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Toxicologic Pathology, 35:436–443, 2007
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ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1080/01926230701302541

Possible Application of Human c-Ha-ras Proto-Oncogene Transgenic Rats in a Medium-Term Bioassay Model for Carcinogens

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ABSTRACT

With the aim of developing a medium-term assay for screening of environmental carcinogens, we exposed mammary carcinogen sensitive human c-Ha-ras proto-oncogene transgenic (Hras128) rats to various carcinogens, including compounds that do not normally induce mammary tumors. Seven-week-old Hras128 rats and wild-type littermates received administrations of 3-methylcholanthrene (3-MC), benzo[a]pyrene (B[a]P), anthracene, pyrene, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), dimethylarsinic acid (DMA), diethylnitrosamine (DEN) or azoxymethane (AOM) and were sacrificed at week 12 (females) (at week 10 for the 3-MC group) or week 20 (males). Female Hras128 rats receiving NNK, DEN, or DMA showed a significant increase in mammary tumor incidence and/or multiplicity compared to the respective values with olive oil or deionized distilled water (DDW) vehicles. In male Hras128 rats, a significant increase in mammary tumors was also observed in groups administered 3-MC, B[a]P, anthracene, IQ, and NNK. Mutations of transgenes were observed in codons 12 and/or 61 in the induced tumors by PCR-RFLP except in the DEN group in female and in the MeIQx group in male Hras128 rats. Thus various carcinogens, not necessarily limited to those normally targeting the breast, were found to induce mammary carcinomas in Hras128 rats, especially in females, pointing to potential use for medium-term screening.

Keywords. Transgenic rat; Hras; mammary tumors; medium-term assay.

INTRODUCTION

We have generated human c-Ha-ras proto-oncogene transgenic (Hras128) rats that are highly sensitive to mammary carcinogens, rapidly developing carcinomas after exposure to *N*-methyl-*N*-nitrosourea (MNU), dimethylbenzo[a]anthracene (DMBA), or PhIP (Asamoto et al., 2000; Tsuda et al., 2001). Furthermore, the Hras128 rats are also highly susceptible to induction of lesions in the esophagus, bladder, skin and tongue (Ota et al., 2000; Asamoto et al., 2002; Park et al., 2004; Suzuki et al., 2006).

The incidence of spontaneous tumors in the mammary gland of Hras128 rats was 52.8% at 40 weeks and slightly increased compared to female Sprague–Dawley wild-type rats (Tsuda et al., 2005). Taking advantage of these characteristics, we have focused on whether our transgenic animals might have advantages for use in short- or medium-term assay systems for screening environmental carcinogens. One problem is that carcinogens generally have specific organotropic actions as initiating agents (Tsuda et al., 1999).

One way to overcome this is to use multi-organ carcinogenesis models (Imaida and Fukushima, 1996; Ito et al., 1988) in which animals are first treated with various carcinogens, initiating carcinogenesis in the major organs and then assaying promotion or other modulating effects. However, the established protocols require upwards of 30 weeks until tumors or preneoplastic lesions are induced. As a single organ model, the Ito approach in the liver has many advantages in terms of cost and duration, at 8 weeks, but requires partial hepatectomy to enhance carcinogenesis (Tsuda et al., 1980; Ito et al., 1989). While transgenic (rasH2) mice bearing a human c-H-ras proto-oncogene have attracted interest for testing purposes (Ando et al., 1992; Yamamoto et al., 1996), the assay takes 26 weeks and cannot be said to be short-term. Our Hras128 rats develop tumors within 8 weeks.

For validation in the present study, a number of known carcinogens were selected. These were genotoxic agents as the results could not be applied directly to nongenotoxic agents. The polycyclic aromatic hydrocarbons 3-methylcholanthrene (3-MC) and benzo[a]pyrene (B[a]P) and their parent nuclear substances anthracene and pyrene are included in exhaust gas and tobacco smoke. 3-MC and B[a]P in particular are known to be causative agents for

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Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; DMBA, dimethylbenzoanthracene; 3-MC, 3-methylcholanthrene; B[a]P, benzo[a]pyrene; IQ, 2-amino-3-methylimidazo[4, 5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4, 5-*f*]quinoxaline; NNK, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone; DEN, diethylnitrosamine; AOM, azoxymethane; DMA, dimethylarsinic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphisms.

lung cancer in humans and mammary cancers in rats (Bolasny et al., 1963; Gingell et al., 1981). The heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3, 8-dimethylimidazo[4, 5-*f*]quinoxaline (MeIQx) are contained in broiled meat and fish (Sugimura, 1985) and 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK) is found in tobacco smoke (Brown et al., 1999). Diethylnitrosamine (DEN) is an N-nitroso compound commonly used in liver cancer experiments (Ito et al., 1989), while azoxymethane (AOM) specifically induces aberrant crypt foci and tumors in the colon (Thorup et al., 1995). Dimethylarsinic acid (DMA) is an arsenic compound present in the environment (Braman and Foreback, 1973), which is known to cause urinary bladder cancers (Wei et al., 1999; Cohen et al., 2001).

In a series of experiments we administered these chemical carcinogens to Hras128 rats and made gross pathological and histopathological assessment of lesion induction. Furthermore, transgene mutations were examined to determine whether the exogenous gene copies were targeted by the carcinogens. The results indicated that the Hras128 rat may indeed have potential for use as a medium-term assay model.

MATERIALS AND METHODS

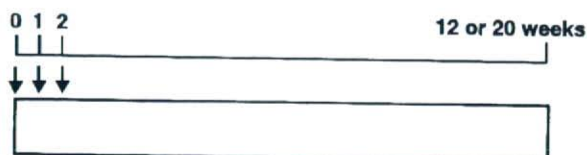
Animals and Chemicals

Sprague–Dawley rats (Clea Japan, Inc., Tokyo, Japan) were used for creating the human c-Ha-ras proto-oncogene transgenic rats (Hras128) (Asamoto et al., 2000), with the human c-Ha-ras proto-oncogene established by Sekiya et al. (1985). The animals were kept under constant conditions with a 12-hour light/dark cycle, a room temperature of 22 ± 2°C, and a humidity of 55 ± 10%. They were allowed access to a basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and tap water. All rats, transgenic and wild-type littermates, were treated the same. 3-MC, B[a]P, pyrene, and AOM were purchased from Sigma Chemical Co., St Louis, USA; IQ and MeIQx from Nard Institute, Osaka, Japan; NNK from Toronto Research Chemicals Inc., Ontario, Canada; DEN from Tokyo Kasei, Co., Tokyo, Japan; and DMA and olive oil from Wako Pure Chemical Industries, Osaka, Japan. Anthracene (purity >99.9%) was provided by Dr. Matsushima of Japan Bioassay Research Center, Hadano, Japan.

The experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center Japan" promulgated by the Committee for Ethics of Animal Experimentation.

Experimental Protocol (Figure 1)

3-MC, B[a]P, anthracene, pyrene, IQ, MeIQx, and NNK were dissolved in olive oil, and DEN, DMA, and AOM in deionized distilled water (DDW). Two hundred mg/kg of 3-MC, B[a]P, anthracene, and pyrene, 80 mg/kg of IQ and MeIQx, and 100 mg/kg of NNK and DMA were administered by gastric intubation to 7-week-old Hras128 rats and their littermates (wild-type) once a week for 3 weeks. One hundred mg/kg of DEN was given once a week for 2 weeks, and 50 mg/kg of AOM once. The control group received 5 ml/kg of olive oil or DDW. Females were sacrificed at week 12, except for the 3-MC treated group (week 10 due to a moribund condition caused by multiple mammary carcinomas), and males at week 20. Numbers, weights, and sizes of all mammary tumors were then recorded.



Animal: 7-week-old male and female Hras128 rats

↓Test compound administration with gastric intubation (i.g.)
Administration by i.g. start at 0 and rats are killed at 12 or 20 weeks

200 kg/kg x 3 of 3-MC, B[a]P, Anthracene and pyrene
80 mg/kg x 3 of IQ and MeIQx
100 mg/kg x 3 of NNK and DMA
100 mg/kg x 2 of DEN
50 mg/kg x 1 of AOM
5 ml/kg x 3 of olive oil or deionized distilled water (DDW)

FIGURE 1.—Experimental protocol for the assay of test compounds using Hras128 and littermate wild-type rats. 0 is the start of administration by i.g. and rats are sacrificed at 12 or 20 weeks of this study.

Histological Study and DNA Isolation

All mammary tumors were removed and, after measurements, were immediately fixed in ice-cold acetone. Tissues were embedded in paraffin and stained with hematoxylin-eosin, followed by histopathological examination. DNA was extracted using DEXPAT (Takara, Otsu, Japan) from paraffin sections 10 μm in thickness.

Mutation Analysis

Mutation analysis of transgene codons 12 and 61 was performed using the PCR-restriction fragment length polymorphism (RFLP) approach (Asamoto et al., 2002). The primers for codon 12 were hHras1F (5′-GC AGGCCCCTGAGGAGCGAT-3′), and hHras1RN (5′-AGC AGCTGCTGGCACCTGGA-3′), and for codon 61 were hHras2F (5′-AGCCCTGTCCTCCTGCAGGAT-3′), hHras2 R (5′-GGCCAGCCTCACGGGGTTCA-3′), and H61/2A2 (5′-CGCATGGCGCTGTACAGCTC-3′). After 5 minutes at 95°C, thermocycling conditions were: 1 minute at 95°C, 1 minute at 60°C, 3 minutes at 72°C for 35 cycles, with a final extension of 10 minutes at 72°C. The thermal cycler was a Gene Amp PCR System 9600 (Perkin-Elmer Corp. Norwalk, USA), with MSP I (Takara, Otsu, Japan) for codon 12 and AlwN I (New England BioLabs, MA, USA) for codon 61 as restriction enzymes. After confirming mutations in codons 12 and 61 with PCR-RFLP, DNA lengths of 167 bp for codon 12 and 93 bp for codon 61 were extracted from 4% agarose gels (NuSieve GTG agarose, BMA, USA) using a Min Elute Gel Extraction Kit (QIAGEN, USA) and sequenced using Big Dye Terminator v3.1 (Applied Biosystems, Japan) and an ABI PRIZM3100-Avant Genetic Analyzer (Applied Biosystems, Japan).

Statistics

Analysis of the incidences of mammary tumors and their sizes and multiplicities was conducted using the JMP software package (version 3.1)(SAS Institute, Cary, NC). Chi

TABLE 1.—Incidence and multiplicity of mammary tumors in Hras128 and nontransgenic female rats.

Treatment	No. of rat	Incidence (%)[a]	Diameter(mm)[b]	Microscopic Data		
				Adenoma[b]	Adenocarcinoma[b]	Total[b]
Hras128						
3-MC	7	7 (100)***	28 ± 8.5**	0.3 ± 0.5	5.3 ± 3.9**	5.6 ± 3.8**
B[a]P	8	8 (100)***	20 ± 8.4**	0.1 ± 0.4	6.8 ± 3.5***	6.9 ± 3.6***
Anthracene	7	4 (57.1)*	9.8 ± 14	0.4 ± 0.5	0.1 ± 0.4	0.6 ± 0.5*
Pyrene	7	3 (42.9)	8.0 ± 8.3	0.1 ± 0.4	0.4 ± 0.8*	0.6 ± 0.8
NNK	10	2 (20)	1.3 ± 9.3	0	0.2 ± 0.6	0.6 ± 0.6
IQ	10	7 (70)**	14 ± 10*	0	2.0 ± 1.9**	2.0 ± 1.9**
MeIQx	10	6 (60)*	7.6 ± 7.1	0	0.8 ± 0.9*	0.8 ± 0.9*
DEN	9	3 (33.3)	4.8 ± 8.3	0.1 ± 0.3	0.2 ± 0.4	0.3 ± 0.5
DMA	9	1 (11.1)	4.5 ± 8.6	0	0.3 ± 0.7	0.2 ± 0.7
AOM	9	6 (66.7)#	13 ± 12#	0	0.6 ± 0.5#	0.6 ± 0.5#
Olive Oil	11	2 (18.2)	6.5 ± 14	0.2 ± 0.4	0	0.2 ± 0.4
DDW	8	1 (12.5)	4.3 ± 8.8	0	0.3 ± 0.7	0.3 ± 0.7
Non-Tg						
3-MC	7	2 (28.6)	7.2 ± 13	0	0.3 ± 0.5	0.3 ± 0.5
B[a]P	7	0	0	0	0	0
Anthracene	8	0	0	0	0	0
Pyrene	7	0	0	0	0	0
NNK	12	0	0	0	0	0
IQ	14	1 (7.1)	0.7 ± 2.7	0.1 ± 0.3	0	0.1 ± 0.3
MeIQx	12	0	0	0	0	0
DEN	9	0	0	0	0	0
DMA	9	0	0	0	0	0
AOM	9	0	0	0	0	0
Olive Oil	12	0	0	0	0	0
DDW	8	0	0	0	0	0

[a]Adenoma and carcinoma combined; [b]Number count / rat, Mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to olive oil of Hras128 rat, #$p < 0.05$ as compared to DDW of Hras128.

squared tests were conducted for tumor incidence data and the Dunnett's t-test with ANOVA for tumor size and multiplicity.

RESULTS

Incidences and Multiplicity of Mammary Tumors

Female Rats. (see Table 1) All tumors taken (larger than 3 mm in longer diameter) were adenocarcinomas with obvious invasion of surrounding mammary and stromal tissue. In female Hras128 rats, mammary tumors developed in 7 of 7 rats (100%) given 3-MC, 8/8 (100%) with B[a]P, 4/7 (57.1%) with anthracene, 3/7 (42.9%) with pyrene, 2/10 (20%) with NNK, 7/10 (70%) with IQ, 6/10 (60%) with MeIQx, 3/9 (33.3%) with DEN, 6/9 (66.7%) with AOM, and 1/9 (11.1%) with DMA. There was a significant increase in the tumor incidence in female Hras128 rats in the 3-MC and B[a]P groups at $p < 0.001$, the IQ group at $p < 0.01$, and the anthracene, MeIQx, and AOM groups at $p < 0.05$. The pyrene group also exhibited a significantly increased number of tumors in comparison with the olive oil group ($p < 0.05$). Among the littermate rats (wild-type), single tumors were found in 2 rats of the 3-MC group and 1 rat of the IQ group, but there were no significant differences from the control (olive oil) group. No tumors other than mammary gland were found in Hras 128 rats. No tumors were detected in any other groups of wild-type rats.

Male Rats (Table 2). The percentages of male rats with mammary tumors for each carcinogen were as follows: 3-MC, 87.5%; B[a]P, 62.5%; anthracene, 42.9%; pyrene, 10%; NNK, 25%; IQ, 16.7%; MeIQx, 8.3%; AOM, 25%; DEN and DMA, 0%. The incidences were significantly increased in the 3-MC, B[a]P ($p < 0.001$), and anthracene ($p < 0.05$) groups. The multiplicity was significantly increased in the NNK ($p < 0.05$) group. Mammary tumor size (7.8 ± 15 mm) was significantly greater in the IQ group than in the olive oil group ($p < 0.05$). No significant difference from controls was seen in tumor development in the littermate wild rats. In Hras128 rats, zymbal gland tumors occurred in 3 rats, colonic polyps in 3 rats and scrotal squamous cell papillomas in 2 rats with AOM (Figure 3A), a scrotal squamous cell papillomas in 1 rat and a malignant lymphoma in 1 rat with DMA, zymbal gland tumors in 2 rats with NNK, scrotal squamous cell papillomas in 2 rats with DEN, a scrotal squamous cell papilloma in 1 rat with IQ and a back skin squamous cell papilloma in 1 rat with pyrene (Table 3). Sarcomas, composed of spindle shaped tumor cells, were found only in male Hras128 rats at lower incidences. These cells were negative for antibodies for pankeratin, S-100 protein and alfa-smooth muscle actin (Figure 3B). Sarcomas occurred in 2 rats with 3-MC, 1 rat with B[a]p, and 1 rat with MeIQx.

Mutation Analysis of the Transgenes

The tumors mutation results of PCR-RFLP for codons 12 and/or 61 in the Hras128 rats are shown in Tables 4 and 5. Codons 12 and/or 61 in female rats were as follows: 3-MC, 84.8%; B[a]P, 75%; anthracene, 66.7%; NNK, 100%; IQ, 83.3%; and AOM, 100%. Mutations in both codons 12 and 61 were present in 18.2% of the 3-MC group and 28.6% of the B[a]P group (Table 4). Codons12 and/or 61 in male rats were 3-MC, 66.7%; B[a]P, 100%; anthracene, 66.7%; and AOM, 100%. Mutations on both codons 12 and 61 were present in 5.6% of the 3-MC group and 50% of the B[a]P group (Table 5).

Direct Sequencing of Mutated Bands

The results of direct sequencing of DNA are summarized in Table 6. Figures in Table 6 show the numbers of mutation type in mammary tumors in Hras128 rats combined for female

TABLE 2.—Incidence and multiplicity of mammary tumors in Hras128 and nontransgenic male rats.

Treatment	No. of rat	Incidence (%)[a]	Diameter (mm)[b]	Microscopic Data		
				Adenoma[b]	Adenocarcinoma[b]	Total[b]
Hras128						
3-MC	8	7 (87.5)***	38 ± 18***	0	4.1 ± 2.9***	4.1 ± 2.9***
B[a]P	8	5 (62.5)***	28 ± 9.8***	0.1 ± 0.4	1.0 ± 0.9**	1.1 ± 1.1**
Anthracene	7	3 (42.9)*	5.5 ± 7.5*	0.3 ± 0.5*	0.1 ± 0.4	0.4 ± 0.5*
Pyrene	10	1 (10.0)	3.0 ± 9.7	0	0.1 ± 0.3	0.1 ± 0.3
NNK	12	3 (25)	3.1 ± 8.1	0.3 ± 0.5*	0	0.3 ± 0.5*
IQ	12	2 (16.7)	7.8 ± 15*	0	0.3 ± 0.6	0.3 ± 0.6
MeIQx	12	1 (8.3)	4.0 ± 14	0	0.1 ± 0.3	0.1 ± 0.3
DEN	10	0	0	0	0	0
DMA	10	0	0	0	0	0
AOM	8	2 (25.0)	13 ± 8.5	0	0.3 ± 0.7	0.3 ± 0.7
Olive Oil	12	0	0	0	0	0
DDW	8	1 (12.5)	14.5	0	0.13	0.13
Non-Tg						
3-MC	7	1 (14.3)	2.7 ± 7.2	0	0.1 ± 0.4	0.1 ± 0.4
B[a]P	8	0	0	0	0	0
Anthracene	6	0	0	0	0	0
Pyrene	10	0	0	0	0	0
NNK	10	0	0	0	0	0
IQ	10	0	0	0	0	0
MeIQx	10	0	0	0	0	0
DEN	10	0	0	0	0	0
DMA	10	0	0	0	0	0
AOM	9	0	0	0	0	0
Olive Oil	10	0	0	0	0	0
DDW	8	0	0	0	0	0

[a] Adenoma and carcinoma combined; [b] Number count / rat, Mean ± SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to olive oil of Hras128 rat.

with male. In codon 12 there were transversion mutations of GGC to GTC and GGC to TGC (mutation underlined) at rates of 95.3% (61/64) and 4.7% (3/61), respectively. In codon 61 there were transition mutations of CAG to CGG and transversion mutation of CAG to CAT, CAG to AAG, CAG to CTG and CAG to CGT (mutation underlined) at rates of 58.3% (21/36), 33.3% (12/36), 2.8% (1/36), 2.8% (1/36) and 2.8% (1/36), respectively.

DISCUSSION

The present study demonstrated that the mammary tissue of our transgenic rats is sensitive to the carcinogenic actions of chemicals such as IQ, MeIQx, NNK, and AOM, the last two not normally inducing breast tumors (Reddy et al., 1975; Thorup et al., 1995; Masumura et al., 2003). Furthermore, positive results were also obtained with 3-MC and B[a]P, along with their parent compounds, pyrene, rated as Group 3 in the IARC Monograph series (1983), and anthracene. It should be noted that anthracene, which has been generally considered as a noncarcinogen, also gave positive results in a 2-year chronic feeding test (personal communication from Dr. Matsushima of the Japan Bioassay Research Center).

TABLE 3.—Tumors other than mammary glands in Hras128 and nontransgenic rats.

		Number of rats with tumor			
Animals	No. of rat	Zymbal gland tumor	Colonic adenoma	Scrotum and back skin papilloma	Malignant lymphoma
Hras128					
Female	105	0	0	0	0
Male	114	5 (4.4%)	3 (2.6%)	7 (6.1%)	1 (0.9%)
Non-Tg					
Female	117	0	0	0	0
Male	108	0	0	0	0

TABLE 4.—Transgene mutation rate of mammary tumors in Hras128 female rats.

Test compound	Codon12 (%)	Codon 61 (%)	Codon 12 and/or 61 (%)
3-MC	20/33 (60.6)	14/33 (42.4)	28/33 (84.8)
B[a]P	17/28 (60.7)	12/28 (42.9)	21/28 (75.0)
Anthracene	1/3 (33.3)	1/3 (33.3)	2/3 (66.7)
Pyrene	1/2 (50.0)	0/2 (0)	1/2 (50.0)
NNK	1/2 (50.0)	1/2 (50.0)	2/2 (100)
IQ	8/12 (66.7)	2/12 (16.7)	10/12 (83.3)
MeIQx	1/3 (33.3)	0/3 (0)	1/3 (33.3)
DEN	0/3 (0)	0/3 (0)	0/3 (0)
DMA	0/2 (0)	1/2 (50.0)	1/2 (50.0)
AOM	3/5 (60.0)	2/5 (40.0)	5/5 (100)

TABLE 5.—Transgene mutation rate of mammary tumors in Hras128 male rats.

Test compound	Codon12 (%)	Codon 61 (%)	Codon 12 and/or 61 (%)
3-MC	2/18 (11.1)	11/18 (61.1)	12/18 (66.7)
B[a]P	4/4 (100)	2/4 (50.0)	4/4 (100)
Anthracene	0/3 (0)	2/3 (66.7)	2/3 (66.7)
Pyrene	1/1 (100)	0/1 (0)	1/1 (100)
NNK	—	—	—
IQ	1/2 (50.0)	0/2 (0)	1/2 (50.0)
MeIQx	0/2 (0)	0/2 (0)	0/2 (0)
DEN	—	—	—
DMA	—	—	—
AOM	4/7 (57.1)	3/7 (42.9)	7/7 (100)

TABLE 6.—Transgene mutation type of codon 12 and 61 in Hras128 rats.

	Codon12 (GGC)		Codon 61 (CAG)				
Test compound	GTC	TGC	CGG	CAT	AAG	CTG	CGT
3-MC	20	2	9	4	1	0	0
B[a]P	20	1	6	4	0	0	0
Anthracene	1	0	3	0	0	0	0
Pyrene	2	0	0	0	0	0	0
NNK	1	0	0	1	0	0	0
IQ	9	0	1	1	0	0	0
MeIQx	1	0	0	0	0	0	0
DEN	0	0	0	0	0	0	0
DMA	0	0	0	0	0	1	0
AOM	7	0	2	2	0	0	1

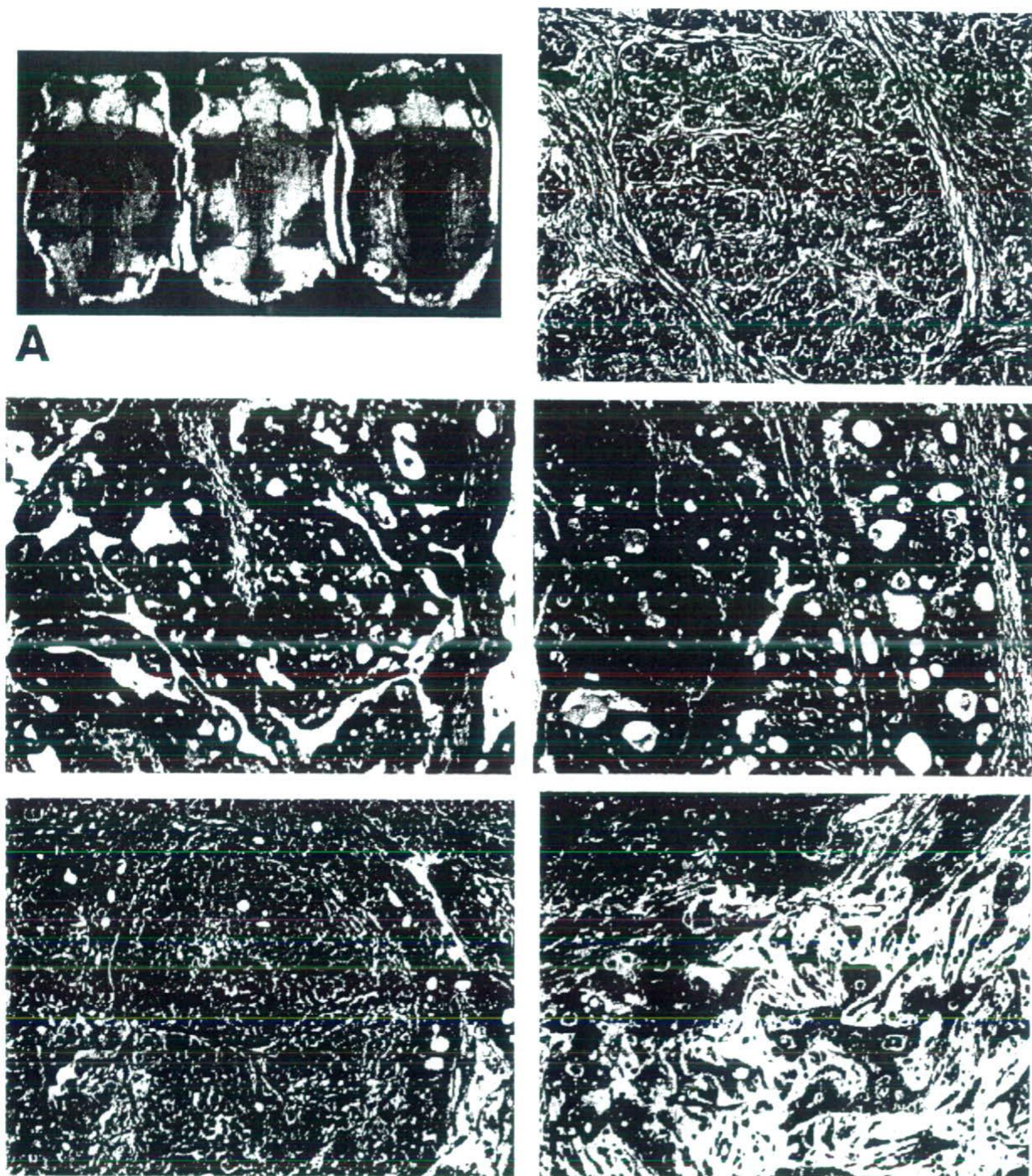


FIGURE 2.—Macroscopic and histological appearance of tumors found in Hras128 rats. (A) From left, B[a]P-, IQ-, and olive oil-treated female rats. (B) Fibroadenoma in a DEN treated female rat. (C) Papillary tubular carcinoma in a B[a]P-treated female rat. (D) Solid tubular (left half) and tubular (right half) carcinoma in an IQ-treated female rat. (E) Solid tubular carcinoma with a cribriform pattern in an IQ-treated female rat. (F) Tubular carcinoma with loose fibrosis in a MeIQx-treated female rat.

Although the mouse model harboring the same human c-Ha-ras proto-oncogene as in our Hras128 rats has been extensively examined for susceptibility to various carcinogens and has found application as a medium-term assay system with lung tumors as the endpoint lesions, the experimental protocol required in 26 weeks (Mitsumori et al., 1998; Yamamoto et al., 1998). The duration with the current model, 12 weeks for females and 20 weeks for males, has clear

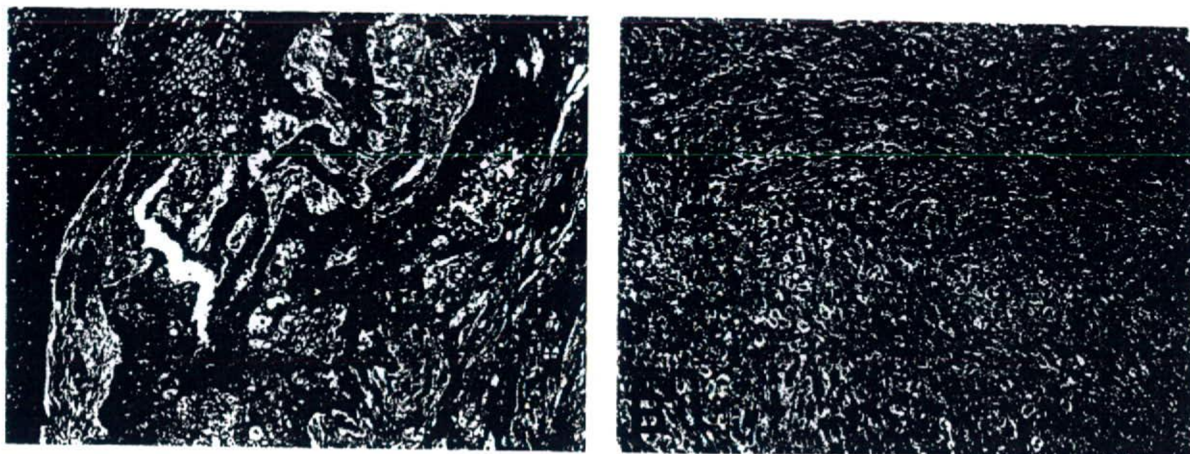


FIGURE 3.—Histological appearance of nonadenocarcinoma tumors. (A) Squamous cell papilloma of the scrotum in a Hras128 rat treated with AOM showing papillary formation and keratinization. (B) Sarcoma of a mammary gland in a male Hras128 rat treated with B[a]P. Note the spindle-shaped tumor cells showing a storiform arrangement.

advantages in terms of practical application. Indeed, based on our recent observation of development of mammary cancers 15 and 20 days after the administration of MNU (Matsuoka et al., 2003), it may be possible to shorten the experimental period by histopathological detection of early carcinomas in abdominal mammary glands.

Tumors observed in Hras128 rats were mammary carcinomas and squamous cell papillomas in the back and the scrotum skin. Histological types of mammary carcinomas were tubular with a cribriform arrangement, solid tubular or papillary tubular (Figure 2), all of which are similarly found after treatment with N-methyl-N-nitrosourea, and, importantly, resemble those found in humans (Asamoto et al., 2000). Acinar cell type tumors were not observed. Areas of differing morphology were often found mixed within the same mammary tumors. Furthermore, there was no tendency for specific types to be localized in different mammary glands. No treatment related incidence of any specific histological type or localization was observed. Fibrosarcomas, composed of spindle-shaped, irregular-shaped tumor cells, were found only in male Hras128 rats at lower incidence. Metastasis from adenocarcinomas was not found. Although we have conducted histological examinations of all major organs, including the esophagus, forestomach, tongue, and urinary bladder, which were also found to be highly susceptible to chemical carcinogens in Hras128 rats, no tumors were found, possibly due to the relatively shorter duration of the observation period and low doses of carcinogens. It appears that carcinomas induced in Hras128 rats are not as variable as those observed in transgenic mice (Cardiff et al., 2000).

Since high incidences of the transgene mutation are observed in the mammary tumors in this transgenic rat induced by typical mammary carcinogens (Asamoto et al., 2000), it is clearly of interest whether the same situation might exist with regard to various other carcinogens. Our present studies clearly indicated that the transgenes, but not the endogenous rat c-Ha-ras gene, demonstrate mutations at relatively high incidence, suggesting an important role in carcinogenesis. Although the number of tumors used for mutation analysis was low except for the B[a]P and 3-MC cases, the results are highly suggestive that the compounds commonly cause mutation of the transgenes. The c-Ha-ras gene was also observed in mice with the same transgene (Ando et al., 1992).

In our recent studies, such mutations were already evident in endbuds (Hamaguchi et al., 2004), postulated tissue targets of carcinogens (Russo et al., 1979, 1983), before obvious proliferative change occurred. Thus, it is possible that test compounds including nonmammary carcinogens might also cause mutation of the transgenes, a possibility which we are presently exploring. In the present study, most mutations were of transversion type in codon 12, GGC to GTC predominating, irrespective of the chemical carcinogen. Clearly, it is necessary to analyze whether transversion clustering is dependent on the carcinogen administered or the organ in which the tumor appears. Establishment of short-term assay models is essential to reduce the cost and increase the number of compounds that can be tested (Tennant et al., 1995; Tsuda et al., 1999). From our present review, the human c-Ha-ras proto-oncogene transgenic rat is a good candidate for this purpose.

The assay model is advantageous because the endpoint is mammary carcinomas that can be grossly observed. Furthermore, this model can be used for the assay of modifying agents including chemopreventive compounds (Matsuoka et al., 2003) and also nongenotoxic promoting agents (Fukamachi et al., 2004; Tsuda et al., 2005). Given the number of compounds released in our environment, further validation studies using Hras128 rats will be necessary.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare, Japan, by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan, and a Grant-in-Aid for Scientific Research (KAKENHI) on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, by a Grant-in Aid for The Long-Range Research

Initiative from Japan Chemical Industry Association (CC05-01), and by Surveillance Study for Appropriate Assessment of Refined Petroleum Products by the Ministry of Economy, Trade and Industry. The authors would like to thank Dr. T. Matsushima of the Japan Bioassay Center, Hadano, for generous provision of purified anthracene and Dr. Samuel M. Cohen of University of Nebraska Medical Center for his kind advice in preparation of the manuscript. T. Ohnishi was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research under the Second Term Comprehensive 10-Year Strategy for Cancer Control, when he was at the Experimental and Chemotherapy Division of National Cancer Center Research Institute.

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Sensitive and Specific New Enzyme-Linked Immunosorbent Assay for N-ERC/Mesothelin Increases its Potential as a Useful Serum Tumor Marker for Mesothelioma

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Abstract **Background:** Because mesothelioma initially progresses on the surface of the pleura and peritoneum without forming masses, it has been difficult to diagnose at an early stage. It would be very useful to identify a tumor marker that could be used for screening to enable more diagnoses to be made at an early, treatable stage.

Materials and Methods: We had previously identified N-ERC/mesothelin as a potential biomarker for mesothelioma. In the current work, we used a newly developed ELISA system to gain data on N-ERC/mesothelin levels in various clinical settings. A total of 102 healthy volunteers were recruited. In addition, 39 patients were diagnosed with mesothelioma, 53 patients were diagnosed with diseases that should be distinguished from mesothelioma, and 201 subjects were diagnosed with asbestos-related nonmalignant diseases (including simple exposure to asbestosis) who were treated at any of the cooperating hospitals were enrolled.

Results: Serum N-ERC/mesothelin levels measured by a new ELISA system showed that the median values from patients with mesothelioma were extremely high compared with levels obtained from other patients. Analysis in terms of histologic type showed that serum levels of N-ERC/mesothelin were elevated in epithelioid type mesothelioma, especially. In four important models of clinical settings, the sensitivity and specificity of N-ERC/mesothelin were about 71% to 90% and 88% to 93%, respectively.

Conclusion: N-ERC/mesothelin is a very promising tumor marker for mesothelioma, especially epithelioid mesothelioma.

Mesothelioma initially progresses along the surfaces of the pleura and peritoneum without forming masses; it is anatomically difficult to diagnose at an early stage and to completely remove with surgery. Moreover, mesothelioma typically has a long incubation period before it becomes clinically evident among high-risk individuals with severe exposure to asbestos. Sugarbaker et al. (1) has reported a groundbreaking result: for patients with early stage disease, 5-year survival after trimodality therapy exceeded 40%. This finding that early disease may be effectively treated emphasizes the importance of identifying a tumor marker that is practical for screening and can allow physicians to make an early diagnosis.

Recently, osteopontin, soluble mesothelin-related protein, and serum mesothelin have been reported as candidates for a mesothelioma tumor marker (2-7). We have postulated (8) that another product may be useful as a tumor marker: N-ERC/mesothelin, a NH_2 terminal 31-kDa fragment of mesothelin gene products that was first cloned as a megakaryocyte-potentiating factor in humans and that is physiologically secreted into blood. Since the time of that report, we have established a new ELISA system that detects the NH_2 terminal fragments of ERC/mesothelin products at a higher sensitivity and specificity. The current work was done to obtain data for

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Received 6/30/07; revised 11/14/07; accepted 11/20/07.

Grant support: Grant-in-aid for Cancer Research and grants-in-aid for Scientific Research from Ministry of Education, Culture, Sports, and Science and Technology of Japan and Ministry of Health, Labor, and Welfare of Japan. This study was partially supported by a consignment expense for Molecular Imaging Program on "Research Base for PET Diagnosis" from Ministry of Education, Culture, Sports, and Science and Technology, Government of Japan.

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doi:10.1158/1078-0432.CCR-07-1613

N-ERC/mesothelin with use of the new ELISA system in various clinical settings and to compare its data with that of the previous ELISA system.

Materials and Methods

Preparation of novel anti-ERC/mesothelin antibodies. The anti–N-ERC/mesothelin monoclonal antibody (MoAb) clone 7E7 has been previously reported (8). We established a novel MoAb clone in the same way. Briefly, N-ERC/mesothelin, expressed in *Escherichia coli* as glutathione S-transferase–tagged and histidine-tagged fusion proteins, was purified and used as an immunogen. Splenocytes from immunized mice were fused with myeloma cell line X63-Ag8.653. Supernatants of the hybridoma cells were screened for reactivity to immunogen using ELISA, and several positive clones were selected by limiting dilution method. One novel clone, 16K16, was chosen for use with the new ELISA system in this study.

Cell culture, protein expression, and Western blot analysis. CHO-K1 cells were cultured in DMEM supplemented with 10% FCS. Full-length cDNA of the ERC/mesothelin coding region was inserted into the pcDNA3.1(+) vector (Invitrogen) to enable expression in CHO-K1 cells. Transfection was done using FuGENE6 transfection reagent (Roche-Diagnostics). A stable transfectant of CHO-K1 cells that expressed ERC/mesothelin protein was screened by G418 resistance and established as the cell line for further study.

Culture supernatants and ERC/mesothelin/CHO-K1 transfectant cells were harvested. CHO-K1 cells containing expressed ERC/mesothelin protein were lysed in a solution containing 2% SDS, 10% glycerol, 50 mmol/L Tris-HCl (pH 6.8), and 100 mmol/L DTT, and then boiled. The crude lysates and culture supernatants were electrophoresed on 12.5% Laemmli gels and transferred to polyvinylidene fluoride membranes. Two identical membranes were blocked in 1% skim milk in PBS with 0.1% Tween 20 (PBS-T) for 1 h at room temperature. Next, one of the two membranes was incubated with 1 μg/mL 7E7 MoAb; the other was incubated with 16K16 MoAb in 1% skim milk with PBS-T for an additional 1 h at room temperature. Secondary antibodies in the form of rabbit anti-mouse immunoglobulin conjugated to peroxidase (IBL) were then added and allowed to react with the membranes for 1 h longer at room temperature. ERC/mesothelin on the membranes was visualized by the enhanced chemiluminescence detection system (Amersham Biosciences).

Epitope mapping of MoAbs against N-ERC/mesothelin. The epitopes of MoAb-7E7 and MoAb-16K16 were searched against a series of deletion mutants of recombinant N-ERC/mesothelin protein expressed in an *in vitro* translation system using wheat germ extract. The cDNA of N-ERC/mesothelin was inserted into pEU vector (CellFree Sciences) as a glutathione S-transferase–tagged protein. A series of cDNA coding deletion mutants of recombinant N-ERC/mesothelin protein was amplified by PCR reactions with a series of antisense primers, each of which was displaced by 18 nucleotides from the COOH terminal region of N-ERC/mesothelin, then transcribed and translated in an *in vitro* protein expression system (ENDEXT Wheat Germ Expression Premium Kit, CellFree Sciences), according to the manufacturer's protocol. A series of recombinant proteins of deletion mutant N-ERC/mesothelin was applied to Dot blotting analysis with MoAb-7E7 and MoAb-16K16 to determine epitopes of each MoAb, as described in the paragraph of Western blotting.

Novel sandwich ELISA using MoAb-16K16. A novel sandwich ELISA system using clone 16K16 was established in the same manner as described previously (8). Microtiter plates (96 wells) were coated with 100 μL/well 100 mmol/L carbonate buffer (pH 9.5) containing purified 7E7 MoAb and allowed to adhere overnight at 4°C. Plates were washed with PBS-T and blocked for 1 h at room temperature with 200 μL/well 1% (w/v) bovine serum albumin in PBS containing 0.05% NaN_3. After three washes with PBS-T, 100-μL aliquots of test samples or recombinant N-ERC/mesothelin as a standard, serially diluted in 1% bovine serum albumin in PBS-T, were added in duplicate to wells and incubated at 37°C for 1 h. After seven washes with PBS-T, 100 μL horseradish peroxidase–conjugated MoAb-16K16 mouse IgG was added to each well and incubated for 30 min at 4°C. Wells were washed nine times with PBS-T, then 100 μL freshly prepared tetramethyl benzidine solution was added to each well as a substrate and incubated in the dark for 30 min at room temperature. The reaction was terminated by addition of 100 μL of 1 N H_2SO_4. Absorbance of the solution at 450 nm was measured in an ELISA reader (E-Max, Molecular Devices Co.). Recombinant N-ERC/mesothelin used as the standard protein in the ELISA system was purified from culture supernatants of

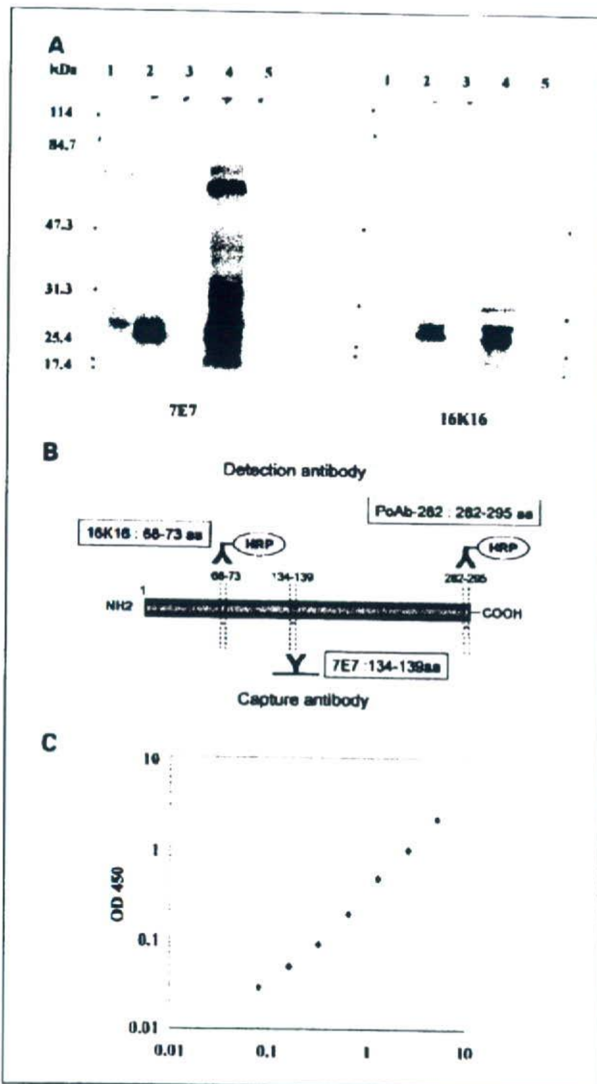

Fig. 1. *A*, characterization of anti–N-ERC/mesothelin antibodies by Western blot analysis. Lanes 2 and 4, culture supernatant and lysate of CHO-K1 cells transfected with ERC/mesothelin cDNA, respectively; lanes 1 and 3, culture supernatant and lysate of mock/CHO cells, respectively. *B*, epitopes of antibodies in N-ERC/mesothelin ELISA system. Epitope mapping for MoAbs revealed that the epitope of MoAb-7E7 was in amino acids 134 to 139 of N-ERC/mesothelin. Similarly, MoAb-16K16 recognized amino acids 68 to 73 of the N-ERC/mesothelin protein. The new ELISA system uses MoAb-7E7 and MoAb-16K16. The previous ELISA system used MoAb-7E7 and polyclonal antibody 282. *C*, standard dose-response curves for new ELISA system (7-16) for N-ERC/mesothelin.

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 abovementioned 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).

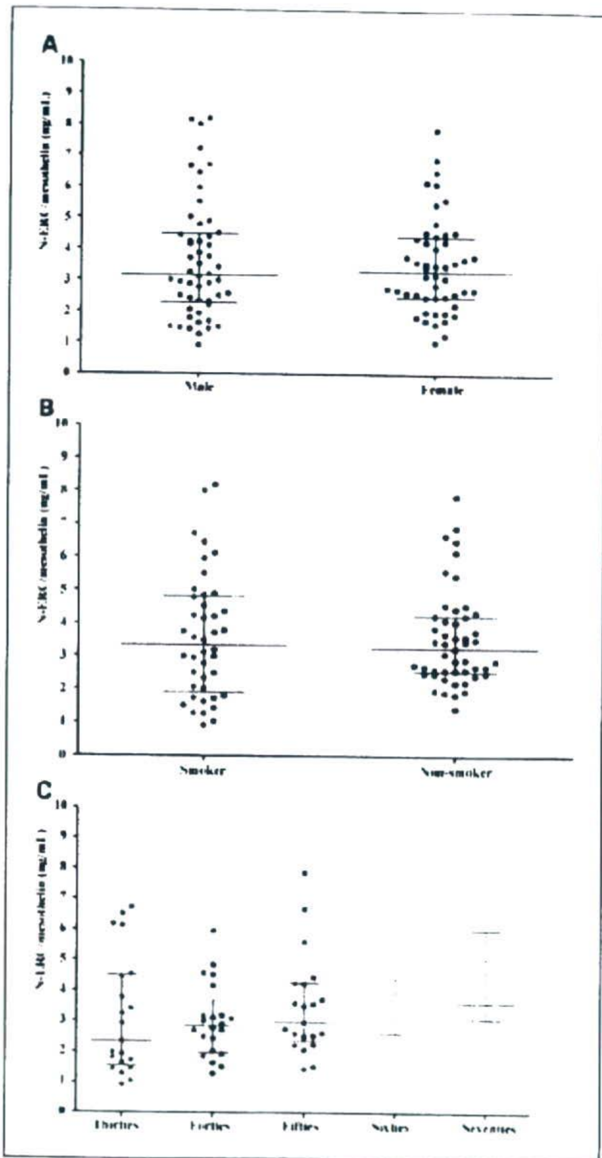

Fig. 2. Scatter plots of values for serum N-ERC/mesothelin (7-16) in healthy volunteers analyzing for possible effects of sex (*A*), smoking status (*B*), and age (*C*).

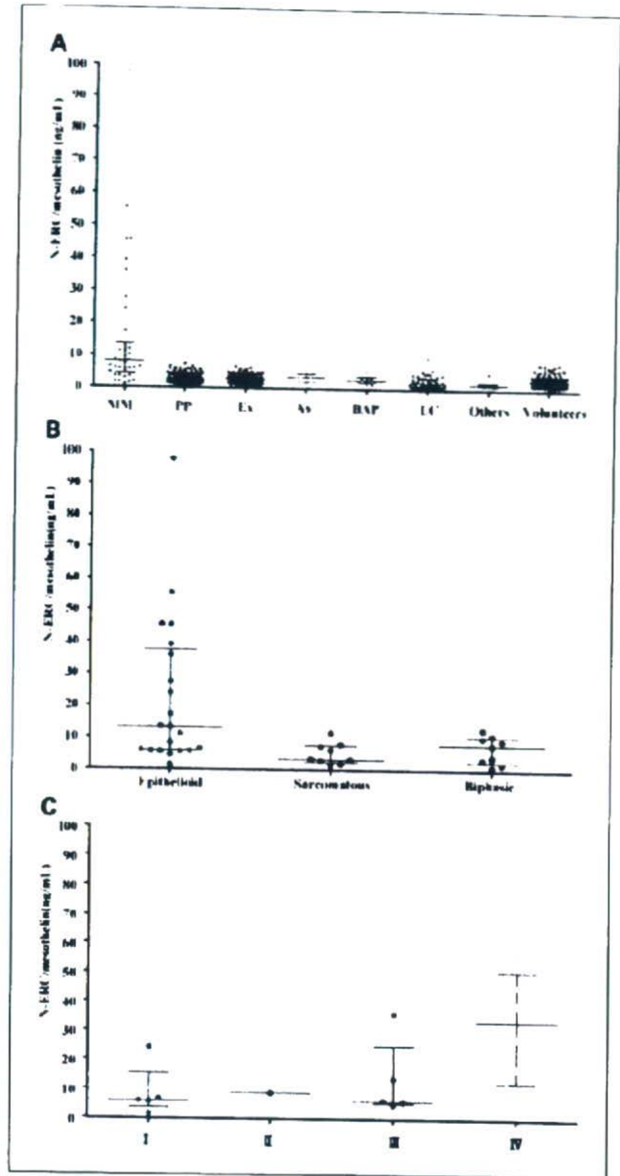

Fig. 3. *A*, scatter plots of values for serum N-ERC/mesothelin (7-16). MM, mesothelioma; As, asbestosis; PP, pleural plaque; BAP/DPT, benign asbestos pleuritis/diffuse pleural thickening; LC, lung cancer; Ex, exposure. *B*, scatter plots of serum N-ERC/mesothelin (7-16) by histologic type. *C*, scatter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

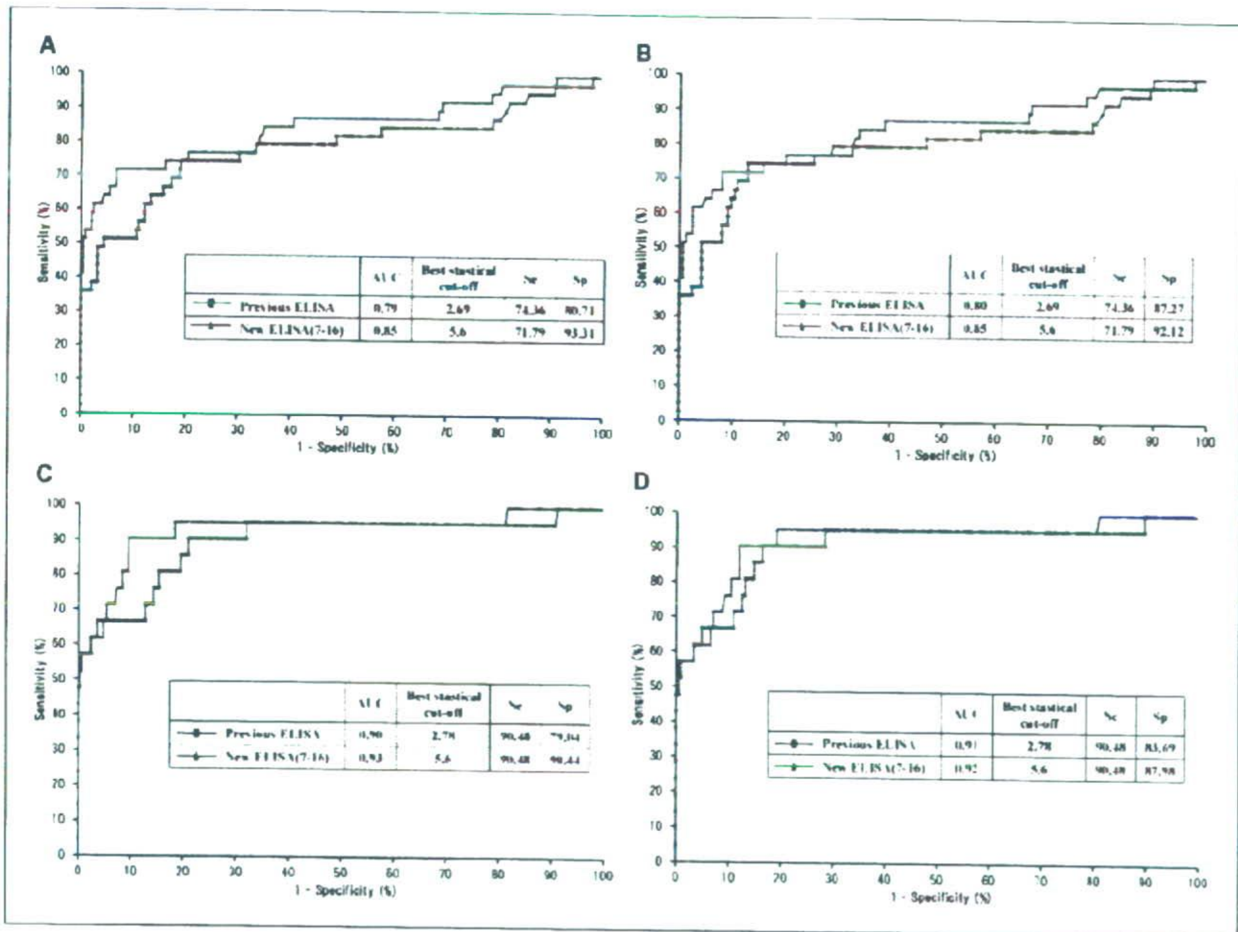

Fig. 4. *A,* screening mesothelioma among patient groups (including *PP, Ex, As, BAP, LC*, others). *B,* differentiating mesothelioma from other conditions in patients with clinically suspected mesothelioma, including patients with pleural effusion and/or pleural masses (including *PP, BAP, LC*, others).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

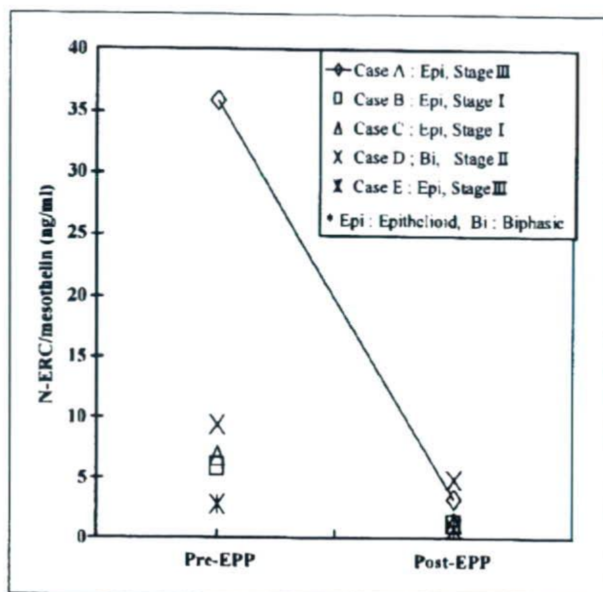

Fig. 5. Monitoring of N-ERC/mesothelin in some patients with confirmed mesothelioma.

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.

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

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

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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.

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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

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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

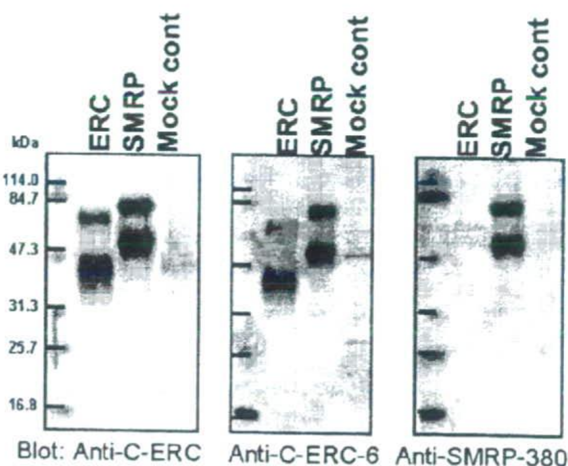

Fig. 1. Characterization of antibodies by Western blot analysis. Total cell lysates of COS-1 cells transfected with cDNA of ERC/mesothelin (ERC), SMRP and vector only (Mock) were subjected to Western blot analysis by anti-C-ERC, anti-C-ERC-6 and anti-SMRP-380 PoAb.