Original Article

Telomerase reverse transcriptase promoter region mutations and the clinical characteristics of pulmonary neuroendocrine tumors

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Background: Pulmonary neuroendocrine tumors (NETs) represent approximately 20–25% of all primary lung tumors. It is occasionally difficult to distinguish between the different types of pulmonary NETs. Telomerase reverse transcriptase (TERT) can effectively maintain the structural integrity of telomeres. Alterations in telomere length, telomerase activity, and the expression of hTERT mRNA may be a useful tool for the differential diagnosis among different kinds of NETs. There is no report about TERT promoter mutations in pulmonary NETs in China. This study aimed to clarify the status of TERT promoter region mutations and the clinical characteristics of pulmonary NETs.

Methods: A total of 41 surgically resected pulmonary NETs were retrospectively collected from the Zhejiang Cancer Hospital in China between 2008 and 2016, including typical carcinoid (TC) cases, atypical carcinoid (AC) cases, large cell neuroendocrine carcinoma (LCNEC) cases, and small cell lung carcinoma (SCLC) cases. TERT promoter mutation was analyzed by polymerase chain reaction (PCR) amplification and Sanger sequencing. The clinical characteristics and treatment data were also collected. The survival time of all patients was followed-up.

Results: Most pulmonary NET patients were male. Most SCLC patients were heavy smokers, while most AC/TC patients were non-smokers. No TERT promoter region 124 (C228T) and 146 (C250T) mutations were found in the 41 cases of pulmonary NET. The prognosis of AC/TC was the best, followed by LCNEC, while SCLC was the worst. Overall survival (OS) of the SCLC patients ≤65 years (n=23) and >65 years (n=6) were 35 and 18 months, respectively, P=0.041. SCLC patients who received ≥3 cycles of adjuvant chemotherapy (n=18) had longer OS as compared to those who received ≤2 cycles of adjuvant chemotherapy (n=11) (not reach *vs.* 22 months, P=0.061).

Conclusions: TERT promoter mutations are rare in pulmonary NET. The prognosis of different histological subtypes of pulmonary NET varies. Age and cycles of adjuvant chemotherapy are related to prognosis of resected SCLC.

Keywords: Pulmonary neuroendocrine tumor; telomerase reverse transcriptase (TERT); overall survival (OS)

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Introduction

Pulmonary neuroendocrine tumors (NETs) represent approximately 20–25% of all primary lung tumors (1,2). They represent a broad spectrum of morphological types that share specific morphological, ultrastructural, immunohistochemical, and molecular characteristics. NETs have been classified into four categories: low grade typical carcinoid (TC), intermediate grade atypical carcinoid (AC), high grade large cell neuroendocrine carcinoma (LCNEC), and small cell lung carcinoma (SCLC) (3). It is occasionally difficult to distinguish between the different types of pulmonary NETs since the clinical and pathological features, and molecular characteristics of these tumors are not fully understood.

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, which can effectively maintain the structural integrity of telomeres. Human TERT gene is located on chromosome 5p. TERT promoter mutations mainly occur in the core promoter region 124 (C228T) and 146 (C250T), and these mutations can increase TERT mRNA, protein and enzyme activities, thereby increasing telomere length. Human TERT siRNA can effectively suppress telomerase and lead to apoptosis in A549 lung adenocarcinoma cells (4). Nishio *et al.* suggested that alterations in telomere length, telomerase activity, and the expression of hTERT mRNA may play roles in the pathogenesis of pulmonary NETs, and can be a useful tool for the differential diagnosis between TCs and LCNECs (5). There is no report about TERT promoter mutations in pulmonary NETs in China. In order to clarify the status of TERT promoter region mutation and the prognosis of pulmonary NET, TERT promoter mutation was analyzed in this study using ARMs-qPCR and Sanger sequencing in resected pulmonary NETs at the Zhejiang Cancer Hospital. The clinical characteristics and prognosis were also analyzed.

Methods

Patients

A total of 41 pulmonary NET specimens obtained from resected tumors were retrospectively collected from the Zhejiang Cancer Hospital in China between 2008 and 2016. The pathological diagnosis was based on the standard criteria defined by the World Health Organization (WHO) (6). The classification of stages was defined by the eighth edition of the TNM classification for lung cancer (7). Clinical characteristics such as gender, age, pathological type, stage, smoking history, and adjuvant chemotherapy cycles received by each patient are listed in *Table 1*. This study was approved by the Medical Ethical Committee of Zhejiang Cancer Hospital.

None of the TC patients received adjuvant chemotherapy, while one AC patient with stage IIIA received adjuvant chemotherapy (four cycles of etoposide and cisplatin) and thoracic radiotherapy. ALL LCNEC cases received >3 cycles of adjuvant chemotherapy. Among the 29 cases of SCLC, 18 cases received >3 cycles of adjuvant chemotherapy (*Table 1*). Nine patients with stage IIIA and one patient with stage IIIB received adjuvant thoracic radiotherapy. Only four patients received prophylactic cranial irradiation (PCI) including three stage IIIA cases and one stage IIIB case.

Analysis of TERT mutations

Mutational analyses of TERT (C228T and C250T) were performed on genomic DNA extracted from paraffin-embedded tumor tissues using ARMs-qPCR (Bio-Rad qPCR, CFX96) and sanger sequencing. The protocol of qPCR was as follows: The reaction mix was prepared with enzyme, primer, probe, $MgCl_2$ (25 mM), dUTP (2 mM), Uracil DNA glycosylase (UNG) and heat-labile. Then, the mix (18 mL/well), sample DNA (10 ng/μL, 2 μL/well) and reference standard DNA (2 μL/well), respectively, were added to a 96-well-plate, which was sealed and centrifuged for 30 s at 3,700 rpm. Sequencing was performed with BIO-RAD CFX96 PCR detection system for one hour, and the 6-Carboxyfluorescein (FAM) and rhodamine-X (ROX) signals were collected. The results were statistically analyzed with matched software. The protocol for Sanger sequencing was as follows: A total of 19 μL of reaction mix including PCR reaction enzyme, primer and ddH_2O was combined with 1 μL of sample DNA (10 ng/μL). PCR was performed with 2,720 Thermal Cycler PCR detection system for 2 hours and 10 minutes, then sequencing was performed with ABI3730. Finally, the results were statistically analyzed with Mutation Surveyor V4.0.8 (demo).

Follow-up

The follow-up deadline was May 05, 2017. The survival time was calculated from the date of pathological diagnosis.

Statistical analyses

The data were analyzed using the statistical package for

Table 1 Clinical features of all patients (n=41)

Clinical features	Total	TC/AC [6]	LCNEC [6]	SCLC [29]	P
Age [median]	58 [38–77]	52 [49–63]	58 [42–70]	58 [38–77]	–
Gender					0.596
Female	9	2	2	5	
Male	32	4	4	24	
Stage					0.248
IA/IB	14	3	2	9	
IIA/IIB	8	2	1	5	
IIA/IIIB	18	1	2	15	
IV	1	0	1	0	
Smoking					0.004
Non-smokers	10	5	3	2	
Light smokers	5	0	0	5	
Moderate smokers	1	0	0	1	
Heavy smokers	25	1	3	21	
Chemotherapy cycles					0.037
≤2	17	5	1	11	
≥3	24	1	5	18	

TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma.

the social sciences (SPSS) software version 15.0. Overall data were screened using the Chi-square test. The survival curves were calculated by the Kaplan-Meier method, and compared using the log-rank test, with $P<0.05$ indicating statistical significance.

Results

Population characteristics

Most of the patients were male (76.2%). The median age of TC/AC, LCNEC and SCLC patients was >50 years. Most of the patients were heavy smokers in the SCLC group (72.4%), but non-smokers in the TC/AC group (83.3%).

Status of TERT mutation

No TERT promoter region 124 (C228T) and 146 (C250T) mutation was found among the 41 cases of pulmonary NET (*Figure 1*).

Survival analyses

No TC/AC patient was lost to follow-up. All TC/AC patients were alive with no relapse, and the follow-up duration was 11–107 months. No LCNEC patient was lost to follow-up, and the follow-up duration was 22–44 months. One patient suffered from cervical vertebra metastasis and died 34 months after pathological diagnosis. One LCNEC patient with brain metastasis underwent surgery and is currently alive (≥29 months). One patient suffered from thoracic vertebra metastasis 10 months after the pathological diagnosis and is currently alive (≥34 months). The other three LCNEC patients are currently alive with no relapse. Three patients were lost to follow-up, 17 patients died and nine patients were alive among the 29 SCLC patients. The follow-up duration was 5–109 months. Overall survival (OS) of the different histological subtypes of pulmonary NET is shown in *Figure 2*. OS of the SCLC patients ≤65 and >65 years old is shown in *Figure 3*.

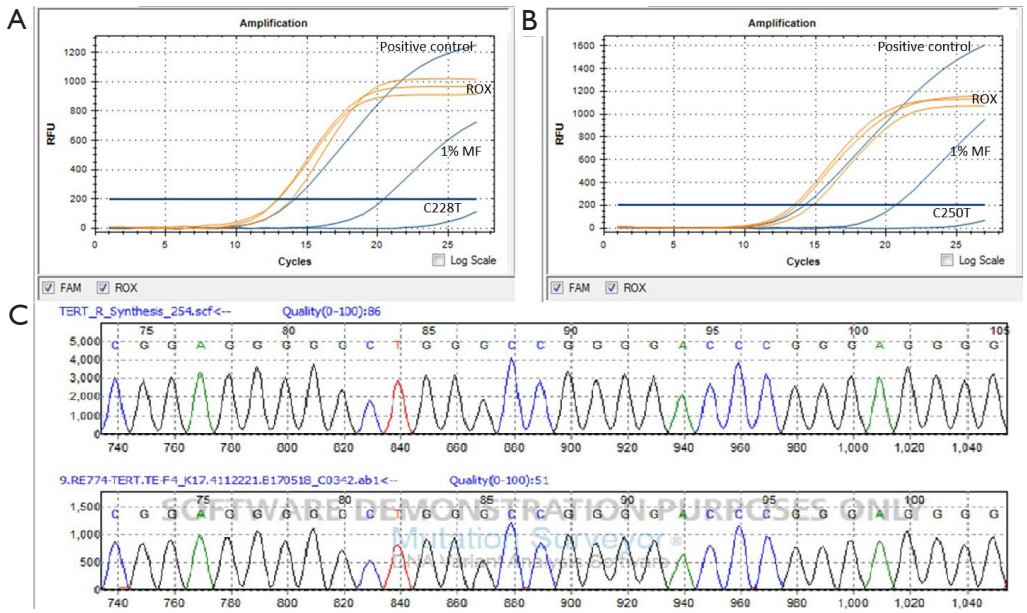

Figure 1 There were no patients with TERT promoter mutations for the core promoter region 124 (C228T) and 146 (C250T). (A) Tumor tissue tested negative for TERT (C228T) mutation by ARMs-qPCR; (B) tumor tissue tested negative for TERT (C250T) by ARMs-qPCR; (C) the core promoter region 124 (C228T) and 146 (C250T) were wild type detected by sanger sequencing for TERT. TERT, telomerase reverse transcriptase; RFU, relative fluorescence unit; ROX, rhodamine-X; MF, mutation frequency.

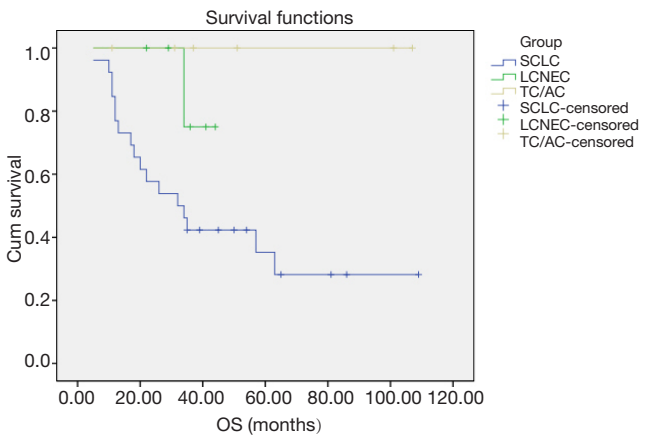

Figure 2 OS of the different histological subtypes of pulmonary NETs (P=0.028) (TC/AC, n=6; LCNEC, n=6; SCLC, n=29). NETs, neuroendocrine tumors; SCLC, small cell lung carcinoma; TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; OS, overall survival.

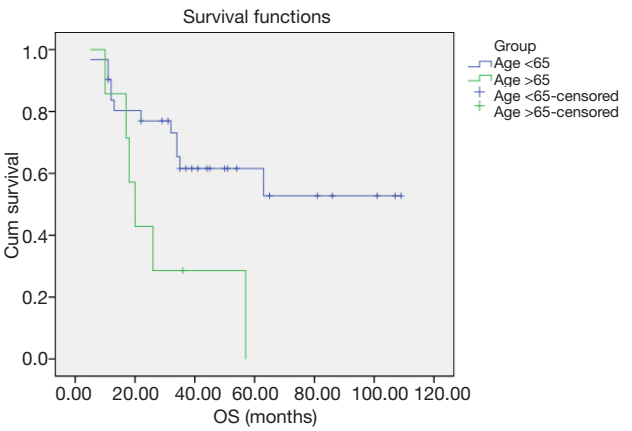

Figure 3 OS of the SCLC patients ≤65 years old (n=23) and >65 years old (n=6) (35 *vs.* 18 months, P=0.041). OS, overall survival; SCLC, small cell lung carcinoma.

OS of the female and male SCLC patients is shown in *Figure 4*. OS of the SCLC patients who received ≤2 cycles of adjuvant chemotherapy and those who received ≥3 cycles of adjuvant chemotherapy is shown in *Figure 5*.

Discussion

TERT is the catalytic component of telomerase, and the rate-limiting determinant of telomerase activity (8). Telomerase activation is essential for carcinogenesis (9). TERT promoter mutations frequently occur in older patients, males, with high tumor grade and stage. It is

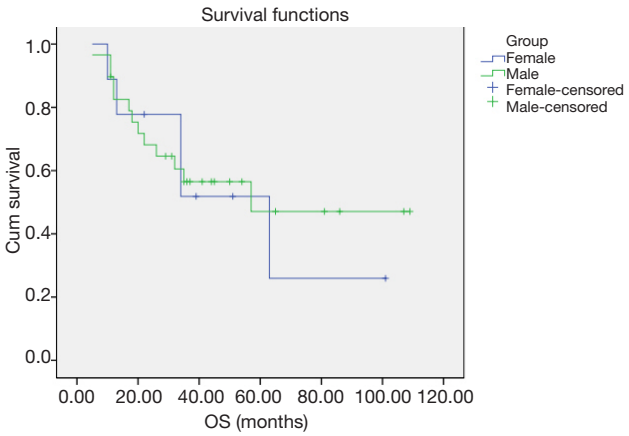

Figure 4 OS of the female SCLC patients (n=5) and male SCLC patients (n=24) (34 *vs.* 32 months, P=0.596). OS, overall survival; SCLC, small cell lung carcinoma.

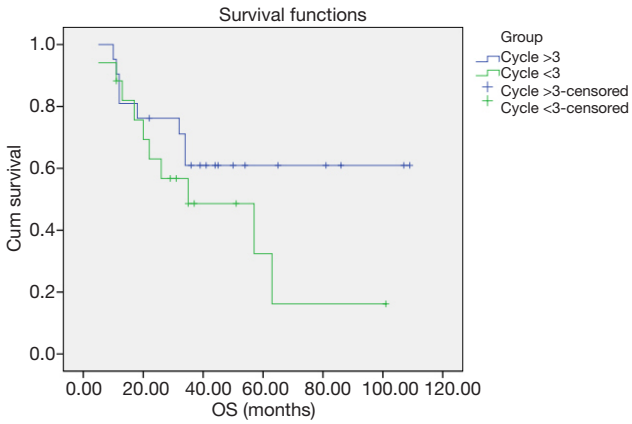

Figure 5 OS of the SCLC patients who received ≤2 cycles of adjuvant chemotherapy (n=11) and ≥3 cycles (n=18) (22 months *vs.* not reach, P=0.061). OS, overall survival; SCLC, small cell lung carcinoma.

significantly associated with distant metastasis and serves as an adverse prognostic factor (10). TERT promoter mutations were seen in NSCLC patients from China, the United States and European countries (11,12). The frequencies of TERT promoter mutations among squamous cell carcinomas (SCCs) at different sites are as follows: ~70% in skin SCC and urothelial carcinoma with squamous differentiation, 16.67% in head and neck SCC, and none in lung and cervical SCC (13). Data from the United States showed that the frequency of TERT promoter mutation in small cell carcinoma is low, except for those originating from the bladder (14). Next generation sequencing (NGS) was applied to 148 pulmonary NET from Italy, comprising of the four WHO classification categories. Multivariate survival analysis revealed RB1 mutation (P=0.0005) and TERT copy gain (P=0.016) as independent predictors of poorer prognosis. No TERT mutation was found in these 148 pulmonary NET patients (15). The incidence of EGFR mutation in NSCLC is higher in China than in the United States and European countries (16,17). TERT polymorphisms of NSCLC are higher in Asians than in Europeans (18,19). In China, TERT polymorphisms are more strongly associated with the risk of NSCLC with EGFR mutation than those without EGFR mutation (19). Whether there is a difference in TERT promoter region 124 (C228T) and 146 (C250T) mutations between Chinese as compared to Americans and Europeans remains unknown. Our study showed no TERT promoter region 124 (C228T) and 146 (C250T) mutations in 41 cases of pulmonary NET. The consistency of TERT promoter region 124 (C228T) and 146 (C250T) mutation of pulmonary NET from China and Italy is different from the inconsistency of EGFR mutation in Chinese and Caucasians (14-17). These data suggested that TERT promoter region 124 (C228T) and 146 (C250T) mutations may not be a useful biomarker to distinguish between the different subtypes of pulmonary NET.

The OS curve of the subtypes of pulmonary NET showed that the prognosis of TC/AC was better than SCLC and LCNEC, which may be due to the different differentiations and malignancies of these subtypes. The prognosis of these subtypes is consistent with other reports (20-23). Yang *et al.* reported the role of adjuvant therapy in a population-based cohort of patients with early stage SCLC. The results showed that the adjuvant chemotherapy was associated with significantly improved survival as compared to surgery alone (23). In our cohort, SCLC patients who received >3 cycles of adjuvant chemotherapy had a longer OS as compared to those with ≤2 cycles of chemotherapy. Kim *et al.* showed that OS was significantly improved in younger than in older patients with local disease SCLC (LD−SCLC) (24), which was consistent with our findings.

Pulmonary NETs tend to occur in the fourth to sixth decade of life (25,26). The median ages of TC/AC, LCNEC, and SCLC were 52, 58 and 58 years in our cohort. Another report from China showed that 215 (72.1%) patients were <65 years old at the time of diagnosis (27). The majority of ACs/TCs occurred in never or light smokers (26). In our cohort, most of the AC/TC patients

were non-smokers. SCLC is a smoking-related disease and most of the patients in our study were heavy smokers. It is consistent with another report from China in which 208 patients (69.8%) had a smoking history (27). The proportion of women with SCLC increased from 28% in 1973 to 50% in 2002 as per the data from the surveillance, epidemiologic, and end results (SEER) database (28). Our study suggested that most of the SCLC patients were male and another report from China also showed that 235 (78.9%) of the 298 SCLC patients were male (27). Male to female ratios of pulmonary TC/AC were 0.8 as recorded in SEER (29), while most of the TC/AC patients were male in our cohort. Another report showed that LCNEC frequently occurred in males and smokers (22,30). In our cohort, 50% of the LCNEC patients were male and heavy smokers.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2017.12.06). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Medical Ethical Committee of Zhejiang Cancer Hospital (IRB-2016-86). Most of the patients in this retrospective study signed the written informed consent before surgery to preserve their specimens in the Biological Sample Bank of Zhejiang Cancer Hospital to be used in research. This study is a retrospective study and a number of patients have died, exempt written informed consent was also approved by the Ethics Committee of Zhejiang Cancer Hospital.

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