KRAS Exon 3 and PTEN Exon 7 Mutations in Small-cell Lung Cancer*

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Summary: Small cell lung cancer (SCLC) is recognized as one of the most aggressive and fatal malignant tumors. No significant improvement has been made to prolong the survival of SCLC patients. This study aimed to examine the mutation status of K-Ras (KRAS) and phosphatase and tensin homolog (PTEN) in SCLC patients in order to identify potential therapeutic targets for SCLC. Nineteen primary SCLC tumor specimens were enrolled in the study. Direct sequencing was performed to detect the mutations of KRAS exon 3 and PTEN exon 7 in the specimens. Kaplan-Meier and Cox regression analysis was performed to determine the overall survival (OS) of these SCLC patients. KRAS exon 3 mutation was found in 4 (21%) SCLC patients, and PTEN exon 7 mutation in only 1 (5%) SCLC patient. Kaplan Meier analysis showed that clinical stage and brain metastasis were significantly associated with OS (both $P<0.05$), but neither KRAS exon 3 mutation nor PTEN exon 7 mutation was significantly associated with OS ($P>0.05$). Cox proportional hazards regression model indicated that extensive stage of disease was the only independent negative prognostic factor for OS in SCLC patients. In conclusion, KRAS exon 3 and PTEN exon 7 mutations had no significant impact on OS of SCLC patients. Further study is still necessary to validate the molecular profiles of SCLC.
Key words: gene; Ras; small cell lung carcinoma; prognosis

Lung cancer is still one of the leading causes of cancer-related deaths throughout the world. It remains to be one of the top 5 leading causes of cancer death among both men and women[1]. Small cell lung cancer (SCLC) is characterized by rapid proliferation and short multiplication time of tumor cells, and early development of remote metastases. Approximately one-third SCLC patients present with limited-stage disease confined to the chest. Although SCLC is highly sensitive to the initial chemotherapy and radiotherapy, most patients die of recurrent disease[2]. In contrast to the obvious progression in the treatment of non-small cell lung cancer (NSCLC), there are hardly any new and effective therapeutic targets for SCLC. The survival rate of SCLC remains very poor. Targeted drugs were found to be superior to conventional combined chemotherapeutic regimens due to their lower toxicity and good compliance of patients for treatment. Identification of potential effective molecular targets for SCLC has always been desirable.

K-Ras (KRAS) mutations are the most frequently seen molecular abnormalities and they happen in one out of four NSCLC patients. However, direct inhibition of RAS activation failed to show any clinical efficacy for NSCLC patients. The exact mechanism of unsatisfactory response of NSCLC patients with KRAS mutations to regular chemotherapy and targeted therapies is still under research[3]. To date, there is no approved targeted therapy for the treatment of SCLC. Understanding KRAS mutational status in SCLC may provide further information regarding the treatment options for SCLC. Kodaz *et al* found more KRAS mutations in exon 3 than in exon 2 (83% *vs.* 16.2%) in Japanese SCLC patients[4]. The prevalence and clinical value of exon 3 in KRAS mutation in Chinese SCLC patients are unknown and worth exploration.

Phosphatase and tensin homolog (PTEN) is a tumor suppressor gene on chromosome 10q23.3 and encodes a 403 amino acid dual-specificity lipid and protein phosphatase[5]. PTEN negatively regulates phosphatidylinositol 3-kinase (PI3K) -Akt- mammalian

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target of rapamycin (mTOR) pathway, leading to G1 cell cycle arrest and apoptosis. It can inhibit cell migration and regulate the spreading of cancer cells via focal adhesion kinase and p53[6]. Frequent somatic mutations in the PTEN gene were found in endometrial and prostate cancers[7, 8], but rarely in NSCLC[9, 10]. A previous study reported that PTEN mutations were relatively common (about 10%) in SCLC than in NSCLC and was strongly associated with cigarette smoking[10]. The truncating and point mutations are distributed over most of the PTEN gene and the mutation in PTEN exon 7 was reported in SCLC patients recently[11]. The frequency and condition of PTEN exon 7 mutation in Chinese SCLC patients, however, have not been well studied yet.

In the present study, the KRAS and PTEN mutations were detected by reverse-transcription polymerase chain reaction (RT-PCR) and direct sequencing technology in 19 SCLC patients in order to identify potential therapeutic targets for SCLC.

1 MATERIALS AND METHODS

1.1 Patient Characteristics

Nineteen pathologically diagnosed SCLC patients were prospectively enrolled in this study from Zhejiang Cancer Hospital from October 2013 to October 2015. The research was approved by the Ethics Review Committee of Zhejiang Cancer Hospital (Hangzhou, China). The clinical stage was categorized according to the Veterans Administration Lung Study Group (VALSG) staging system[12], and patients were classified into the limited-stage disease (LD) or extensive-stage disease (ED). Median follow-up was 9 months (range: 3–26 months). The follow-up period was defined as the time from diagnosis to the last visit or death. Overall survival (OS) was defined as the time from diagnosis until the last visit or death. The SCLC-related clinical data were collected, including gender, age at diagnosis, clinical stage, smoking history, bone or brain metastatic lesions, abnormal serum tumor markers and chemotherapy and/or radiotherapy. Eighteen patients were diagnosed by fine-needle aspiration biopsy and one patient by postoperative pathological examination. Six patients refused intravenous chemotherapy, and received oral etoposide capsule instead. Thirteen patients intravenously received etoposide plus cisplatin regiment for six cycles. Serum tumor markers were detected before treatment, including neuron-specific enolase (NSE), carcinoembryonic antigen (CEA), squamous cell carcinoma (SCC), carcinoma antigen 125 (CA125), carcinoma antigen 153 (CA153), and Cyfra 21-1 (CYF211). The detailed characteristics of patients are presented in table 1.

Table 1 Clinical and gene mutation characteristics of 19 SCLC patients

Parameters	*n*	%
Age (years)		
≤70	13	68.4
>70	6	31.6
Gender		
Male	18	94.7
Female	1	5.3
Smoking history		
≤40 packs year	7	36.8
>40 packs year	12	63.2
Serum tumor marker		
Normal	3	15.8
Abnormal	16	84.2
Clinical stage		
LD	7	36.8
ED	12	63.2
Bone metastasis		
No	16	84.2
Yes	3	15.8
Brain metastasis		
No	16	84.2
Yes	3	15.8
Chemotherapy		
Oral	6	31.6
Vein	13	68.4
Radiotherapy		
No	16	84.2
Yes	3	15.8
Surgery		
No	18	94.7
Yes	1	5.3
KRAS mutation		
No	15	78.9
Yes	4	21.1
PTEN mutation		
No	18	94.7
Yes	1	5.3

LD: limited disease; ED: extensive disease

1.2 Direct Sequencing

KRAS exon 3 and PTEN exon 7 mutations were detected using RT-PCR and direct sequencing technology as described by our previous study[13]. The primer sequences of KRAS and PTEN are shown in table 2.

1.3 Data Analysis

Data were analyzed using SPSS software version 21.0 (SPSS Inc., USA). The relationship between nonparametric variables was analyzed by Chi-square test. The Kaplan-Meier method and Cox regression analysis were used to determine the factors that had impacts on the OS.

Table 2 Primer sequences of KRAS and PTEN

Site	Sequence
KRAS-61AF	tcatctttggagcaggaaca
KRAS-61AR	tgcatggcattagcaaagac
PTEN7-AF	tttatttcagttgatttgcttga
PTEN7-AR	ccagagtaagcaaaacacctgca

2 RESULTS

The intermediate survival time was 9 months (range: 3–26 months, *n*=19). At the end of follow-up, there were 17 deaths among 19 SCLC patients after diagnosis. Exon 3 of KRAS mutation was found in 4 (21%) SCLC patients, and the point mutations were in codon 56 (CTC>CCC), 57 (GAC>CAC), 58 (ACA>CCC), 59 (GCA>GCC), 60 (GGT>AGT), 61 (CAA>CCA), 62 (GAG>GTG), 63 (GAG>GTG), 74 (ACT>ACA>ACC), 76 (GAG>GTG), 80 (TGT>GGT >AGT) (table 3, fig. 1A–1C). Exon 7 of PTEN mutation was found in only one (5%) SCLC patient with the point mutation in codon 238 (TTC>TTT) (table 3, fig. 1D). Kaplan Meier analysis showed that neither mutations of KRAS exon 3 nor PTEN exon 7 was significantly associated with OS (P>0.05). There was significant association between OS and clinical stage (LD *vs*. ED) or brain metastasis (with *vs*. without) (both P<0.05) (fig. 2).

Cox proportional hazards regression model demonstrated that extensive stage of disease was the only independent negative prognostic factors for OS in SCLC patients after adjusting for the age at diagnosis, smoking history, bone metastasis, brain metastasis, radiotherapy, abnormal serum tumor markers, and mutations of KRAS or PTEN (table 4).

Table 3 KRAS and PTEN mutations detected in SCLC tissues

Sample number	Age (years)	Clinical stage	KRAS mutation	PTEN mutation
1	48	LD	Codon 60(GGT/AGT); 74(ACT/ACA/ACC); 76(GAG/GTG); 80(TGT/CGT/AGT)	Wild type
2	35	ED	Codon 56(CTC/CCC); 57(GAC/CAC); 58(ACA/CCC); 59(GCA/GCC); 61(CAA/CCA)	Wild type
3	65	ED	Codon 60(GGT/AGT); 62(GAG/GTG); 63(GAG/GTG)	Wild type
4	59	ED	Wild type	Condon 238 TTC>TTC/TTT
5	35	ED	Codon 56(CTC/CCC); 57(GAC/CAC); 58(ACA/CCC); 59(GCA/GCC); 61(CAA/CCA)	Wild type

LD: limited disease; ED: extensive disease

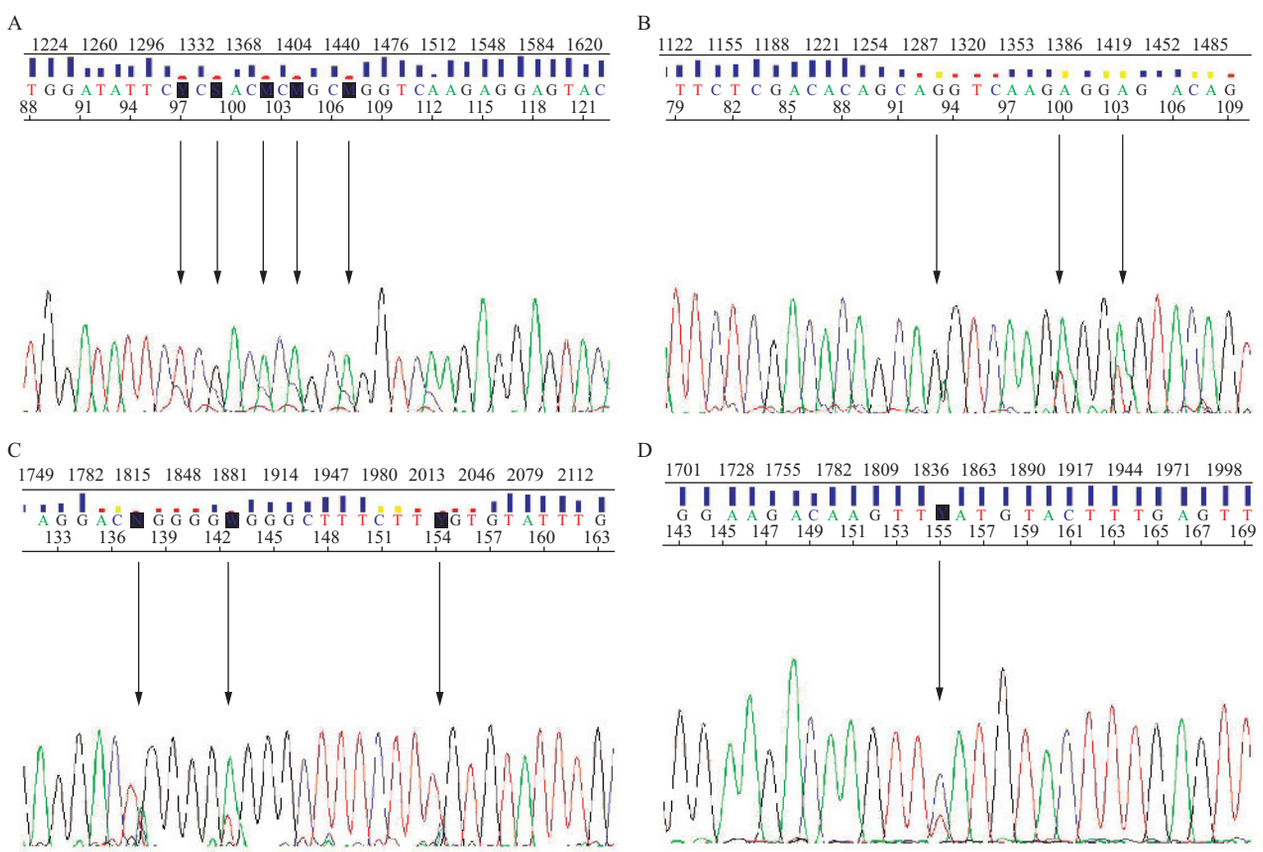

Fig. 1 A: KRAS codon 56 (CTC/CCC), 57 (GAC/CAC), 58 (ACA/CCC), 59(GCA/GCC), 61 (CAA/CCA) mutations were detected in case 2. B: KRAS codon 60 (GGT/AGT), 62 (GAG/GTG), 63 (GAG/GTG) mutations were found in case 3. C: KRAS codon 74 (ACT/ACA/ACC), 76 (GAG/GTG), 80(TGT/CGT/AGT) mutations were detected in case 1. D: PTEN 7 condon 238 TTC>TTC/TTT mutation was detected in case 4.

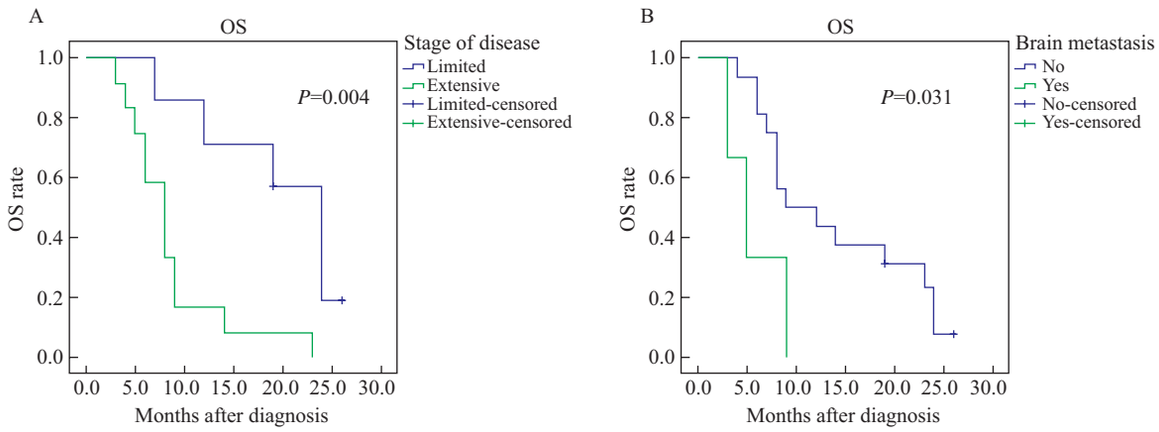

Fig. 2 A: overall survival (OS) rate of limited and extensive stages of SCLC patients. Log-rank test showed that the extensive stage of SCLC patients had worse OS than the limited stage patients (χ^2=8.405, P=0.004). B: OS rate in patients with and without brain metastasis lesions when diagnosed as SCLC. Log-rank test showed that SCLC patients with brain metastasis lesions had worse OS than those without (χ^2=4.648, P=0.031).

Table 4 Cox proportional hazards regression model test for OS of all patients[§]

	B	SE	Wald	Df	Sig.	Exp (B)	Exp (B) 95.0% CI	
							Lower	Upper
Age >70 years	–0.226	1.086	0.043	1	0.835	0.798	0.095	6.699
Clinical stage	3.204	1.198	7.154	1	0.007	24.643	2.354	257.948
Smoking history	1.767	1.359	1.691	1	0.194	5.851	0.408	83.888
Bone metastasis	–1.487	1.186	1.572	1	0.210	0.226	0.022	2.310
Brain metastasis	1.141	1.514	0.568	1	0.451	3.129	0.161	60.841
Radiotherapy	1.279	1.163	1.208	1	0.272	3.593	0.367	35.136
Abnormal serum tumor marker	1.603	0.915	3.069	1	0.080	4.970	0.827	29.879
KRAS mutation	–0.599	0.757	0.626	1	0.429	0.549	0.125	2.423
PTEN mutation	0.764	1.590	0.231	1	0.631	2.148	0.095	48.477

[§]Clinical stage: limited *vs.* extensive stages of SCLC patients; Abnormal serum tumor marker: any higher than the upper limit of normal range of tumor markers

3 DISCUSSION

SCLC is a fatal, smoking-related, multiple mutant disease and accounts for 10%–15% of all lung cancers[14]. Clinically it is divided into two subgroups: “limited disease” (LD-SCLC) and “extensive disease” (ED-SCLC). LD-SCLC is defined as tumor and nodes confined to 1 hemithorax and able to be encompassed within a single radiotherapy port, whereas “extensive disease” is defined as disease beyond these boundaries. Cisplatin combined with etoposide has been the standard treatment for SCLC since 2002 and there is no obvious progression made in the systemic treatment of SCLC. Although significant progress has been made in the molecular characterization and identification of molecular targets for SCLC, few therapies have improved the OS of SCLC patients. It seems that targeting one genetic mutation, as that in EGFR-driven NSCLC or HER2-positive breast cancer, could not achieve satisfactory clinical outcomes for SCLC, and more therapeutic targets should be identified.

The Ras oncogene controls several cellular functions, such as cell proliferation, apoptosis, migration, and differentiation[15]. KRAS mutations are present in over 20% of all cancers[16]. They are commonly present in lung adenocarcinoma patients who smoke. More than 80% of KRAS mutations are exon 2 mutations in NSCLC and colorectal cancers with ethnic and geographical differences noted[17–19]. The percentage of KRAS mutation in SCLC patients ranged from 0–16.2%[4, 20, 21]. Kodaz *et al*[4] reported 6 in 37 (16.2%) cases of SCLC with KRAS mutation and, among them, 5 had (83%) exon 3 mutation. Abdelraouf *et al* evaluated the mutation rate of a 4-gene panel [epidermal growth factor receptor (EGFR), N-ras (NRAS), KRAS, and B-Raf (BRAF)], anaplastic lymphoma kinase (ALK) rearrangement and MET amplification in patients with SCLC, but the results showed no KRAS mutation (both exons 2 and 3) in the 35 SCLC patients[21]. A combination of sequencing data from 375 SCLC cases (NGS, nZ65; Sanger, nZ100) revealed a wider spectrum of mutations, with variants detected in TP53 (57%), RB1 (11%), ATM, cMET, PTEN (6%–7%), BRAF, SMAD4, KRAS (3%–

4%) and ABL1, APC, CTNNB1, EGFR, FBXW7, FGFR2, HNF1A, HRAS, JAK3, MLH1 and PIK3CA (1%–2%)[20]. In the present study, we found KRAS exon 3 mutation in 21% SCLC patients, which is higher than that reported by literature before[4, 20, 21] and we speculate that the discrepancy may be caused by racial and geographic differences or mutation detection methods.

SCLC is an aggressive malignancy with much higher loss of growth control due to loss of tumor suppressors, including PTEN[22–24]. Alterations (point mutations, small deletions) in the PTEN, which is located on chromosome 10q23.3, are observed in 10% to 18% of SCLC tumors[10]. PTEN deletion could not only accelerate the development of SCLC but also abrogate the loss of Chr19 in TP53 in engineered murine model[25]. Also, retinoblastoma 1 (RB1) is convinced to be associated with the initiation of SCLC by genetically engineered mouse model experiment[25]. Malkoski *et al* found that tumor with PTEN deletion displayed more malignant conversion than KRAS (G12D) initiated tumors[26]. NGS testing of PTEN mutation in 98 SCLC cases[27] showed 1 case of substitution/indel mutation, 2 of deletion and 2 of truncated mutation. According to the comprehensive genomic profiles of 152 fresh-frozen tumor specimens obtained from 110 SCLC patients, the frequency of PTEN mutation was 9%[28]. PTEN mutation rate in our research was 5%, which is similar to that published previously[20, 27]. The patient with a point mutation of PTEN in our study was a heavy smoker with LD-SCLC. Although the patient was treated on a routine basis by chemotherapy of EP regimen for six cycles, his OS time was only eight months.

More than half of NSCLC and SCLC patients with mediastinal lymph node metastasis would eventually develop to have brain metastasis[29]. According to the data from 1973 to 2011 using the SEER database, nearly 18% SCLC patients without previous metastasis would develop brain metastasis after disease progresses and the propensity is almost doubled compared with that in NSCLC patients[30]. SCLC patients who are initially diagnosed as having distant spread of disease and brain metastasis are considered to have poor prognosis[31]. In our study, 12/19 (63%) were ED SCLC patients and 3/12 (25%) presented with brain metastasis during the first consultation. Extensive stage and brain metastasis were found to be the negative factors affecting the OS of SCLC patients.

Although our study found no positive association of mutations of KRAS and PTEN with the prognosis and clinical characteristics of SCLC patients, the mutant status still provides information regarding the complex genetic phenotypes of SCLC. Identification of useful biomarkers and potential molecular targets is necessary for effective targeted therapies in SCLC.

There are several limitations in our research. Firstly, a comprehensive panel of genomic aberrations was not analyzed in SCLC. Secondly, our study employed an exon-specific PCR-based direct sequencing method to examine KRAS and PTEN mutations, by which large intragenic deletions couldn't be detected. While few specific targeted therapies have been approved for the treatment of SCLC, knowing KRAS and PTEN mutational status may help understand the development of this deadly disease and contribute to the identification of more targeted therapies for SCLC.

In conclusion, our study demonstrated that there was no significant association of KRAS exon 3/ PTEN exon 7 mutations with OS of SCLC patients; brain metastasis and extensive disease stage were the prognostic factors for OS of SCLC patients. Further studies are still warranted to validate our understanding of the molecular profiles of SCLC.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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