ethidium bromide (EtBr) gels to verify the RNA quantity. Subsequently, RNA concentration was determined by UV spectrophotometry. Total RNA (1.5 µg) was then reversely transcribed utilizing the specific primers.

The following PCR amplification was carried out in a Thermal Cycler (Takara, Tokyo, Japan). In each case, the PCR cycles were optimized to confirm amplification within the linear phase. For each sample, the relative mRNA level was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer pairs were used: MetAP2 sense, 5'ATGGCGGGTGTGGAGGAGGTAGCGGCCT-3' (nucleotides 135-162) and anti-sense 5'TTAATAGTCATCTC CTCTGCTGACAACT-3' (nucleotides 1544-1571). The expected PCR product for MetAP2 was 1440 bp. The internal control was GAPDH. For quantification of the PCR bands, we used densitometry with Quantity One Software (Bio-Rad Labs, Hercules, CA, USA) relative to GAPDH. The results were considered as the mean \pm SE of three experiments.

Immunostainings for MetAP2

Primary cultures of RERF-LC-AI and LC-1/sq human SCC cell lines were stained using an indirect immunofluorescence method. Cells (2.5×10^4) in chamber slides (Nalge Nunc Int., Naperville, Illinois) were cultured overnight. Two chamber slide sets from LSCC were prepared, one of which received 1 ug/mL fumagillin (BIOMOL Research Lab, Inc, USA) treatment. After 24 h culture, we fixed the cells in cold acetone, washed in PBS, and treated for 30 min with 10% normal horse serum to eliminate non-specific reaction. The cells were treated with 1 : 50 dilution of polyclonal anti-human MetAP2 antibody overnight followed by PBS washes and reaction with FITC-conjugated goat anti-rabbit IgG at 1 : 100 dilution. Immunoperoxidase reaction for metAP2 was done using frozen or deparaffinized sections and a streptavidin-biotin peroxidase complex method. In brief, endogenous peroxidase was blocked by 10% normal goat serum and the sections were reacted with 1 : 50 polyclonal rabbit anti-human MetAP2 antibody (Zymed Lab Inc, CA) overnight. After PBS washes, the sections were treated with biotinylated goat anti-mouse or anti-rabbit IgGs, washed and processed using a streptavidin-biotin-peroxidase kit (Histofine, Nichirei, Japan). The chromogenic reaction was with diaminobenzidine. Counterstaining was done with hematoxylin. For negative control, the primary antibody was omitted or substituted with non-immune rabbit serum.

MetAP2 Expression in Lung Adenocarcinomas

We studied the relationships between MetAP2 expression and pathological and clinical features (tumor grade, tumor stage, tumor size and nodal metastasis). MetAP2 staining was graded by the

percentage of staining area of tumor cells as follows: 0: 0%, 1: 1~25%, 2: 26~50%, 3: 51~75% and 4: 76~100%. Staining intensity was graded 0: negative, 1: mild, 2: moderate and 3: strong staining. The staining score (0 to 12) was estimated by multiplying the staining area 0 to 4 (%) by the staining intensity 0 to 3. We set the low expression of MetAP2 intensity as a score of 0 to < 6 and high expression as a score of 6 to 12.

Western Blot Analysis

We extracted proteins from the primary cultures of three cases each of adenocarcinoma and SCC of the lung. Each protein sample (20 µg/lane) was run on SDS gels, transferred onto Immobilon polyvinylidene difluoride membranes (Bio-Rad Lab, Hercules, CA, USA) in a transfer buffer consisting of 0.02% SDS, 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% v/v methanol, then incubated overnight at 4°C with a blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl, pH 7.5, 0.1% Tween-20, 150 mM NaCl). Two sets of membranes were made and incubated with 1 : 500 polyclonal rabbit anti-human MetAP2 antibodies (Zymed Lab Inc, CA, USA) overnight at room temperature. Proteins were extracted from the primary cultures of three cases of ADC and SCC of the lung and normal lung tissues. The samples were washed with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween 20 and then were treated with corresponding secondary antibodies conjugated with alkaline phosphatase. The reaction was developed with the ProtoBlot NBT and BICP System (Promega, Madison, WI, USA).

Caspase-3 Fluorometric Measurement

Caspases-3 production requires that the active enzymes undergo folding from their large and small subunit constituents after expressing separately in *Escherichia coli*. The methodological details have been described previously [23]. The active enzyme was obtained under optimal conditions for each enzyme; each subunit from the purified inclusion bodies were solubilized in 6 M guanidine HCl and subsequently diluted to a 100 µg/mL final concentration at room temperature.

Measurement of the fluorescence counts in the wells was done with a 400 nm excitation filter and 505 nm emission filter. The levels of released 7-amino-4-trifluoromethyl coumarin were measured with a BioLumin 960 spectrofluorometer (Molecular Dynamics Japan, Tokyo, Japan). The specific activity of caspase-3 present was calculated in each sample. The relative absorbance was calculated as described according to the manufacturer's instructions (MBL, Co, LTD, Nagoya, Japan).

Flow Cytometric Assay

RERF-LC-AI and LC-1/sq human SCC cells obtained from the low-passage cultures (passages at 4–5) were utilized in this study. To measure propidium iodide (PI) staining, lung cancer cells (1×10^4) were harvested and stained with FITC-labeled PI (Molecular Probes, Eugene, OR, USA) as specified by the supplier. Briefly, cancer cells (1×10^4) in 1 mL of medium were cultured as indicated for 21h, washed and then stained with PI-FITC in a binding buffer and analyzed with CellQuest software (BD Bioscience, San Jose, CA, USA) with FACSCalibur within 1 h. The data was expressed as the mean of three experiments before and after treatment.

Statistical Analysis

Statistical analyses for comparisons between the clinicopathological findings were performed using Fisher's exact test and Kaplan-Meier survival analyses. The difference between two related groups was examined for statistical significance using the Student's *t*-test for paired data. A $p < 0.05$ was recorded as statistically significant.

Results

MetAP2 Expression in Normal Lung and NSCLC Tissues

Immunoperoxidase staining for MetAP2 in a paraffin section of normal alveolus showed negative staining (Fig. 1A). Sections from bronchial epithelium showed a weak degree of staining (Fig. 1B). ADC (well differentiated) and SCC cells showed moderate to strong intensity (Fig. 1C, D). The intensity of MetAP2 expression was greater in carcinoma cells as compared to bronchial epithelial tissues. SCC tissues showed stronger staining than ADC tissues.

Pathological Features of Lung Adenocarcinoma Patients and Expression of MetAP2

We studied paraffin-embedded lung tissue sections of 41 cases of histologically classified ADCs. The relationships between pathological and clinical features are shown in Table 1. A comparison between the low expression of MetAP2 intensity and high expression of MetAP2 intensity in ADCs showed that 21 cases had low expression and 20 cases had high expression of MetAP2. There was

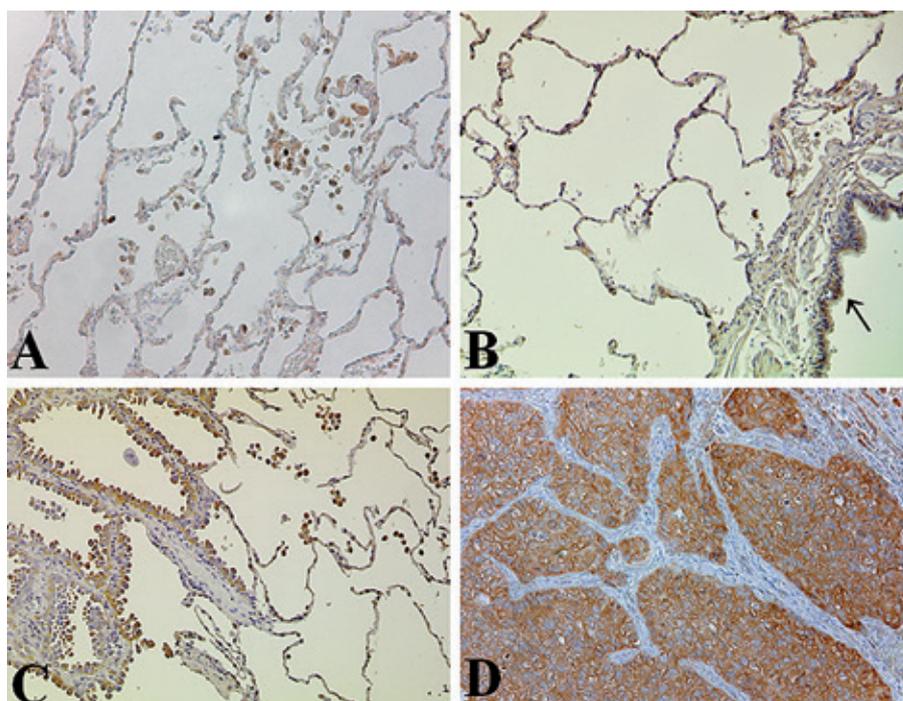


Fig. 1. Immunohistochemical analysis of human normal lung and cancer tissues (A-D, magnification $\times 100$). MetAP2 in paraffin section from normal alveolus showed negative staining (Fig. 1A). Bronchial epithelium showed a weak degree of staining (Fig. 1B, arrow). ADC (well-differentiated) and SCC cells showed moderate to strong intensity (Fig. 1C, D)

Table 1. Pathological features of lung adenocarcinoma patients and expression of MetAP2

Variables	No.	MetAP2 expression		
		low	high	p-value
Age				
≤ 70	24	13	11	0.756
> 70	17	8	9	
Gender		15		1.000
male	30	6	15	
female	11		5	
Tumor size				0.751
≤ 30 mm	16	9	7	
> 30 mm	25	12	13	
Tumor differentiation				1.000
w/d & m/d	28	14	14	
p/d	13	7	6	
Node metastasis				0.197
negative	27	16	11	
positive	14	5	9	
Pathological stage				0.350
stage I	28	14	10	
stage II & III	13	7	10	
Patient outcome				0.043
alive	10	3	9	
dead	31	18	11	

* w/d & m/d – well differentiated & moderately-differentiated; ** p/d – poorly differentiated.

no significant association between the low and high expression of MetAP2 and clinical features (tumor size, tumor differentiation, nodal metastasis and

staging), but patient outcome revealed a significant difference between low and high MetAP2 expressing patients (Table 1; $p < 0.04$).

MetAP2 Activities in Normal Lung and NSCLC Tissues

Using methionine-*p*-nitroanilide as a substrate, we measured the spectrophotometric determination of the hydrolysis of methionine *p*-nitroanilide in their soluble and membrane-bound forms in surgically removed lung carcinoma tissues and normal lung tissues freshly obtained from resected benign lesions, serving as the control. The mean aminopeptidase activity was 80.6 ± 13.4 in normal lung tissue vs. 156.9 ± 20.8 in lung carcinoma tissue. Compared to normal lung tissue, carcinoma tissue had a remarkably higher activity (Table 2). In 4 cases of ADC, each case showed a significantly higher aminopeptidase activity than an unaffected normal tissue counterpart (Fig. 2A) and overall, ADC cases had 2 times higher activity than normal tissue counterparts (97.3 ± 16.51 vs. 45.4 ± 8.6 ; $p < 0.03$, Fig. 2B). Likewise, 6 cases of SCC tissues had 196.7 ± 20.1 aminopeptidase activity vs. 104.2 ± 15.2 activity in unaffected adjacent normal lung tissues (Fig. 2C) with an overall significant difference ($p < 0.04$; Fig. 2D).

Table 2. Aminopeptidase activities in normal lung and NSCLC* tissues

Case**	Age/Sex	Aminopeptidase activity (unit/L)	
		normal	tumor
1	82 / M	29.311.8	73.3 ± 3.3
2	77 / F	38.7+1.8	129.3 ± 5.2
3	65 / F	69.7± 1.5	122 ± 5.3
4	71 / M	43.7± 2.0	64.7+3.2
5	77 / M	102.7± 5.5	115.7± 5.5
6	64 / M	149.7 ± 2.6	174 ± 3.5
7	78 / M	50± 1.7	247 ± 7.8
8	76 / M	102 ± 3.6	226.7± 9.5
9	60 / M	141 ± 3.8	182.3 ± 4.6
10	74 / M	79.7 ± 2.0	234.3 ± 7.4

* NSCLC – non-small cell lung cancer; ** cases 1–4 – adenocarcinoma and 5–10 – squamous cell carcinoma (SCC); the data presented as mean ± S.E.

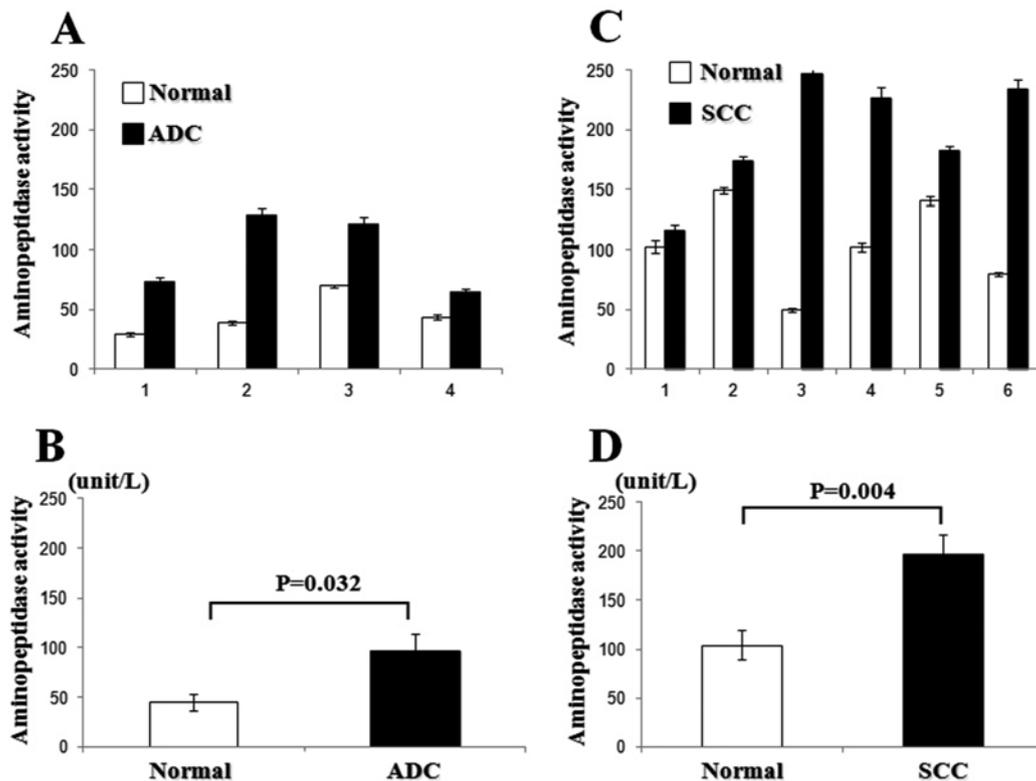


Fig. 2. Spectrophotometric determination of hydrolysis of methionine *p*-nitroanilide in their soluble and membrane-bound forms in surgically-removed lung carcinoma tissues and unaffected adjacent surrounding tissues. Comparison of the expression level of MetAP2 between 4 cases of ADC and 4 cases of normal lung tissues separately (A) and in combination (B). Comparison of the expression level of MetAP2 between 6 cases of SCC and 6 cases of unaffected adjacent normal lung tissues separately (C) and in combination (D)

Semi-Quantitative RT-PCR and Western Blot Analysis of MetAP2 in Normal Lung and NSCLC Tissues

To assess the mRNA expression levels of MetAP2, we studied surgical operating tissues from 7 cases of histologically classified primary NSCLC as 4 ADC and 3 SCC patients. Normal lung tissues were obtained from surgically resected benign lesions.

The mRNA levels of MetAP2 in the four cases of ADC were significantly different compared to normal lung tissues (Fig. 3A; $p < 0.008$). Also, the three cases of SCC had a significant upregulation of the MetAP2 gene as compared to normal lung tissues (Fig. 3B; $p < 0.002$).

Proteins were extracted from subconfluent primary cultures of three representative cases of ADC and SCC and normal lung tissue counterparts. The levels of MetAP2 in the three cases of

ADC (Fig. 3C) and three cases of SCC (Fig. 3D) showed a significant difference in the quantitative measurement of the protein expression compared to normal lung tissues ($p < 0.05$; respectively).

MetAP2 Immunofluorescence Intensity, Caspase-3 Activity and Cell Death Effect of Fumagillin in Cultured SCC Cell Lines

We assessed the effect of fumagillin as an inhibitor of MetAP2 on cell proliferation activity and cell death population using caspase-3 fluorometric and PI (propidium iodide) flow cytometric analysis. We used the primary cultures of RERF-LC-AI (well differentiated) and LC-1/sq (moderately differentiated) human SCC cell lines.

The immunofluorescence intensity of MetAP2 in moderately differentiated SCC cells was decreased after fumagillin (1 $\mu\text{g/mL}$) treatment for 24h (Fig. 4A). Also, caspase-3 activity was markedly

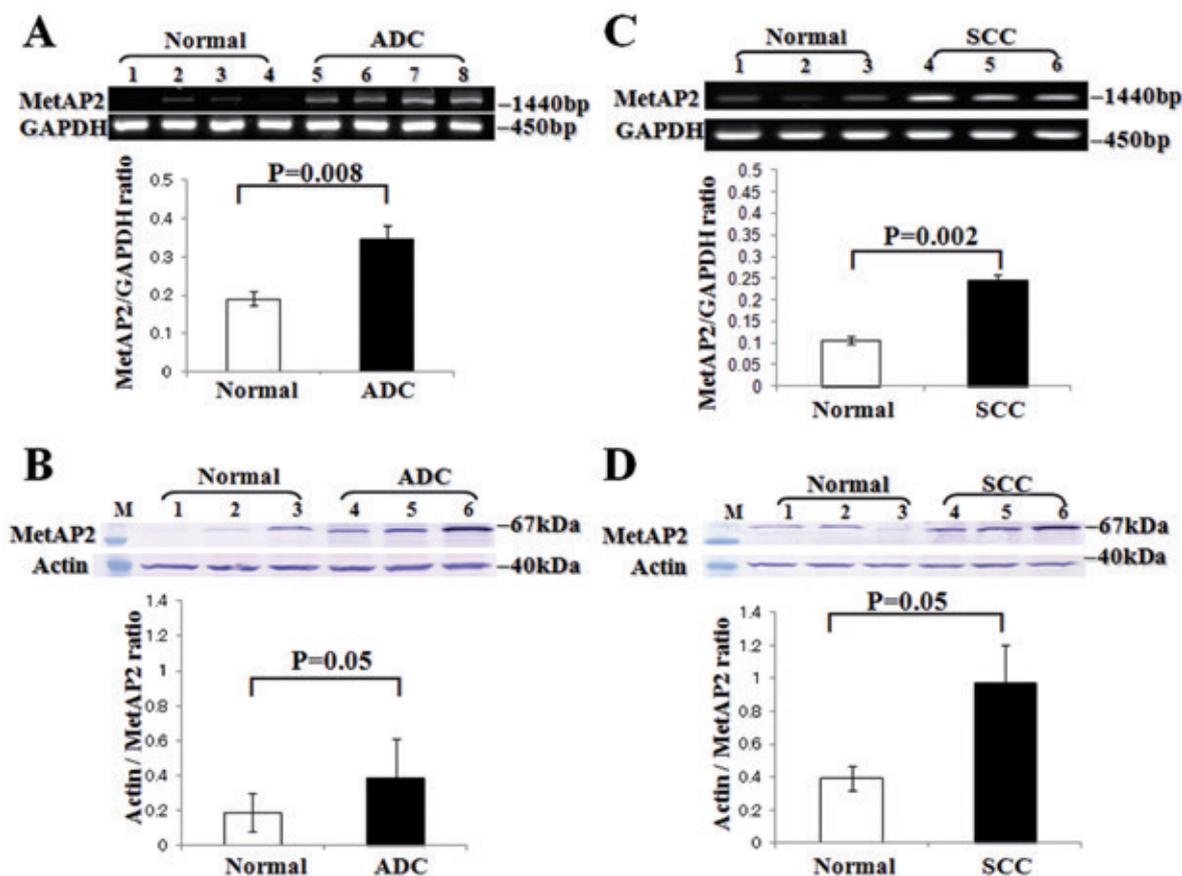


Fig. 3. MetAP2 mRNA and protein expression in ADC and SCC. (A) The mRNA levels of MetAP2 by RT-PCR in 4 cases of ADC compared to 4 cases of normal lung tissues showed significant increase in ADC ($p < 0.008$). (B) The protein levels of MetAP2 by Western blot in 3 cases of ADC compared to 3 cases of normal lung tissues showed significant increase in ADC ($p < 0.05$). (C) The mRNA levels of MetAP2 by RT-PCR in 3 cases of SCC compared to 3 cases of normal lung tissues showed significant increase in SCC ($p < 0.002$). (D) The protein levels of MetAP2 by Western blot in 3 cases of SCC compared to 3 cases of normal lung tissues showed significant increase in SCC ($p < 0.05$)

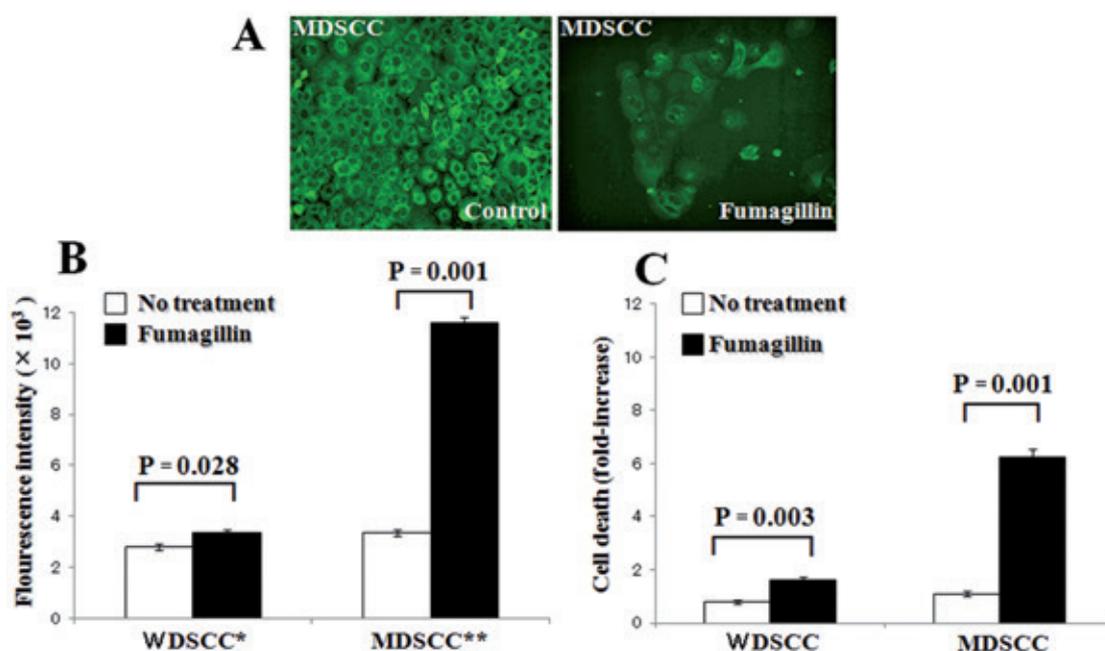


Fig. 4. Inhibition of MetAP2 by addition of fumagillin in WDSCC and MDSCC cell cultures. (A) Immunofluorescence staining of MetAP2 in untreated and treated (plus fumagillin, 1 $\mu\text{g}/\text{mL}$) MDSCC cell culture showed decrease in fluorescence intensity in treated cells (magnification $\times 200$). (B) Caspase-3 fluorometric assay after exposure of MDSCC cells to fumagillin. Both well-differentiated SCC and moderately-differentiated SCC cells showed significantly increased caspase-3 activity. (C) Cell death effect using propidium iodide stain and flow cytometry on MDSCC cells. Both WDSCC and MDSCC cells showed significantly increased cell death by propidium iodide staining

increased in fumagillin-treated well and moderately differentiated SCC cells (Fig. 4B) ($p < 0.028$ and $p < 0.001$, respectively) compared to no-treatment control SCC cells. In the moderately differentiated SCC cells, caspase-3 activity was more increased than in the no-treatment SCC control cells (Fig. 4B). In addition, regarding cell death effect, the fumagillin-treated well and moderately differentiated SCC cells showed significantly increased cell death (Fig. 4C; $p < 0.003$ and $p < 0.001$, respectively).

Discussion

Despite aggressive therapy, patients with advanced stage NSCLC demonstrate a poor survival with significant long-term morbidity in disease survivors. High-risk disease features are strongly correlated with tumor vascularity, suggesting that angiogenesis inhibitors may be a useful addition to current therapeutic strategies [24].

In the past, NSCLCs were seen together without paying attention to the more specific molecular pathological types. This was thought to be appropriate, because different therapeutic procedures were not available for the treatment of the subtypes of NSCLC such as ADC and squamous cell carcinomas. Since the time the first special EGFR mutation was identified, the situation has changed and

nowadays this rapidly evolving field provides new results [25]. EGFR mutations have been reported as a predictive factor for favorable prognosis of gefitinib-treated patients with lung ADC. Patient's sex and smoking status were not significantly associated with longer overall survival and progression-free survival according to EGFR mutation status [26]. Patients with advanced NSCLC who were selected on the basis of EGFR mutations improved progression-free survival with acceptable toxicity but those with no EGFR mutation did not.

In all living cells, protein synthesis is initiated with either methionine (in the cytosol of eukaryotes) or formylmethionine. MetAP activity is essential for cellular growth and viability. In yeasts, knockout of either MetAP1 or MetAP2 causes a decrease in growth rates while elimination of both genes is lethal, indicating that the two MetAPs play essential functions and are together essential for yeast proliferation [27, 28]. MetAP2 has attracted more attention than MetAP1 by the discovery of MetAP2 as a target molecule of the anti-angiogenic compounds, fumagillin and ovalicin [13]. A novel MetAP2 inhibitor, fumagillin, strongly inhibits the growth of human colon cancer HT29 cells, melanoma B16F10 cells and neuroblastoma CHP-134 cells [29–31]. MetAP2 is the molecular target of angiogenesis inhibitors, such as fumagillin, which can also inhibit cancer cell proliferation, implying that MetAP2 may play a quite

complex role in tumor progression under which MetAP2 enzyme activity is inactivated through covalent modification [32, 33].

Several studies have demonstrated that the metastasis-associated gene product S100A4 interacts with the angiogenesis-related protein MetAP2. Other studies also support this conclusion [34]. Recently, small molecule protein p67/MetAP2 was shown to have a greater affinity toward ERK1/2 kinases, and its N-terminal p26 segment will mask the phosphorylation sites on ERK1/2 to block the activation and activity of ERK1/2. This will then lead to inhibition of the cell cycle activated through the growth factor-mediated cell signaling pathway and thus cell growth and proliferation [35].

Major components of the cell signaling pathways, the *ras*/mitogen-activated protein kinase (MAPK) systems are altered in lung cancer cells by oncogenes through overexpression or mutation, leading to dysregulated cell signaling and cell proliferation [36]. It has been reported that NCI-H-460 (large cell carcinoma), H1299, A549 (ADC) and Calu6 (anaplastic carcinoma) cell lines cause inhibition of tumor cell growth on three distinct chemical classes of MetAP2 inhibitors as follows: TNP-470, A800141, and A-357300.

This data prompted us to examine the possible new treatment roles of MetAP2 in lung cancers. There was no significant association of low and high expression of MetAP2 and clinical features (tumor size, tumor cell differentiation, nodal metastasis and staging) (Table 1). But median survival time of the low and high expression of MetAP2 disclosed a significant difference (Table 1, $p = 0.043$). Survival rate was more increased in low MetAP2 expression patients (85.7%) than high MetAP2 expression patients (55%). MetAP2 may play quite a complex role in tumor progression, which inactivates MetAP2 enzyme activity through covalent modification [32, 33].

Additionally, we measured the spectrophotometric determination of the hydrolysis of methionine *p*-nitroanilide in their soluble and membrane-bound forms in surgically removed lung carcinoma tissues and unaffected adjacent normal surrounding tissues. Total average aminopeptidase activity was 80.6 ± 13.4 in normal lung tissue versus 156.9 ± 20.8 in lung carcinoma tissue (Table 2). Compared to normal lung tissue, carcinoma tissue had significantly higher activities. Histologically, SCC demonstrated higher MetAP2 activity than ADC. These results were further confirmed by the semi-quantitative RT-PCR analysis of MetAP2 mRNA expression in normal lung and NSCLC tissues. The mRNA levels of MetAP2 in four cases of ADC showed significant differences in the quantitative extent of MetAp2 gene expression as

compared to normal lung tissues ($p = 0.008$). Also, three cases of squamous cell carcinomas had significant upregulation of the MetAP2 gene as compared to normal lung tissues ($p = 0.002$). In seven cases of NSCLC, MetAP2 mRNA expression levels were upregulated as compared to normal lung tissues ($p = 0.001$) (data not shown). Also, the levels of MetAP2 in three cases of ADC and three cases of squamous cell carcinomas showed significant differences in the quantitative extent of protein expression compared to normal lung tissues ($p = 0.05$, respectively). MetAP2 proteins were increased in ADC and SCC cases compared to normal lung tissues, according to Western blot analysis. Higher expression of the MetAP2 protein in human cancers further supports the contention that MetAP2 plays a role in cancer development. We saw moderate-to-strong staining of MetAP2 in all ADC cases examined [37]. S100a4 protein is a calcium-binding agent that regulates tumor metastasis and a variety of cellular processes *via* interaction with different target proteins, including MetAP2, a main regulator of the proliferative and apoptotic pathways in mesothelioma cells [33, 34].

Interestingly, the normal bronchial epithelium showed a weak degree of staining; therefore, the frequent MetAP2 expression in NSCLC seems to be categorized as an aberrant expression. This finding correlates to the finding that colorectal normal mucosa far from the cancer shows a mild degree of MetAP2 staining [11].

The frequent and aberrant MetAP2 expressions in biliary epithelial cells might have acquired MetAP2 through their dysplasia-carcinoma sequence. Positive MetAP2 expression was observed in a small population of non-dysplastic biliary epithelial cells. Thus, it has been reported that MetAP2 is a novel biomarker for the early detection of cholangiocarcinoma [19].

In the present study, we asked whether MetAP2 is activated and expressed in human NSCLC tissues or inactivation of MetAP2 activity with fumagillin (an angiogenesis inhibitor) may potentially inhibit proliferation of lung cancer cell lines.

The expression level of MetAP2 was significantly higher in the 6 cases of SCC than 4 cases of ADC. Also, semi-quantitative MetAP2 mRNA and protein levels showed higher expression in SCC than in ADC. Thus, we chose the SCC cell lines to assess the effect of fumagillin as an angiogenesis inhibitor on cell proliferation activity and cell death population using caspase-3 flow cytometric analysis. The immunofluorescence intensity of MetAp2 in the moderately differentiated SCC cell line was decreased after treatment with fumagillin for 24 h (Fig. 4A) and caspase-3 activity was markedly increased (Fig. 4B; $p = 0.001$) as compared to

well-differentiated SCC cells (Fig. 4B; $p = 0.028$). In addition, the cell death effect was also increased in fumagillin-treated moderately differentiated SCC cells (Fig. 4C; $p = 0.001$) as compared to well-differentiated SCC cells (Fig. 4C; $p = 0.003$). These results indicate that fumagillin as an angiogenesis inhibitor has potentially inhibited lung cancer cell proliferation.

The mechanism by which inhibition of MetAP2 might lead to growth inhibition may be through both tumor cell intrinsic and extrinsic mechanisms. A defect in the removal of N-terminal methionine caused by metAP2 inhibition might lead to aberrant levels of proteins important for cell proliferation and apoptosis [33]. In a mechanism that remains to be completely elucidated, inhibition of MetAP2 by small molecule inhibitors led to the transcriptional activation of p53, which in turn activates the expression of p21 that inhibits cyclin E/Cdk2, accounting for the cell cycle blockade by these inhibitors [38]. Fumagillin is likewise a eukaryotic initiation factor 2-associated glycoprotein, p67. Fumagillin increases the stability of p67 and affinity to ERKs 1 and 2 and causes the inhibition of the phosphorylation of ERKs 1 and 2 [38, 39]. MetAP2 inhibitors IDR-803, IDR-804, IDR-805 and CDK-732, as well as fumagillin analogs, strongly inhibit the growth of cancers in a model of nude mouse xenograft. Inhibition of angiogenesis is emerging as a promising strategy for the treatment of cancer [41]. Choosing the most appropriate time of day for TNP-470, a synthetic analogue of fumagillin, administration will aid in the treatment of tumors.

The transcription of MetAP2 mRNA is regulated by clock gene proteins of the mCLOCK: mBMAL1 heterodimer in sarcoma180-bearing mice [42]. Interestingly, MetAP2 was expressed in many cell types, including fibroblasts in idiopathic pulmonary fibrosis. In the bleomycin induced acute lung injury in mice, fumagillin attenuated the deposition of collagen [43]. This finding was further confirmed when it was found that PPL-2458, a member of the fumagillin class of irreversible MetAP2 inhibitors, potently inhibits the proliferation of human fibroblast-like synoviocytes derived from rheumatoid arthritis in the late G1 phase of the cell cycle [44]. Further, we report the first study on MetAP2 expression and function in NSCLC tissues and cell lines.

A high MetAP2 mRNA and protein expression as well as activity was found in NSCLC tissues and cell lines and the expression of MetAP2 correlated with patient's outcome. The higher concentration of MetAP2 in NSCLC tissues than the corresponding normal bronchial epithelial cells suggests a greater dependence on this enzyme by malignant cells for their function and proliferation. Hence, a reduction in the enzyme activity may be more harmful to cancer cells than normal epithelial cells. The evaluation of specific inhibitors of MetAP2 in animal models should provide justification for future selection and evaluation of MetAP2 inhibitors in clinical trials for NSCLC patients. MetAP2 is an important regulator of proliferative and apoptotic pathways and its inhibition may provide a potential therapeutic intervention for lung cancer.

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References

- [1] Spira A, Ettinger DS: Multidisciplinary management of lung cancer. *N Engl J Med* 2004, 350, 379–392.
- [2] Shimizu H, Sugino T, Chiba H: Interphase cytogenetic analysis of lung adenocarcinomas with bronchioloalveolar pattern. *Fukushima J Med Sci* 2012, 58, 66–73.
- [3] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isoke H: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR *N Engl J Med* 2010, 362, 2380–2388.
- [4] Bo H, Ghazizadeh M, Shimizu H, Kurihara Y, Egawa S, Moriyama Y: Effect of ionizing irradiation on human esophageal cancer cell lines by cDNA microarray gene expression analysis. *J Nippon Med Sch* 2004, 71, 172–180.
- [5] Tucker LA, Zhang Q, Sheppard GS, Lou P, Jiang F, McKeegan E: Ectopic expression of methionine aminopeptidase-2 causes cell transformation and stimulates proliferation. *Oncogene* 2008, 26, 3967–3976.
- [6] Selvakumar P, Lakshmikuttyamma A, Kanthan R, Kanthan SC, Dimmock JR, Sharma RK: High expression of methionine aminopeptidase 2 in human colorectal adenocarcinomas. *Clin Cancer Res* 2004, 10, 2771–2775.
- [7] Walsh CT, Garneau-Tsodikova S, Gatto GJ Jr: Protein posttranslational modifications: The chemistry of proteome diversifications. *Angew Chem Int Ed Engl* 2005, 44, 7342–7372.
- [8] Sawanyawisuth K, Wongkham C, Pairojkul C, Saeseow OT, Riggins GJ, Araki N: Methionine aminopeptidase 2 over-expressed in cholangiocarcinoma: potential for drug target. *Acta Oncol* 2007, 46, 378–385.
- [9] Walker KW, Bradshaw RA: Yeast methionine aminopeptidase I. Alteration of substrate specificity by site-directed mutagenesis. *J Biol Chem* 1999, 274, 13403–13409.
- [10] Bernier SG, Taghizadeh N, Thompson CD, Westlin WF, Hannig G: Methionine aminopeptidases type I and type II are essential to control cell proliferation. *J Cell Biochem* 2005, 95, 1191–1203.

- [11] Selvakumar P, Lakshmikuttyamma A, Dimmock JR, Sharma RK: Methionine aminopeptidase 2 and cancer. *Biochim Biophys Acta* 2006, 1765, 148–154.
- [12] Turk BE, Griffith EC, Wolf S, Biemann K, Chang YH, Liu JO: Selective inhibition of amino-terminal methionine processing by TNP-470 and ovalicin in endothelial cells. *Chem Biol* 1999, 6, 823–833.
- [13] Griffith EC, Su Z, Niwayama S, Ramsay CA, Chang YH, Liu JO: Molecular recognition of angiogenesis inhibitors fumagillin and ovalicin by methionine aminopeptidase 2. *Proc Natl Acad Sci USA* 1998, 95, 15183–15188.
- [14] Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* 1990, 6, 555–557.
- [15] Liu S, Widom J, Kemp CW, Crews CM, Clardy J: Structure of human methionine aminopeptidase-2 complexed with fumagillin. *Science* 1998, 282, 1324–1327.
- [16] Chun E, Han CK, Yoon JH, Sim TB, Kim YK, Lee KY: Novel inhibitors targeted to methionine aminopeptidase 2 (MetAP2) strongly inhibit the growth of cancers in xenografted nude model. *Int J Cancer* 2005, 10, 124–130.
- [17] Martínez JM, Prieto I, Ramírez MJ, Cueva C, Alba F, Ramírez M: Aminopeptidase activities in breast cancer tissue. *Clin Chem* 1999, 45, 1797–1802.
- [18] Selvakumar P, Lakshmikuttyamma A, Kanthan R, Kanthan SC, Dimmock JR, Sharma RK: High expression of methionine aminopeptidase 2 in human colorectal adenocarcinomas. *Clin Cancer Res* 2004, 10, 2771–2775.
- [19] Sawanyawisuth K, Wongkham C, Pairojkul C, Saeseow OT, Riggins GJ, Araki N: Methionine aminopeptidase 2 over-expressed in cholangiocarcinoma: potential for drug target. *Acta Oncol* 2007, 46, 378–385.
- [20] Yoshida T, Kaneko Y, Tsukamoto A, Han K, Ichinose M, Kimura S: Suppression of hepatoma growth and angiogenesis by a fumagillin derivative TNP470: possible involvement of nitric oxide synthase. *Cancer Res* 1998, 58, 3751–3756.
- [21] Travis WD, Brambilla E, Noguchi M: International association for the study of lung cancer/American thoracic society/European respiratory society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol* 2011, 6, 244–285.
- [22] Nirasawa S, Nakajima Y, Zhang ZZ, Yoshida M, Hayashi K: Intramolecular chaperone and inhibitor activities of a propeptide from a bacterial zinc aminopeptidase. *Biochem J* 1999, 341, 25–31.
- [23] Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M: A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* 1997, 272, 17907–17911.
- [24] Shusterman S, Maris JM: Prospects for therapeutic inhibition of neuroblastoma angiogenesis. *Cancer Lett* 2005, 228, 171–179.
- [25] Bittner N, Ostoros G, Géczi L: New treatment options for lung adenocarcinoma – in view of molecular background. *Pathol Oncol Res* 2013, 5. (Epub ahead of print).
- [26] Toyooka S, Takano T, Kosaka T, Hotta K, Matsuo K, Ichihara S: Epidermal growth factor receptor mutation, but not sex and smoking, is independently associated with favorable prognosis of gefitinib-treated patients with lung adenocarcinoma. *Cancer Sci* 2008, 99, 303–308.
- [27] Li X, Chang YH: Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. *Proc Natl Acad Sci USA* 1995, 92, 12357–12361.
- [28] Chang YH, Teichert U, Smith JA: Molecular cloning, sequencing, deletion, and overexpression of a methionine aminopeptidase gene from *Saccharomyces cerevisiae*. *J Biol Chem* 1992, 25, 8007–8011.
- [29] Selvakumar P, Lakshmikuttyamma A, Das U, Pati HN, Dimmock JR, Sharma RK: NC2213: a novel methionine aminopeptidase 2 inhibitor in human colon cancer HT29 cells. *Mol Cancer* 2009, 8, 65.
- [30] Hannig G, Lazarus DD, Bernier SG, Karp RM, Lorusso J, Qiu D: Inhibition of melanoma tumor growth by a pharmacological inhibitor of MetAP-2, PPI-2458. *Int J Oncol* 2006, 28, 955–963.
- [31] Morowitz MJ, Barr R, Wang Q, King R, Rhodin N, Pawel B: Methionine aminopeptidase 2 inhibition is an effective treatment strategy for neuroblastoma in preclinical models. *Clin Cancer Res* 2005, 11, 2680–2685.
- [32] Wang J, Lou P, Henkin J: Selective inhibition of endothelial cell proliferation by fumagillin is not due to differential expression of methionine aminopeptidases. *J Cell Biochem* 2000, 77, 465–473.
- [33] Catalano A, Romano M, Robuffo I, Strizzi L, Procopio A: Methionine aminopeptidase-2 regulates human mesothelioma cell survival: role of Bcl-2 expression and telomerase activity. *Am J Pathol* 2001, 159, 721–731.
- [34] Endo H, Takenaga K, Kanno T, Satoh H, Mori S: Methionine aminopeptidase 2 is a new target for the metastasis-associated protein. S100A4. *J Biol Chem* 2002, 19, 26396–26402.
- [35] Datta B: Roles of P67/MetAP2 as a tumor suppressor. *Biochim Biophys Acta* 2009, 1796, 281–292.
- [36] Dy GK, Adjei AA: Novel targets for lung cancer therapy: part I. *J Clin Oncol* 2002, 15, 2881–2894.
- [37] Tucker LA, Zhang Q, Sheppard GS, Lou P, Jiang F, McKeegan E: Ectopic expression of methionine aminopeptidase-2 causes cell transformation and stimulates proliferation. *Oncogene* 2008, 27, 3967–3976.
- [38] Lu J, Chong CR, Hu X, Liu JO: Fumarranol, a rearranged fumagillin analogue that inhibits angiogenesis *in vivo*. *J Med Chem* 2006, 49, 5645–5648.
- [39] Datta B, Majumdar A, Datta R, Balusu R: Treatment of cells with the angiogenic inhibitor fumagillin results in increased stability of eukaryotic initiation factor 2-associated glycoprotein, p67, and reduced phosphorylation of extracellular signal-regulated kinases. *Biochemistry* 2004, 43, 14821–14831.
- [40] Datta B: Roles of P67/MetAP2 as a tumor suppressor. *Biochim Biophys Acta* 2009, 1796, 281–292.
- [41] Chun E, Han CK, Yoon JH, Sim TB, Kim YK, Lee KY: Novel inhibitors targeted to methionine aminopeptidase 2 (MetAP2) strongly inhibit the growth of cancers in xenografted nude model. *Int J Cancer* 2005, 10, 124–130.

- [42] Nakagawa H, Koyanagi S, Takiguchi T, Kuramoto Y, Soeda S, Shimeno H: 24-hour oscillation of mouse methionine aminopeptidase2, a regulator of tumorprogression, is regulated by clock gene proteins. *Cancer Res* 2004, 64, 8328–8333.
- [43] Kass D, Bridges RS, Borczuk A, Greenberg S: Methionine aminopeptidase-2 as a selective target of myofibroblasts in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2007, 37, 193–201.
- [44] Bernier SG, Lazarus DD, Clark E, Doyle B, Labenski MT, Thompson CD, Westlin WF, Hannig G: A methionine aminopeptidase-2 inhibitor, PPI-2458, for the treatment of rheumatoid arthritis. *Proc Natl Acad Sci USA* 2004, 101, 10768–10773.

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