Concomitance of P-gp/LRP Expression with EGFR Mutations in Exons 19 and 21 in Non-Small Cell Lung Cancers

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Purpose: Traditional chemotherapy is the main adjuvant therapy for the treatment of non-small cell lung cancer (NSCLC). However, the emergence of multi-drug resistance (MDR) has greatly restricted the curative effect of chemotherapy. Therefore, it is necessary to find a method to treat MDR NSCLC clinically. It is worth investigating whether NSCLCs that are resistant to traditional chemotherapy can be effectively treated with tyrosine kinase inhibitors targeting epidermal growth factor receptor (EGFR). **Materials and Methods:** The expression of P-glycoprotein (P-gp) and lung resistance-related protein (LRP) was detected by immunohistochemistry, and mutations in EGFR (exons 19 and 21) and Kirsten rat sarcoma viral oncogene homolog (KRAS) (exon 2) were detected by high-resolution melting analysis (HRMA) of surgical NSCLC specimens from 127 patients who did not undergo traditional chemotherapy or radiotherapy. A Pearson chi-square test was performed to analyze the correlations between the expression of P-gp and LRP and mutations in EGFR and KRAS. **Results:** The expression frequencies of P-gp and LRP were significantly higher in adenocarcinomas from non-smoking patients; the expression frequency of LRP was significantly higher in cancer tissue from female patients. The frequency of EGFR mutations was significantly higher in well to moderately differentiated adenocarcinomas from non-smoking female patients. The frequency of EGFR mutations in the cancers that expressed P-gp, LRP, or both P-gp and LRP was significantly higher than that in cancers that did not express P-gp or LRP. **Conclusion:** NSCLCs expressing P-gp/LRP bear the EGFR mutation in exon 19 or 21 easily.

Key Words: Chemotherapy, multi-drug resistance, epidermal growth factor receptor-tyrosine kinase inhibitor, non-small cell lung cancer

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. Until now, the main therapeutic method for

NSCLC has been surgical resection with adjuvant chemotherapy, and for patients with unresectable, recurrent, or metastatic tumors, chemotherapy is the predominant treatment method.[1] Unfortunately, the use of traditional chemotherapy in the treatment of NSCLC is greatly limited by primary or secondary multi-drug resistance (MDR).[2] MDR, which is defined as a resistance to one drug that is accompanied by a resistance to many other drugs, is believed to be a major reason for the primary drug resistance of NSCLC and can lead to the failure of traditional chemotherapy.[3,4] Therefore, determining how to treat NSCLC patients with primary drug resistance has become an urgent problem. Tyrosine kinase inhibitors targeting epidermal growth factor receptor (EGFR-TKIs) comprise one class of molecular target drugs and have achieved remarkable

success in the treatment of patients with NSCLCs bearing activating mutations of EGFR in exons 19 and 21.[5,6] Moreover, these activating EGFR mutations appear more frequently in adenocarcinomas from Asian, female, and non-smoking patients.[7-10] Primary drug resistance to EGFR-TKIs still exists in NSCLC patients bearing the activating mutations in exon 2 of the Kirsten rat sarcoma viral oncogene homolog (KRAS) gene.[11] Activating mutations in EGFR exons 19 and 21 have been used as biomarkers of NSCLC sensitivity to EGFR-TKIs, while activating mutations in KRAS exon 2 have been used as biomarkers of resistance to EGFR-TKIs. Clinically, many NSCLCs with MDR are well differentiated carcinomas, and well differentiated adenocarcinomas are generally sensitive to EGFR-TKIs. Therefore, it could be hypothesized that some NSCLCs with primary MDR may be sensitive to EGFR-TKIs or, in other words, that NSCLC patients with primary MDR may benefit from EGFR-TKIs. Both P-glycoprotein (P-gp) and lung resistance-related protein (LRP) are drug transporters in cells, and the expression of these factors is often observed in cancers that have developed MDR.[4,12-16] Recently, the expression levels of these factors have been recognized as major biomarkers of MDR in NSCLC.

In this study, the expression of P-gp and LRP as biomarkers of MDR was detected in NSCLCs by immunohistochemistry, and mutations in EGFR exons 19 and 21 and KRAS exon 2 (as biomarkers of whether the patients can benefit from EGFR-TKIs, respectively) were analyzed by high-resolution melting analysis (HRMA) in 127 NSCLCs. By comparing the expression of P-gp/LRP and EGFR mutation status, we investigated whether patients with NSCLC resistant to traditional chemotherapy would benefit from treatment with EGFR-TKIs.

MATERIALS AND METHODS

Patients and tissue samples
The NSCLC samples were collected from 127 patients who underwent surgical procedures without any traditional chemotherapy or radiotherapy at the affiliated hospitals of Dalian Medical University from July 2007 to May 2009. Seventy-five of these patients were male and 52 were female. The patients had a mean±SD age of 62.1±9.1 years (range 36−80 years); 47 patients had a history of smoking, and 80 patients had no history of smoking. Histologically, the tumor samples consisted of 42 squamous cell carcinomas and 85 adenocarcinomas. Lymph node metastases were found in 64 of the patients. There were 74 patients with well to moderately differentiated tumors and 53 patients with poorly differentiated tumors. All of the tissue samples were collected with the approval of the Ethics Committee of the affiliated hospitals of Dalian Medical University and with the informed consent of the patients or families before the surgical procedures.

Immunohistochemistry
Monoclonal antibodies (Abs) against human P-gp (1:200; clone F4; Sigma-Aldrich Co., LLC, St. Louis, MO, USA) and LRP (1:100; clone 1032; Beijing Golden Bridge Biotechnology Company Ltd., Beijing, China) were used as primary antibodies. Biotin-streptavidin-peroxidase staining with 3, 3′-diamino-benzidine-tetrahydrochloride detection was used. Tumor cells with staining in the cell membrane or cytoplasm were considered to be positive. Each slide was graded blindly according to the percentage of positive tumor cells. The immunoreactivity of P-gp and LRP was scored as negative (-) when the proportion of positive tumor cells was less than 10% or as positive (+) when the number of positive tumor cells was between 10% and 100%.

DNA extraction and gene mutation detection
Tumor-enriched areas were selected and cut from the stained frozen sections that were marked by two pathologists. Genomic DNA was extracted from these areas and purified using the TIANamp Genomic DNA kit according to the manufacturer's protocol (Tiangen, Beijing, China). EGFR exons 19 and 21 and KRAS exon 2 were amplified in triplicate from each sample in a 10-µL reaction volume with a 15-µL mineral oil overlay in each well of a 96-well PCR plate on a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The primers were 5′-TG GATCCCAGAAGGTGAGAA-3′ and 5′-AGCAGAAACTCACAT CGAGGA-3′ (EGFR exon 19); 5′-CGCAGCATGTCAAGATCA-3′ and 5′-CCTCCTTACTTTGCCTCC-3′ (EGFR exon 21); and 5′-A GGCCTGCTGAAAATGACT-3′ and 5′-AATGGTCCTGCACCA GTAA-3′ (KRAS exon 2). The reaction conditions were described previously. The mutations were detected with HRMA on a LightScanner® 96 (Biofire Diagnostics, LLC, Salt Lake City, UT, USA). Melting curves were acquired at temperatures ranging from 60°C to 95°C and analyzed using LightScanner software (version 2.0, Biofire Diagnostics, LLC, Salt Lake City, UT, USA) according to the manufacturer's instructions.[17,18]

Statistics
Pearson chi-square tests were used to analyze correlations for the expression of P-gp and LRP and mutations in EGFR and KRAS with the clinical and pathological characteristics of the patients; similar tests evaluated the correlation between the expression of P-gp and LRP and mutations in EGFR and KRAS. Statistical significance was defined as $p<0.05$. Data analysis was performed using the SPSS 11.5 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

P-gp and LRP expressions and their correlations with the clinicopathological characteristics of NSCLC patients
Of the 127 NSCLC samples analyzed by immunohistochemis-

try, the expression of P-gp was detected in 56 samples, corresponding to a positive expression frequency of 44.1%. LRP was expressed in 84 samples, corresponding to a positive expression frequency of 66.1% of the tested NSCLCs.

The frequency of positive P-gp expression in patients aged >60 years (52.1%, 37/71) was significantly higher than that in the patients aged ≤60 years (33.9%, 19/56) (*p*=0.004), and the frequency of positive P-gp expression in non-smokers was 51.3% (41/80), which was significantly higher than that in smokers (31.9%, 15/47) (*p*=0.034). The frequency of positive P-gp expression in adenocarcinomas was 57.6% (49/85), significantly higher than that in squamous cell carcinomas (16.7%, 7/42) (*p*=0.000). Significant relationships were observed between protein expression and patient age, smoking history, and tumor histology, but no significant relationships were found with patient gender, tumor differentiation, or lymph node metastasis (Table 1).

The frequency of positive LRP expression in female patients was 80.8% (42/52), significantly higher than that observed in male patients (56.0%, 42/75) (*p*=0.004), and the frequency of positive LRP expression in non-smokers was 80.0% (64/80), significantly higher than that observed in smokers (42.6%, 20/47) (*p*=0.000). The frequency of positive LRP expression in adenocarcinomas was 85.9% (73/85), significantly higher than that in squamous cell carcinomas (26.2%, 11/42) (*p*=0.000). We concluded that significant relationships exist between protein expression and patient gender, smoking history, and tumor histology, but not between protein expression and patient age,

tumor differentiation, or lymph node metastasis (Table 1).

EGFR and KRAS mutations detected by HRMA in NSCLC

Of the 127 NSCLC samples analyzed by HRMA, EGFR mutations were detected in 52 samples, corresponding to a mutation frequency of 40.9%; 19 samples contained mutations in exon 19 (Fig. 1) and 33 samples contained mutations in exon 21 (Fig. 2). However, mutations in exon 2 of KRAS were detected in seven samples, corresponding to a mutation frequency of 5.5%.

Correlations between EGFR and KRAS mutations and the clinicopathological characteristics of NSCLC patients

The EGFR mutation frequency in female patients (59.6%, 31/52) was significantly higher than that in male patients (28.0%, 21/75) (*p*=0.000), and the frequency of EGFR mutations in non-smokers (55.0%, 44/80) was significantly higher than that in smokers (17.0%, 8/47) (*p*=0.000). The frequency of EGFR mutations in adenocarcinomas was 58.8% (50/85), which was significantly higher than that in squamous cell carcinomas (4.8%, 2/42) (*p*=0.000); and the frequency of EGFR mutations in well to moderately differentiated tumors (50.0%, 37/74) was significantly higher than that in poorly differentiated tumors (28.3%, 15/53) (*p*=0.014). We concluded that significant relationships exist between EGFR mutation status and patient gender, smoking history, tumor histology, and degree of differen-

Table 1. The Associations between Clinicopathological Characteristics and P-gp or LRP Expression in NSCLC

Clinicopathological characteristics	No.	P-gp			LRP		
		Positive number	Positive frequency (%)	*p* value	Positive number	Positive frequency (%)	*p* value
Gender				0.264			0.004
Male	75	30	40.0		42	56.0	
Female	52	26	50.0		42	80.8	
Age				0.004			0.695
≤60	56	19	33.9		36	64.3	
>60	71	37	52.1		48	67.6	
Smoking history				0.034			0.000
Non-smokers	80	41	51.3		64	80.0	
Smokers	47	15	31.9		20	42.6	
Lymph node metastasis				0.131			0.901
Absent	63	32	50.8		42	66.7	
Present	64	24	37.5		42	65.6	
Histology				0.000			0.000
Adc	85	49	57.6		73	85.9	
SCC	42	7	16.7		11	26.2	
Differentiation grade				0.390			0.435
Well-moderate	74	35	47.3		51	68.9	
Poor	53	21	39.6		33	62.3	

Adc, adenocarcinoma; SCC, squamous cell carcinoma; P-gp, P-glycoprotein; LRP, lung resistance-related protein; NSCLC, non-small cell lung cancer.

tiation, but not between EGFR mutation status and patient age or lymph node metastasis (Table 2).

The KRAS mutation frequency in male patients was 9.3% (7/75), significantly higher than that in female patients (0.0%, 0/52) (p=0.041). We concluded that a significant relationship exists between KRAS mutation status and patient gender, but not between KRAS mutation status and patient age, smoking history, lymph node metastasis, histological type, or differentiation (Table 2).

Correlations of P-gp and LRP expression with EGFR mutation

The EGFR mutation frequency in P-gp-positive samples was 57.1% (32/56); this was significantly higher than that in the P-gp-negative samples (28.2%, 20/71) (p=0.001). The EGFR mutation frequency in the LRP-positive samples was 53.6% (45/84), significantly higher than that in the LRP-negative samples (16.3%, 7/43) (p=0.000). We then selected samples that were positive for P-gp or LRP and analyzed the correlation between the expression in these samples and the presence of EGFR

mutations. We found that the EGFR mutation frequency in these samples was 52.1% (49/94), significantly higher than that in the negative samples (9.1%, 3/33) (p=0.000). We then selected the samples that coexpressed both P-gp and LRP and analyzed the correlation between the coexpression of these proteins and EGFR mutations. We found that the EGFR mutation frequency in these samples was 60.9% (28/46), significantly higher than that in the P-gp- and LRP-negative samples (29.6%, 24/81) (p=0.001). Because the samples with positive expression of either P-gp or LRP could be considered to have MDR, we concluded that the NSCLCs with MDR always bear the EGFR mutations (Table 3, Fig. 3).

Correlations between P-gp and LRP expression and KRAS mutations

The KRAS mutation frequency in the P-gp-positive samples was 5.4% (3/56), lower than that in the negative samples (5.6%, 4/71), although this difference was not significant (p=1.000). The KRAS mutation frequency in the LRP-positive samples was 6.0% (5/84), higher than that in the LRP-negative samples
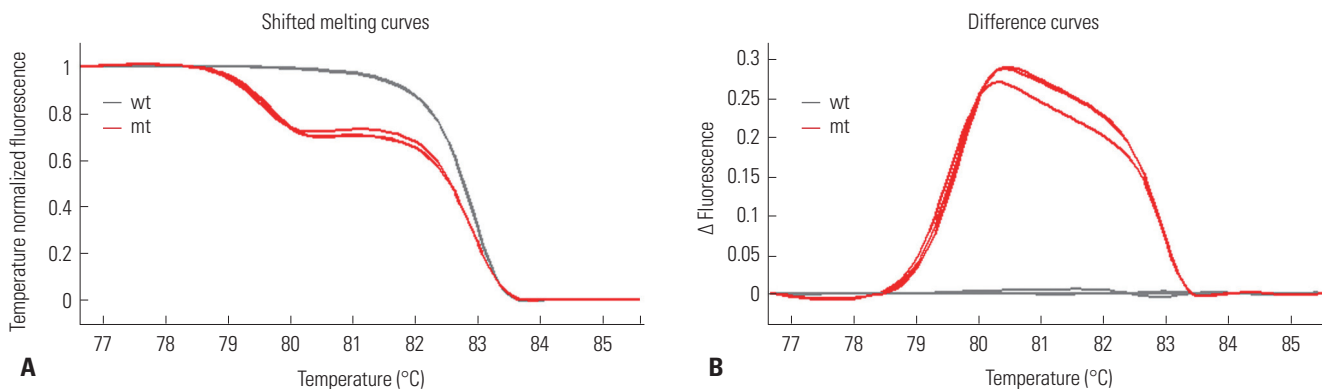
Fig. 1. High-resolution melting curves for exon 19 of the EGFR gene. (A) Temperature shift observed in the melting curves of a NSCLC sample with a mutation in exon 19 of the EGFR and a NSCLC sample with a wild-type EGFR. Each sample was analysed in triplicate. (B) Fluorescence difference curves of the same samples depicted in A. The NSCLC sample with a mutation in EGFR exon 19 is a well differentiated adenocarcinoma from a non-smoking woman. NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.
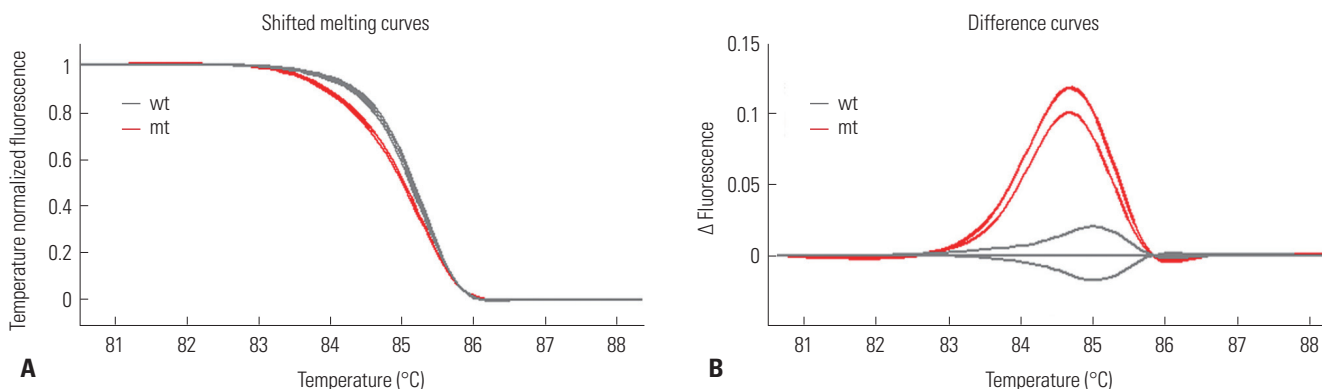
Fig. 2. High-resolution melting curves for exon 21 of the EGFR gene. (A) Temperature shift observed in the melting curves of a NSCLC sample with a mutation in exon 21 of the EGFR and a NSCLC sample with a wild-type EGFR. Each sample was analysed in triplicate. (B) Fluorescence difference curves of the same samples depicted in A. The NSCLC sample with a mutation in EGFR exon 21 is a moderately differentiated adenocarcinoma from a non-smoking woman. NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.

(4.7%, 2/43), although this difference was also not significant (*p*=1.000). Moreover, we selected the samples that were positive for P-gp or LRP and analyzed the correlation between the expression of these proteins and KRAS mutations. We found that the KRAS mutation frequency in these samples was 5.3% (5/94), lower than that in the negative samples (6.1%, 2/33), although this difference was not significant (*p*=1.000). We then selected the samples that coexpressed both P-gp and LRP and analyzed the correlation between the coexpression of these

proteins and the presence of KRAS mutations. We found that the KRAS mutation frequency in these samples was 6.5% (3/46), higher than that in the P-gp- and LRP-negative samples (4.9%, 4/81), although this difference was not significant (*p*= 1.000). Thus, we concluded that no significant relationship exists between MDR and the presence of KRAS mutations in NSCLC (Table 4).

Table 2. The Associations between Clinicopathological Characteristics and EGFR or KRAS Mutations in NSCLC

Clinicopathological characteristics	No.	EGFR status			KRAS status		
		Positive number	Positive frequency (%)	*p* value	Positive number	Positive frequency (%)	*p* value
Gender				0.000			0.041
Male	75	21	28.0		7	9.3	
Female	52	31	59.6		0	0.0	
Age				0.979			0.746
≤60	56	23	41.1		4	7.1	
>60	71	29	40.8		3	4.2	
Smoking history				0.000			0.124
Non-smokers	80	44	55.0		2	2.5	
Smokers	47	8	17.0		5	10.6	
Lymph node metastasis				0.517			0.424
Absent	63	24	38.1		5	7.9	
Present	64	28	43.8		2	3.1	
Histology				0.000			0.327
Adc	85	50	58.8		3	3.5	
SCC	42	2	4.8		4	9.5	
Differentiation grade				0.014			0.648
Well-moderate	74	37	50.0		3	4.1	
Poor	53	15	28.3		4	7.5	

Adc, adenocarcinoma; SCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma viral oncogene homolog; NSCLC, non-small cell lung cancer.

Table 3. The Association of P-gp or LRP Expression with EGFR Mutations in NSCLC

MDR protein	No.	EGFR mutation		
		Number	Frequency (%)	*p* value
P-gp				0.001
Positive	56	32	57.1	
Negative	71	20	28.2	
LRP				0.000
Positive	84	45	53.6	
Negative	43	7	16.3	
P-gp/LRP				0.000
Positive*	94	49	52.1	
Negative	33	3	9.1	
P-gp and LRP				0.001
Positive†	46	28	60.9	
Negative	81	24	29.6	

P-gp, P-glycoprotein; LRP, lung resistance-related protein; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.
*The positive expression of P-gp/LRP was defined as the expression of P-gp or LRP in a sample, †The positive expression of P-gp and LRP was defined as the co-expression of both P-gp and LRP in a sample.
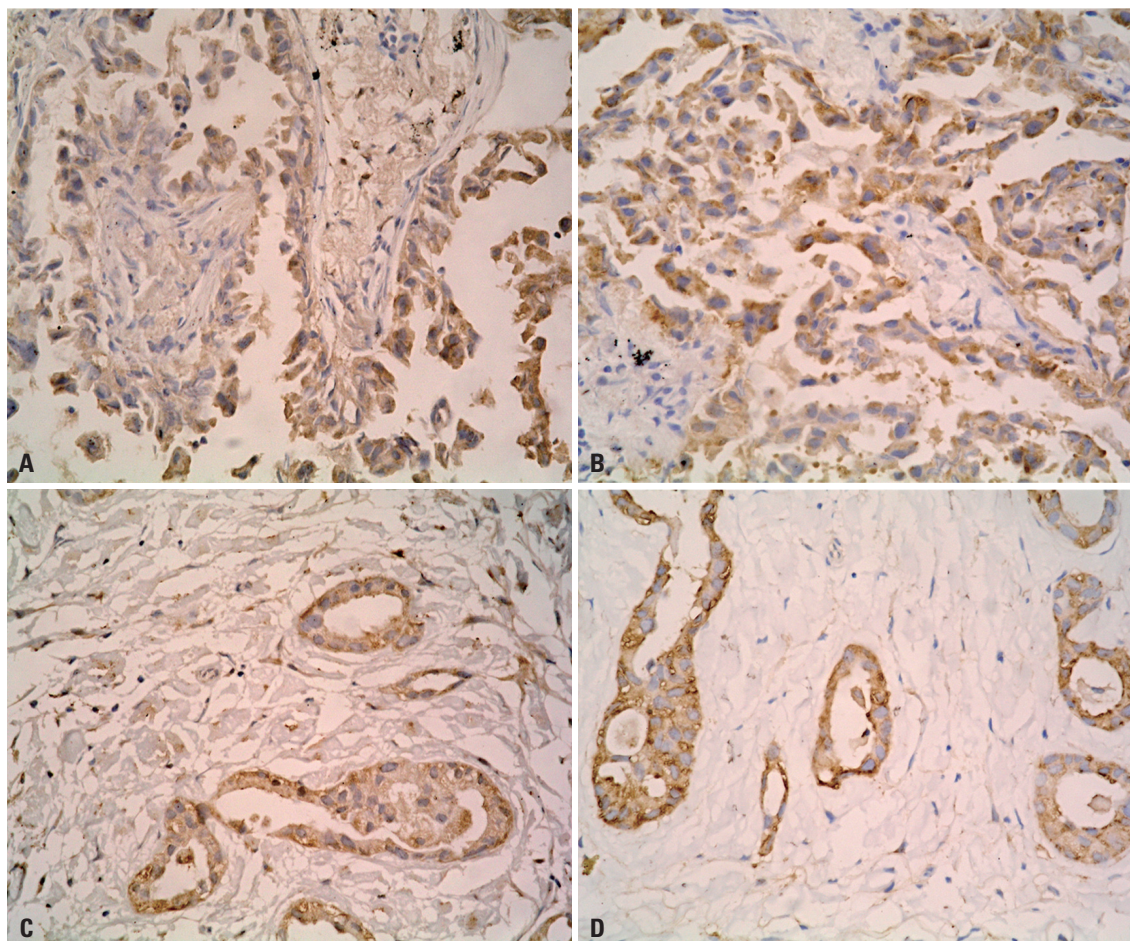
Fig. 3. Immunohistochemical staining of P-glycoprotein and lung resistance-related protein expression in NSCLC samples. Immunohistochemical staining of P-glycoprotein expression (A) and lung resistance-related protein expression (B) in the same sample (a well differentiated adenocarcinoma from a female non-smoker). This was the NSCLC sample with a mutation in exon 19 of the epidermal growth factor receptor, as detected by high-resolution melting analysis, shown in Fig. 1. Immunohistochemical staining of P-glycoprotein expression (C) and lung resistance-related protein expression (D) in the same sample (a moderately differentiated adenocarcinoma from a non-smoking woman). This sample was the NSCLC sample that carried a mutation in exon 21 of the epithemal growth factor receptor, as detected by high-resolution melting, shown in Fig. 2. Original magnification: ×400. NSCLC, non-small cell lung cancer.

Table 4. The Association between the Expression of P-gp or LRP and Mutations in KRAS in NSCLC

| MDR protein | No. | KRAS mutation | | *p* value |
		Nnumber	Frequency (%)	
P-gp				1.000
Positive	56	3	5.4	
Negative	71	4	5.6	
LRP				1.000
Positive	84	5	6	
Negative	43	2	4.7	
P-gp/LRP				1.000
Positive*	94	5	5.3	
Negative	33	2	6.1	
P-gp and LRP				1.000
Positive[†]	46	3	6.5	
Negative	81	4	4.9	

P-gp, P-glycoprotein; LRP, lung resistance-related protein; KRAS, Kirsten rat sarcoma viral oncogene homolog; NSCLC, non-small cell lung cancer.
*The positive expression of P-gp/LRP was defined as the expression of P-gp or LRP in a sample, [†]The positive expression of P-gp and LRP was defined as the co-expression of both P-gp and LRP in a sample.

DISCUSSION

NSCLC accounts for approximately 85% of lung cancers.[19] Traditional chemotherapy has played an important role in the treatment of NSCLC, although its curative effect has been greatly limited by MDR. It is well known that the occurrence of MDR is closely related to the expression of P-gp/LRP in NSCLCs. P-gp is a 170-kDa transmembrane protein that was first discovered in 1976. P-gp can decrease the concentration of various drugs in the cytoplasm by extruding them out of cells and promoting the development of MDR.[20-23] LRP, which was initially identified in a non-P-gp MDR lung cancer cell line, is a major component of the human major vault protein.[24-28] LRP can decrease the concentration of chemical drugs in the nucleus by regulating nucleocytoplasmic transport and the drugs that are transported to the cytoplasm can then be extruded from the cancer cell by exocytosis.[16,29-31] Previous studies have reported that P-gp is expressed in 35−52% of NSCLCs and LRP is expressed in 65−88% of NSCLCs.[29,32,33] The expression of P-gp/LRP could be regarded as a biomarker of primary MDR in NSCLCs. In this study, we found that the frequencies of positive expression of P-gp and LRP detected by immunohistochemistry were 44.1% and 66.1% respectively, which was consistent with the frequencies reported previously, thus the expressions of them were reliable for used as biomarkers to indicate primary MDR of the patients in this study.

In this research, clinicopathological characteristics of the patients with NSCLCs expressing P-gp/LRP positively were similar to those bearing EGFR mutation in exon 19 or 21. Generally, patients with NSCLCs bearing these mutations could benefit from EGFR-TKI. Therefore, one could speculate that patients with NSCLCs expressing P-gp/LRP would also benefit from EGFR-TKIs. Recently, mutation in EGFR exon 19 or 21 has been used as a biomarker for sensitivity of NSCLCs to EGFR-TKIs.[34-37] Thus, we analyzed correlations of P-gp/LRP expression with EGFR mutation status to investigate whether patients with NSCLCs expressing P-gp/LRP would bear the EGFR mutation in exon 19 or 21. We found that the EGFR mutation frequency in cancers expressing P-gp/LRP was significantly higher than in those without. These results suggest that NSCLCs expressing P-gp/LRP bear the EGFR mutation in exon 19 or 21. As P-gp/LRP expression was found to be a biomarker for MDR, we discerned that NSCLC patients who are not fit for traditional chemotherapy due to cancers with MDR may benefit from EGFR-TKIs.

ACKNOWLEDGEMENTS

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