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Expression and mutation of the c-kit gene and correlation with prognosis of small cell lung cancer

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Abstract. Small cell lung cancer (SCLC) is a highly aggressive and lethal type of cancer in humans. SCLC is sensitive to chemotherapy and radiotherapy, but long-term survival is low and the majority of patients eventually develop progressive disease. With the success of imatinib mesylate in the treatment of gastrointestinal stromal tumors expressing c-kit, its use in SCLC serves as a novel molecular therapeutic approach. The activity of imatinib mesylate is correlated with the mutation of c-kit gene exons 9 and 11 in gastrointestinal stromal tumors. The incidence of epidermal growth factor receptor mutation in non-small cell lung cancer is higher in China than in the United States of America and European countries. There may be also differences in the incidence of c-kit mutation between China and European countries. At present, no study examining imatinib mesylate treatment for SCLC in China is available. To investigate the expression and mutation of c-kit and the correlation with prognosis of SCLC in China, immunohistochemistry was used to detect the expression of c-kit, and a pyrosequencing assay was used to detect mutations in c-kit exons 9 and 11 of 36 SCLC patients who received surgical treatment at the Zhejiang Cancer Hospital, Hangzhou, China, between 1998 and 2010. All 36 patients were followed up to analyze the correlation between prognosis and expression and mutation of c-kit. The incidence of c-kit-positive expression was 83.3%, including 25.0% weak staining, 22.2% moderate staining and 36.1% strong staining. The overall survival of patients with c-kit strong staining was shorter compared to patients with c-kit not strong staining. No mutation in c-kit exons 9 and 11 was detected. In conclusion, the findings showed that the expression of c-kit is high, and strong staining is a prognostic factor for worse survival.

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Abbreviations: SCLC, small cell lung cancer; EGFR, epidermal growth factor receptor; PCR, polymerase chain reaction; NSCLC, non-small cell lung cancer; MST, median survival time; OS, overall survival

Key words: small cell lung cancer, c-kit, expression, mutation, prognosis, immunohistochemistry, pyrosequencing assay

Introduction

Small cell lung cancer (SCLC) is a highly aggressive and lethal type of cancer in humans. It constitutes approximately 15% of all cases of primary lung cancer (1). SCLC is sensitive to chemotherapy and radiotherapy, but long-term survival is low and the majority of patients eventually develop progressive disease. There is a high rate of relapse even among patients who achieve a complete response. High levels of expression of c-kit and its ligand, stem cell factor (SCF), have been widely found in both SCLC tumors and established cell cultures (2).

Imatinib mesylate (STI571) is an oral inhibitor of a number of tyrosine kinases, which acts through occupation of the highly conserved structure of tyrosine kinase at the ATP binding site. With the success of imatinib mesylate in the treatment of gastrointestinal stromal tumors expressing c-kit, its use in SCLC serves as a novel molecular therapeutic approach. Autocrine or paracrine activation of c-kit by its ligand has been postulated for lung cancer, but this receptor can also be activated by mutations of c-kit. The activity of imatinib mesylate is associated with mutation of the c-kit gene exons 9 and 11 in gastrointestinal stromal tumors (3,4). Certain studies have reported that imatinib mesylate failed to demonstrate clinical activity in SCLC (5-7). Boldrini *et al* (8) examined 60 SCLC samples to determine mutations of the c-kit coding region. Expression of c-kit was demonstrated in approximately 40% of SCLC samples. Their results showed that two patients had mutations in exon 9 and three patients had mutations in exon 11.

The expression of c-kit and its mutational status did not appear to be relevant to or have a significant impact on survival (8). The cause of this negative result with imatinib used in SCLC may be due to the low incidence of c-kit exon 9 or 11 mutation.

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been widely used in non-small cell lung cancer (NSCLC) (9-13), but have failed to treat relapsed SCLC (14). The incidence of EGFR mutation in NSCLC is higher in China than in the United States of America and European countries (15,16). There may be also differences in the incidence of c-kit gene mutations between China and European countries. However, at present no study examining imatinib mesylate treatment for SCLC in China is available. To determine the expression and mutation of c-kit and its correlation with prognosis of SCLC in China, we examined c-kit expression and mutation status in 36 SCLC patients.

Patients and methods

Patient characteristics. A total of 36 SCLC surgical specimens were retrospectively collected at the Zhejiang Cancer Hospital, Hangzhou, China between 1998 and 2010. The median age of the patients was 54 years and the range was 22-73 years. There were 4 female and 32 male patients. Samples were derived from the primary tumor. The pathological type of all patients was conventional SCLC based on the standard criteria defined by WHO classification. The median pack-years of smoking history was 30. The cancer stage was determined according to the seventh edition of TNM classification for lung cancer: T1 8 cases, T2 14 cases, T3 10 cases and T4 4 cases; N0 12 cases, N1 10 cases, N2 14 cases and N3 0 cases (Table I); IA 4 cases, IB 2 cases, IIA 6 cases, IIB 5 cases, IIIA 17 cases and IIIB 2 cases. A total of 23 patients (64%) were successfully followed-up. The median age was 56 years and the range was 38-73 years. There was 1 female and 22 male patients. The cancer stage was determined according to the seventh edition of TNM classification for lung cancer: T1 6 cases, T2 7 cases, T3 7 cases and T4 3 cases; N0 10 cases, N1 5 cases, N2 8 cases and N3 0 cases (Table I); IA 4 cases, IB 2 cases, IIA 3 cases, IIB 2 cases, IIIA 11 cases and IIIB 1 case. The median pack-years of smoking history was 30 (Table I). A total of 82.6% of patients received first-line chemotherapy after surgery; the most common treatment was a combination regimen of etoposide and cisplatin. The follow-up deadline was November 01, 2011. The survival time was calculated from the date of histological diagnosis. The use of tissue samples was approved by the Ethics Review Committee of Zhejiang Cancer Hospital.

Immunohistochemistry. Slide sections (4 μm) of the specimens were heated in a pressure cooker for 4 h at 56˚C. Paraffin wax sections were de-waxed and 0.01 mmol/l pH 9.0 TE buffer was added before slides were placed in a water bath for 20 min at 95˚C. After antigen retrieval, the sections were rinsed under distilled water, and 3% H_2O_2 was used to block endogenous-peroxidase activity. Each section had 50 μl polyclonal rabbit anti-c-kit antibody (1:400; Dako, Glostrup, Denmark) added for 60 min at 37˚C, and 50 μl Envision complex was added for 40 min at room temperature. After each step the slides were rinsed 3 times with 0.01 mmol/l PBS (pH 7.4) for 5 min. The slides were colored by 0.04% DAB-0.03% H_2O_2 colored water, rinsed under distilled water, and counterstained in hematoxylin solution for 1 min. The slides were then dehydrated, made transparent and covered with a coverslip. The sections incubated in PBS instead of the primary antibody were used as negative controls, while the known positive sections were used as positive controls. C-kit expression of tumors were scored as negative (0) if <5% of cells were positive, weak staining (+) if 5-25% of cells were positive, moderate (++) if 26-50% of cells were positive and strong (+++) if >50% of cells were positive (7).

Table I. Baseline characteristics of the study population.

Characteristic	No. of patients (n=36)	Follow-up no. of patients (n=23)
Age (years) (median)	54	56
Gender (female/male)	4/32	1/22
Smoking history (median pack-years)	30	30
Stage		
I	6	6
II	11	5
III	19	12
IV	0	0
T stage		
T1	8	6
T2	14	7
T3	10	7
T4	4	3
N stage		
N0	12	10
N1	10	5
N2	14	8
N3	0	0

DNA preparation. Genomic DNA was isolated and purified from formalin-fixed paraffin-embedded tissues using the GT pure FFPE Tissue DNA Extraction kit (Gene Tech, Shanghai, China).

C-kit pyrosequencing assay. For the amplification of the exon 9 and 11 fragments of the c-kit gene isolated from the genomic DNA, polymerase chain reaction (PCR) amplification primers were designed for pyrosequencing: c-kit-9, forward: 5'-ATGGCACGGTTGAATGTAAGG-3' and reverse biotinylated primer: 5'-CAGAGCCTAAACATCCCCTT AAA-3'; and c-kit-11, forward: 5'-AGGTGATCTATTTTTCCC TTTCTC-3' and reverse biotinylated primer: 5'-GGAAACT CCCATTTGTGATCAT-3'. Each PCR reaction contained forward and reverse primers (each 4 pmol), 2 μl template DNA solution and 2 unit hotstart Taq DNA Polymerase (Takara, Shiga, Japan) in a 40 ml volume. PCR conditions consisted of initial denaturing for 3 min at 95˚C; annealing at 50 cycles of 15 sec at 95˚C, 30 sec at 56˚C, 30 sec at 72˚C; and a final exten-

sion of 5 min at 72˚C. The PCR products were sequenced by the Pyrosequencing PyroMark ID system (Qiagen, Hilden, Germany) according to the manufacturer's instructions.Using the two pyrosequencing primers (5'-3' orientation): c-kit-9, CGATGTGGGCAAGACT; and c-kit-11, TCCCTTTCTCCC CAC, pyrosequencing was performed using PyroMark Gold Q96 reagents (Qiagen) containing an enzyme and substrate mixture, dATP-S, dCTP, dGTP and dTTP.

Statistical analysis. Data were analyzed using the statistical software SPSS (version 15). Kaplan-Meier life-table curves were compared using the log-rank test to estimate survival. To compare the survival pattern of the different variables, logistic regression analysis was applied. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The incidence of c-kit-positive expression was 83.3%, including 25.0% weak staining, 22.2% moderate staining and 36.1% strong staining (Table II). The status of c-kit expression in the follow-up patients was negative staining in 6 cases, weak in 6 cases, moderate in 5 cases and strong staining in 6 cases (Fig. 1). The median survival time (MST) of the follow-up patients was 20 months. There was no difference in overall survival (OS) between the SCLC patients with c-kit negative expression and SCLC patients with c-kit positive expression. There was also no difference in OS between the SCLC patients with c-kit negative and weak staining and SCLC patients with c-kit moderate and strong staining. The OS of SCLC patients with c-kit strong staining was shorter than those with c-kit not strong staining (P=0.030) (Fig. 2). No mutation in c-kit exons 9 and 11 was detected (Fig. 3).

Table II. Clinical characteristics of patients with c-kit-positive or c-kit-negative expression.

	c-kit expression			
	Negative	+	++	+++
No. of patients	6	9	8	13
Age (median)	64	52	50	53
Gender (female/male)	0/6	0/9	0/8	4/9
History of smoking (median pack-year)	22	30	31	20

Discussion

The c-kit protein is a member of the type III receptor tyrosine kinase family. A positive c-kit expression has been observed in 37% of SCLC patients, and c-kit expression has been demonstrated to be associated with decreased survival (17). C-kit expression is of particular clinical relevance for patients with advanced disease and poor response to chemotherapy. C-kit serves as a new prognostic factor for SCLC. Given the limited therapeutic options and unfavorable prognosis of SCLC, clinical studies aimed at targeting c-kit are necessary (17). In their study, Blackhall *et al* demonstrated c-kit expression in 51% of tumors, and concluded that c-kit expression is not predictive of survival (18). A number of different studies have classified c-kit-positive expression using a variety of standards. The standard of c-kit positivity as identified in a study by Micke *et al* (17) was determined when positive slides demonstrated c-kit-positive cells including membrane staining in at least 10% of all tumor cells. Slides demonstrating a weak cytoplasmic signal without membrane staining were defined as negative. The standard of c-kit positivity by Blackhall *et al* (18) was determined when c-kit expression was greater than 35%. The standard of c-kit positivity by Boldrini *et al* (8) was determined when tumors with immunoreactive cells had a threshold value above 1%. 'Positive' was classified into three scales, as follows: weak staining as +, moderate staining as ++ and strong staining as +++. These different standards may lead to varying results of whether c-kit expression may serve as a prognostic factor of SCLC. Therefore, it is significant to incorporate the standard of c-kit positivity in all studies. Our standard of c-kit positivity was in accordance with Dy *et al* (7). In this standard, c-kit positivity was classified as weak staining (+), moderate staining (++) and strong staining (+++).

Combined SCLC has been reported to account for less than 1-3.2% of all SCLC (19,20). The surgical specimens reflect the clinicopathological status; a high percentage of cases (28%) demonstrated that SCLC combined with NSCLC in surgical specimens (21). The specimens used in our study were obtained from surgical resection, and pathological type of all our patients was conventional SCLC. The specimens reported by Blackhall *et al* were from SCLC biopsies (18). Our study has shown that c-kit expression is high in SCLC, and strong staining correlates with a worse survival prognosis.

Imatinib mesylate failed to demonstrate any clinical activity despite patients being selected for c-kit-expressing SCLC, and efficacy prediction of imatinib mesylate should not be based on c-kit expression (8). Patients of gastrointestinal stromal tumor with mutations in c-kit exons 9 and 11 are sensitive to imatinib mesylate. The incidence of EGFR mutation of NSCLC in China is higher than in the United States of America and European countries (15,16), but no mutation of c-kit exons 9 or 11 was detected in our study.

Compared to other genotyping and genetic detection methods, pyrosequencing technology is unique. It delivers the 'gold standard' of genetic analysis: the sequence itself. Other methods only provide a 'Yes/No' signal. Unlike a fluorescent signal, sequence information is intelligible. It is easy to communicate these results in literature, and easy to transfer meaningful data between research labs. Pyrosequencing assays are mutation-tolerant. Unlike hybridisation-based assays, pyrosequencing analysis generates a correct sequence regardless of the appearance of a new, unexpected mutation. This is crucial to microbiological applications: hybridisation-based assays can give false negatives in the presence of a new mutation. With pyrosequencing analysis, the sequence information is obtained, and the data is fully quantitative; ideal for measuring the relative amounts of alleles. This property allows the quantification of DNA methylation, heterozygosity, ploidy levels, multi-copy genes, pooled DNA samples, hematopoeitic chimerism and mixed

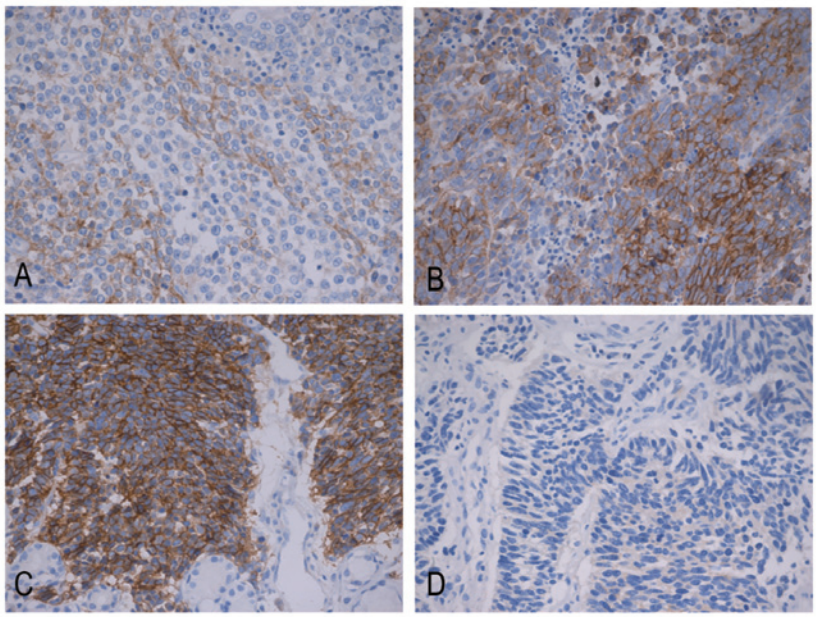

Figure 1. Immunohistochemical staining of SCLC using polyclonal rabbit antibodies against c-kit. (A) Weak staining (+); (B) moderate staining (++); (C) strong staining (+++); (D) negative staining. SCLC, small cell lung cancer.

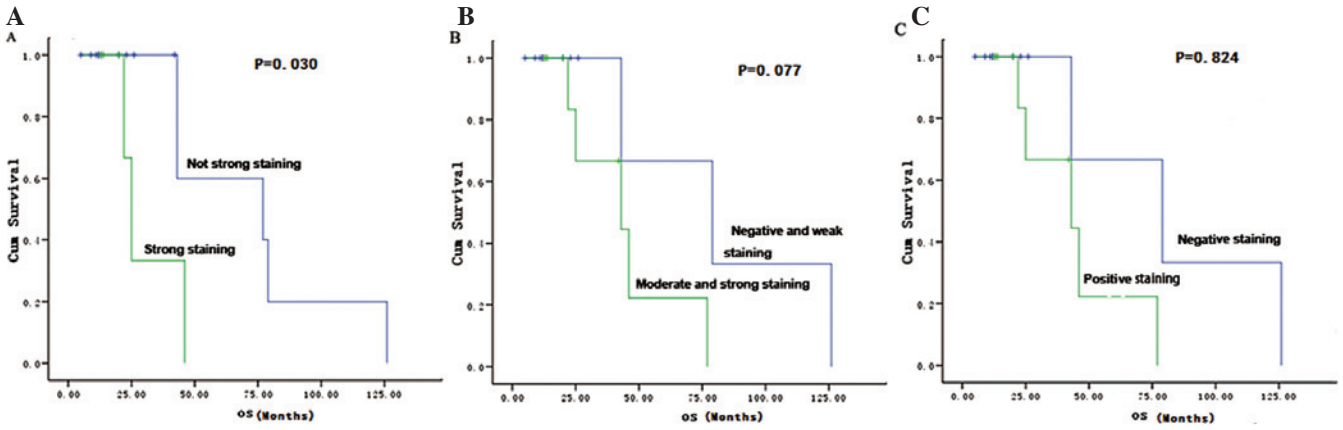

Figure 2. Association between c-kit expression in patients with SCLC (n=23) with survival time. (A) Survival curves of c-kit strong staining vs. not strong staining; (B) survival curves of c-kit strong staining and moderate staining vs. negative and moderate staining; (C) survival curves of c-kit positive vs. negative staining. SCLC, small cell lung cancer.

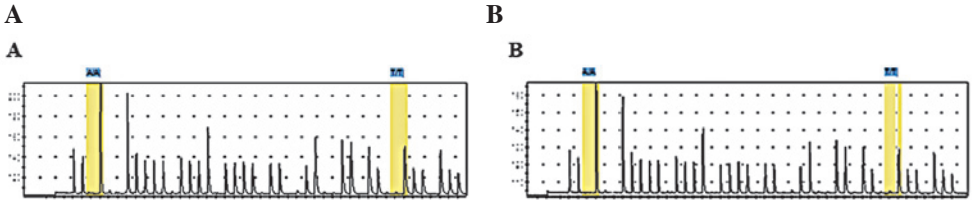

Figure 3. Wild-type pyrogram of (A) c-kit exon 9 (501-507) and (B) c-kit exon 11 (549-562).

genotypes in heterogeneous samples (e.g., tumor and normal cells). In the study by Boldrini *et al*, c-kit exons 9 and 11 were analyzed by PCR-single-strand conformational polymorphism and automated sequencing (8), while in our study pyrosequencing technology was used to detect c-kit exons 9 and 11. Boldrini *et al* (8) demonstrated that the expression of c-kit and its mutational status failed to appear relevant or to have a significant impact on survival.

No mutation of c-kit exons 9 or 11 was detected in our study. C-kit expression is high in SCLC, and strong staining correlates to to worse survival prognosis. The incidence of EGFR exons 19 and 21 mutation in SCLC is extremely low in China and Japan (22-24), and gefitinib failed to demonstrate benefit in relapsed SCLC patients (14). Given the limited therapeutic options and unfavorable prognosis of SCLC, increased interest and studies are required to develop targeted therapies to improve survival.

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