Original Article

Establishment of lung cancer cell lines and tumorigenesis in mice from malignant pleural effusion in patients with lung cancer

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Background: Lung cancer was often diagnosed by malignant pleural effusion (MPE). Excessive MPE is generally discarded. The establishment of cell lines and the generation of cancer mouse models have the potential to be directly linked to personalized medicine. This study aimed to establish cell lines and generate mouse models using MPE.

Methods: Cells derived from 5 mL of MPE were cultured in several conditions, including 100% MPE supernatant and Roswell Park Memorial Institute-1640 supplemented with 10% fetal bovine serum (FBS) or 10% MPE supernatant. When steady cell growth was observed, fewer cells were spread and the colonies were selected to establish the cell line. Cells derived from 10 mL of MPE were inoculated subcutaneously into non-obese diabetic-severe combined immunodeficiency (NOD-*scid*) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tmlWjl}/SzJ (NSG) mice to assess tumorigenic potential.

Results: MPEs were obtained from 28 lung cancer patients, 23 of whom had adenocarcinoma. Cell lines were established from 5 patients (18%). Tumorigenesis was observed in 6 of 28 cases (21%). However, in 7 cases, the mice (7 NSG and 1 NOD-*scid* mice) became progressively weaker, lost their hair, and died within 12 weeks without tumorigenesis. The appearance and pathological findings were consistent with graft-versus-host disease. Cell line establishment and tumorigenesis in mice were associated with a lower response to first-line therapy and poorer prognosis of patients.

Conclusions: When MPEs were simply utilized, the cell line establishment rate was 18% and the engraftment rate in mice was 21%. The prognosis of patients who underwent cell line establishment and engraftment in mice was poor.

Keywords: Cell line; lung cancer; malignant pleural effusion (MPE); prognosis; xenograft

Submitted Feb 13, 2024. Accepted for publication Aug 20, 2024. Published online Sep 25, 2024.
doi: 10.21037/tlcr-24-143
View this article at: https://dx.doi.org/10.21037/tlcr-24-143

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Introduction

Lung cancers are often accompanied by malignant pleural effusion (MPE), that is, pleural effusion in which cancer cells are detected. In clinical settings, approximately 5% and 15% of patients are diagnosed with lung cancer by MPE for non-small cell lung cancer (NSCLC) and SCLC, respectively (1,2). Pleural effusions are often examined to confirm the pathological diagnosis of lung cancer, and pleural effusion drainage is sometimes selected as a treatment option for improvement from progressive respiratory and circulatory disturbances. Thus, excessive MPEs are usually discarded.

Cancer research in humans is limited by difficulties in accessing cancer lesions and ethical issues. Cancer cell lines and cancer mouse models have been generated to overcome these limitations and have become essential for elucidating the mechanisms of carcinogenesis, growth, and metastasis, as well as to pursue the causes of drug resistance or develop cancer therapies. Several studies have reported the establishment of lung cancer and other malignant tumor cell lines from MPEs, including pancreatic carcinoma, malignant mesothelioma and soft tissue sarcoma (3-6). However, large-sample investigations of the methods and establishment rates of lung cancer cell lines derived from MPEs have rarely been reported (7).

Most NSCLC patient-derived xenograft (PDX) animal models are derived from fresh, surgically resected tissues of early-stage NSCLC (8-14). Two studies utilized bronchoscopy-guided biopsy tissues of NSCLC patients to establish xenograft models (15,16).

The absence of the interleukin-2 receptor -chain in mice leads to severe impairments in B-, T-, and natural killer cell development (17-19). NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tmlWjl}/SzJ mice (abbreviated NOD/LtSz-*scid Il2rg*$^{-/-}$ and often referred to as NSG mice) are a strain of immunodeficient *Il2rg*$^{-/-}$ mice. NSG mice are more susceptible to xenotransplanted lung cancer cell lines (20). Few studies have examined tumorigenicity in NSG mice using resected or biopsied tissues (14,15). Although one study reported tumorigenicity derived from MPE in rag2/IL2 knockout mice (21), the generation of mouse models utilizing MPE has not yet been established and remains in the trial and error stage.

The establishment of cell lines and the generation of cancer mouse models from MPE may lead to the development of personalized therapy. Based on this background, we attempted to establish cell lines and generate mouse models using MPEs that were no longer needed and should have been discarded. We present this article in accordance with the STROBE and ARRIVE reporting checklists (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-143/rc).

Highlight box

Key findings

- When malignant pleural effusions (MPE) from patients with advanced lung cancer were utilized, the cell line establishment rate was 18% and the engraftment rate in mice was 21%. The prognosis of patients who underwent cell line establishment and engraftment in mice was poor. Cell line establishment and tumorigenesis in mice were associated with a lower response to first-line therapy and poorer prognosis of patients.

What is known and what is new?

- The excessive MPE is generally discarded. Large-sample investigations of the methods and establishment rates of lung cancer cell lines derived from MPEs have rarely been reported.
- The rates of cell line establishment and tumor formation in mice from MPEs were shown. The relationship between these *in vitro* conditions and the clinical response to treatment and prognosis of patients were investigated.

What is the implication, and what should change now?

- MPEs that are no longer needed can be used effectively. The establishment of cell lines and the generation of cancer mouse models are linked to response to treatment and patients' prognosis and might have the potential of development of personalized medicine.

Methods

Patients and collections of MPE

This study was approved by the Institutional Review Board of Kagawa University (No. H27-037) in 2015 and was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from all individual patients. When thoracentesis was performed for diagnosis in patients with pleural effusion who were highly suspected of having lung cancer, an additional 50 mL pleural effusion was collected. In some patients who had already been pathologically confirmed to have lung cancer, 50 mL pleural effusion was collected when massive pleural effusions were drained as a treatment.

Establishment of lung cancer cell lines

Five milliliters of MPE were centrifuged and cell pellets

were cultured in 6-cm dishes in Roswell Park Memorial Institute (RPMI)-1640 supplemented with (I) 10% fetal bovine serum (FBS) or (II) 10% MPE supernatant from the same patient. Cells from 6 individuals were also cultured in other conditions including 100% MPE supernatant without RPMI-1640. The medium was replaced with fresh medium every 2 to 3 days. When sufficient cell growth was observed after two passages of culture, 100 to 10,000 cells were replaced into new culture dishes. If colony formation was observed, cells were pipetted from the colony under visualization using a microscope and transferred to a 96-well plate. Cells were further cultured until they reached 100% confluency, at which point they were detached with trypsin and transferred to larger dishes.

Animals and xenotransplantation

The non-obese diabetic-severe combined immunodeficiency (NOD-*scid*) and NSG mouse strains were purchased from the Jackson Laboratory Japan (Yokohama, Japan) and were maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University (Kagawa, Japan). The protocols of the animal experiments were approved by the Animal Care and Use Committee at Kagawa University (No. 18654) in 2015, in compliance with the Institutional Regulations for Animal Experiments for the care and use of animals. A protocol was prepared before the study without registration. Twenty milliliters of MPE were centrifuged, and cell pellets were frozen immediately after collection from patients. Cell pellets were thawed before one week of inoculation into mice and immediately incubated in RPMI-1640 supplemented with 10% FBS. After one week of incubation, cells were subcutaneously inoculated into 2 female mice (NOD-*scid* and NSG) when the mice were approximately 6 weeks of age; that is, cells derived from 10 mL MPE were inoculated into NOD-*scid* and NSG mice. The tumor sizes were measured every week with a caliper. The tumor volume was calculated by $1/2 \times A \times B^2$ (where A = length and B = width), as previously described (20). The criteria for successful engraftment were continuous nodule growth at the site of inoculation and tumor volumes greater than 10 mm^3. Mice were monitored for up to 12 weeks after inoculation, and then euthanized. The enlarged tumors were then resected and fixed with 10% phosphate-buffered formalin, and paraffin-embedded sections were stained with hematoxylin and eosin.

Statistical analysis

Progression-free survival (PFS) was defined as the time between the start of first-line therapy and disease progression or death. Overall survival (OS) was defined as the time between the date of diagnosis with lung cancer and date of death. PFS and OS curves were constructed with the Kaplan-Meier method, and differences in survival were assessed by the log-rank test. Fisher's exact test was used to analyze response rates to first-line therapy. All statistical analyses were conducted using Ekuseru-Toukei 2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Establishment of cell lines from MPE

Cells derived from the initial 6 MPEs were cultured in several conditions including 100% MPE supernatant and RPMI-1640 supplemented with 10% FBS. MPEs include many types of cells such as neutrophils, lymphocytes, histiocytes, and mesothelial cells, in addition to cancer cells. The appearance and cell proliferation differed depending on the culture medium (*Figure 1*). Cells that appeared to be histiocytes with a broad cytoplasm tended to be more prominent in culture media containing MPE supernatant. However, the proliferation of all types of cells gradually diminished in MPE supernatant-containing conditions in all 6 cases. Conversely, cells in 2 of 6 cases proliferated in RPMI-1640 supplemented with 10% FBS, and finally, cell lines were established (*Figure 1D,1H*). Therefore, only the two following conditions were adopted as the culture method: RPMI-1640 supplemented with 10% FBS or 10% MPE supernatant. MPEs were obtained from 28 lung cancer patients in total (*Table 1*). Of them, 23 were adenocarcinoma, 4 were SCLC, and one was NSCLC that was not otherwise determined. Cell lines were established from 5 patients (18%) (*Table 2*). One MPE (named PE17-05) led to the establishment of a cell line in RPMI-1640 with both 10% FBS and 10% MPE supernatant. The other 4 cell lines were established in RPMI-1640 with 10% FBS but not 10% MPE supernatant.

Tumorigenesis in mice by primary cells of MPE

Cells (cancer cells and other types of cells) in MPE were subcutaneously inoculated into two types of mice (NOD-

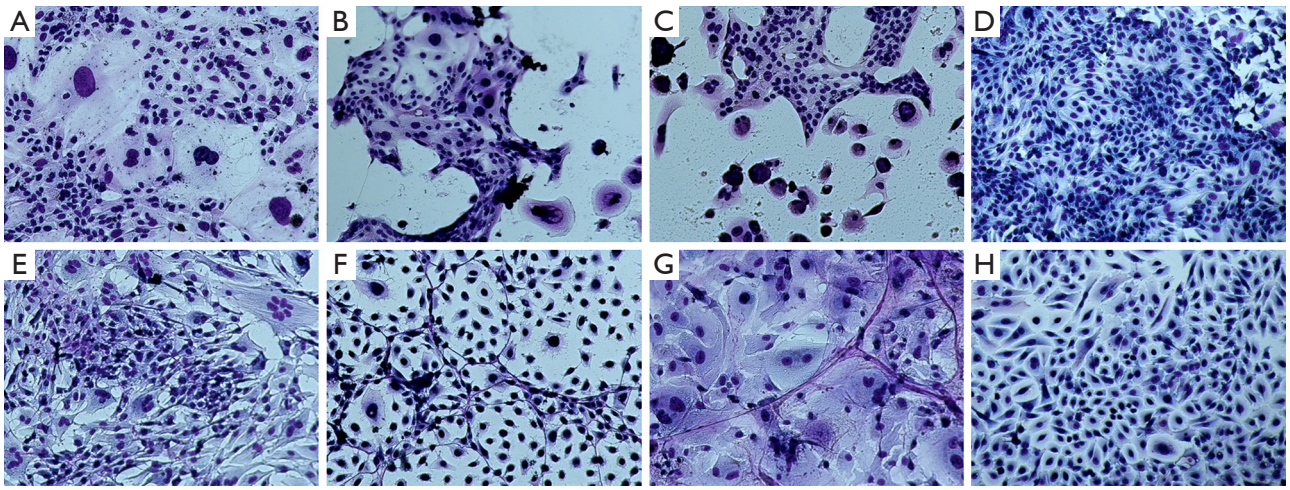

Figure 1 Culture conditions of cells derived from MPE. Photographs were taken after Diff-Quik staining (Sysmex Corporation, Kobe, Japan) (original magnification ×200). (A-D) PE15-01, (E-H) PE15-05, (A,E) culture in RPMI-1640 supplemented with 10% FBS, (B,F) culture in RPMI-1640 supplemented with 10% MPE supernatant, (C,G) culture in 100% MPE supernatant, and (D,H) cell lines established in RPMI-1640 supplemented with 10% FBS. MPE, malignant pleural effusion; RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum.

scid and NSG). The number of cells inoculated ranged from 400,000 to 4,300,000 cells per mouse. At just after inoculation with MPE-derived cells, mass formation is unrecognizable. The subsequent process of tumorigenesis varied from case to case, with the fastest case becoming recognizable after 2 weeks and the slowest after 12 weeks. The tumor subsequently increased over time. During the 12-week period, tumorigenesis was observed in 6 of 28 cases: 3 in both NOD-*scid* and NSG, 2 in only NSG, and 1 (PE17-05) in only NOD-*scid* mice (*Table 2*). PE15-05 obtained from a patient diagnosed with NSCLC showed tumorigenesis and the establishment of cell lines (*Figure 1E-1H* and *Figure 2*). Pathological findings of PE15-05-derived tumors formed in mice showed features of both squamous cell carcinoma and signet ring cell carcinoma (*Figure 2*). In PE17-08, cell line establishment was unsuccessful. However, tumorigenesis was observed in both NOD-*scid* and NSG mice. The pathological findings of tumors derived from PE17-08 showed small cell carcinoma which is the same as its clinical diagnosis (*Figure 3*).

Debilitating death of mice without tumorigenesis

PE17-05 was the only strain that showed successful tumor formation in NOD-*scid* mice but not in NSG mice. However, NSG mouse in which PE17-05 was inoculated progressively weakened, lost the hair and died within 3 weeks. Similarly, several other mice gradually weakened and died within 12 weeks. Thus, in 7 cases, the mice (7 NSG and 1 NOD-*scid* mice) died early without tumorigenesis. Furthermore, some mice were markedly debilitated, although they did not die within 12 weeks. The NSG mouse inoculated with PE18-06 also became progressively weaker and lost the hair (*Figure 4A*) at 12 weeks, which is the common visual finding in NSG mice that die early. The pathological findings of the skin showed a decrease in hair follicles, subcutaneous fibrosis, inflammatory cell infiltration, and lengthened hair roots, suggesting chronic inflammation (*Figure 4B*). In the liver, neutrophil-dominated inflammatory cell infiltration around the Gleason sheath, perivascular hepatocellular necrosis and mild fibrosis were observed (*Figure 4C*). These findings were consistent with graft-versus-host disease (GVHD). NOD-*scid* mouse inoculated with PE18-05 did not show signs of GVHD during the 12-week course (*Figure 4D*). Pathologically, the skin was normal (*Figure 4E*). However, inflammatory cell infiltration, which consisted mainly of lymphocytes, was observed around the Gleason sheath in the liver (*Figure 4F*).

Association of clinical signs of patients with establishment of cell lines or tumorigenicity

Twenty-five patients received first-line therapy, 17 received chemotherapy and 8 received epidermal growth factor

Table 1 Patient and tumor characteristics

No.	Malignant pleural effusion		Tumor characteristics					Patient characteristics					
	Name	Timing of collection	Histology	Stage at diagnosis of lung cancer	CYFRA (ng/mL)	CEA (ng/mL)	Driver mutation	Age (years)	Gender	1st line therapy		PFS (days)	OS (days)
										Agent	Response		
1	PE15-01	At PD	Ad	cT2aN3M1b (OSS, LYM, PUL, PLE), IVB	29	732	EGFR	62	F	TKI	PR	246	332
2	PE15-02	At PD	Ad	cT2aN3M1b (PUL, OSS), IVB	7.2	32.4		61	M	Chemo	PR	245	724
3	PE15-03	At LC diagnosis	Ad	cT2aN0M1b (PUL, OSS, BRA, PLE), IVB	74.8	393	EGFR	81	F	TKI	PD	5	18
4	PE15-04	At LC diagnosis	Ad	cTXN3M1b (OSS, PLE), IVB	8.2	29		63	M	Chemo	SD	129	177
5	PE15-05	At PD	NSCLC	cT1bN3M0, IIIB	6	3.8		66	M	Chemo	PD	52	89
6	PE15-06	At LC diagnosis	Ad	cT2aN3M1b (PLE, OTH), IVB	5.2	93.2		87	M	BSC	NA	NA	11+
7	PE16-01	At PD	Ad	cT4N1M0, IIIA	2.6	13.8	EGFR	74	F	Chemo	PR	198	3,195+
8	PE16-02	At LC diagnosis	Ad	cT3N3M1b (OSS, PLE, OTH), IVB	26.5	77.1		65	M	Chemo	PR	277	597
9	PE16-03	At LC diagnosis	Ad	cT3N3M1b (OSS, PLE, OTH), IVB	3.6	4.8		63	M	BSC	NA	NA	18
10	PE16-04	At PD	Ad	cT4N3M1b (OSS, LYM, PUL, BRA), IVB	7.7	17.3		58	F	Chemo	PR	228	486
11	PE16-05	At PD	Ad	cT2aN3M1b (BRA, PUL, OSS), IVB	22.7	90.4	EGFR	75	F	TKI	PR	472	983
12	PE16-06	At LC diagnosis	Ad	cT2aN0M1b (BRA, PLE), IVB	NE	NE		84	F	BSC	NA	NA	48
13	PE16-07	At LC diagnosis	Small	cT2aN3M1b (ADR, LYM, OSS, PLE, PER), IVB	8.4	16.2		69	M	Chemo	PR	154	237
14	PE16-09	At PD	Ad	cT2aN3M1a (PLE), IVA	9.8	149	ALK	54	F	Chemo	PR	186	1522
15	PE16-10	At LC diagnosis	Ad	cT4N3M1b (PLE, OSS, BRA, HEP, PER, LYM, ADR, OTH), IVB	130	318		61	M	Chemo	PD	62	105
16	PE17-01	At PD	Ad	cT4N3M1b (BRA, PLE, OTH), IVB	11	21		84	M	Chemo	PD	41	334
17	PE17-02	At PD	Small	cT2aN2M1a (PLE), IVA	6.3	160.5		83	M	Chemo	SD	191	243
18	PE17-03	At LC diagnosis	Ad	cT4N3M1a (PLE), IVA	10.9	30.5	EGFR	79	F	TKI	PR	49	476

Table 1 *(continued)*

Table 1 *(continued)*

No.	Malignant pleural effusion		Tumor characteristics					Patient characteristics					
	Name	Timing of collection	Histology	Stage at diagnosis of lung cancer	CYFRA (ng/mL)	CEA (ng/mL)	Driver mutation	Age (years)	Gender	1st line therapy		PFS (days)	OS (days)
										Agent	Response		
20	PE17-06	At LC diagnosis	Ad	cT4N2M1c (PUL, BRA, OSS, PLE), IVB	3.9	14.2		75	F	Chemo	SD	71	139
21	PE17-08	At LC diagnosis	Small	cT2aN3M1c (OSS, HEP, PLE), IVB	4.8	3		60	M	Chemo	PD	55	115
22	PE17-09	At LC diagnosis	Ad	cT4N3M1c (PUL, OSS, ADR, HEP, BRA, PLE), IVB	20.3	3.4	EGFR	78	F	TKI	PR	304	384
23	PE18-01	At PD	Ad	cT1aN2M1a (OTH), IVA	4.3	7.4	EGFR	49	F	TKI	PR	501	1,908+
24	PE18-02	At PD	Ad	cT2bN3M1a (PLE), IVA	4.7	12.6		77	M	Chemo	PR	197	826
25	PE18-03	At LC diagnosis	Ad	cT4N3M1c (PLE, LYM), IVB	29.6	109		48	M	Chemo	SD	124	370
26	PE18-04	At PD	Ad	pT2N2M0, IIIA	1	6.8	EGFR	70	F	TKI	PR	295	3,303
27	PE18-05	At PD	Small	cT3N3M1b (OSS), IVA	3.6	5.8		79	F	Chemo	PR	370	567
28	PE18-06	At LC diagnosis	Ad	cT2aN0M1a (PLE), IVA	1.8	134	EGFR	62	F	TKI	PR	133	1,506+

+ (plus) means non-fatal state. PD, progressive disease; LC, lung cancer; Ad, adenocarcinoma; NSCLC, non-small cell lung cancer; OSS, bone; LYM, lymph node; PUL, pulmonary; PLE, pleural; BRA, brain; OTH, others; PER, peritoneal; HEP, liver; ADR, adrenal; CYFRA, cytokeratin 19 fragment; CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; M, male; F, female; TKI, tyrosine kinase inhibitor; BSC, best supportive care; PR, partial response; SD, stable disease; NA, not applicable; PFS, progression-free survival; NE, not evaluated; OS, overall survival.

receptor (EGFR)-tyrosine kinase inhibitor (*Table 1*). The remaining 3 patients received the best supportive care. The response rate to first-line therapy was 60% (15 of 25 patients). Although the response to first-line therapy was not associated with the establishment of a cell line from MPE (P=0.27), first-line therapy was associated with tumorigenesis in mice (P=0.02). Patients whose MPEs were able to establish cell lines showed a tendency toward shorter PFS (*Figure 5A*, P=0.14), and shorter OS (*Figure 5B*, P=0.006) than patients whose MPEs failed to establish cell lines. Patients whose MPEs exhibited tumorigenesis in mice had shorter PFS (*Figure 5C*, P<0.001) and OS (*Figure 5D*, P<0.001) than patients whose MPEs failed to establish tumors.

Discussion

The current study showed that (I) cell lines could be established from MPEs in 5 of 28 patients (18%); (II) RPMI-1640 supplemented with 10% FBS was most suitable to establish cell lines among tested media including MPE supernatant-containing conditions; (III) tumorigenesis in mice utilizing MPE was observed in 6 cases (21%); (IV) some mice were debilitated and died within 12 weeks without tumorigenesis, and the appearance and histology were consistent with GVHD; and (V) cell line establishment and tumorigenesis in mice were associated with a lower response to first-line therapy and poorer prognosis.

The establishment of cell lines and tumorigenesis in mice would be useful to investigate the characteristics of each tumor and determine treatment strategies for individual patients. Even if a large amount of tumor volume is needed for genetic testing, sufficient quantities can be obtained from these proliferated cells or tumors without rebiopsy from the patient. Before administering anticancer drugs to

Table 2 Establishment of cell lines and tumorigenesis in mice

No.	Malignant pleural effusion	Establishment of cell line		Tumorigenesis using primary cells		Early death without tumorigenesis	
		RPMI-1640 + 10% FBS	RPMI-1640 + 10% MPE	NOD-*scid* mouse	NSG mouse	NOD-*scid* mouse	NSG mouse
1	PE15-01	Yes	–	–	–	–	Yes
2	PE15-02	–	–	–	–	–	Yes
3	PE15-03	–	–	–	–	–	–
4	PE15-04	–	–	–	–	–	–
5	PE15-05	Yes	–	Yes	Yes	–	–
6	PE15-06	–	–	–	–	–	–
7	PE16-01	–	–	–	–	–	Yes
8	PE16-02	–	–	–	–	–	–
9	PE16-03	Yes	–	-	Yes	–	Yes
10	PE16-04	–	–	–	–	–	–
11	PE16-05	–	–	–	–	–	Yes
12	PE16-06	–	–	–	Yes	–	-
13	PE16-07	–	–	–	–	Yes	Yes
14	PE16-09	–	–	–	–	–	–
15	PE16-10	Yes	-	Yes	Yes	–	–
16	PE17-01	–	–	–	–	–	–
17	PE17-02	–	–	–	–	–	–
18	PE17-03	–	–	–	–	–	–
19	PE17-05	Yes	Yes	Yes	–	–	Yes
20	PE17-06	–	–	–	–	–	–
21	PE17-08	–	–	Yes	Yes	–	–
22	PE17-09	–	–	–	–	–	–
23	PE18-01	–	–	–	–	–	–
24	PE18-02	–	–	–	–	–	–
25	PE18-03	–	–	–	–	–	–
26	PE18-04	–	–	–	–	–	–
27	PE18-05	–	–	–	–	–	–
28	PE18-06	–	–	–	–	–	–

RPMI, Roswell Park Memorial Institute; FBS, fetal bovine serum; MPE, malignant pleural effusion; NOD-*scid*, non-obese diabetic-severe combined immunodeficiency; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ.

the patient, drug sensitivity can be examined to estimate the most effective drug. A report attempted to determine drug sensitivity using primary cells obtained from MPEs (21). Although this strategy likely does not yield accurate results due to the mixture of multiple cell types, the fact that it can be performed in most cases at an early stage is a great advantage. Conversely, the establishment of cell lines takes a long time, and these cells might no longer faithfully

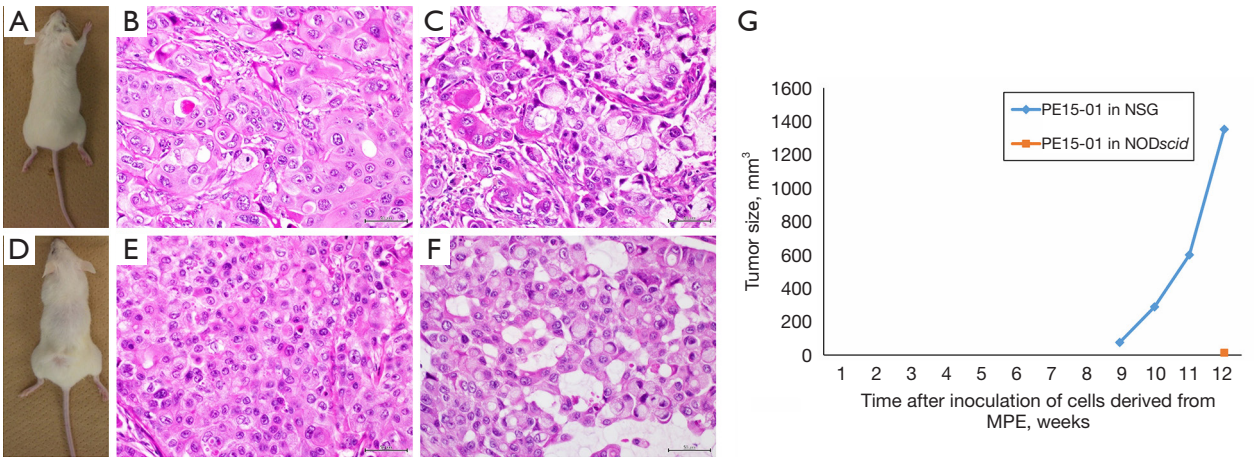

Figure 2 Xenograft mouse models derived from PE15-05. (A-C) NOD-*scid* mouse, (D-F) NSG mouse, (A,D) appearance of mice at 12 weeks after inoculation; tumor diameters were (A) 3 mm and (D) 13 mm, (B,E) histology suggesting squamous cell carcinoma which was observed mainly, and (C,F) histology suggesting signet ring cell carcinoma which was observed partly (hematoxylin and eosin staining). Scale bars indicate 50 µm. (G) Time courses of tumor growth. NOD-*scid*, non-obese diabetic-severe combined immunodeficiency; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tmlWjl}/SzJ; MPE, malignant pleural effusion.

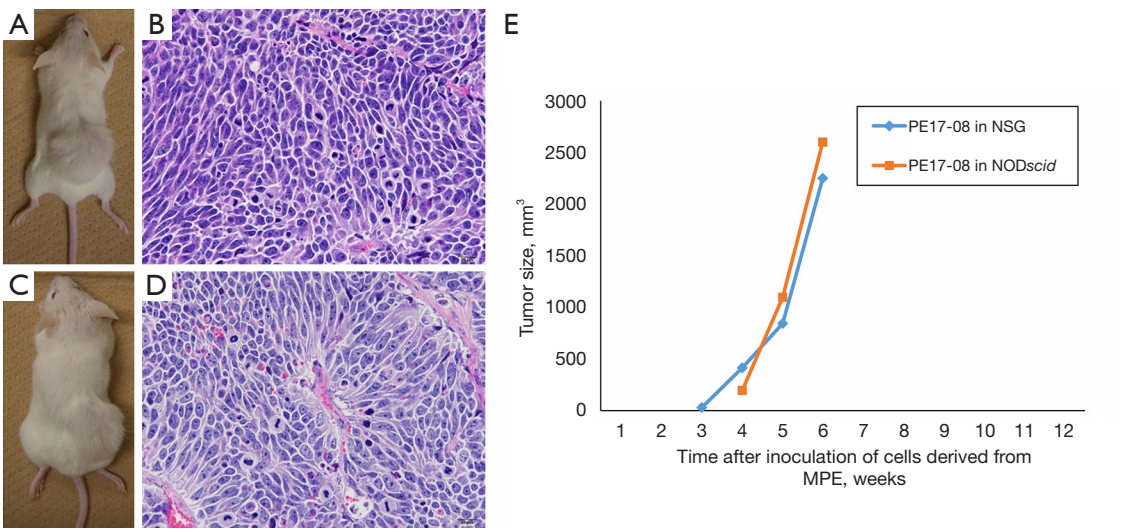

Figure 3 Xenograft mouse models derived from PE17-08. (A,B) NOD-*scid* mouse, (C,D) NSG mouse, (A,C) appearance of mice at 6 weeks after inoculation; tumor diameters were (A) 18 mm and (C) 20 mm, (B,D) histology suggesting small cell carcinoma (hematoxylin and eosin staining). Scale bars indicate 20 µm. (E) Time courses of tumor growth. At 6 weeks, mice were sacrificed. NOD-*scid*, non-obese diabetic-severe combined immunodeficiency; NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tmlWjl}/SzJ; MPE, malignant pleural effusion.

represent the molecular heterogeneity of primary patient tumors once established (22).

Regarding PDX mouse models, resected tumor fragments were subcutaneously xenografted into CD1 nude and CB17-*scid* mice within 24 hours post-surgery (11). The tumor formation rate was 35%, with higher rates for squamous cell carcinoma (60%) than for adenocarcinoma (13%). In other studies in which surgically resected NSCLC tumors were implanted into NOD-*scid* mice, engraftment rates ranged from 18% to 42% (8-10,12,13). In a study utilizing bronchoscopy-guided biopsy tissues, 30 (26.3%) of 114 NSCLC PDX models were successfully generated (16).

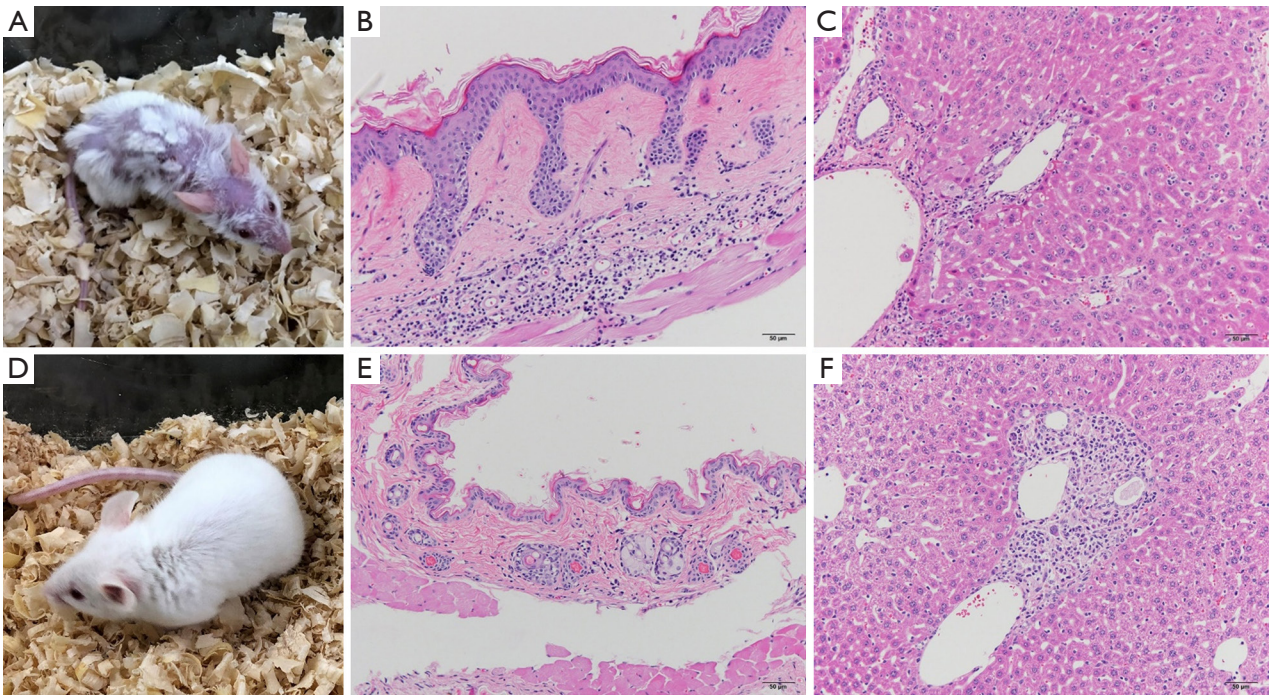

Figure 4 Appearance of mice at 12 weeks after inoculation and pathological findings consistent with graft-versus-host disease. (A-C) NSG mouse that PE18-06 was inoculated, (D-F) NOD-*scid* mouse that PE18-05 was inoculated, (B,E) histology of skin, (C,F) histology of liver (hematoxylin and eosin staining). Scale bars indicate 50 μm. NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tmlWjl}/SzJ; NOD-*scid*, non-obese diabetic-severe combined immunodeficiency.

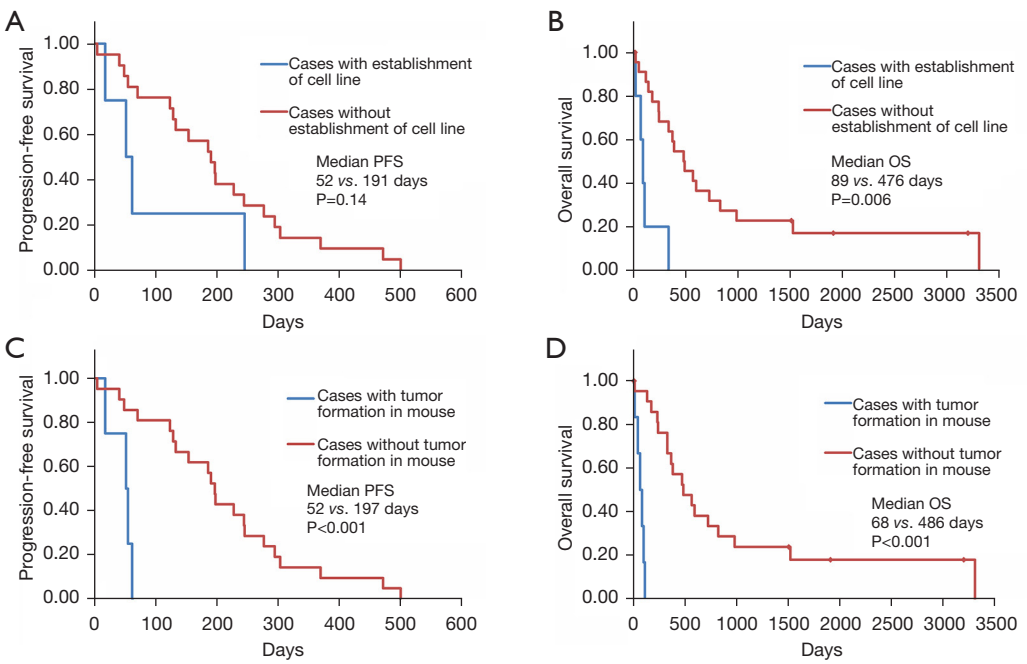

Figure 5 Kaplan-Meier curves of (A,C) progression-free survival and (B,D) overall survival in patients according to (A,B) establishment of cell line and (C,D) tumorigenesis in mouse using with malignant pleural effusion. PFS, progression-free survival; OS, overall survival.

In the current study, tumorigenesis in mice utilizing MPE was observed in 21% of cases. The difference in tumor formation rates was not solely due to whether the specimens were MPEs or resected specimens but also likely due to differences in the storage conditions of specimens prior to use, culture and inoculation methods, type of mouse, and tumor biological characteristics. The freeze-thawing performed in the current study clearly reduces the cell viability and increases dead cells. NSG mice are superior to NOD-*scid* mice in generating mouse models of lung cancer by cell lines (20). The current study also showed that NSG mice had a tendency toward a higher rate of tumorigenesis than NOD-*scid* mice. Roscilli *et al.* optimized isolation procedures and culture conditions to expand primary cultures from MPEs and increased the rate of tumorigenesis (10 of 16 cases) in rag2/IL2 knock-out mice (21).

The association of PDX engraftment with patient prognosis has yielded consistent results. Successful PDX engraftment obtained by surgical resection was associated with worse disease-free survival and OS compared with non-PDX-engraftment patients (11,12). The current study showed that cell line establishment and tumorigenesis in mice using MPE were also associated with a lower response to first-line therapy and poorer prognosis. Similarly, the survival rates of patients corresponding to the successful establishment of MPE-derived PDXs were lower (7). Regardless of the type of PDX, poorly differentiated, i.e., more malignant cancer cells, likely lead to the establishment of cell lines and engraftment in mice. EGFR- or ALK-positive lung adenocarcinomas are generally not poorly differentiated. In the 10 individuals with this driver gene mutation, the cell line was established in only one case (10%), and none formed tumors in mice.

NOD-*scid* mice have B- and T-cell dysfunction, but unlike NSG mice, NK cell activity is preserved in NOD-*scid* mice. Due to this severe combined immunodeficiency, tumorigenesis is overwhelmingly higher in NSG mice than in NOD-*scid* mice (20). However, the present study showed early death among mice without tumor growth and abnormal visual and pathological findings of the skin and liver consistent with GVHD, which is more frequent in NSG mice. Why was GVHD more common in NSG mice than in NOD-*scid* mice? Few studies report GVHD in severely immunodeficient mice xenotransplanted from lymphodominant tumor xenografts (23). This type of GVHD is reportedly due to engraftment and the expansion of primary graft-originated tumor-infiltrating lymphocytes (TILs) in the animal body (23). Several solutions have been suggested to prevent GVHD in PDX models of solid tumors: use of the NSG-2m^{null} strain, generation of humanized models, blocking of IL-21 signaling, azacytidine therapy, and lymphodepletion of the tumor xenograft (23). In the cases of transplanted xenografts without a high tumor cell ratio, such as MPE, the NK cell activity of NOD-*scid* mice might be beneficial to eliminate many types of cells that are detrimental to the survival of the mice and to suppress the development of GVHD. However, using NOD-*scid* mice instead of NSG mice is not a fundamental solution, and the use of NSG mice with a higher cancer cell ratio is desired based on the methods described above.

For cells living in MPE, MPE seems to be a more suitable environment for survival than blood. However, in the current study, despite the use of MPE-derived cancer cells, FBS was more useful to establish cell lines than MPE supernatant. In the microenvironment of MPEs, cancer cells have complex interactions with non-cancer cells, and various cytokines and growth factors are continuously secreted by non-cancer cells. For example, the population of Th22 cells is increased by cytokines and chemokines such as CCL20-CCR6, and the produced IL-22 promotes the proliferation of lung cancer cell lines (24). In the *in vitro* experiments performed in the current study, MPE supernatant was replaced periodically, but new non-cancer cells were not supplied. The lack of reproduction in the true MPE environment might be the reason why cell line establishment is not always successful, even with fresh MPE supernatant.

The limitations of this study include the following. First, some aspects of the management of MPE can be improved, such as inoculation methods into mice, to increase the engraftment rate. This strategy includes removing non-cancer cells, such as TILs, as much as possible, not cryopreserving the cells, increasing the cell number for inoculation, using Matrigel, etc. We injected unselected cell populations into mice. We chose the simplest possible method for inoculating MPE-derived cells. We took into consideration the fact that it is not easy to completely remove all non-tumor cells in a short culture period, that some tumor cells in pleural effusion have a tendency to float, and that we wanted to reduce the amount of labor involved. Second, some mice could not be evaluated for PDX engraftment due to early death. Third, the details of the origin of inflammatory cell infiltration consistent with GVHD have not been evaluated. Fourth, the number of mice used was two per each MPE sample. The small number of mice could have resulted in inaccurate results.

Although tumorigenesis was observed in 6 of 28 cases, it is conceivable that this probability would increase if more mice were used. Fifth, the differentiation of adenocarcinoma has not been evaluated.

Conclusions

When MPEs were used as PDXs, the cell line establishment rate was 18%, and the successful engraftment rate in mice was 21%. The methods can be improved to increase the successful PDX engraftment rate and prevent the development of GVHD. The prognosis of patients whose MPEs were able to establish cell lines and engraft in mice was poor.

Acknowledgments

Funding: None.

Footnote

Reporting Checklist: The authors have completed the STROBE and ARRIVE reporting checklists. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-143/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-143/dss

Peer Review File: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-143/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-24-143/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki as revised in 2013. The study was approved by the Institutional Review Board of Kagawa University (No. H27-037) and informed consent was obtained from all individual patients. Experiments were performed under a project license (No. 18654) approved by the Animal Care and Use Committee at Kagawa University, in compliance with the Institutional Regulations for Animal Experiments for the care and use of animals.

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Cite this article as: Kanaji N, Yokohira M, Inoue T, Watanabe N, Mizoguchi H, Komori Y, Kawada K, Kadowaki N. Establishment of lung cancer cell lines and tumorigenesis in mice from malignant pleural effusion in patients with lung cancer. Transl Lung Cancer Res 2024;13(9):2340-2351. doi: 10.21037/tlcr-24-143

