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ORIGINAL PAPER

Molecular profiles of EGFR, K-ras, c-met, and FGFR in pulmonary pleomorphic carcinoma, a rare lung malignancy

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Abstract
Background Pulmonary pleomorphic carcinoma (PPC) is a rare type of lung cancer characterized by the poor response to conventional chemotherapy and subsequent disappointing outcomes. Therefore, it is paramount to delineate the molecular characteristics of this disease entity.
Methods In this study, we retrospectively examined the surgical specimens of 61 patients who underwent lung surgery. Mutational or gene amplification statuses of epidermal growth factor receptor (EGFR), *k-ras*, *c-kit*, *c-met*, and fibroblast growth factor receptor (FGFR) were examined using genomic DNA sequencing, real-time PCR and/or fluorescence in situ hybridization (FISH).
Results The median age was 61 years, and 50 patients were men and 11 were women. In the histologic review of epithelial component, adenocarcinoma were in 44 cases (72%), squamous cell carcinoma in 15 (25%) and large cell carcinoma in 2 patients (3%). Overall, 30 cases (49%) had any molecular alterations. Nine patients (15%) possessed EGFR deletion in exon 19 ($n = 8$) or L858R mutations in exon 21 ($n = 1$), while 3 other cases having atypical EGFR mutations. Six patients (9.8%) had *k-ras* mutations in exon 12, and 3 had *c-kit* mutations. High gene copy number of *c-met* was found in 11 patients (18.0%) and that of FGFR was in 6 patients (9.8%). No significant relationships were identified among the occurrence and type of mutations and patient survival or any other clinicopathological variables.
Conclusions Given the diverse repertoire of mutational profiles observed in PPC samples, clinical trials based on accurate cancer-genotyping should be considered as a legitimate treatment scheme for this rare disease entity in the future.

Keywords Lung cancer · Pulmonary pleomorphic carcinoma · EGFR · k-ras · c-kit · c-met · FGFR

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Background

Pulmonary pleomorphic carcinoma (PPC) is a rare pulmonary epithelial malignant tumor that represents 0.1–0.4% of all malignant tumors of the lung (Ito et al. 2010; Pelosi et al. 2010). According to the World Health Organization's classification of lung tumors, PPC is a group of poorly differentiated tumors characterized pathologically by a combination of epithelial and mesenchymal elements and is defined as either non-small-cell carcinoma combined with at least 10% of neoplastic spindle or giant cell (Beasley et al. 2005).

The clinical characteristics and the behaviors of these tumors have not been well known due to a small series of data. In general, however, PPC has been reported to occur predominantly in men who smoke heavily, with an average age at diagnosis of 60 years, and is characterized by the poor response to conventional chemotherapy and subsequent disappointing clinical outcomes (Bae et al. 2007; Fishback et al. 1994; Hong et al. 2009; Mochizuki et al. 2008; Rossi et al. 2003).

Compared with other common epithelial cancers, current knowledge about the biology of PPC is very limited, and as a result, little is known about its molecular pathology. Some genetic alterations appear frequently in non-small-cell lung cancer and drugs targeting these genetic pathways are clinically available. Some selected genes, such as epidermal growth factor receptor (EGFR), *k-ras, p53*, etc., have been analyzed in small cohorts of PPC patients (Arita et al. 2005; Blaukovitsch et al. 2006; Italiano et al. 2009; Kaira et al. 2010; Pelosi et al. 2004; Ushiki et al. 2009). A previous study reported that EGFR mutation was positive in about 20% of PPC, which give some chance to apply target agents to PPC (Kaira et al. 2010). To date, however, extensive molecular researches on PPC have been largely hampered by the rarity of the tumor and a lack of established cell lines and animal models.

To gain more extensive insights into the oncogenic nature of PPC, we have characterized the mutational or amplification status of several well-known oncogenic target genes, such as EGFR mutation, *K-ras* mutation, c-kit mutation, c-met amplification, and fibroblast growth factor receptor (FGFR) amplification in 61 surgically resected PPC samples. The target genes were selected based on the possibility that these are the most frequent genes in NSCLC and are correlated with clinical outcomes of available target agents (Han et al. 2006; Heinrich et al. 2002; Ma et al. 2003; Noble et al. 2004).

Materials and methods

Patient samples

Tumors specimens from 61 patients with PPC who underwent surgical resection at Samsung Medical Center from January 1995 to March 2009 were identified. All tumors were retrieved from paraffin-embedded blocks. All slides from the lung resection specimens were reviewed by two expert pathologists (JHH and YLC). In all cases, the diagnosis of PPC was established according to the 2004 World Health Organization's classification of tumors. Clinical staging was determined according to the 6th International Association for the Study of Lung Cancer.

Biomarker analysis

Biomarker analyses were retrospectively performed on formalin fixed paraffin-embedded (FFPE) archival tumor tissues after pathologic review. Sections from tissue blocks containing more than 80% representative malignant cells were used for all analyses.

The mutational analyses of EGFR (exon 18–21), *k-ras* (exon 2, 3), and *c-kit* (exon 9, 11, 13, and 17) were performed using directional sequencing of PCR fragments amplified from genomic DNA. The results were marked as mutation-positive if a mutation was detected in both the forward and reverse DNA strand.

Gene copy numbers of *c-met* per cell were determined using fluorescence in situ hybridization (FISH) with the Kreathech Poseidon MET/SE 17-probe kit. Copy numbers of FGFR were determined using bacterial artificial chromosome clones #669(10q26.13) and #493(10q26.13) containing $FGFR_2$ (Macrogen, Seoul, Korea) labeled with rhodamine fluorescent dye. The centromere clones labeled with FITC fluorescent dye were #5331, #5344, and #46 (10q12.1). Briefly, for FISH procedures, 4-μm-thick sections of FFPE tissue blocks were deparaffinized, dehydrated, and immersed in 0.2N HCl, and then boiled in a microwave in citrate buffer (pH 6.0). The probe mixtures were added to the slides, and then the slides were incubated in a humidified atmosphere at 73°C for 5 min to denature the probe and target DNA, after which they were sequentially cooled and incubated at 37°C for 19 h to allow for hybridization. The slides were then washed with 0.4 × SSc/0.3% NP-40 for 2 min at room temperature, followed by 2 × SSc/0.1% NP-40 for 5 min at 73°C. For the nuclei counterstaining, 4,6-diamidini-2-phenylindone (DAPI) and anti-face compound (p-phenylenediamine) were added. Signals for each probe were evaluated under a microscope equipped with a triple-pass filter (DAPI/Green/Orange; Vysis). At least 100 tumor cell nuclei were counted per case. FISH analysis was performed independently by two pathologists who were blinded to the patients' clinical characteristics and to all other molecular variables.

High gene copy number was defined as high polysomy (≥cutoff copies in >40% of cells using Colorado system) or gene amplification (presence of tight gene clusters; a gene: chromosome ratio per cell ≥2 or ≥15 copies of target probes per cell in ≥10% of cells analyzed).

All biomarkers were independently determined with blinding to clinical outcome prior to any statistical analysis.

Statistical methods

All patients were included in the statistical calculations. Follow-up was obtained in all cases and was ended on

November 1, 2009. Categorical variables were compared using the Chi-squared test. The overall survival (OS) was the interval from lung surgery to death or the last follow-up. Survival was assessed using the Kaplan–Meier method. Results were considered significant at the 0.05 level. Statistical analyses were performed using the SPSS software program, version 18.0.

Results

Patient characteristics and survival

A total of 61 PPC specimens were available, and the clinical characteristics of patients are described in Table 1. The median age was 61.4 years (range, 36.0–85.0 years), and the male ($n = 50$)/female ($n = 11$) ratio was 5:1. Thirteen patients were current smokers, 27 patients were ex-smokers, and 21 had never been smokers. Tumor size ranged between 14 and 153 mm (median, 46 mm).

Table 1 Patients' characteristics ($N = 61$)

	Number of patients (%)
Median age, years (range)	61.4 (36.0–85.0)
Sex	
Male	50 (82.0)
Female	11 (18.0)
Smoking status	
Never smoker	21 (34.4)
Ex-smoker	27 (44.2)
Current smoker	13 (21.3)
Performance status (ECOG)	
0	29 (47.5)
1	29 (47.5)
2	3 (4.9)
Adjuvant chemotherapy	14 (23.0)
Adjuvant radiotherapy	10 (16.4)
Surgery type	
Pneumonectomy	5 (8.2)
Bilobectomy	10 (16.4)
Lobectomy	43 (70.5)
Others	3 (4.9)
Stage	
I	26 (41.1)
II	19 (31.1)
III	13 (21.3)
IV	3 (4.9)
Recurrence	
Yes	22 (36.1)
No	39 (43.9)
Median follow-up (months)	63.5 (8.0–165.6)
OS (months)	33.9 (0.0–69.3)

All patients underwent curative resection except three patients who received palliative surgery for metastatic disease. Pathological stages were stage I in 26 patients (41.0%), stage II in 19 (31.1%), stage III in 13 (21.3%), and stage IV in 3 patients. The median survival time was 33.9 months (95% CI, 0.0–69.3 months).

Of the 61 patients, 22 (36%) relapsed after surgery with 16 recurrences in distant sites. The other 39 patients (63.9%) experienced no recurrence at the time of analysis, including 11 patients who died of non-cancer-related disease (pneumonia, heart disease, etc.).

Among the various clinical parameters, univariate analyses indicated that early disease stage ($P < 0.001$), good ECOG performance status ($P < 0.001$), and receipt of adjuvant radiotherapy ($P = 0.012$) or palliative chemotherapy ($P < 0.001$) were related with significantly increased OS. The multivariate analysis showed that clinical parameters including stage, ECOG performance status, and receipt of palliative chemotherapy were independent prognostic factors in terms of OS (Table 2).

Epithelial/mesenchymal components and survival

In the histologic review of malignant epithelial components, adenocarcinoma were in 44 cases (72%), squamous cell carcinoma in 15 (25%), and large cell carcinoma in 2 patients (3%). For the mesenchymal components, 44 cases (72%) had spindle cell tumors and 17 (28%) had giant cell tumors. Therefore, 5 subgroups were categorized according to the epithelial/mesenchymal component: adenocarcinoma/spindle cell tumors were in 29 cases (48%), adenocarcinoma/giant cell tumors in 15 (25%), squamous cell carcinoma/spindle cell tumors in 13 (21%), squamous cell carcinoma/giant cell tumors in 2 (3%), and large cell carcinoma/spindle cell tumors in 2 cases (3%).

The median OS in patients with adenocarcinoma ($n = 44$) and non-adenocarcinoma ($n = 17$) component were 30.2 months (95% CI, 0.0–70.8 months) and 33.9 month (95% CI, 13.2–54.6 months), respectively, and there was no statistical difference ($P = 0.86$). Patients with spindle cell tumor component ($n = 44$) showed longer median OS (38.9 months: 95% CI cannot be calculated) than those with giant cell tumor component (24.7 months: 95% CI, 5.9–43.5 months), but the difference was short of the statistical significance ($P = 0.37$). In the analyses of 5 subgroups categorized by the epithelial/mesenchymal component, there was no significant survival difference among 5 subgroups (data not shown).

Table 2 Univariate and multivariate analysis for overall survival

Variables	Univariate analysis			Multivariate analysis		
	Survival (mon)	95% CI	*P*-value	Survival (mon)	95% CI	*P*-value
Age						
<61.4	NA	NA	0.157			
≥61.4	30.2	9.3–51.2				
Sex						
Male	38.9	7.8–70.1	0.462			
Female	18.0	1.6–34.4				
Smoking						
Never smoker	18.0	0.0–37.0	0.163			
Ex-smoker	NA	NA				
Current smoker	38.9	0.0–84.8				
Performance status (ECOG)						
0	NA	NA	<0.001	2.586	1.304–5.128	0.007
1	24.7	13.5–35.9				
2	2.4	1.9–2.8				
Adjuvant chemotherapy						
No	30.2	14.4–46.1	0.642			
Yes	NA	NA				
Adjuvant radiotherapy						
No	94.7	14.2–175.1	0.012			
Yes	9.5	4.1–14.9				
Surgery						
Pneumonectomy	18.1	0.0–43.1	0.218			
Bilobectomy	12.0	0.0–27.7				
Lobectomy	94.7	12.2–177.1				
Others	NA	NA				
Stage						
1	NA	NA	<0.001	1.577	1.100–2.261	0.013
2	33.9	17.9–49.9				
3	7.8	0.0–15.5				
4	8.9	4.9–12.9				
Recurrence						
No	94.7	NA	<0.001			
Yes	12.0	2.3–21.7				
Palliative chemotherapy						
No	94.7	0.9–188.5	<0.001	0.393	0.173–0.859	0.020
Yes	9.5	3.9–15.2				

Molecular profiles and survival

Of the 61 clinical tumor specimens profiled, 30 cases contained any genetic mutations or increased gene dosages. The molecular profiles, combined with the histologic review of epithelial/mesenchymal components and smoking status, of these 30 cases were shown in Table 3.

Somatic EGFR mutations were found in 12 out of 61 patients (19.6%). Nine patients (14.8%) possessed well-established activating EGFR mutation signatures, with exon 19 partial deletions in 8 patients and an exon 21 L858R substitution mutation in one patient. The other three mutations resided in either exon 20 or exon 21, the targetabilities by clinically available EGFR inhibitors of which are currently not well established. From the profiling analysis of the tumor specimens, we identified 6 patients who possessed *k-ras* mutations. A heterozygous G to T substitution at nucleotide position 34 (Gly to Cys) was

Table 3 Molecular profiles in PPCs ($N = 30$)

Sample No	EGFR	KRAS	c-kit	c-met	FGFR
2				High polysomy	
4				High polysomy	
7	Exon 19 c.2240-2257del		Exon 9 p.Thr488Ala		
8	Exon 19 c.2240-2257del				
9				High polysomy	
11	Exon 21 p.Leu858Arg				
15	Exon 19c.2240-2257del				
	Exon 21 c.2561insC		High polysomy		
16				High polysomy	
17				High polysomy	
21				High polysomy	
25		c.35G > T;p.Gly12Val			
26	Exon 20 p.Arg776Cys			High polysomy	
28		c.34G > T;p.Gly12Cys			
32		c.34G > T;p.Gly12Cys			
34	Exon 20 c.2370_2371insAG, c.2298G > C(p.Ala800Pro)				
36	Exon 19 c.2240-2257del	c.35G > A;p.Gly12Asp			+
37	Exon 19 c.2240-2257del				
38				High polysomy	
40				High polysomy	
41		c.35G > C;p.Gly12Ala			
42	Exon 19 c.2240-2257del		Exon 9 p.Pro468Leu, p.Val489Asp		+
46					+
47	Exon 19 c.2240-2257del		Exon 9 p.Phe483Leu,p.Cys491Arg		
50	Exon 19 c.2240-2257del				
51					+
52		c.34G > T;p.Gly12Cys			
55	Exon 20 p.Ser768_Asp770dupSerValAsp				
58				Amplification	
59					+
60					+

found in 3 patients. The other three mutations also corresponded to the *k-ras* amino acid 12 substitution mutation, including a heterozygous G to T substitution at nucleotide position 35 (Gly to Val), a G to A substitution at 35 (Gly to Asp), and a G to C substitution at 35 (Gly to Ala). Interestingly, one patient (patient number 36) with k-ras mutation (c.35G>;p Gly12Ala) also had EGFR mutation (exon 19 deletion). In the analysis of *c-kit*, we identified 3 patients who possessed *c-kit* mutations.

Further, we analyzed the gene dosage statuses of FGFR and *c-met* using fluorescence in situ hybridization (FISH). The median gene dosage of FGFR was 1.06 (range, 0.33–5.76), and, with the cutoff value set at 2 points, six patients showed increased FGFR gene dosage (Fig. 1). In the case of *c-met*, 11 patients demonstrated increased gene dosage; *c-met* amplification was observed in one patient and high polysomy was seen in 10 patients (Fig. 2). There was no statistically significant correlation between high gene copy number of the *c-met* gene and patient survival.

There were no statistically significant correlations between the type of gene mutations and overall survival (Table 4).

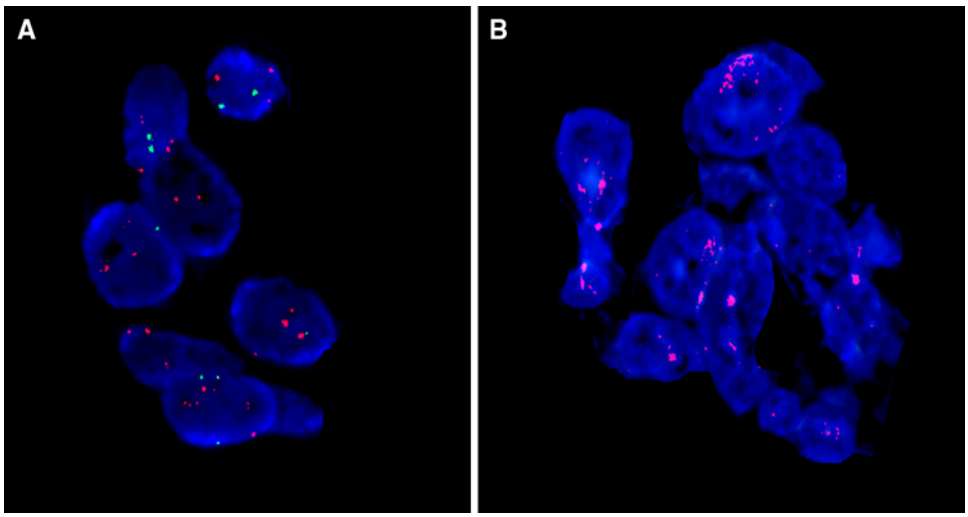

Fig. 1 Fluorescence in situ hybridization (FISH) assay for the detection of the FGFR **a** No amplification of the FGFR **b** Amplification of the FGFR

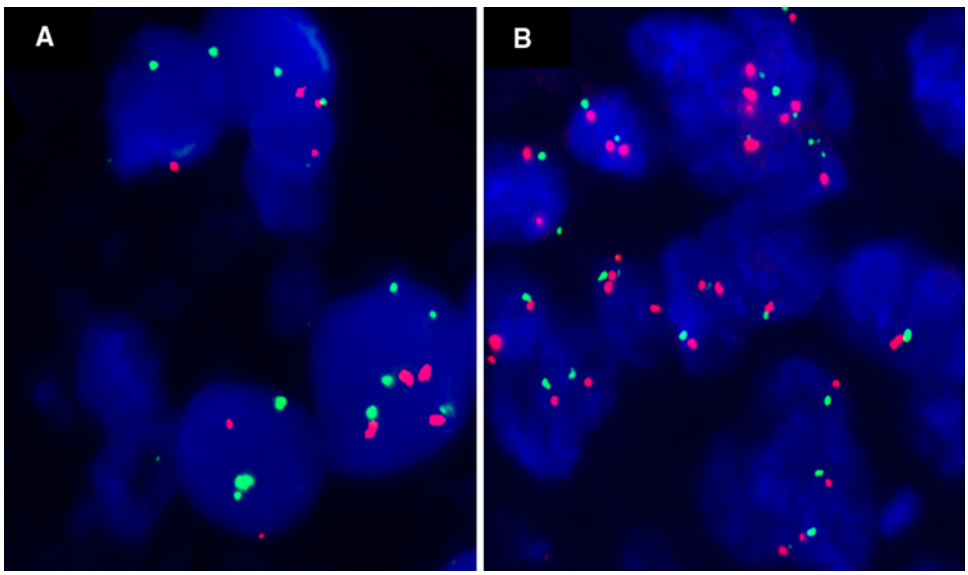

Fig. 2 FISH assay for the detection of c-Met **a** No amplification of the c-Met **b** High polysomy of the c-met

The distribution of activating EGFR mutation in association with clinicopathologic factors

Of 44 tumors with adenocarcinoma components, 6 (13.6%) had activating EGFR mutations, and 3 (17.6%) of 17 tumors with non-adenocarcinoma component had activating EGFR mutations. Therefore, the distribution of activating EGFR mutation was not associated with the type of malignant epithelial components ($P = 0.69$). The distribution of activating EGFR mutation was also insignificantly associated with other clinicopathologic factors, such as gender (all 9 in men, $P = 0.13$), smoking status (never- vs ever-smoker, $P = 0.94$), and the type of mesenchymal malignant components (spindle cell vs giant cell, $P = 0.23$).

Discussion

PPC is a rare aggressive disease characterized by a high rate of distant recurrence after initial surgery for localized disease (Hong et al. 2009). There is presently no available data indicating the optimal management of patients with this advanced disease. In particular, the role of chemotherapy remains unclear, and these patients are often treated according to the results of trials of enrolled patients with NSCLC, such as squamous cell carcinoma or adenocarcinoma. Many targeted agents are widely used for the treatment of patients with advanced NSCLC. However, whether these drugs have some activity in PPC is not yet clear.

EGFR, K-ras, c-met, and FGFR are receptor tyrosine kinases (RTKs) frequently activated by gene amplification

Table 4 Association of EGFR, KRAS, c-kit, C-met, and FGFR mutations with overall survival using univariate analysis

Molecular markers	Univariate analysis		
	Survival (months)	95% CI	*P*-value
EGFR			
No	30.2	0.0–70.1	0.879
Yes	33.9	NA	
k-ras			
No	33.9	0.0–71.4	0.348
Yes	10.1	0.0–26.8	
c-kit			
No	30.2	13.6–46.8	0.575
Yes	NA	NA	
FGFR			
No	33.9	0.0–72.3	0.744
Yes	10.1	NA	
c-Met			
No	26.0	9.2–42.8	0.374
Yes	94.7	12.0–177.3	

or mutation in a variety of human neoplasm especially lung cancer. EGRF and K-ras have been already well-known target genes in NSCLC. The occurrence of MET amplification and EGFR/K-ras mutation might be mutually exclusive suggesting several distinct mechanisms in the development of NSCLC (Onitsuka et al. 2010). FGFR-mediated signals in cooperation with those transmitted by EGFR are involved in growth and survival of human NSCLC cells and should be considered as targets for combined therapeutic approaches (Fischer et al. 2008). Various RTKs have come into the focus of therapeutic considerations since they can be targeted by pharmaceutical intervention and because tumors bearing oncogenic RTK mutations generally show a good response to targeted therapy.

To gain further insight into the clinical targetabilities of PPCs, we performed a molecular analysis of 61 resected PPCs. Previous studies have analyzed only a small number of tumors and have mostly focused on histological and clinical characteristics of this rare disease entity. Thus, this study is one of the first attempts to genetically characterize PPCs, with the underlying premise of rapidly extending this knowledge to clinical oncology practices. Several characteristics of PPCs emerged from these analyses. Mutational analysis of PPCs demonstrated that about half of the patients possess one or multiple combinations of the above five genetic abnormalities. The activating EGFR mutations were seen in 9 patients (14.8%) (19.8%, including 3 atypical mutations), whereas *k-ras* oncogenic mutations were identified in six patients (9.8%); the EGFR positive rates is largely consistent with that observed for other lung cancers in East Asians (Ahn et al. 2008), and with the recently evaluated value (18%, 3 out of 17) in Japanese PPC patients (Kaira et al. 2010). However, the mutation rate found here is in contrast to results of Italiano et al., who reported that PPCs are characterized by a lack of EGFR mutation and a high rate of *k-ras* mutations (Italiano et al. 2009; Pelosi et al. 2004). It is well established that ethnic differences heavily affect EGFR etiology in lung cancer, and PPCs might be under similar ethnic influences in their ontogeny. In the present study, however, the distribution of EGFR mutation was not in line with the well-established phenomena. Namely, the EGFR mutations were detected, irrespective of clinicopathologic factors such as the component of adenocarcinoma, smoking status, and gender. Although this distribution of EGFR mutation can be explained in part by the specific characteristics of PPC, it should be cautious to interpret these data due to the small number of samples.

In the case of *c-met*, 11 patients demonstrated increased gene dosage; a *c-met* amplification and 10 high polysomies were detected using FISH. The *c-met* receptor tyrosine kinase has been associated with NSCLC as well as with SCLC (Chang et al. 2001; Jagadeeswaran et al. 2007; Maulik et al. 2002), suggesting that the *c-met* receptor may play an important role in cellular fate changes, including the epithelial to mesenchymal transition (Kubo et al. 2009; Salgia 2009). Increased *c-met* gene dosage was observed to be largely independent of EGFR and *k-ras* mutations in PPCs, positioning its blockade as an interesting monotherapy target.

Remarkably, the five genetic factors we have chosen to study in the analysis resulted in an unexpectedly high ratio of PPC samples detected with this defined probe set of genetic alterations. The fact that these patients who possess druggable or actionable molecular targets should be considered as candidates for mono or combinational targeted cancer therapy leads to an immediate clinical implication that warrants further validation in prospective clinical trials. Especially, EGFR mutation can be a possible predicitive marker for gefitinib or erlotinib and it is important to investigate where EGFR-TKI is effective for advanced PPC patients.

The molecular origin of PPC still remains largely obscure. It is the definition of a disease unit based on the histological observation, and whether there exists a singular genetic event leading to the emergence of this rare tumor type is still unknown. It is possible that a minute proportion of non-small-cell lung cancer will undergo a series of secondary genetic changes during the course of tumor evolution, reprogramming the cellular fates of primary tumors and propelling rare transitions in cellular fate through transdifferentiation or dedifferentiation. This redirected cellular fate specification may culminate in

histologically mixed cell populations observed in patient samples. The mutation profiling study presented here indicates that, at least, the oncogenic origins of this cancer type may be rather diverse, encompassing several well-known pulmonary oncogenic genetic factors.

The limitation of this study, as with many other mutation profiling studies, is that we can only detect a set of mutations in a subset of oncogenes with pre-established oncogenecity. Thus, other sets of mutations critical to the oncogenic process of PPCs might be missing in the analysis. Moreover, the percentages of these mutations detected from this preliminary screening that correspond to oncogenic or driver mutations need to be determined in future prospective studies. Given the rarity of this disease type, it will take a significant amount of time to accumulate sufficient clinical cases to properly determine the benefit the patients will experience from prospective oncogeno-chemo trials compared with that of histology-based conventional chemotherapeutics. To resolve this issue, further systemic and hypothesis-driven or a priori knowledge-free genomics-guided patient stratification is warranted in the laboratory to help more accurately describe each patient's onco-genetics map. Until then, however, this preliminary attempt to characterize the mutational space of PPCs, previously thought to be an extremely malignant cancer type, will provide measurable molecular guidance to the alternative molecular treatment of this rare disease.

In summary, we have characterized the mutational statuses of several well-known oncogenic targets in a large set of PPC tumors. Given the diverse repertoire of mutational profiles observed from PPC samples, clinical trials based on accurate cancer-genotyping should be considered as a legitimate treatment scheme for this rare and hard-to-cure disease entity.

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Conflict of interest None declared.

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