Abstract. Bronchopulmonary neuroendocrine tumours (BP-NETs) comprise a large spectrum of tumours including typical carcinoids (TCs), atypical carcinoids (ACs), large-cell neuroendocrine carcinomas (LCNECs) and small-cell lung carcinomas (SCLCs) that exhibit considerably different biological aggressiveness and clinical behaviours. The phosphatidylinositol-3-kinase α catalytic subunit (PIK3CA) gene is known to be involved in the pathogenesis of several types of human cancers through gene amplification, deletions or somatic missense mutations within the helical and catalytic domains. However, the PIK3CA gene status in BP-NETs has yet to be explored. This study aimed to investigate the PIK3CA gene status in a large series of BP-NETs by direct gene sequencing and to analyse its correlation with the main clinicopathological parameters. To the best of our knowledge, we demonstrated for the first time a high frequency of somatic missense mutations (23.2%) in the PIK3CA gene in a series of 190 BP-NETs, including 75 TCs, 23 ACs, 17 LCNECs and 75 SCLCs. The frequency of the PIK3CA gene mutation in the kinase domain was higher (17.9%) than that in the helical domain (5.3%). When the mutational status of the PIK3CA gene was compared with the main clinical and pathological characteristics of the BP-NET patients, we found a significant association between PIK3CA gene mutations and BP-NET histology (P=0.011). Interestingly, the frequency of PIK3CA gene mutations increased with the biological aggressiveness of all BP-NETs, except LCNECs. In conclusion, our results suggest that PIK3CA gene mutations may play a key role in tumourigenesis and aggressiveness of BP-NETs. The PIK3CA gene may represent a favourable candidate for an effective therapeutic strategy in the treatment of patients with BP-NETs.

Introduction

Bronchopulmonary neuroendocrine tumours (BP-NETs) comprise approximately 20% of lung cancers and represent a large spectrum of tumours arising from neuroendocrine cells of the bronchopulmonary epithelium. Although they share structural, morphological, immunohistochemical and ultrastructural features, BP-NETs exhibit considerably different biological characteristics and clinical behaviours. Based on their increasing biologic aggressiveness, BP-NETs are separated into four different subgroups: low-grade typical carcinoids (TCs), intermediate-grade atypical carcinoids (ACs) and two high-grade malignancies, large-cell neuroendocrine carcinomas (LCNECs) and small-cell lung carcinomas (SCLCs) (1-3).

TCs and ACs account for ~1-2% of primary lung cancers. The slow-growing TCs exhibit a fairly good prognosis, although ~10-23% of cases when diagnosed metastasize to the regional lymph nodes, with a 5-year overall survival rate ranging from 82 and 100% (2-5). In contrast, ~40-50% of ACs metastasize to the regional lymph nodes when diagnosed, with a 5-year overall survival rate of ~50% (2-5).

SCLCs are the most common BP-NETs and account for 15-20% of invasive lung malignancies, while LCNECs are a very rare neoplasia and represent <1% of lung cancers. LCNECs are currently considered a distinct subtype of non-small cell lung cancer (NSCLC) and are classified as a variant of large-cell carcinomas (LCCs), whose diagnosis may be difficult to establish because of the difficulties in distinguishing LCNECs from poorly differentiated adenocarcinomas, squamous cell carcinomas and basaloid carcinomas. In spite of the several differences between high-grade LCNECs and SCLCs, both progress rapidly, are aggressively metastatic and have a very poor prognosis with a 5-year overall survival rate of 15 to 57% and <5%, respectively (2-5).

The management of BP-NETs is largely dependent on the grade of differentiation (low-to-intermediate grade vs. high-grade) and clinical stage when diagnosed (localized vs. metastatic disease). Surgical resection is the treatment of...
choice for low-to-intermediate grade and localized tumours, whereas chemotherapy is generally preferred for high-grade and/or disseminated lesions. However, traditional therapies, including radiotherapy and systemic chemotherapy with DNA-damaging cytotoxic agents, are not effective and offer limited benefits to patients with advanced disease (6,7). The lack of effectiveness of traditional agents for BP-NETs has led to an extensive exploration of the molecular profile of these tumours in order to clarify the molecular mechanisms of BP-NET carcinogenesis and progression and to identify new targets for innovative therapies.

The phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway plays a key role in essential cellular processes, such as cell proliferation, cell growth, apoptosis, metabolism, transcription, protein synthesis, angiogenesis, and tissue invasion, and is involved in the pathogenesis of several types of human cancers (8-11).

Class IA PI3K is a heterodimeric lipid kinase composed of a regulatory subunit (p85α, p85β, p50α, p55α, or p55γ, collectively called p85) and a catalytic subunit (p110α, p110β, or p110δ, collectively called p110). Upon activation by multiple receptor kinase families, including receptor tyrosine kinases and G protein-coupled receptors, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), a process that is reversed by the lipid and protein phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10). PIP3 acts as a docking site and consists of 20 exons encoding for the p110 catalytic subunit of PIK3CA (Reference sequence ENSG00000121879).

DNA isolation. Genomic DNA was isolated from 10 µm sections of formalin-fixed and paraffin-embedded tissues. Tissue digestion was preceded by xylene treatment to remove paraffin, rehydration through a graded series of alcohol and manual macrodissection of the tumour area to obtain at least 70% of the tumour cells. Then, genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions for paraffin-embedded tissues. The DNA quality and quantity were evaluated using a NanoDrop ND-1000 spectrophotometer.

To date, the mutational status of the PIK3CA gene in BP-NETs remains unknown. The aim of this study was to analyse the mutational profile of the PIK3CA gene in a large series of BP-NETs and to correlate the PIK3CA gene status with the main clinicopathological parameters.

Materials and methods

Patient selection and tumour characteristics. One hundred and ninety consecutive BP-NETs were retrospectively collected from patients who had undergone surgery at the Department of Cardio-Thoracic Surgery of the University of Pisa between 2000 and 2009. Patients enrolled in this study did not receive chemotheraphy and/or radiotherapy before the surgery.

Histological diagnoses and pathological features were reviewed independently by two pathologists (G.A. and G.F.) and formulated according to the 2004 World Health Organization (WHO) classification. Discrepent diagnoses were re-evaluated jointly and discussed until an agreement was reached. Neuroendocrine differentiation was detected by a positive immunohistochemical staining for chromogranin A, synaptophysin and/or CD56 markers.

The selection of patients did not require approval by the Institutional Ethics Committee since all samples were coded and the names of the patients were not revealed.

DNA isolation. Genomic DNA was isolated from 10 µm sections of formalin-fixed and paraffin-embedded tissues. Tissue digestion was preceded by xylene treatment to remove paraffin, rehydration through a graded series of alcohol and manual macrodissection of the tumour area to obtain at least 70% of the tumour cells. Then, genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions for paraffin-embedded tissues. The DNA quality and quantity were evaluated using a NanoDrop ND-1000 spectrophotometer.

Mutational analysis of the PIK3CA gene. Mutational analysis of the PIK3CA gene (Reference sequence ENSG00000121879) was performed by PCR amplification and direct gene sequencing of the helical and kinase domains of PI3K encoded by exons 9 and 20, respectively.

Primer pairs flanking PIK3CA exons 9 and 20 were selected to avoid the frequent cross-amplification of chromosome 22q (a known PIK3CA pseudogene) using the software Primer3 (http://frodo.wi.mit.edu/primer3/). The PIK3CA gene was amplified for exon 9 with the primers 5'-ATCATCTGTG AATCCAGA-3' (forward) and 5'-TTAGCACTTACCTGTG AC-3' (reverse) and for the exon 20 with the primers 5'-GTGGCTGATTGTGACAAAGACC-3' (forward) and 5'-GTGGAGAAT

PCR amplification was performed in a total volume of 25 µl containing 100 ng of genomic DNA, 12.5 µl of HotStarTaq master mix (Qiagen), 0.5 µl of each primer (20 µM), and water as follows. After an initial denaturation step of 15 min at 95°C, the reaction mixture was run for 40 cycles at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. The efficiency and the quality of the PCR amplification were confirmed by running the PCR products on a 1.5% agarose gel.
The PCR products were subsequently subjected to a purification procedure to remove primers, nucleotides, enzymes and salts using the QIAquick PCR Purification kit (Qiagen).

Cycle sequencing analysis of the purified PCR products was performed with the ABI BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions using the PCR amplification primers for bidirectional sequencing, and the reaction products were purified by ethanol precipitation. Finally, sequence determination was performed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and data were analysed using the Sequencing Analysis 5.0 Software (Applied Biosystems).

Statistical analysis. Statistical analyses were performed using the StatView 5.0 Software (Abacus Concepts, Inc., Berkeley, CA). The contingency tables and the Chi-square test were used to analyse the association between the different biological parameters. All the tests were two-tailed and a P-value <0.05 was considered to indicate a statistically significant difference.

**Table I.** Characteristics of the BP-NET patients.

| Clinicopathological features | TC | AC | LCNEC | SCLC |
|-----------------------------|----|----|------|------|
| Age (years)                 |    |    |      |      |
| Median (range)              | 61 (24-82) | 58 (23-82) | 68 (45-84) | 69 (49-83) |
| Gender                      | n=75 | n=23 | n=17 | n=75 |
| Male                        | 31 (41.3) | 6 (26.0) | 13 (76.5) | 60 (80.0) |
| Female                      | 44 (58.7) | 17 (74.0) | 4 (23.5) | 15 (20.0) |
| Tumour size                 | n=74 | n=23 | n=16 | n=65 |
| T1 (T1a-T1b)                | 50 (67.6) | 9 (39.1) | 4 (25.0) | 21 (32.3) |
| T2 (T2a-T2b)                | 19 (25.7) | 11 (47.8) | 7 (43.7) | 29 (44.6) |
| T3                          | 5 (6.7) | 3 (13.1) | 5 (31.3) | 15 (23.1) |
| Lymph node status           | n=65 | n=19 | n=13 | n=56 |
| Negative                    | 62 (95.4) | 13 (68.4) | 11 (84.6) | 34 (60.7) |
| Positive                    | 3 (4.6) | 6 (31.6) | 2 (15.4) | 22 (39.3) |

*Values are shown as n (%). TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma; BP-NET, bronchopulmonary neuroendocrine tumour.

The PCR products were subsequently subjected to a purification procedure to remove primers, nucleotides, enzymes and salts using the QIAquick PCR Purification kit (Qiagen).

Cycle sequencing analysis of the purified PCR products was performed with the ABI BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions using the PCR amplification primers for bidirectional sequencing, and the reaction products were purified by ethanol precipitation. Finally, sequence determination was performed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and data were analysed using the Sequencing Analysis 5.0 Software (Applied Biosystems).

**Results**

**PI3KCA gene mutation in BP-NETs.** To assess the frequency of PI3KCA gene mutations in BP-NETs we sequenced genomic DNA isolated from formalin-fixed and paraffin-embedded tissues in a large series of lung neuroendocrine tumours, including 75 low-grade TCs, 23 intermediate-grade ACs, 17 high-grade LCNECs and 75 high-grade SCLCs. Clinical and pathological characteristics of the patients, including patient age, gender, histological type, tumour size, and lymph node status were recorded whenever available and summarized in Table I.

Targeted sequencing of the helical and kinase domains of PI3K revealed that PI3KCA gene mutations were present in 44 of the 190 analysed tumours (23.2%). The distribution of mutations between the kinase and the helical domains of PI3K showed that the mutation frequency of the kinase domain (34/190, 17.9%) was approximately three times the mutation frequency of the helical domain (10/190, 5.3%) (Table II).

All the mutations identified in our series of BP-NETs were single nucleotide substitutions that lead to non-synonymous mutations, including 36 transversions (G ↔ A or C ↔ T) and 8 transitions (A ↔ T, G ↔ T or G ↔ C) (Table II). The most frequent genetic alteration was the E547K mutation in the helical domain accounting for 15.9% (7/44) of the PI3KCA gene mutations identified in our study (Table II and Fig. 1A). In the kinase domain we identified 3 different mutational hotspots at codons 1021, 1047, and 1049. The most prevalent genetic anomalies observed in these codons were the mutations Y1021N/F/C (5/44, 11.4%), H1047R (2/44, 4.5%) and G1049S (4/44, 9.1%) (Table II and Fig. 1B-D).

**Correlation between the presence of PI3KCA gene mutations and clinical and pathological parameters.** The mutational status of the PI3KCA gene was compared with the main clinical and pathological characteristics of the BP-NET patients. The PI3KCA gene mutations did not correlate with the clinical and pathological characteristics of the patients, such as age, gender, or lymph node status (Table III). However, statistical analysis showed a significant association between the PI3KCA gene mutations in the helical and kinase domains and BP-NET histology (Chi-square test, P=0.011). Our results showed a relatively lower prevalence of PI3KCA gene mutations in the low-grade TCs (10/75, 13.3%) compared to the intermediate-grade ACs (9/23, 39.1%) and high-grade SCLCs (23/75, 30.7%). In contrast to the SCLCs, the high-grade LCNECs showed a lower frequency of PI3KCA gene mutations (2/17, 11.8%) compared with the other types of BP-NETs (Table III).
Discussion

BP-NETs comprise a large spectrum of lung cancers ranging from low-grade TCs, to intermediate-grade ACs, to high-grade LCNECs and SCLCs that exhibit considerably different biological aggressiveness and clinical behaviour. At present, the only curative treatment for BP-NETs is radical surgery since traditional therapies are not effective (1,2,5,7).

Recently, successful clinical trials of imatinib, gefitinib/erlotinib and trastuzumab, which are specific for \textit{BCR/ABL} translocations (18), epidermal growth factor receptor (\textit{EGFR}) mutations (19,20) and \textit{HER-2/neu} amplifications (21), respectively, have illustrated the ability to develop drugs that target genetic abnormalities and lead to potential streamlined therapies based on the genomic landscape of an individual's cancer.

Table II. \textit{PIK3CA} gene status in BP-NET patients.

| Exon | \textit{PIK3CA} Domain | ID | Sample | Histology | Nucleotide substitution | Amino acid substitution | No. of cases |
|------|------------------------|----|--------|-----------|------------------------|------------------------|-------------|
| 9    | Helical                | N145| SCLC   | c.1573 G>A | p.E525K                | 1                      |
| 9    | Helical                | N56 | TC     | c.1576 A>G | p.N526D                | 1                      |
| 9    | Helical                | N170| SCLC   | c.1624 G>A | p.E542K                | 1                      |
| 9    | Helical                | N20, N46, N50| TC| c.1639 G>A | p.E547K                | 3                      |
| 9    | Helical                | N34 | AC     | c.1639 G>A | p.E547K                | 1                      |
| 9    | Helical                | N18, N33, N59| SCLC| c.1639 G>A | p.E547K                | 3                      |
| 20   | Kinase                 | N188| SCLC   | c.2944 G>C | p.E982Q                | 1                      |
| 20   | Kinase                 | N101| SCLC   | c.2949 G>A | p.M983I                | 1                      |
| 20   | Kinase                 | N172| TC     | c.2993 T>C | p.F998S                | 1                      |
| 20   | Kinase                 | N173| SCLC   | c.2998 A>G | p.N1000D               | 1                      |
| 20   | Kinase                 | N116| SCLC   | c.3007 T>C | p.S1003P               | 1                      |
| 20   | Kinase                 | N129| AC     | c.3007 T>C | p.S1003P               | 1                      |
| 20   | Kinase                 | N97 | SCLC   | c.3012 G>T | p.M1004I               | 1                      |
| 20   | Kinase                 | N8  | AC     | c.3017 T>C | p.L1006P               | 1                      |
| 20   | Kinase                 | N35 | SCLC   | c.3016 C>T | p.L1006F               | 1                      |
| 20   | Kinase                 | N167| SCLC   | c.3022 T>C | p.S1008P               | 1                      |
| 20   | Kinase                 | N176| AC     | c.3022 T>C | p.S1008P               | 1                      |
| 20   | Kinase                 | N125| SCLC   | c.3032 C>T | p.P1011L               | 1                      |
| 20   | Kinase                 | N26 | TC     | c.3034 G>A | p.E1012K               | 1                      |
| 20   | Kinase                 | N95 | AC     | c.3041 A>G | p.Q1014R               | 1                      |
| 20   | Kinase                 | N182| AC     | c.3050 A>T | p.D1017V               | 1                      |
| 20   | Kinase                 | N67 | AC     | c.3062 A>G | p.Y1021C               | 1                      |
| 20   | Kinase                 | N143| SCLC   | c.3062 A>T | p.Y1021F               | 1                      |
| 20   | Kinase                 | N88, N157| SCLC| c.3061 T>A | p.Y1021N               | 2                      |
| 20   | Kinase                 | N159| TC     | c.3061 T>A | p.Y1021N               | 1                      |
| 20   | Kinase                 | N17 | SCLC   | c.3068 G>A | p.R1023Q               | 1                      |
| 20   | Kinase                 | N119| AC     | c.3068 G>A | p.R1023Q               | 1                      |
| 20   | Kinase                 | N138| SCLC   | c.3074 C>T | p.T1025I               | 1                      |
| 20   | Kinase                 | N115| SCLC   | c.3085 G>C | p.D1029H               | 1                      |
| 20   | Kinase                 | N14 | SCLC   | c.3110 A>G | p.E1037G               | 1                      |
| 20   | Kinase                 | N11 | SCLC   | c.3115 T>C | p.F1039L               | 1                      |
| 20   | Kinase                 | N115| TC     | c.3133 G>A | p.D1045N               | 1                      |
| 20   | Kinase                 | N152| TC     | c.3140 A>G | p.H1047R               | 1                      |
| 20   | Kinase                 | N102| LCNEC  | c.3140 A>G | p.H1047R               | 1                      |
| 20   | Kinase                 | N62, N82| TC| c.3154 G>A | p.G1049S               | 2                      |
| 20   | Kinase                 | N114| LCNEC  | c.3154 G>A | p.G1049S               | 1                      |
| 20   | Kinase                 | N121| SCLC   | c.3154 G>A | p.G1049S               | 1                      |
| 20   | Kinase                 | N186| AC     | c.3155 C>T | p.T1052I               | 1                      |

Total \textit{PIK3CA} gene mutations\textsuperscript{a} 44/190 (23.2%)

Total mutations in the helical domain of the \textit{PIK3CA} gene\textsuperscript{a} 10/190 (5.3%)

Total mutations in the kinase domain of the \textit{PIK3CA} gene\textsuperscript{a} 34/190 (17.9%)

\textsuperscript{a}Frequencies of mutation are shown as n (%). TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma; BP-NET, bronchopulmonary neuroendocrine tumour.
Several studies have shown that the dysregulation of the PI3K/Akt pathway is involved in cancer pathogenesis and prognosis, and PIK3CA gene mutations have been reported in several types of human cancers, including colorectal, breast and hepatocellular carcinomas (10-14).

The functional activation of the PI3K/Akt pathway has been investigated in the spectrum of pulmonary or other neuroendocrine tumours only by the indirect evidence of the expression of functionally related molecules, such as PTEN (22), tuberous sclerosis complex (TSC) (23) and mammalian target of rapamycin (mTOR) (24,25). However, the mutational status of the PIK3CA gene in BP-NETs remains unknown. This study aimed to explore the mutational profile of the PIK3CA gene in a large series of BP-NETs and to determine the correlation of the PIK3CA status with the main clinico-pathological parameters.

Figure 1. Most frequent mutations of the phosphatidylinositol-3-kinase α catalytic subunit (PIK3CA) gene in a series of 190 bronchopulmonary neuroendocrine tumours (BP-NETs). (A) PIK3CA helical domain mutation p.E547K (c.1639 G>A). (B) PIK3CA kinase domain mutation p.Y1021N (c.3061 T>A). (C) PIK3CA kinase domain mutation p.H1047R (c.3140 A>G). (D) PIK3CA kinase domain mutation p.G1049S (c.3145 G>A).
A number of the somatic mutations involving the PIK3CA gene are clustered in exons 9 and 20, which encodes for PI3K helical and kinase domains, respectively (16,17). Previous studies have analysed PIK3CA gene mutation in NSCLC and have demonstrated that its frequency is relatively low (3.4-4.3%) compared to that observed in other tumours, such as breast, colon and ovarian cancers. (12-14).

To the best of our knowledge, we demonstrated for the first time a high prevalence of somatic missense mutations (23.2%) in the PIK3CA gene in human BP-NETs that is comparable to the mutational frequency of the PIK3CA gene in other types of human cancers (12-14,26,27). Moreover, our data are in agreement with the results obtained by Shibata et al (28) who have shown three mutations of the PIK3CA gene in 13 SCLC cell lines (23%) in an extensive mutational screening.

The high frequency of the PIK3CA gene mutation in our series of BP-NETs as in several other aggressive human tumours, highlights that somatic mutations of this gene are an important genetic event in BP-NET tumourigenesis and may represent a potentially effective therapeutic target for these types of tumours.

The analysis of the distribution of mutations between the kinase and the helical domains of PI3K in our series of BP-NETs revealed that the frequency of the kinase domain mutations (17.9%) was approximately three times the frequency of mutations in the helical domain (5.3%) according to the findings reported by other authors in different types of human cancers (12-14). We identified four different mutational hotspots: the codon 547 in the helical domain accounting for 15.9% of the PIK3CA gene mutations identified in our study and codons 1021, 1047 and 1049 in the kinase domain that together represent 25% of the identified mutations. Several studies have demonstrated a direct connection between mutations in the helical and kinase domain of PI3K and carcinogenesis as well as the prognosis of colorectal and breast cancers (29,30). The probable mechanisms for the oncogenicity of these mutations are the disruption of an inhibitory charge-charge interaction between p110α and the N-terminal SH2 domain of the p85 regulatory subunit and the increased binding affinity of p110α for the negatively charged phosphatidylinositol substrate, as it has been demonstrated by crystallographic and biochemical studies (17,31).

In our current study, a statistically significant correlation was not observed between PIK3CA mutations and the main clinical and pathological characteristics of the patients, such as age, gender or lymph node status. However, we found that the frequency of PIK3CA gene mutations was significantly associated with BP-NET histology (P=0.011). Interestingly, the prevalence of PIK3CA gene mutations increases parallel to the biological aggressiveness of the BP-NETs, since it was relatively lower in the low-grade TCs (13.3%) compared to the intermediate-grade ACs (39.1%) and high-grade SCLCs (30.7%). Notably, the high-grade LCNECs demonstrated an unexpected lower frequency of PIK3CA gene mutations

| Clinicopathological features | PIK3CA wild-type | PIK3CA mutant | P-value |
|------------------------------|------------------|---------------|---------|
| Gender                       |                  |               |         |
| Male                         | 84 (76.4)        | 26 (23.6)     | 0.992   |
| Female                       | 62 (77.5)        | 18 (22.5)     |         |
| Age (years)                  |                  |               |         |
| <66                          | 69 (77.5)        | 20 (22.5)     | 0.979   |
| ≥66                          | 72 (76.5)        | 22 (23.5)     |         |
| Tumour size                  |                  |               |         |
| T1 (T1a-T1b)                 | 67 (79.8)        | 17 (20.2)     | 0.972   |
| T2 (T2a-T2b)                 | 50 (75.7)        | 16 (24.3)     |         |
| T3                           | 21 (75.0)        | 7 (25.0)      |         |
| Lymph node status            |                  |               |         |
| Negative                     | 95 (79.2)        | 25 (20.8)     | 0.641   |
| Positive                     | 24 (72.7)        | 9 (27.3)      |         |
| Histology                    |                  |               |         |
| TC                           | 65 (66.6)        | 10 (13.3)     |         |
| AC                           | 14 (60.9)        | 9 (39.1)      | 0.011   |
| LCNEC                        | 15 (88.2)        | 2 (11.8)      |         |
| SCLC                         | 52 (69.3)        | 23 (30.7)     |         |

Values are shown as n (%); P-values were assessed by the Chi-square test and significant P-values are in bold. TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma; BP-NETs, bronchopulmonary neuroendocrine tumours.
(11.8%) compared with the other types of lung neuroendocrine tumours. LCNECs are relatively uncommon tumours, accounting for ~1% of the resected primary lung cancers and represent a controversial entity from both the diagnostic and clinical point of view (2-5). The diagnosis of high-grade neuroendocrine tumours of the lung requires to demonstrate the histopathologic neuroendocrine morphology and the neuroendocrine differentiation using immunohistochemistry or electron microscopy. However, LCNECs are a poorly recognized and underdiagnosed entity as a result of the difficulty in recognizing neuroendocrine morphology and are frequently mistaken for poorly differentiated NSCLCs, ACs, and intermediate cell-type SCLCs (5,32,33). Furthermore, although a previous retrospective report demonstrated that the survival rate of patients with surgically resected LCNECs and SCLCs were closely related to each other and inferior to that of patients with TCs and ACs (5), there is no clear-cut evidence concerning the optimal treatment for LCNECs, and therapeutic approaches adopted for SCLCs are not considered effective for patients with LCNEC, thus questioning whether LCNECs are best classified and treated as SCLC or LCC patients (5,32,33).

Our results demonstrated that PIK3CA gene mutations were more common in ACs and SCLCs than in LCNECs, suggesting that LCNECs, although sharing several similarities with SCLCs on morphological, immunohistochemical and molecular grounds, represent a distinct biological entity. In a wide retrospective study, Varlotto et al, demonstrated that clinical as well as histopathologic and biological characteristics of LCNECs more closely resemble LCCs than SCLCs (33). The differences in the PIK3CA gene mutational status between LCNECs and ACs or SCLC observed in our study may suggest that a different signalling pathway such as the MAPK pathway or other receptor tyrosine kinases (RTKs), but not PI3K, may play a key role in the tumourigenesis of the former type of BP-NETs. In support of this hypothesis, Rossi et al demonstrated that LCNECs overexpress several RTKs, including KIT, the platelet-derived growth factor receptor α (PDGFRα) and β (PDGFRβ), and MET in a number of patients, whereas they failed to find a significant expression in other NSCLCs and carcinoids (32,34).

An important implication of this study is the possibility of applying our results in the clinics. PIK3CA mutations have been associated with paclitaxel resistance in breast epithelial cells and the PI3K/Akt pathway has been linked with resistance to a number of other cancer therapies (35). In experimental models of human pulmonary carcinoid and SCLC cells, the inhibition of the PI3K/Akt pathway by LY294002 and Tricribine, respectively, significantly reduced cellular growth and neuroendocrine marker expression in vitro and increased apoptosis and sensitivity to chemotherapeutic treatments (28,36). Since mutations in the PIK3CA gene result in constitutively active PI3K activity, the presence of PIK3CA mutations may allow for the selection of patients with a high response rate to novel and targeted strategies of treatment based on the development of compounds designed to target PI3K or more feasible downstream effectors within the PI3K/Akt signalling pathway.

In conclusion, our results strongly suggest that PIK3CA genetic alterations may play an extensive and fundamental role in the tumourigenesis and aggressiveness of BP-NETs and specific-based targeting at the PI3K/Akt signalling pathway may be an effective therapeutic strategy for BP-NET treatment.

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