Association of EGFR and KRAS mutations with expression of p-AKT, DR5 and DcR1 in non-small cell lung cancer

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The activation of AKT is one of the causes of resistance to epidermal growth factor receptor (EGFR)– tyrosine kinase inhibitors (TKIs). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) combines with related receptors to trigger apoptosis or protect the cells against TRAIL apoptosis. This research focused on the association of EGFR and KRAS mutations with expression of AKT, p-AKT, DR5 and DcR1 in non-small cell lung cancer. 82 NSCLC patients were included in the study. xTAG liquichip techonolgy (xTAG-LCT) was applied to investigate the genetic mutation of EGFR and KRAS, Quantitative Real-time PCR was used to test the mRNA expression of AKT, DR5 and DcR1 and Western Blot was applied to test the protein expression of AKT, p-AKT, DR5, and DcR1. We found that of 82 patients, 31 cases had EGFR-activating mutations, more common in female, adenocarcinoma, and non-smoker patients; 9 cases had KRAS mutations, frequently found in patients with smoking history. The expression of AKT and p-AKT correlated with staging, tumor differentiation, and lymph node metastasis. The expression of DR5 in phase III and low differentiation tumor was significantly higher than that in phase I+II and high and median differentiation tumor; the expression of DcR1 in phase III and low differentiation tumor was significantly lower than that in phase I+II and high and median differentiation tumor. Compared with EGFR and KRAS wild type, in NSCLC tissue with EGFR and KRAS mutations, the expression of AKT and p-AKT was significantly higher. These results suggest that EGFR and KRAS mutation status was associated with the expression of AKT and p-AKT. AKT, p-AKT, DR5, and DcR1 all took part in the occurrence and development of NSCLC, and may become a reference index to evaluate the prognosis of NSCLC.

Key words: epidermal growth factor, KRAS, p-AKT, receptor, carcinoma, non-small-cell lung

So far non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide. An important therapeutic strategy of advanced NSCLC is the multidisciplinary synthetic therapy including traditional chemotherapy and targeted drug therapy. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have shown significant therapeutic effect in NSCLC with EGFR mutations [1]. But the drug resistance of EGFR TKIs remains one of the difficulties to be solved urgently in clinical practice. EGFR is a transmembrane receptor with tyrosine kinase activity encoded by oncogene erbB. Under the stimulation of ligands such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), the receptor is autophosphorylated, leading to the activation of three downstream signal transduction pathways, PI3K/AKT, RAS/RAF-MAPK, and STAT3, which transduct signal into cells, so that regulate cell proliferation, anti-apoptosis, and angiogenesis, and promote the formation and development of tumor [2, 3]. Preliminary studies have indicated that the activation of PI3K/AKT/mTOR signaling pathway is one of the causes of resistance to EGFR-TKIs, but the specific mechanism is not clear [4], may be related to the higher activation of mutant EGFR than wild type EGFR, which further induces the higher activation of AKT and STAT [5]. Through phosphorylation of a series of downstream signaling pathways, the activated AKT can promote the growth and proliferation of tumor cells, inhibit cell apoptosis, and promote

the invasion and metastasis of tumor cells [6, 7]. Dysregulation of EGFR/PI3K/AKT/mTOR pathway has an important effect in the formation of lung cancer. AKT was reported to be activated in most of the lung cancers and its phosphorylation or overexpression is related to the poor prognosis of NSCLC patient. Researches that combine conventional chemotherapy with AKT kinase inhibitory have been proven valid in delaying chemotherapeutic resistance in NSCLC [8].

KRAS gene is an important downstream molecule in the EGFR signal transduction pathway, and about 15%– 30% NSCLC has KRAS mutations [9]. The mutations mostly occur in the point mutation of exon 2 or exon 3 of KRAS, which are common in patients with heavy smoking, and seldom co-exist with EGFR mutations. Studies have reported that KRAS mutation can be the negative factor for the prognosis of NSCLC patient, and KRAS mutation has been proven to be negative related to the treatment effects of Erlotinib and Gefitinib. The cause may be that KRAS mutation results in ERK and/or AKT activation which is associated with drug resistance to EGFR TKIs [10].

Tumor necrosis factor superfamily (TNFSF) plays an important role in the induction of apoptosis. TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL-R are the essential ligand and its associated receptors of TNFSF, respectively [11]. TRAIL combines with death receptors DR4 and DR5, and initiates apoptosis. TRAIL combines with decoy receptors DcR1 and DcR2 and protects cells against TRAIL apoptosis [12, 13]. The intracellular region of DR5 contains a death domain, which is highly expressed in a variety of tumor tissues including NSCLC. Studies have shown that at 37 °C TRAIL has the maximum binding force with DR5 [14]. This characteristic allows DR5 to surpass other receptors and has the highest affinity with TRAIL when the cells of body are at the most suitable survival temperature of 37 °C, thus maximizing the function of TRAIL-induced apoptosis. Lacking intracellular death domain, DcR1 competitively prevents the combination of TRAIL and DR5 and plays a role in inhibiting apoptosis [15]. The change of DR/DcR expression level will regulate the sensitivity of cells to TRAIL-mediated apoptosis. The expression of TRAIL receptors and their intensities will to a large extent affect the future clinical application of TRAIL. In NSCLC, the expression of DR4, DR5, and TRAIL in poorly differentiated tumors is normally increased, and the expression of DR5 is associated with patient's death [16]. This provides a basis for TRAIL and agonistic antibodies of targeted DR4 or DR5 in treating NSCLC. Studies have shown that a variety of NSCLC cells are resistant to TRAIL [17]. TRAIL resistance largely comes from the downward expression of DR5 as well as the activation of AKT phosphorylation. Shrader etc. reported that Gefitinib could regulate the activity of AKT, down-regulate the expression of XIAP, and further enhance TRAIL-induced apoptosis in patients with NSCLC [18]. The activation of death receptor pathway can directly induce the apoptosis of tumor cells, which may provide a new mode of treating NSCLC, and further overcome drug resistance in cells. But the expression and distribution of TRAIL receptor in NSCLC are still not clear, and need further research.

In this study, we detected EGFR or KRAS mutation and the protein expression of p-AKT, DR5, and DcR1 in patients with NSCLC. In addition, the correlations of the above genetic mutations and protein expressions to the clinicopathological features were also investigated to provide a certain basis for the clinical application of TRAIL and death receptor agonistic antibody.

Patients and methods

Study population. 82 cases of NSCLC confirmed by surgery and pathology were collected from the Second Affiliated Hospital of Harbin Medical University from March 2012 to December 2015. All patients joined this study were approved by the ethics committee of the Second Affiliated Hospital of Harbin Medical University. Each sample was divided into two parts. Tumor tissue was taken from one part of the sample in vitro within 30 min, and quickly put into -180 °C liquid nitrogen, and then preserved at -80°Clow-temperature refrigerator. The other part of the sample was fixed in 10% formalin solution. The specimen was embedded in paraffin, and sectioned at 4μm thickness. The above two parts of the specimen all used pericarcinous lung tissue as the control (cancer margin distance > 2 cm, microscopy showed no cancerous invasion and metastasis). Patients had not received chemotherapy or radiotherapy before surgery. Patients aged 33-72 years old, average age 52.35 years old. The TNM classification was determined according to the 7th version of the International Association for the Study of Lung Cancer Staging System [19]. Detailed clinical and pathological data was shown in Table 1.

Detection of EGFR and KRAS genetic mutation by xTAG Liquichip Technology (xTAG-LCT). DNA extraction kit was used to extract DNA from tissue sample. Gene segments with common alleles in various gene exons were obtained by using multiplex polymerase chain reaction (PCR). PCR reaction system contained 13.8 μl H_2O, 10 μl 5×PCR buffer, 4 μl 25 mM $MgCl_2$, 2 μl dNTP mixture, various gene exon primers mixture 10 μl, 0.2 μl DNA polymerase, and 10μl sample. PCR reaction condition was annealing temperature at 52 °C for 30 cycles. Then PCR product was digested by exonuclease and hydrolysed by alkaline phosphatase to remove redundant primers and dNTP. The reaction system contained 0.5 μl exonuclease, 0.5 μl alkaline phosphatase, 10 μl 2× buffer, 9 μl PCR product, and enzyme hydrolysis reaction at 37 °C for 2 hours. Then with the specific sequence on the allele specific primer extension (ASPE), the product specifically identified each allele and formed the extension product. ASPE reaction system contained 7.8 μl H_2O, 2 μl 10×PCR buffer, 0.5 μl 25mM $MgCl_2$, 1 μl dNTP (without dCTP) mixture, 0.5 μl biotinylated dCTP, 5 μl various allele primers mixture, 0.2 μl DNA polymerase, and 3 μl enzyme digestion hydrolysis product. ASPE reaction condition was annealing temperature at 56 °C for 30 cycles. Tag sequence on ASPE primer specifically combined with

Table 1. Clinicopathological Characteristics of patients with NSCLC (N=82)

Characteristics	No. of patients (%)
Age(years)	
Median(range), y	54(33-72)
≥60	36(43.90)
<60	46(56.10)
Gender	
Male	45(54.88)
Female	37(45.12)
Stage	
I	27(32.93)
II	30(36.59)
III	25(30.49)
Histopathology	
AD	47(57.32)
SC	35(42.68)
Tumor differentiation	
Well differentiated	16(19.51)
Moderately differentiated	29(35.37)
Poorly differentiated	37(39.9)
Lymph node metastasis	
Yes	44(53.66)
No	38(46.34)
Smoking status	
Yes	42(51.22)
No	40(48.78)

AD, adenocarcinoma; SC, squamous cell carcinoma

Anti-Tag sequence on polystyrene microsphere, which was hybridization reaction. The hybridization reaction system contained 12 µl microspheres, 33 µl hybridization solution, and 5 µl ASPE product. The hybrid system went through denaturation at 95 °C for 3 minutes. After hybridization at 37 °C for 30 minutes, 25 µl SA-PE working solution were added to the system, reaction was continued at 37 °C for 10 minutes. Luminex 200 system was applied to analyze the fluorescence value of each sample.

Primers for EGFR and KRAS gene target sequence were designed and synthesized by Guangzhou Surexam Biotechnology Company. Primer sequences were shown in Supplementary Tables 1 and 2.

Quantitative real-time PCR. Total RNA from the human tissue samples were extracted using TRIzol reagent (Invitrogen). Reverse transcription reaction with 1 µg of total RNA in 100 µl was carried out using M-MLV Reverse Transcriptase (D2640A,Takara, Japan). For quantitative PCR, 10 µl SYBR Premix Ex Taq (Takara, Japan) with 10 µM of each primer and 10 ng cDNA template in 20 µl of reaction volume was applied. Primers used for amplification were designed as: AKT, forward, 5′- CTTGCTTTCAGGGGCT-GCTCA-3′ and reverse, TACACGTGCTGCCACACGATAC; DR5, forward, 5′-CACCACGACCAGAAACACAG-3′

and reverse, 5′-AATCACCGACCTTGACCATC-3; DcR1, forward, 5′-TGCCTGAATCATACTCCTT-3′ and reverse, 5′-CCTGTGAATGTTCCTGGGT-3′; β-actin (as an internal control), forward, 5′-CCTGTACGCCAACACAGTGC-3′ and reverse, 5′- ATACTCCTGCTTGCTGATCC-3′. All reactions were performed on the TaKaRa PCR Thermal Cycler Dice. To calculate values of mRNA levels, 2(-Delta Delta C(T)) method was used, where CT is the threshold cycle. Target gene mRNA levels were normalized to that of actin mRNA in the same sample.

Antibodies and western blotting. Anti-AKT, anti-phospho-AKT, anti-DR5, anti-DcR1 and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse and goat anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA, USA). Total protein lysates were obtained from tissue using lysis buffer including 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and one Complete Mini Protease Inhibitor Cocktail tablet (Roche, Indianapolis, IN). The lysates were centrifuged at 10,000 x g for 30 minutes at 4 °C and the supernatant was collected. Then total proteins were extracted and protein concentrations were determined by Bradford assay. Took 100 µg sample, denatured for 5 minutes, and used 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. The separated proteins were transferred onto PVDF membranes (IPVH00010, Millipore). After electro-transfer, membranes were blocked with 5% nonfat milk in TBS-T buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Added the first antibody, incubated, and shook overnight at 4 °C. After washing three times with TBST, added the second antibody labeled with horseradish peroxidase, and incubated at room temperature for 1 hour. Optical density of the bands was determined using the LabWork software (UVP Laboratory Products, CA). All experiments were repeated at least three times.

Statistical analysis. The association between EGFR-activating mutations or KRAS mutations and clinicopathological factors was evaluated using the χ^2 test. The correlations between the mRNA and protein expression of factors examined and clinicopathological features were evaluated by Student's t-test. In order to assess the significant differences between mutation status and mRNA or protein expression of factors, we performed one-way analysis of variance (ANOVA). Tukey's test was used for post-hoc analysis. The measurement data was expressed as means with standard deviation (SD). A value of $P<0.05$ was considered as statistically significant.

Results

EGFR and KRAS mutation analysis. 36 patients out of 82 patients had EGFR mutations, accounting for 43.90%, among which exon 18 mutation 2 cases (2.44%): G719A 1 case, G719S 1 case; exon 19 deletion 15 cases (18.29%); exon 20 threonine to methionine codon 790 (T790M) mutation

3 cases (3.66%); exon 21 leucine to arginine codon 858 (L858R) missense mutation 16 cases (19.51%). No patient with multiple EGFR mutations was found. EGFR-activating mutations (exon 19 deletion and L858R missense mutation in exon 21) accounted for 86.11% (31/36) of all patients, and other rare mutations accounted for 13.89%. The analysis of relationship between EGFR-activating mutations and clinicopathological data showed that EGFR-activating mutations were associated with gender, pathological type, and smoking status (Table 2). 67.57% (25/37) women had EGFR-activating mutations, which was significantly higher than that of male patients [13.33% (6/45)] (χ^2=25.401, P<0.001). The EGFR-activating mutation rate of patient with adenocarcinoma was significantly higher than that of squamous cell carcinoma (51.06% $vs.$ 20.00%, χ^2=8.233, P=0.004). The EGFR-activating mutation rate of patient with smoking history was significantly lower than that of patient with non-smoking history (23.81% $vs.$ 52.50%, χ^2=7.172, P=0.007) (Table 2).

Nine patients out of 82 patients had KRAS mutations (10.98%), among which exon 2 mutation 4 cases (4.88%), exon 3 mutation 5 cases (6.10%). KRAS mutations were relevant to the smoking status of patient. KRAS mutation ratio was significantly higher in patients with smoking history than those without smoking history (19.05% $vs.$ 2.50%, χ^2=5.742, P=0.017) (Table 2).

The mRNA expression of AKT, DR5 and DcR1 in NSCLC tissue and their relationships with clinicopathological factors. The mRNA expression of AKT, DR5 and DcR1 in 82 cases of lung cancer and pericarcinous tissue was detected by Real-time PCR. As shown in Figure 1, the relative amount of AKT mRNA in NSCLC tissue was 0.107±0.009, while the relative amount of AKT mRNA in pericarcinous tissue was 0.099±0.008, so the difference was statistically significant (t=6.372, P<0.001). The expression of AKT mRNA in NSCLC tissue was associated with lung cancer staging, tumor differentiation, and lymph node metastasis (Table 3). The relative amount of DR5 mRNA in NSCLC tissue was 0.093±0.008, which was significantly higher than the relative amount of DR5 mRNA in pericarcinous tissue (0.088±0.007) (t=4.391, P<0.001) (Figure 1). The expression of DR5 mRNA in phase III NSCLC and poorly differentiated tumor was significantly higher than that in phase I + II and high and median differentiated tumor (Table 3), whereas the relative amount of DcR1 mRNA in NSCLC tissue was 0.091±0.009, which was significantly lower than that in the paired pericarcinous tissue (0.097±0.006) (t=5.234, P<0.001) (Figure 1). The expression of DcR1 mRNA in phase III NSCLC and poorly differentiated tumor was significantly lower than that in phase I + II and high and median differentiated tumor (Table 3).

Table 2. The correlation between EGFR active mutation (exons 19 and 21) or KRAS mutation and clinicopathological factors

Characteristics	N	EGFR			KRAS		
		Mutation (%)	χ^2	P	Mutation (%)	χ^2	P
Age(years)							
≥60	36	12(33.33)			4(11.11)		
<60	46	19(41.30)	0.546	0.460	5(10.87)	0.001	0.972
Gender							
Male	45	6(13.33)			6(13.33)		
Female	37	25(67.57)	25.401	<0.001	3(8.11)	0.567	0.451
Stage							
I	27	10(37.04)			3(11.11)		
II	30	12(40.00)			4(13.33)		
III	25	9(36.00)	0.103	0.950	2(8.00)	0.398	0.820
Histopathology							
AD	47	24(51.06)			5(10.64)		0.204
SC	35	7(20.00)	8.233	0.004	4(11.43)	0.013	0.910
Tumor differentiation							
Well differentiated	16	6(39.5)			2(12.50)		
Moderately differentiated	29	10(34.48)			4(13.79)		
Poorly differentiated	37	15(40.54)	0.255	0.881	3(8.11)	0.585	0.746
Lymph node metastasis							
Yes	44	17(38.64)			4(9.09)		
No	38	14(36.84)	0.028	0.867	5(13.16)	0.345	0.557
Smoking status							
Yes	42	10(23.81)			8(19.05)		
No	40	21(52.50)	7.172	0.007	1(2.50)	5.742	0.017

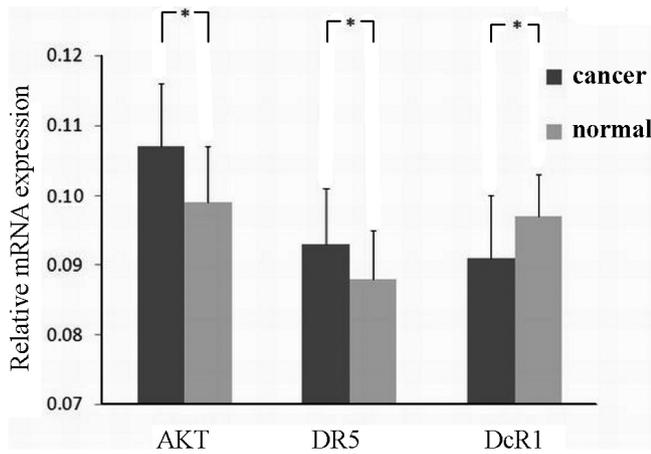AD, adenocarcinoma; SC, squamous cell carcinoma

Figure 1. The mRNA expression of AKT, DR5 and DcR1 in NSCLC tissue. The mRNA expression was detected by realtime PCR. Cancer refers lung cancer tissue. Normal refers to pericarcinous tissue. The figure is representative of one of three independent experiments. This panel reflects the ratios of AKT, DR5 and DcR1 to β-actin, respectively. Compared with the normal group, *P<0.01.
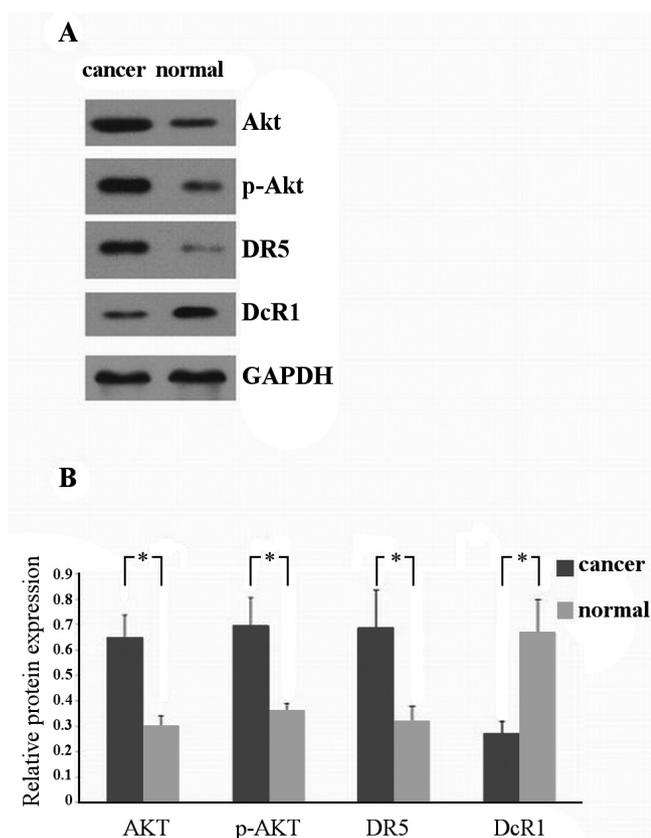
The protein expression of AKT, p-AKT, DR5 and DcR1 in NSCLC tissue and their relationships with clinicopathological factors. The protein expression of AKT, p-AKT, DR5 and DcR1 in 82 cases of frozen lung cancer and pericarcinous tissue was detected by Western Blotting.

As shown in Figure 2A, the expression of AKT, p-AKT and DR5 in lung cancer tissue was significantly higher than that in pericarcinous tissue, while the expression of DcR1 was significantly lower than that in pericarcinous tissue. Film scanning analysis showed that the relative amount of AKT protein in NSCLC tissue was 0.65±0.09, while the relative amount of AKT protein in pericarcinous tissue was 0.30±0.04, so the difference was statistically significant (t=3.007, P=0.004) (Figure 2B). The relative amount of p-AKT protein in NSCLC tissue was 0.70±0.11, while the relative amount of p-AKT protein in pericarcinous tissue was 0.36±0.03, so the difference was statistically significant (t=2.786, P=0.007) (Figure 2B). The expression of AKT and p-AKT protein in NSCLC tissue was associated with lung cancer staging, tumor differentiation, and lymph node metastasis (Table 4). The relative amount of DR5 protein in NSCLC tissue was 0.69±0.11, which was significantly higher than the relative amount of DR5 protein in pericarcinous tissue (0.32±0.06) (t=2.774, P=0.007) (Figure 2B). The expression of DR5 protein in phase III NSCLC and poorly differentiated tumor was significantly higher than that in phase I + II and high and median differentiated tumor (Table 4), whereas the relative amount of DcR1 protein in NSCLC tissue was 0.27±0.05, which was significantly lower than that in the paired pericarcinous tissue (0.67±0.13) (t=2.738, P=0.008) (Figure 2B). The expression of DcR1 protein in phase III NSCLC and poorly differentiated tumor

Table 3. The correlation between AKT, DR5 and DcR1 mRNA expression and clinicopathological factors

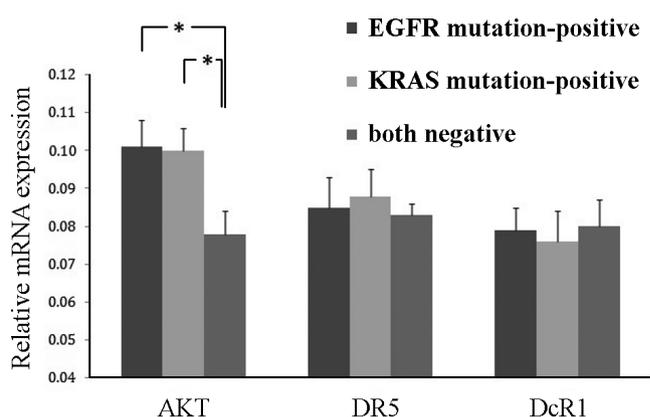| Characteristics	N	AKT			DR5			DcR1		
		$\bar{x}_{\pm s}$	t	P	$\bar{x}_{\pm s}$	t	P	$\bar{x}_{\pm s}$	t	P
Age(years)										
≥60	36	0.071±0.002			0.084±0.005			0.103±0.006		
<60	46	0.072±0.003	1.722	0.089	0.083±0.003	1.124	0.265	0.101±0.006	1.498	0.138
Gender										
Male	45	0.093±0.008			0.101±0.008			0.095±0.007		
Female	37	0.091±0.002	1.482	0.142	0.104±0.009	1.597	0.114	0.098±0.008	1.811	0.074
Stage										
I+II	57	0.071±0.006			0.067±0.006			0.084±0.007		
III	25	0.075±0.004	3.046	0.003	0.072±0.006	3.476	0.001	0.079±0.005	3.225	0.002
Histopathology										
AD	47	0.093±0.007			0.075±0.008			0.106±0.011		
SC	35	0.095±0.008	1.204	0.232	0.077±0.002	1444	0.153	0.103±0.009	1.318	0.191
Tumor differentiation										
Well – Moderately differentiated	45	0.068±0.007			0.065±0.004			0.083±0.007		
Poorly differentiated	37	0.072±0.006	2.745	0.007	0.069±0.006	3.607	0.001	0.078±0.004	3.858	<0.001
Lymph node metastasis										
Yes	44	0.086±0.008			0.098±0.007			0.106±0.006		
No	38	0.081±0.005	3.332	0.001	0.096±0.006	1.378	0.172	0.103±0.009	1.798	0.076
Smoking status										
Yes	42	0.094±0.005			0.105±0.007			0.086±0.008		
No	40	0.096±0.006	1.643	0.104	0.108±0.009	1.690	0.095	0.084±0.009	1.065	0.290

AD, adenocarcinoma; SC, squamous cell carcinoma

Figure 2. The protein expression of AKT, p-AKT, DR5 and DcR1 in NSCLC tissue. A: The protein expression was detected by western blot with corresponding antibodies. Cancer refers lung cancer tissue. Normal refers to pericarcinous tissue. The figures are representatives of one of three independent experiments. B: This panel reflects the ratios of AKT, p-AKT, DR5 and DcR1 to GAPDH, respectively. Compared with the normal group, *P<0.01.

Figure 3. The mRNA expression of AKT, DR5 and DcR1 according to different EGFR and KRAS mutation status. This panel reflects the ratios of AKT, DR5 and DcR1 to β-actin, respectively. Compared with the both negative group, *P<0.01, respectively.
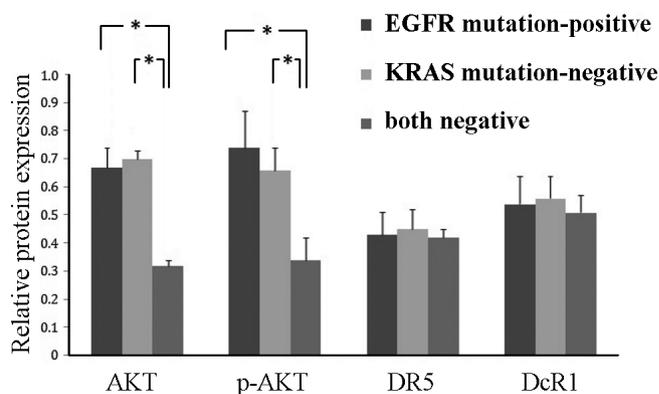
Figure 4. The protein expression of AKT, p-AKT, DR5 and DcR1 according to different EGFR and KRAS mutation status. This panel reflects the ratios of AKT, p-AKT, DR5 and DcR1 to GAPDH, respectively. Compared with the both negative group, *P<0.01, respectively.

was significantly lower than that in phase I + II and high and median differentiated tumor (Table 4).

The relationship of EGFR or KRAS mutation Status to expression of AKT, p-AKT, DR5 and DcR1. According to the genetic state of EGFR and KRAS in patients with lung cancer, NSCLC tissue samples were divided into three groups: an EGFR-activating mutation group, a KRAS mutation group, and a group that was negative for both biomarkers. Figure 3 showed that the expression of AKT mRNA in NSCLC tissue with different EGFR/KRAS mutation status had significant difference (F=54.065, *P*<0.001). The expression of AKT mRNA in NSCLC tissue with EGFR-activating mutations (*P*<0.001) and KRAS mutations (*P*<0.001) was significantly higher than that in NSCLC tissue with EGFR/KRAS wild type. There was no significant difference between the mRNA expression of AKT in NSCLC tissue with EGFR-activating mutations and KRAS mutations (*P*=0.211). However, the mRNA expression of DR5 and DcR1 in NSCLC tissue with different EGFR or KRAS mutation status showed no significant difference (Figure 3). Figure 4

showed that the expression of AKT and p-AKT protein in NSCLC tissue with different EGFR/KRAS mutation status had significant difference (F=95.951, P<0.001; F=40.086, P<0.001, respectively). The expression of AKT and p-AKT protein in NSCLC tissue with EGFR-activating mutations (both P<0.001) and KRAS mutations (both P<0.001) was significantly higher than that in NSCLC tissue with EGFR/KRAS wild type. There was no significant difference between the protein expression of AKT and p-AKT in NSCLC tissue with EGFR-activating mutations and KRAS mutations (*P*=0.104). However, the protein expression of DR5 and DcR1 in NSCLC tissue with different EGFR or KRAS mutation status showed no significant difference (Figure 4). The results indicated that EGFR and KRAS mutation status was associated with the expression of AKT and p-AKT, and had nothing to do with the expression of DR5 and DcR1.

Table 4. The correlation between AKT, p-AKT, DR5 and DcR1 protein expression and clinicopathological factors

Characteristics	N	p-AKT			DR5			DcR1		
		$\bar{x}_{\pm s}$	t	P	$\bar{x}_{\pm s}$	t	P	$\bar{x}_{\pm s}$	t	P
Age(years)										
≥60	36	0.53±0.11			0.48±0.07			0.53±0.15		
<60	46	0.56±0.09	1.359	0.178	0.46±0.05	1.509	0.135	0.55±0.14	0.622	0.536
Gender										
Male	45	0.50±0.12			0.55±0.14			0.46±0.12		
Female	37	0.51±0.12	0.376	0.708	0.52±0.13	0.997	0.322	0.43±0.13	1.085	0.281
Stage										
I+II	57	0.44±0.08			0.49±0.04			0.67±0.14		
III	25	0.79±0.14	14.405	<0.001	0.77±0.15	13.215	0.000	0.40±0.08	9.023	<0.001
Histopathology										
AD	47	0.61±0.15			0.56±0.08			0.56±0.10		
SC	35	0.58±0.09	1.050	0.297	0.59±0.10	1.509	0.135	0.59±0.11	1.288	0.202
Tumor differentiation										
Well – Moderately differentiated	45	0.34±0.08			0.55±0.09			0.63±0.07		
Poorly differentiated	37	0.74±0.13	17.194	<0.001	0.61±0.06	3.471	0.001	0.58±0.06	2.745	0.007
Lymph node metastasis										
Yes	44	0.65±0.14			0.58±0.04			0.59±0.04		
No	38	0.57±0.07	3.194	0.002	0.56±0.05	1.006	0.318	0.60±0.03	1.264	0.210
Smoking status										
Yes	42	0.45±0.12			0.51±0.06			0.53±0.07		
No	40	0.40±0.14	1.739	0.086	0.54±0.10	1.657	0.102	0.55±0.06	1.386	0.170

AD, adenocarcinoma; SC, squamous cell carcinoma

Discussion

The treatment effect and prognosis of NSCLC patients, which were of the same clinical stage, the same pathological type, and the same therapy, were commonly significantly different. The difference of treatment effect and prognosis may be due to the existence of genetic heterogeneity, such as the different mutation status of EGFR and KRAS genes. Researches showed that after applying Gefitinib and Erlotinib on behalf of TKI therapy, the remission rate of NSCLC patients with EGFR exon 19 deletion and exon 21 L858R point mutation was significantly higher than that of EGFR wild-type patients [20]. However, similar to chemotherapy, TKI therapy also inevitably faced the issue of drug resistance. Moreover, resistant to various TKI, NSCLC patients with KRAS mutations often predict worse treatment effect and prognosis. This research selected NSCLC patients with different EGFR and KRAS genetic state, analyzed the relationship of EGFR and KRAS genetic state in lung cancer tissue to the expression of p-AKT and TRAIL receptor, and investigated further opportunities for improving the treatment effect and prognosis of NSCLC.

Our research found that 36 patients out of 82 patients had EGFR mutations at different loci, accounting for 43.90%, which was in accordance with the reported Asian EGFR mutation rate (39%-64%) [21]. EGFR mutation mainly focused on the deletion of exon 19 and L858R missense

mutation of exon 21 (about 86%), that was relatively close to the literature's reported 90% [22]. Further analysis of the relationship between EGFR-activating mutations and clinicopathological characteristics showed that the incidence of EGFR-activating mutations in female, adenocarcinoma and non-smoking patients was significantly increased, which was consistent with previous studies [23, 24]. However, although many researches have shown that EGFR mutation was common in Asian female NSCLC patients, and the treatment effectiveness of EGFR TKIs in Asian female NSCLC patients was better than that of male patients with NSCLC [25], so from the aspect of clinical sign, it seemed that EGFR TKIs had better treatment effect in Asian female patients. However, further analysis showed that, in Europe and the United States, more than 80% of the female patients with NSCLC were smokers, while the situation in China was different from that in Western countries, in China most of the women were non-smokers, indicating that the difference of EGFR TKIs treatment effect between men and women might mainly be due to the impact of whether smoking or not, and not much associated with gender. In addition, in NSCLC patients, KRAS gene mutation was a common gene abnormality associated with smoking. Our research also confirmed the correlation between KRAS gene mutation and smoking in NSCLC patients. Since KRAS mutation was relevant to the poor prognosis of NSCLC [26], therefore, it

might be speculated that among the many clinicopathological factors associated with the prognosis of NSCLC patients, smoking seemed to play a more vital role. The smoking history of patient with NSCLC had a serious impact on the treatment effect of EGFR TKIs.

EGFR in lung cancer cells was often overexpressed or mutated, and was in an abnormal state of activation. EGFR was activated or autophosphorylated by the ligand EGF, activating the intracellular signaling proteins, mainly the RAS– RAF– MEK– MAPK and PI3K- AKT signal pathway. The inhibition of phosphorylation of ERK or AKT can block MAPK, AKT signaling pathway, respectively, which can further blocklung cancer cell proliferation, angiogenesis, invasion, metastasis, and other processes induced by EGFR activation [27]. Besides, the phosphorylation of AKT was also one of the important causes of chemoresistance. Studies have shown that the combined application of PI3K/AKT pathway inhibitors and conventional chemotherapy can delay the occurrence of chemotherapy resistance [28]. EGFR TKIs can inhibit the activity of this proliferation pathway and restart cell apoptosis. So we detected the expression of AKT and its relevant phosphorylated form p-AKT in lung cancer and normal tumor-adjacent lung tissues, respectively, and analyzed the relationship among gene expression, clinical pathological factors, and driver gene status. Our research indicated that no matter the mRNA level or protein level of AKT expression in lung cancer tissues was significantly higher than that in the normal cancer-adjacent tissues, and the expression was much higher in the NSCLC tissues of late stage, poorly differentiation, and with lymph node metastasis, suggesting that the AKT gene exerted its biological functions through transcriptional regulation; while in lung cancer tissues, the phosphorylation level of AKT protein was significantly higher than that in adjacent tissues, and also the expression was much higher in the NSCLC tissues of late stage, poorly differentiation, and with lymph node metastasis, indicating the expression of AKT and its phosphorylated form may be positively correlated with tumor invasion, metastasis, and prognosis. EGFR activity mutation or KRAS mutation could significantly enhance the expression of mRNA and protein in AKT and the phosphorylation of AKT in NSCLC tissues, indicating that the activation of EGFR signaling pathway, which was mediated by the mutation of EGFR or KRAS, played an important role in the occurrence and development of NSCLC.

The sensitivity of tumor cells to TRAIL depended on the ratio of DR to DcR on the target cell membrane. High expression of DR contributed to the apoptosis of tumor cells, and high expression of DcR helped the cells escape TRAIL attack [16]. There was still much controversy over the complexity of TRAIL receptor system. But one thing in common was that no matter how the apoptotic signal was transduced in cells, TRAIL needed to combine with its receptors first, in order to induce cell apoptosis. Therefore, the expression level and function of TRAIL receptors on cell surface were particularly important

for TRAIL-mediated apoptosis. Our research showed that in lung cancer tissues on the aspect of mRNA or protein level, the expression of DR5 was significantly higher than that in normal tumor-adjacent tissues, while the expression of DcR1 was significantly lower than that in tumor adjacent tissues, and the expression of these two were all relevant to the staging and differentiation of tumor. With higher NSCLC staging, DR5 mRNA and protein expression showed an increasing trend. The lower the degree of differentiation the higher the level of DR5 expression, suggesting that the overexpression of DR5 in NSCLC may be a vital cause for the promotion of tumor development and invasion. On the contrary, the mRNA and protein expression of DcR1 in NSCLC tissues decreased with the increasing stage of NSCLC, and the lower the degree of differentiation, the lower the expression level of DcR1, which indicated that due to DcR1 the relatively late stage and relatively low differentiated tumor cells showed decreased ability to escape apoptosis. This kind of tumor may be more sensitive to cytotoxic drugs. Further study showed that the mRNA and protein expression levels of DR5 and DcR1 in NSCLC tissue were not associated with the mutation status of EGFR and KRAS, which indicated that the combination of TRAIL and TRAILR was not affected by EGFR and KRAS signaling pathway.

In conclusion, our research suggested that among various clinicopathological factors smoking might have a bigger impact on the prognosis of patients with NSCLC. The mutative state of EGFR and KRAS gene was associated with the expression of AKT and AKT phosphorylation, and had nothing to do with the expression of TRAIL receptor. This phenomenon had some reference value to the comprehensive treatment strategy and the prognosis of NSCLC.

Supplementary information is available in the online version of the paper.

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Supplementary Table 1 The sequences of ASPE primers used for the 56plex

Genes	Type	Universal tage –Primer, 5'→3'
EGFR	WT	ATACTTCATTCATTCATCAATTCA CCGAACGCACCGGAGC
Exon18	G719A	ATACAATCTAACTTCACTATTACA GTGCCGAACGCACCGGAGG
	G719C	CTTTAATCTACACTTTCTAACAAT CCGAACGCACCGGAGCA
	G719S	ATACTACATCATAATCAAACATCA CCGAACGCACCGGAGCT
EGFR	WT	CTTCTCATTAACTTACTTCATAAT CTATCAAGGAATTAAGAGAAGC
Exon19	E746-A750 (1)	TCAATTACTTCACTTTAATCCTTT AATTCCCGTCGCTATCAAAAC
	E746-A750 (2)	CTTTTCATCAATAATCTTACCTTT TTCCCGTCGCTATCAAGACA
	L747-E749 ins P	AAACAAACTTCACATCTCAATAAT CGTCGCTATCAAGGAACCAA
	L747-A750 ins P	TCATTTCAATCAATCATCAACAAT CGTCGCTATCAAGGAACCAT
	L747-T751	TCAATCATCTTTATACTTCACAAT CGTCGCTATCAAGGAATCT
	L747-T751 ins A	TTACTCAAAATCTACACTTTTTCA CCCGTCGCTATCAAGGCAT
	L747-S752	CTTTTTCAATCACTTTCAATTCAT CCGTCGCTATCAAGGAACAT
	L747-S752 ins V	CAATATCATCATCTTTATCATTAC CCCGTCGCTATCAAGGTTC
	L747-S752 ins D	AATCTACAAATCCAATAATCTCAT CCGTCGCTATCAAGGATCC
	L747-S752 ins Q	ATCAAATCTCATCAATTCAACAAT CGTCGCTATCAAGGAACAG
	L747-S752 ins S	TTCATAACTACAATACATCATCAT CGTCGCTATCAAGGAATCG
	E749-S752 ins D	AAACTAACATCAATACTTACATCA CTATCAAGGAATTAAGAGATCC
	E746-T751 ins T	TCAAAATCTCAAATACTCAAATCA TCCCGTCGCTATCAAGACC
	E746-P753 ins VS	CTACTAATTCATTAACATTACTAC CCCGTCGCTATCAAGGTCT
	E746-T751 ins Y	CTACAAACAAACAAACATTATCAA CCGTCGCTATCAAGGAACAA
	E746-T751 ins A	TAACATTACAACTATACTATCTAC CCCGTCGCTATCAAGGCTT
	E746-T750 ins AP	TATATACACTTCTCAATAACTAAC TCGCTATCAAGGCCCCAAC
	E747-T751 ins V	CTTTTCATCTTTTCATCTTTCAAT CGTCGCTATCAAGGTATCT
	L747-S752 ins E	CTTTAATCCTTTATCACTTTATCA CGTCGCTATCAAGGAACCG
	R748-T751 ins LS	TTACCTTTATACCTTTCTTTTTAC CGCTATCAAGCTATCAAGCC
EGFR	WT (1)	TCAATTACCTTTTCAATACAATAC CACCGTGCAGCTCATCAC
Exon20	WT (2)	TACACTTTCTTTCTTTCTTTCTTT AAGCCTACGTGATGGCCAG
	T790M	TCATTTACCAATCTTTCTTTATAC CCACCGTGCAGCTCATCAT

	S768I	AATCTAACAAACTCATCTAAATAC AAGCCTACGTGATGGCCAT
	V769_D770 ins ASV	TACAAATCATCAATCACTTTAATC GATGGCCAGCGCCAGCGTG
	D770_N771 ins G	TACATACACTAATAACATACTCAT GTGATGGCCAGCGTGGGT
	H773_V774 ins H	CAATAAACTATACTTCTTCACTAA CGTGGACAACCCCCACCA
EGFR	WT (1)	TAATTATACATCTCATCTTCTACA CAAGATCACAGATTTTGGGCT
Exon21	WT (2)	CTACTATACATCTTACTATACTTT GATTTTGGGCTGGCCAAACT
	L858R (1)	CTTTTCAATTACTTCAAATCTTCA AAGATCACAGATTTTGGGCG
	L858R (2)	CAATATACCAATATCATCATTTAC AGATCACAGATTTTGGGCGT
	L861Q	CTACATATTCAAATTACTACTTAC GATTTTGGGCTGGCCAAACA
KRAS	WT (1)	AATCAATCTTCATTCAAATCATCA TTGTGGTAGTTGGAGCTGGT
Exon2	WT (2)	AATCTTACCAATTCATAATCTTCA GTAGTTGGAGCTGGTGGC
	G12A	CTTTTCAAATCAATACTCAACTTT TGGTAGTTGGAGCTGCT
	G12C	TTCAATCATTCAAATCTCAACTTT TTGTGGTAGTTGGAGCTT
	G12D	CTTTCTACATTATTCACAACATTA TTGTGGTAGTTGGAGCTGAT
	G12R	TCATTTACTCAACAATTACAAATC TGTGGTAGTTGGAGCTCG
	G12S	TCATAATCTCAACAATCTTTCTTT CTTGTGGTAGTTGGAGCTA
	G12V	CAATTTACTCATATACATCACTTT TTGTGGTAGTTGGAGCTGTT
	G13A	TACACATCTTACAAACTAATTTCA GGTAGTTGGAGCTGGTGC
	G13C	TCAATCATAATCTCATAATCCAAT TGGTAGTTGGAGCTGGTTG
	G13D	CAATTAACTACATACAATACATAC TGGTAGTTGGAGCTGGTGA
	G13R	CTACTTCATATACTTTATACTACA GGTAGTTGGAGCTGGTCG
	G13S	CTTTCAATTACAATACTCATTACA TGGTAGTTGGAGCTGGTAG
	G13V	ATACCAATAATCCAATTCATATCA TGGTAGTTGGAGCTGGTGT
KRAS	WT	AATCATACCTTTCAATCTTTTACA ATTGCACTGTACTCCTCTTG
Exon3	Q61H	TTCACTTTTCAATCAACTTTAATC CATTGCACTGTACTCCTCA
	Q61K	TTACTTCACTTTCTATTTACAATC CATTGCACTGTACTCCTCTTT
	Q61L	AATCTTACTACAAATCCTTTCTTT TCATTGCACTGTACTCCTCTA

Supplementary Table 2 PCR primers used for the 56plex

Gene	Exon	Sequence, 5'→3'	PCR product length (bp)	Sensitivity, %
EGFR	18	TGGAGCCTCTTACACCCAGT CTTATACACCGTGCCGAACG	123	5
	19	CCAGAAGGTGAGAAAGTTAAAAT ACCCCCACACAGCAAAGCAG	115	5
	20	TCCAGGAAGCCTACGTGATG TGAGCAGGTACTGGGAGCCA	168	5
	21	ACCGCAGCATGTCAAGATCAC CCCTGGTGTCAGGAAAATGCT	131	5
KRAS	2	ATAAACTTGTGGTAGTTGGAGCT GTATCAAAGAATGGTCCTGCAC	153	5
	3	ATCCAGACTGTGTTTCTCCCTT AGTCCTCATGTACTGGTCCCT	138	1