A comprehensive study on the oncogenic mutation and molecular pathology in Chinese lung adenocarcinoma patients

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Research

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Abstract

Background: Several driver genetic alterations have been identified in micropapillary lung adenocarcinoma (MPA). However, the frequency of ROS1 rearrangements is yet unclear. Herein, we investigated the associations between clinicopathological and molecular characteristics of MPA compared with non-micropapillary lung adenocarcinoma (LA).

Methods: Formalin fixed paraffin-embedded (FFPE) sections derived from lung adenocarcinoma patients who never received adjuvant chemotherapy or radiation therapy prior to surgical resection were collected from October 2016 to June 2019. EGFR mutations, ROS1 rearrangement and EML4-ALK fusion were identified in a set of 131 MPA and LA cases by using the Amplification Refractory Mutation System (ARMS).

Results: EGFR mutations had occurred in 42 (76.4%) MPA patients and 42 (55.3%) LA patients. But interestingly, ROS1 rearrangement was present in 10.9% (6/55) MPA cases and 1.3% (1/76) LA cases. Moreover, 7.3% (4/55) MPA samples had multiple gene mutations, while only 1.3% (1/76) LA cases had double gene alterations. Of 5 patients with harbouring two driver oncogenes mutations, four patients (80%) obtained partially response and one patient (20%) suffered recurrence.

Conclusions: A higher prevalence of ROS1 rearrangements or combined mutations of ROS1 and EGFR or EML4-ALK may play a critical role in the tumorigenesis of MPA. These findings provide a novel therapeutic strategy for the patients with malignant MPA through combining TKIs than one TKI.

Background

Lung cancer remains to be the leading cause of cancer-related deaths worldwide, and the most frequent histological subtype is lung adenocarcinoma[1]. Lung adenocarcinoma usually includes various histological subtypes, such as solid, lepidic, acinar, papillary, and micropapillary pattern[2] according to the International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS)[3, 4]. Numerous studies have reported that lung adenocarcinoma with micropapillary pattern (MPA) shows more aggressive behavior and worse prognostic value than lung adenocarcinoma without micropapillary pattern (LA)[5-7].

Several oncogenic drivers have been identified in lung adenocarcinoma, including mutations in the epidermal growth factor receptor (EGFR)[8], rearrangements of anaplastic lymphoma kinase (ALK)[9], and ROS proto-oncogene 1 receptor tyrosine kinase (ROS1)[10]. Accumulating evidence demonstrated that mutations of *EGFR* were identified in 15-30% of lung adenocarcinomas in Caucasians[11] and 40%-60% of lung adenocarcinomas in East Asians[12-14], indicating the frequency of activating mutations of *EGFR* depends on ethnic. In addition, *ALK* fusions were firstly identified in 2007 and approximately occurred in 3-7% of all lung adenocarcinoma patients, and the most common form was echinoderm microtubule associated protein-like 4/anaplastic lymphoma kinase (*EML4-ALK*)[15]. In the same year, an additional novel oncogenic fusion gene-*ROS1* was identified, which accounted for 1-2% of all lung adenocarcinoma

patients, but 5%-7% of the lung adenocarcinoma patients with *EGFR*/ *KRAS*/ *BRAF*/ *ALK* negative[16]. With the development of tyrosine kinase inhibitors (TKIs), TKIs are served as the first-line option for patients harboring *EGFR*-sensitive mutations or *ALK* and *ROS1* fusions. Therefore, the discovery of TKIs against *EGFR* gene activation mutations (for example, gefitinib, erlotinib), and *ALK* or *ROS1* gene rearrangement (for example, crizotinib) has significantly improved the outcomes of patients. For the detection of *ROS1* and *EML4-ALK* fusions, immunohistochemistry (IHC), next generation sequencing (NGS), ARMS-ploymerase chain reaction (ARMS-PCR), and fluorescence in situ hybridization (FISH) have been widely used[17]. Although FISH is the gold standard test, it is expensive and time-consuming. In contrast, ARMS-PCR is a more sensitive and feasible approach compared to FISH and IHC[18].

Increasing evidence indicates that *EGFR* gene mutations are more frequent in MPA than other histological types of lung adenocarcinoma or LA, while *ROS1* gene rearrangement has not been clearly demonstrated in MPA patients[19-21]. Moreover, co-existence of *EGFR* gene mutations with *ALK* or *ROS1* gene fusions has been reported in a few lung adenocarcinoma cases[22-24], but the co-alteration of *ROS1* and *EGFR* or *EML4-ALK* in MPA remains unclear.

The molecular features of micropapillary lung adenocarcinoma may differ from other histopathological subtypes of lung adenocarcinoma[25], however, the determinate information is not available. In this study, we investigate the relationship between the most common driver mutations and the pathology features in Chinese lung adenocarcinoma patients.

Materials And Methods

Patient selection

A total of 131 lung adenocarcinoma patients were enrolled in the First People' Hospital of Huzhou from January 2016 to June 2019. Of them, 55 cases harbored at least 5% micropapillary component[19], who were represented as MPA, and the remaining cases (43 solid, 20 acinar and 13 lepidic) were defined as LA. All of them were initially diagnosed with lung adenocarcinoma, and had not received neoadjuvant or adjuvant chemotherapy or radiation therapy prior to surgical resection. The pathological diagnosis was confirmed and classified by two certified pathologists (Qilin Shi and Xiaolan Zhang from the First People's Hospital of Huzhou) based on the IASLC/ATS/ERS multidisciplinary classification system[3]. All specimens contained 60% of tumor cells and sufficient tissues for further mutational analysis. Clinical information is to collect, including age, gender, tumor differentiation, tumor size, smoking history, lymphatic invasion, pleural invasion, tumor node metastasis (TNM) stage, micropapillary pattern, and prognostic data. This study was undertaken with the agreement of our hospital ethics committee, and the informed consent signature was provided by all patients.

Mutational analysis

For *EGFR* mutation analysis, DNA from FFPE tissue sections was extracted by using a QIAamp DNA FFPE tissue kit (cat. no. 56404, Qiagen, Germany) according to the manufacturer's instructions. *EGFR*

mutations within exons 18-21 were identified with commercial kits (Human EGFR mutation Detection Kit, cat. no. YZYMT-002-A, YZY Medical Co., Ltd., Wuhan, China) using ARMS. It was capable of detecting the following mutations: three in exon 18 (G719S, G719C and G719A), one deletion in exon 19, two mutations in exon 20 (T790M and S768I), three insertions in exon 20 and two mutations in exon 21 (L858R and L861Q). Briefly, the ARMS-PCR amplification (37˚C for 10 minutes; 95˚C for 5 minutes; 40 cycles of 95˚C for 15 seconds and 60˚C for 60 seconds) was performed using the 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). For *EML4-ALK* and *ROS1* fusions analysis, RNA was isolated from FFPE sections by applying an RNeasy FFPE tissue kit (cat. no. 73504, Qiagen, Germany) according to the standard procedure. Complementary DNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. RNA (500 ng), 2 µl PrimeScript^{TM} RT Master Mix (Perfect Real Time) (Takara Biotechnology Co., Ltd.) and RNase free water (up to 10µl), were mixed together and incubated at 37˚C for 15 minutes and 85˚C for 5 seconds. Then, the ARMS-PCR amplification (37˚C for 10 minutes; 95˚C for 5 minutes; 40 cycles of 95˚C for 15 seconds and 60˚C for 35 seconds) was performed using the 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). *ROS1* fusions (fusion partners for *ROS1*: *CD74*, *SLC34A2*, *SDC4*, *EZR*, *LRIG3*, *TPM3* and *GOPC*) and *EML4-ALK* rearrangement were identified with commercial kits (Human ROS1 fusion Detection Kit, cat. no. YZYMT-022 and Human EML4-ALK fusion Detection Kit, cat. no. YZYMT-021, YZY Medical Co., Ltd., Wuhan, China).

Statistical analysis

Comparisons between two or more categorical variables were investigated using the Chi square test and Fisher's exact test. Data were statistically performed on *SPSS22.0* (Chicago, IL, USA). The results were considered statistically significant at $p<0.05$.

Results

Clinicopathologic characteristics

In MPA, 36 patients fell into stage I, 11 into stage II, 6 into stage III, and 2 into stage IV. In LA, 39 (16 solid, 16 acinar and 7 lepidic) fell into stage I, 12 (10 solid, 1 acinar and 1 lepidic) into stage II, 8 (4 solid, 1 acinar and 3 lepidic) into stage III, and 17 (13 solid and 4 lepidic) into stage IV. About 72 people had no smoking history, and 59 were smokers, including former smokers and current smokers. The histological images of MPA and LA are represented in Fig. 1.

The MPA group consisted of 24 (43.6%) women and 31 (56.4%) men with an age at diagnosis ranging from 31 to 82 years (mean 62). In comparison, the LA group composed with 38 (50.0%) women and 38 men (50.0%) with a mean age of 65 years (range, 46-81 years). Compared to other histological lung adenocarcinoma, MPA patients had significantly younger age at diagnosis ($p=0.008$), positive lymph node metastasis ($p=0.03$), positive pleural invasion ($p=0.036$) and earlier disease staging ($p=0.024$)

(Table 1). However, the micropapillary pattern was not significantly associated with sex, smoking history, tumor size and tumor differentiation (Table 1).

Mutational status of classic oncogenes

The MPA and LA groups had 42 (76.4%) and 42 (55.3%) *EGFR* mutations respectively. But interestingly, *ROS1* rearrangements were highly presented only in the MPA group (6/55) and basically not in the LA group (1/76) (p=0.041, Table 1). Interestingly, we also discovered that the combination of driver genetic alterations existed in MPA group, eg. *EGFR* combined *ROS1* (n=2), and *EML4-ALK* combined *ROS1* (n=2), while only one LA case harbored *EGFR* combined *ROS1*, suggesting co-existent alterations of *EGFR*, *ROS1* and *EML4-ALK* were more frequent in MPA than other histological lung adenocarcinomas (p=0.043, Table 2) and indicating the potential combined treatments of MPA with two different TKIs targeted to *EGFR* and *ROS1*.

To further explore the association between clinicopathologic characteristics and driver genetic alterations, we analyzed the general information and therapeutic outcomes in 5 MPA or LA patients with combined mutations during one year follow-up. The 5 patients were provided with a combination of surgical resection, chemotherapy and targeted therapy. Four patients partially responded and one patient suffered recurrence (Table 3).

Discussion And Conclusions

Accumulating evidence indicates the co-existence of classic oncogenes, involving *EGFR*, *ALK*, *ROS1* and *MET*, has identified in lung adenocarcinoma patients, especially younger and women patients without smoking history. However, few studies have focused on the frequency of *ROS1* rearrangement or co-alterations of *EGFR*, *ROS1* and *EML4-ALK* in MPA and LA. Therefore, we investigated the relationship between the most common oncogenic mutations and molecular characteristics in Chinese lung adenocarcinoma patients.

Like in previous reports[26, 27], we here discovered that MPA has positive lymph node metastasis, positive pleural invasion and earlier disease staging compared with LA (Table 1). Increasing studies have showed that an micropapillary component was associated with lymph node metastasis, pleural invasion and an early recurrence in stage I patients, suggesting MPA had a poorer prognosis compared with those without micropapillary component or other histological subtypes[28-35]. Our results further implied that the higher prevalence of lymph node metastasis and pleural invasion may be a valuable poor prognosis marker for MPA.

An investigation of 15 MPAs revealed that the mutational status of *EGFR*, *KRAS* and *BRAF* harbored 73% mutually exclusive mutations in the Western population[36]. A study involving 21 micropapillary predominant lung adenocarcinoma patients showed that oncogenic mutations in *EGFR*, *HER2* and *RET* were apparently frequent in 95.2% Chinese people[19]. Here, our results manifested the majority (47 out of 55, 85.5%) of MPA harbored the driver genetic alterations of *EGFR* (76.4%), *ROS1* (10.9%), or *EML4-ALK*

(5.5%) from a Chinese cohort. The previous cohort detected no *ROS1* fusions[19], but two other independent teams found *ROS1* rearrangements in MPA[20, 21]. Therefore, there are no consistent conclusions about *ROS1* rearrangements in MPA patients. Here, our cohort reported that 6 MPA cases possessed *ROS1* rearrangements. In the past, oncogenic mutations involving *EGFR*, *KRAS*, *ALK*, *RET*, *ROS1* and *MET* were regarded as mutually independent events. However, two or more cancer-associated genes were recently found in lung adenocarcinoma cases[37-40]. Our study indicated that 3.8% lung adenocarcinoma cases harbored two-driver alterations of *EGFR*, *ROS1* or *EML4-ALK*, including 7.3% MPA cases and 1.3% LA cases, and this result was consistent with previous reports[37-41].

According to previous reports, the patients with co-alterations of *EGFR*, *ALK*, *ROS1* and other oncogenic drivers showed distinctive clinical responses to TKIs in lung adenocarcinoma[40, 42-44]. Yang et al demonstrated that the median progression-free survival of gefitinib was 11.2 months in patients with concomitant *EGFR* and *ALK* alteration[45]. Mao et al indicated that the median progression-free survival of *EGFR*-TKIs and/or *ALK/ROS1* inhibitor was 6.6 months in patients with concomitant *EGFR* and *ALK* alteration[40]. However, 75% patients with crizotinib treatment obtained disease control[40]. In the present study, all patients undertook the operation and chemotherapy initially and undertook subsequently targeted therapy. In addition, Katsuya et al showed that an micropapillary component was associated with an early recurrence in stage I patients but not in advanced-stage patients, indicating MPA retained a high risk of early recurrence after one year surgery[35]. In the present study, among five patients with harbored two-driver alterations of *EGFR*, *ROS1* or *EML4-ALK*, four patients partially responded and one patient suffered recurrence during one year follow-up. Our study provided evidence that lung adenocarcinoma patients with co-alterations of *EGFR*, *ROS1* or *EML4-ALK* may benefit from TKIs treatment.

So far, there is little progress on digging the pathogenic mechanism of MPA or the treatment of this subtype by TKIs. Therefore, based on our finding, we will focus of elucidating the function on *ROS1* rearrangement and *EGFR* mutations in MPA by establishing the cell and animal models both *in vitro* and *in vivo*. In addition, we will test the efficacy of one targeted TKI or combined TKIs for MPA, and provide the potential treatment strategy.

In summary, we report for the first time the relationship between the most common oncogenic mutations and pathology characteristics in Chinese lung adenocarcinoma patients. We also discover the higher incidence of *ROS1* rearrangements and the coexistence of genetic alterations involving *EGFR*, *ROS1* and *EML4-ALK* in MPA cases, indicating that targeting of *ROS1* rearrangements and/or *EGFR* mutations may provide a novel strategy and potential prognosis marker for these patients. However, these results still should be confirmed by further studies with larger cases and more clinical information, especially prognosis data and mechanism.

Declarations

Abbreviations

MPA: Micropapillary Lung Adenocarcinoma; LA: Lung Adenocarcinoma without micropapillary component; EGFR: Epidermal Growth Factor Receptor; ROS1: ROS proto-Oncogene 1 Receptor Tyrosine Kinase; ALK: Anaplastic Lymphoma Kinase; EML4-ALK: Echinoderm Microtubule associated protein-Like 4-Anaplastic Lymphoma Kinase; KRAS: Kirsten Rat Sarcoma viral oncogene; HER2: Erb-B2 receptor tyrosine kinase 2; RET: Rearranged during transfection; MET: Mesenchymal-Epithelial Transition; FFPE: Formalin Fixed Paraffin-Embedded; TKI: Tyrosine Kinase Inhibitor; TNM: Tumor Node Metastasis; IASLC: International Association for the Study of Lung Cancer; ATS: American Thoracic Society; ERS: European Respiratory Society; ARMS: Amplification Refractory Mutation System; PCR: Ploymerase Chain Reaction; IHC: Immunohistochemistry; NGS: Next Generation Sequencing; FISH: Fluorescence In Situ Hybridization.

Ethics approval

This study was approved by the ethics committee of the First People's Hospital of Huzhou (grant no. 2015-012).

Conflicts of Interest

All authors declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author's contributions

XW designed and conceived this study. HX provided and evaluated the tumor tissues. HMY performed the follow-up study. XLZ and YJ performed the experiments. XLZ and XW analyzed the data. All authors wrote and read the manuscript.

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Consent for publication

The informed consent signature was provided by all patients.

Availability of data and materials

All data generated and analyzed during the present study are available from the corresponding author on reasonable request.

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Tables

Table 1. Clinicopathologic and molecular characteristics of MPA and LA cases

Variables	MPA (n=55)	LA (n=76)	χ^2	*p*
Gender			0.518	0.485
Female	24 (43.6%)	38 (50.0%)		
Male	31 (56.4%)	38 (50.0%)		
Age (years)			7.452	0.008**
>65	20 (36.4%)	46 (60.5%)		
≤65	35 (63.6%)	30 (39.5%)		
Smoking history			0.191	0.723
Ever	26 (47.3%)	33 (43.4%)		
Never	29 (52.7%)	43 (56.6%)		
Tumor size (cm)			0.058	0.851
>3.0	17 (30.9%)	25 (32.9%)		
≤3.0	38 (69.1%)	51 (67.1%)		
Lymphovascular invasion			1.645	0.225
Present	17 (30.9%)	16 (21.1%)		
Absent	38 (69.1%)	60 (78.9%)		
Tumor differentiation			0.179	0.835
Well/Moderate	43 (78.2%)	57 (75.0%)		
Poor	12 (21.8%)	19 (25.0%)		
N status			4.291	0.03*
N0	31 (56.4%)	56 (73.7%)		
N1/N2	24 (43.6%)	20 (26.3%)		
Pleural invasion			4.117	0.036*
Present	15 (27.3%)	10 (13.2%)		
Absent	40 (72.7%)	66 (86.8%)		
Stage			5.701	0.024*
I/II	47 (85.5%)	51 (67.1%)		
III/IV	8 (14.5%)	25 (32.9%)		
Mutation status				
EGFR mutation	42 (76.4%)	42 (55.3%)	6.175	0.016*
EML4-ALK fusion	3 (5.5%)	1 (1.3%)	1.846	0.309
ROS1 fusion	6 (10.9%)	1 (1.3%)	5.806	0.041*

Abbreviations: MPA, micropapillary lung adenocarcinoma; LA, lung adenocarcinoma without micropapillary component.

Table 2. Coexistent genetic alterations including *EGFR*, *ROS1* and *EML4-ALK* in MPA and LA cases

Variables	MPA（n=55）	LA（n=76）	*p*
Single alteration			0.028*
EGFR +	40（72.7%）	41（53.9%）	
ROS1+	2（3.6%）	0	
EML4-ALK+	1（1.8%）	1（1.3%）	
Double alteration			0.043*
EGFR+; ROS1+	2（3.6%）	1（1.3%）	
ROS1+; EML4-ALK+	2（3.6%）	0	
No alteration			0.001**
EGFR-; ROS1-; EML4-ALK-	8（14.5%）	33（43.5%）	

Abbreviations: MPA, micropapillary lung adenocarcinoma; LA, lung adenocarcinoma without micropapillary component. EGFR+: EGFR mutation; EGFR-: EGFR wild type; ROS1+: ROS1 fusion; ROS1-: no ROS1 fusion; EML4-ALK+: EML4-ALK fusion; EML4-ALK-: no EML4-ALK fusion.

Table 3. General information and therapeutic outcomes on 5 patients with coexistent genetic alterations including *EGFR*, *ROS1* and *EML4-ALK* in MPA and LA patients

Patients	1	2	3	4	5
Gender	female	male	female	male	female
Age (y)	52	31	45	71	75
Smoking history	never	never	never	ever	never
Lymphovascular invasion	absent	present	absent	absent	absent
Tumor differentiation	moderate	moderate	moderate	moderate	moderate
Stage	T1bN0M0	T1cN1M0	T1cN0M0	T1bN0M0	T1cN0M0
Pathological type	MPA	MPA	MPA	MPA	LA
Mutation status	ROS1+; EML4-ALK+	ROS1+; EML4-ALK+	EGFR L858R+; ROS1+	EGFR L858R+; ROS1+	EGFR 19-del+; ROS1+
Therapeutic intervention	Surgery; Chemotherapy; Crizotinib	Surgery; Chemotherapy; Crizotinib	Surgery; Chemotherapy; EGFR inhibitors	Surgery; Chemotherapy; EGFR inhibitors	Surgery; Chemotherapy; EGFR inhibitors
Outcomes	Partial response	Partial response	Partial response	Recurrence	Partial response

Abbreviations: MPA, micropapillary lung adenocarcinoma; LA, lung adenocarcinoma without micropapillary component. EGFR+: EGFR mutation; ROS1+: ROS1 fusion; EML4-ALK+: EML4-ALK fusion.

Figures

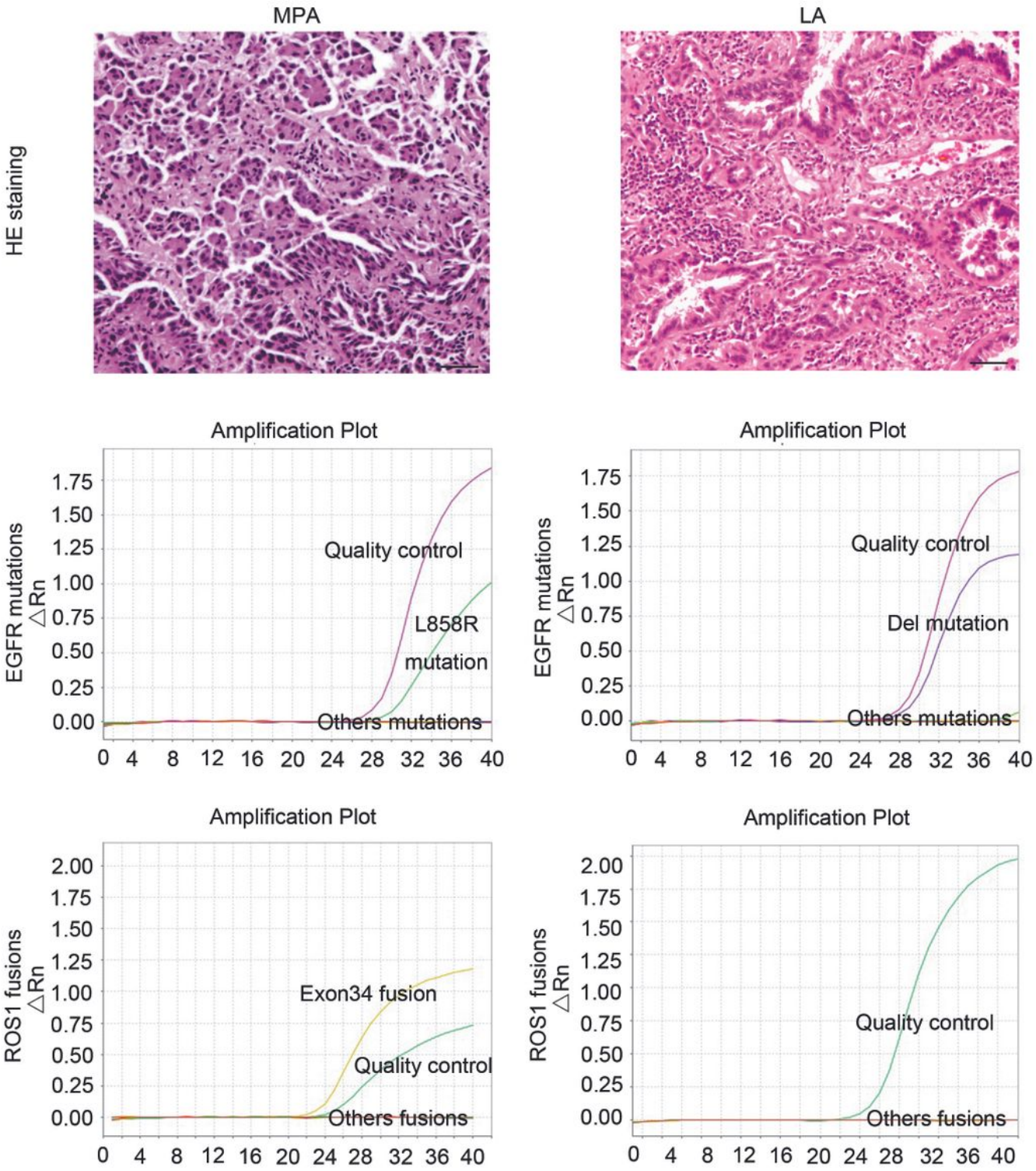

Figure 1

Immunohistochemical staining and mutation analysis of MPA and LA patients. 3μm FFPE sections of MPA (patient 1) and LA (patient 16) were immunostained with Hematoxylin and Eosin (100x magnification). Scale bar: 20μm. Abbreviations: MPA, micropapillary lung adenocarcinoma; LA, lung adenocarcinoma without micropapillary component.