Table 1. Characteristics of patients and volunteers

	Mesothelioma (n = 39)	Healthy volunteers (n = 102)	ARD/E (n = 201)	Lung cancer (±pleuritis; n = 45)	Others (n = 8)
Age	65 ± 18 (47-81)	52 ± 13 (30-79)	62 ± 11 (29-80)	65 ± 10 (35-83)	62 ± 13 (33-62)
Sex (n)					
Male	29	52	171	30	6
Female	10	50	30	15	2
Smoker (%)	68	46	73	67	100
Histology (n)					
Epithelioid	21				
Biphasic	9				
Sarcomatous	9				
Stages					
I	7				
II	5				
III	7				
IV (recurrence)	20				

Abbreviations: ARD/E, asbestos-related disease (pleural plaque, benign asbestos pleuritis, asbestosis) and exposure.

CHO-K1 cells transfected with ERC/mesothelin cDNA using a formylcellulofine affinity column coupled with anti-ERC/mesothelin PoAb-282. The concentration of affinity-purified N-ERC/mesothelin was determined by protein assay using Bradford methods (Bio-Rad).

Estimation of tumor marker effectiveness in clinical settings. Study design for the evaluation of N-ERC/mesothelin as a tumor marker of mesothelioma was approved by the Institutional Review Board of Juntendo University School of Medicine, National Organization Tokyo Hospital, Hyogo Prefectural Tsukaguti Hospital, Japan Antituberculosis Association Fukujuji Hospital, Yokosuka Kyosai Hospital, St. Marianna University School of Medicine, Tohoku University School of Medicine, Hirano Kameido Himawari Clinic, and Immunobiological Laboratories. Patients and healthy volunteers gave signed informed consent before enrollment.

To study the diagnostic value of N-ERC/mesothelin, 102 healthy volunteers were recruited, coming close to the goal of 10 men and 10 women in each 10-year age category from 30 to 70 years (e.g., 30-39, 40-49, 50-59, 60-70 years). Patients treated at any of the above-mentioned hospitals from August 2005 through October 2006 who had mesothelioma, asbestos-related disease, or significant pleural effusion or chest wall mass were prospectively enrolled. This study included both consecutive patients attending the above hospitals who were clinically suspected of the target condition because of presenting symptoms or referred by another health care professional because of diagnostic suspicions and a small number of patients who had already been diagnosed with the target condition. Blood sampling to determine the diagnostic value of N-ERC/mesothelin as a tumor marker was conducted in daily clinical practice, before and independent of final diagnosis. Furthermore, sample tubes were sent to Juntendo University School of Medicine; measurement of serum N-ERC/mesothelin was done there by one specialist in a blinded fashion.

Tissue sections were obtained from archival paraffin-embedded tumor blocks from thoracoscopic biopsies or surgical resection and sent to Juntendo University School of Medicine.

Mesothelioma was diagnosed by immunohistochemistry using antibodies against the following molecules: calretinin, Wilm's tumor 1, mesothelin, cytokeratin5/6, D2-40, vimentin, AE1/AE3, epithelial membrane antigen, carcinoembryonic antigen, Ber-EP4, and thyroid transcription factor-1. Other diseases were diagnosed comprehensively, including both pathologic and clinical information.

Statistical analysis. We analyzed ELISA data using JMP and SAS version 8.1.3 (SAS institute) and GraphPad Prism 4.0 (GraphPad Software). To compare serum levels between groups, the Mann-Whitney test was used. To analyze the trend of increasing serum level with increasing age, we used the linear trend test using a general linear model with linear contrast. $P < 0.05$ was considered statistically significant. Area under the curve of the receiving operating characteristics curve was calculated by the trapezoidal method. To examine the cutoff values of serum levels, we first calculated the total value of the specificity and sensitivity for each cutoff value and then chose the best cutoff values (e.g., the values that maximized total value).

Results

Characterization of anti-ERC/mesothelin antibodies. The novel MoAb-16K16 detected N-ERC/mesothelin in culture supernatants and N-ERC and full-length ERC/mesothelin in the cell lysates of CHO-K1 cells transfected with full-length cDNA of ERC/mesothelin (Fig. 1A).

Epitope mapping of MoAbs against N-ERC/mesothelin. MoAb-7E7 was able to detect the deletion mutant N-ERC/mesothelin protein that consisted of 139 amino acids, but not the mutant with 133 amino acids, suggesting that the epitope of MoAb-7E7 was in the region of 134 to 139 amino acids of N-ERC/mesothelin (data not shown). Similarly, we found that MoAb-16K16 recognized the sequence from 68 to 73 amino acids of N-ERC/mesothelin protein (Fig. 1B).

Establishment of ELISA system using novel MoAb-16K16 for N-ERC/mesothelin. To detect N-ERC/mesothelin in clinical samples, we developed ELISA combinations using MoAb-7E7 and PoAb-282, as described previously (8). The new ELISA system using MoAb-7E7 and the novel MoAb-16K16 for N-ERC/mesothelin was established and evaluated by measuring N-ERC/mesothelin against the standard ELISA system (MoAb-7E7 and PoAb-282). The new ELISA system was designated as N-ERC/mesothelin ELISA system (7-16) to distinguish it from the previous ELISA system. The standard dose-response curve of ELISA system (7-16) exhibited a linear shape when plotted on a log/log scale over a range from 0.081 to 5.2 ng/mL or 2.62 to 168 pmol/L calculated with N-ERC/mesothelin as a standard protein expressed in CHO-K1 cells transfected with ERC/mesothelin cDNA (Fig. 1C).

Serum levels of N-ERC/mesothelin in various clinical populations. We recruited a total of 293 patients. Of them, 39 had confirmed mesothelioma, 98 had pleural plaque, 83 had

exposure to asbestos, 14 had benign asbestos-related pleuritis, 6 had asbestosis, 45 had lung cancer (including eight patients who had carcinomatous pleuritis), and 8 patients whose disease could be distinguished from mesothelioma by chest computed tomography, with diagnoses including tubercular pleuritis, metastatic malignant melanoma, empyema, cardiac failure, and postmediastinal tumors. In addition, there were 102 healthy volunteers. Characteristics of both groups are reported in Table 1.

When serum N-ERC/mesothelin levels were measured with ELISA system (7-16), there were no significant differences among healthy volunteers based on sex or smoking status ($P = 0.96$ and $P = 0.87$, respectively; Fig. 2A and B). However, serum level did increase with increasing age ($P = 0.013$; Fig. 2C).

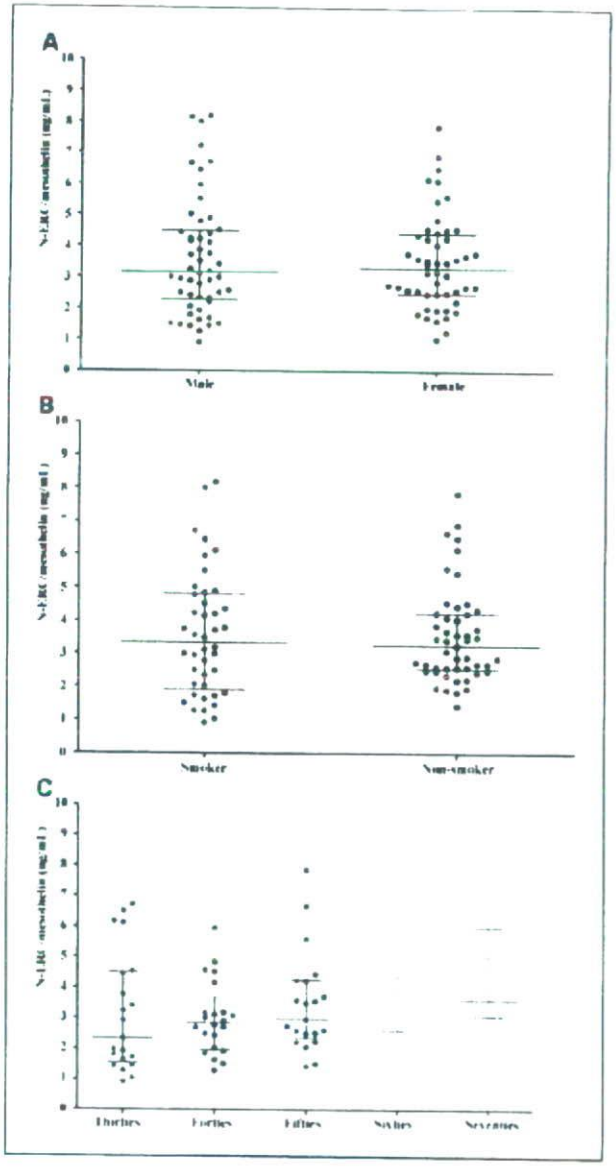

Fig. 2. Scatter plots of values for serum N-ERC/mesothelin (7-16) in healthy volunteers analyzing for possible effects of sex (*A*), smoking status (*B*), and age (*C*).

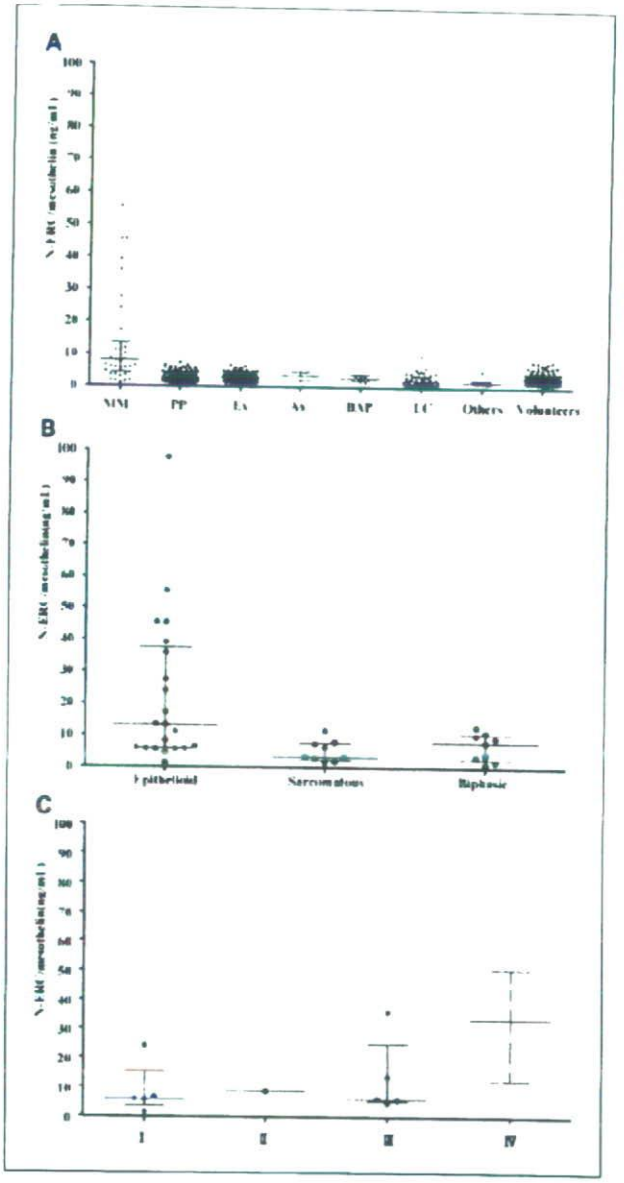

Fig. 3. *A*, scatter plots of values for serum N-ERC/mesothelin (7-16). MM, mesothelioma; As, asbestosis; PP, pleural plaque; BAP/DPT, benign asbestos pleuritis/diffuse pleural thickening; LC, lung cancer; Ex, exposure. *B*, scatter plots of serum N-ERC/mesothelin (7-16) by histologic type. *C*, scatter plots of serum N-ERC/mesothelin (7-16) by stage in epithelioid type.

Scatter plots of serum N-ERC/mesothelin level measured by ELISA system (7-16) showed that the median values from patients with mesothelioma were extremely high compared with levels obtained from other patients (Fig. 3A). Furthermore, analysis in terms of histologic type showed that serum levels of N-ERC/mesothelin were significantly elevated in epithelioid-type mesothelioma than other types ($P = 0.039$; Fig. 3B). Moreover, the scatter plots of the N-ERC/mesothelin in epithelioid

type showed the tendencies for the N-ERC/mesothelin value to increase as stage went up (Fig. 3C).

Receiving operating characteristics analysis, comparing ELISA system (7-16) with the previous ELISA system, was done in four models of clinical settings (Fig. 4A-D). The first setting (A) was the context of screening of at-risk individuals (mesothelioma versus other patient groups; excluding volunteers from the above groups). The second setting (B) tested differentiation (mesothelioma versus patient groups excluding asbestos exposure, asbestosis, and volunteers). The same comparisons were done with epithelioid mesothelioma instead of all mesothelioma [screening (C) and differentiation (D)]. Unfortunately, the only type which we can get the benefits from treatments is epithelioid mesothelioma, at present. So, to know the data focused on epithelioid mesothelioma is very important for its effective treatment strategy. In all clinical settings, the area under the curve values of the new ELISA system (7-16) were higher than those of the previous one. Furthermore, we compared not only the area under the curve but also statistical best sensitivities and specificities. In all settings, the new ELISA system (7-16) was superior to the previous one.

We were able to gather only a few samples to assess the value of N-ERC/mesothelin for monitoring patients with mesothelioma; there seemed to be a pattern that serum levels of N-ERC/mesothelin varied sensitively with tumor volume (Fig. 5).

Discussion

Sugarbaker and colleagues have shown that patients with early-stage mesothelioma have the potential for prolonged survival if treated with multimodality therapies (1, 9, 10). Increased use of thoracoscopy has permitted earlier diagnosis for more patients with mesothelioma. However, only a very small percentage of patients with mesothelioma can currently benefit from aggressive, potentially curative intervention because the vast majority has locally advanced disease, advanced age, or significant comorbid illnesses at time of diagnosis. Furthermore, extrapleural pneumonectomy, which is the center of trimodality therapy, is a high-risk procedure (11). Therefore,

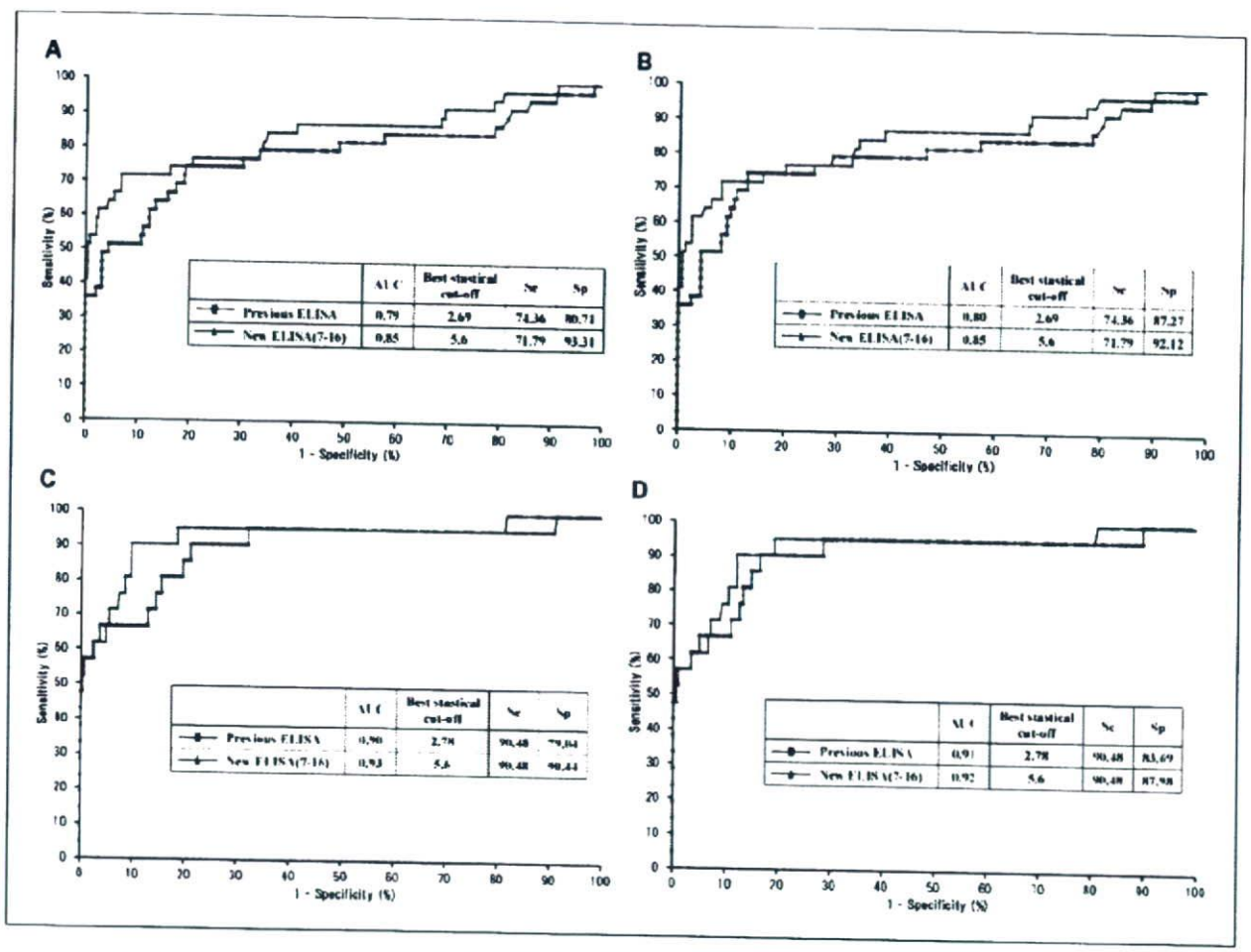

Fig. 4. *A*, screening mesothelioma among patient groups (including *PP, Ex, As, BAP, LC*, others). *B*, differentiating mesothelioma from other conditions in patients with clinically suspected mesothelioma, including patients with pleural effusion and/or pleural masses (including *PP, BAP, LC*, others). *C*, screening epithelioid mesothelioma from other conditions in patient groups (including *PP, Ex, As, BAP, LC*, others). *D*, differentiating epithelioid mesothelioma from other conditions in patients with clinically suspected mesothelioma, including patients with pleural effusion and/or pleural masses (including *PP, BAP, LC*, others).

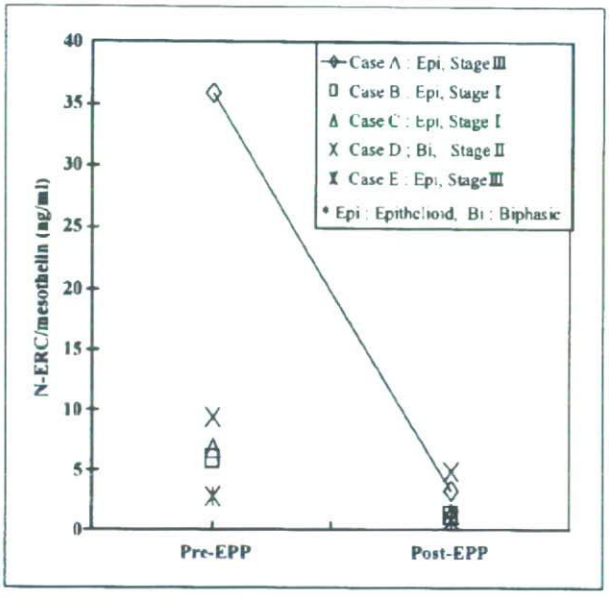

Fig. 5. Monitoring of N-ERC/mesothelin in some patients with confirmed mesothelioma.

efforts toward diagnosing mesothelioma at early stages (stages IA and IB for IMIG staging) are important to give more patients the option to be treated with not only less invasive therapies, but also locally effective therapeutic options; the combination of which may result in improved prognosis.

Several potential tumor markers for mesothelioma have previously been described, including Cyfra, tissue polypeptide antigen, carcinoembryonic antigen, CA-125, and hyaluronic acid (12–20), but none currently provides satisfactory reliability. These studies included small numbers of patients and insufficient analysis about diagnostic values for the potential serum tumor markers. Recently, osteopontin, soluble mesothelin-related protein, and serum mesothelin have been reported to have potential utility for early diagnosis and monitoring of mesothelioma (3–7). Among these markers, soluble mesothelin-related protein and serum mesothelin are particularly interesting because, like N-ERC/mesothelin, they are mesothelium-specific.

On the other hand, we identified the ERC gene, the homologue of the human mesothelin gene, in the course of the research on carcinogenesis in Eker rats in 1995 (21–26). Very recently, we reported initial data on serum N-ERC/mesothelin measured by a previous ELISA system (8, 27). Now, we have developed a more sensitive and stable ELISA system (7-16) for use in measuring serum N-ERC/mesothelin levels. The data about N-ERC/mesothelin measured with the ELISA system (7-16) that we presented in this paper are exciting compared with previous reports. In contrast with COOH terminal fragments, such as soluble mesothelin-related protein and serum mesothelin, most of which remain on the cell surface, N-ERC/mesothelin is found mainly in blood. Therefore, we think the new system can detect our target proteins with high reliability and identify the inflection point more easily.

Moreover, we noticed in this research that the affinity of antibodies used in the ELISA system and the stability of the molecule itself in the blood are very important. Therefore, we will continue to develop more precise ELISA systems. As a next step, we are examining the combination and comparison of other tumor markers for mesothelioma to see whether there is a possibility of early diagnosis using not only tumor markers but also other diagnostic modalities and development of a new, more sensitive ELISA system.

Conclusion

We have developed a new sandwich ELISA system (7-16), which is more sensitive than the previous system. Using this new system, we showed that N-ERC/mesothelin shows promise as a tumor marker for mesothelioma, especially for epithelioid type.

Acknowledgments

We thank Masaaki Abe, Naoko Aoki, Toshiyuki Kobayashi, the members of the National Institute of Radiological Science, and Naoya Katsuragi, for helping in management of this study.

References

1. Sugarbaker DJ, Flores RM, Jaklitsch MT, Richards WG, Strauss GM, DeCamp JM. Resection margins, extrapleural nodal status, and cell type determine postoperative long-term survival in trimodality therapy of malignant pleural mesothelioma: results in 183 patients. J Thorac Cardiovasc Surg 1999;117:54–63; discussion 63–5.
2. Robinson BWS, Creaney J, Lake R, Nowak A, Musk AW, de Klerk N. Mesothelin-family proteins and diagnosis of mesothelioma. Lancet 2003;362:1612–6.
3. Robinson BWS, Creaney J, Lake R, Nowak A, Musk AW, de Klerk N. Soluble mesothelin-related protein-a blood test for mesothelioma. Lung Cancer 2005;49 Suppl 1:S109–11.
4. Hassan R, Remaley AT, Sampson ML, Zhang J, Cox DD, Pingpank J. Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer. Clin Cancer Res 2006;12:447–53.
5. Onda M, Nagata S, Ho M, Bera TK, Hassan R, Alexander RH. Megakaryocyte potentiation factor cleaved from mesothelin precursor is a useful tumor marker in the serum of patients with mesothelioma. Clin Cancer Res 2006;12:4225–31.
6. Scherpereel A, Grigoriu B, Conti M, Gey T, Gregoire M, Copin M-C. Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. Am J Respir Crit Care Med 2006;173:1155–60.
7. Pass HI, Lott D, Lonardo F, Harbut M, Liu Z, Tang N. Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. N Engl J Med 2005;353:1564–73.
8. Shiomi K, Miyamoto H, Segawa T, Hagiwara Y, Ota A, Hino O. Novel ELISA system for detection of N-ERC/mesothelin in the sera of mesothelioma patients. Cancer Sci 2006;97:928–32.
9. Sugarbaker DJ, Norberto JJ, Swanson SJ. Extrapleural pneumonectomy in the setting of multimodality therapy for diffuse malignant pleural mesothelioma. Semin Thorac Cardiovasc Surg 1997;9:373–82.
10. Sugarbaker DJ, Strauss GM, Lynch TJ, Richards W, Mentzer SJ, Lee TH. Node status has prognostic significance in the multimodality therapy of diffuse, malignant mesothelioma. J Clin Oncol 1993;11:1172–8.
11. Sugarbaker DJ, Jaklitsch MT, Bueno R, Richards W, Lukanich J, Mentzer SJ. Prevention, early detection, and management of complications after 328 consecutive extrapleural pneumonectomies. J Thorac Cardiovasc Surg 2004;128:138–46.
12. Frebourg T, Lerebours G, Delpech B, Benhamou D, Bertrand P, Maingonnat C. Serum hyaluronate in malignant pleural mesothelioma. Cancer 1987;59:2104–7.
13. Pluygers E, Baidewyns P, Minette P, Beauduin M, Gourdin P, Robinet P. Biomarker assessments in asbestos-exposed workers as indicators for selective prevention of mesothelioma or bronchogenic carcinoma: rationale and practical implementations. Eur J Cancer Prev 1992;1:129–38.
14. Almudevar Bercero E, Perez GM-R, Garcia Bragado F, Jimenez C. Prognostic value of high serum levels of CA-125 in malignant secretory peritoneal mesotheliomas affecting young women. A case report with

differential diagnosis and review of the literature. Histopathology 1997;31:267–73.
15. Bonfrer JMG, Schouuwink JH, Korse CM, Baas P. Cyfra 21-1 and TPA as markers in malignant mesothelioma. Anticancer Res 1997;17:2971–3.
16. Schouwink H, Korse CM, Bonfrer JMG, Hart AAM, Bass P. Prognostic value of the serum tumour markers Cyfra 21-1 and tissue polypeptide antigen in malignant mesothelioma. Lung Cancer 1999;25:25–32.
17. Paganuzzi M, Onetto M, Marroni P, Filiberti R, Tassara E, Parodi S. Diagnostic value of CYFRA 21-1 tumor marker and CEA in pleural effusion due to mesothelioma. Chest 2001;119:1138–42.
18. Lee YC, Knox BS, Garrett JE. Use of cytokeratin fragments 19.1 and 19.21 (Cyfra 21-1) in the differentiation of malignant and benign pleural effusions. Aust N Z J Med 1999;29:765–9.
19. Thylen A, Hjerpe A, Martensson G. Hyaluronan content in pleural fluid as a prognostic factor in patients with malignant pleural mesothelioma. Cancer 2001; 92:1224–30.
20. Pettersson T, Froseth B, Riska H, Klockars M. Concentration of hyaluronic acid in pleural fluid as a diagnostic aid for malignant mesothelioma. Chest 1988; 94:1037–9.
21. Hino O, Kobayashi E, Nishizawa M, Kubo Y, Kobayashi T, Hirayama Y. Renal carcinogenesis in the Eker rat. J Cancer Res Clin Oncol 1995;121:602–5.
22. Yamashita Y, Yokohama M, Kobayashi E, Takai S, Hino O. Mapping and determination of the cDNA sequence of the Erc gene preferentially expressed in renal cell carcinoma in the Tsc2 gene mutant (Eker) rat model. Biochem Biophys Res Commun 2000; 275:134–40.
23. Hino O. Multistep renal carcinogenesis in the Eker (Tsc 2 gene mutant) rat model. Curr Mol Med 2004; 4:807–11.
24. Nakaishi M, Kajino K, Ikesue M, Hagiwara Y, Kuwahara M, Hino O. Establishment of the enzyme-linked immunosorbent assay system to detect the amino terminal secretory form of rat Erc/Mesothelin. Cancer Sci 2007;98: 659–64.
25. Maeda M, Hino O. Molecular tumor markers for asbestos-related mesothelioma: serum diagnostic markers. Pathol Int 2006;56:649–54.
26. Maeda M, Hino O. Blood tests for asbestos-related mesothelioma. Oncology 2006;71:26–31.
27. Hino O, Shiomi K, Maeda M. Diagnostic biomarker of asbestos-related mesothelioma: Example of translational research. Cancer Sci 2007;98: 1147–57.

Biochemical and Biophysical Research Communications 369 (2008) 915–918

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MESOMARK kit detects C-ERC/mesothelin, but not SMRP with C-terminus

Tatsuya Segawa [a], Yoshiaki Hagiwara [a,b], Kiyoshi Ishikawa [a], Naoko Aoki [a], Masahiro Maeda [a], Kazu Shiomi [c], Okio Hino [b,*]

[a] Immuno-Biological Laboratories Co. Ltd., 5-1 Aramachi, Takasaki, Gunma 370-0831, Japan
[b] Department of Pathology and Oncology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of General Thoracic Surgery, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 16 February 2008
Available online 5 March 2008

Keywords:
ERC/mesothelin
SMRP
Soluble forms
ELISA
Mesothelioma

ABSTRACT

ERC/mesothelin is expressed on the normal mesothelium and some cancers such as mesothelioma or ovarian carcinoma. A splicing isoform of ERC/mesothelin (known as SMRP), which has an 82-bp insertion and codes for a C-terminus with a hydrophilic, presumably soluble, tail instead of a GPI-anchoring signal, has been reported as a useful marker for the diagnosis of mesothelioma. However, the existence of SMRP has not yet been demonstrated in the serum of mesothelioma patients. To elucidate the existence of SMRP, we have established a new enzyme-linked immunosorbent assay (ELISA) system for SMRP. The ELISA study revealed that N- and C-ERC/mesothelin were detected in sera from mesothelioma patients, but not SMRP, even in these samples. This result showed that the SMRP detected with MESOMARK kit should be lack of soluble C-terminus and indistinguishable from C-ERC/mesothelin. Further study might be necessary to demonstrate the relationship between SMRP and mesothelin.

ERC/mesothelin is expressed on the normal mesothelium and in some cancers such as mesothelioma or ovarian carcinoma, and its use is anticipated as a promising diagnostic marker for malignant mesothelioma [1–4]. A 71-kDa precursor protein of human ERC/mesothelin can be cleaved into a 40-kDa C-terminal fragment as a surface GPI-anchored glycoprotein, and a 31-kDa N-terminal fragment as a secreted protein.

Moreover, a splicing isoform of ERC/mesothelin (known as SMRP), which has an 82-bp insertion and lacks a GPI-anchoring signal, has been reported. Scholler et al. [5] reported that the antigen protein recognized by mAb OV569 generated by immunization with ovarian carcinoma cells had an identical N-terminal amino acid sequence to that of the membrane-bound portion of mesothelin and MPF; this 42- to 45-kDa protein was referred to as a soluble member(s) of the mesothelin/megakaryocyte potentiating factor family-related protein (SMRP). SMRP has an 82-bp insertion in the membrane-associated part and codes for a C-terminus with a hydrophilic, presumably soluble, tail.

An enzyme-linked immunosorbent assay (ELISA) system using mAb-OV569 and another mAb-4H3 with another epitope on the same SMRP, called MESOMARK, revealed that the sera of most mesothelioma, ovarian and lung carcinoma patients were positive for SMRP.

Robinson et al. [6,7] and Creaney and Robinson [8] reported that the serum concentrations of SMRP measured using this ELISA system could be a useful marker for the diagnosis of mesothelioma and for monitoring the disease progression in cases of mesothelioma.

However, the existence of SMRP (82-bp inserted splicing isoform) has not yet been demonstrated in the serum of mesothelioma patients. Hellstrom et al. [9] reported the establishment of specific mAbs and ELISA systems for each of the three variants of ERC/mesothelin, 1, 2 and 3, and analyzed the serum samples of patients with ovarian carcinoma. ERC/mesothelin variant 2 had a 24-bp insert, and variant 3 had an 82-bp insert within the C-ERC/mesothelin region, consistent with the report by Scholler et al. [5]. Furthermore, purification and analysis of the ERC/mesothelin variants from the ascitic fluid of ovarian cancer patients by immunoaffinity chromatography with mAb 569 yielded detection of the ERC/mesothelin variants 1 and 2, but not 3.

The mAbs in the MESOMARK kit are capable of recognizing SMRP, but their epitopes in the SMRP molecule are not clear, and the antibodies may also react with C-ERC/mesothelin. This suggests that MESOMARK and the ELISA system developed by Hassan et al. [10] may detect the same molecule.

In this study, we have established a novel specific ELISA system for SMRP using a specific Ab against SMRP and employed it to take measurements to elucidate the existence of SMRP in samples from mesothelioma patients.

Materials and methods

Polyclonal antibodies against SMRP and C-ERC/mesothelin. Synthetic peptides VPPQENSRSVNGNMPPADT and VEGLKAEERHRPVRDWIL, which correspond to the V380–T399 of amino acid sequences of human SMRP and the V547–L564 of amino

* Corresponding author.
E-mail address: ohino@med.juntendo.ac.jp (O. Hino).

0006-291X/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2008.02.114

acid sequences of human ERC/mesothelin, respectively, were coupled with thyroglobulin, and immunized to rabbits. Also, the C-ERC/mesothelin protein, expressed in *Escherichia coli* as GST-fused protein, was purified and used as an immunogen for the rabbit polyclonal antibody (PoAb). Antisera from immunized rabbits were applied to thiol-sepharose beads (Amersham Biosciences) coupled with synthetic peptide or GST-fused protein, and IgG fractions were eluted. The eluted polyclonal IgG was designated as anti-SMRP-380, anti-C-ERC-6 and anti-C-ERC PoAb, respectively. The characterization of antibodies was determined against ERC/mesothelin or SMRP expressed in COS-1 cells by Western blotting.

Detection of the SMRP transcripts in various cultured cell lines with the reverse transcription-polymerase chain reaction (RT-PCR). HeLa S3, SMOV-1, MKN-45, MKN-74, PC-9, PC-3, HPC-y9, MSTO-211H (mesothelioma), NCI-H2452 (mesothelioma) and NCI-H28 cell lines (mesothelioma) were maintained in TIL medium (IBL, Fujioka, Japan) supplemented with 10% fetal bovine serum. Total RNA was extracted from the cultured cell using RNeasy Mini (QIAGEN, Chatsworth, CA), and cDNA was generated with a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). Human *ERC/mesothelin* cDNA was amplified with PCR using forward (1746 primer; 5′-GCGGAGGAGCGGCACCGCCCGGTGCGGGAC-3′) and reverse (3′ERC primer; 5′-CATGCCCCCTGCAGACACGT-3′) primers for its detection, which correspond from nucleotides 1746 to 1775 and from nucleotides 2074 to 2093 of the human *ERC/mesothelin* gene (#D49441), respectively. These primer sets could give 342 bp of PCR product for *ERC/mesothelin* and 424 bp for SMRP (*ERC/mesothelin variant 3*), respectively.

Western blotting to detect the overexpressed and endogenous ERC/mesothelin in the cultured cells. A C-terminal cDNA fragment (nucleotides 797–1104) of *SMRP* (#AF180951) was chemically synthesized, digested with BamHI, ligated with the BamHI digested N-terminal region of human ERC/mesothelin full-length cDNA inserted in pcDNA3.1(+) vector, and expressed in COS-1 cells.

Transfected COS-1 cells and various cell line cells, as described above, were lysed, boiled and subjected to Western blot analysis by anti-SMRP-380, anti-C-ERC-6 and anti-C-ERC PoAbs.

Establishment of the ELISA system to detect the C-ERC/mesothelin and SMRP. As a standard protein for the ELISA system, the C-ERC/mesothelin protein expressed in *E. coli* as described above, was used for the C-ERC/mesothelin assay system. In addition the culture supernatant of COS-1 cells transfected with SMRP cDNA was used as a standard protein for the SMRP ELISA system. The concentration of purified C-ERC/mesothelin protein was measured with a Protein Assay kit (Bio-Rad, Tokyo, Japan). The purity of the protein was demonstrated densitometrically with a Densitograph (ATTO, Tokyo, Japan) and gel-filtration chromatography (data not shown). The concentration of the SMRP protein was calculated by comparison with the C-ERC/mesothelin protein.

For the SMRP ELISA system, Immuno Module Plates (Nalge Nunc, Rochester, NY) were coated with anti-C-ERC PoAb (in 0.1 M carbonate buffer, pH 9.5) at 4 °C overnight, then blocked with 1% bovine serum albumin in PBS containing 0.05% NaN_3 at 4 °C overnight. sample and standard proteins were diluted with 1% Triton-X in PBS, added to each well and incubated at 37 °C for 1 h. After nine washes with washing buffer, 100 μL of horseradish peroxidase-labeled anti-SMRP-380 PoAb was added to each well and incubated for 30 min at 4 °C. After nine washes with washing buffer, 100 μL of tetramethyl benzidine buffer as a substrate was added to each well and incubated for 30 min at room temperature in the dark. Color development was stopped by addition of 100 μL of stop solution (1 N H_2SO_4). Optic density of each sample was measured at 450 nm. For the C-ERC/mesothelin ELISA system, an anti-C-ERC-6 PoAb was used as precoated antibody, and anti-C-ERC PoAb was used for the labeled antibody.

Patients (blood samples). Our study for the tumor marker of mesothelioma was approved by the Institutional Review Board of Juntendo University School of Medicine and its Hospital. Patients signed informed consent. We recruited 11 mesothelioma patients and 10 healthy volunteers whose characteristics are reported in Table 1.

Detection and quantification of N- and C-ERC/mesothelin in the culture supernatant of cell line cells and sera of mesothelioma patients by specific ELISA systems. Each batch of cells (5×10^4) was seeded into 24-well culture plates, and incubated for 3 days. Supernatants were harvested and measured. Also, the sera from mesothelioma patients were measured. The concentration of N-ERC/mesothelin was measured with a specific ELISA system as previously reported Shiomi et al. [11,12]. The C-ERC/mesothelin and SMRP concentration were measured with our new ELISA system.

Table 1
Characteristics of patients and healthy volunteers

	Patients				Healthy volunteers
Age	64.0 ± 10.4				57.9 ± 11.0
Sex	Male (7)		Female (4)		Male (4) Female (7)
Stage	I (1)	II (3)	III (3)	IV (4)	–
Histology	Epithelioid (6)	Biphasic (3)	Sarcomatous (2)		–

Results

Characterization of antibodies against SMRP and ERC/Mesothelin

The reactivities of antibodies were analyzed with Western blotting against COS-1 cells transfected with cDNA of SMRP or ERC/mesothelin (Fig. 1). Anti-C-ERC-6 and anti-C-ERC PoAbs were able to detect SMRP and ERC/mesothelin. On the other hand, anti-SMRP-380 PoAb reacted to SMRP, specifically.

Detection of the SMRP transcripts in various cultured cell lines by RT-PCR

SMRP and *ERC/mesothelin* mRNA expression in various cell lines were analyzed with RT-PCR using specific primers (Fig. 2A). All of the cells tested except PC-3 and NCI-H28 cells expressed *ERC/mesothelin* mRNA (342 bp product). An *SMRP* mRNA (424 bp product) was detected clearly in NCI-H2452 and NCI-H28 cells and faintly in HeLa-s3, SMOV-1, MKN-45, MKN-74 and PC-9 cells. NCI-H28 cell expressed only *SMRP* mRNA.

Establishment of the ELISA system for the C-ERC/mesothelin and SMRP

In order to detect C-ERC/mesothelin and SMRP, we developed C-ERC/mesothelin ELISA system combinations using anti-C-ERC-6 and anti-C-ERC PoAbs, and SMRP ELISA system combinations using anti-C-ERC and anti-SMRP-380 PoAbs. The standard dose-response curve of the C-ERC/mesothelin ELISA system and the SMRP ELISA system exhibited a linear shape when plotted on a log/log scale over a range from 0.078 to 5.0 ng/ml and 0.016 to 1.0 ng/ml, respectively. The specificities of the ELISA systems were confirmed against recombinant SMRP and ERC/mesothelin protein expressed in COS-1 cells. The SMRP ELISA system had no cross reactivity to C-ERC/mesothelin (data not shown).

Detection and quantification of N- and C-ERC/mesothelin in the culture supernatant of cell line cells, and sera from mesothelioma patients by the specific ELISA systems

The presence of ERC/mesothelin and SMRP in the culture supernatant of various cell lines was analyzed with the ELISA systems and Western blotting (Fig. 2B). Many cell lines secreted N- and

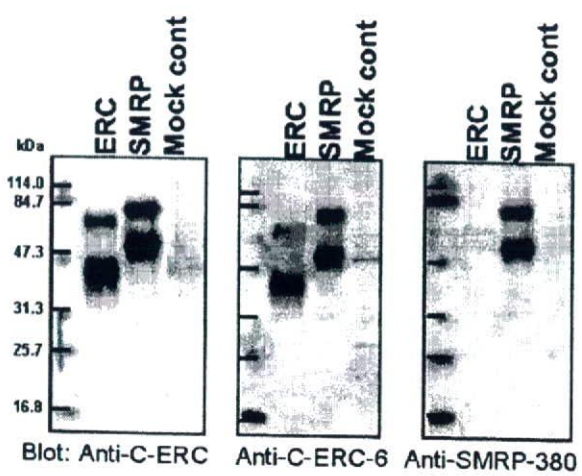

Fig. 1. Characterization of antibodies by Western blot analysis. Total cell lysates of COS-1 cells transfected with cDNA of ERC/mesothelin (ERC), SMRP and vector only (Mock) were subjected to Western blot analysis by anti-C-ERC, anti-C-ERC-6 and anti-SMRP-380 PoAb.

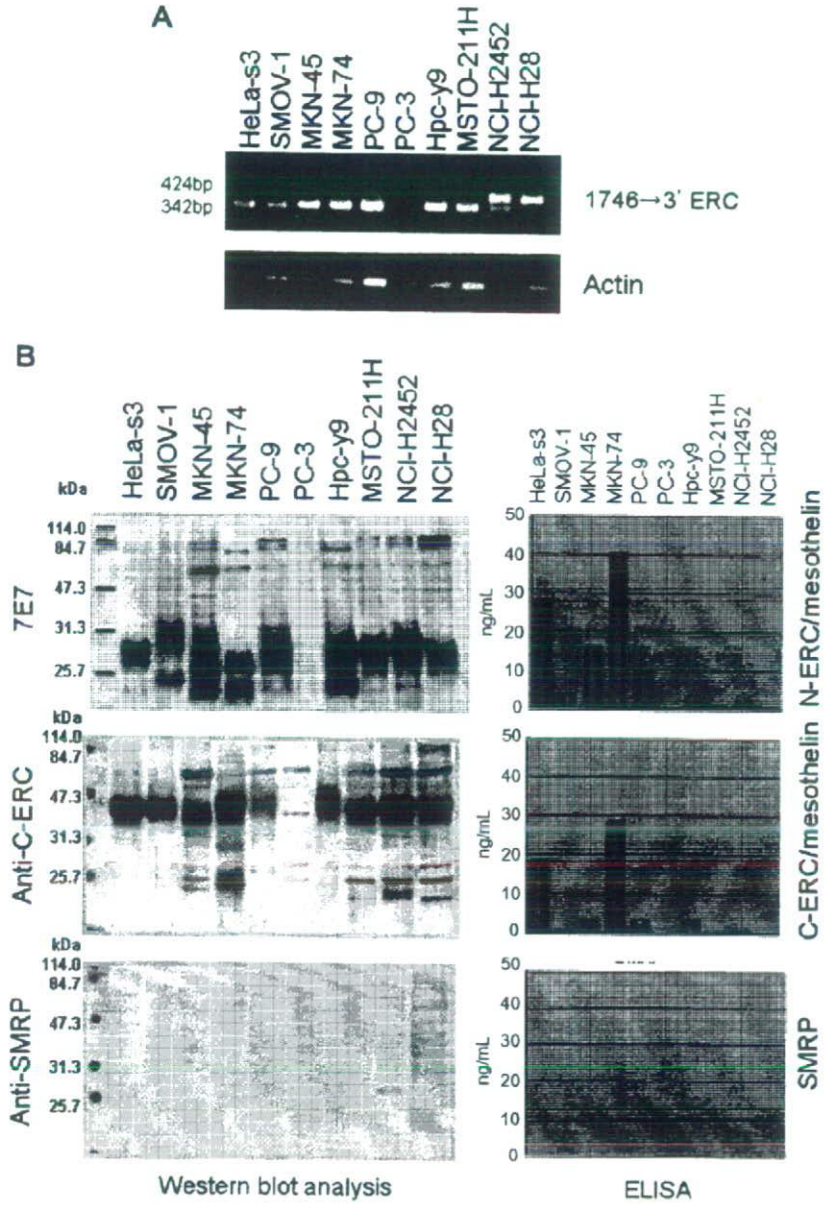

Fig. 2. Detection of N- and C-ERC/mesothelin and SMRP in various cell line cells. (A) *SMRP* and *ERC/mesothelin* mRNA expression in various cell lines were analyzed with RT-PCR using specific primers. The 424 bp product is derived from *SMRP* mRNA and the 342 bp is derived from *ERC/mesothelin* mRNA. (B) The presence of N- and C-ERC/mesothelin and SMRP protein in various cell lines were analyzed with Western blotting and ELISA systems. Total cell lysates were subjected to Western blot analysis by anti N- ERC/mesothelin (7E7), anti-C-ERC and anti-SMRP PoAb. The culture supernatants were measured with ELISA systems for N- and C-ERC/mesothelin and SMRP.

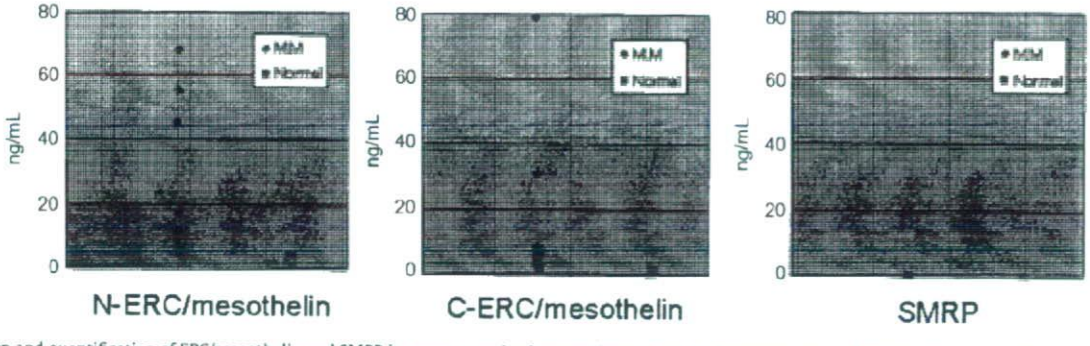

Fig. 3. Detection and quantification of ERC/mesothelin and SMRP in serum samples from malignant mesothelioma patients and healthy volunteers. The serum samples were analyzed with the specific ELISA systems for N- and C-ERC/mesothelin and SMRP.

C-ERC/mesothelin into the culture supernatant, but SMRP was not detected in any cells.

Also, the presence of ERC/mesothelin and SMRP in serum samples from mesothelioma patients was analyzed with the ELISA systems (Fig. 3). Although the serum concentrations of mesothelioma patients ranged from 3.86 to 67.97 ng/ml (average: 22.18 ng/ml) for N-ERC/mesothelin (These data were referred in previous report [12]), and from 1.59 to 79.17 ng/ml (average: 13.51 ng/ml) for C-ERC/mesothelin, SMRP was not detected in any samples. In healthy volunteers, N- and C-ERC/mesothelin were detected (1.42–4.36 ng/ml for N-ERC/mesothelin, and 0.56–2.41 ng/ml for C-ERC/mesothelin), but SMRP was not measurable.

Discussions

Although the usefulness of SMRP, which has an 82-bp insertion, has been as a diagnostic marker for mesothelioma [13,14], the expression of SMRP in the cells and body fluids from mesothelioma or other carcinoma patients has not been elucidated.

Among the tested cells in this study, cells which expressed only *ERC/mesothelin* mRNA secreted a detectable amount of N- and C-ERC/mesothelin with the ELISA system, but the SMRP molecule was not detected even in cells which expressed *SMRP* mRNA.

Even in serum samples from mesothelioma patients, N- and C-ERC/mesothelin were detected, but SMRP was not detected. These results predict that even if SMRP could be translated, it could be degraded in its C-terminal region during the expression process, be undetectable by the specific ELISA system and be indistinguishable from C-ERC/mesothelin with MESOMARK kit.

Sapede et al. [15] reported about the mechanisms involved in soluble mesothelin production by tumor cells. They explored two alternative possibilities: release of an aberrant RNA splicing product (mesothelin variant 3, *SMRP*) or enzyme-mediated shedding of membrane-bound mesothelin (phospholipases, proteases as MMP- and ADAM-family proteases).

According to the present study, accurately, not SMRP but SMRP and/or C-ERC/mesothelin without C-terminus should be considered as a marker for mesothelioma. Further study might be necessary to demonstrate the relationship between SMRP and mesothelin.

Acknowledgments

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan, and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science. Furthermore, this study was partially supported by a consignment expense of the Molecular Imaging Program "Research Base for PET Diagnosis" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Government of Japan.

References

[1] M. Maeda, O. Hino, Molecular tumor markers for asbestos-related mesothelioma: serum diagnostic markers, Pathol. Int. 56 (2006) 649–654.
[2] M. Maeda, O. Hino, Blood tests for asbestos-related mesothelioma, Oncology 71 (2006) 26–31.
[3] O. Hino, K. Shiomi, Diagnostic biomarker of asbestos-related mesothelioma: example of translational research, Cancer Sci. 98 (2007) 1147–1157.
[4] M. Onda, S. Nagata, M. Ho, T.K. Bera, R. Hassan, R.H. Alexander, I. Pastan, Megakaryocyte potentiation factor cleaved from Mesothelin precursor is a useful tumor marker in the serum of patients with mesothelioma, Clin. Cancer Res. 12 (2006) 4225–4231.
[5] N. Scholler, N. Fu, Y. Yang, Z. Ye, G.E. Goodman, K.E. Hellström, I.N. Hellström, Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma, Proc. Natl. Acad. Sci. USA 96 (1999) 11531–11536.
[6] B.W. Robinson, J. Creaney, R. Lake, A. Nowak, A.W. Musk, N. de Klerk, P. Winzell, K.E. Hellstrom, I. Hellstrom, Mesothelin-family proteins and diagnosis of mesothelioma, Lancet 362 (2003) 1612–1616.
[7] B.W. Robinson, J. Creaney, R. Lake, A. Nowak, A.W. Musk, N. de Klerk, P. Winzell, K.E. Hellstrom, I. Hellstrom, Soluble mesothelin-related protein—A blood test for mesothelioma, Lung Cancer 49S1 (2005) S109–S111.
[8] B.W. Robinson, J. Creaney, Detection of malignant mesothelioma in asbestos-exposed individuals: the potential role of soluble mesothelin-related protein, Hematol. Oncol. Clin. North Am. 19 (2005) 1025–1040.
[9] I. Hellstrom, J. Raycraft, S. Kanan, N.Y. Sardesai, T. Verch, Y. Yang, K.E. Hellstrom, Mesothelin variant 1 is released from tumor cells as a diagnostic marker, Can. Epid. Biomarker Prev. 15 (2006) 1014–1020.
[10] R. Hassan, A.T. Remaley, M.L. Sampson, Detection and quantitation of serum mesothelin, a tumor marker for patients with mesothelioma and ovarian cancer, Clin. Cancer Res. 12 (2006) 447–453.
[11] K. Shiomi, H. Miyamoto, T. Segawa, Y. Hagiwara, A. Ota, M. Maeda, K. Takahashi, K. Masuda, Y. Sakao, O. Hino, Novel ELISA system for detection of N-ERC/Mesothelin in the sera of mesothelioma patients, Cancer Sci. 97 (2006) 928–932.
[12] K. Shiomi, Y. Hagiwara, K. Sonoue, T. Segawa, K. Miyashita, M. Maeda, H. Izumi, K. Masuda, M. Hirabayashi, T. Moroboshi, T. Yoshiyama, A. Ishida, Y. Natori, A. Inoue, M. Kobayashi, Y. Sakao, H. Miyamoto, K. Takahashi, O. Hino, Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma, Clin. Cancer Res., accepted for publication.
[13] H.I. Pass, A. Wali, N. Tang, A. Ivanova, S. Ivanov, M. Harbut, M. Carbone, J. Allard, Soluble mesothelin-related peptide level elevation in mesothelioma serum and pleural effusions, Ann. Thorac. Surg. 85 (2008) 265–272.
[14] D.G. Weber, D. Taeger, B. Pesch, T. Kraus, T. Brüning, G. Johnen, Soluble mesothelin-related peptides (SMRP)—High stability of a potential tumor marker for mesothelioma, Cancer Biomark 3 (2007) 287–292.
[15] C. Sapede, A. Gauvrit, I. Barbieux, M. Padieu, L. Cellerin, C. Sagan, A. Scherpereel, G. Dabouis, M. Grégoire, Aberrant splicing and protease involvement in mesothelin release from epithelioid mesothelioma cells, Cancer Sci. 99 (2008) 590–594.

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 268 (2008) 278–285
CANCER Letters
www.elsevier.com/locate/canlet

siRNA-mediated *Erc* gene silencing suppresses tumor growth in *Tsc2* mutant renal carcinoma model

Osamu Imamura [a], Hiroaki Okada [b], Yuuki Takashima [b], Danqing Zhang [a], Toshiyuki Kobayashi [a], Okio Hino [a,*]

[a] *Department of Pathology and Oncology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-842, Japan*
[b] *Department of Pharmaceutics and Drug Delivery, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

Received 9 January 2008; received in revised form 28 March 2008; accepted 2 April 2008

Abstract

Silencing of gene expression by small interfering RNAs (siRNAs) is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. However, there are no reports of systemic delivery of siRNAs for stable treatment except short hairpin RNAs (shRNAs). On the other hand, there are many reports of systemic delivery of siRNAs for transient treatment using liposome carriers and others. With regard to shRNAs, a report showed fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Therefore, we decided to use original siRNA microspheres instead of shRNA for stable treatment of disease. In this study, we designed rat-specific siRNA sequences for *Erc/mesothelin*, which is a tumor-specific gene expressed in the Eker (*Tsc2* mutant) rat model of hereditary renal cancer and confirmed the efficacy of gene silencing in vitro. Then, by using siRNA microspheres, we found that the suppression of *Erc/mesothelin* caused growth inhibition of *Tsc2* mutant renal carcinoma cells in tumor implantation experiments in mice.

Keywords: *Erc* gene; *Tsc2*; siRNA microspheres

1. Introduction

The Eker (*Tsc2*) gene mutant rat model of hereditary renal carcinoma (RC) is an example of Mendelian dominantly-inherited predisposition to a specific cancer in an experimental animal [1]. The germline mutation is like an 'initial gene' (*Tsc2* gene) of the abnormal networks of gene expression that are involved in tumor formation (federal headship of carcinogenesis) [2]. We previously isolated the *Erc* gene from the RC tissue of Eker rats by representational difference analysis [3]. The *Erc* gene (NM_031658) is more preferentially expressed in renal cell carcinoma (RCC) of the Eker rats than in normal kidney. We subsequently confirmed that *Erc* is a homolog of the human mesothelin gene (*MSLN*) because rat *Erc* and human *MSLN* are localized on chromosomes 10q12-21 and 16p13.3,

* Corresponding author. Tel.: +81 3 5802 1038; fax: +81 3 5684 1646.
E-mail address: ohino@med.juntendo.ac.jp (O. Hino).

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.04.002

respectively, both of which coincided with the locus of the *Tsc2/TSC2* gene [4].

Recently, Shiomi et al. already showed that N-ERC/mesothelin is a very promising tumor marker for mesothelioma, especially epithelioid mesothelioma [5]. Mesothelioma is an aggressive tumor arising from the mesothelium, and is usually associated with previous exposure to asbestos [6]. On the other hand, Hassan R. et al. reported on mesothelin as a target for cancer therapy and summarized the available pre-clinical data as well as on-going and planned clinical trials [7]. We are now characterizing the role of *Erc* in carcinogenesis.

The siRNA technology has rapidly become an important tool for gene therapy to target several pathological conditions such as cancer and viral infectious diseases [8]. Many researchers have examined how to effectively deliver siRNA to target cells and tissues using gene carriers such as cationic liposomes, polymers and lipids [8–16]. It was reported that the cationized-gelatin microspheres containing siRNA for VEGF could inhibit tumor growth in mice [17]. However, a clinically viable delivery system for siRNA has not been developed yet. The effects of siRNA are transient even though they are highly suppressive. Therefore, it is necessary to develop methods to sustainably deliver siRNA to target cells for continuous cleavage of the target mRNA.

In the present study, we prepared biodegradable microspheres for long-term sustained release of siRNA that target and continuously suppress the production of rat *Erc*. We used poly (DL-lactic/glycolic acid, PLGA) as a biodegradable and biocompatible polymer [18–22]. The gene carrier is important to deliver siRNA into the cells, therefore arginine and polyethyleneimine were used as carriers. We examined the efficacy of the siRNA in vitro as well as the physical properties of microspheres [20,21]. Then, we injected microspheres carrying *Erc* siRNA into rat *Tsc2* mutant tumors in tumor implantation experiments in mice to determine their effects upon tumor growth.

2. Materials and methods

2.1. Cell culture and siRNA transfection for Erc silencing in vitro

Anti-rat *Erc* siRNA (siRNAE244) and (siRNAE1393) was purchased from Ambion Inc. (Austin, TX). The sense and antisense sequences of siRNAE244 were 5′-GAC CGG CAC CGU UGA CUU UGC-3′ and 5′-CAC UGG CCG UGG CAA CUG AAA-3′, respectively. The sense and antisense sequence of siRNAE1393 were 5′-GGACAACAUCCCCUUAAGUUA-3′ and 5′-ACU UAAGGGGAUGUUGUCCAG-3′, respectively. A non-silencing siRNA (sense, 5′-CAG UUC CGC CAC UUG CCA A dTdT-3′; antisense, 5′-UUG GCA AGU GGC GGA ACU G dTdT-3′) served as a mock double-stranded RNA (dsRNA) control. Mouse MKOC1-277 and rat ERC33 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Co., USA) containing 10% FBS (Invitrogen, Carlsbad, California) and 1% penicillin/streptomycin (stock 10,000 U/mL, 10,000 μg/mL, Invitrogen, Carlsbad, CA). Cells were seeded into 12-well plates at a density of 6×10^4 cells per well with 1 mL of FBS(+) DMEM and incubated at 37 °C in a humidified 5% CO_2 chamber for 24 h before the transfection study. Then the medium was discarded and replaced with antibiotic-free medium. After 12 h, cells were transfected with 10 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent as per manufacturer's instructions.

2.2. Reverse transcription polymerase chain reaction and quantitative real-time PCR

Total RNA was extracted by using Absolutely RNA Reverse Transcription-PCR Miniprep kits (Stratagene, La Jolla, CA). The concentration of total RNA was adjusted to 200 μg/mL using a spectrophotometer. For quantitative reverse transcription-PCR, the following reagents were used according to the manufacturer's instructions: One Tube Reverse transcription-PCR system (Roche Applied Science, Alameda, CA) and SYBR Green PCR Core Reagents. Primer sequences used were as follows: ERC13930, sense; 5′-ATGCTCAACTGTGACATCTC-3′, antisense; 5′-TGTGACAAATTAGCGCT TCC-3′, ERC2440, sense; 5′-CCTAGAAGGACAGATGTTCC-3′, antisense; 5′-TAGCAACAGAAAT AGGCCCC-3′.

For real-time PCR, conversion of RNA to cDNA was performed first, by incubating 50 ng of total RNA, random hexamer primers (Roche Applied Science, Alameda, CA), and SuperScript II enzyme (Invitrogen, Carlsbad, CA) at 48 °C for 60 min and 95 °C for 5 min. TaqMan probes for rat (Rn00581388_m1, ABI) and mouse *Erc/mesothelin* (Mm00450770_m1, ABI), and SYBR Green PCR Core Reagents were purchased from ABI. Quantitative real-time PCR amplification of the cDNA template corresponding to 50 ng total RNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and ABI PRISM 7500 (Applied Biosystems, Foster City, CA). PCR conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The glyceraldehyde-3-phosphate dehydrogenase gene (*G3pdh*) transcripts served as the internal control. The primer and probe mixtures for ZIP6, ZIP10 and G3pdh were pur-

chased from Applied Biosystems. Primer sequences for *G3pdh* used were as follows: sense; 5′-AACGGCACAGT CAAGGCTGAGAACG-3′ antisense; 5′-CAACATACT CGGCACCGGCATCG-3′.

2.3. Soft agar assay

Rat ERC33 cells were treated with 10 nM siRNAE244 or scramble siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, California) reagent as per manufacturer's instructions. After 24 h, cells were trypsinized, mixed with agar (final concentration, 0.3%; Becton–Dickinson and Company, Franklin Lakes, New Jersey) and RPMI 1640 containing 10% fetal bovine serum, and overlaid onto an underlayer of 0.53% Difco agar containing the same medium in 6-well plates. To assist *Erc* suppression, siRNAs (10 nM) were included in top gel. Cells (3000 per well) were seeded and allowed to grow for >10 days before counting the number of colonies [23].

2.4. Preparation of siRNA microspheres

PLGA with a 75:25 lactic/glycolic acid ratio and a molecular weight of 14,400 was purchased from WAKO (Japan). Branched polyethylenimine (PEI, Mw 25,000, Sigma–Aldrich, St. Louis, MO) and arginine (WAKO, Japan) were used as gene carriers.

As shown in Fig. 1, we prepared PLGA microspheres containing siRNA using a w/o/w in-water drying method [18,19]. Briefly, 100 μL of 0.4% polyvinyl alcohol (PVA) (W_1), containing 0.13 μg of siRNA and 5 μg of PEI carrier, and 0.5 g of PLGA was dissolved in 3 mL of CH_2Cl_2 (O) and then homogenized (Polytron PT3100, Kinematica AG, Littau Lucerne, Switzerland) at 10,000 rpm for 2 min. The resulting W_1/O emulsion was homogenized with 500 mL of 0.25% PVA (W_2) [21,22]. The resulting $W_1/O/W_2$ emulsion was stirred gently for 3 h to evaporate the organic solvent. The microspheres were passed through a 75-μm sieved to remove large particles and then sedimented by centrifugation at 3000 rpm for 15 min. The PLGA microspheres containing siRNA encapsulated in PEI were collected by centrifugation, rinsed with distilled water three times, and then lyophilized [21,22].

2.5. Physicochemical characterization of siRNA microspheres

Microspheres were observed using an S-2250N scanning electron microscope (Hitachi, Tokyo, Japan). The samples were coated with 25-nm-thick gold using a quick carbon coater (SC-701, Sanyu Electronics, Tokyo, Japan). The particle diameter (the horizontal fret diameter) and size distribution of the microspheres were determined by image analysis of 1000 particles using WinROOF image analysis software (Mitani, Tokyo, Japan). The mean particle diameter was defined as the arithmetic mean of the number based on particle size distribution.

The siRNA encapsulation efficiency into microspheres was determined by HPLC. In brief, microspheres (25 mg) were dissolved in 0.5 mL of acetonitrile and then 0.5 mL of phosphate buffer (pH 6.0) was added. After vigorous shaking for 2 h, the supernatants were collected by centrifugation (5000 rpm, 20 min) and then analyzed using HPLC (Prominence, SHIMADZU, Kyoto, Japan) with an ultraviolet (UV) detector under the following conditions: column, TSKgel Oligo DNA RP (4.6 mm × 15 cm, TOSOH, Japan); mobile phases, (A) 0.1 M triethylamine–acetic acid and (B) acetonitrile [and] 10–30% B (45 min, linear); flow rate, 1.0 mL/min; wave length, 260 nm; and

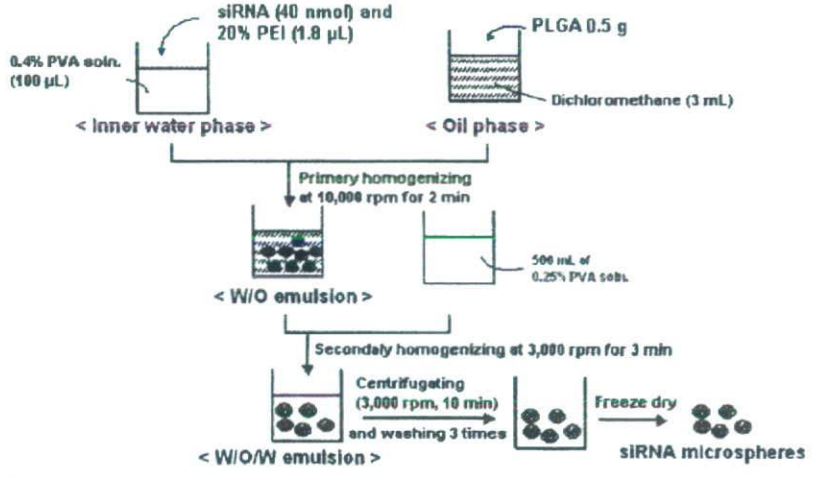

Fig. 1. We prepared biodegradable microspheres for long-term sustained release of siRNA that target and continuously suppress the production of rat *Erc/mesothelin*. We used poly (DL-lactic/glycolic acid, PLGA) as a biodegradable and biocompatible polymer. We prepared PLGA microspheres containing siRNA using a w/o/w in-water drying method.

injection volume, 10 μL. The ratio (%) of the measured to the formulated amount of siRNA was defined as the encapsulation efficiency of siRNA into microspheres.

2.6. Injection of tumor cells into mice

For implantation, a cocktail of ERC33 cells (1×10^6 cells per mice) suspended in 0.5 mL of serum-free medium and 10 mg of microspheres containing siRNA were used as carrier. It was injected into the flank of BALB/c nude mice. Tumor volume was calculated as length × width × height in mm^3 every week after injection [24]. ERC33-bearing mice were followed up for 6 weeks after implantation.

2.7. Statistical analysis

We analyzed all of the data using the Statcel statistical program (OMC, Tokyo, Japan), then evaluated the statistical significance using Mann–Whitney's U test. We considered $P < 0.05$ to be statistically significant.

3. Results

3.1. Physicochemical characterization of siRNA microspheres

The siRNA encapsulation efficiency in microspheres was shown by using HPLC (data not shown). We confirmed that the formulated amount of siRNA; the encapsulation efficiencies of siRNA were 64.3% when PEI were used as carrier.

3.2. Efficacy of specific rat Erc gene silencing in Tsc2 mutant renal carcinoma cell

To establish a system to specifically suppress rat *Erc*, without affecting the endogenous mouse homolog in the tumor implantation experiments in nude mice, we designed rat-specific siRNAs. We confirmed which siRNA sequence, siRNAE244 or si1RNAE1393, was the most effective for rat *Erc* gene silencing ($n = 5$, Fig. 2) by performing quantitative real-time PCR analysis using Taqman probes. *Erc* gene silencing using siRNAE244 showed approximately 80% reduction in the mRNA levels in rat ERC 33 cells (Fig. 3A). On the contrary, siRNAE244 exhibited minimal suppression in mouse MKOC1-277 cells, indicating that it could act as a rat-specific siRNA (Fig. 3B). Therefore, we selected siRNAE244 for our investigation in vivo ($P = 0.02$). So far, no apparent effect of siRNAE244 on cellular proliferation of ERC33 was detected in soft agar assay (data not shown).

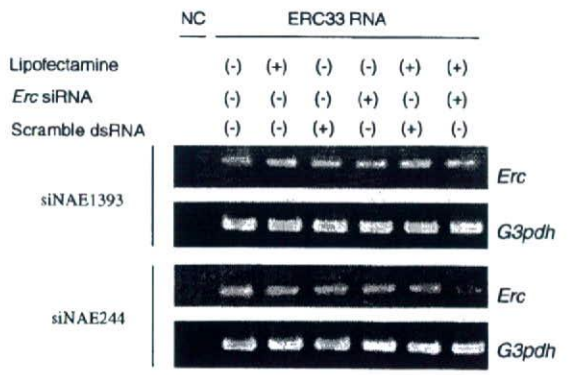

Fig. 2. Evaluation of Erc gene silencing in rat ERC33 cell line by using reverse transcription-PCR. Cells were transfected with siRNA for Erc (siRNAE244 or siRNAE1393) or scramble dsRNA. After 72 h, total cellular RNAs was subjected to RT-PCR for Erc or G3pdh. Lane NC: no template cDNA.

3.3. Erc gene silencing suppressed tumor growth of rat renal carcinoma cells in vivo

To investigate the efficacy of specific siRNAE244 microspheres in a *Tsc2* mutant renal carcinoma model, we simultaneously injected ERC33 cells and siRNA containing microspheres into nude mice, and then measured tumor volumes. As shown in (Fig. 4), siRNAE244 microspheres suppressed tumor growth relative to scramble dsRNA microspheres from the 4th week (42.7 mm^3 vs. 66.3 mm^3 $P = 0.018$) to 5th week (49.8 mm^3 vs. 181.0 mm^3, $P = 0.018$) after injection. At the 6th week, considerable growth of siRNAE244-injected ERC33 cells was observed, although there was a significant difference in volume compared with control (170.0 mm^3 vs. 290.0 mm^3, $P = 0.018$). Such a growth might reflect an exhaustion of siRNAE244. We did observe a difference between the tumor volume in mice after treatment with scramble dsRNA microspheres and siRNAE244, as shown in (Fig. 5A and B).

4. Discussion

We are exploring the role of *Erc* during tumorigenesis. As an approach to characterize the effect of *Erc* suppression on tumor growth in vivo, we aimed to establish an in vivo knockdown system. For this purpose, we selected rat-specific sequence for siRNA against *Erc* and successfully achieved rat-specific *Erc* gene silencing in vitro. We then performed a trial for siRNA-mediated rat *Erc* gene silencing in vivo by using the *Tsc2* mutant RC model and we observed a reduction in tumor growth following treatment. In this study, for implantation, we used a cocktail of ERC33 cells and microspheres containing siRNA. The implantation by using cocktail can provide excellent exposure of necessary drug delivery for tumor cells. On the other hand, it is difficult for us to provide rapid, excellent microspheres containing siRNA in implanted tumor cells because

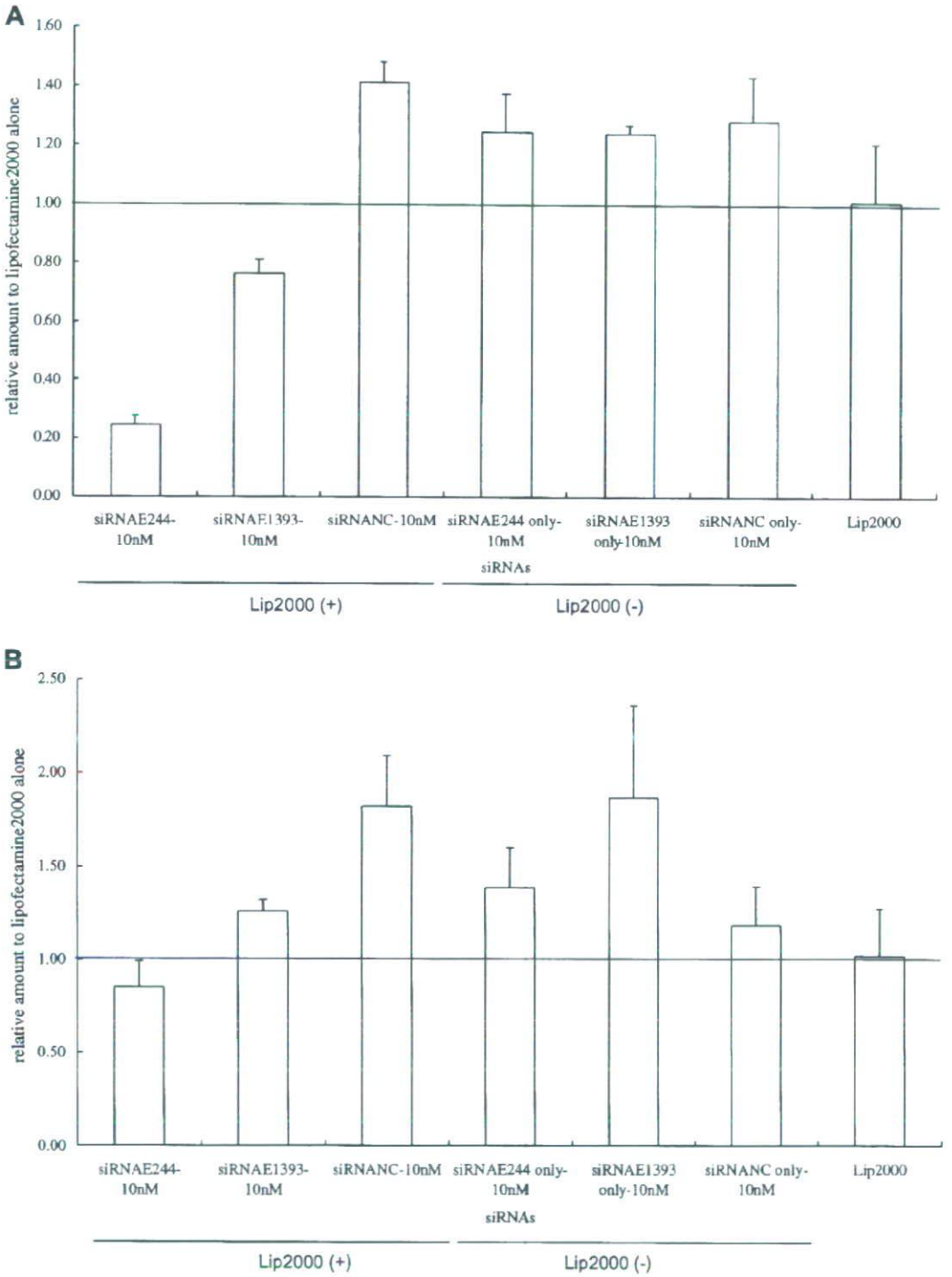

Fig. 3. Evaluation of mRNA levels of Erc by real-time PCR. (A) Rat ERC33. (B) Mouse MKOC1-277. Cells were treated either with Lipofetcamine only (Lipo2000), with control dsRNA (NC), or siRNAs for Erc (siRNAE244 or siRNAE1393) in the presence (+) or absence (−) of Lipofectamine.

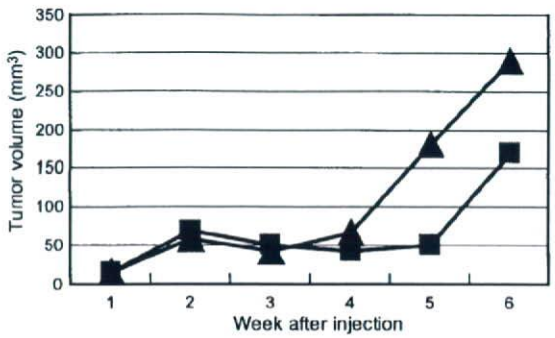

Fig. 4. Evaluation of tumor volume after *Erc* gene silencing in vivo. ERC33 cells were injected with microspheres containing either scramble dsRNA (triangle) or siRNAE244 (square) into nude mice ($n = 5$).

we are in preliminary stage to need experience of using microspheres containing siRNA.

With regard to siRNA delivery systems in vivo, at present, the main obstacle to the development of therapeutic products using RNAi technologies is the absence of a suitable delivery method. We are interested in a stable treatment regimen because in transient treatment, lipid delivery of synthetic siRNAs can induce immune activation in vivo [25,26]. On the other hand, using viral delivery systems for stable treatment, are efficient but cause concerns over serious side effects [26]. A side effect was associated with the downregulation of microRNAs (miRNAs), indicating possible competition of the latter with shRNAs for limiting cellular factors required for the processing of various small RNAs. In vitro and in vivo shRNA transfection studies suggest that one such factor, shared by both shRNA and miRNA pathways, which is readily saturated, is the nuclear karyopherin exportin-5 [26]. Therefore, shRNA-induced toxicity requires global miRNA inhibition [26]. For effective siRNA delivery systems, monitoring and controlling intracellular shRNAs are imperative for achieving stable in vivo gene silencing while mitigating adverse effects. However, it is very difficult to optimize shRNA dosage for minimizing the risk of oversaturation of the endogenous small RNA pathways [26]. On the other hand, Okada et al. confirmed to release of siRNA with PEI from microspheres in vitro instead of using shRNA [20]. Besides, Okada et al. showed anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice [21]. Therefore, we prepared long-term sustained release biodegradable microspheres encapsulating siRNA that target and continuously suppress the production of rat *Erc/mesothelin* [21,22]. We confirmed that siRNAE244-induced Erc gene silencing suppressed tumor growth of rat renal carcinoma cells in vivo, as shown in Fig. 5. Therefore, we are very interested in *Erc/mesothelin* function which may play a role in tumor growth. Although we have not observed specific effects of siRNAE244 on cellular phenotype in vitro, several functions have been ascribed to mesothelin [28–30]. CA125 is a tumor antigen originally defined by the monoclonal antibody OC125 that is routinely used for diagnosis of ovarian cancer and to monitor the recurrence after therapy [27]. Miyajima et al. show CA125 and mesothelin are co-expressed in advanced grade ovarian adenocarcinoma. Taken together, their data indicate that mesothelin is a novel CA125-binding protein and that CA125 might contribute to the metastasis of ovarian cancer to the peritoneum by initiating cell attachment to the mesothelial epithelium via binding to mesothelin [27]. Chang et al. reported that mesothelin may play a role in cellular

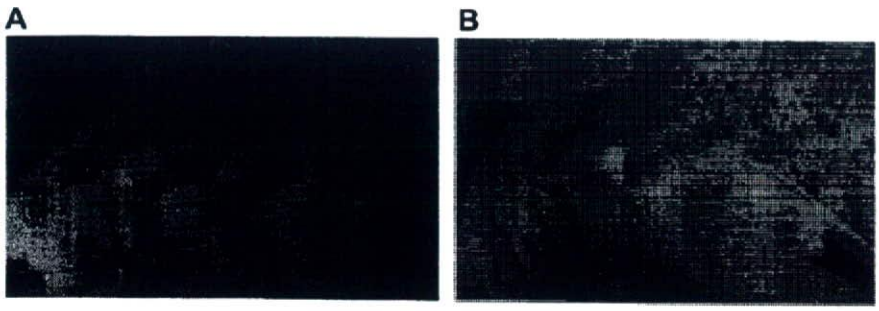

Fig. 5. Macroscopic appearance of tumors. Representative tumors (5 week) treated with siRNAE244 (A) or scramble dsRNA (B) are shown. Tumor positive areas are highlighted by black dotted lines. Pale-colored mass is less apparent in (A) indicating that remarkable attenuation of tumor growth was caused by using siRNAE244.

adhesion [28]. A recent report indicated that mesothelin expression modulates cellular anoikis [23]. The mucin MUC16 carries the peptide epitope CA125, which is a prominent molecular marker for monitoring the progression and recurrence of epithelial ovarian cancer [29]. Gubbels et al. reported the strong binding kinetics of the mesothelin–MUC16 interaction and the cell adhesion between ovarian tumor cells [29]. Frierson et al. reported mesothelin expression in endometrioid and undifferentiated ovarian carcinoma in a large scale tissue microarray study [30]. Hino et al. established the rat ELISA system to detect the soluble form of *Erc/MPF/Mesothelin*, and demonstrated the high concentration of this form in the sera of rats bearing mesohelioma. [3]. These reports in correlation with our results suggest that *Erc/mesothelin* may have a role in tumor growth. Further investigation with respect to the role of *Erc/mesothelin* in new anti-cancer therapy is currently in progress in our laboratory.

Acknowledgements

We thank Dr. Toshiaki Osawa, emeritus professor of the University of Tokyo, for providing us with information on microsphere-mediated siRNA delivery systems and Mr. Kosei Toyoshima, of Tokyo University of Pharmacy and Life Science, for technical support. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan, and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science.

Furthermore, this study was partially supported by a consignment expense of the Molecular Imaging Program "Research Base for PET Diagnosis" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Government of Japan.

References

[1] O. Hino, T. Kobayashi, M. Nishizawa, Y. Kubo, T. Kobayashi, Y. Hirayama, Y. Kikuchi, K. Orimoto, Renal carcinogenesis in the Eker rat, J. Cancer Res. Clin. Oncol. 121 (1995) 602–605.

[2] O. Hino, K. Shiomi, M. Maeda, Diagnostic biomarker of asbestos-related mesothelioma: example of translational research, Cancer Sci. 98 (2007) 1147–1151.

[3] M. Nakaishi, K. Kajino, M. Ikesue, Y. Hagiwara, M. Kuwahara, H. Mitani, Y. Horikoshi-Sakuraba, T. Segawa, S. Kon, M. Maeda, T. Wang, M. Abe, M. Yokoyama, O. Hino, Establishment of the enzyme-linked immunosorbent assay system to detect the amino terminal secretory form rat Erc/mesothelin, Cancer Sci. 98 (2007) 659–664.

[4] K. Shiomi, H. Miyamoto, T. Segawa, Y. Hagiwara, A. Ota, M. Maeda, K. Takahashi, K. Masuda, Y. Sakao, O. Hino, Novel ELISA system for detection of N-ERC/mesothelin in the sera of mesothelioma patients, Cancer Sci. 97 (2007) 928–932.

[5] K. Shiomi, Y. Hagiwara, K. Sonoue, T. Segawa, K. Miyashita, M. Maeda, H. Izumi, K. Masuda, M. Hirabayashi, T. Moroboshi, T. Yoshiyama, A. Ishida, Y. Natori, A. Inoue, M. Kobayashi, Y. Sakao, H. Miyamoto, K. Takahashi, O. Hino, Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma, Clin. Cancer Res. 14 (2008) 1431–1437.

[6] M. Maeda, O. Hino, Blood tests for asbestos-related mesothelioma, Oncology 71 (2006) 26–31.

[7] R. Hassan, M. Ho, Mesothelin targeted cancer immunotherapy, Eur. J. Cancer 44 (2008) 46–53.

[8] A. De Fougerolles, M. Manoharan, R. Meyers, H.P. Vornlocher, RNA interference in vivo: toward synthetic small inhibitory RNA-based therapeutics, Methods Enzymol. 392 (2005) 278–296.

[9] Z. Hassani, G.F. Lemkine, P. Erbacher, K. Palmier, G. Alfama, C. Giovannangeli, J.P. Behr, B.A. Demeneix, Lipid-mediated siRNA delivery down-regulates exogenous gene expression in the mouse brain at picomolar levels, J. Gene Med. 7 (2005) 198–207.

[10] B. Urban-Klein, S. Werth, S. Abuharbeid, F. Czubayko, A. Aigner, RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo, Gene Ther. 12 (2005) 461–466.

[11] M. Leirdal, M. Sioud, Gene silencing in mammalian cells by preformed small RNA duplexes, Biochem. Biophys. Res. Commun. 295 (2002) 744–748.

[12] D. Sorensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.

[13] T. Ishii, K. Ohnuma, A. Murakami, N. Takasawa, T. Yamochi, S. Iwata, M. Uchiyama, N.H. Dang, H. Tanaka, C. Morimoto, SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production, J. Immunol. 170 (2003) 3653–3661.

[14] M. Ito, S. Yamamoto, K. Nimura, K. Hiraoka, K. Tamai, Y. Kaneda, Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin, J. Gene Med. 7 (2005) 1044–1052.

[15] W.J. Kim, L.V. Christensen, S. Jo, J.W. Yockman, J.H. Jeong, Y.H. Kim, S.W. Kim, Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma, Mol. Ther. 14 (2006) 343 350.

[16] C. Zhang, N. Tang, X. Liu, W. Liang, W. Xu, V.P. Torchilin, siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene, J. Control Release 112 (2006) 229–239.

[17] S.H. Kim, J.H. Jeong, S.H. Lee, S.W. Kim, T.G. Park, PEG conjugated VEGF siRNA for anti-angiogenic gene therapy, J. Control Release 116 (2006) 123–129.

[18] A.C. Grayson, A.M. Doody, D. Putnam, Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro, Pharm. Res. 23 (2006) 1868–1876.

[19] S. Werth, B. Urban-Klein, L. Dai, S. Hobel, M. Grzelinski, U. Bakowsky, F. Czubayko, A. Aigner, A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes, J. Control Release 112 (2006) 257–270.

[20] G. Matsumoto, T. Kushibiki, Y. Kinoshita, U. Lee, Y. Omi, E. Kubota, Y. Tabata, Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma, Cancer Sci. 97 (2006) 313–321.

[21] N. Murata, Y. Takashima, K. Toyoshima, M. Yamamoto, H. Okada, Anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice, J. Control Release 126 (2008) 246–254.

[22] H. Okada, H. Toguchi, Biodegradable microspheres in drug delivery, Crit. Rev. Ther. Drug Carrier Syst. 12 (1995) 1–99.

[23] N. Uehara, Y. Matsuoka, A. Tsubura, Mesothelin promotes anchorage-independent growth and prevents anoikis via extracellular signal-regulated kinase signaling pathway in human breast cancer cells, Mol. Cancer Res. 6 (2008) 186–193.

[24] K. Narita, J. Staub, J. Chien, K. Meyer, M. Bauer, A. Friedl, S. Ramakrishnan, V. Shridhar, HSulf-1 inhibits angiogenesis and tumorigenesis in vivo, Cancer Res. 66 (2006) 6025–6032.

[25] A. Bridge, S. Pebernard, Induction of an interferon response by RNAi vectors in mammalian cells, Nat. Genet. 34 (2003) 263–264.

[26] D. Grimm, K.L. Streetz, C.L. Jopling, T.A. Stom, K. Pandey, C.R. Davis, P. Marion, F. Salazar, M.A. Kay, Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways, Nature 25 (2006) 537–541.

[27] A. Rump, Y. Morikawa, M. Tanaka, S. Minami, N. Umesaki, M. Takeuchi, A. Miyajima, Binding of ovarian cancer antigen CA125/MUC16 to Mesothelin mediates cell adhesion, J. Biol. Chem. 279 (2004) 9190–9198.

[28] K. Chang, I. Pastan, Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, meotheliomas, and ovarian cancers, Proc. Natl. Acad. Sci. USA 93 (1996) 136–140.

[29] J. Gubbels, J. Belisle, M. Onda, C. Rancourt, M. Migneault, M. Ho, T.K. Bera, J. Connor, B.K. Sathyanarayana, B. Lee, I. Pastan, M.S. Patankar, Mesothelin-MUC16 binding is a high affinity, *N*-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors, Mol. Cancer 5 (2006) 50–65.

[30] H. Frierson, C. Moskaluk, Large-scale molecular and tissue microarray analysis of mesothelin expression in common human carcinomas, Hum. Pathol. 34 (2003) 605–609.

ONCOLOGY REPORTS 20: 1375-1380, 2008

Secretion of N-ERC/mesothelin and expression of C-ERC/mesothelin in human pancreatic ductal carcinoma

KOICHI INAMI[1,2], KAZUNORI KAJINO[1], MASAAKI ABE[1], YOSHIAKI HAGIWARA[1,3], MASAHIRO MAEDA[3], MASAFUMI SUYAMA[2], SUMIO WATANABE[2] and OKIO HINO[1]

Departments of [1]Pathology and Oncology and [2]Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421; [3]Immuno-Biological Laboratories, 5-1 Aramachi, Takasaki-shi, Gunma 370-0831, Japan

Received July 28, 2008; Accepted September 12, 2008

DOI: 10.3892/or_00000155

Abstract. ERC/mesothelin gene (*MSLN*) encodes a precursor protein, which is cleaved by proteases to generate N-ERC/mesothelin and C-ERC/mesothelin. N-ERC/mesothelin is a soluble protein, also known as megakaryocyte-potentiating factor, which is released into extracellular space. N-ERC/mesothelin is known to be a serum marker of mesothelioma. We have previously developed an enzyme-linked immunosorbent assay system for N-ERC/mesothelin, which can detect mesothelioma. C-ERC/mesothelin is expressed in normal mesothelial cell, pancreatic cancers, ovarian cancers, mesotheliomas and some other cancers. Pancreatic ductal carcinoma remains a fatal disease because its diagnosis often occurs very late. In this study, we examined ERC/mesothelin expression in human pancreatic cancer cell lines (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) by reverse transcription-polymerase chain reaction and immunoblotting and N-ERC/mesothelin concentration in the supernatant of cultured cancer cells by the ELISA system. We also investigated C-ERC/mesothlein expression in human pancreatic ductal carcinoma tissues by immunostaining using 5B2 anti-mesothelin monoclonal antibody and N-ERC/mesothelin concentration in sera obtained from patients with pancreatic ductal carcinoma via ELISA. *In vitro*, N-ERC/mesothelin concentration in cell culture medium nearly correlated with the expression level of C-ERC/mesothelin. Although C-ERC/mesothelin was frequently expressed in human pancreatic ductal carcinoma, serum N-ERC/mesothelin concentration of cancer patients was equivalent to healthy controls. N-ERC/mesothelin was not useful as a serum marker of pancreatic ductal carcinoma, but because of frequent expression, C-ERC/mesothelin might be useful as a target of molecular imaging and immunotherapy.

Correspondence to: Dr Okio Hino, Department of Pathology and Oncology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
E-mail: ohino@med.juntendo.ac.jp

Key words: ERC/mesothelin, pancreatic ductal carcinoma, ELISA, immunohistochemistry

Introduction

ERC/mesothelin gene (*MSLN*) encodes a 71 kDa precursor protein, which is cleaved by proteases to yield 31 kDa N-terminal (N-ERC/mesothelin) and 40 kDa C-terminal (C-ERC/mesothelin) proteins (1,2). N-ERC/mesothelin, originally identified as megakaryocyte-potentiating factor (MPF), is soluble and released into extracellular space (1-9). C-ERC/mesothelin is a glycoprotein tethered to the cell surface by glycosyl-phosphatidyl-inositol (GPI) anchor. Some forms of C-ERC/mesothelin are released into extra-cellular space by aberrant splicing or proteases (1,2,10-13).

N-ERC/mesothelin/MPF was isolated from the medium of cultured pancreatic cancer cells (3,4) and is known to be a serum marker of mesothelioma (5-9). C-ERC/mesothelin is expressed not only in normal mesothelial cells of the pleura, pericardium and peritoneum, but also in malignant cells of pancreatic ductal carcinomas, ovarian cancers, mesotheliomas and some other cancers (1,14-17). C-ERC/mesothelin can be detected in the sera of patients with ovarian carcinoma and mesothelioma (10-12,18). Previously, we discovered *Erc*, which is expressed in renal cell cancers of Eker rats. We also confirmed that *Erc* is a homolog of human *MSLN* (19-21).

Pancreatic ductal carcinoma remains a fatal disease because of its poor prognosis. Unfortunately, the diagnosis of pancreatic ductal carcinoma often occurs very late and consequently, <40% of patients are candidates for tumor resection (22-24). Thus, the overall 5-year survival rate of these patients is <10% (23,24). On the other hand, those of patients with stage I disease is 58.1% (25). Novel strategy for early diagnosis of pancreatic ductal carcinoma is warranted.

ERC/mesothelin is expressed in human pancreatic ductal carcinoma and not expressed in normal pancreatic tissue (15,16). Previous studies showed the usefulness of N-ERC/mesothelin and C-ERC/mesothelin as diagnostic markers for C-ERC/mesothelin expressing tumors (5-12). To date, there has been no report about the effectiveness of N-ERC/Meosthelin and C-ERC/mesothelin as serum markers of pancreatic ductal carcinoma. We have previously devised a novel enzyme-linked immunosorbent assay (ELISA) system for N-ERC/mesothelin and showed that it is useful for diagnosis of human mesothelioma (5-7). In this study, we

examined the expression of C- and N-ERC/mesothelin in cultured pancreatic cancer cell lines and human pancreatic ductal carcinomas and investigated the usefulness of our ELISA system as a diagnostic procedure of human pancreatic ductal carcinoma.

In the cultured cells, the concentration of N-ERC/mesothelin in the medium nearly correlated with the expression of C-ERC/mesothelin. C-ERC/mesothelin was frequently expressed in human pancreatic ductal carcinoma. There was, however, no increase in N-ERC/mesothelin concentration in the sera of pancreatic cancer patients compared with that of normal controls. Although N-ERC/mesothelin is established as a reliable marker for mesothelioma, N-ERC/mesothelin is not useful as a diagnostic marker of pancreatic ductal carcinoma. As for C-ERC/mesothelin, it might be useful as a target of molecular imaging and immunotherapy, because of its frequent expression.

Materials and methods

Pancreatic cancer cell lines. MIA-PaCa2 and PK-1 were provided by Cell Resource Center for Biomedical Research, Tohoku University, Sendai-shi, Miyagi, Japan. KP-3 and TCC-PAN2 were provided by Health Science Research Resources Bank, Sennan-shi, Osaka, Japan. PK-59 and PK-45H were provided by RIKEN CELL BANK, Tsukuba-shi, Ibaraki, Japan. MIA-PaCa2 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Culture supernatants and cells were harvested 48 h after culturing at 37˚C and 5% CO_2 atmosphere, upon reaching >80% confluency.

Reverse transcription-polymerase chain reaction (RT-PCR). mRNA levels of ERC/mesothelin in the cultured cells (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) were analyzed by RT-PCR. Cells in petri dishes were lysed by the acid guanidinium thiocyanate-phenol-chloroform extraction method (26) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNAs were extracted from these lysates following manufacturer's instructions. Total RNA (1 μg) was reverse transcribed for 30 min at 50˚C and subjected to polymerase chain reaction amplification. The primers used to amplify the ERC/mesothelin were: sense 5'-CAAGAA GTGGGAGCTGGAAG-3' and antisense 5'-GTCTCCAGG GACGTCACATT-3'. As a control for RT-PCR, ß-actin mRNA was amplified using the following ß-actin-specific primers: sense 5'-CCGCGAGAAGATGACCCAGA-3'; and antisense 5'-CAGGAGGAGCAATGATCTTG-3'. All primers were purchased from Operon (Tokyo, Japan). RT-PCR was carried out in an MBS Satellite 0.2 (Thermo Fisher Scientific, Kanagawa, Japan), using Titan RT-PCR System (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. After an initial denaturation step of 4 min at 94˚C, each sample was subjected to 25 cycles of amplification (denaturation, 30 sec at 94˚C; annealing, 30 sec at 50˚C; and elongation, 1 min at 68˚C) followed by a final elongation step for 10 min at 68˚C. PCR product (10 μl) was analyzed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Immunoblotting. MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H cells in petri dishes were lysed in a solution containing 2% sodium dodecylsulfate, 10% glycerol, 50 mM Tris-HCl (pH 6.8) and 100 mM dithiothreitol, followed by boiling for 2 min. These lysates were electrophoresed in 10% Laemmli gels and transferred onto nitrocellulose membranes. Membranes were blocked in 1% skim milk in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) for 1 h at room temperature. Next, membranes were incubated with 5B2 anti mesothelin antibody (Novocastra Laboratory Vision BioSystems, Boston, MA, USA, 1:100 dilution) or AC15 anti ß-actin antibody (Sigma, St. Louis, MO, USA, 1:5000 dilution) in PBS-T with 1% skim milk for 1 h at room temperature. EnVision+ system labeled polymer-horseradish peroxidase (HRP) (K4000 or K4001 purchased from Dako, Glostrup, Denmark) at a 100-fold dilution in PBS-T with 1% skim milk was added and allowed to react with the membrane at room temperature for 1 h. ECL detection system (GE Healthcare, Buckinghamshire, UK) was used to visualize ERC/mesothelin on the membrane.

Human subjects. Patients with ductal pancreatic carcinoma, treated in Juntendo hospital between April 1, 2006 and November 30, 2007, were evaluated in this study. Pathological diagnosis was based on the histological analysis of tissue samples obtained from pancrearectomy or endoscopic ultrasonography guided fine needle aspiration biopsy (FNA), using sterile 21-gauge needles. This study was approved by the Institutional Review Board of Juntendo University School of Medicine and its hospital. Patients gave their signed informed consent. Nineteen healthy controls were sampled at random from a database, as described (6), with an age range of 50-79 years.

Immunohistochemistry. Tissue sections, 3 μm thick, were prepared from archival formalin-fixed, paraffin-embedded specimens. After deparaffinization, the tissue sections were heated in 10 mM citrate buffer (pH 6.0) for antigen retrieval and then treated with 3% hydrogen peroxide. Next, the sections were incubated with primary antibody solutions diluted in Tris-buffered saline with 0.1% Tween-20 (TBS-T) overnight at 4˚C. We used mouse monoclonal anti human C-ERC/mesothelin antibody 5B2 (1:50 dilution) as the primary antibody and EnVision+ system labeled with polymer-HRP (Dako) as the secondary antibody. Diaminobenzidine was used as the substrate for peroxidase. For immunostained slides, the intensity of staining was semiquantitatively graded on a scale of 1+ to 3+ and the proportion of stained ducts of cancer gland was graded as 0%, 1 to <10%, 10-50% and >50%.

ELISA. N-ERC/mesothelin concentration in sera and cell culture supernatants (MIA-PaCa2, PK-1, KP-3, TCC-PAN2, PK-59 and PK-45H) were analyzed by sandwich ELISA method. Sandwich ELISA method was performed as

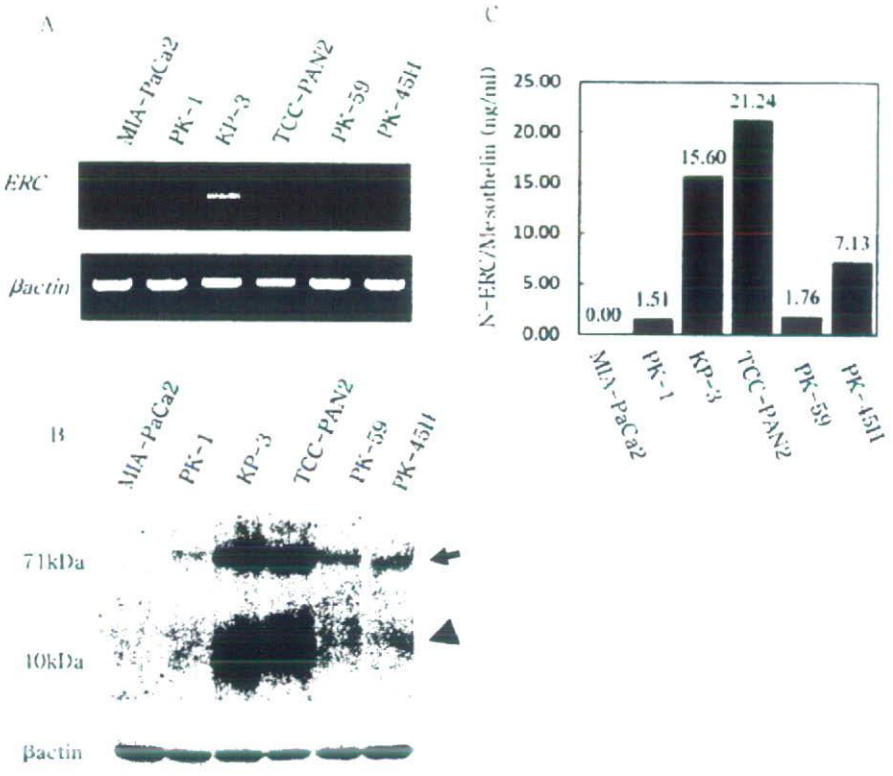

Figure 1. ERC/mesothelin expression in human pancreatic cancer cell lines. (A) ERC/mesothelin transcript in human pancreatic cancer cell lines detected by RT-PCR. (B) ERC/mesothelin protein in human pancreatic cancer cell lines detected by immunoblotting. Arrow, precursor ERC/mesothelin (71 kDa); Arrowhead, C-ERC/mesothelin (40 kDa). (C) Secreted N-ERC/mesothelin in the cell culture medium of human pancreatic cancer cell lines detected by ELISA.

previously described (5,6), using 7E7 monoclonal antibody and HRP-conjugated polyclonal antibody-282. Absorbance at 450 nm was measured in an ELISA reader (E-MAX; Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis. We analyzed ELISA data using JMP and SAS version 8.1.3 (SAS Institute, Cary, CA, USA). To compare serum concentration between groups, the Mann-Whitney test was used. $P<0.05$ was considered statistically significant.

Results

ERC/mesothelin expression in human pancreatic cancer cell lines. RT-PCR revealed ERC/mesothelin mRNA expression in most of the investigated pancreatic cancer cell lines, except for MIA-PaCa2 (Fig. 1A). Immunoblotting showed strong ERC/mesothelin and C-ERC/mesothelin expression in 2 of 6 cell lines (KP-3 and TCC-PAN2) and weak expression in PK-1, PK-59 and PK-45H. MIA-PaCa2 did not demonstrate any ERC/mesothelin and C-ERC/mesothelin expression (Fig. 1B). N-ERC/mesothelin was secreted into the culture supernatants of 5 cell lines, of which 2 (KP-3 and TCC-PAN2) showed high N-ERC/mesothelin concentration. PK-1, PK-59 and PK-45H secreted N-ERC/mesothelin moderately. MIA-PaCa2 did not secrete N-ERC/mesothelin at all (Fig. 1C).

C-ERC/mesothlein expression in human pancreatic ductal carcinoma. Of 19 tissue samples, 8 were obtained from pancrearectomy and 11 from FNA. They included 10 men and 9 women with age range of 40-78 years (mean 69.8) and consisted of one stage I, 14 stage III and 4 stage IV patients (the International Union against Cancer classification). The immunostaining results are shown in Table I. Positive staining for C-ERC/mesothelin was seen in 14 of the 19 samples. Six of 11 samples from FNA and all of the samples from pancreatic resections showed positive staining. The staining pattern was often focal and cytoplasmic with polarity to apical membrane (Fig. 2A and B). In some cases, polarity of the signal was weak or none (Fig. 2C).

Serum N-ERC/mesothelin levels in patients with pancreatic ductal carcinoma. Serum samples from 19 patients were obtained before surgery, chemotherapy, or any other therapies. The sera were evaluated for N-ERC/mesothelin. The 19 age-matched healthy control samples, from the database as described (6), included 10 men and 9 women with age range of 50-79 years (mean 65). There was no significant difference in serum N-ERC/mesothelin