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Immunohistochemistry of cytokeratins 7, 8, 17, 18, and 19, and GLUT-1 aids differentiation of desmoplastic malignant mesothelioma from fibrous pleuritis

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Summary. It is difficult to distinguish desmoplastic malignant mesothelioma (DMM) from fibrous pleuritis (FP). We investigated the utility of immunohistochemistry as a way of differentiating between DMM and FP. We examined 11 DMMs and 46 FPs with the aid of antibodies against 18 cytokeratin (CK) subtypes, calponin, caldesmon, desmin, and GLUT-1. The best sensitivity and specificity cut-off values in the receiver operating characteristic curves (ROC) for CKs 7, 8, 17, 18, and 19, and GLUT-1 were each above 60%. When cases with either DMM or FP were partitioned by the staining score associated with the best sensitivity and specificity cut-off values in ROC, the incidence of a positive expression for CKs 7, 8, 17, 18, and 19, and GLUT-1 was significantly higher in DMM than in FP. In conclusion, immunohistochemistry for CKs 7, 8, 17, 18, and 19, and GLUT-1 may be useful, alongside histological characteristics, for separating DMM from FP.

Key words: Immunohistochemistry, Desmoplastic malignant mesothelioma, Fibrous pleurisy, Cytokeratins, GLUT-1

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Introduction

Malignant mesothelioma is a relatively rare tumor that originates from the serosal membrane of the pleura, peritoneum, pericardium, or tunica vaginalis. The latent period between asbestos exposure and onset of mesothelioma is reported to be between 15 and 60 years (Bianchi et al., 1997; McElvenny et al., 2005). In Japan, the Ministry of Health, Labor, and Welfare has disclosed that the number of deaths due to mesothelioma increased gradually from 500 in 1995 to 1156 in 2009 (Ministry of Health, Labour and Welfare 2010). On the above basis, mesothelioma cases are expected to continue to increase in Japan, and to peak in about the year 2025 because large amounts of asbestos were used in Japan between 1960 and 1975.

In the 2004 WHO classification, malignant mesotheliomas were essentially classified as epithelioid, biphasic, sarcomatoid, or desmoplastic. Although the desmoplastic type had been classified as a subtype of sarcomatoid mesothelioma until 2004, it is now considered a new entity because it is characterized by a shorter survival than either epithelioid or sarcomatoid mesothelioma (Churg et al., 2004). To qualify for a diagnosis of desmoplastic mesothelioma (DMM), the paucicellular collagen-rich tissue must occupy at least 50% of a tissue specimen. In addition to the above findings, a diagnosis of DMM requires a storiform pattern or the "patternless pattern" of Stout (Stout,

1965), plus one or more of the following four findings: invasion of chest wall or lung, bland necrosis, frankly sarcomatoid areas, and distant metastases. However, it is difficult to distinguish the histological features of DMM from those of fibrous pleuritis (FP) because the inflammation and hyperplasia of connective tissue cause a change in the form of the epithelioid cell so that it shows cytological atypia.

In recent years, a number of immunohistochemical markers -- including antibodies to cytokeratin (CK) 5/6, calretinin, Wilm's tumour-1 (WT-1), and thrombomodulin -- have become available for the diagnosis of mesothelioma. These markers have proven very useful for differentiating epithelioid mesothelioma from lung adenocarcinoma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Ordóñez 2003; Suster and Moran 2006; Addis and Roche 2009; Husain et al., 2009). In contrast, the frequency and degree of expression of these markers in DMM has not been characterized, and the effectiveness of immunohistochemistry for differentiating DMM from FP is unclear. To address these issues, we used antibodies against 18 cytokeratins, calponin, caldesmon, desmin, and GLUT-1 to examine a series of 11 DMMs and 46 FPs.

Materials and methods

We obtained surgically resected or autopsied specimens from patients with DMM (11 specimens) or FP (46 specimens). The DMM specimens were collected in 7 different hospitals across Japan. The DMM patients were 9 men and 2 women (mean age 68.3 years; range 57 to 83). Among them, seven patients had had previous occupational or environmental exposure to asbestos. Ten patients had pleural effusion with a high hyaluronic acid concentration, and 2 cases had positive cytology. Seven patients (excluding the 4 autopsy cases) died after an operation, such as panpleuropneumonectomy, pleurectomy or pleural biopsy. The four autopsy cases died at 1 to 8 months after the onset of symptoms.

Macroscopically, pleural thickening and adhesion were seen in DMM. Microscopically, DMM was characterized by the presence, in at least 50% of the tumor, of dense collagenized tissue separated by atypical tumor cells arranged in a storiform or "patternless" pattern. These areas included micronodular proliferation and foci of bland necrosis. For the definitive diagnosis of mesothelioma, we performed immunohistochemistry using 8 antibodies. Tumor cells were positive for

Table 1. Antibodies used in this study.

Antibody/ antigen	Type*/clone	Source	Dilution	Pretreatment
CK† 1	G	Acris Antibodies GmbH, Herford, Germany	1/25	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 2	M/BM5091	Acris Antibodies GmbH, Herford, Germany	1/100	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 3	M/AE5	Enzo Life Sciences, Plymouth Meeting, PA	1/1,000	boil in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 60 min
CK 4	M/EP1599	Novus Biologicals, Littleton, CO	1/100	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 5	M/XM26	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/300	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 6	M/LHK6B	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/20	boil in 0.01M Tris buffer, pH 10.0, for 60 min
CK 7	M/OV-TL 12/30	Nichirei Biosciences Inc, Tokyo, Japan	1/5	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 8	M/DE-K	DAKO, Glostrup, Denmark	1/2	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 9	M/Ks9.70+Ks9.216	Progen Biotechnik GmbH, Heidelberg, Germany	1/10	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 10	M/LHP1	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/400	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
CK 12	R	TransGenic Inc, Hyogo, Japan	1/500	autoclave in 0.05M Tris buffer, pH10.0, for 20 min
CK 13	M/KS-1A3	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/500	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 14	M/LL002	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 15	M/LHK15	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/300	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 16	M/LL025	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 17	M/E3	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	autoclave in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 20 min
CK 18	M/DC-10	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/200	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
CK 19	M/b170	Novocastra Laboratories Ltd, Tyne and Wear, UK	1/1000	boil in 0.01M Tris-0.001M EDTA buffer, pH 9.0, for 60 min
Calponin	M/ CALP	DAKO, Glostrup, Denmark	1/400	incubate in 0.05M Tris-0.01% protease (Sigma type XXIV), for 30 min at room temperature
Caldesmon	M/ h-CD	DAKO, Glostrup, Denmark	1/200	boil in 0.05M citrate-0.002M EDTA buffer, pH6.0, for 60 min
Desmin	M/ D33	Nichirei Biosciences Inc, Tokyo, Japan	ready to use	No pretreatment
GLUT-1	R	Immuno-Biological Laboratories Co. Ltd, Fujioka, Japan	1/50	No pretreatment

*: G, guinea pig: M, mouse: R, rabbit; †: cytokeratin.

calretinin and AE1/AE3 in all cases, D2-40 in 9 cases, and WT-1 in 7 cases, but negative for CEA, BerEP4, MOC31, and TTF-1 in all cases. Since the first four antibodies yield positive reactions in mesothelioma, while the last four yield positive reactions in pulmonary adenocarcinoma, we diagnosed DMM in all 11 cases.

Immunohistochemistry

We used the polymer-peroxidase method (EnVision+/HRP; Dako Cytomation, Denmark) on deparaffinized sections of DMM and FP. All antibodies were incubated overnight at 4 degree Celsius. The monoclonal and polyclonal antibodies used are listed in Table 1, together with the antigen-retrieval conditions.

For the analysis of immunoreactivity, the extent of moderate-to-strong staining was scored as: 0, indicating negative reaction of tumor cells; 1, ≤10% of tumor area stained; 2, 11 to 25% stained; 3, 26 to 50% stained; 4, 51 to 75% stained; or 5, ≥76% stained. For each antibody, a

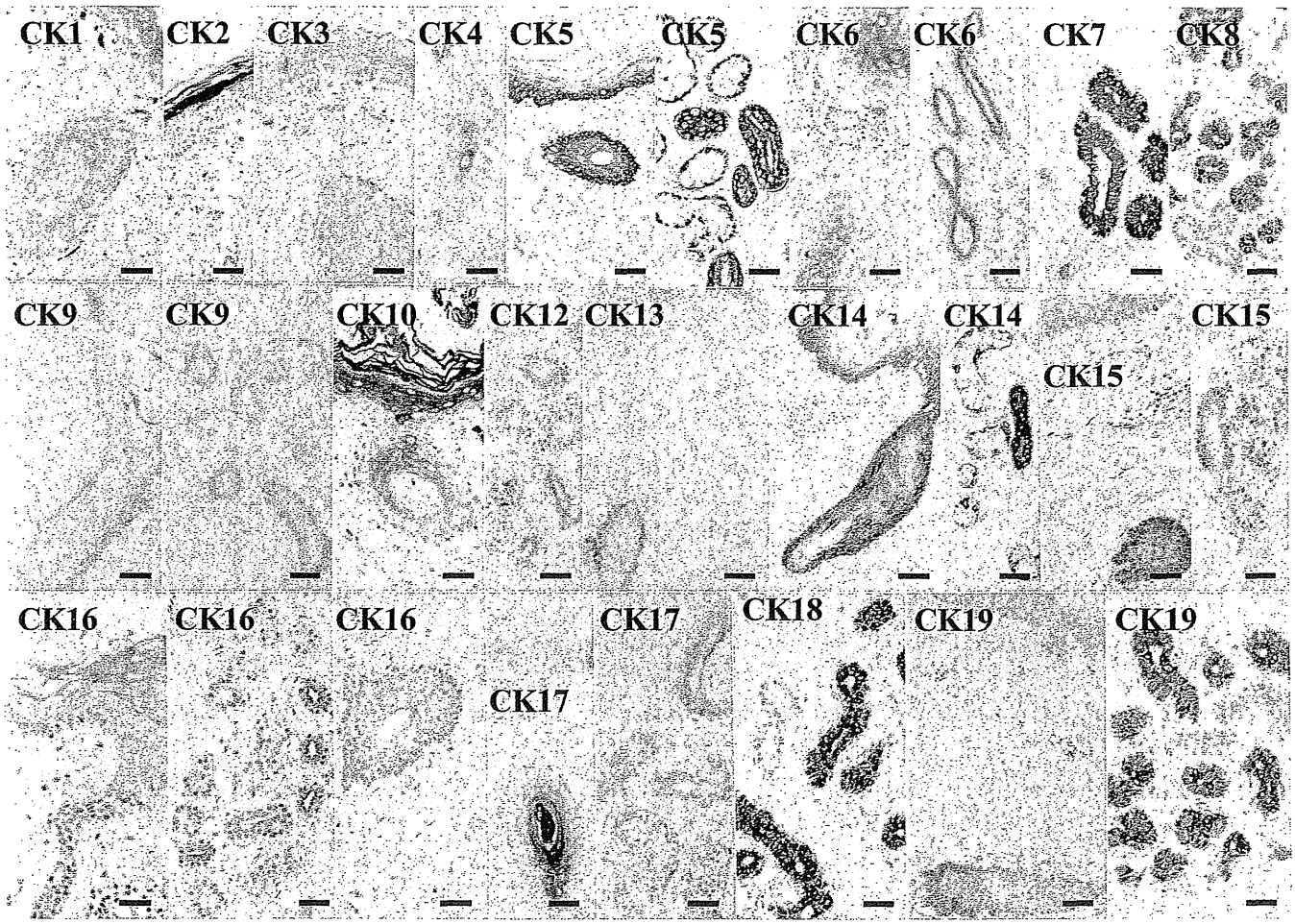

Fig. 1. Immunohistochemistry of cytokeratins within normal skin. CK1 was revealed in squamous, granular, and cornified layers of the epidermis, and inner root hair sheath, CK2 in granular and cornified layers of the epidermis, CK3 in squamous, granular, and cornified layers of the epidermis, and inner root hair sheath, CK4 in inner root hair sheath, CK5 in basal, squamous, and granular layers of the epidermis, outer root hair sheath, inner and outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK6 in squamous, granular, and cornified layers of the epidermis, inner root hair sheath, and inner layer of the eccrine ducts, CK7 in inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands, CK8 in secretory and myoepithelial cells of the secretory glands, CK9 in basal and cornified layers of the epidermis, outer root hair sheath, outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK10 in granular and cornified layers of the epidermis, and inner root hair sheath, CK12 in inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands, CK13 in outer root hair sheath, CK14 in basal and squamous layers of the epidermis, outer root hair sheath, inner and outer layers of the eccrine ducts, and myoepithelial cells of the secretory glands, CK15 in outer root hair sheath, and secretory and myoepithelial cells of the secretory glands, CK16 in cornified layer of the epidermis, inner root hair sheath, and inner layers of the eccrine ducts, CK17 in inner root hair sheath, inner layer of the eccrine ducts, and myoepithelial cells of the secretory glands, CK18 in secretory and myoepithelial cells of the secretory glands, and CK19 in outer root hair sheath, inner layer of the eccrine ducts, and secretory and myoepithelial cells of the secretory glands. Bars: 50 μm.

Table 2. Cytokeratin (CK) expression in the skin.

	CK 1	CK 2	CK 3	CK 4	CK 5	CK 6	CK 7	CK 8	CK 9	CK 10	CK 12	CK 13	CK 14	CK 15	CK 16	CK 17	CK 18	CK 19
Epidermis																		
Basal layer	-	-	-	-	+	-	-	-	+	-	-	±	+	-	-	-	-	-
Squamous layer	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-
Granular layer	+	+	+	-	±	+	-	-	-	+	-	-	-	-	-	-	-	-
Cornified layer	+	+	+	-	-	+	-	-	+	+	+	-	-	-	±	-	-	-
Hair sheath																		
Inner root sheath	+	-	±	±	-	+	-	-	-	±	+	-	-	-	+	+	-	-
Outer root sheath	-	-	-	-	+	-	-	-	+	-	-	+	+	+	-	-	-	+
Eccrine duct																		
Inner layer	-	-	-	-	+	+	±	-	-	-	+	-	+	-	+	±	-	+
Outer layer	-	-	-	-	±	-	-	-	+	-	-	-	±	-	-	-	-	-
Secretory gland																		
Secretory cells	-	-	-	-	-	-	+	+	-	-	±	-	-	±	-	-	+	+
Myoepithelial cells	-	-	-	-	+	-	±	+	±	-	±	-	+	±	-	+	+	+

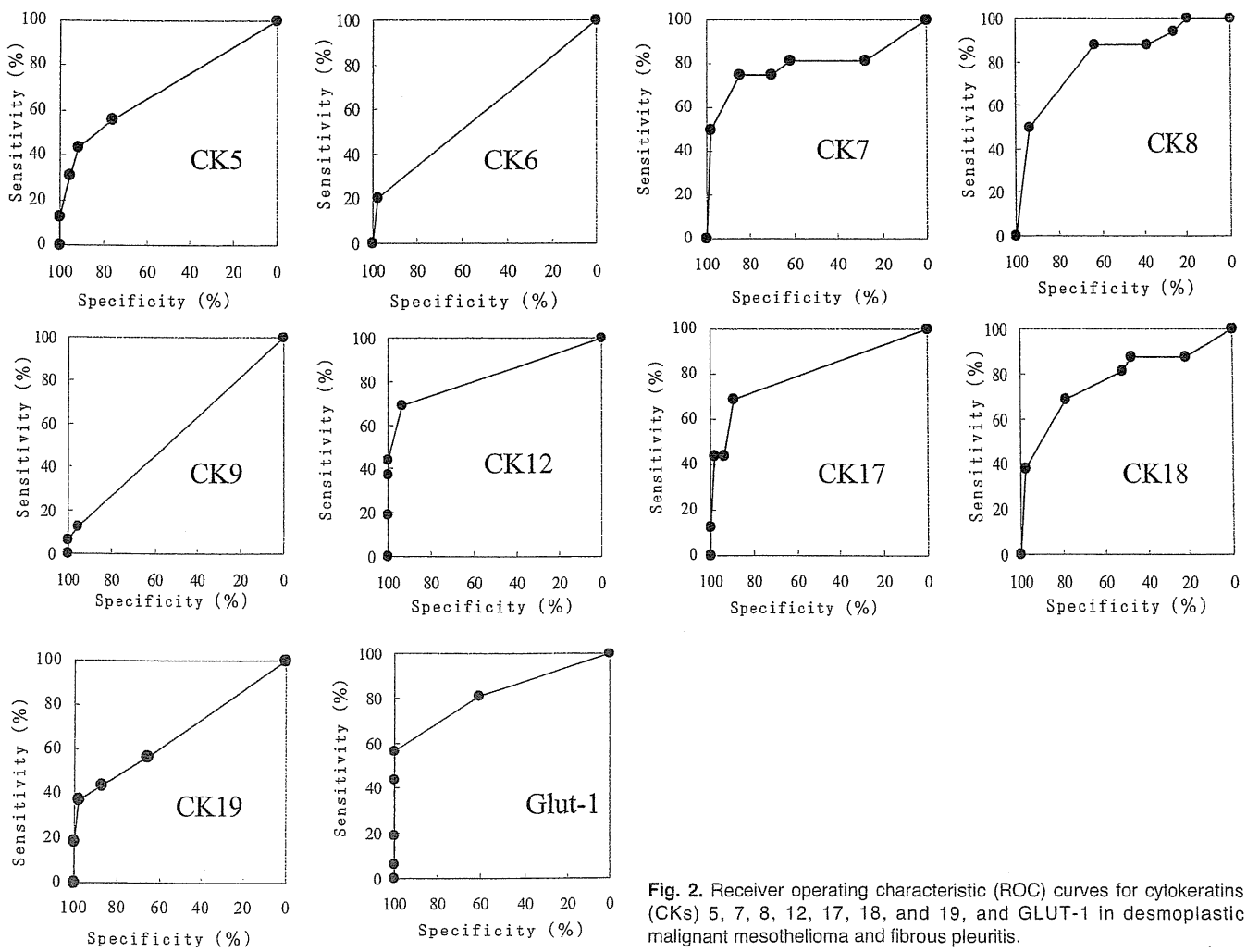

Fig. 2. Receiver operating characteristic (ROC) curves for cytokeratins (CKs) 5, 7, 8, 12, 17, 18, and 19, and GLUT-1 in desmoplastic malignant mesothelioma and fibrous pleuritis.

receiver operating characteristic (ROC) curve was employed to identify the best cut-off values for sensitivity and specificity (13). Then, tumors with a staining score equal to or above that associated with the best sensitivity and specificity were graded as positive.

Data analysis

Statistical analysis of the difference in incidence between two groups was performed using the Chi-square analysis. A p value of less than 0.05 was considered significant.

Results

By immunohistochemistry, each CK was revealed in the cytoplasm of positive cells in the epidermis, dermis, eccrine ducts, and/or secretory glands within normal skin (Table 2, Fig. 1). Calponin, caldesmon, and desmin were detected in the cytoplasm of both vascular smooth muscle cells and bronchial surface epithelial cells within the normal lung. GLUT-1 was found in the membrane of red blood cells within blood vessels and in the membrane and cytoplasm of bronchial surface epithelial cells, each within the normal lung. Expressions of CKs 5, 7, 8, 9, 12, 17, 18, and 19, calponin, caldesmon, and desmin were confined to the cytoplasm of DMM tumor cells and of reactive spindle cells in FP, while GLUT-1 expression was detected in the membrane and cytoplasm of DMM tumor cells and reactive spindle cells in FP (Fig. 2). However, the staining intensity of their proteins sometimes varied within a given case. No ROC curve could be obtained for CKs 1, 2, 3, 4, 6, 9, 10, 13, 14, 15, or 16, or for calponin, caldesmon, or desmin.

Among CKs 5, 7, 8, 12, 17, 18, and 19, and GLUT-1, the best sensitivity and specificity cut-off values in the ROC curves were above 60% for each of CKs 7, 8, 17, 18, and 19, and GLUT-1 (Table 3, Fig. 3). The incidence of a positive expression for CK5, CK12, or CK17 was significantly higher in DMM than in FP (tumors were graded as positive if 1% or more of their cells showed staining) (Table 4; p=0.046, p=0.001, or p<0.0001, respectively). The incidence of a positive expression for CK7 or CK18 was significantly higher in DMM than in FP (tumors were graded as positive if 51% or more of their cells showed staining) (p<0.0001 or p=0.0001, respectively). The incidence of a positive expression for CK18 was significantly higher in DMM than in FP (tumors were graded as positive if 76% or more of their cells showed staining) (p=0.0001). Further, the incidence of a positive expression for GLUT-1 was significantly higher in DMM than in FP (tumors were graded as positive if 11% or more of their cells showed staining) (p<0.0001). Given the best sensitivity and specificity cut-off values in the ROC curves, and the statistical analysis of the difference in incidence between the two groups, CKs 7, 8, 17, 18, and 19, and GLUT-1 were identified as potentially useful markers for diagnosis between DMM and FP.

Table 3. Sensitivity and specificity data for cytokeratins (CKs) and GLUT-1.

	CK5	CK7	CK8	CK12	CK17	CK18	CK19	GLUT-1
Sensitivity*	55	82	64	55	91	82	64	73
Specificity*	76	85	94	94	89	78	87	100

*: CK5, CK12, CK17: positive if staining score >1, CK19, GLUT-1: positive if staining score ≥2, CK7, CK18: positive if staining score >4, CK8: positive if staining score >5

Discussion

Although several proteins -- such as CK5/6, calretinin, WT-1, thrombomodulin, and mesothelin -- are useful markers for distinguishing epithelioid mesothelioma from pulmonary adenocarcinoma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Ordóñez 2003), no immunohistochemical marker for

Table 4. Expressions of cytokeratins (CKs) and GLUT-1 in desmoplastic malignant mesothelioma (DMM) and fibrous pleuritis (FP).

	DMM	FP	p value*
CK5			
>1%	6	11	0.046
0%	5	35	
CK7			
>51%	9	7	<0.0001
<50%	2	39	
CK8			
>76%	7	3	<0.0001
<75%	4	43	
CK12			
>1%	6	3	0.001
0%	5	43	
CK17			
>1%	10	5	<0.0001
0%	1	41	
CK18			
>51%	9	10	0.0001
<50%	2	36	
CK19			
>11%	7	6	0.0047
<10%	4	40	
GLUT-1			
>11%	8	0	<0.0001
<10%	3	46	

*: Statistical analysis of the difference in incidence between two groups was performed using the Chi-square analysis.

differential diagnosis between DMM and FP has been reported. In the present study, in which we examined 18 CKs, calponin, caldesmon, desmin, and GLUT-1 in 11 DMMs and 46 FPs, we observed that the best sensitivity and specificity cut-off values in the ROC curves were above 60% for each of CKs 7, 8, 17, 18, and 19, and GLUT-1, and that for each of these, the incidence of a positive expression was significantly higher in DMM than in FP. On that basis, immunohistochemistry for CKs 7, 8, 17, 18, and 19, and GLUT-1 may provide useful markers for separating DMM from FP, alongside such histological characteristics as cellular atypia, storiform

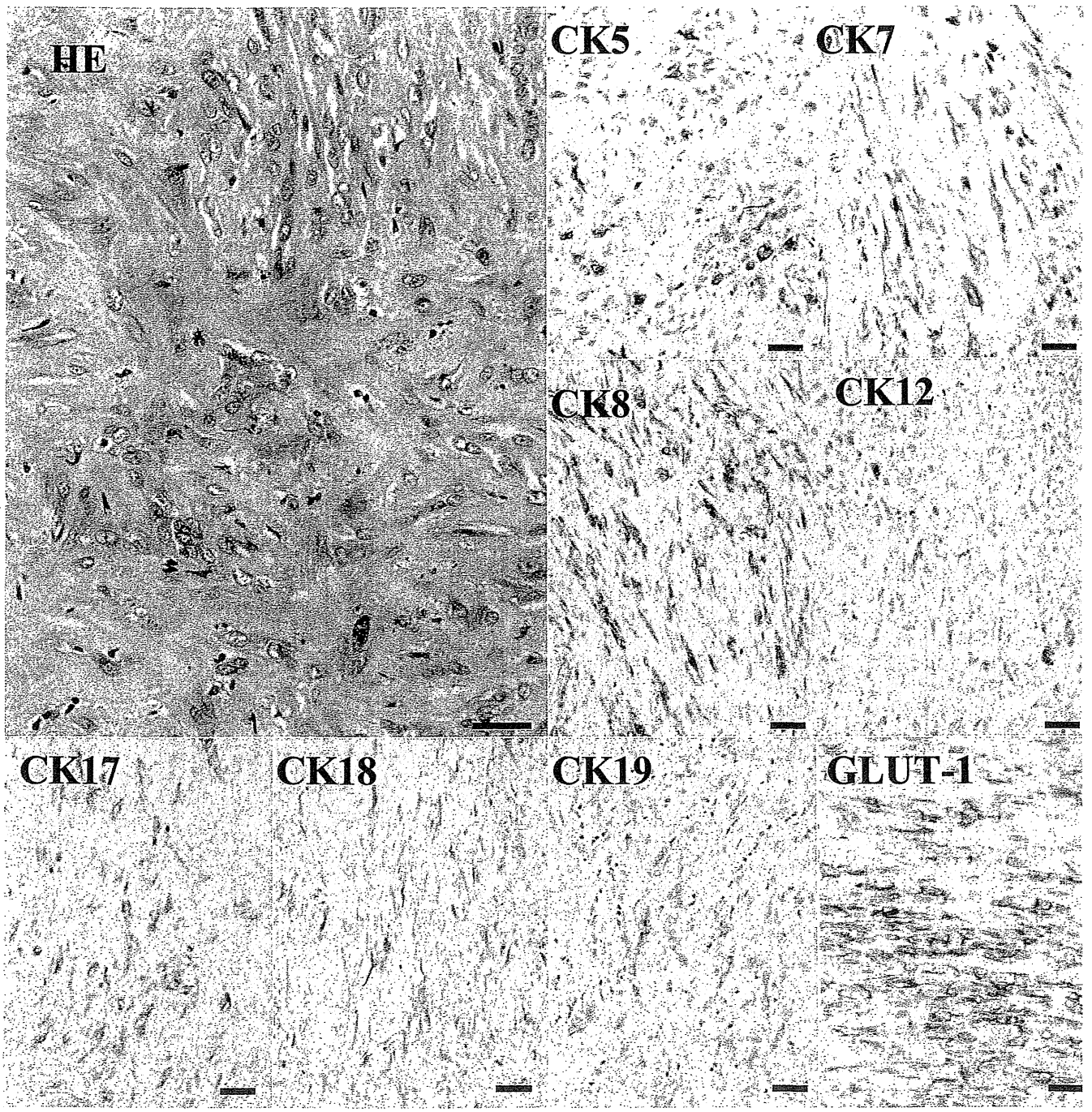

Fig. 3. Hematoxylin-eosin (HE) staining and immunohistochemistry of cytokeratins (CKs) 5, 7, 8, 12, 17, 18, and 19, and GLUT-1 in desmoplast malignant mesothelioma (DMM). Bars: 50 μm.

pattern and/or bland necrosis.

It was previously reported that immunohistochemical staining for CK5/6, calretinin, WT-1, and mesothelin was useful in the diagnosis of sarcomatoid mesothelioma, as well as of epithelioid mesothelioma (Cury et al., 2000; Oates and Edwards 2000; Carella et al., 2001; Lucas et al., 2003; Ordóñez 2003; Suster and Moran 2006; Addis and Roche 2009; Husain et al., 2009). In epithelioid mesothelioma, CK5/6, calretinin, WT-1, and mesothelin have been found to be positive in 55-100%, 42-100%, 43-95%, and 100% of cases, respectively (Ordóñez 2003; Suster and Moran 2006). As far as we can tell, however, these positive expressions are lower in incidence in sarcomatous mesothelioma than in epithelioid mesothelioma, since CK5/6, calretinin, and WT-1 were detected in 0-29%, 39-100%, and 0-50%, respectively, in sarcomatoid mesothelioma (Suster and Moran 2006). However, there is little reported immunohistochemistry for DMMs (Lucas et al., 2003), although in routine practice pathologists frequently have need to differentiate DMM from FP.

It is well known that CKs are a family of intermediate filaments involved in epithelial differentiation, and several CKs are useful tools for differential diagnosis in surgical pathology (Quinlan et al., 1985; Moll et al., 2008; Klebe et al., 2010). So far, at least 20 distinct CKs have been identified. In mesothelioma, Bolen et al (1986), who examined 9 epithelial, 5 sarcomatoid, and 2 desmoplastic malignant mesotheliomas, using antibodies of both low molecular weight CKs (2 antibodies of 44 and 54 kDa, and 46, 52, and 54 kDa) and high molecular weight CKs (one antibody of 57 and 66 kDa), demonstrated that epithelial mesotheliomas were all positive for low and high molecular weight CKs, while sarcomatoid and desmoplastic mesotheliomas were all positive for low molecular weight CKs, but negative for high molecular weight CKs, except for one sarcomatous mesothelioma. In the present study, we investigated 18 CKs for their utility in differentiating DMM from FP. In the ROC curves for low molecular weight CKs (less than 55kDa), such as CKs 7, 8, 17, 18, and 19, sensitivity and specificity tended to be above 60%, while for high molecular weight CKs (equal or more than 55kDa), such as CKs 1, 2, 3, 4, 5, 6, 9, 10, 11, and 12, one or both of them tended to be below 60%. When tumors were graded as positive if 1% or more of their cells showed staining for CK17, if 11% or more of their cells showed staining for CK19, if 51% or more of their cells showed staining for CK7 or CK18, or if 76% or more of their cells showed staining for CK8 (criteria based on the sensitivity and specificity data in their ROC curves), we demonstrated that positive expressions of CKs 7, 8, 17, 18, and 19 were significantly more frequent in DMMs than in FPs. To judge from that finding, immunostaining for CKs 7, 8, 17, 18, and 19 may not only help to identify the presence of invasion into adipose tissue or invasion into the underlying lung in DMM, but also aid the diagnosis of DMM.

In the present study, we demonstrated that GLUT-1 immunohistochemistry was useful for separating DMM from FP. It is generally accepted that GLUT-1 is one of 14 members of the mammalian facilitative glucose transporter (GLUT) family of passive carriers that function as an energy-independent system for the transport of glucose down a concentration gradient (Olson and Pessin, 2005). Although GLUT-1 is not detectable in a large proportion of cells within normal tissues or benign lesions, it is expressed in various cancers (Macheda et al., 2005). In recent studies, GLUT-1 expression has been found to be useful for distinguishing malignant mesothelioma from reactive mesothelial hyperplasia (Kato et al., 2007; Acurio et al., 2008). In fact: (a) Kato et al. (2007) found that its immunoreactivity was negative in 40 reactive mesothelial cases, but positive in all of 40 malignant mesotheliomas, and (b) Acurio et al. (2008) found that 40 benign mesothelial tissues (20 normal, 20 reactive cases) were negative, while 34 of 45 malignant mesotheliomas were positive (unpublished observations). On the basis of the present data and those from the above two studies, GLUT-1 may be a useful marker for separating DMM from FP, as well as for separating malignant mesothelioma from FP or reactive mesothelial hyperplasia.

In conclusion, immunohistochemistry for CKs 7, 8, 17, 18, and 19, and GLUT-1 may be useful for differentiating DMM from FP, alongside their characteristic histological features. Even so, accurately diagnosing DMM in routine practice will require careful consideration of all the available findings by the pathologists.

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Naftopidil Induces Apoptosis in Malignant Mesothelioma Cell Lines Independently of α_1-Adrenoceptor Blocking

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Abstract. *Background: Naftopidil, an α_1-adrenoceptor blocker, has been clinically used for the treatment of benign prostate hyperplasia and hypertension. Emerging evidence has shown that naftopidil exhibits an antitumor effect on a variety of cancer types including prostate cancer. The aim of the present study was to investigate naftopidil-induced apoptosis in human malignant mesothelioma cells and to shed light on the underlying mechanism. Materials and Methods: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) staining, western blotting, and enzymatic assay of caspase-3, -8, and -9 activities were carried out on human malignant mesothelioma cell lines NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells. To knock-down α_{1D}-adrenoceptor, siRNA to silence human α_{1D}-adrenoceptor-targeted gene was constructed and transfected into cells. Results: Naftopidil induced apoptosis in all the investigated malignant mesothelioma cells, and a similar effect was obtained with prazosin, another α_1-adrenoceptor blocker. α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving activation of protein kinase C (PKC). Naftopidil-induced reduction in cell viability was inhibited by GF109203X, while prazosin-induced in cell viability was less affected. Knocking-down α_{1D}-adrenoceptor promoted malignant mesothelioma cell proliferation. Both naftopidil and prazosin activated caspase-3 and -8 in all the investigated malignant mesothelioma cells. Conclusion: Naftopidil, as well as prazosin, has the potential to induce apoptosis in malignant mesothelioma cells by activating caspase-8 and the effector caspase-3, regardless of α_1-adrenoceptor blocking.*

Adrenaline/noradrenaline play a pivotal role in the autonomic nervous system by activating adrenoceptors, *e.g.*, regulation of cardiac motility and blood pressure. Adrenoceptors are classified into three groups: α_1-, α_2-, and β-receptors (1-3). α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving phospholipase C activation, and further divided into α_{1A}-, α_{1B}-, and α_{1D}-subtypes. α_2-Adrenoceptor is linked to G_i protein involving adenylate cyclase inhibition, and further divided into α_{2A}-, α_{2B}-, and α_{2C}-subtypes. β-Adrenoceptor is linked to the G_s protein involving adenylate cyclase activation, and further divided into β_1-, β_2-, β_3-, and β_4-subtypes.

Intriguingly, α_1-adrenoceptor antagonists such as prazosin, doxazosin and terazosin have the potential to inhibit cell growth by arresting the cell cycle or induce apoptosis of malignant and non-malignant cells (4-20). In explanation of the apoptotic action of α_1-adrenoceptor antagonists, a variety of pathways have been proposed, such as mitochondria-mediated activation of caspase-3/-9 and c-Jun N-terminal kinase (JNK)1/2; recruitment of Fas-associated death domain (FADD) and the ensuing activation of caspase-8; transforming growth factor-β1 (TGF-β1) activation followed by IκBα induction; or antagonistic effect of BCL-2. α_1-Adrenoceptor antagonists, alternatively, exhibit anti-angiogenic effects, resulting in suppression of cell growth in human prostate cancer and human bladder cancer (21-25).

Naftopidil, an antagonist for α_1-adrenoceptor, with higher selectivity for α_{1A}- and α_{1D}-receptors, has been developed as a drug for treatment of benign prostate hyperplasia and hypertension (26). Naftopidil is still capable of inhibiting prostate cancer cell growth by arresting cells at the G_1 phase of cell cycling (27, 28). Moreover, a study showed that an

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asymptomatic meningioma markedly regressed two years after oral intake of naftopidil (29). Naftopidil might be available for the treatment of various malignant tumors. Malignant pleural mesothelioma, a highly aggressive neoplasm, has been increasing in incidence and is strongly associated with asbestos exposure (30). No efficient therapy or drug for malignant mesothelioma has been yet established, and therefore, patients with malignant mesothelioma cannot escape death.

The present study investigated the antitumor action of naftopidil on human malignant mesothelioma cell lines. We showed that naftopidil induces apoptosis of malignant mesothelioma cells by a mechanism independent of α_1-adrenoceptor blocking.

Materials and Methods

Cell culture. Human malignant pleural mesothelioma cell lines such as NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.003% L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Cell viability assay. Cell viability was evaluated by the method of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described (31).

Terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) staining. TUNEL staining was performed to detect *in situ* DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37°C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510; Carl Zeiss Co., Ltd., Oberkochen, Germany).

Construction and transfection of siRNA. siRNA to silence human α_{1D}-adrenoceptor-targeted gene (α_{1D}R siRNA) and negative control siRNA (NC siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The α_{1DR} siRNA or the NC siRNA was reverse-transfected into cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Western blotting. Western blotting was carried out on cells using an antibody to α_{1D}-adrenoceptor (Santa Cruz Biotechnology) and to β-actin (Sigma, St. Louis, MO, USA) by the method previously described (31).

Enzymatic assay of caspase-3, -8, and -9 activities. Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) by the method described previously (31). Briefly, cells were harvested before and after treatment with naftopidil or prazosin, and then centrifuged at 1,200 rpm for 5 min at 4°C. The cell pellet was incubated on ice in cell lysis buffer for 10 min, and reacted with the fluorescently-labeled tetrapeptide at 37°C for 2 h. The fluorescence was measured at an excitation of wavelength of 380 nm and an emission wavelength of 460 nm with a fluorometer (Fluorescence Spectrometer F-4500; Hitachi High-Tec, Tokyo, Japan).

Statistical analysis. Statistical analysis was carried out using unpaired *t*-test.

Results

Naftopidil induces apoptosis of malignant mesothelioma cells. For all the malignant mesothelioma cell lines examined here, naftopidil reduced cell viability in a concentration (1-100 μM)- and treatment time (24-48 h)-dependent manner, the extent reaching almost 0% of basal levels at 100 μM (Figure 1A-D). Likewise, prazosin, another α_1-adrenoceptor antagonist, reduced cell viability in a fashion mimicking the effect of naftopidil (Figure 1E-H).

In the TUNEL staining, naftopidil significantly increased the number of TUNEL-positive cells as compared with that for the untreated control in all the malignant mesothelioma cell lines (Figure 2A-D). A similar effect was obtained with prazosin (Figure 2A-D). These results indicate that α_1-adrenoceptor blockers, such as naftopidil and prazosin, are capable of inducing apoptosis of malignant mesothelioma cells.

Naftopidil induces apoptosis of malignant mesothelioma cells, regardless of α_{1D}-adrenoceptor blocking. α_1-Adrenoceptor is linked to $G_{q/11}$ protein involving phospholipase C activation, to hydrolyze phosphatidylinositol into inositol 1,4,5-trisphosphate (IP_3) and diacyl-glycerol allowing protein kinase C (PKC) activation. We postulated that naftopidil and prazosin induce apoptosis of malignant mesothelioma cells by inhibiting PKC as a result of α_1-adrenoceptor blocking. If this was true, then PKC inhibitors should enhance the cell death-inducing effect of naftopidil and prazosin. Unexpectedly, the effect of naftopidil on malignant mesothelioma cell death was attenuated by GF109203X, an inhibitor of PKC, for all the cell types examined here (Figure 3A-D). Naftopidil, thus, appears to induce apoptosis of malignant mesothelioma cells by a mechanism independent of α_1-adrenoceptor blocking and PKC inhibition.

GF109203X, in contrast, enhanced the effect of prazosin on cell viability, only for NCI-H28 cells (Figure 3E) or inhibited it for NCI-H2052 and NCI-H2452 cells (Figure 3F,G), but did not alter the effect for MSTO-211H cells (Figure 3H). It is even less likely that prazosin induces apoptosis of malignant mesothelioma cells by inhibiting α_1-adrenoceptor bearing PKC activation.

To obtain further evidence for naftopidil- or prazosin-induced apoptosis independent of α_1-adrenoceptor blocking,

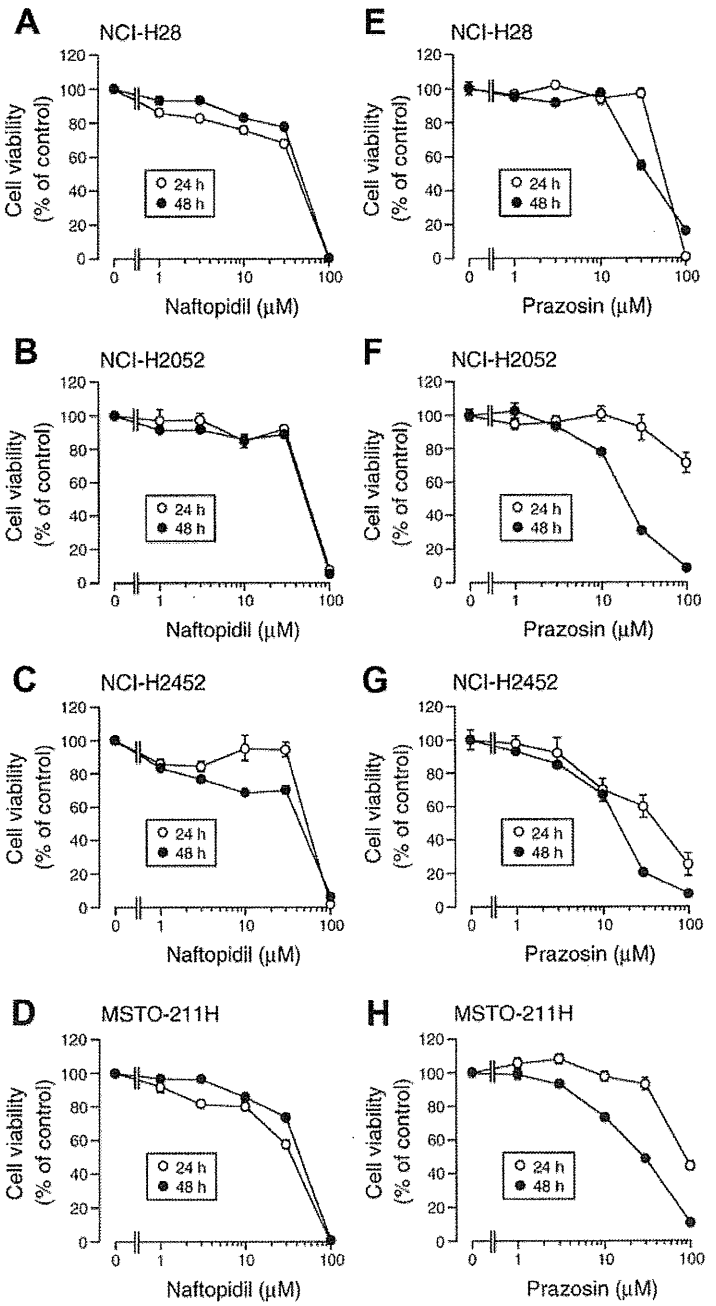

Figure 1. *The effects of naftopidil and prazosin on cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out on NCI-H28 (A, E), NCI-H2052 (B, F), NCI-H2452 (C, G), and MSTO-211H cells (D, H) untreated and treated with naftopidil or prazosin, at concentrations, as indicated for 24-48 h. In the graphs, each point represents the mean (±SEM) percentage of control cell viability (MTT intensities of cells untreated with naftopidil or prazosin) (n=4 independent experiments).*

an α_{1D}R siRNA was constructed and transfected into malignant mesothelioma cells. Expression of α_{1D}-adrenoceptor protein for cells transfected with α_{1D}R siRNA significantly decreased as compared with the expression for cells transfected with the NC siRNA (Figure 4A-D), confirming α_{1D}-adrenoceptor knock-down. Cell viability for all the malignant mesothelioma cell lines was not reduced by knocking-down α_{1D}-adrenoceptor, but conversely, it was

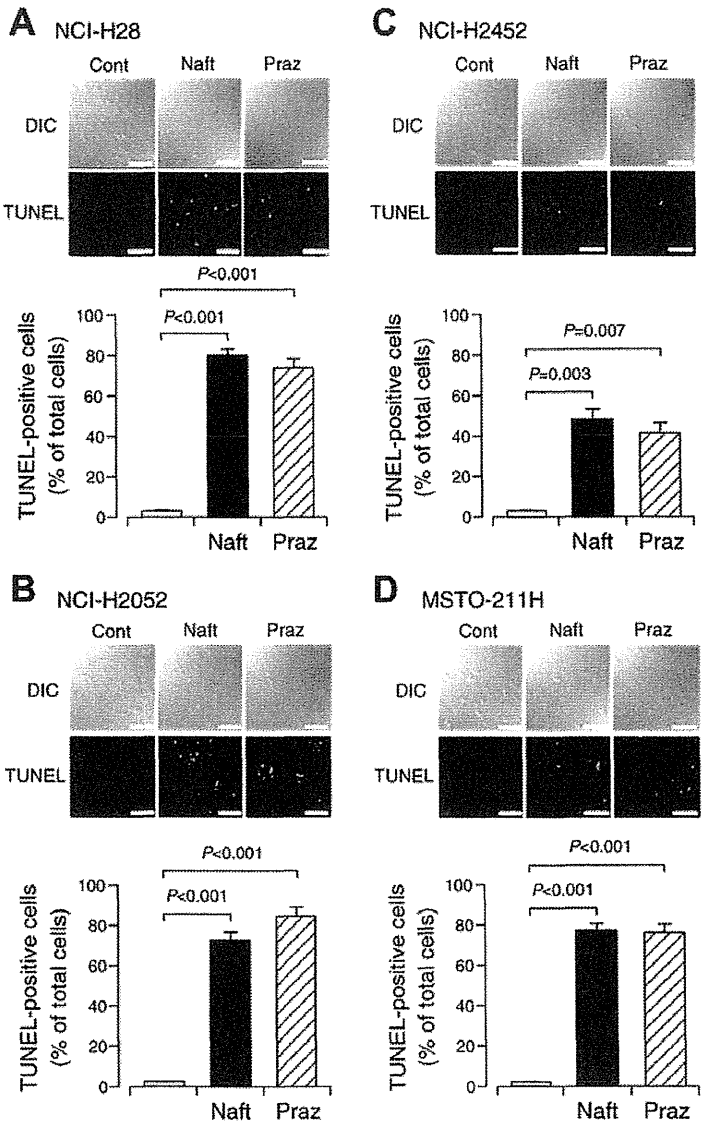

Figure 2. *Terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) staining. TUNEL staining was carried out on NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) untreated (Cont) and treated with naftopidil (Naft) (100 μM) or prazosin (Praz) (100 μM) for 12 and 24 h, respectively. DIC, Differential interference contrast. Bars=100 μm. TUNEL-positive cells were counted in an area (0.4 mm × 0.4 mm) selected randomly. In the graphs, each column represents the mean (±SEM) percentage of TUNEL-positive cells relative to total cells (n=4 independent experiments). p-Values were defined from unpaired t-test.*

increased (Figure 5A-D). This indicates that malignant mesothelioma cell apoptosis is not induced by blocking α_{1D}-adrenoceptor; in other words, naftopidil- and prazosin-induced apoptosis of malignant mesothelioma cells is not due to α_1-adrenoceptor blocking action.

Naftopidil activates caspase-3/-8 in malignant mesothelioma cells. For all the malignant mesothelioma cell lines examined here, naftopidil significantly activated caspase-3 and -8, but otherwise did not activate caspase-9 except for NCI-H28 cells (Figure 6A-D). Prazosin also activated caspase-3 and -8 without affecting caspase-9 activity except for MSTO-211H cells (Figure 6E-H). These results imply that naftopidil and prazosin activate caspase-8 and the effector caspase-3, thereby inducing apoptosis of malignant mesothelioma cells. The results also suggest that naftopidil and prazosin could still activate caspase-9 followed by caspase-3 for certain types of malignant mesothelioma cell.

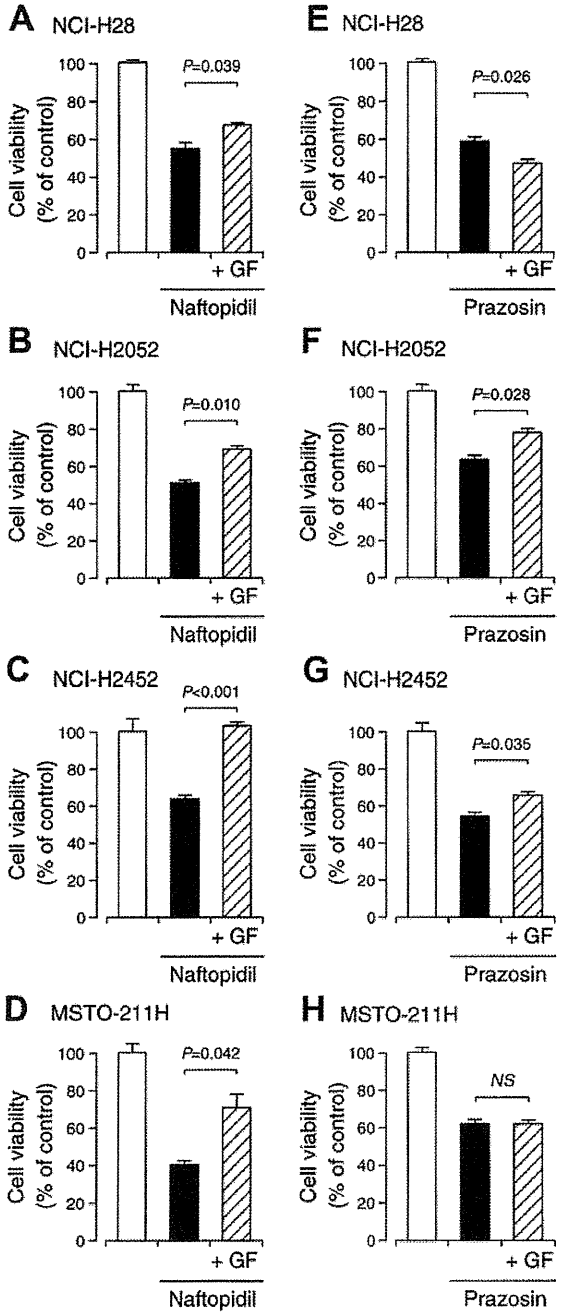

Figure 3. *The effect of protein kinase-C inhibitor GF109203X on cell death induced by naftopidil or prazosin. NCI-H28 (A, E), NCI-H2052 (B, F), NCI-H2452 (C, G), and MSTO-211H cells (D, H) were treated with naftopidil (50 μM) or prazosin (50 μM) for 24 h in the presence and absence of GF109203X (GF) (100 nM), and then 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out. In the graphs, each column represents the mean (±SEM) percentage of control cell viability (MTT intensities of cells untreated with naftopidil or prazosin in the absence of the inhibitor) (n=4 independent experiments). p-Values were defined from unpaired t-test. NS, Not significant.*

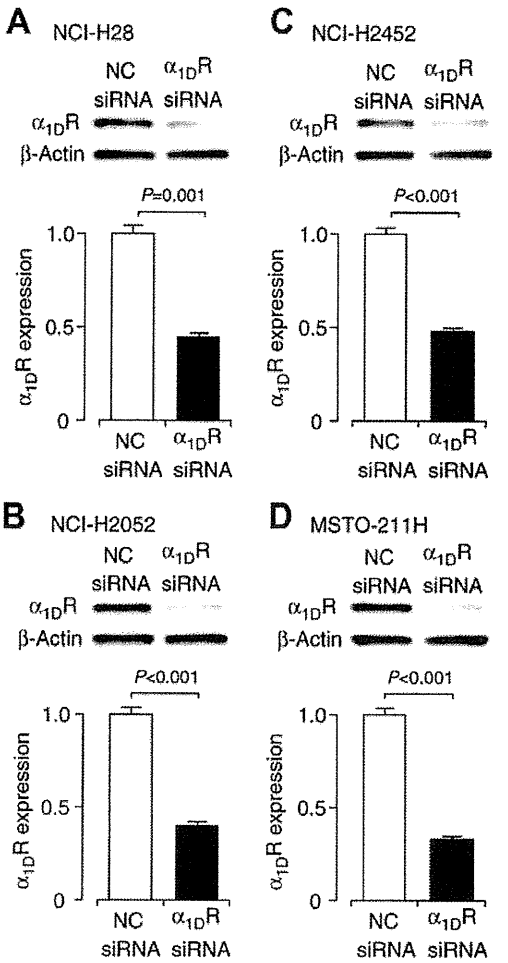

Figure 4. *α_{1D} Adrenoceptor knock-down. Western blotting for NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) transfected with negative control (NC) siRNA or α_{1DR} siRNA 48 h after transfection. Signal intensities for α_{1D}-adrenoceptor protein were normalized by those for β-actin. In the graphs, each column represents the mean (±SEM) α_{1D}-adrenoceptor protein intensity (n=4 independent experiments). p-Values were defined from unpaired t-test.*

Discussion

Naftopidil, an inhibitor of α_{1A}- and α_{1D}-adrenoceptors, has been clinically used for the treatment of benign prostate hyperplasia and hypertension (26). Interestingly, recent evidence has shown that naftopidil exerts an antitumor action on prostate cancer cells (27, 28). In the present study, naftopidil induced apoptosis in the human malignant mesothelioma cell lines NCI-H28 and NCI-H2052 sarcomatoid cells, NCI-H2452 epithelioid cells, and MSTO-211H biphasic cells. A similar effect was obtained with prazosin, another α_1-adrenoceptor blocker. α_1-

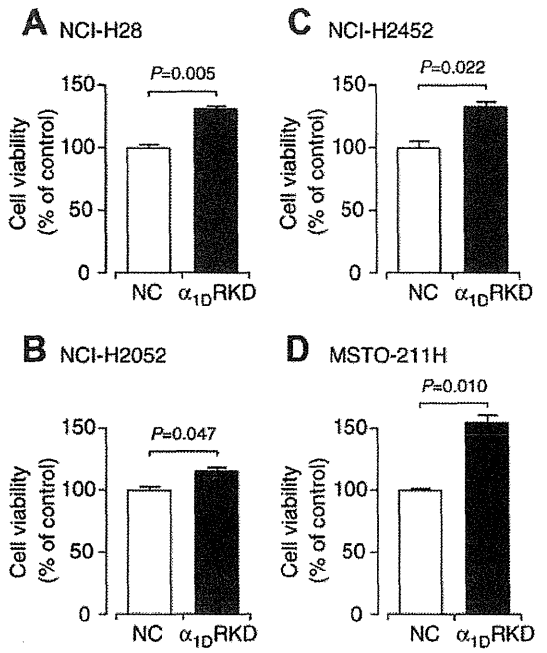

Figure 5. *The effect of α_{1D}-adrenoceptor knock-down on cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) transfected with negative control siRNA (NC) or α_{1DR} siRNA (α_{1DR} KD) 48 h after transfection (n=4 independent experiments). In the graphs, each column represents the mean (±SEM) percentage of control cell viability (MTT intensities for cells transfected with NC siRNA at 48 h after transfection)(n=4 independent experiments). p-Values were defined from unpaired t-test.*

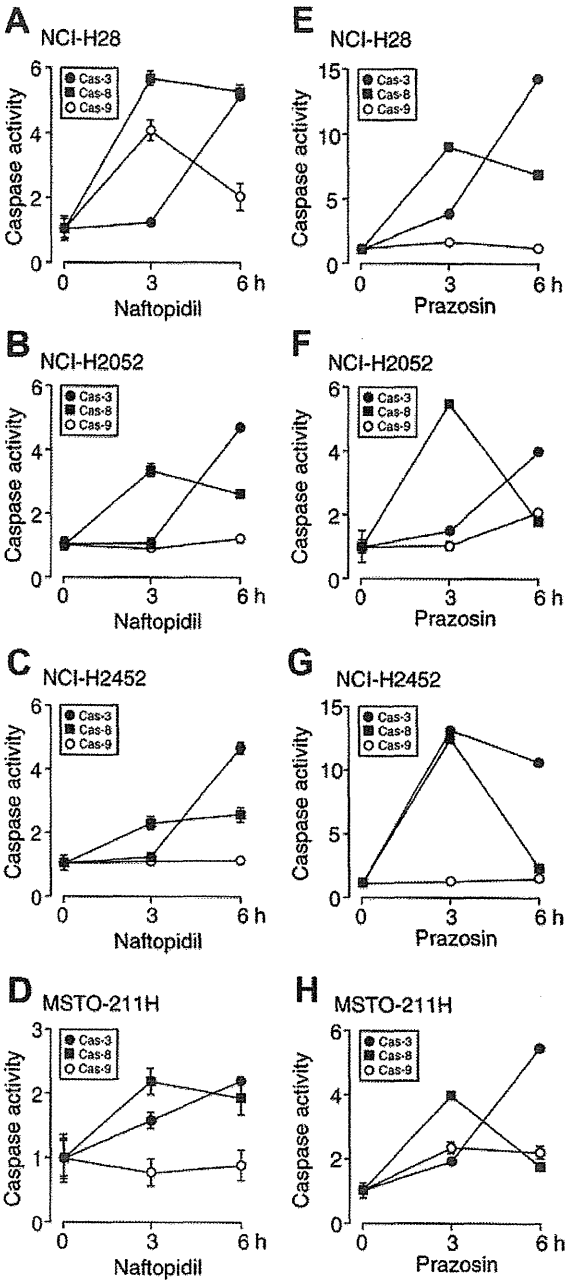

Figure 6. *Activities of caspase-3, -8, and -9. NCI-H28 (A, E), NCI-H2052 (B, F), NCI-H2452 (C, G), and MSTO-211H cells (D, H) were treated with naftopidil (100 μM) or prazosin (100 μM) for 3-6 h, and then activities of caspase-3, -8, and -9 were enzymatically assayed. In the graphs, each point represents the mean (±SEM) ratio to basal caspase activities (before treatment with naftopidil or prazosin) (n=4 independent experiments).*

Adrenoceptor blockers such as naftopidil and prazosin, thus, might exert an antitumor action on malignant mesothelioma cells.

α_1-Adrenoceptor linked to $G_{q/11}$ protein engages PKC activation. Naftopidil and prazosin, therefore, should inhibit PKC following α_1-adrenoceptor blocking. Surprisingly, naftopidil- and prazosin-induced cell death of all the malignant mesothelioma cell lines and some cell lines, respectively, was attenuated by the PKC inhibitor GF109203X. In addition, malignant mesothelioma cell death was not induced by knocking-down the α_{1D}-adrenoceptor; conversely, cell proliferation was promoted. Taken together, these results show that naftopidil and prazosin are likely to induce apoptosis of malignant mesothelioma cells by a mechanism independent of α_1-adrenoceptor blocking. In support of this, α_1-adrenoceptor antagonists have been shown to modulate differentiation and death of human erythroleukemia cells, regardless of α_1-adrenoceptor blocking (32).

Naftopidil and prazosin activated caspase-3 and -8 in all the investigated malignant mesothelioma cell lines. This indicates that they activate caspase-8 and the effector caspase-3 to induce apoptosis of malignant mesothelioma cells. Naftopidil and prazosin also activated caspase-9 for some

malignant mesothelioma cell lines. This suggests that naftopidil and prazosin could still ultimately activate caspase-3. Caspase-8 is recognized as being activated through death receptors such as tumor necrosis factor receptor-1 (TNFR1), FAS/apoptosis antigen-1 (APO1)/CD95, death receptor-3 (DR3)/APO3/WSL-1/lymphocyte-associated receptor of death (LARD)/TRAMP, DR4/TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-R1), DR5/TRAIL-R2/TNF-related apoptosis-inducing ligand receptor inducer of cell killing-2 (TRICK2)/KILLER, and DR6 (33). FAS, activated by FASL, recruits the adaptor protein FAS-associated protein with death domain (FADD) to aggregate procaspase-8, which cleaves to initiate the active form of caspase-8 (34). TNFR1, activated by TNF-α, alternatively, forms a complex of TNFR1-associated death domain (TRADD)/receptor interacting protein-1 (RIP1)/FADD/procaspase-8 to activate caspase-8 (35). In contrast, caspase-9 is activated in concert with mitochondrial damage, allowing cytochrome c efflux from the mitochondria into the cytosol, to form an apoptosome complex with apoptotic protease activating factor 1 (APAF-1) or dATP (36-38). How naftopidil or prazosin activates caspase-8 or caspase-9 in malignant mesothelioma cells remains to be explored. To address this question, we are currently carrying out further experiments.

Conclusion

The results of the present study show that naftopidil and prazosin have the potential to induce apoptosis of malignant mesothelioma cells by activating caspase-8 and the effector caspase-3, regardless of α_1-adrenoceptor blocking. Naftopidil, which has been permitted for clinical use, may have a future role in treatment of human malignant mesothelioma.

Conflicts of Interest

None of the Authors have any potential conflicts of interest.

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Clinical Trial Note

A Feasibility Study of Induction Pemetrexed Plus Cisplatin Followed by Pleurectomy/Decortication Aimed at Macroscopic Complete Resection for Malignant Pleural Mesothelioma

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A prospective multi-institutional study has been initiated in Japan to evaluate the feasibility of induction chemotherapy using pemetrexed plus cisplatin, followed by pleurectomy/decortication aimed at macroscopic complete resection in patients with resectable malignant pleural mesothelioma. The study was initiated on September 2012, for which 24 patients will be recruited over a period of 2 years. The primary endpoint is the macroscopic complete resection rate, regardless of the surgical technique employed (i.e. pleurectomy/decortication or extrapleural pneumonectomy). The secondary endpoints are the pleurectomy/decortication rate, macroscopic complete resection rate by pleurectomy/decortication, pulmonary function at 3 months after surgery, adverse events, treatment-related mortality, response rate to chemotherapy and 3-year overall survival rate.

Key words: extrapleural pneumonectomy – induction chemotherapy – malignant pleural mesothelioma – macroscopic complete resection

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an extremely poor-prognosis malignant tumor caused by asbestos exposure. The number of cases of this tumor in Japan is expected to rise in the future (1–3). MPM is very difficult to cure. While extrapleural pneumonectomy (EPP) is performed with radical intent, the outcome is not very good in patients treated with surgery alone (4). The current standard for possible cures for this disease has shifted to a multidisciplinary approach combining induction chemotherapy with cisplatin and pemetrexed followed by EPP and radiation therapy (trimodality therapy).

In recent years, another operative method, known as pleurectomy/decortication (P/D), has come into the spotlight. EPP is a very invasive surgery and shows cardiorespiratory depression and high rates of mortality and complications. P/D is less invasive than EPP. As of yet, it is not apparent which risk-benefit ratio of P/D and EPP is better as a part of multimodality therapy. It has been reported that the survival rate of P/D is higher than or equal to that of EPP (5–8). The possible reasons for this are as follows:

(1) The perioperative mortality rate of P/D is lower than that of EPP.
(2) Patients who had P/D receive better treatment than those who received EPP at the time of recurrence.

Postoperative quality of life is maintained to a larger extent in those patients who have undergone P/D rather than EPP (9). The results of major clinical trials for trimodality therapy, including EPP, have been reported by cancer study groups in North America, the University of Toronto and Europe (10–12). In all clinical trials, only around 50% of patients completed trimodality therapy, thus suggesting that trimodality therapy, including EPP, poses major difficulties even at some of the world's most experienced and top-ranking facilities. In addition, both a high complication rate and a number of treatment-related deaths were reported in a Japanese multi-institutional clinical trial for trimodality therapy conducted in 2008. Considering this, the survival benefits of this therapy reported from clinical trials in Europe and the USA are not high. Therefore, the risk-benefit ratio of this treatment is not satisfiable.

There is no good evidence of multimodality therapy involving P/D. However, the benefit of adding induction chemotherapy to P/D may be speculated in the light of that for EPP (13–15). The study protocol is a clinical trial to evaluate induction chemotherapy with pemetrexed plus cisplatin followed by P/D aimed at macroscopic complete resection (MCR) for resectable MPM (16). The study protocol was approved by the protocol review committee and activated on 12 October 2012. The study has been registered at the UMIN Clinical Trials Registry as UMIN000009092 (http://www.umin.ac.jp/ctr/index.htm).

PROTOCOL DIGEST OF THE STUDY

Purpose

The aim of this study is to evaluate the feasibility of multimodality therapy for resectable MPM, comprised induction chemotherapy using pemetrexed plus cisplatine (PC) followed by P/D aimed at MCR.

Study Setting

This is a multi-institutional, single-arm study.

Study Method

Figure 1 shows a flow chart of the study.

Endpoints

The primary endpoint is MCR rate regardless of the surgical technique employed (i.e. P/D or extrapleural pneumonectomy). MCR is defined as the surgical removal of all gross tumor tissue (16,17). Secondary endpoints are as follows: (i) P/D rate, (ii) MCR rate by P/D, (iii) pulmonary function

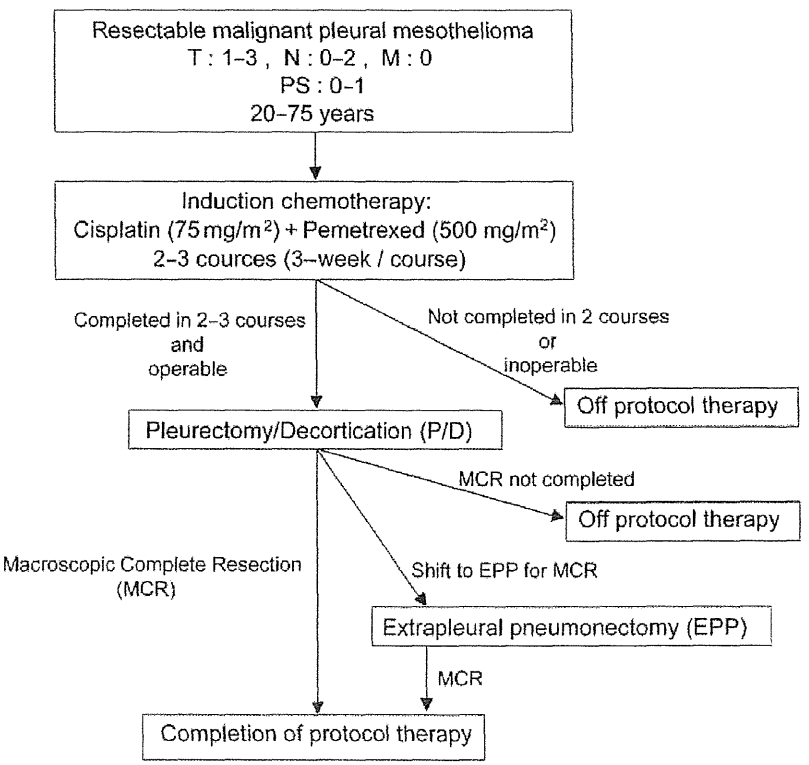

Figure 1. Flow chart of the study.

at 3 months after surgery, (iv) incidence of treatment-related adverse events according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 guidelines (18), (v) treatment-related mortality, (vi) response rate for induction chemotherapy evaluated by a modified version of the Response Evaluation Criteria in Solid Tumors [modified RECIST (19)], (vii) 3-year overall survival rate in all eligible patients with MCR.

Eligibility/Inclusion Criteria

Patients are eligible for the trial if they have a histologically confirmed diagnosis of MPM, including all subtypes and clinical T1–3, N0–2, M0 disease considered to be resectable. Other requirements are as follows: no prior treatment with chemotherapy, surgery or radiation therapy (RT) for the disease; age between 20 and 75 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; a predicted postoperative forced expiratory volume of >1000 ml in 1 s; adequate bone marrow, hepatic, renal, cardiac and respiratory functions; a life expectancy of >12 weeks; and written informed consent.

Exclusion Criteria

Patients are excluded if they meet any of the following criteria: serious systemic complications including poorly controlled diabetes or hypertension, active infectious diseases, interstitial pneumonia or lung fibrosis; simultaneous or metachronous (within 5 years) double cancers; serious drug allergy or hypersensitivity to any drugs; pregnancy or breast-feeding; Grade 2 or greater peripheral neuropathy at registration; or considered as clinically inappropriate for registration.

TREATMENT METHODS

Induction Chemotherapy

Induction chemotherapy consists of three cycles of pemetrexed at 500 mg/m^2 followed by cisplatin 75 mg/m^2 on Day 1, given every 21 days. Folic acid (0.5 mg per daily oral administration) and vitamin B12 (1 mg intramuscularly every 9 weeks) are administered a week before the first dose of chemotherapy and continue to be administered throughout the induction chemotherapy. Dose adjustments of chemotherapy are required for renal and nonhematologic toxicity as well as hematologic effects. Dose delays of up to 42 days are permitted for recovery from drug toxicity. Tumor response is assessed through computed tomography (CT) following the completion of induction chemotherapy using unidimensional measurement of the pleural thickness perpendicular to the chest wall or mediastinum and modified RECIST criteria.

Pleurectomy/Decortication and Extrapleural Pneumonectomy

All patients undergo P/D or EPP within 42 days of the last dose of induction chemotherapy unless there is deterioration of organ functions that would make the surgery intolerable. P/D complies with the definition of the International Association for the Study of Lung Cancer (IASLC) staging committee and the International Mesothelioma Interest Group (IMIG). The above report does not prescribe whether P/D mandates the removal of a part of the pleura without macroscopic disease. Therefore, in this study, it is stipulated that P/D requires mandatory removal of all the parietal pleura and removal of all the area of the visceral pleura with macroscopic disease. If it is necessary to achieve MCR, P/D permits resecting either of the diaphragm, pericardium, chest wall and lung parenchyma. EPP is defined as an en-bloc resection of the entire pleura, lung, ipsilateral diaphragm and pericardium (20). Also, while it is impossible to achieve MCR through P/D, EPP is performed in cases where operators deem that MCR can be achieved through EPP. If lymph node metastasis is confirmed by pathological examination, excision of this is also a prerequisite for MCR. Mediastinal nodal dissection is recommended in all patients having either P/D or EPP.

Study Design and Statistical Methods

The primary analysis of this study was to estimate the MCR rate and 95% confidence interval (CI). If the lower limit of the 95% CI exceeds 0.5, the protocol treatment will be considered feasible. Thus, 24 patients were planned to be enrolled onto this study, with planned accrual of 2 years and follow-up of 3 years after the accrual completion. This sample size was considered sufficient to estimate 95% confidence intervals for the true MCR rate within a width of ± 0.2, when the true MCR rate is expected to be 70%.

Study Monitoring

The Data and Safety Monitoring Committee (DSMC) will make independent recommendations to investigators regarding the continuation, termination or modification of the trial. Protocol compliance, safety and study progress will also be monitored by the DSMC.

Participating Institutions

A total of 24 institutions in Japan with certified specialists in oncology and surgery will participate in this trial.

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Conflict of interest statement

None declared.

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