

Somatic mutation of KIT is rare in small cell lung cancer patients from Northeast China

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Summary. Studies have confirmed that protein overexpression or mutations of KIT are involved in growth and development of a variety of cancers. however, little is known about data of gene mutation and protein expression in small cell lung cancer (SCLC) patients from northeast China. The aim of study is to investigate gene mutation and protein expression in such patients with small cell lung cancer (SCLC) and analyse their clinical significance. The expression of c-Kit protein was analyzed by immunohistochemistry in 77 SCLC samples and 22 normal lung samples. KIT mutations were screened in exons 9, 11, 13, 14, 17 and 18 by DNA direct sequencing. The study showed that positive staining for c-Kit was observed in 28 of 77 SCLC patients. There was no correlations between expression of c-Kit and sex, ages, smoking status, stage. only 1 case was found to have known T801I mutation in exon 17. The median survival (13.9 months) of cases with c-Kit-positive was shorter than that (19.9 months) of cases with c-Kit-negative. The finding revealed that stages was identified as an independent predictive factor for SCLC patients. Our finding reveals that somatic mutation of KIT is rare in SCLC patients from the northeast China and there is no enough evidence confirming KIT inhibitors for treatment in SCLC.

Key words: Small cell lung cancer, KIT, Mutation, Protein expression

Introduction

Lung cancer is the most common cancer worldwide, and was the leading cause of cancer-related death in males and the fourth in females in 2008 globally (Jemal et al., 2011). Lung cancer includes non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), differing pathological types. Although SCLC accounts for only about 15% of all lung cancers, it is an aggressive malignant disease, characterized by rapid growth and a high prevalence of widespread metastases (Brigham et al., 1978). For those 80% with limited-stage (LS) SCLC, who are not cured, and for all patients with extensive-stage (ES) SCLC, the outcome remains poor despite a typical initial temporary response to chemoradiotherapy (Dowell, 2010). Thus, there is a continuing need to develop new treatments and drugs, including molecular-targeted drugs.

The proto-oncogene KIT encodes a transmembrane tyrosine kinase receptor for stem cell factor (SCF), also known as c-Kit and CD117. SCF binding to the extracellular domain of c-Kit results in c-Kit heterodimerization and activation of the intracellular tyrosine kinase (Lev et al., 1992). Activation of c-Kit activates several downstream signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K) (Krishnan et al., 2012), “Janus activated kinase/signal transduction and activators of transcription” (JAK/STAT) (Omholt et al., 2011), RAS/RAF/MEK/ERK (Chaix et al., 2011), and Src (Lennartsson et al., 1999), and plays a role in the growth, proliferation, and differentiation of cells. Studies have confirmed that abnormal activation of KIT can be caused by c-Kit overexpression, gene amplification, or

mutations in a variety of cancers, such as gastrointestinal stromal tumors (GIST) (Bachet et al., 2009), melanoma (Kong et al., 2011), and breast cancer (Charpin et al., 2009). Thus, multi-target inhibitors, such as Imatinib, against abnormal activation of KIT have achieved great success in GIST (Heinrich et al., 2003).

Several studies have shown that there is reduced (27.9-87.7%) (Potti et al., 2003; Rohr et al., 2004; Camps et al., 2006) protein expression and, rarely, mutations in non-Asian patients with SCLC (Burger et al., 2003; Tamborini et al., 2004; Mojica et al., 2005; Sihto et al., 2005). Despite the failure of several clinical trials in non-Asian SCLC, KIT is still considered to be promising for the treatment of SCLC. While it is known that there are differences in gene profiles among ethnic groups, little is known about gene mutations and protein expression levels in Chinese patients with SCLC, especially in patients from northeast China. Thus, we hypothesized that KIT mutations might occur in Chinese patients with SCLC and that KIT mutations and/or c-Kit protein expression levels may be prognostic factors. In the present study, to clarify the status of KIT gene mutations and protein expression levels in SCLC patients from northeast China, we collected 77 SCLC tissue samples and retrospectively analyzed mutation status in exons 9, 11, 13, 14, 17, and 18 of the KIT gene by direct sequencing and c-Kit protein expression levels by immunohistochemistry (IHC).

Materials and methods

Patients and samples

We collected 77 tumor samples, diagnosed histologically as SCLC, from May 2000 to June 2009 and 22 normal lung samples from January 2011 to June 2011 at the Tumor Hospital of Harbin Medical University. Clinical data including gender, age, smoking status, stage, and survival were collected. A representative formalin-fixed, paraffin-embedded block from 77 tumor samples was selected and used for directed sequencing of polymerase chain reaction (PCR)-amplified products generated with specific primers, as well as immunohistochemistry. To determine somatic mutations, matched normal tissues from patients with KIT mutations were selected for resequencing. This study was approved by the medical ethics committee of the Tumor Hospital of Harbin Medical University. All patients provided written informed consent.

DNA extraction and mutational analysis

Genomic DNA was extracted from eight unstained 10- μ m sections using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocol. We performed DNA sequence analysis by direct sequencing of polymerase chain reaction (PCR)-amplified products generated with specific primers designed to include KIT exons 9, 11, 13, 14, 17, and 18.

The primers used were as follows: KIT-9F: ctactaggtcaccacaaagtgtct; KIT-9R: gcctaaacatccccttaaattg; KIT-11F: tagctggcatgatgtgcatt; KIT-11R: gacatggaaagccctgtt; KIT-13F: gaggcaactgcttatggctta; KIT-13R: tgacagacaataaaaaggcagctt; KIT-14F: agtctgatccac-tgaagctgaa; KIT-14R: gccttgattgcaaacctta; KIT-17F: tgaacatcatcaaggcgtg; KIT-17R: tgcaggactgtcaagcagag; KIT-18F: ggtaaaaggtttttgtgagatgg; KIT-18R: aatgaag-ttgtctttggcaag. The PCR cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, then a final extension for 5 min at 72°C and hold at 4°C. The PCR products were purified using a PureLink PCR Purification Kit (Invitrogen, USA). Direct sequencing of PCR products was performed using an ABI 3730XL automated sequencer (Applied Biosystems, USA).

Immunohistochemistry

Unstained sections (4 μ m) were mounted on glass slides and used for immunohistochemistry. Immunohistochemical staining was performed according to the manufacturer's instructions using the standard streptavidin-peroxidase biotin technique with a SP kit (Zhongshan Co., China). After deparaffinization, the tissue sections were heated at 120°C for 15 min in 10 mM Tris-HCl with 1 mM EDTA (pH 9.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were incubated overnight at 4°C with antibodies against c-Kit (Santa Cruz Biotechnology, USA) at a 1:100 dilution. Then, biotinylated immunoglobulin and streptavidin conjugated to peroxidase were added. Finally, 3,3'-diaminobenzidine was added for color development, and hematoxylin was used for counterstaining. Negative controls processed without the primary antibody were performed in each experiment. The mean percentage of positive tumor cells was determined in at least five areas at x400 magnification. The slides were evaluated independently by two experienced pathologists who reached consensus. Membrane or cytoplasm staining was evaluated as positive. The percentage of positive cells was categorized as follows: 0: <5%, 1: 5-25%, 2: 25-50%, 3: 50-75%, 4: >75%. The staining intensity was scored as follows: 0: negative, 1: weak, 2: moderate, 3: strong. The scores for the percentage of positive cells and staining intensity were multiplied to achieve a weighted score for each case, and cases with scores ≥ 2 were deemed positive.

Statistical analysis

The SPSS software (ver. 18.0) was used for all calculations and statistical analyses. Associations between clinical characteristics and KIT mutations or c-Kit expression were analyzed using the χ^2 test. Correlations between KIT gene mutation and protein expression were identified using Pearson's correlation analysis. Survival curves were plotted according to the

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Kaplan-Meier method and statistical significance was assessed using the log-rank test. A two-tailed P value <0.05 was considered to indicate statistical significance.

Results

Clinical characteristics

Among the 77 patients, there were 49 males and 28 females; the median age was 52 years (mean, 51.7, SD, 9.64, range, 24-75). There were 43 non-smokers and 34 smokers. According to the Veterans Administration Lung Study Group (VALG) classification, 65 patients were confirmed as limited-stage and 12 patients were extensive-stage. Follow-up was complete for all patients up to June 2011. The overall 12-, 24-, and 30-month survival rates were 70%, 29%, and 17%, respectively.

c-Kit expression in patients with SCLC

In this study, positive staining for c-Kit was observed in 28 of 77 SCLC patients (36%), while no positive staining was observed in 22 normal lung tissues (Fig. 1). The expression of c-Kit in SCLC tissues was significantly higher than in normal lung tissue ($P=0.004$). Next, correlations between expression of c-Kit and clinical characteristics were analyzed. However, we did not find significant relationships between expression of c-Kit and gender, age, smoking status, or stage (Table 1).

Genetic alterations of KIT in patients with SCLC

Of the 77 SCLC cases, only one case was found to have a known missense mutation (T801I) in exon 17; this was a point mutation resulting in a single amino acid substitution (Fig. 2). Matched normal tissue from the mutant case was analyzed again and no mutation was found, indicating that it was a somatic mutation. The subject had limited-stage SCLC, had a smoking history of more than 20 years, and was a 48-year-old male. His

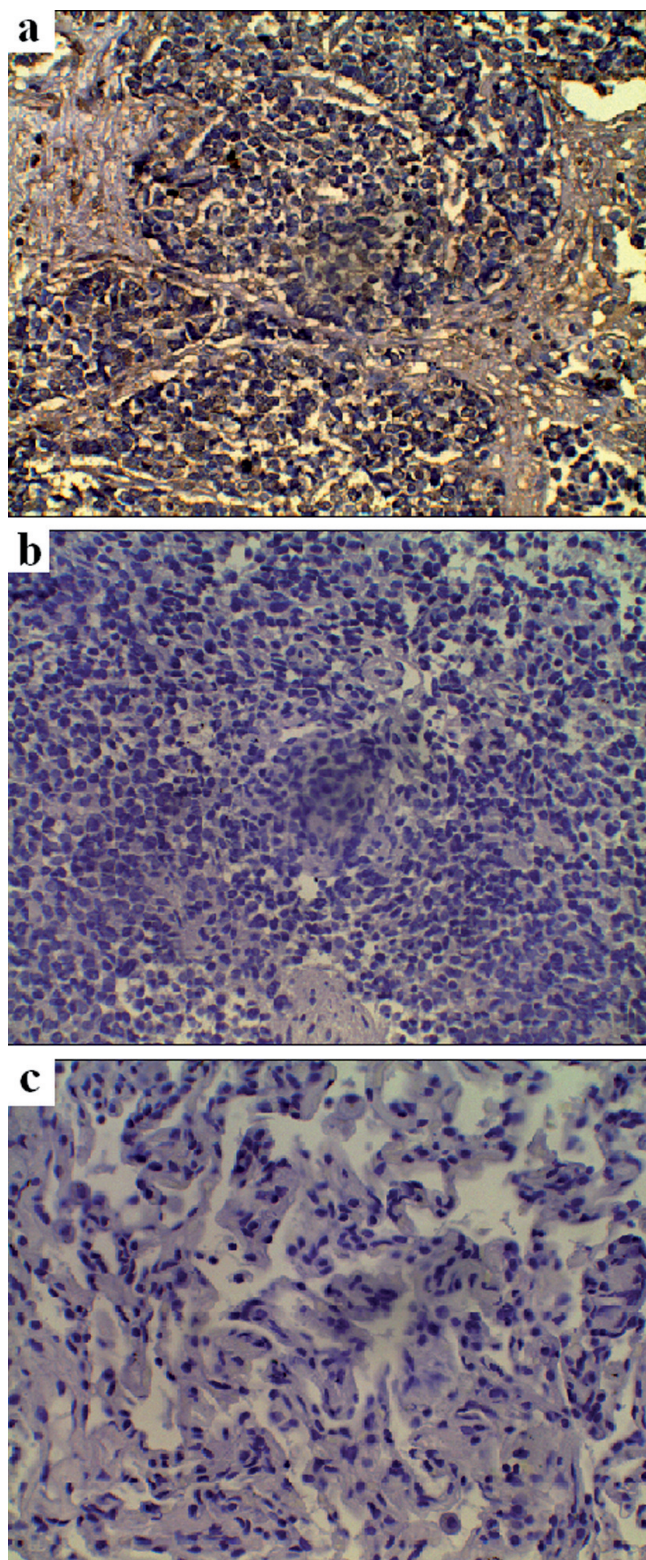


Fig. 1. Immunohistochemical expression of c-Kit protein in SCLC and normal lung tissues. **A.** Brown staining in cytomembrane or cytoplasm of SCLC cells indicates c-Kit positive expression. **B.** No staining in SCLC cells indicates negative expression of c-Kit protein. **C.** No staining of c-Kit protein is observed in normal lung tissues. x 400

Table 1. Correlation between expression of c-Kit and clinical/pathological features in SCLC.

Characteristics	N	c-Kit Expression		P-value
		Positive (%)	Negative (%)	
Sex				
Male	49	19 (38.8)	30 (61.2)	0.561
Female	28	9 (32.1)	19 (67.9)	
Age				
<60	47	17 (36.2)	30 (63.9)	0.965
≥60	30	11 (36.7)	19 (63.3)	
Smoking Status				
Never	25	7 (28.0)	18 (72.0)	0.290
Current	52	21 (40.4)	31 (59.6)	
Stage				
Limited	65	23 (35.4)	42 (64.6)	0.678
Extensive	12	5 (41.7)	7 (58.3)	

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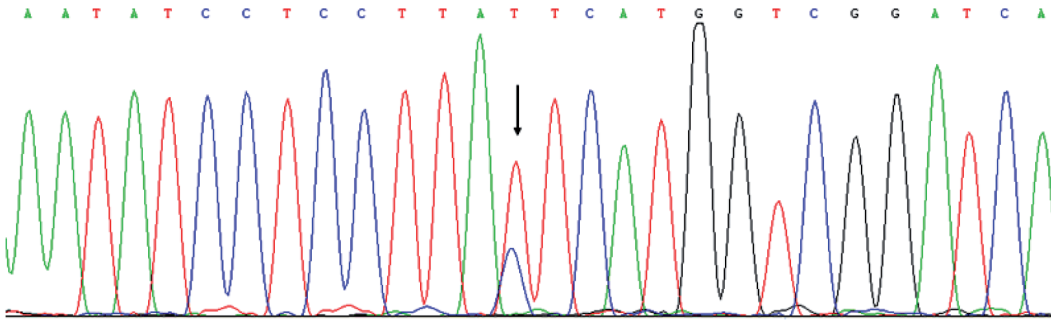


Fig. 2. The sequencing chromatogram shows T8011 in exon 17 of KIT. The mutated peak of T8011 2489C>T is marked with an arrow.

Table 2. Cox proportional hazard regression model analysis for survival of SCLC.

Variates	Univariate analysis			Multivariate analysis		
	HR	95%CI	P	HR	95%CI	P
Age (<60 vs. ≥60)	1.577	0.649-3.835	0.315	1.527	0.613-3.803	0.363
Gender (male vs. female)	1.137	0.492-2.628	0.763	1.031	0.366-2.904	0.954
Smoking Status (never vs. smoking)	0.859	0.379-1.948	0.716	1.112	0.434-2.851	0.825
Stage (limited vs. extensive)	3.143	0.131-0.774	0.012	0.317	0.124-0.810	0.016
c-Kit Expression (negative vs. positive)	0.967	0.418-2.234	0.861	0.912	0.374-2.228	0.840

HR, hazard ratio; CI, confidence interval.

survival time was more than 5 years and, finally, he was lost to follow-up. The IHC results were consistent in that no positive staining was observed in the tumor tissue. No novel mutation was found in this study.

Overall survival and prognostic significance analyses

Kaplan-Meier survival analysis revealed that the median survival (13.9 months) of cases who were c-Kit-positive was shorter than the survival time (19.9 months) of cases who were c-Kit-negative, although no statistically significant difference was found (P=0.861; Fig. 3).

A multivariate analysis using the Cox proportional hazards model was performed and gender, age, smoking, stage, and c-Kit protein expression were included as variables. The findings revealed that stage was an independent predictive factor for SCLC patients (Table 2; P=0.016), consistent with a previous report that patients with extensive-stage disease had shorter survival (Micke et al., 2002).

Discussion

In the present study, we found that positive expression of c-Kit was common and no expression was found in normal lung tissues, indicating involvement of

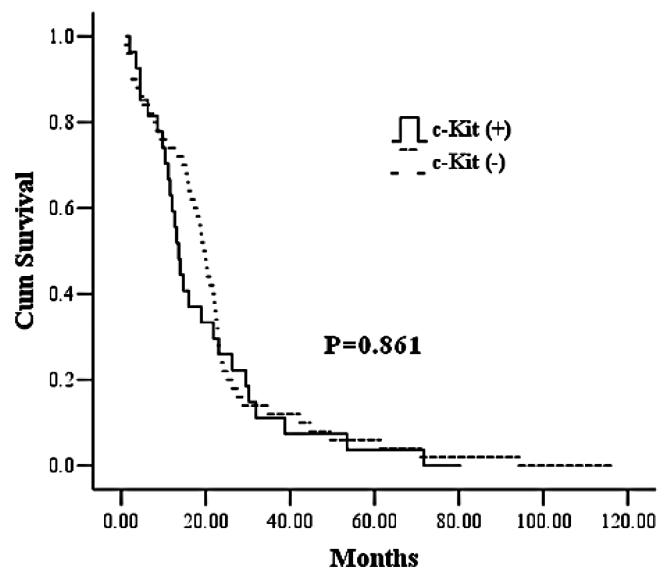


Fig. 3. Kaplan-Meier overall survival analysis of SCLC patients according to the expression levels of c-Kit protein expression. The statistical analysis reveals there is no difference between the survival time of cases with c-Kit-positive and c-Kit-negative (P=0.861).

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c-Kit in carcinogenesis and the development of SCLC. However, c-Kit expression was not significantly correlated with prognosis in SCLC. Although patients negative for c-Kit expression survived longer than c-Kit-positive patients, no statistically significant association was found. Importantly, our results confirmed that KIT mutations are extremely rare in patients of northeast China with SCLC.

As an important transmembrane tyrosine kinase receptor involved in the growth and development of cancers, KIT and its encoded protein have been of interest to researchers. Initially, high c-Kit expression was found in gastrointestinal stroma tumors (GIST) and Imatinib or STI571, a selective inhibitor of BCR/ABL, PDGFR, and c-Kit kinase activity, was effective in patients who had c-Kit-positive GIST (Verweij et al., 2003). Subsequently, c-Kit overexpression was found in variety of solid tumors, including SCLC. Studies in Western countries showed that c-Kit was expressed in 27.9-87.7% of SCLC tumors (Potti et al., 2003; Rohr et al., 2004; Camps et al., 2006). There have been few prior reports of c-Kit expression in Asians. Recently, another study in 36 Chinese SCLC patients showed the rate of positive c-Kit expression was 83.3% (Lu et al., 2012). Our findings revealed an expression rate of only 36%; this difference is likely related to the small number of samples. Based on the overexpression of c-Kit in SCLC, researchers have attempted to develop KIT inhibitors for SCLC. Although STI571 and SU5416 (another multi-targeted kinase inhibitor) inhibit tumor cell growth in a large proportion of SCLC cell lines and human small cell lung cancer xenografts (Litz et al., 2004; Decaudin et al., 2005), STI571 failed to demonstrate clinical activity despite patient selection for c-Kit-positive SCLC in two phase II trials (Dy et al., 2005; Schneider et al., 2010). Thus, previous studies and our findings indicate that c-Kit overexpression may not be a potent therapeutic target or prognostic factor for SCLC.

Apart from c-Kit overexpression, KIT gene mutations are considered another cause of the abnormal activation of c-Kit. KIT-activating mutations as a target for KIT inhibitors have been found in several types of solid tumors, especially in GIST and melanoma (Beadling et al., 2008; Bachet et al., 2009; Guo et al., 2011). Moreover, several mutations in KIT were identified to be drug-resistant markers for STI571 or SU5416 (Gajiwala et al., 2009). The results of several small-sample studies revealed that mutations in KIT were rare in non-Asian SCLC patients. However, one study in India found two mutations in exon 9 and three in exon 11 in 60 SCLC samples (Boldrini et al., 2004). Other studies subsequently focused on mutations in exons 9 and 11. In the present study, we analyzed mutation status in exons 9, 11, 13, 14, 17, and 18 of the KIT gene, encoding the extracellular domain, juxtamembrane domain, TK1, and TK2. Moreover, studies have shown that mutations in these functional domains correlated with sensitivity to KIT inhibitors (Gajiwala et al., 2009). In the present study, we found only a single case with a point mutation, T801I, which

was first identified in a testicular seminoma (Kemmer et al., 2004). A previous study found that T801I KIT mutation was dependent on SCF for kinase activation, similar to wild-type KIT. Compared with mutations that confer sensitivity or resistance to KIT inhibitors, the biological significance of T801I has not been determined. Thus, unlike the situation with GIST and melanoma, KIT mutations are rare in SCLC, suggesting that KIT is unlikely to be useful as a target for targeted therapy in SCLC patients.

Compared with previous studies in SCLC, this is the first report to focus on mutation status in exons 9, 11, 13, 14, 17, and 18 of the KIT gene, and the sample size was larger than those in previous studies. However, 77 samples in the present study is insufficient for analysis of rare gene aberrations. Most patients with SCLC are diagnosed with inoperable disease and a bronchoscope biopsy is the method used most commonly to obtain specimens, but tissues obtained in this way are not sufficient for genetic analysis, so obtaining a sufficient number of suitable specimens is difficult. Thus, there is a need to obtain more operative specimens, likely by means of multi-center cooperation, for the analysis of KIT aberrations in SCLC.

In conclusion, our results provide data on the protein expression and mutation status of KIT in 77 northeast Chinese patients with SCLC. However, our findings show that KIT mutations are rare in SCLC patients from northeast China, and that the evidence is insufficient to confirm the value of KIT inhibitors for treatment of SCLC.

Acknowledgements. This work was supported by the Natural Science Foundation of Heilongjiang Province (No. D201111), the Education Department Foundation of Heilongjiang Province (No. 12521193) and the Health Department Foundation of Heilongjiang Province (No. 2012-652).

Conflict of Interest: We declare that we have no conflict of interest

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