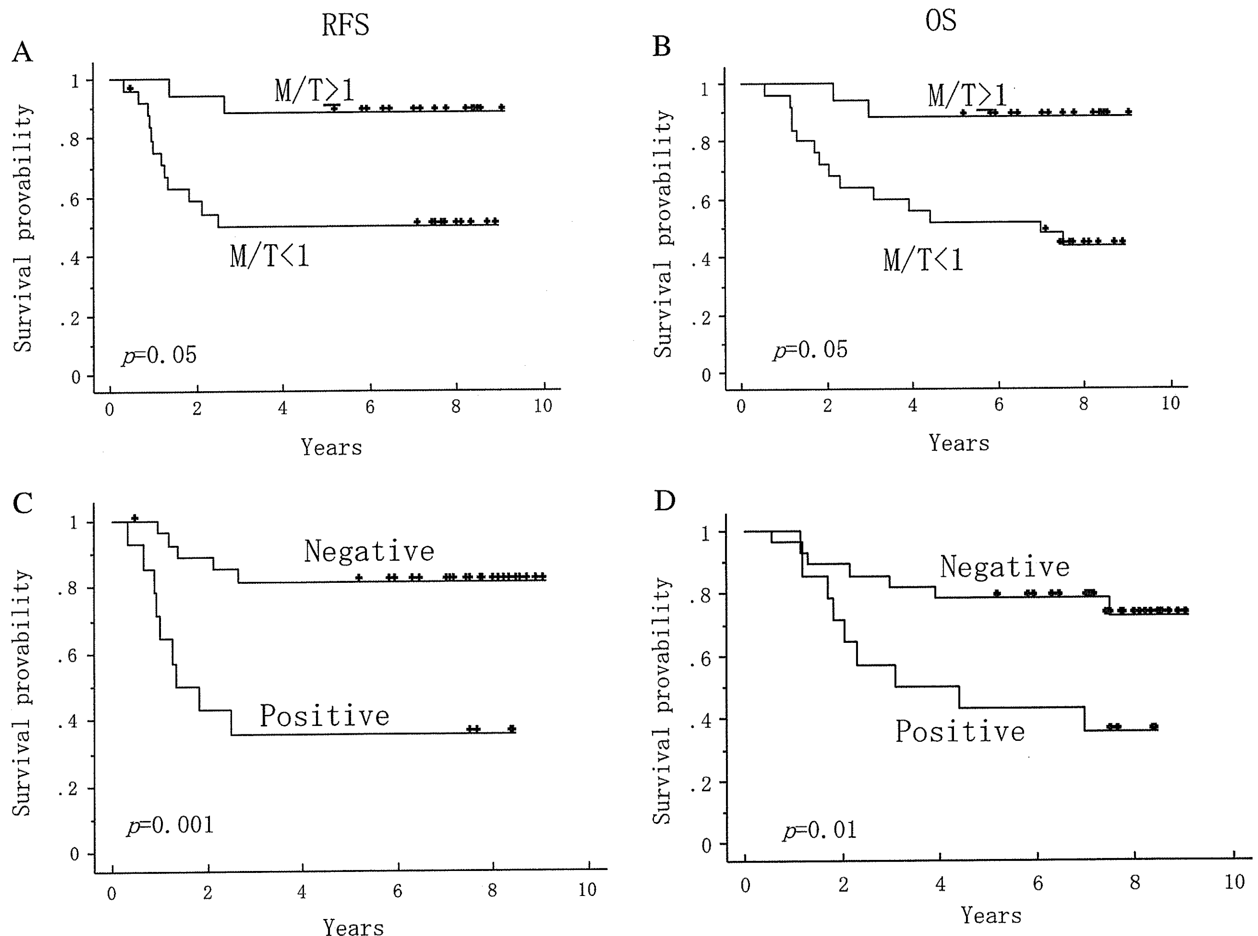

Fig. 3 Survival curves according to margin/tumor size ratio (*M/T*) and the status of the surgical margin. **a, c** The 5-year disease-free survival (*DFS*) rate according to *M/T* was 52.3% ($n = 24$) for $M/T < 1$ and 84.6% for $M/T \geq 1$ ($n = 13$, $P = 0.05$), while it was 30.8% for cases with positive ($n = 13$) and 82.6% for those with negative ($n = 24$) cytology findings ($P = 0.001$). **b, d** The 5-year overall survival (*OS*) rate according to *M/T* was 54.2% ($n = 24$) for $M/T < 1$ and 84.6% for $M/T \geq 1$ ($n = 13$, $P = 0.05$), while it was 38.5% for cases with positive ($n = 13$) and 79.2% for those with negative ($n = 24$) cytology findings ($P = 0.01$)

A malignant positive surgical margin indicates that residual tumor cells remain in the margin area, which is related to the *M/T* [7]. Few cases with an $M/T > 1$ have positive margin cytology findings, thus removal of a greater amount of pulmonary parenchyma is associated with a lower potential for relapse. The reported ratio of local recurrence in cases of pulmonary excision for NSCLC varies between 14 and 25% [12–15], with a median of 19%. By contrast, that ratio varies from 2 to 21% in cases of segmentectomy for LSCLC [3, 16–21], with a median of 9%. A segmentectomy provides a lower potential of local recurrence in comparison to a wedge resection, which may contribute to the anatomical sufficiency of a segmentectomy, because a greater volume of pulmonary tissue is removed. Therefore, a segmentectomy provides a greater *M/T* value than a wedge resection and has a lower potential of leaving residual tumor cells at the surgical margin, which may lead to a lower chance of local recurrence. A recent analysis of 188 cases of anatomical segmentectomy for NSCLC by Schuchert et al. [22] reported that 23 (85.2%) of 27 patients with recurrence had an *M/T* value of less than 1 and recurrence rates in those cases were significantly higher than in those with a value greater than 1 (25.0 vs. 6.2%, $P = 0.0014$).

The present study of cases of wedge resection for NSCLC found a 5-year survival rate of 79.6% for those with malignant negative cytology findings, in contrast to 38.5% in those with positive findings. Malignant negative cytology findings can be attributed to a sufficient ratio of margin distance to tumor size ($M/T > 1$), thus pulmonary excision for peripheral NSCLC should result in a malignant negative margin.

The current study is limited by its retrospective nature, and the clinical impact of the margin cytology findings

Table 4 Independent Cox regression hazard model

Variable	*n*	HR	95% CI	*P* value
Age (years)				
≥75	12	Ref.		
<75	25	1.7	0.1–1.9	0.4
Sex				
Female	20	Ref.		
Male	17	1.7	0.6–5.0	0.2
pT				
1	32	Ref.		
2	7	2.0	0.6–6.3	0.2
pN				
N0	28	Ref.		
N1 or N2	1	4.5	0.7–55.6	0.1
Tumor location				
Easy	25	Ref.		
Difficult	12	3.1	1.1–9.0	0.04
Stapling pattern				
A	24	Ref.		
B or C	13	2.0	0.7–5.6	0.2
M/T				
<1	13	Ref.		
≥1	24	0.3	0.06–1.1	0.07
Margin cytology				
Negative	24	Ref.		
Positive	13	4.7	1.6–14.3	0.006

The stapling pattern is the same as that shown in Fig. 1. Easy, easily resectable region (apex, edge, lingual); Difficult, difficult to resect region (large ovoid surface, deep in fissure, basal)

HR hazard ratio, *CI* confidence interval

Table 5 Multivariate Cox regression hazard model

Variable	HR	95% CI	*P* value
Tumor location			
Easy	Ref.		
Difficult	2.1	0.7–6.5	0.2
Margin cytology			
Negative	Ref.		
Positive	3.8	1.2–12.0	0.02

Easy, easily resectable region (apex, edge, lingual); Difficult, difficult to resect region (large ovoid surface, deep in fissure, basal)

should be further investigated in a prospective study. The protocol for a sublobar resection states that it is mandatory to obtain an *M/T* greater than 1 [8], while margin cytology findings have been examined in a prospective study of brachytherapy cases that underwent a wedge resection [23]. Therefore, the importance of obtaining a sufficient margin distance and malignant negative margin is expected to be confirmed in the near future.

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Overexpression of SOCS3 exhibits preclinical antitumor activity against malignant pleural mesothelioma

Kota Iwahori[1,2], Satoshi Serada[1], Minoru Fujimoto[1], Shintaro Nomura[3], Tadashi Osaki[2], Chun Man Lee[4], Hiroyuki Mizuguchi[5], Tsuyoshi Takahashi[1], Barry Ripley[6], Meinoshin Okumura[7], Ichiro Kawase[2], Tadamitsu Kishimoto[6] and Tetsuji Naka[1]

[1] Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan
[2] Department of Respiratory Medicine, Allergy, and Rheumatic Diseases, Osaka University Graduate School of Medicine, Osaka, Japan
[3] Faculty of Animal Bioscience, Nagahama Institute of Bio-Science and Technology, Shiga, Japan
[4] Medical Center for Translational Research, Osaka University Hospital, Osaka, Japan
[5] Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan
[6] Laboratory of Immune Regulation, Osaka University Graduate School of Frontier Biosciences, Osaka, Japan
[7] Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

Malignant pleural mesothelioma (MPM) is an aggressive tumor with poor prognosis for which an effective therapy remains to be established. Our study investigated the therapeutic potential of the suppressor of cytokine signaling 3 (SOCS3), an endogenous inhibitor of intracellular signaling pathways, for treatment of MPM. We infected MPM cells (H226, EHMES-1, MESO-1 and MESO-4) with an adenovirus-expressing SOCS3 (AdSOCS3) to examine the effect of SOCS3 overexpression on MPM cells. SOCS3 overexpression reduced MPM proliferation and induced apoptosis and partial G0/G1 arrest. SOCS3 also inhibited the proliferation of MPM cells *via* multiple signaling pathways including Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), focal adhesion kinase (FAK) and p53 pathways. Notably, AdSOCS3 treatment inhibited tumor growth in an MPM pleural xenograft model. These findings demonstrate that overexpression of SOCS3 has a potent antitumor effect against MPM both *in vitro* and *in vivo* and indicate the potential for clinical use of SOCS3 for MPM treatment.

Malignant pleural mesothelioma (MPM) is an aggressive tumor arising from the mesothelial cells of serosal cavities. MPM may be asymptomatic at the early stage and is sometimes observed incidentally during routine chest radiography. Common symptoms include chest pain and dyspnea, which are caused by tumor invasion of the chest wall or pleural effusion and occur late during disease progression. Although chemotherapy with the drug pemetrexed improves survival time for unresectable MPM patients, the overall median survival time is only 12 months.[1] MPM is often associated with past exposure to asbestos, in which case there is a long latency period, often exceeding 20 years, between first exposure to asbestos and diagnosis of MPM.[2] The number of deaths from MPM is expected to increase in the next 20 years world-wide where heavy use of asbestos has occurred.[2–5] There is thus a growing need for the development of new therapies to treat this disease.

A growing number of studies of MPM tumor biology have established important roles for cytokines involved in tumor growth or the spread of this disease.[6–10] A reported potential molecular target for MPM therapeutics is the interleukin-6 (IL-6)/Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway and

Cancer Therapy

Key words: malignant pleural mesothelioma, suppressor of cytokine signaling 3, gene therapy, signal transducer and activator of transcription 3, p53

Abbreviations: 7-AAD: 7-amino-actinomycin D; DAPI: 4′,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; FCS: fetal calf serum; IL-6: interleukin-6; JAK: Janus kinase; MPM: malignant pleural mesothelioma; MOI: multiplicity of infection; MRA: anti-IL-6 receptor antibody; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; pfu: plaque-forming units; PI: propidium iodide; SH2: Src homology 2; SHP: SH2-domain-containing tyrosine phosphatase; siRNA: small interfering RNA; SOCS3: suppressor of cytokine signaling 3; STAT3: signal transducer and activator of transcription 3; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Grant sponsor: Ministry of Health, Labour and Welfare, Japan, Ministry of Education, Culture, Sports, Science and Technology, Japan
DOI: 10.1002/ijc.25716
History: Received 15 Jun 2010; Accepted 22 Sep 2010; Online 14 Oct 2010
Correspondence to: Tetsuji Naka, Laboratory for Immune Signal, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan, Tel.: +81-72-641-9843, Fax: +81-72-641-9837, E-mail: tnaka@nibio.go.jp

high-level expression of IL-6 in the pleural fluid of MPM patients.[11,12] The binding of IL-6 to its cognate receptor leads to a conformational change in the receptor that initiates the activation of JAK, which in turn activates the transcription factor STAT3 to dimerize and translocate into the nucleus, thus leading to the initiation of target gene transactivation. This pathway is crucial for the occurrence of hematopoiesis, immune response and oncogenesis.[13] Moreover, dysfunction of the regulatory system for the JAK/STAT3 pathway has been demonstrated to be involved in the development of cancer.[13]

The suppressor of cytokine signaling (SOCS) family proteins[14–16] participate in the negative regulation of multiple signaling pathways including the IL-6/JAK/STAT3 signaling pathway,[17–19] while SOCS3 can bind both the cytokine receptor and JAK, thus facilitating inhibition of the JAK molecule.[20] The restoration of SOCS3 expression in several cancer cell lines was found to effectively suppress tumorigenicity.[21] Because the JAK/STAT3 signaling pathway is frequently activated in a wide variety of human malignancies,[22] SOCS3 gene delivery may represent a novel therapeutic strategy for the treatment of human cancers, including mesothelioma.

In addition to the IL-6/JAK/STAT3 signaling pathway, various other signaling pathways are associated with tumorigenesis in MPM. Among these pathways, activators of oncogenic molecules such as the extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK) have been implicated.[23,24] It was reported that SOCS3 participated in the inhibition of ERK phosphorylation and the degradation of FAK.[25–27] SOCS3 can competitively block receptor recruitment of SH2-domain-containing tyrosine phosphatase (SHP-2) to Tyr759 of gp130, thus inhibiting ERK activation. Interactions of SOCS3 with FAK through the Src homology 2 (SH2) domain have been reported to promote polyubiquitination and subsequent degradation of FAK. Because there are multiple abnormalities in signal transduction and genetic differences in individual patients, SOCS3, which, as seen above, is involved in the regulation of multiple signals, is expected to be effective for the treatment of MPM. However, the potential therapeutic benefits of SOCS3 for MPM have not yet been investigated. In the study presented here, we investigated the efficacy of SOCS3 gene delivery for the treatment of MPM.

Material and Methods

Cell lines

Mesothelioma cell lines H28, H226 and H2452 were purchased from American Type Culture Collection (Manassas, VA). Mesothelioma cell line EHMES-1 was kindly provided by Dr. Hironobu Hamada (Ehime University, Ehime, Japan). ACC-MESO-1 (MESO-1) and ACC-MESO-4 (MESO-4) cell lines were purchased from RIKEN BRC cell bank (Tsukuba, Japan). All the cells were cultured in RPMI 1640 (Wako, Osaka, Japan) with 10% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT), 100 IU/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan). Human adult mesothelial cells were purchased from Zen-Bio (Research Triangle Park, NC) and cultured in Mesothelial Cell Growth Medium (Zen-Bio). HEK293 cells were obtained from the JCRB Cell Bank (Tokyo, Japan) and cultured in DMEM (Wako) with 10% FCS (HyClone), 100 IU/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque).

Reagents

Recombinant human IL-6 was kindly provided by Dr. Kazuyuki Yoshizaki (Osaka University, Osaka, Japan), recombinant soluble IL-6 receptor (sIL-6R) and anti-IL-6R monoclonal antibody (tocilizumab, currently known as MRA) were obtained from Chugai Pharmaceutical Co. (Tokyo, Japan). Purified human IgG, purchased from Sigma (St. Louis, MO), was used as control for MRA. JAK inhibitor I and PD98059 were purchased from Calbiochem (La Jolla, CA) and doxorubicin from Wako.

Preparation of adenoviruses

Replication-defective recombinant adenoviral vectors were constructed with the cosmid–adenoviral DNA terminal protein complex method.[28] Adenoviral vectors AdLacZ and adenovirus-expressing SOCS3 (AdSOCS3) were designed to express the LacZ gene and the human SOCS3 gene, respectively, under the control of the CAG promoter (a modified chicken β-actin promoter with a cytomegalovirus immediate early enhancer).[29–31] Solutions of these adenoviral vectors were prepared as described previously and stored at −80°C until use.[32] Adenoviral vectors containing the genes for HA-tagged Y705F dominant-negative STAT3 (AddnSTAT3) were kindly provided by Dr. Akihiko Yoshimura (Keio University, Tokyo, Japan).

X-gal staining

The transduction efficiency of adenoviral vectors was assessed by means of X-gal staining. Cells were cultured in 6-well plates at a density of 1×10^5 cells per well and infected with AdLacZ at the indicated multiplicities of infection (MOIs). X-gal staining was performed 24 hr after infection according to the protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO).

Reverse transcription-polymerase chain reaction analysis

Total RNA of cultured cells was isolated with Sepasol-RNA I (Nacalai Tesque) and cDNAs were synthesized from 500 ng of each total RNA preparation with a Quantitect Reverse Transcription kit (QIAGEN, Valencia, CA), all according to the manufacturers' instructions. The TaKaRa Ex Taq (Takara Bio, Otsu, Shiga, Japan) was used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. β-actin was used as a housekeeping gene to evaluate and compare the quality of different cDNA samples. Primers for β-actin (67°C annealing, 33 cycles) were: forward, 5′-AGCCTCGCCTTTGCCGA-3′;

reverse, 5′-CTGGTGCCTGGGGCG-3′. Primers for SOCS3 (55°C annealing, 33 cycles) were: forward, 5′-TCAAGACCTT-CAGCTCCAAG-3′; reverse, 5′-TTGACGCTGAGCGTGAAGAA-3′. And primers for p53 (60°C annealing, 33 cycles) were: forward, 5′-CCCCAGCCAAAGAGAAACC-3′; reverse, 5′-TCCAAGGCCTCATTCAGCTCT-3′. PCR products were detected by means of 1% agarose gel electrophoresis with ethidium bromide staining.

Small interfering RNA transfection

Commercial JAK1 small interfering RNA (siRNA) was obtained from QIAGEN. Cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nonspecific siRNA (QIAGEN) was used as a negative control, and the selective silencing of JAK1 was confirmed by Western blot analysis.

Measurement of IL-6 and sIL-6R concentrations in culture supernatant

MPM cells were cultured in 6-well plates at a density of 1×10^5 cells per well and incubated in RPMI 1640 medium containing 0.5% FCS. The concentrations of IL-6 and sIL-6R in the 24-hr culture supernatant were measured by means of Quantikine Colorimetric Sandwich ELISA (R&D Systems, Minneapolis, MN).

SDS-PAGE and western blot analysis

Whole cell protein extract was prepared from MPM cells in RIPA buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mmol/L Na_3VO_4, and 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. Extracted proteins were resolved on SDS-PAGE and transferred to an Immobilon-P Transfer membrane (Millipore, Bedford, MA). The following antibodies were used: antiphospho-STAT3, 1:1,000; anticleaved caspase-3, 1:500; antiphospho-ERK, 1:1,000; anti-ERK, 1:1,000; antiphospho-p53 (Ser46), 1:1,000; antiphospho-p53 (Ser392), 1:1,000 (all from Cell Signaling Technology, Danvers, MA), anti-STAT3, 1:1,000; anti-p53, 1:500; anti-GAPDH, 1:1,000 (all from Santa Cruz Biotechnology, Santa Cruz, CA), antiphospho-FAK (Tyr397) (1:1,000; Biosource, Camarillo, CA), anti-JAK1, 1:1,000; anti-FAK, 1:1,000, anti-p21, 1:1,000 (all from BD Transduction Laboratories, San Jose, CA), antiphosphotyrosine (clone 4G10), 1:1,000 (Upstate Biotechnology, Lake Placid, NY) and anti-SOCS3 antibody (1:500; IBL, Gunma, Japan), followed by a 1:5,000 dilution of donkey antirabbit or 1:5,000 dilution of sheep antimouse horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Bio-Sciences, Piscataway, NJ) and visualized with the Western Lightning ECL reagent (Perkin-Elmer, Boston, MA).

Immunoprecipitation

Cells were lysed in ice-cold RIPA buffer. After clearing of the lysate, anti-p53 antibody (Cell Signaling Technology) was added to the lysate followed by overnight incubation at 4°C. Protein G Sepharose (GE Healthcare Bio-Sciences) was then added and incubated by end-over-end mixing for 2 hr. The beads were washed five times in RIPA buffer and analyzed by Western blotting.

Phospho-kinase array

Expression of phosphorylated proteins was detected with the Proteome Profiler^{TM} Human Phospho-Kinase Array kit (R&D Systems). The procedures were performed according to the manufacturer's protocol using 400 μg protein lysate per array.

MTS assay

MPM cell lines were plated in 96-well plates at a density of 1,000 cells per well and incubated in RPMI 1640 medium containing 10% FCS. After a three-day culture, cell proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 aqueous nonradioactive cell proliferation assay; Promega, Madison, WI). MTS color development was measured and analyzed with a microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 450 nm with a reference wavelength of 750 nm. This assay was performed in triplicate.

Apoptosis assay

MPM cells were grown to confluence to attain synchronization in G1 and subcultured at a lower density (1×10^5 cells in a six-well plate) for 24 hr so that most of the cells were in the S phase. Adenoviral vectors were infected by distributing suspensions of AdSOCS3 or AdLacZ onto cells at a MOI of 40, followed by incubation at 37°C for an additional 72 hr. The cells were then trypsinized and collected with the supernatants, followed by determination of cell viability by means of annexin V and 7-amino-actinomycin D (7-AAD) staining (BD Biosciences, San Jose, CA) using the FACSCanto flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR). This assay was performed in duplicate.

Cell cycle assay

MPM cells were grown to confluence to attain synchronization in G1 and subcultured at a lower density (1×10^5 cells in a six-well plate) for 24 hr so that most of the cells were in the S phase. Adenoviral vectors were infected by distributing suspensions of AdSOCS3 or AdLacZ onto cells at an MOI of 40, followed by incubation at 37°C for an additional 24 hr. The cells were then trypsinized and collected with the supernatants, after which the cell cycle was determined by means of propidium iodide (PI) staining according to the instructions for the Cycle Test Plus DNA Reagent kits (BD Biosciences) using the FACSCanto flow cytometer. This assay was performed in duplicate.

TABLE 3. Relationship between ground-glass opacity/tumor disappearance rate and revised maxSUV as predictors for Ly factor, V factor, P factor, N factor, and recurrence status

	Revised maxSUV	Ly permeation (+)/(-)	V invasion (+)/(-)	P invasion (+)/(-)	N metastasis (+)/(-)	Recurrence (+)/(-)
GGO ≤50% and TDR ≤50%	≤1.5	2/46	1/47	3/45	0/48	0/48
(n = 259)	(n = 48)	(4%)	(2%)	(6%)	(0)	(0)
	<1.5 ≤2.5	10/58	14/54	8/60	8/60	3/65
	(n = 68)	(15%)	(21%)	(12%)	(12%)	(4%)
	2.5<	59/84	73/70	43/100	27/116	22/121
	(n = 143)	(41%)	(51%)	(30%)	(19%)	(15%)
>50% GGO or >50% TDR	≤1.5	2/178	1/179	0/180	1/179	2/178
(n = 243)	(n = 180)	(1%)	(1%)	(0)	(1%)	(1%)
	<1.5 ≤2.5	1/46	0/47	1/46	1/46	2/45
	(n = 47)	(2%)	(0)	(2%)	(2%)	(4%)
	2.5<	2/14	3/13	1/15	1/15	0/16
	(n = 16)	(13%)	(19%)	(6%)	(6%)	(0)

maxSUV, Maximum standardized uptake value; *Ly,* lymphatic; *V,* blood vessel; *P,* pleural; *N,* lymph node; *GGO,* ground-glass opacity; *TDR,* tumor disappearance rate.

for overall survival, whereas TDR ($P = .056$) was marginally significant (Figure 4).

We examined the relationships between HRCT findings and maxSUV for predicting tumor invasiveness, nodal metastasis, and recurrence (Table 3). Generally, solid tumors on HRCT with GGO 50% or less and TDR 50% or less had high maxSUV and were more frequently associated with high malignant grade, nodal metastasis, and recurrence. However, solid tumors with lower maxSUV were associated with low malignant grade and far less nodal metastasis and recurrence. Among patients with tumors showing GGO 50% or less and TDR 50% or less, 19% and 15% of those with a revised maxSUV greater than 2.5 had nodal metastasis and recurrence, respectively,

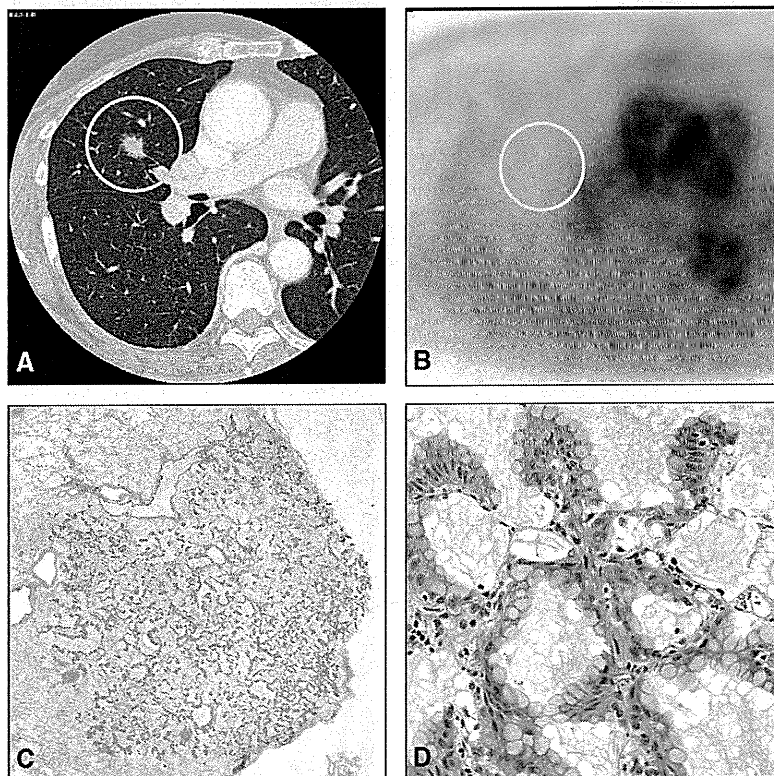

FIGURE 5. Tumor (1.5 cm in diameter) located at right middle lobe. A, HRCT findings show GGO ratio of 5%. B, PET/CT findings show no accumulation. Microscope findings show BAC with mucin formation (C and D, staining with hematoxylin–eosin at ×25 and ×200 magnification, respectively).

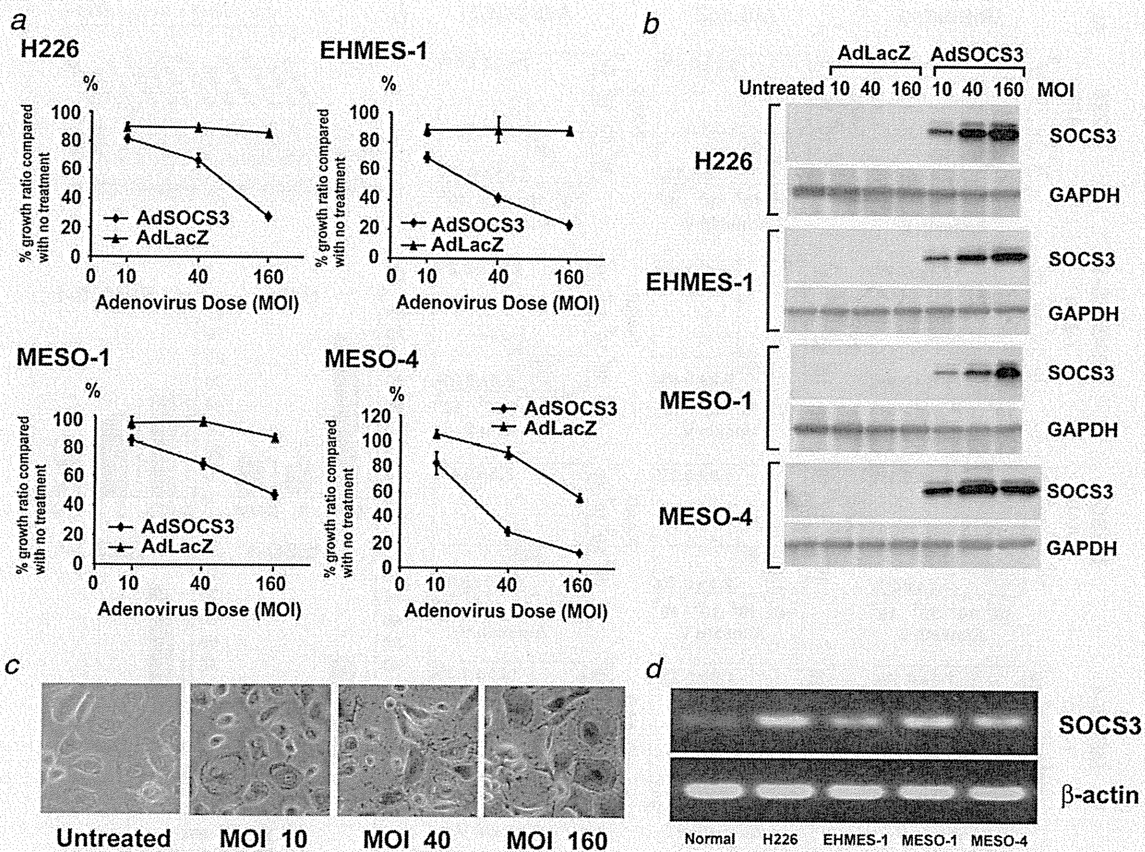

Figure 2. Overexpression of SOCS3 inhibits the growth of MPM cells. (*a*) Growth curves of MPM cells treated with AdSOCS3. Cells were infected with either AdSOCS3 or AdLacZ as control at an MOI of 10–160. Cells were cultured in RPMI 1640 medium containing 10% FCS. After a 3-day culture, viable cell numbers were counted with the MTS assay. Figures show the average (points) of triplicate wells ± SD (bars). (*b*) Expression of SOCS3 as a result of transduction of AdSOCS3. Cells were infected with either AdSOCS3 or AdLacZ as control at an MOI of 10–160. Cells were cultured in RPMI 1640 medium containing 10% FCS. After a 24-hr culture, the cell lysates were analyzed by means of Western blotting using anti-SOCS3 antibody and subsequently with anti-GAPDH antibody. (*c*) Transduction efficiency of the adenoviral vector in H226 cells. H226 cells were infected with AdLacZ at the indicated MOI and stained with X-gal 24 hr after infection. (*d*) Expression of endogenous SOCS3. RT-PCR was used for the assessment of SOCS3 expression.

150 μL of AdSOCS3 or AdLacZ was injected into the thoracic space with the same technique. Twenty-eight days after cell inoculation, the mice were sacrificed and their thoracic spaces examined macroscopically for growths, and tumors detected in the thoracic spaces were removed and weighed.

Immunohistochemistry

Tumors in the thoracic spaces were harvested and paraffin embedded for immunohistochemical analysis using anti-SOCS3 antibody (IBL). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (with DAPI nuclear counterstaining) for apoptosis was performed using the ApopTag® Fluorescein *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions.

Statistical analysis

Data are shown as mean ± SD for the number of experiments indicated. Student's *t*-test was used for comparison of the data. Differences were considered significant at $p < 0.05$.

Results

MRA treatment has no inhibitory effect on the growth of MPM cells

Reports of high-level expression of IL-6 in the pleural fluid of MPM patients prompted us to investigate the role of this signaling pathway in MPM. To characterize IL-6/sIL-6R levels secreted by MPM cell lines, we used sandwich ELISA for quantitation of IL-6/sIL-6R levels in 24-hr culture supernatants. As shown in Figure 1*a*, H226 and EHMES-1 were identified as cell lines with high IL-6/sIL-6R secretion.

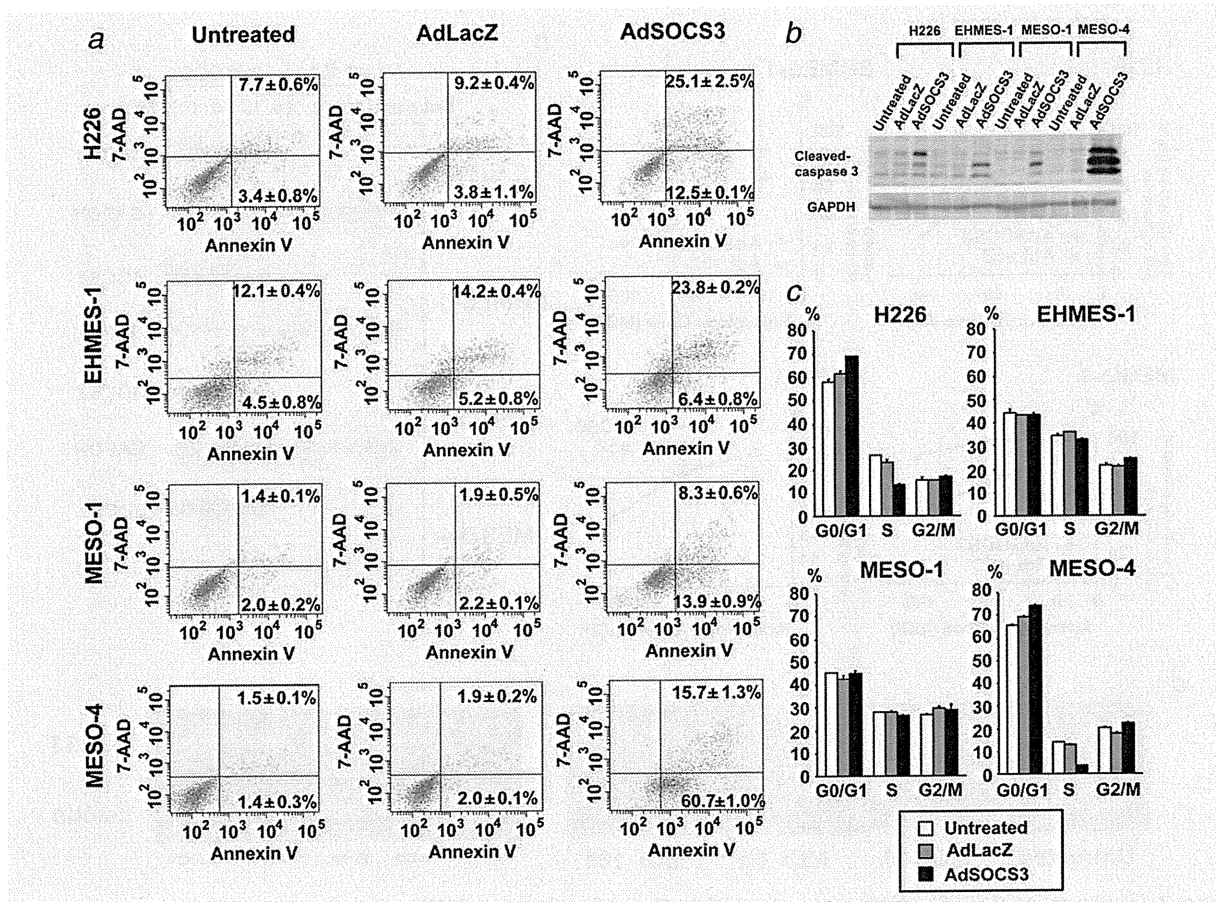

Figure 3. SOCS3 induces apoptosis and G0/G1 arrest in MPM cells. (*a*) Cells were infected with either AdSOCS3 or AdLacZ as control at an MOI of 40. Cells were cultured in RPMI 1640 medium containing 10% FCS for 3 days. Apoptosis was determined by means of annexin V and 7-AAD staining using flow cytometry. Figures show the average of duplicate wells ± SD. (*b*) Cells were infected with either AdSOCS3 or AdLacZ as control at an MOI of 40. Cells were cultured in RPMI 1640 medium containing 10% FCS for 3 days. Whole cell extracts were prepared and immunoblotted with anticleaved caspase-3 antibody. (*c*) Cells were infected with either AdSOCS3 or AdLacZ as control at an MOI of 40. Cells were cultured in RPMI 1640 medium containing 10% FCS for 24 hr. The cell cycle was determined by means of propidium iodide (PI) staining using flow cytometry. Figures show the average (columns) of duplicate wells + SD (bars).

Because it has been reported that IL-6 may represent a therapeutic target for tumorigenesis in MPM cells,[12] we subsequently tested the effect of anti-IL-6R monoclonal antibody (MRA) treatment. For this analysis, we used H226 and EHMES-1 cells which secrete high levels of IL-6. Figure 1*b* shows that MRA did not inhibit cell growth in H226 and EHMES-1 cells. On the other hand, it has been reported that 25 μg/mL of MRA is required for sufficient growth suppression of Lennert's lymphoma-derived T cells which show IL-6–dependent cell growth[12] and we demonstrated that MRA inhibited IL-6-stimulated STAT3 phosphorylation in MPM cells (Fig. 1*c*). These results suggest that, although H226 and EHMES-1 cells secreted high levels of IL-6, IL-6 signaling had little effect on the growth of these cells. However, the role of STAT3 on cell growth in H226 and EHMES-1 cells was not clear since MRA treatment did not inhibit endogenous phosphorylated STAT3 levels in these cells.

Overexpression of SOCS3 inhibits the growth of MPM cells

The JAK/STAT3 pathway is an important signaling pathway negatively regulated by SOCS3. We therefore used a replication-defective recombinant adenoviral vector to investigate the regulation of MPM cell growth by SOCS3. As shown in Figure 2*a*, AdSOCS3 strongly inhibited cell growth in H226, EHMES-1, MESO-1 and MESO-4 cells, while the endogenous SOCS3 expression levels of MPM cells were higher than those of normal mesothelial cells (Fig. 2*d*). This indicates that overexpression of SOCS3 was required for growth inhibition of MPM cells. Because sufficient transduction efficiency of the adenovirus vector and strong expression of

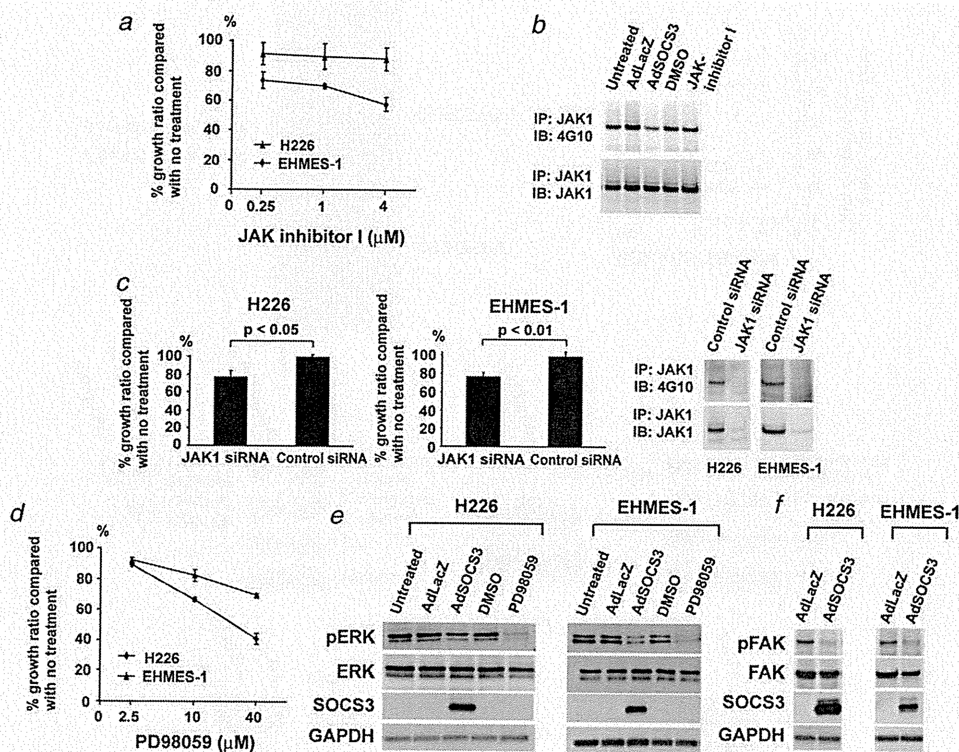

Figure 4. SOCS3 inhibits JAK1, ERK and FAK signaling. (*a*) Growth curves of H226 and EHMES-1 cells treated with JAK inhibitor I. Cells were cultured in RPMI 1640 medium containing 10% FCS with 0.25–4 μM JAK inhibitor I or dimethyl sulfoxide (DMSO) (untreated). After a 3-day culture, viable cell numbers were counted with the MTS assay. Figures show the average (points) of triplicate wells ± SD (bars). (*b*) Inhibition of JAK1 phosphorylation by AdSOCS3. H226 cells were cultured in RPMI 1640 medium containing 0.5% FCS with 1 μM JAK inhibitor I or AdSOCS3 at an MOI of 40. After 24 hr of culturing, 100 ng/mL of IL-6 and 100 ng/mL of sIL-6R were added for 10 min and protein extracts were immunoprecipitated with anti-JAK1 antibody and blotted with antiphosphotyrosine antibody (clone 4G10). (*c*) Growth of H226 and EHMES-1 cells treated with JAK1 siRNA. Cells were treated with either JAK1 siRNA or nonspecific siRNA as control. Cells were cultured in RPMI 1640 medium containing 0.5% FCS, 100 ng/mL of IL-6 and 100 ng/mL of sIL-6R. After a 3-day culture, viable cell numbers were counted with the MTS assay. Figures show the average (columns) of triplicate wells + SD (bars). Protein extracts were immunoprecipitated with anti-JAK1 antibody and blotted with antiphosphotyrosine antibody (clone 4G10) and subsequently with anti-JAK1 antibody. (*d*) Growth curves of H226 and EHMES-1 cells treated with PD98059. Cells were cultured in RPMI 1640 medium containing 10% FCS with 2.5–40 μM PD98059 or dimethyl sulfoxide (DMSO) (untreated). After a 6-day culture, viable cell numbers were counted with the MTS assay. Figures show the average (points) of triplicate wells ± SD (bars). (*e*) Inhibition of ERK phosphorylation by AdSOCS3. H226 and EHMES-1 cells were cultured in RPMI 1640 medium containing 0.5% FCS with 4 μM PD98059 or AdSOCS3 at an MOI of 40. After 24 hr of culturing, protein extracts were blotted with antiphospho-ERK antibody. (*f*) Negative regulation of FAK signaling by SOCS3. H226 and EHMES-1 cells were cultured in RPMI 1640 medium containing 10% FCS with AdSOCS3 at an MOI of 40. After 24 hr of culturing, cells were cultured in RPMI 1640 medium containing 0.5% FCS for an additional 24 hr. Protein extracts were blotted with antiphospho-FAK antibody.

SOCS3 were detected at an MOI of 40 in MPM cells (Figs. 2*b* and 2*c*), we performed subsequent experiments using AdSOCS3 at an MOI of 40.

SOCS3 induces apoptosis and G0/G1 arrest in MPM cells

Next, we investigated the mechanism by which AdSOCS3 inhibited cell growth in H226, EHMES-1, MESO-1 and MESO-4 cells. Since light microscopy findings suggested poor cell viability, apoptosis in these cells was tested by means of annexin V and 7-AAD staining using flow cytometry three days after the addition of AdSOCS3 to the culture. The results of flow cytometric analysis led to the identification of two types of cells: early apoptotic (AnnexinV$^+$7-AAD$^-$) and late apoptotic (Annexin V$^+$7-AAD$^+$). Compared to treatment with AdLacZ, treatment with AdSOCS3 resulted in elevated

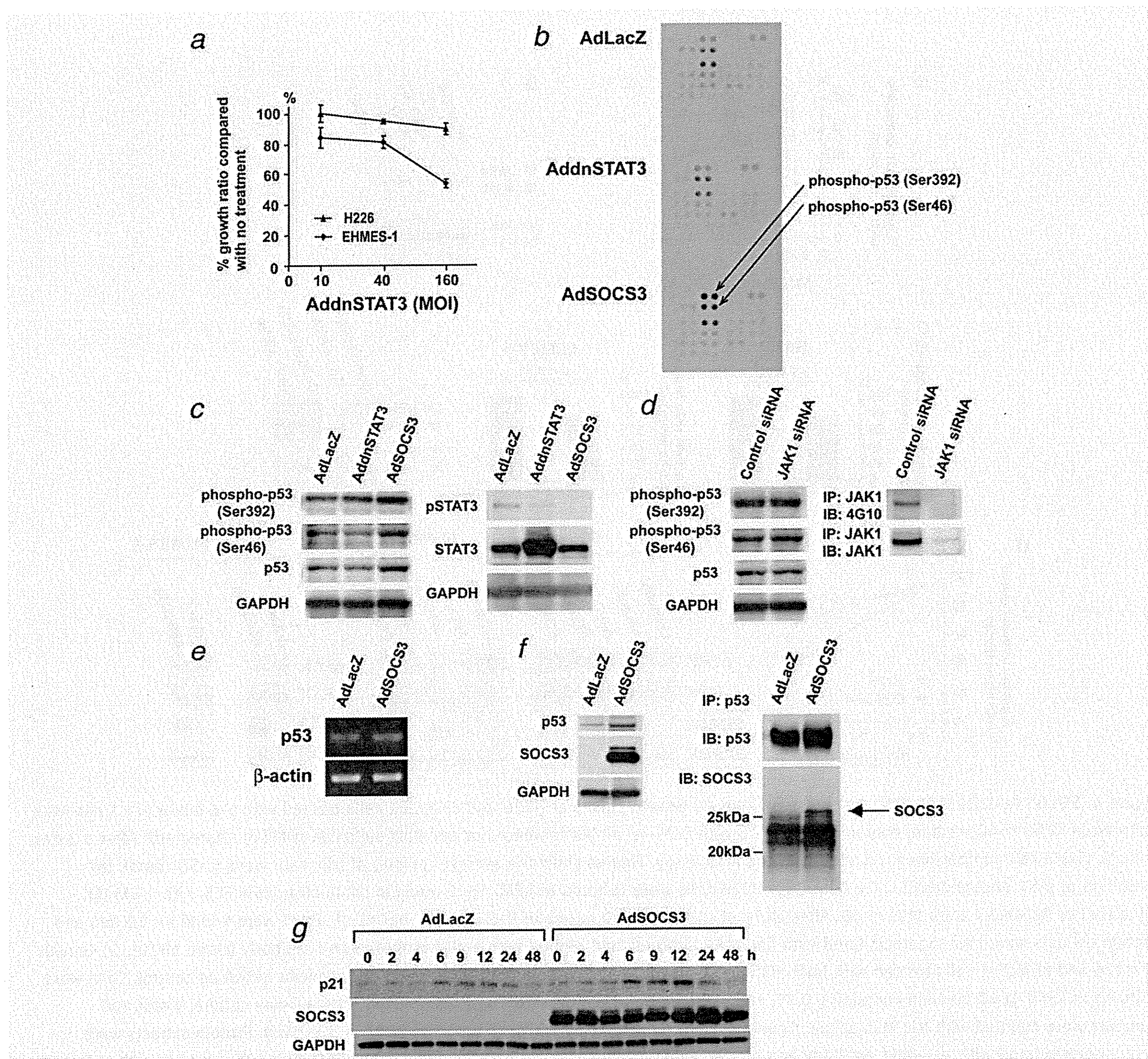

Figure 5. SOCS3 regulates p53 expression in a STAT3-independent manner. (*a*) Growth curves of H226 and EHMES-1 cells treated with AddnSTAT3. Cells were infected with either AddnSTAT3 or AdLacZ as control at an MOI of 10–160. Cells were cultured in RPMI 1640 medium containing 10% FCS. After a 3-day culture, viable cell numbers were counted with the MTS assay. Figures show the average (points) of triplicate wells ± SD (bars). (*b*) Phospho-kinase array revealed that H226 cells treated with AdSOCS3 showed higher levels of phospho-p53 (Ser 392) and phospho-p53 (Ser 46) expression than those treated with AddnSTAT3 or AdLacZ. H226 cells were cultured in RPMI 1640 medium containing 0.5% FCS with AdSOCS3 at an MOI of 40. After 24 hr of culturing, protein extracts were examined with a phospho-kinase array with each phosphorylated protein identified in duplicate. The double-labeled spots in the upper right corner represent the positive controls. (*c*) p53 expression was induced by AdSOCS3. H226 cells were cultured in RPMI 1640 medium containing 0.5% FCS with AdSOCS3 at an MOI of 40. After 24 hr of culturing, protein extracts were probed with antiphospho-p53 (Ser 392), phospho-p53 (Ser 46), p53, phospho-STAT3, STAT3 and GAPDH antibody. (*d*) Influence of JAK1 siRNA on p53 expression. Either JAK1 siRNA or nonspecific siRNA as control was added to H226 cells. Cells were cultured in RPMI 1640 medium containing 0.5% FCS. After 48 hr of culturing, 100 ng/mL of IL-6 and 100 ng/mL of sIL-6R were added for 10 min. The protein extracts were probed with antiphospho-p53 (Ser 392), phospho-p53 (Ser 46), p53 and GAPDH antibody or immunoprecipitated with anti-JAK1 antibody and blotted with antiphosphotyrosine antibody (clone 4G10) and subsequently with anti-JAK1 antibody. (*e*) Expression of p53. RT-PCR was used to determine levels of p53 expression. (*f*) SOCS3 interacts with p53. H226 cells were cultured in RPMI 1640 medium containing 10% FCS with AdSOCS3 at an MOI of 40. After 24 hr of culturing, protein extracts were immunoprecipitated with anti-p53 antibody and immunoblotted with anti-SOCS3 antibody. (*g*) SOCS3 enhances p21 expression. H226 cells were cultured in RPMI 1640 medium containing 10% FCS with AdSOCS3 at an MOI of 40. After 12 hr of culturing, H226 cells were treated with doxorubicin (300 ng/mL) for 0–48 hr. Protein extracts were immunoblotted with anti-p21 antibody.

apoptosis in both early and late apoptotic subsets in H226, EHMES-1, MESO-1 and MESO-4 cells (Fig. 3*a*). Furthermore, cleaved caspase-3, one of the key molecules in apoptosis, was detected in whole cell extracts of H226, EHMES-1, MESO-1 and MESO-4 cells treated with AdSOCS3 (Fig. 3*b*). We therefore conclude that AdSOCS3 induced apoptosis in MPM cells.

In addition to apoptosis, cell cycle regulation is an important mechanism for inhibition of cell growth. To analyze the effect of AdSOCS3 on cell cycle regulation, we infected H226, EHMES-1, MESO-1 and MESO-4 cells with AdSOCS3 and then analyzed cell cycle distribution by means of flow cytometry. When H226 and MESO-4 cells were treated with AdSOCS3, the G0/G1 cell population increased more than in those treated with AdLacZ (Fig. 3*c*).

SOCS3 inhibits JAK1, ERK and FAK signaling

One of the important signaling pathways regulated by SOCS3 is the JAK/STAT3 pathway. We used JAK inhibitor I, which suppresses JAK2 signaling, to investigate the regulation of cell growth by JAK signaling pathways. For this assay, we used H226 and EHMES-1 cell lines (on which MRA had no growth inhibitory effect) to investigate the IL6-independent growth inhibitory effect by SOCS3. As shown in Figure 4*a*, only the growth of EHMES-1 cells, and not of H226 cells, was inhibited by JAK inhibitor I. This suggests that AdSOCS3 inhibited cell growth in H226 cells *via* signaling pathways which were not inhibited by JAK inhibitor I.

To identify the differences between the inhibitory effects of AdSOCS3 and JAK inhibitor I on JAK signaling pathways, we studied changes in the phosphorylation status of JAK1 in H226 cells treated with AdSOCS3 and JAK inhibitor I. Figure 4*b* shows that AdSOCS3 suppressed IL-6–stimulated JAK1 phosphorylation, but that JAK inhibitor I did not affect it. These results lead us to propose that SOCS3 inhibited cell growth in H226 in part by means of negative regulation of JAK1.

We used JAK1 siRNA to evaluate the role of JAK1 signaling pathways in cell growth. As seen in Figure 4*c*, JAK1 siRNA inhibited cell growth in H226 and EHMES-1 cells, suggesting that the growth of H226 cells was partially regulated by JAK1 signaling.

Because it has been reported that SOCS3 inhibits the ERK and FAK signalling pathways, we examined these pathways in H226 and EHMES-1 cells. Figure 4*d* demonstrated that the growth of H226 and EHMES-1 cells was inhibited by the ERK inhibitor PD98059, while ERK phosphorylation was inhibited by AdSOCS3 in these cells (Fig. 4*e*). In addition, AdSOCS3 inhibited phospho-FAK and FAK in H226 and EHMES-1 cells (Fig. 4*f*). We therefore conclude that overexpression of SOCS3 regulates multiple signaling pathways in MPM cells.

SOCS3 regulates p53 expression

We next investigated the role of STAT3 in H226 and EHMES-1 cells and used AddnSTAT3 to examine the regulation of cell growth by STAT3 signaling pathways. As seen in Figure 5*a*, AddnSTAT3 inhibited cell growth in EHMES-1 cells but not in H226 cells. Since this suggests that STAT3 is not involved in the growth of H226 cells, we focused our study on signaling pathways independent of STAT3.

To identify the target molecules of SOCS3 in STAT3-independent pathways, we used a phospho-kinase array to evaluate the expression profile of phosphorylated proteins in H226 cells. Figure 5*b* shows that expression of phospho-p53 (Ser 392) and phospho-p53 (Ser 46) in H226 cells after treatment with AdSOCS3 was higher than in those treated with either AddnSTAT3 or AdLacZ. In addition to phospho-p53, p53 was also highly expressed when treated with AdSOCS3 (Fig. 5*c*). These results suggest that SOCS3 regulated p53 expression.

We next investigated whether JAK1 regulates p53 expression. To this end, we used JAK1 siRNA for the transfection of H226 cells and Western blotting for the examination of p53 expression. As shown in Figure 5*d*, silencing of JAK1 did not influence p53 expression, indicating that JAK1 and p53 are regulated by SOCS3 *via* independent pathways.

Since transfection with AdSOCS3 did not enhance transcription of p53 in H226 cells (Fig. 5*e*), we next investigated whether SOCS3 interacts with p53. After transfection of AdSOCS3 into H226 cells, we detected SOCS3 in the immunoprecipitate of a p53-specific antibody (Fig. 5*f*). In addition, SOCS3 enhanced the expression of p21 which was the target of p53 (Fig. 5*g*). Taken together, these findings suggest that SOCS3 interacts with p53 protein.

SOCS3 exhibits antitumor activity in a mesothelioma xenograft model

We also evaluated the therapeutic effect of AdSOCS3 injection on the growth of subcutaneously or intrathoracically implanted MPM cells in ICR *nu*/*nu* mice. Of the MPM cell lines used in our study, we were able to establish H226 and MESO-4 xenograft models. Injection of AdSOCS3 vector (1×10^8 pfu/50 μL) intratumorally twice per week reduced tumor volumes compared to tumor volumes in control AdLacZ-injected animals (Fig. 6*a*). Preliminary experiments revealed that when 1×10^6 MPM cells (H226 or MESO-4) were inoculated into the thoracic space, dissemination of tumors was observed in all mice 28 days after cell implantation. Injection of the AdSOCS3 vector (5×10^7 pfu/150 μL) into the thoracic cavity 7, 14 and 21 days after the implantation of 1×10^6 MPM cells (H226 or MESO-4), reduced the weight of tumor nodules compared to the weight of those in control AdLacZ-injected animals (Figs. 6*b* and 6*c*). Finally, Western blot and immunohistochemical analysis indicated that SOCS3 was overexpressed and induced apoptosis in the

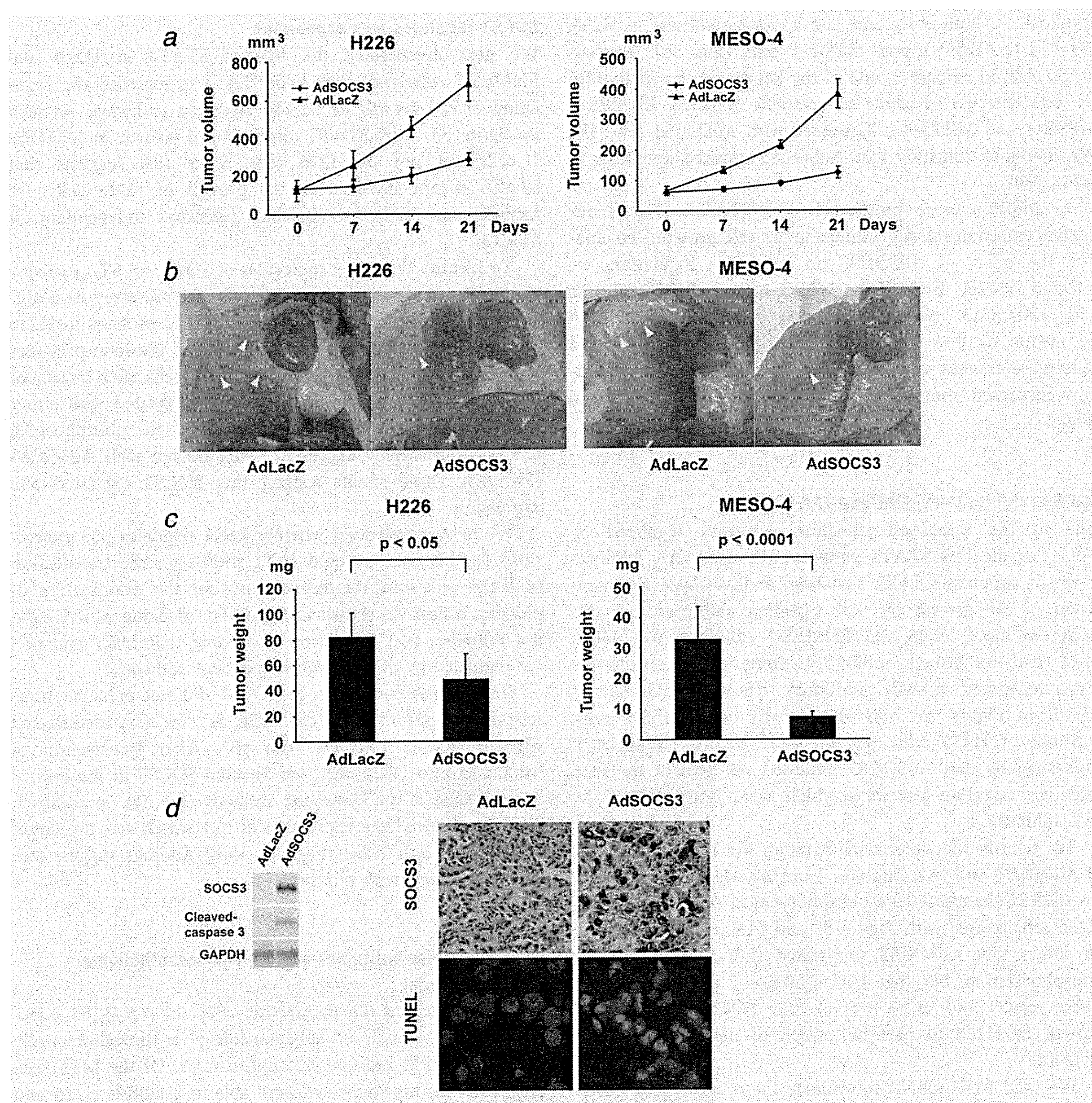

Figure 6. SOCS3 exhibits antitumor activity in a mesothelioma xenograft model. (*a*) Female ICR *nu*/*nu* mice were intratumorally treated with 1×10^8 pfu of AdSOCS3 or AdLacZ twice per week after the implantation of 3×10^6 H226 or MESO-4 cells subcutaneously in the flank of mice. Tumor volumes were determined weekly. Figures show the average (points) for five animals ± SD (bars). (*b*) Gross appearance of H226 and MESO-4 tumors grown orthotopically in the thoracic spaces. Female ICR *nu*/*nu* mice were intrathoracically treated with 5×10^7 pfu of AdSOCS3 or AdLacZ for 7, 14 and 21 days after the implantation of 1×10^6 H226 or MESO-4 cells into the pleural space. After 28 days of tumor cell inoculation, the animals were sacrificed and pleural dissemination of the tumor cells was assessed. (*c*) Each tumor nodule found in the thoracic spaces was also weighed. Figures show the average (columns) for eight animals + SD (bars). (*d*) Western blot analysis of SOCS3 and cleaved caspase-3 in H226 tissue from AdSOCS3-injected animals (left panel). The H226 cell bearing animals were treated with AdSOCS3 on day 7 and sacrificed on day 10. The thoracic tumors were analyzed by Western blot. Immunohistochemical analysis of SOCS3 and TUNEL (blue fluorescence = DAPI staining for nuclei; cyan fluorescence = TUNEL positivity) in H226 tissue from AdSOCS3-injected animals (right panel). The animals were treated in the same way as described above.

H226 tissue from AdSOCS3-injected animals (Fig. 6*d*). Immunohistochemical analysis of SOCS3 overexpression and apoptosis in MESO-4 tissue could not be clearly determined, however Western blot analysis showed identical results to that observed in H226 tissue (data not shown). From these results, we conclude that SOCS3 exhibits antitumor activity not only *in vitro* but also *in vivo* in the MPM model. We hope that these findings may lead to the successful clinical application of SOCS3 for MPM treatment.

Discussion

Malignant mesothelioma represents a great challenge to both clinicians and researchers due to its poor prognosis and remarkable resistance to current therapies. Although there have been some improvements in treatment over the past few years, a better understanding of the molecular basis of the disease and of how to improve treatment is required. Among molecular targeted therapies, recently developed tyrosine kinase inhibitors have been tested for MPM but without therapeutic benefit. This is partially explained by the fact that multiple receptor tyrosine kinases are frequently activated in most MM cells.[23] In our study, we showed that SOCS3 inhibited the proliferation of MPM cells through multiple signaling pathways including JAK/STAT3, ERK, FAK and p53 pathways. We observed that SOCS3 did not influence the expression and activation of p38, JNK, Akt or GSK3β proteins in H226 and EHMES-1 cell lines (data not shown). Specifically, we were able to demonstrate that AdSOCS3 inhibits MPM progression in a mouse pleural xenograft model. These data provide new insights into the clinical application of SOCS3 gene delivery for the treatment of MPM.

We also provided evidence that MRA had little effect on proliferation of MPM cells. A recent study by Adachi *et al.*[12] found that MRA is capable of blocking IL-6 signaling and suppresses the cell growth of MPM induced by IL-6/sIL-6R. We hypothesize that MRA was not able to inhibit proliferation of these cells because it did not inhibit signals from other cytokines acting through gp130 or endogenous activated molecules downstream of gp130 involved in proliferation of these cells.

There are several JAK inhibitors, including JAK inhibitor I,[33] but these inhibitors inhibit JAK1 less than they do other JAK family molecules. This may explain why, although JAK1 is involved in H226 cell proliferation, JAK inhibitor I had little effect on proliferation of H226 cells. SOCS3, however, is an effective JAK1 inhibitory molecule and also inhibits proliferation of H226 cells. It has further been reported that JAK2 inhibitors have antitumor effects on various cancer cells.[34] Because of its pan-JAK inhibitory effect,[20] SOCS3 appears to be a promising antitumor molecule.

We were able to show that SOCS3 regulated phospho-p53 (Ser392 and Ser46) and total p53 expression. Functional inactivation of the p53 pathways appears to be a critical requirement for the development of several human cancers.[35] In spite of the fact that mutations in p53 are among the most commonly acquired genetic lesions seen in cancers, p53 mutations are rarely seen in MPM including H226 cells.[36] It has been reported that Ser392 phosphorylation may regulate the oligomerization of p53 and thus its sequence-specific DNA binding,[37,38] while phosphorylation of Ser46 has been implicated in the activation of p53-dependent apoptotic responses.[39,40] The most thoroughly characterized downstream target of p53 activation is the induction of p21 expression, with p21 playing a critical role in the cell cycle checkpoint.[41] In our study, moreover, SOCS3 enhanced p21 expression and induced apoptosis and G0/G1 arrest in MPM cells. In view of these results, we propose that SOCS3 induces apoptosis as well as G0/G1 arrest partially through the p53 pathways in MPM cells.

Recently, it has been reported that SOCS1 activates p53 *via* a direct interaction between the SH2 domain of SOCS1 and the N-terminal transactivation domain of p53,[42] while we were able to show that SOCS3 did not enhance transcription of p53 but interacted with p53. It is thus conceivable that the observed interaction of SOCS3 with p53 may in turn enhance p53 protein stability. Such a mechanism may therefore involve the inhibition of interaction of p53 with other proteins that promote p53 protein degradation.

In our study, we used adenoviral SOCS3 gene transfer to the thoracic cavity in a mouse xenograft pleural tumor model to provide evidence of a potent antitumor effect of SOCS3 *in vivo*. Because MPM locates within the thoracic cavity and rarely displays widespread metastasis, gene transfer to the thoracic cavity makes this tumor uniquely accessible, thus facilitating the direct administration of novel therapeutic agents and subsequent analysis of treatment effects. Clinical trials involving intrapleural administration of adenoviral vectors to MPM patients have demonstrated that intrapleural gene therapy using adenoviral vectors is safe and well tolerated by MPM patients.[43,44]

In conclusion, we demonstrated the antitumor effect of SOCS3 against MPM both *in vitro* and *in vivo*. The results of clinical application of SOCS3 for MPM treatment are eagerly anticipated.

Acknowledgements

This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan (T. Naka), a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (K. Iwahori) and a grant from the Kansai Biomedical Cluster Project in Saito, which is promoted by the Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology, Japan (T. Naka). We wish to thank Akihito Yokoyama (Kochi University, Kochi, Japan) and Hironobu Hamada (Ehime University, Ehime, Japan) for providing helpful comments on this article, and Namiko Kawakami and Yukako Ito for their secretarial assistance.

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A Review of Prognostic Factors in Thymic Malignancies

Frank Detterbeck, MD, Samuel Youssef, MD,* Enrico Ruffini, MD,† and Meinoshin Okumura, MD‡*

Key Words: Prognostic factor, Thymoma, Thymic carcinoma.

(*J Thorac Oncol.* 2011;6: S1698–S1704)

Prognosis is a central piece of information we would like to know when someone is diagnosed with a disease. Prognosis is affected by many factors. For a cancer, one important aspect is the anatomic extent of the tumor, typically described by a stage classification. Nevertheless, a stage classification system should not be confused with a system to predict prognosis—tumor stage is only one aspect. Prognosis is affected by the treatment given, by patient-specific factors (i.e., comorbidities), tumor-related factors, and others, collectively known as prognostic factors.

The International Thymic Malignancy Interest Group (ITMIG) is currently engaged in the development of a validated formal stage classification system for thymic malignancies, together with the International Association for the Study of Lung Cancer and under the auspices of the Union International Contre le Cancer (UICC) and American Joint Commission on Cancer (AJCC). Assessment of prognostic factors is an important corollary to development of an anatomic stage classification system. A review of the current literature identifying prognostic factors is an important baseline to have for this initiative.

The study and classification of prognostic factors, in general, is very rudimentary. Often there is confusion because people are not clear about the outcome in question: a factor may be prognostic relative to overall survival, to cure from the disease, to response to treatment, etc. Prognostic factors are often specific to a subgroup of patients and a particular setting: factors associated with prognosis for a patient with thymoma undergoing surgery may be different from that of a patient with thymic carcinoma undergoing chemotherapy and radiation. Prognostic factors can be divided into "domains" of tumor-related factors, host factors (i.e., that would be present in the patient even if there was no tumor), and environmental factors (including things such as access to optimal care). Furthermore, once we identify a prognostic factor, we immediately begin trying to change the outcome to be what we want to see (i.e., to undermine the predictive value of the prognostic factor). Finally, there are many statistical pitfalls and errors in the definition of prognostic factors, as discussed in another article in this issue.[1]

Despite deficiencies in our level of sophistication about the science of prognostic factors, the need to estimate future outcomes for patients is great. This article sets out to provide a review of the current state of affairs in the area of thymic malignancies.

METHODS

A search was conducted for English language articles reporting prognostic factors for survival or recurrence for thymoma or thymic carcinoma published from January 1, 1980, to December 31, 2010. This was supplemented with a review of reference lists of retrieved articles, recent book chapters and review articles, and articles identified independently by the authors. We did not evaluate prognostic factors relative to outcomes other than overall survival or recurrence mainly because there are only a few isolated studies.

Recurrence is probably the best measure of outcomes for thymic malignancies, as discussed in a recent landmark ITMIG article.[2] Unfortunately, few studies have addressed this. We included studies reporting disease-free survival in the analysis for recurrence, even though it is problematic to view a recurrence and (unrelated) death as equal end points.[2] Overall survival has been the most commonly used end point because it is easy to measure. Nevertheless, is not an ideal end point, because many patients with thymoma die of unrelated causes, and patients may survive for many years with recurrent disease.[2] Different studies have used different measures (e.g., overall survival and thymoma-specific survival), which can give quite different survival results. Nevertheless, as the analyses were designed to evaluate the relative prognostic value of different factors within a study, it is reasonable to combine the prognostic factor results from studies involving various survival measures.

A valid prognostic factor must have independent significance and, therefore, must be evaluated in conjunction with other factors. Because of this, only studies that reported multivariate analysis (MVA) were included. No restriction was placed on the method of MVA or the number or nature of factors considered. Nevertheless, only articles reporting on ≥75 patients were included. This is because a rough estimate suggests that at least 75 patients are needed to be

*Yale University School of Medicine, New Haven, Connecticut; †Department of Thoracic Surgery, University of Torino, Torino, Italy; and ‡Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Frank C. Detterbeck, MD, Yale Thoracic Surgery, 330 Cedar St. BB205, PO Box 208062, New Haven, CT 06520-8062. E-mail: frank.detterbeck@yale.edu

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ISSN: 1556-0864/11/0607-1698

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able to assess three factors with a medium effect size at a power of 80%.[2]

We included studies reporting on a broad group of patients, who were mostly treated with surgery and sometimes additional modalities. To have an assessment of the patients involved, we included the rates of treatment with different modalities in the tables. Separate consideration of particular subgroups and settings in these studies is not possible, partly because of how the reported studies were done, and partly because the rarity of the disease precludes an appropriate statistical analysis of many subgroups. Furthermore, studies reporting on a specific cohort are so limited that it is difficult to draw any conclusions. Nevertheless, several studies reported an analysis including all patients and only resected patients; data from both were included.

We excluded duplicate publications or articles involving smaller cohorts that were subsequently also included in a larger updated cohort. Some institutions reported results on partially overlapping cohorts in which each article included some unique patients; in this case, we included both articles even though there was substantial overlap.

Data were extracted into tables, and factors were recorded as positive if reported to have a p value of ≤ 0.05 by MVA. Depending on how a MVA is conducted, there may be a high chance of false-positive results.[2] For example, the way a variable is divided (e.g., dichotomized) into groups for analysis also often results in an overly optimistic assessment of statistical significance (e.g., choosing the threshold by the best separation and then entering the variable into the multivariate model this way, which is known as "double-dipping").[1,2] As a crude approach to assessment of this risk, we grouped those studies in the tables in which dichotomization seems to have been done in a way associated with a risk of false-positive designation of a prognostic factor. It was not feasible to go beyond this to assess the actual risk.

There is also a chance of false-negative results, especially in studies of limited size, because the power of detection is low. Data were recorded as negative if reported as such with a p value of more than 0.05. A crude estimate of the ability to detect a difference according to the size of the study, number of factors analyzed, and the magnitude of the difference can be made.[2] We established a priori that to be counted as a negative result the study should have, as a minimum by this crude estimate, a power of detection of 80% for a medium effect size. In the end, however, we found that all studies of more than 75 patients satisfied this requirement.

Not all studies analyzed the same factors. Although the assessment of significance can be strongly influenced by which factors are left out of the analysis, no attempt was made to correct for this. We sought to be as inclusive as possible, and therefore, data were retrieved and accepted as reported. The reported data were recorded as positive, negative, or not assessed to provide a general overview of reported results. Furthermore, most reported series chose to dichotomize variables but not always at the same threshold. In an effort to be inclusive, we tried to combine the data if the thresholds were reasonably similar (and noted differences in the footnotes of the tables).

Some studies reported several multivariate analyses (e.g., by including or not including some factors or using different definitions). Again, to be inclusive and in the absence of criteria to define one as more valid than another, we included each as a separate entry in the tables. In addition, multivariate analyses in several of the studies were specifically done or redone excluding a particular factor because it had a dominant effect or was correlated to another factor, to study the value of a particular factor of interest.[3–8] This would seem to undermine the purpose of MVA to sort out which factors have the most important influence on survival and bias the study toward finding prognostic value (falsely?) in the factor of interest. When such studies did not report an analysis including all factors, we were forced to include all subset analyses (e.g., stage without histology or histology without stage). By including all subset analyses, we hoped to represent a more unbiased overall picture. Nevertheless, this illustrates the potential for bias in how studies are conducted. One study was excluded because a separate MVA was reported for practically each factor, tailored to exclude other factors that "hid" the significance of the factor of interest.[3] Because this study reported no overall MVA, the reported results were felt to be biased and were reported without sufficient detail to even be sure what had or had not been included in each.

To summarize the data, we calculated the percentage of multivariate studies that found a factor to be significant out of the number that analyzed it. This should be interpreted cautiously and qualitatively, because of the many reasons for false-positive and false-negative results that we cannot sort out from this literature review. Factors that were addressed in less than five and less than three studies of overall survival or recurrence, respectively, are not listed in the tables because the data are too limited to draw any meaningful conclusions.

RESULTS

The literature search and inclusion criteria outlined earlier resulted in a total of 29 studies reporting a MVA of prognostic factors for survival and 12 reporting this for recurrence. A few studies[4,8,9] reported more than one analysis; each of these analyses were included in the tables (in accordance with the criteria outlined in the Methods section). The results from the included studies are summarized in Tables 1 and 2 and Figures 1 and 2.

The patients included are not clearly described in most of studies. Nevertheless, they can be loosely thought of as the patients presenting to an institution for curative-intent treatment. Data regarding the treatment received are summarized in the tables. The majority of patients underwent surgery, approximately half also received radiotherapy (RT), and approximately 25% chemotherapy. A few studies deserve special mention: de Jong et al.[11] involved a population-based cohort in the Netherlands, Cowen et al.[22] involved only patients treated with RT, and in the series by Lucchi et al.,[25] all patients had myasthenia gravis (MG).

The factor most consistently identified as significant—for both recurrence and survival—is the stage. In most of studies, this was dichotomized as stages I and II versus III

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TABLE 1. Multivariate Analysis of Factors Predicting Better Survival

Study	n	Treatment (%)			Factors Predicting Better Survival								No. of Additional Factors
		R_0	Ch	RT	Stage I, II	R_0	Hist Thym	Hist w/TC	Older Age[a]	Small Size	Male Gender	MG	
Studies with more robust statistical approaches													
Ruffini 10[10,b]	255	87	2	45	0.001	—	NS	—	—	—	—	NS	—
de Jong 08[11,c]	232	41	10[d]	33[d]	0.01	—	—	<0.001	*<0.001*[e]	—	NS	NS	1
Rieker 02[6]	218	77	14	39	<0.001	—	—	<0.03	NS	NS	NS	NS	2
Park 04[12]	150	69	—	—	<0.001	—	—	<0.02	NS	—	NS	NS	—
Venuta 97[13,b]	148	—	33[d]	—	0.0001	—	NS[f]	—	0.001	—	—	—	2
Park 04[12]	133	77	—	—	0.006	NS	—	NS	NS	—	<0.04	NS	—
Kim 05[7,g]	108	82	16	28	<0.03	NS	—	NS	NS	NS	NS	NS	—
Studies with less robust or unclear statistical approaches													
Kondo 03[14,b]	1093	—	—	—	<0.001	<0.001	NS[f]	—	NS	—	NS	NS	3
Margaritoria 10[15]	317	93	—	38	NS	0.001	—	NS	NS	—	NS	NS	2
Regnard 96[9]	307	85	6	52	NS	0.00001	—	NS[f]	—	—	—	NS	—
Lewis 87[16,b,g]	283	83	2	26	<0.05	<0.05	NS[f]	—	<0.05	NS	NS	NS	5
Regnard 96[9]	260	100	6	52	0.00001	—	—	NS[f]	—	—	—	NS	—
Okumura 02[17,b,g]	243	95	10[d]	60[d]	<0.0001	NS	0.05	—	NS	—	NS	NS	1
Ströbel 04[18,g]	228	67	17	32	<0.05	<0.05	—	<0.05	NS	NS	NS	NS	1
Fang 05[19]	204	88	—	—	<0.001	0.004	—	0.001	—	—	—	NS	—
Lee 07[20]	195	83	5	40	<0.001	NS	—	<0.001	NS	—	NS	NS	—
Rena 05[21,b]	178	84	13	43	<0.04[d]	<0.02	<0.03	—	NS	—	NS	NS	1
Cowen 95[22,b,h]	149	42	50	100	NS	(0.003)[i]	—	—	0.013	0.001[j]	—	NS	1
Wilkins 99[23]	136	68	7	37	NS[k]	<0.001	—	0.02[f]	*0.036*[e]	—	NS	0.005	4
Nakagawa 03[4,b,g]	130	95	4	5	<0.01	NS	—	—	NS	0.01	NS	NS	—
Nakagawa 03[4,b,g]	130	95	4	5	—	0.002	0.01	—	NS	0.001	NS	NS	—
Rea 04[24]	132	82	18	47	0.003	NS	—	0.0001	NS	—	NS	NS	2
Lucchi 09[25,b,l]	123	95	17	73	0.04[m]	—	NS	—	NS	NS	NS	—	2
Blumberg 95[26]	118	73	32	58	0.003	0.0006	—	0.004[f]	NS	0.0001	NS	NS	—
Pan 94[27,b]	112	80	—	—	<0.05	—	NS	—	—	—	—	—	—
Quintanilla 94[28,b]	105	100	0	24	<0.05	—	<0.05[f]	—	NS	NS	NS	NS	1
Zisis 05[8,b]	104	100	14	63	—	—	0.05	—	NS	—	NS	NS	2
Zisis 05[8,b]	104	100	14	63	<0.05	—	—	—	*<0.02*[e]	—	NS	NS	2
Kondo 04[29]	100	84	28	37	0.04	<0.05	—	NS	NS	—	NS	NS	—
Kim 10[30,n]	100	79	7	67	NS	—	NS	—	—	NS	—	—	—
Kim 08[31,h]	100	78	45	100	0.04	NS	—	0.02	<0.03	—	NS	NS	4
Chalabreysse 02[5]	90	67	3	12	—	—	—	<0.001	NS	—	—	NS	—
Rieker 07[32]	77	74	30[d]	62	NS	0.001	—	0.001	NS	—	NS	NS	1
Summary: % positive[o]					**83%**	**63%**	**42%**	**67%**	**15/11%**[p]	**36%**	**4%**	**3%**	

Inclusion criteria: Studies from 1980 to 2010 of ≥75 patients reporting multivariate analysis of prognostic factors. Factors evaluated by <5 studies are not listed. Studies with "less robust statistical approaches" have used "double-dipping" methods to define dichotomization that carry a risk of a false-positive identification of a statistically significant result.

[a] Variously defined as >30 (Lewis, Cowen), >60 (Venuta), >47 (Park), >57 (Wilkins, Kondo, Rieker), >52 (Rena) or unspecified.
[b] Thymic carcinoma excluded.
[c] Population-based study.
[d] Estimated, not specifically reported.
[e] In this series, older age groups had significantly worse survival.
[f] Older, non-World Health Organization classification.
[g] Thymoma-specific survival.
[h] RT-based series.
[i] Biopsy only vs. resection .
[j] Defined as no mediastinal compression.
[k] Stage I vs. II–IV.
[l] MG-based series.
[m] Definition unclear.
[n] For thymoma type B only.
[o] Excluding values in parentheses.
[p] Associated with better survival in 15% and worse survival in 11%.
Ch, chemotherapy; Hist, histologic type; MG, myasthenia gravis; NS, not significant; R_0, complete resection; RT, radiotherapy; w/TC, with thymic carcinoma; Thym, thymoma.

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TABLE 2. Multivariate Analysis of Factors Predicting Lower Rates of Recurrence or Disease-Free Survival

		Treatment (%)			Factors Predicting a Lower Recurrence Rate								
Study	***n***	**% R_0**	**Ch**	**RT**	**Stage I, II**	**R_0**	**Hist Thym**	**Hist w TC**	**Older Age**	**Small Size**	**Gender**	**MG**	**No. of Additional Factors**
Studies with more robust statistical approaches													
Ruffini 10[10a,b]	255	87	2	45	0.001	—	NS	—	—	—	—	NS	—
Rieker 02[6a]	218	77	14	39	<0.05	—	—	<0.05	NS	NS	NS	NS	2
Wright 05[33]	179	90	—	—	<0.0001	NS	—	0.003	NS	0.001	NS	NS	3
Huang 09[34]	112	73	67	43	(NS)[c]	<0.001	—	0.006	NS	NS	NS	—	2
Studies with less robust or unclear statistical approaches													
Margaritoria 10[15a]	317	93	—	38	<0.001	NS	—	NS	NS	—	NS	NS	2
Margaritoria 10[15a]	295	100	—	36	<0.001	—	—	0.003	NS	—	NS	<0.0001	2
Rena 05[21a,b]	178	84	13	43	0.01	0.0001	<0.02	—	NS	—	NS	NS	1
Cowen 95[22b,d]	149	42	50	100	0.04	(0.003)[e]	—	—	0.006	0.001[f]	—	NS	1
Blumberg 95[26]	118	73	32	58	0.03	NS	—	NS	NS	NS	NS	NS	—
Quintanilla-Martinez[28a,b]	105	100	0	24	0.03	—	0.03[g,h]	—	NS	NS	NS	NS	1
Kondo 04[29a]	100	84	28	37	0.002	0.05	—	NS	NS	—	NS	NS	—
Kim 10[30a,b,i]	100	79	7	67	0.002	—	NS	—	—	0.002	—	—	—
Kim 08[31a,d]	100	78	45	100	0.04	NS	—	0.01	NS	—	NS	NS	3
Summary: % positive[j]					**100%**	**43%**	**50%**	**63%**	**9%**	**43%**	**0%**	**9%**	

Inclusion criteria: studies from 1980 to 2010 of ≥75 patients reporting multivariate analysis of prognostic factors. Factors evaluated by <3 studies are not listed. Studies with "less robust statistical approaches" have used "double-dipping" methods to define dichotomization that carry a risk of a false-positive identification of a statistically significant result.

[a] Disease-free survival.
[b] Thymic carcinoma excluded.
[c] III vs. IV.
[d] RT-based series.
[e] Biopsy only vs. resection.
[f] Defined as no mediastinal compression.
[g] Older, non-WHO classification.
[h] Medullary, mixed, or cortical thymomas had lower recurrence rates than well-differentiated thymic carcinoma (undifferentiated thymic carcinoma was excluded from the analysis).
[i] For thymoma type B only.
[j] Excluding values in parentheses.

Ch, chemotherapy; Hist, histologic type; MG, myasthenia gravis; NS, not significant; R_0, complete resection; RT, radiotherapy; w/TC, with thymic carcinoma; Thym, thymoma.

and IV. In most of studies, Masaoka or Masaoka-Koga staging was used, and, in fact, in all staging systems, this dichotomization yields essentially the same cohorts of patients. It should be explicitly noted, however, that most of these multivariate analyses have not defined prognostic significance for stage I versus II, II versus III, III versus IVa, or for a sequential progression to worsening outcomes from I to IVb. Nevertheless, multiple studies do show progressively worse rates of recurrence and survival,[35] although questions have been raised whether the difference between stages I and II is significant.[36] Nevertheless, despite the limitations of these prognostic factor studies, it seems reasonable to view the stage of disease as a validated prognostic factor.

Another fairly consistent prognostic factor for both recurrence and survival is a complete resection. Obviously, it only makes sense to assess this factor in patients who are treated with surgery (i.e., a subgroup of all patients). Despite the fact that the rate of complete resection is clearly associated with tumor stage,[35] an R0 resection seems to carry independent significance by MVA.

The histologic subtype of thymic malignancy also seems to be important but is a bit more difficult to fully assess. First, different histologic classification schemes have been used, although more recently the World Health Organization system[37,38] has been the predominant one. Second is the issue of interobserver variation in assigning the histologic class.[39–41] The greatest problem comes from the fact that most studies reporting prognostic value for histologic typing have not reported exactly which subtypes account for the difference. Furthermore, the dichotomization used in most studies has been first chosen to maximize the difference and then tested for significance (i.e., double dipping). Uneasiness about the strength of the results is also fostered by the fact that the "best" dichotomization has yielded many different cutpoints in these studies. Thymic carcinoma seems quite consistently to have the worst survival, but whether this subtype has independent prognostic significance cannot be assessed from the data reported. We attempted to explore this by separating the MVA in cohorts that included or excluded thymic carcinoma. This shows that more studies including thymic carcinoma found prognostic significance (67%) for overall survival than if only thymoma was included (42%). Nevertheless, there is less difference when assessing recurrence.

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