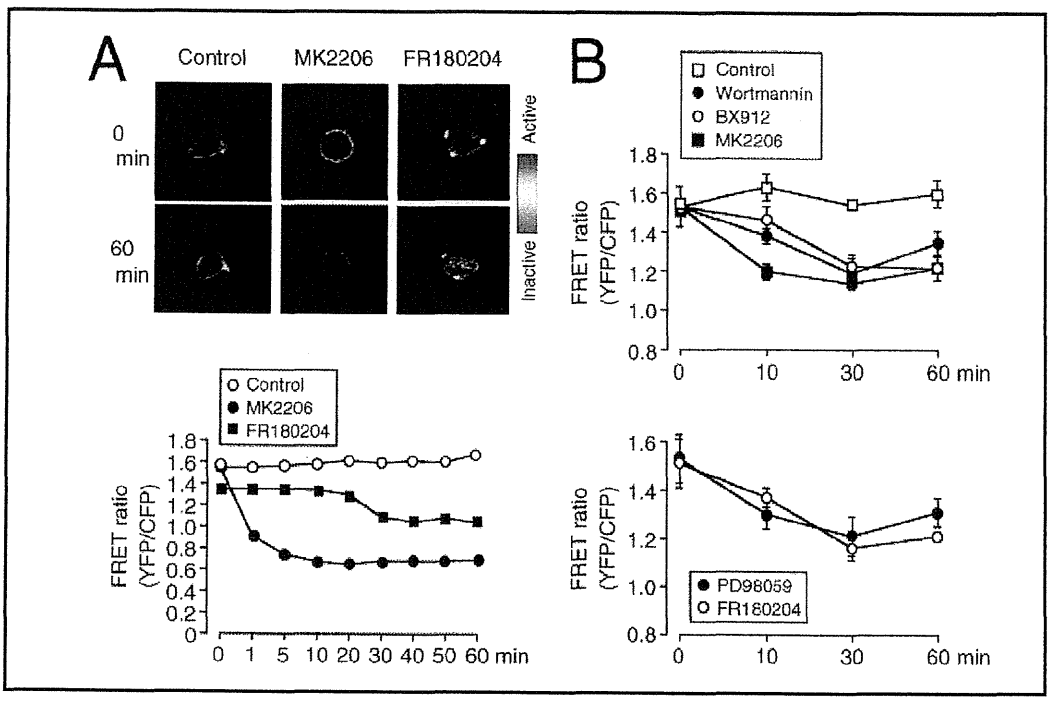

Fig. 5. Rac1 activation due to MEK and ERK. (A) FRET monitoring was carried out on living MSTO-211H cells in the absence (Control) and presence of MK2206 (5 μM) or FR180204 (10 μM), and the FRET ratio (YFP signal intensity/CFP signal intensity) was calculated. The FRET ratio images are shown in the upper panel and time-course FRET ratio profiles are shown in the graph. Note that a similar result was obtained with 6 independent experiments. (B) Cells were untreated and treated with wortmannin (10 μM), BX912 (100 nM), MK2206 (5 μM), PD98059 (50 μM), or FR180204 (10 μM) for periods of time as indicated. Subsequently, cells were fixed followed by FRET monitoring. In the graphs, each point represents the mean (± SEM) FRET ratio (n=5-10 independent experiments).

An established pathway is that ERK is activated through a pathway along a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis as mediated by receptor tyrosine kinase including PDGF-ββ receptor. In the present study, ERK phosphorylation in MSTO-211H cells was prevented by inhibitors for PI3 kinase, PDK1, Akt, and Rac1 or by knocking-down PI3 kinase, PDK1, Akt, and Rac1. This indicates that ERK could be still activated through a pathway along a PDGF-ββ receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis, regardless of a PDGF-ββ receptor/ (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis (Fig. 6). In other words, this shows a crosstalk activation of ERK in PDGF-ββ receptor signaling pathways (Fig. 6).

It is well-recognized that Akt is activated through a pathway along an IRS/PI3 kinase/PDK1/Akt axis as mediated by receptor tyrosine kinase including PDGF-ββ receptor. In support of this pathway,

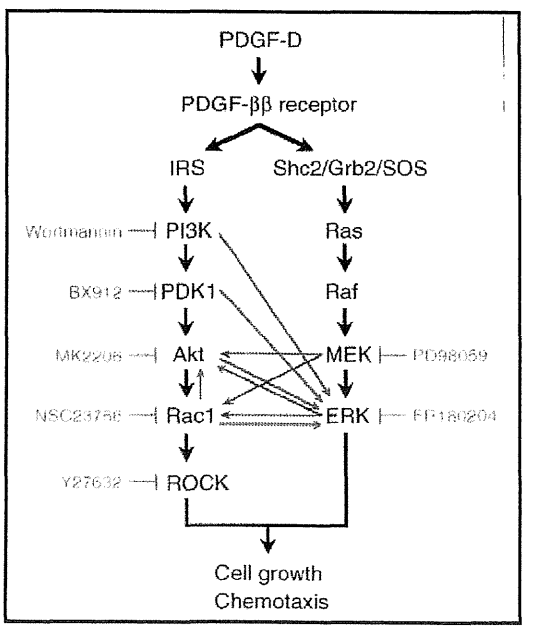

Fig. 6. A schematic diagram for crosstalk in PDGF-ββ receptor signaling pathways.

Akt phosphorylation in MSTO-211H cells was inhibited by inhibitors for PI3 kinase and PDK1 or by knocking-down PI3 kinase and PDK1. Notably, Akt phosphorylation was still inhibited by an Akt inhibitor, suggesting that Akt might be activated through its autophosphorylation. Of particular interest is the finding that Akt phosphorylation was suppressed by knocking-down Rac1. This raises the possibility that Rac1 serves as a positive feedback activator of Akt.

Akt phosphorylation was also prevented by a MEK inhibitor or an ERK inhibitor. This indicates that MEK and ERK could activate Akt via a pathway along a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis as mediated by receptor tyrosine kinase including PDGF-ββ receptor (Fig. 6). In the FRET analysis, Rac1 was activated under the basal conditions in MSTO-211H cells. Expectedly, Rac1 activation was suppressed by a PI3 kinase inhibitor, a PDK1 inhibitor, and an Akt inhibitor. This provides further evidence for Rac1 activation through a pathway along a PDGF-ββ receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis (Fig. 6). Moreover, Rac1 activation was also inhibited by a MEK inhibitor and an ERK1/2 inhibitor. This indicates that MEK and ERK could also activate Rac1 via a PDGF-ββ receptor/(Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK signaling pathway (Fig. 6). Overall, these results may represent fresh insight into activation of Akt/Rac1 and ERK through crosstalk between PDGF-ββ receptor-linked two signaling pathways.

In summary, the results of the present study show that ERK could be activated through a pathway along a PDGF-ββ receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis and that alternatively, Akt and Rac1 could be activated through a pathway along a PDGF-ββ receptor/(Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis in MSTO-211H cells.

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Dipalmitoleoyl-phosphatidylethanolamine Induces Apoptosis of NCI-H28 Malignant Mesothelioma Cells

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Abstract. *Background/Aim: The phospholipid phosphatidylethanolamine regulates a wide range of cellular processes. The present study investigated the antitumor effect of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) on malignant pleural mesothelioma cells. Materials and Methods: Activities of protein phosphatases (PPs) such as PP1, PP2A, and protein tyrosine phosphatase 1B (PTP1B) were assayed under cell-free conditions. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, and western blotting were carried out on the human Met5A non-malignant mesothelial cell line and NCI-H28 malignant mesothelioma cell line. Results: DPPE significantly enhanced PP2A and PTP1B activities. DPPE tended to attenuate activity of extracellular signal-regulated kinase-1 (ERK1)/ERK2, with the greater efficacy for NCI-H28 cells than that for Met5A cells. DPPE reduced NCI-H28 cell viability in a concentration (1-100 μM)-dependent manner, while it had no effect on Met5A cell viability. DPPE markedly increased TUNEL-positive cells in the NCI-H28 cell line, but otherwise induced few TUNEL-positive cells in the Met5A cell line. Conclusion: The results of the present study clearly demonstrate that DPPE induces apoptosis of NCI-H28 malignant pleural mesothelioma cells. DPPE-induced enhancement of PP2A and PTP1B activities might at least in part contribute to the apoptotic effect of DPPE.*

Malignant mesothelioma is an aggressive form of cancer that arises from mesothelial cells and may develop in the pleural space, pericardium, peritoneum, tunica vaginalis testis and ovarian epithelium (1). This type of cancer is often known to be resistant to chemotherapy. The median survival period of patients diagnosed with malignant mesothelioma is usually less than a year. Some factors have been associated with the development of malignant mesothelioma, which include asbestos, erionite, and simian virus 40 (SV40). Moreover, several genes are involved in the pathogenesis of this cancer: $p16^{INK4A}$, $p14^{ARF}$, and *neurofibromatosis type 2* (*NF2*). The genes *tumor protein 53* (*TP53*) and *phosphatase and tensin homolog deleted from chromosome 10* (*PTEN*) are still under investigation for their role in the development of malignant mesothelioma. In spite of extensive and intensive studies, no beneficial therapy or drug for malignant mesothelioma has been established and challenges for such development are continuing.

Receptor tyrosine kinase (RTK) is implicated in the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), a mitogen-activated protein (MAP) kinase (MAPK), as one of the major signaling pathways (Figure 1). When activated, RTK phosphorylates its own receptor and Src-homology and collagen homology-2 (SHC2). Phosphorylated SHC2 recruits the adaptor protein growth factor receptor binding protein-2 (GRB2) and forms a complex with the exchanger factor son of sevenless (SOS); SHC2/GRB2/SOS, which activates the small G-protein RAS by exchanging GDP with GTP and activated RAS activates the effector RAF, a serine/threonine protein kinase. Activated RAF phosphorylates and activates MAP kinase kinase (MAPKK=MEK), which in turn, phosphorylate and activate MAPK (ERK1/2). ERK1/2 is recognized to promote cancer cell growth and protect cells from apoptosis. In this pathway, protein tyrosine phosphatase 1B (PTP1B), a tyrosine phosphatase, de-phosphorylates phosphorylated RTK and SHC2; in other words, PTP1B down-regulates RTK signaling (Figure 1). Protein phosphatase-2A (PP2A), a serine/threonine phosphatase, de-phosphorylates phosphorylation of MAPKKK, MAPKK, and ERK1/2; in other words, PP2A inhibits ERK1/2 activation (Figure 1).

In our preliminary study, we have found that phosphatidylethanolamines (PEs) such as 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine (DAPE), 1,2-dilinoleoyl-*sn*-

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glycero-3-phosphoethanolamine (DLPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) enhance PP2A and PTP1B activities, with the highest potential for DPPE. Then, we postulated that DPPE could exhibit an anti-tumor effect by suppressing MEK and ERK1/2 activities in association with PP2A and PTP1B activation. To address this hypothesis, the present study investigated the anti-tumor effect of DPPE on human malignant pleural mesothelioma cell lines.

Materials and Methods

Assay of protein phosphatase activities. Activities of protein phosphatases PP1, PP2A, and PTP1B under cell-free conditions were assayed as previously described (2). Human recombinant PP1 was purchased from New England BioLabs Inc. (Ipswich, MA, USA) and human recombinant PP2A from Millipore (Billerica, MA, USA). Human PTP1B was cloned into pGEX-6P-3 vector with a glutathione S-transferase (GST) tag at the NH_2 terminus, and expressed in competent *Escherichia coli* BL21 (DE3), suitable for transformation and protein expression. GST-fusion PTP1B was affinity-purified using glutathione sepharose 4B (GE Healthcare, Piscataway, NJ, USA). Activity of each phosphatase was assayed by reacting with *p*-nitrophenyl phosphate (pNPP)(Sigma-Aldrich, St. Louis, MO, USA) as a substrate. PP1 (1 U/well), PP2A (0.2 U/well), or PTP1B (1 μg/well) was pre-incubated at 30°C (for PP1) or 37°C (for PP2A and PTP1B) for 30 min in a reaction medium [50 mM HEPES, 100 mM NaCl, 2 mM dithiothreitol, 0.01% (v/v) Brij-35, 1 mM $MnCl_2$, pH 7.5 for PP1; 50 mM Tris-HCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, pH 7.0 for PP2A; and 50 mM HEPES, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, pH 7.2 for PTP1B] in the presence and absence of phosphatase inhibitors or DPPE. pNPP at a concentration of 5 mM for PP1, 0.5 mM for PP2A, and 10 mM for PTP1B was then added to the reaction medium followed by 60-min incubation, and the reaction was terminated by adding 0.1 N of NaOH. De-phosphorylated pNPP was quantified at an absorbance of 405 nm with a SpectraMax PLUS384 (Molecular Devices, Sunnyvale, CA, USA).

Cell culture. NCI-H28 cell, a human malignant pleural mesothelioma cell line, and Met5A, a human non-malignant mesothelial cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured by the method previously described (3). Briefly, cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 0.003% (w/v) 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 using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) as previously described (4).

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).

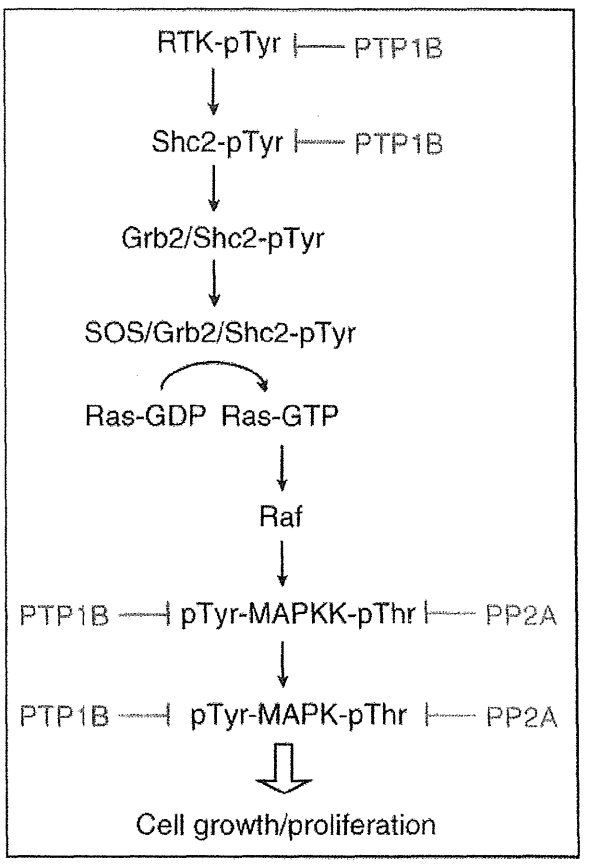

Figure 1. *A schematic pathway for receptor tyrosine kinase (RTK)-related mitogen-activated protein (MAP) kinase (MAPK) activation pathway. SHC2, Src-homology and collagen homology 2; GRB2, growth factor receptor binding protein 2; SOS, son of sevenless; MAPKK, MAPK kinase; PP2A, protein phosphatase 2A; PTP1B, protein tyrosine phosphatase 1B.*

Western blotting. Cells were treated or not with DPPE, and then lysed in a lysate solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 7.5] containing 1% (v/v) protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail. The lysates were centrifuged at 3,000 rotation per minute (rpm) for 5 min at 4°C. Proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes. Blotted membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against phospho-MEK (pMEK) (Cell Signaling Technology, Inc., Danvers, MA, USA), MEK (Cell Signaling Technology), phospho-ERK1/2 (pERK1/2)(Santa Cruz Biotechnology, Santa Cruz, CA, USA), and ERK (Santa Cruz Biotechnology). After

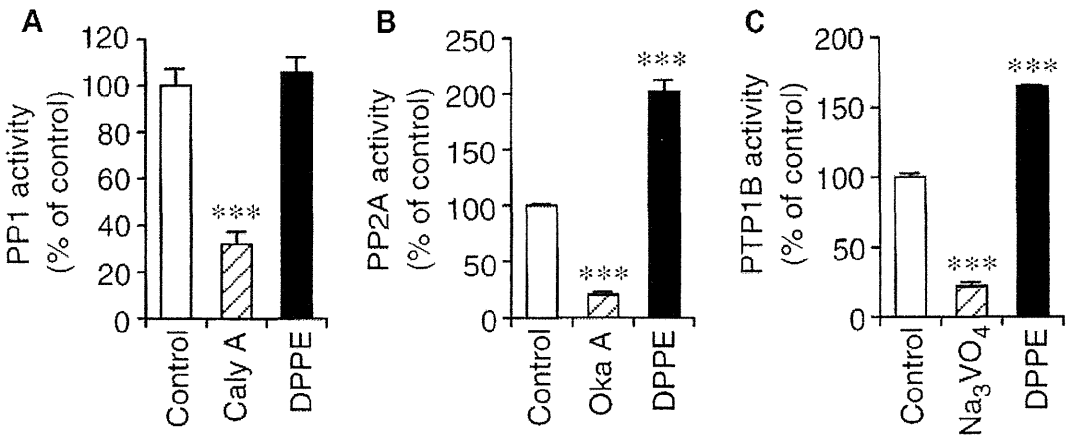

Figure 2. *Effects of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) on activities of protein phosphatase 1 (PP1) (A), protein phosphatase 2A (PP2A) (B), and protein tyrosine phosphatase 1B (PTP1B) (C). PP1, PP2A, or PTP1B was reacted separately with p-nitrophenyl phosphate (pNPP) in the presence and absence of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) (100 μM) with and without calyculin A (Caly A) (20 nM), okadaic acid (Oka A) (2 nM), or sodium orthovanadate (Na_3VO_4) (1 μM), and dephosphorylated pNPP was quantified. In the graphs, each value represents the mean (±SEM) percentage of basal phosphatase activity (control) (n=4 independent experiments). ***p<0.0001 as compared with control, Dunnett's test.*

washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence LAS-4000 mini detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis. Statistical analysis was carried out using Dunnett's test, and $p<0.01$ was regarded as significance.

Results

DPPE enhances PP2A and PTP1B activities. We initially examined the effects of DPPE (100 μM) on protein phosphatases. In the PP1 assay, calyculin A (20 nM), an inhibitor of PP1, clearly reduced PP1 activity (Figure 1A), confirming a reliable PP1 assay. No significant effect on PP1 activity was obtained with DPPE (Figure 2A).

In the PP2A assay, okadaic acid (2 nM), an inhibitor of PP2A, clearly reduced PP2A activity (Figure 2B), confirming a reliable PP2A assay. DPPE significantly enhanced PP2A activity, reaching nearly twice that of the control levels (Figure 2B).

In the PTP1B assay, sodium orthovanadate (Na_3VO_4) (1 μM), an inhibitor of PTP1B, clearly attenuated PTP1B activity (Figure 2C), confirming a reliable PTP1B assay. DPPE significantly enhanced PTP1B activity, reaching approximately 1.6 fold that of the control levels (Figure 2C).

DPPE tends to attenuate ERK1/2 activity in NCI-H28 cells. If DPPE enhances PP2A and PTP1B activities, then the lipid should suppress activities of MEK and ERK1/2. To address whether DPPE affects protein phosphatase activities, we monitored phosphorylation of MEK and ERK1/2 in Met5A non-malignant mesothelial cells and NCI-H28 malignant mesothelioma cells.

DPPE (100 μM) reduced phosphorylation of MEK in a treatment time (10-60 min)-dependent manner in Met5A cells, but phosphorylation of ERK1/2 was little affected (Figure 3A). DPPE (100 μM) had little effect on phosphorylation of MEK, but reduced phosphorylation of ERK1/2 in a treatment time (10-60 min)-dependent manner in NCI-H28 cells (Figure 3B). The potential for the inhibitory effect of DPPE on phosphorylation of MEK and ERK1/2, however, was much lower than expected and not significant.

DPPE induces apoptosis of NCI-H28 cells. In the MTT assay, treatment with DPPE for 24-48 h reduced NCI-H28 cell viability in a concentration (1-100 μM)-dependent manner, but had no effect on Met5A cell viability (Figure 4). This indicates that DPPE induces cell death in NCI-H28 malignant mesothelioma cells, but not Met5A non-malignant mesothelial cells.

To determine whether the effect of DPPE was due to apoptosis, we finally carried out TUNEL staining. DPPE (30 and 100 μM) significantly increased the number of TUNEL-positive cells as compared with those for untreated control NCI-H28 cells (Figure 5). DPPE at a concentration of 100 μM significantly increased the number of TUNEL-positive Met5A cells, but to a much lesser extent than that for NCI-H28 cells (Figure 5). Taken together, these results indicate that DPPE induces apoptosis of NCI-H28 malignant mesothelioma cells rather than Met5A non-malignant mesothelial cells.

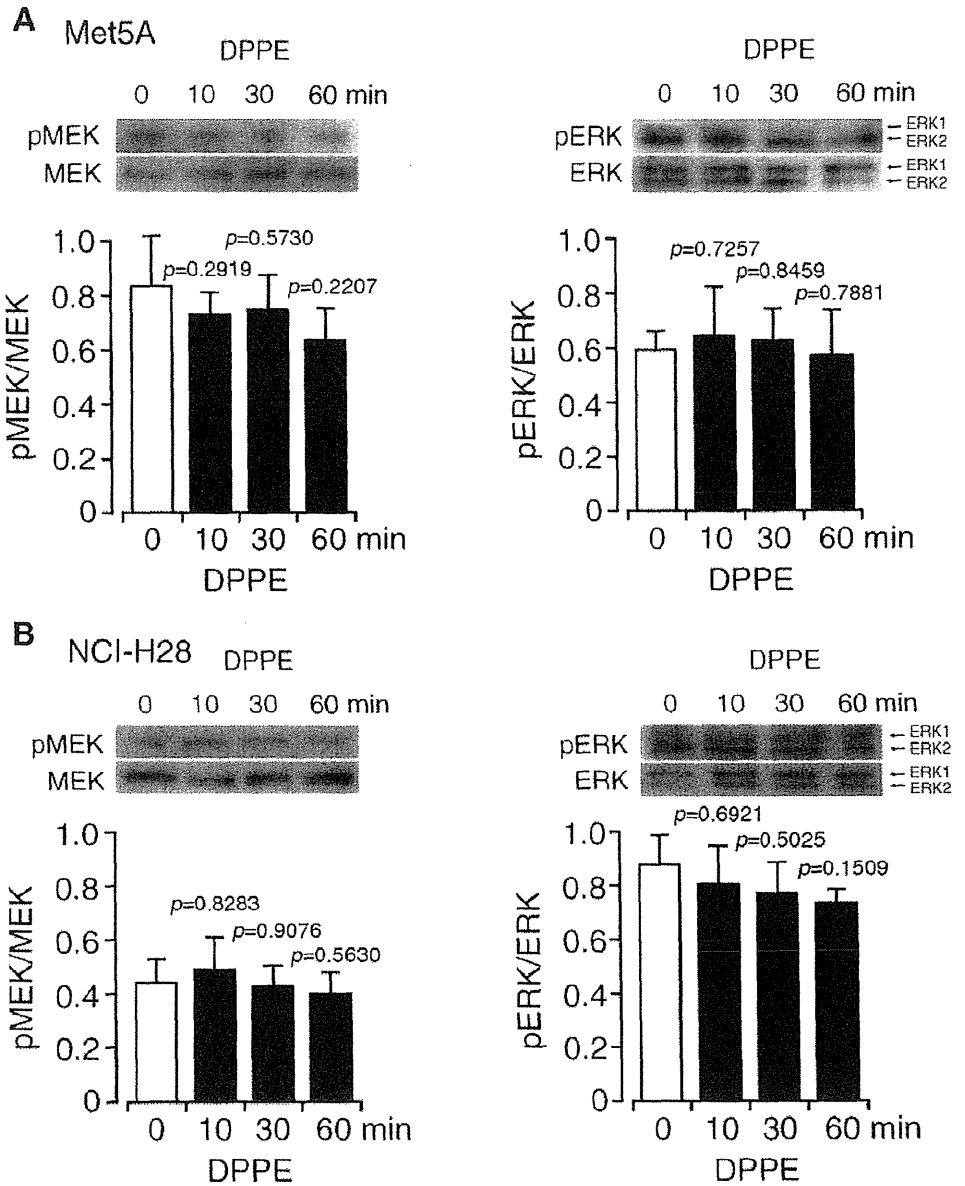

Figure 3. *Effects of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) on activity of mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK). Met5A (A) and NCI-H28 (B) cells were treated with DPPE (100 μM) for the indicated periods of time, followed by western blotting using antibodies against pMEK, MEK, pERK1/2, and ERK1/2. In the graphs, each column represents the mean (±SEM) ratio of signal intensity of phosphorylated protein relative to MEK signal intensity or that of non-phosphorylated protein (n=4 independent experiments). p-Values were defined by Dunnett's test.*

Discussion

The phospholipid PE is the most abundant lipid in the cytoplasmic layer of cellular membranes and PE is implicated in a wide range of cellular processes such as membrane fusion, cell cycle, autophagy, apoptosis, and cognitive function (5-9).

PE is produced through three main pathways: the cytidine 5'-diphosphate (CDP)-ethanolamine Kennedy pathway, mitochondrial phosphatidylserine (PS) decarboxylation pathway catalyzed by PS decarboxylase, and acylation of lysoPE catalyzed by lysophosphatidylethanolamine acyltransferase. The CDP-ethanolamine Kennedy pathway is the only route for *de novo* synthesis of PE (10).

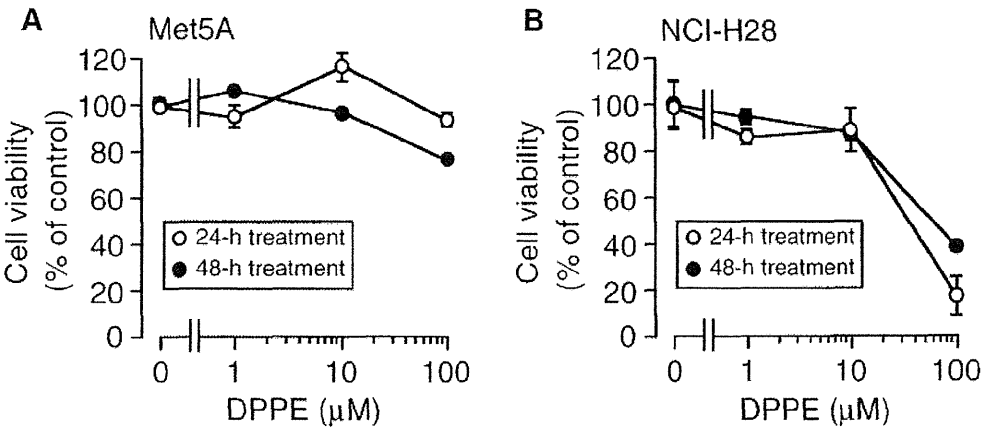

Figure 4. *Effects of 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) on cell viability. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out on Met5A (A) and NCI-H28 (B) cells treated with DPPE at the concentrations indicated for 24-48 h. In the graphs, each point represents the mean (±SEM) percentage of control cell viability (MTT intensity of cells not treated with DPPE) (n=4 independent experiments).*

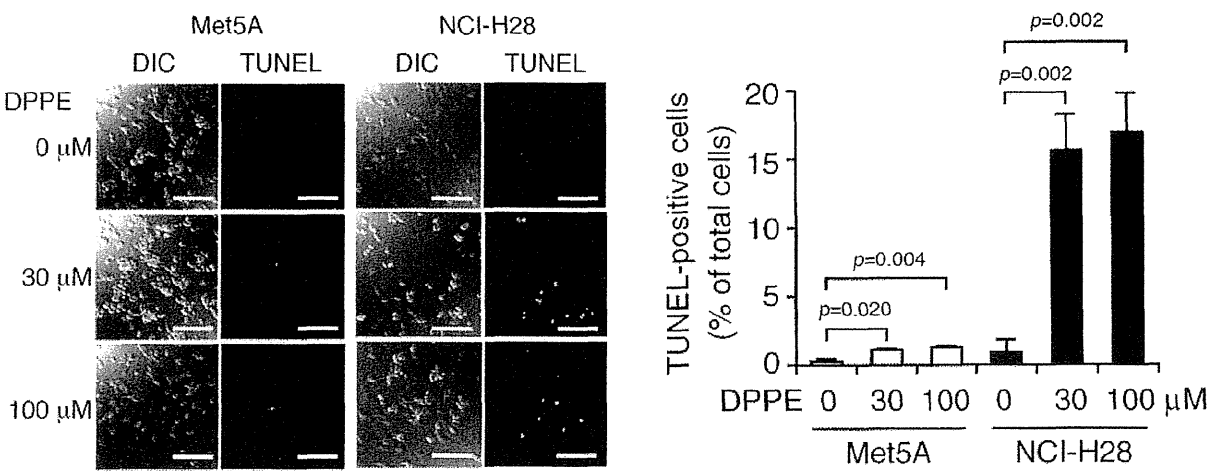

Figure 5. *Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. TUNEL staining was carried out on Met5A and NCI-H28 cells treated with 1,2-dipalmitoleoyl-sn-glycero-3-phosphoethanolamine (DPPE) at the concentrations indicated for 48 h. DIC, Differential interference contrast. Bars=100 μm. TUNEL-positive cells were counted in an area (0.4 mm × 0.4 mm) selected at random. In the graph, each column represents the mean (±SEM) percentage of TUNEL-positive cells relative to the total number of cells (n=4 independent experiments). p-Values were defined from Dunnett's test.*

Phosphorylation of ethanolamine by ethanolamine kinase is followed by the CTP:choline cytidylyltransferase 2 (PCyt2)-mediated production of CDP-ethanolamine, and PE production is catalyzed by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase. The analogous enzymes of the CDP-choline branch of the Kennedy pathway include choline kinase, PCyt1, and CDP-choline:1,2-diacylglycerol choline phosphotransferase. In the liver, PE is transformed into phosphatidylcholine (PC) catalyzed by PE N-methyltransferase. PE is also produced in mitochondria by PS decarboxylase-catalyzed decarboxylation of PS. Mammals do not synthesize PS *de novo*, and therefore, PS is produced by head-group exchange from PE catalyzed by PS synthase-2 or PC catalyzed by PS synthase-1. PE, on the other hand, is produced by lysoPE acyltransferase-catalyzed fatty acid esterification of lysoPE.

In the present study, DPPE, a PE, enhanced PP2A and PTP1B activities. To our knowledge, this is the first to show the new action of PE on protein phosphatases. How DPPE enhances PP2A and PTP1B activities, however, remains to be determined. Activation of ERK, a MAPK, leads to promotion of cell growth and proliferation not only of normal cells but also of cancer cells. Activation of ERK1/2 through a pathway along the RTK/(SHC2/GRB2/SOS)/RAS/RAF/MAPKK/MAPK axis is initiated by tyrosine phosphorylation of RTK and SHC2 (Figure 1). PTP1B dephosphorylates RTK, SHC2, MAPKK, and MAPK, thereby negatively-regulating RTK signaling (Figure 1). PP2A dephosphorylates and inactivates, MAPKK, and MAPK (Figure 1). Accordingly, DPPE, in order to enhance PP2A and PTP1B activities, should attenuate RTK signaling and inhibit ERK activation. Indeed, DPPE tended to reduce pMEK in Met5A cells and pERK1/2 in NCI-H28 cells at 60-min treatment, but the potential was much lower than expected.

Strikingly, DPPE clearly reduced cell viability and markedly increased TUNEL-positive NCI-H28 malignant mesothelioma cells. In contrast, DPPE had little effect on cell viability and the number of TUNEL-positive cells for Met5A non-malignant mesothelial cells. DPPE, thus, appears to preferentially induce apoptosis of NCI-H28 cells, but not of Met5A cells. This raises the possibility that DPPE could be developed as an anticancer drug for treatment of malignant mesothelioma.

Conclusion

The results of the present study demonstrate that DPPE preferentially induces apoptosis of NCI-H28 malignant mesothelioma cells rather than Met5A non-malignant mesothelial cells. DPPE enhanced PP2A and PTP1B activities in a cell-free system, and this action might, at least in part, contribute to DPPE-induced apoptosis of malignant mesothelioma cells.

Conflicts of Interest

None of the Authors have any potential conflict of interest.

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ORIGINAL ARTICLE

Frequency of epidermal growth factor receptor mutations in Bangladeshi patients with adenocarcinoma of the lung

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Abstract
Background Worldwide studies on lung adenocarcinoma have demonstrated a genetic divergence of the epidermal growth factor receptor (EGFR) pathway according to ethnicity, such as higher frequency of activated EGFR mutations among East Asian patients. However, such information is still lacking in some developing countries.
Methods We investigated the frequency of EGFR mutations among Bangladeshi patients with adenocarcinoma of the lung. Fine-needle aspiration tissue samples were collected from 61 Bangladeshi patients. Polymerase chain reaction–single-strand conformation polymorphism was performed on extracted DNA for mutational analysis of EGFR exons 19 and 21.
Results EGFR mutations were found in 14 of 61 (23.0 %) Bangladeshi patients. There was no significant difference in EGFR mutation rate with regard to patient's age, sex, smoking history, clinical stage of lung cancer, subtypes of adenocarcinoma, and tumor differentiation.
Conclusion The present study revealed that the EGFR mutation rate in Bangladeshi patients with adenocarcinoma of the lung was higher than in African–American, Arabian, and white Caucasian patients, and was lower than in East Asia.

Keywords Lung cancer · Epidermal growth factor receptor · EGFR mutation · Tyrosine kinase inhibitors · Single-strand conformation polymorphism · Ethnic difference

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Introduction

The frequency of epidermal growth factor receptor (EGFR) mutation in non-small cell lung cancer is documented to differ across ethnic groups, with a notably higher occurrence observed in East-Asian trials (30–60 %) and a lower occurrence in North-American studies (10–20 %) [1–4]. The reasons for ethnic influence on mutation incidence still remain poorly understood.

Several reports suggest that EGFR mutations provide survival benefit independent of treatment [5, 6]. More recent information focussing on East Asia also suggests that the presence of classical EGFR mutations is predictive of survival benefit after EGFR tyrosine kinase inhibitor (TKI) therapy [7].

Since there are no reports of genetic backgrounds of lung cancer in Bangladesh, we examined the frequency of major types of activating mutations (exons 19 and 21) of EGFR in Bangladeshi patients with adenocarcinoma of the lung.

Patients and methods

Clinical characteristics of patients

A total of 61 patients underwent computed tomography (CT)-guided fine-needle aspiration (FNA) biopsy at the National Institute of Diseases of the Chest and Hospital (NIDCH), National Institute of Cancer Research and Hospital (NICRH), and related diagnostic centers in Bangladesh from 2009 to 2011. Written informed consent was obtained from each patient.

The clinical information on these patients is shown in Table 1. None of the patients were exposed to any chemotherapy before FNA. Pathological diagnoses were performed by pathologists, unaware of the clinical information, and finally confirmed by one pathologist (M.G.M.) according to the WHO classification system [8].

Table 1 Characteristics of the 61 patients included in the study

Variable	All patients
Age (years)	Median 56.5 (range 18–95)
Gender	
Male	47 (77 %)
Female	14 (23 %)
Smoking status	
Never-smoker	23 (38 %)
Former-smoker	8 (13 %)
Current-smoker	30 (49 %)
Brinkman index	
Former-smoker	593
Current-smoker	878
Clinical stage	
Stage I	7 (11 %)
Stage II	17 (28 %)
Stage III	17 (28 %)
Stage IV	20 (33 %)

FNA sample and DNA

CT-guided FNA biopsies were performed using 23-gauge needles in all patients. Suitability for DNA extraction was recognized when pathologists confirmed that more than 70 % of each sample consisted of tumor cells.

FNA samples were immediately preserved in tubes containing 99.5 % ethanol at −80 °C. Genomic DNA was isolated from tissue by discarding the ethanol after centrifugation at high speed. Genomic DNA was extracted using the EZ1 DNA Tissue Kit with the EZ1 instrument (QIAGEN, Germany).

Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) was used to detect mutations in exons 19 and 21 of the EGFR gene. PCR was performed using the AmpliTaq Gold PCR Master Mix (Life Technologies, USA). The primers used and PCR conditions are shown in Table 2. After denaturing PCR products, electrophoresis was performed with the GenePhor System and GeneGel Excel 12.5/24 (GE Healthcare, Sweden) at 18 °C, 650 V for 80 min. The gels were stained using the DNA Silver Staining Kit (GE).

Statistical analysis

The incidence of EGFR mutation status along with the corresponding important predictors of incidence of EGFR mutations were additionally identified by logistic regression with a forward-model selection procedure. The factors included in the model selection were age, sex, histology, and tumor stage. The differences in continuous measurements between two groups were examined by the t test or Fisher's exact test, and the Mann–Whitney U test was used to determine the differences between continuous variables. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Out of 61 tumors, mutations in exons 19 and 21 of the EGFR gene were detected in 8 (13.1 %) and 6 (9.8 %)

Table 2 PCR primers and parameters

EGFR gene	Forward (5′–3′)	Reverse (5′–3′)	Product size (bp)	Number of cycle	Annealing condition
Exon19	CGTCTTCCTTCTCTCTCTGT	CCACACAGCAAAGCAGAAAC	148	35	55 °C, 15 s
Exon21	AGGGCATGAACTACTTG	CCTCCTTACTTTGCCTCCTTC	167	35	55 °C, 15 s

Table 3 Relationship of EGFR gene mutations to clinicopathological characteristics

Variable	All patients ($n = 61$)	EGFR gene mutation status		
		Mutated ($n = 14$)	Wild-type ($n = 47$)	*P* value
Age, median	56.5	61.6	55.0	
Age, range	18–95	45–76	18–95	
Age <60 years	35	6 (17.1 %)	29	0.10
Age ≥60 years	26	8 (30.8 %)	18	
Gender				0.37
Male	47	12 (25.5 %)	35	
Female	14	2 (14.3 %)	12	
Smoking status				0.97
Never-smoker	23	5 (21.7 %)	18	
Former-smoker	8	3 (37.5 %)	5	
Current-smoker	30	6 (20.0 %)	24	
Clinical stage				0.40
Stage I	7	3 (42.9 %)	4	
Stage II	17	5 (29.4 %)	12	
Stage III	17	3 (17.6 %)	14	
Stage IV	20	3 (15.0 %)	17	
Subtypes of adenocarcinoma				0.60
Bronchio-alveolar	1	0	1	
Papillary	3	0	3	
Solid	2	0	2	
Mixed	55	14	41	
Acinar component				0.50
Present	31	6 (19.4 %)	25	
Absent	30	8 (26.7 %)	22	
Bronchio-alveolar component				0.88
Present	8	2 (25.0 %)	6	
Absent	53	12 (22.6 %)	41	
Papillary component				0.04
Present	32	4 (12.5 %)	28	
Absent	29	10 (34.5 %)	19	
Solid component				0.22
Present	11	1 (9.1 %)	10	
Absent	50	13 (26.0 %)	37	
Tumor differentiation				0.21
Well	5	1 (20.0 %)	4	
Moderate	21	3 (14.3 %)	18	
Poor	35	10 (28.6 %)	25	

tumors, respectively. The overall mutation rate was 23.0 % (14 of 61). The mutation rate in male/female patients was 25.5 % (12 of 47) and 14.3 % (2 of 14), respectively. The clinical characteristics and the mutation patterns of these patients are summarized in Table 3. A significantly higher incidence of EGFR mutation was seen in adenocarcinomas with papillary component. No other significant relationship between EGFR mutation rate and patient characteristics was found.

Discussion

To our knowledge, the present study is the only published series to date to comprise information on EGFR mutations in a fair number of Bangladeshi patients. Our samples were collected from major hospitals and diagnostic centers for lung cancer and respiratory diseases, to which patients come from widespread areas of Bangladesh. Therefore, it is likely that our series closely reflects the overall frequency of EGFR mutations in the population of Bangladesh.

The present study revealed that the EGFR mutation rate in Bangladeshi patients with adenocarcinoma of the lung was higher than in African–American [9], Arabian [10], and white Caucasian patients [2, 3, 11, 12], and was lower than in patients in East Asia [2, 13–16] and other countries of South Asia [17]. This is probably related to inherent differences between cohorts. Patient characteristics for the different cohorts are listed in Table 4 [2–4, 9–12, 15–17].

It is notable that tumor stage at diagnosis may have affected the reported ethnic difference in EGRF mutation rate. As Bangladesh has no mandatory national health insurance policy yet, Bangladeshi patients with lung cancer mostly present at advanced stage.

With regard to the relationship between EGFR mutation rate and clinicopathological characteristics of the patients, we found a higher rate only in tumors with papillary component. We have no explanation for the above result.

Although the number of samples is not large enough to draw any conclusion, the results of our study may predict a higher possibility of major responses to EGFR-TKIs in Bangladeshi patients with adenocarcinoma of the lung than in African–American, Arabian, and white Caucasian patients. This assumption is good news particularly for patients in Bangladesh, where most lung cancer cases are in advanced stages at diagnosis and surgery is not a treatment option. In addition, the findings of the present study may help to expand the pool of patients eligible for future prospective clinical trials to fully investigate the relationship between the presence of activating EGFR mutation and response to TKIs.

Table 4 Previous studies examining EGFR mutation in non-small-cell lung cancer

Reference	Patient group (ethnicity/country of origin)	Total number of patients	Frequency		Prediction in never/former-smokers	Detection methods	Exons
			Overall	Adencarcinoma			
This study	Bangladesh	61	–	14/61 (23 %)	No	PCR–SSCP	19 and 21
Sahoo et al. [17]	India	220	114/220 (52 %)	–	Yes	ARMS-PCR	18–21
Al-Kuraya et al. [10]	Saudi Arabia	34	1/34 (3 %)	–	Yes	Direct sequencing	18–21
Leidner et al. [9]	African-American	53	1/53 (2 %)	–	Yes	Direct sequencing	18–21
Marchetti et al. [11]	White Caucasian (Italian)	860	37/860 (4 %)	37/375 (10 %)	Yes	Direct sequencing and SSCP	18, 19, and 21
Pao et al. [3]	USA	96	11/96 (11 %)	–	Yes	Direct sequencing	2–28
Yang et al. [12]	USA	219	26/219 (12 %)	25/164 (15 %)	Yes	Direct sequencing	18–21
Paez et al. [2]	USA	61	1/61 (2 %)	1/29 (3 %)	Yes	Direct sequencing	18–21
	Japan	58	15/58 (26 %)	14/41 (34 %)			
Huang et al. [3]	Taiwanase	101	39/101 (39 %)	38/69 (55 %)	Yes	Direct sequencing	18–21
Sonobe et al. [4]	Japanese	154	60/154 (39 %)	60/108 (55 %)	Yes	PCR–SSCP and direct sequencing	18–21
Kosaka et al. [15]	Japanese	277	111/277 (40 %)	110/224 (49 %)	Yes	Direct sequencing	18–21
Han et al. [16]	Korean	90	17/90 (19 %)	14/65 (21 %)	No	Direct sequencing	18, 19, 21, and 23

ARMS amplification refractory mutation system

Conclusion

Epidermal growth factor receptor mutations were found in 14 of 61 (23.0 %) Bangladeshi patients.

Conflict of interest All of the authors declare that they have no financial relationship with the organization that sponsored the research. All of the authors also declare that they have full control of all primary data and that they agree to allow the journal to review their data if requested.

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CURRENT TOPICS REVIEW ARTICLE

Extrapleural pneumonectomy or pleurectomy/decortication for malignant pleural mesothelioma

Seiki Hasegawa

Received: 19 February 2014

Abstract Malignant pleural mesothelioma (MPM) is associated with a very poor prognosis. Unlike other solid tumors, any type of planned surgery for MPM would be cytoreductive rather than radical. There are two types of surgery for MPM. Extrapleural pneumonectomy (EPP) involves en bloc resection of the lung, pleura, pericardium, and diaphragm. Pleurectomy/decortication (P/D) is a lung-sparing surgery that removes only parietal/visceral pleura. In comparison with EPP, P/D is theoretically less radical but is associated with less perioperative mortality/morbidity and less postoperative deterioration of cardiopulmonary function. It still remains unclear which surgical technique is superior in terms of the risk/benefit ratio. In this context, selection between EPP and P/D has been a matter to debate.

Keywords Malignant pleural mesothelioma · Surgery · Extrapleural pneumonectomy · Pleyrectomy/decortication · Multimodality treatment

Introduction

Malignant pleural mesothelioma (MPM) is associated with a very poor prognosis, and its incidence is expected to increase in Asia and developing countries [1–6]. Because any type of planned surgery would be cytoreductive rather than radical [7], an optimal outcome via surgery alone is unlikely [8]. Accordingly, the current strategy for curing this disease has shifted to multimodal therapy with chemotherapy and/or radiation therapy (RT).

There are two types of surgery for MPM. Extrapleural pneumonectomy (EPP) involves en bloc resection of the lung, pleura, pericardium, and diaphragm. Pleurectomy/decortication (P/D) is a lung-sparing surgery that removes only parietal/visceral pleura. EPP leaves less residual tumor cells compared with P/D; however, it often results in high mortality/morbidity, severe depression of cardiorespiratory function, and poor quality of life. Till date, the risk–benefit ratios of P/D and EPP as part of multimodal therapy have not been clearly elucidated.

Furthermore, the decision to perform either EPP or P/D in studies on multimodal approaches has been solely based on surgical conjecture and bias, rather than scientific data [9].

EPP and P/D surgical procedures

The first set of procedures are common between EPP and P/D [10] (shown as Step 1 in Fig. 1). Step 1 involves thoracotomy, extrapleural dissection of the parietal pleura, with diaphragm and/or pericardium resection if required, and systematic lymph node dissection. Therefore, after completing step 1, the lung/pleura block is connected to the body only by hilar components, namely the main bronchus, main pulmonary artery, and pulmonary veins. The second set of procedures involve en bloc extirpation of lung, parietal/visceral pleura, diaphragm, and pericardium in EPP (Step 2a) and visceral pleurectomy in P/D (Step 2b).

Microscopic complete resection (R0) is theoretically impossible in Step 1 and Step 2b, but not in Step 2a. Step 2b is more likely to leave residual tumor cells compared

This review was submitted at the invitation of the editorial committee.

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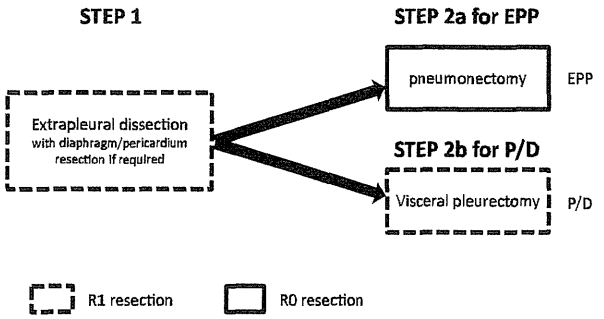

Fig. 1 Diagram of surgical procedures in EPP and P/D. Step 1 comprises the common procedures in EPP and P/D, including thoracotomy, extrapleural dissection of the parietal pleura, with diaphragm and/or pericardium resection if required, and systematic lymph node dissection. Steps 2a and 2b represent other surgical options

Fig. 2 Comparison of disadvantages between EPP and P/D. EPP is associated with high perioperative mortality/morbidity and severe deterioration of postoperative cardiopulmonary function and quality of life. On the other hand, P/D leaves more residual tumor cells because of visceral pleurectomy. Selection between EPP and P/D ultimately leads to the selection of the radicality of Step 2a over that of Step 2b or the selection of less surgical insult from P/D over that from EPP

with Step 1 because connection between the visceral pleura and lung parenchyma is usually tighter than that between the parietal pleura and chest wall. Therefore, P/D is less radical compared with EPP [11].

On the other hand, EPP has several disadvantages such as higher perioperative mortality/morbidity, severe deterioration of postoperative cardiopulmonary function and quality of life, and poorer tolerance to chemotherapy in case of recurrence.

Therefore, selection between EPP and P/D leads to the selection of the radicality of Step 2a over that of Step 2b or the selection of less surgical insult from P/D over that from EPP (Fig. 2).

Confusion and unanswered questions regarding MPM treatment

An element of extreme confusion exists with regard to MPM treatment, particularly surgery. The proposed reasons are mentioned below.

Questionable survival benefit of surgery

Different surgical procedures with curative intent can exist for malignant disease, such as lobectomy and segmentectomy for primary lung cancer. However, the situation is quite different for MPM surgery. Unlike the goal of surgery for other solid tumors, the goal of MPM surgery is not radical resection but macroscopic complete resection (MCR) because of the lack of surgical margins [7, 12]. Recently, Treasure et al. [13] concluded from the Mesothelioma and Radical Surgery (MARS) feasibility study that radical surgery in the form of EPP within trimodality therapy offered no benefit. However, apt interpretation of the MARS study remains debatable [14–19].

Why is survival after P/D equal to or even better than that after EPP

Theoretically, P/D is less radical than EPP, even though both are only cytoreductive procedures. However, most hospitals have reported equal or even better survival after P/D than after EPP [20–23]. In the context of multimodal therapy, Cao et al. [24], on the basis of their meta-analysis, concluded that selected patients who underwent extended P/D had lower perioperative morbidity and mortality with similar, if not superior, long-term survival compared with those who underwent EPP. Furthermore, Lucklatz and others [22] reported that P/D combined with postoperative adjuvant therapy provided better survival compared with EPP, irrespective of factors such as advanced disease or surgically less fit patients.

Other than nonprospective settings and patient selection bias, there may be several explanations for this contradiction.

First, EPP is associated with higher perioperative mortality/morbidity. Cao et al. conducted a systematic analysis and demonstrated that perioperative mortality (2.9 vs. 6.8 %, $p = 0.02$) and morbidity (27.9 vs. 62.0 %, $p < 0.0001$) were significantly lower for patients who underwent extended P/D than for those who underwent EPP [24]. Second, patients who undergo P/D have more opportunities for additional therapy after recurrence compared with patients who undergo EPP. Bolukbas et al. [25] found that additional chemotherapy after recurrence was

acceptable in 64 % patients who initially underwent P/D and 25 % patients who initially underwent EPP. Accordingly, survival after recurrence was longer in patients who underwent P/D than in those who underwent EPP [15, 23]. Third, because of better cardiopulmonary reserve, patients who undergo P/D are more equipped to fend off postoperative nononcological disorders such as pneumonia and cardiac failure compared with those who undergo EPP.

Because there is no randomized study comparing EPP and P/D, it remains unclear whether postoperative survival in P/D patients is really equal to or better than that in EPP patients.

Ambiguity surrounding the definition of P/D

Although P/D has been performed for more than 30 years, confusion still surrounds the actual meaning of pleurectomy/decortication. Recently, the International Mesothelioma Interest Group (IMIG), in collaboration with the International Association for the Study of Lung Cancer (IASLC), published a Consensus Report that classified pleurectomy into three categories according to surgical technique [26].

1. Extended P/D: parietal and visceral pleurectomy to remove all gross tumor, with resection of the diaphragm and/or pericardium.
2. P/D: parietal and visceral pleurectomy to remove all gross tumor, without resection of the diaphragm or pericardium.
3. Partial pleurectomy: partial removal of parietal and/or visceral pleura for diagnostic or palliative purposes, leaving gross tumor behind.

However, several critical points remain unclear.

First, does P/D allow part of the pleura to be left behind as long as it contains no macroscopic tumor? The consensus report does not mandate that P/D include 100 % visceral pleurectomy; it requires only MCR or complete resection of macroscopic tumors. The National Comprehensive Cancer Network (NCCN) guidelines clearly define P/D as complete removal of involved pleura and all gross tumor [27]. This distinction is particularly important in cases of early MPM, in which 100 % resection of almost intact visceral pleura is technically difficult. Second, the consensus states that resection of the diaphragm and/or pericardium is not mandatory in extended P/D; however, it should be performed if required. If so, what does P/D indicate? In cases involving the diaphragm and/or pericardium, pleurectomy without resection of the diaphragm and/or pericardium should be categorized as partial pleurectomy instead of P/D. I would propose that extended P/D and P/D be redefined as P/D, which involves parietal and visceral pleurectomy to remove all gross tumor, with resection of the diaphragm and/or pericardium if required. By changing the meaning of P/D in terms of diaphragm and/or pericardium involvement, a more comprehensible and consistent definition will be realized.

Third, does P/D allow the resection of pulmonary parenchyma? Lang-Lazdunski and colleagues [28] reported that 12 % (5/41) P/D patients required either lobectomy or segmentectomy. Also, an ongoing multicenter phase II study in Japan permits the resection of pulmonary parenchyma [29].

Discrepancy among guidelines

The NCCN guidelines recommend surgical resection for patients with clinical stage I–III MPM who are medically fit for and can tolerate surgery [27]. The NCCN guidelines also recommend that P/D should be the first option for early disease (confined to the pleural envelope, no N2 lymph node involvement) with favorable histology (epithelioid).

In Europe, both the European Respiratory Society (ERS)/European Society of Thoracic Surgery (ESTS) [30] and British Thoracic Society (BTS) [31] guidelines state that the role of surgical resection in MPM is very uncertain and that radical surgery should only be performed in clinical trials, in specialized centers, and as part of a multimodal treatment plan. They also state that P/D should not be proposed with a curative intent. Italian guidelines recommend EPP to achieve adequate local control of MPM and P/D for patients with minimal, early-stage disease [32].

Therefore, discrepancies concerning performance practices and recommendations for P/D and EPP clearly exist. Furthermore, many MPM centers in Europe and some in North America and Japan are currently performing P/D with curative intent [20, 21, 28, 29, 33–37].

Should the surgical techniques for MPM ever be refined, the arrant inconsistencies cited above must be identified and resolved as soon as possible.

Very recently, the attendees of the 2012 International Mesothelioma Interest Group Congress agreed that the type of surgery (EPP or P/D), as long as it pertains to MCR, shall depend on clinical factors and the surgeon's individual judgment and expertise [17]. This concept would seem to hold much promise.

Scarcity of prospective clinical studies on P/D

With regard to EPP, one phase III study [13] and several phase II studies have been reported till date [38–42]. Therefore, the MCR completion rate and overall survival for intent-to-treat patients can be calculated.

With regard to P/D, however, there are few completed phase II studies [43, 44] and a few ongoing phase II studies [29, 45]. Rusch et al. [43] reported in their phase II study that MPM was resectable in 78 % (28/36) patients. However, they did not describe the MCR completion rate. An ongoing Japanese phase II study is designed to observe the feasibility of induction chemotherapy using pemetrexed plus cisplatin followed by P/D in patients with resectable MPM [29]. This study appears promising in that it will clarify the MCR completion rate as well as the conversion rate from P/D to EPP.

RT after P/D

Unlike in EPP, external beam radiation therapy following P/D has been contraindicated because of possible damage to the preserved ipsilateral lung [30, 46, 47].

Very recently, however, a few authors reported successful RT after P/D. Minatel et al. administered 50 Gy of hemithoracic radiation with helical tomotherapy following radical P/D. This protocol resulted in a median survival time of 33 months, progression-free survival of 29 months, and a 3-year survival rate of 49 %, with no fatal toxicity. [48] There is an ongoing phase II study at Memorial Sloan-Kettering Cancer Center in which hemithoracic pleural intensity-modulated radiation therapy (IMRT; 50.4 Gy in 28 fractions) is administered after induction chemotherapy and P/D [45]; an interim analysis found that this protocol had an acceptable toxicity [49].

From these observations, one can speculate that the reintroduction of RT after P/D can result in better local control and longer postoperative survival.

Selection between EPP and P/D

There exist some cases for which only one type of surgery is indicated. For example, patients with poor cardiopulmonary function are only fit for P/D. In patients with bulky and deep invasion to the pulmonary parenchyma, MCR can be achieved only by EPP. In the remaining cases, surgeons have to choose either EPP or P/D. Two different approaches are currently employed in patients with stage I–III resectable MPM who can tolerate aggressive surgery.

Selection of surgery on an individual basis

Some surgeons recommend tailoring of the surgical procedure to intraoperative findings, with the ultimate goal of achieving MCR using the procedure with the least morbidity [9, 50]. These surgeons elect to perform P/D in patients with minimal disease [9, 51]. P/D is also recommended if essential mediastinal structures (e.g., aorta and vertebral bodies) are found to be involved at thoracotomy [52].

This approach is accepted by most MPM centers in North America and Japan, as well as by some European centers [17].

Preference of P/D

Although European guidelines advise that P/D should not be proposed with a curative intent [30, 31], an increasing number of centers have abandoned EPP and consider P/D with a curative intent as their basic approach toward resectable MPM [28, 35, 37, 53]. The feasibility of P/D in

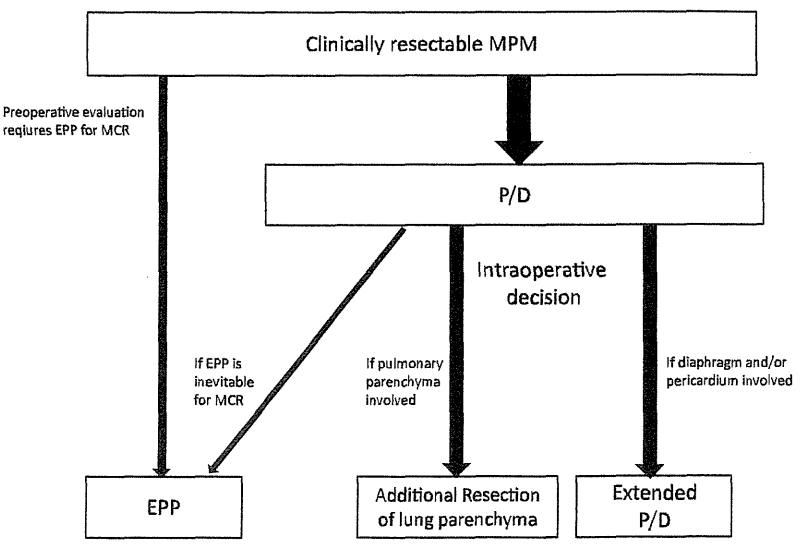

Fig. 3 Current approach to resectable MPM at Hyogo College of Medicine. We are currently choosing the least invasive surgical procedures to achieve MCR. P/D is indicated in most cases, except those with extensive tumor invasion to the pulmonary parenchyma. Resection of the diaphragm, pericardium, and lung parenchyma is performed if required. Conversion to EPP from P/D is decided on the basis of intraoperative findings

patients with advanced MPM may be questionable. Friedberg and others reported an MCR rate of 97 % (37/38) and a median survival of 21 months in their series of radical pleurectomy with intraoperative photodynamic therapy for advanced MPM. On the basis of their results, they theorized that MCR could be achieved with radical pleurectomy in all MPM cases in which MCR could be achieved with EPP [53]. Bolukbas et al. [54] reported that an MCR rate of 61.9 %, a surgical mortality of 4.8 %, a median survival of 21 months, and a 5-year survival of 28 % were achieved in patients with stage III MPM treated by trimodality therapy with radical pleurectomy.

Current approach to resectable MPM at Hyogo College of Medicine (Fig. 3)

As mentioned above, we are currently selecting the least invasive surgical procedures for achieving MCR. Therefore, surgery is initiated with the intention of performing P/D, with the exception of some cases with extensive invasion of MPM to the pulmonary parenchyma. Resection of the diaphragm and/or pericardium is performed only after all efforts to preserve them fail. Although an ongoing Japanese feasibility study permits the sparing of the visceral pleura as long as it does not contain macroscopic tumor [29], we remove all the parietal/visceral pleura irrespective of the presence of macroscopic lesions. Lung resection is frequently performed during P/D to achieve MCR and/or decrease air leakage.

Conflict of interest The authors have declared that no conflict of interest exists.

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