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ORIGINAL PAPER

Prediction of epidermal growth factor receptor mutations in the plasma/pleural effusion to efficacy of gefitinib treatment in advanced non-small cell lung cancer

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Abstract
Purpose Recently, mutations in the epidermal growth factor receptor (EGFR) gene were reported to correlate with EGFR tyrosine kinase inhibitor response in advanced non-small cell lung cancer (NSCLC). In this study, we attempted to detect EGFR mutations in plasma and pleural effusion samples and to make clear its correlations with gefitinib response and survival in NSCLC patients.
Methods The free DNA was isolated from the plasma of 56 cases and pleural effusion of another 32 cases of advanced NSCLC. Five common types of EGFR mutations were analyzed by LightCycle PCR with Taqman-MGB probes.
Results EGFR gene mutations were found in 22 of all the 88 (25%) NSCLC patients (23.2% of 56 plasma samples, 28.1% of another 32 pleural effusion samples). EGFR mutations were more frequently present in females, never-smokers and adenocarcinomas ($P < 0.01$). It also showed that patients with EGFR mutations had a significantly better response rate when compared with that of the wild-type patients ($P < 0.001$). The median progression-free survival (11.2 vs. 2.7 months $P = 0.005$) and overall survival (21.8 vs. 5.8 months $P = 0.003$) were significantly higher in patients with EGFR mutations than in patients with wild-type EGFR.
Conclusions The EGFR mutations in the serum and the pleural effusion from advanced NSCLC patients can be detected with LightCycle PCR using Taqman-MGB probes. The mutations highly predict the efficacy of gefitinib in advanced NSCLC.

Keywords Non-small cell lung cancer · Plasma · Pleural effusion · Epidermal growth factor receptor mutation · PCR

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Introduction

Lung cancer is the leading cause of cancer death in the world for both men and women. It causes more death than colorectal, breast and prostate cancer. What is more, its incidence is increasing in many parts of the world with over one million new cases diagnosed each year (Parkin 2001). Non-small cell lung cancer (NSCLC) constitutes about 80% of lung cancer. It is usually asymptomatic at its early stage. Hence, the majority of NSCLC patients have advanced or metastatic disease at the time of diagnosis. Systematic, palliative chemotherapy becomes their major therapeutic option. Platinum-based doublets prove effective and could improve modest but statistically significant overall survival when compared with best supportive care in the group of patients (Breathnach et al. 2001; Schiller et al. 2002). Disease-free survival is about 4–6 months, suggesting rapid disease progression soon after first-line chemotherapy, and increasing the need for second-line treatment.

Gefitinib, a small molecular epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), was approved for salvage treatment for advanced NSCLC (Fukuoka et al. 2003; Kris et al. 2003). The original rationale for its use was the observation that EGFR is expressed more abundantly in carcinoma tissue than in adjacent

normal lung (Rusch et al. 1993). Recently, an association between somatic mutations in the tyrosine kinase domain of the EGFR gene with clinical efficacy of EGFR TKIs has been reported (Lynch et al. 2004; Paez et al. 2004). The EGFR mutations consisted of either in-frame deletions of exon 19 or amino acid substitutions of exons 18, 20 and 21 clustered around the ATP-binding pocket of the tyrosine kinase domain. The mutations of EGFR may be a good biomarker for the selection of advanced NSCLC patients for clinical use of EGFR TKI. Indeed, some prospective phase II studies showed that the progression-free survival and overall survival were significantly improved in EGFR mutant NSCLC patients receiving EGFR TKI treatment (Mitsudomi et al. 2005; Han et al. 2005). The samples used for detecting EGFR mutation in these trials were lung cancer tissues using DNA sequencing. Although DNA sequencing is still a gold-standard method for mutation detection, it is expensive, time-consuming and not sensitive enough for non-tissue samples. Molecular biomarkers could be stably and easily examined from resected tumor samples. However, for advanced NSCLC, it is often difficult to obtain enough cancer tissue for the EGFR mutation examination. Approaches that can detect molecular biomarkers from other samples than cancer tissues are therefore important for individualized therapy in the future.

Some investigators have shown that the free DNA in plasma or pleural effusion could be a unique and valuable source of biomarkers used to predict response and prognosis (Kimura et al. 2006a, b). The amount of free DNA in plasma or pleural effusion is greatly increased in lung cancer patients when compared with normal subjects or tuberculosis patients. It may mainly come from cancer cells (Dong et al. 2002; Sonobe et al. 2004). On the one hand pleural effusion and blood sample can be obtained easily, non-invasively and repeatedly. On the other hand, the same alterations including mutations, loss of heterozygosity and methylation in genomic DNA have been observed from both tumor cells and blood samples (Sánchez-Céspedes et al. 1998; Esteller et al. 1999). For known mutations, real-time polymerase chain reaction (PCR) followed by melting curve analysis, using hybridization probes, is highly sensitive, rapid, and an efficient alternative approach for mutation detection (Wittwer et al. 1997; Pals et al. 1999; Sasaki et al. 2005). Previously, we reported our results of application of PCR using Taqman-MGB probes to detect mutant EGFR in lung cancer tissues and found its good speciality and sensitivity of detecting the mutations when compared with DNA sequencing (Zhou et al. 2007). In the present study, we used the approach to detect the EGFR mutations in plasma or pleural effusion from 88 advanced NSCLC patients and compared the relationship of EGFR mutations with gefitinib's efficacy.

Materials and methods

Specimens

Eighty-eight patients with advanced NSCLC proven cytologically or pathologically were enrolled into the study from May 2006 to October 2007 in Shanghai Pulmonary Hospital, Tongji University. Eligibility criteria included patients with measurable disease, stage IIIB/IV, ECOG performance status (PS) 0–3, life expectancy of about 3 months, adequate functions of important organs, such as liver, bone marrow, kidney, heart and lungs, aged over 18 years old and failure after or intolerable to chemotherapy with informed consent. Each patient was treated by gefitinib 250 mg daily up to disease progression or intolerable toxicity. Before gefitinib therapy, 5 ml of pleural effusion or blood was collected from the subjects for detecting EGFR mutations.

DNA extractions

Prior to the initiation of gefitinib therapy, 5 ml of blood or pleural effusion was collected and centrifuged at 4,000/min for 10 min. The supernatants were collected and stored at −80°C until DNA extraction. DNA was extracted from 2 ml of the supernatant with a Watson DNA Kit (China) according to the serum/plasma DNA isolation and purification protocol. The extracted DNA was stored at −20°C until used.

Detection of EGFR mutations

Previously, we reported EGFR mutation types in 80 resected NSCLC tissues by PCR sequencing. We found five types of EGFR mutations and designed the method for rapid detection of the mutations with PCR combined with Taqman probes (Zhou et al. 2007). Four deletion mutational probes in exon 19, one mutational probe in exon 21 (L858R) and the corresponding wild types were designed (Table 1). The 3′ end of each probe was labeled with different fluorescent molecules as FAM, TET, HEX and the 5′ end with quenching molecule MGB. All reactions were done in 23-μL volumes using 2 μL of template DNA, 6.25 μL of re Taqman polymerase action buffer mix, 0.4 μL of each primer, 0.7 μL of Taqman-MGB probes and 0.25 μL of Taqman polymerase. Real-time PCR was carried out using LightCycler 2.0 (Roche, Germany) under the following conditions: initial denaturation at 94°C for 3 min, 50 cycles of 94°C for 5 s, and 60°C for 30 s with fluorescence reading at the end of each cycle. This instrument measures fluorescence during PCR. When the primers are extended, fluorescent molecule (at 3′ end of the probes) will be hydrolyzed by Taq polymerase and emit measurable light as the inhibition of quenching molecule is eliminated.

Table 1 Sequences of TaqMan-MGB probes

Exon	Type of probe	Sequence	T_m (°C)
21	Wild-type	5′-FAM-TTT GGC CAG CCC AAA-MGB-3′	67.6
	L858R sub	5′TET-TTT-GGC CCG CCC AA-MGB-3′	67.8
19	Wild-type	5′FAM-AGGAATTAAGAGAAGCAA C-MGB-3′	68.1
	Del2236-2250	5′TET-CTA TCA AGA ACA TCT CCG MGB-3′	68.3
	Del2240-2257	5′FAM-ATCAAGGAATCGAAAGC-MGB-3′	67.8
	Del2235-2249	5′HEX-TCGCTATCAAAACATCT-MGB-3′	67.8
	Del2254-2277	5′HEX-AAGCAACACTCGATGTGA-MGB-3′	68.4

del deletion; *sub* substitution

Statistical analysis

Non-smokers were defined as those who had smoked <100 cigarettes in their lifetime. Lung cancer histology was defined according to the World Health Organization pathology classification. Disease stage was determined in accordance with the International Union against Cancer tumor-node-metastasis classification of malignant tumors. Efficacy was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST)'guidelines (Therasse and Arbuck 2000). Every 2 months, response was evaluated after gefitinib treatment. The relationship between EGFR mutation and clinical features was analyzed using Pearson χ^2 test, including age, gender, histological type, PS, smoking status, disease stage, number of prior chemotherapy and response to gefitinib. Two-sided P values <0.05 were considered statistically significant. Overall survival (OS) and progression-free survival (PFS) were plotted by the Kaplan–Meier method and compared by log-rank test. All analyses were performed using the SPSS software (version 13.0).

Results

Patient's characteristics

The clinical characteristics of these 88 patients were summarized in Table 2. Their median age was 58 years (range 38–84 years). Forty-eight (54.5%) of the patients were male and 40 (45.5%) were females. Thirty-seven patients (42.0%) were current or previous smokers. The most common histological subtype was adenocarcinoma (69 patients, 78.4%) and the majority of patient (97.7%) had stage IV disease. Gefitinib was used in the first-line setting in 23 patients (26.1%), and in second or third-line setting in 65 patients (73.9%). Fifty-one patients (58.0%) had good PS (0–1).

Table 2 Relationships between EGFR mutation and patient's characteristics

Parameter	Cases detected	Cases of mutations	Rates of mutations (%)	P value
Age				
≤70	76	20	26.3	$P = 0.24$
>70	12	2	16.7	
Gender				
Male	48	3	6.3	$P < 0.001$
Female	40	19	47.5	
Histology				
Adenocarcinoma	69	22	31.9	$P < 0.001$
Squamous cell carcinoma	11	0	0	
Smoking history				
Yes	37	2	5.4	$P < 0.001$
No	51	20	39.2	
Prior chemotherapy				
No	23	4	17.4	$P = 0.15$
Yes	65	18	27.7	
Stage				
IIIb	2	0	0	$P = 0.56$
IV	86	22	25.6	
PS				
0–1	51	13	25.5	$P = 0.2$
2–4	37	9	24.3	
Gefitinib response				
Partial response	26	20	76.9	$P < 0.01$
Stable disease	30	2	6.7	
Progressive disease	32	0	0	

EGFR mutations in plasma or pleural effusions

In total, EGFR gene mutations were found in 22 patients (25%). There were 21.6% of EGFR exon 19 deletions including E746_A750del in 15 patients and L747–S752del in four patients. L858R of exon 21 was detected in three patients (3.4%) (Table 3).

There were 56 plasma samples and 32 pleural effusion sample for genotyping of EGFR. The EGFR mutation rate was 23.2% (13/56) in plasma and 28.1% (9/32) in pleural effusion, respectively. The difference in EGFR mutation rate was not significant between plasma and pleural effusion samples ($P = 0.17$).

Table 3 EGFR mutant status detected in plasma or pleural effusions samples using LightCycle PCR with Taqman-MGB probes

Response	Gender	Histology	Smoking history	Exon 19			Exon 21	
				Wild	Del2236-2250	Del2240-2257	Wild	L858R
PR	F	Ad	N	+	_	+	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	M	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	M	Ad	F/C	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	M	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	+	_	+	_
PR	F	Ad	N	+	_	+	+	_
PR	F	Ad	N	+	_	+	+	_
PR	M	Ad	F/C	+	_	+	+	_
PR	F	Ad	N	+	_	_	+	+
PR	F	Ad	N	+	_	_	+	+
PR	F	Ad	N	+	_	_	+	+

SD stable disease, *PD* progressive disease, *PR* partial response, *M* male, *F* female, *Ad* adenocarcinoma, *N* never smoker, *F/C* former or current smoker, + curve detected by LightCycle, − curve not detected by LightCycle

Correlation between EGFR mutations and clinical features

The EGFR mutations were seen more frequently in the never-smokers (20/51, 39.2% vs. 2/37, 5.4% in current/former smokers, $P < 0.001$), in females (19/40, 47.5% vs. 3/48, in male 6.3%, $P < 0.001$), and in adenocarcinoma (22/69, 31.9% vs. 0/11 in squamous carcinoma, $P = 0.01$) (Table 2). We examined the relationship between the presence of EGFR mutation and other patients' demographics. The mutations of EGFR were not affected by patients' age, disease stages, prior chemotherapy number and ECOG PS.

EGFR mutation and response to gefitinib

The analysis of the EGFR mutant status and the response to gefitinib disclosed that 20 of 26 patients with partial response, 2 of 30 patients with stable disease and 0 of 32 with disease progression had EGFR mutations (Table 2). Incidence of EGFR mutations was significantly higher in those patients responsive than in those resistant to gefitinib ($P < 0.01$). Among 22 patients with mutant EGFR, 20 achieved partial response, 2 stable disease, while in 66 patients with wild-type EGFR, 6 patients achieved partial response, 30 stable disease, and another 32 developed disease progression. Response rate in those with mutant EGFR was significantly higher than in those with wild-type EGFR (90.9 vs. 9.1%, $P < 0.01$).

Effect of EGFR mutations on survival

The patients were followed up to March 31, 2008 and about 56 patients died. The median PFS and OS were 4.4 months (95% CI, 2.4–6.4 months) and 9.0 months (95% CI, 6.0–12.0 months) in the whole group, respectively. Median PFS was significantly longer in those with mutant EGFR than in those with wild-type EGFR (11.2 vs. 2.7 months $P = 0.005$, by log-rank test) (Fig. 1a). The overall survival was also significantly improved in those with mutant EGFR (21.8 vs. 5.8 months $P = 0.003$, by log-rank test) (Fig. 1b).

Discussion

In this study, we have testified the feasibility of using free DNA from plasma or pleural effusion as an alternative to tumor samples for the detection of EGFR mutations by PCR using Taqman-MGB probes. We also showed that patients with mutant EGFR had better outcomes of EGFR TKI treatment.

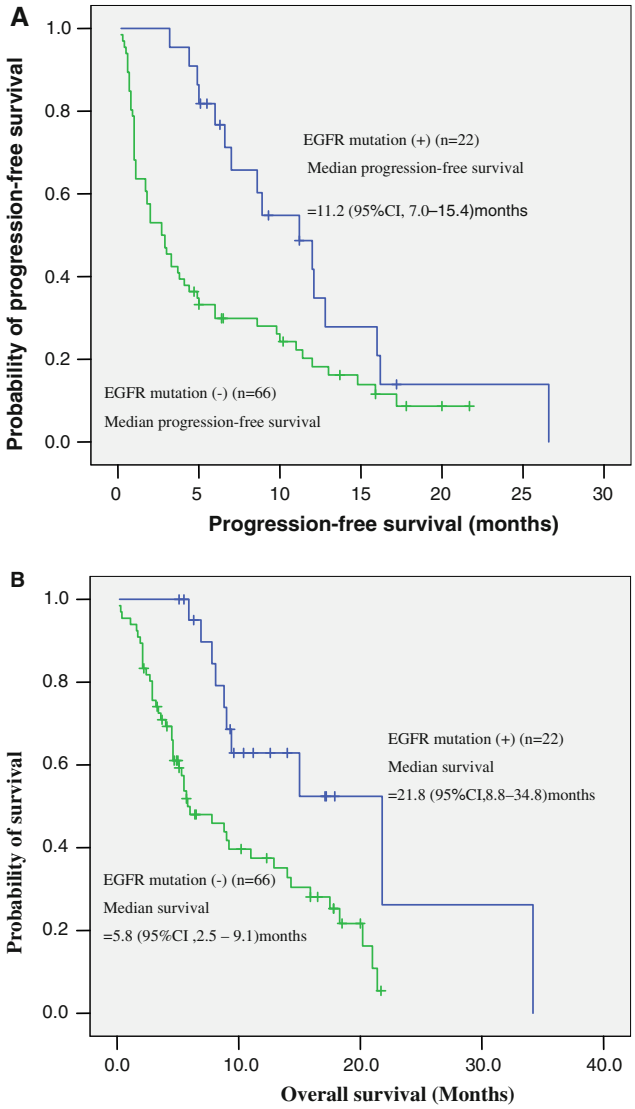

Fig. 1 Progression-free survival (**a**) and overall survival (**b**) with respect to the EGFR mutation status

Gefitnib or erlotinib has been approved in many countries for salvage therapy for advanced NSCLC patients. However, not all patients can get response or survival benefit from EGFR TKI treatment. Response rate is usually <10% and disease progression was seen in about 50% of patients receiving gefitinib. Females, adenocarcinomas, never-smokers and Asian patients got more survival benefit from EGFR TKI treatment. Biomarkers predicted for gefitinib efficacy are under study. The mutations or amplification of EGFR first attracted a great deal of attention. It is well considered that gene amplification of EGFR can predict both response and survival benefit to EGFR TKI, while the EGFR mutations only predict response, but not survival benefit in many studies in the western countries. Recently, studies from Asian countries showed that the EGFR mutations are good biomarkers for response and survival in Asian NSCLC patients (Mitsudomi et al. 2005; Han et al. 2005). These studies suggest that EGFR mutations can be used to guide EGFR TKI use in Asian NSCLC populations.

In our study, the response rate was 91% in EGFR mutation patients, while EGFR wild-type patients had only 9% response rate. Response rate in those with mutant EGFR was significantly higher than in those with wild-type EGFR ($P < 0.01$). Further, we confirmed that EGFR mutation is a prognostic factor for both progression-free survival (11.2 vs. 2.7 months $P = 0.005$) and overall survival (21.8 vs. 5.8 months $P = 0.003$) of NSCLC patients. These dates are equivalent to the reported literature (Lynch et al. 2004; Paez et al. 2004; Mitsudomi et al. 2005; Han et al. 2005).

Gene sequence is still gold-standard method for detecting gene mutations. In the studies mentioned above, investigators used gene sequencing for detecting EGFR mutations. The method had to use cancer tissue as a sample. However, in advanced NSCLC, it is usually difficult to get enough tissue for gene sequencing. After failure of first-line or second-line chemotherapy, many patients in poor conditions could not tolerate invasive biopsy or refused biopsy. Moreover, gene sequence is expensive, time-consuming and technically demanded. It cannot be widely spread into clinical use. Therefore, the development of detection method for EGFR mutations in other samples than cancer tissue is urgently needed for individualized therapy of EGFR TKI.

Free DNA from plasma or pleural effusion may be a good source for genomic DNA and substitute for cancer tissue. The amount of free DNA from plasma of lung cancer patients is much higher than from normal subjects. The advantage of collecting free DNA from plasma or pleural effusion is its simple, noninvasive and repeatable technique. Some studies showed that the same alterations, including mutations, loss of heterozygosity and methylation have been observed in DNA from both tumor cells and blood samples (Sánchez-Céspedes et al. 1998; Esteller et al. 1999) Recently two groups have evaluated the consistency between EGFR mutations in NSCLC tissue and matched serum DNA. One group detected EGFR mutations in pairs of tumour and serum samples from 42 Japanese patients treated with gefitinib and showed that the mutation status between them was consistent in 39 of the 42 pairs (92.9%) (Kimura et al. 2007). The other obtained pairs of tumor and serum samples from 121 Spanish NSCLC patients treated with erlotinib and showed the mutation status in serum was consistent with that in the tumor tissue of 82/121 patients (68%) and of 15/16 patients (93.8%) with PS 2 (Moran et al. 2007).

We previously reported EGFR mutation types in 80 resected NSCLC tissues by PCR sequencing and our results of LightCycle PCR using Taqman-MGB probes for detection of EGFR mutations in the lung cancer. We found the

consistency between the results of EGFR mutations detected by sequencing and PCR. Further for evaluation of sensitivity and speciality of mutant EGFR detection, we mixed PC-9 cells and A549 cells at different ratios and performed PCR as mentioned above. The results showed that PCR with TagMan MGB probes could detect EGFR mutation in as rare as 50 EGFR mutant cells and in a proportion of 10% of mutant cells in a cell population (not reported). Based on these findings, five types of EGFR mutations and the method for rapid detection with PCR combined with Taqman-MGB probes were applied to detect the mutations of EGFR in serum or pleural effusion fluid samples. The results showed EGFR gene mutations were detected in 22 of 88 (25%) in total. There were 21.6% of EGFR exon 19 deletions including E746_A750del in 15 patients and L747-S752del in 4 patients. L858R of exon 21 was detected in 3 patients (3.4%). These mutation frequencies were similar to those found in the results of our previous studies that examined 80 cases of surgical resection specimens.

Some other alternative methods for detecting EGFR mutations have been reported. One group used the Scorpion Amplified Refractory Mutation System technology (Kimura et al. 2006a, b) and the other adopted the SSCP assay (Marchetti et al. 2005). The former postulated that the Scorpion method was superior to direct sequencing for detecting EGFR mutation in serum as a predictive marker. In their study, the EGFR mutation status in serum DNA by direct sequencing did not correlate with the responsiveness to and survival benefit of gefitinib while by the EGFR Scorpion method, the EGFR mutation status could be a prognostic factor and the latter demonstrated SSCP assay was rapid and more sensitive than direct sequencing. Our study also showed that patients with mutant EGFR detected with LightCycle PCR method had better outcomes of EGFR TKI treatment. Further studies are needed to clarify the most sensitive assay for detecting EGFR mutations in non-tissue samples.

We did not compare the results of EGFR mutation status with light-cycle assay to the mutation status with direct sequencing method. The studies mentioned above have shown that sequencing is unable to provide satisfactory results for detection of serum or pleural effusion fluid samples which contain mixtures of DNA from normal and malignant cells (Kimura et al. 2006a, b). Our study also found that in most cases, the concentration of DNA extracted from these specimens was below the minimum concentration detectable by spectrophotometry (data not shown). Small amounts and low proportion of tumor-derived DNA may be misdiagnosed as wild-type EGFR by direct sequencing. Some studies have shown that detection of the mutation by sequencing requires at least 30% of the mutated DNA in a sample (Bosari et al. 1995; Fan et al. 2001). While our previous study demonstrated that LightCycle PCR with TagMan MGB probes could detect EGFR mutation in as rare as 50 EGFR mutant cells and in a proportion of 10% of mutant cells.

Two limitations of the study have to be mentioned. One is that the study population in the current study contained a higher proportion of adenocarcinoma (78 vs. 13%) which was the known factor associated with higher EGFR mutation rate and those with potential response to gefitinib such as the subgroup of female patients, adenocarcinomas and non-smokers may be chosen by the doctors (Fukuoka et al. 2003; Kris et al. 2003). The other limitation is that LightCycle PCR using Taqman-MGB probes method was only able to detect specific mutations by probes designed in advance. Although the five types of EGFR mutations we detected were based on the findings in 80 resected NSCLC tissues as mentioned above and approximately 90% *EGFR* mutations in NSCLC consist of these mutations (Pao et al. 2004; Kosaka et al. 2004), other mutations may be misdiagnosed as negative. Moreover, the secondary mutation, a substitution of methionine for threonine at position 790 (T790 M), which leads to resistance in patients who are initially responsive to gefitinib therapy may also be critical factors (Kobayashi et al. 2005; Kwak et al. 2005). Further study using these probes needs to be designed to detect these mutations.

In conclusion, EGFR mutations can be detected in genomic DNAs extracted from serum or pleural effusion samples using LightCycle PCR with TagMan MGB probes. We also anticipate to realizing personalized therapy in lung cancer patients based on the relationship between biological markers and the better outcomes in terms of response, progression-free survival and overall survival.

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