Clinic and genetic similarity assessments of atypical carcinoid, neuroendocrine neoplasm with atypical carcinoid morphology and elevated mitotic count and large cell neuroendocrine carcinoma

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Abstract
Background: Pulmonary neuroendocrine neoplasms can be divided into typical carcinoid, atypical carcinoid, large cell neuroendocrine carcinoma, and small cell (lung) carcinoma. According to the World Health Organization, these four neoplasms have different characteristics and morphological traits, mitotic counts, and necrotic status. Importantly, “a grey-zone” neoplasm with an atypical carcinoid-like morphology, where the mitotic rate exceeds the criterion of 10 mitoses per 2 mm², have still not been well classified. In clinical practice, the most controversial area is the limit of 11 mitoses to distinguish between atypical carcinoids and large cell neuroendocrine carcinomas.

Methods: Basic and clinical information was obtained from patient medical records. A series of grey-zone patients (n = 8) were selected for exploring their clinicopathological features. In addition, patients with atypical carcinoids (n = 9) and classical large cell neuroendocrine carcinomas (n = 14) were also included to compare their similarity to these neoplasms with respect to tumour morphology and immunohistochemical staining.

Results: We found that these grey-zone tumour sizes varied and affected mainly middle-aged and older men who smoked. Furthermore, similar gene mutations were found in the grey-zone neoplasms and large cell neuroendocrine carcinomas, for the mutated genes of these two are mainly involved in PI3K-Akt signal pathways and Pathways in cancer, including a biallelic alteration of TP53/RB1 and KEAP1.

Conclusions: Our findings indicate that neuroendocrine neoplasm with atypical carcinoid morphology and elevated mitotic counts is more similar to large cell neuroendocrine carcinoma than atypical carcinoid. Furthermore, this study may help improve diagnosing these special cases in clinical practice to avoid misdiagnosis.

Keywords: Atypical carcinoid morphology, Elevated mitotic count, Atypical carcinoid, Large cell neuroendocrine carcinoma

Background
The World Health Organization (WHO) has added large cell neuroendocrine carcinoma (LCNEC) to the classification of pulmonary neuroendocrine neoplasms (pNENs) for the first time [1]. The 2017 consensus conference of the International Agency for Research on
Recent studies have investigated the role of AUTOX1 in neuroendocrine neoplasms (NETs) [1,2]. However, these findings need further validation, and the role of AUTOX1 in NETs remains unclear.

**Methods**

**Sample selection**

Forty-four samples of surgical resected primary untreated ACs and LCNECs diagnosed between January 1, 2016 and January 1, 2021 were collected from the specimen bank of a tertiary referral hospital, with the approval of the Institutional Ethics Committee (No. 2020-120). All specimens were reviewed by two experienced pathologists, and a multi-head microscope was used for joint judgment with the participation of a third professional respiratory diagnostic pathologist.

**DNA extraction and next-generation sequencing**

According to the manufacturer’s instructions, DNA was extracted by a QIAamp DNA FFPE Tissue Kit (Qiagen, Carlsbad, CA, USA) after twice of de-paraffinized by xylene. Extracted DNA was purified and qualified using the Nanodrop2000 (Thermo), and then using Qubit3.0 (Life Technology) to quantify DNA.

**Bioinformatics analysis**

Base calling analysis was used to transfer original image data into raw sequence data, which contained sequence information and corresponding sequencing quality information. Single nucleotide variants (SNVs) and short insertions or deletions (indels) were identified by VarScan2. In-house-developed software was used to detect Copy number variations (CNVs).

**Statistical analysis**

Statistical Package for the Social Sciences version 25.0 statistical software (SPSS Inc., Chicago, IL, USA) and the Kyoto Encyclopedia of Genes and Genomes website (KEGG web) were used to conduct statistical analysis and
query the gene mutation pathways, respectively. Continuous data were evaluated by one-way analysis of variance (ANOVA). Categorical data were assessed by Pearson’s chi-squared test or Fisher’s exact test. The Kaplan–Meier method was used for survival analysis. \( P < 0.05 \) was considered statistically significant.

**Results**

**Clinical information**

Basic information of the patients in the cohort is presented in Table 1 and Fig. 1A. There was a significant difference in average age at diagnosis between the three groups \((P = 0.048)\) and smoking status \((P = 0.028)\). In all 31 patients, asymptomatic patients were most commonly seen in the AC group. More than half the patients with AC-h or LCNEC were symptomatic; coughing was the most common symptom, followed by expectoration. In 77.8% of patients, the ACs were clinically staged I or II, far greater than that of the other two groups of tumours (Table 2). Moreover, the follow-up analyses of 28 patients showed no recurrence, metastasis, or death among the AC group (Fig. 1B). Patient’s postoperative treatment and prognosis are shown in Table 3.

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**Table 1** The demographic characteristics and smoking status of 31 samples

| Characteristics | AC  | AC-h | LCNEC | P-value |
|----------------|-----|------|-------|---------|
| Age (years)    |     |      |       |         |
| <40            | 2   | 0    | 0     | 0.048   |
| 40–49          | 2   | 0    | 1     |         |
| 50–59          | 3   | 3    | 6     |         |
| 60–69          | 1   | 4    | 3     |         |
| >70            | 1   | 1    | 4     |         |
| Range          | 23–74 | 50–74 | 42–78 |         |
| Mean           | 49  | 61   | 61    |         |
| M:F            | 5:4 | 7:1  | 13:1  | 0.074   |
| Smoking        |     |      |       | 0.028   |
| Never          | 6   | 2    | 2     |         |
| Has/Had        | 3   | 6    | 12    |         |

**Abbreviations:** AC Atypical carcinoid, AC-h Atypical carcinoid morphology with increased mitotic counts, LCNEC Large cell neuroendocrine carcinoid, \( P \)-value

The associations of age was assessed by One-Way ANOVA, meanwhile, other information were assessed by Pearson’s chi-squared test or Fisher’s exact test and \( P < 0.05 \) was considered statistically significant for all results; Age: at diagnosed; Range: the range of diagnosed age; M:F: male: female; Smoking: smoking status; Never: never smoker; Has/Had: still smoking/previous smoker

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**Fig. 1** Abbreviations: AC Atypical carcinoid, AC-h Atypical carcinoid morphology with increased mitotic counts, LCNEC Large cell neuroendocrine carcinoid, A) the age-specific box diagram of the three groups of cases, B) the overall survival in 31 patients with AC, AC-h and LCNEC, C) the tumour size-specific box diagram of the three groups of cases
Imaging data
Preoperative chest CT scans were reviewed to determine tumour location (Table 2). Tumours involving the carina or a main segmental bronchus were defined as central, while the others were defined as peripheral. The primary tumour mass occurred preferentially in the periphery in these three groups. In addition, AC-h showed a clearer tendency than AC to occur in the upper lobe ($P = 0.030$) and a more stable range of tumour size fluctuations (Fig. 1C).

Pathological findings
Histopathological analysis of AC-h revealed classical features of NET (tumour cells were relatively uniform, featuring moderate to abundant cytoplasm and finely nuclear chromatin) and NEC (focal necrosis, and even extensive necrosis in four cases) (Fig. 2). IHC for neuroendocrine (NE) markers (CD56, Syn, and CgA), TTF-1 and Ki67 was performed on 31 samples. The most sensitive neuroendocrine marker was CD56 (93.5%), followed by Syn and CgA (both were 67.7%). All ACs exhibited strong positivity for the three NE markers, while the expression mode was more variable in AC-hs and LCNECs. After reviewing all the slices, mitosis in AC-hs (average, 25 per 2 mm$^2$) was found to differ significantly from that of ACs (average, 4 per 2 mm$^2$) and LCNEC (average, 45 per 2 mm$^2$) ($P < 0.001$). Similar results could be observed using Ki67 ($P < 0.001$).

Genomic features
The 425-exon sequencing was performed by unsupervised clustering in 28 pure primary tumour samples (Nanjing Geneseeq Technology Inc.), revealing 113 altered genes (Fig. 3). Tumour mutational burden (TMB) is defined as the number of somatic cells, coding number, base subsets, and index of each detected genome. Our analysis revealed that the TMB value in ACs (average, 0.7 mutations/MB) contrasted with those of AC-hs (average, 8.5 mutations/MB) and LCNECs (average, 10.8 mutations/MB) ($P < 0.001$) (Fig. 4A).

At the single gene level, among the commonly mutated genes, differences in $P$-values were found between these three groups. The most commonly mutated genes were $TP53$ ($P < 0.001$) and $RB1$ ($P = 0.039$), with a significant bi-alteration rate ($P = 0.039$), followed by $PTEN$ ($P = 0.011$), $RICTOR$ and $MCL1$ ($P = 0.040$), $APC$ ($P = 0.054$), $KEAP1$ ($P = 0.132$), $NTRK1$ ($P = 0.265$), $ROS1$ ($P = 0.284$) and $PKHD1$ ($P = 0.293$) (Fig. 4B). Furthermore, we matched

| Variable | AC | AC-h | LCNEC | $P$-value |
|----------|----|------|-------|-----------|
| Lung lobe |    |      |       |           |
| Left lung | 5 (55.6%) | 4 (50.0%) | 6 (42.9%) | 0.833 |
| Right lung | 4 (44.4%) | 4 (50.0%) | 8 (57.1%) | |
| Upper lobe | 2 | 6 | 7 | 0.030 |
| Others | 7 | 2 | 7 | |
| Type |    |      |       | 0.183 |
| Central | 3 (33.3%) | 2 (25.0%) | 4 (28.6%) | |
| Peripheral | 6 (66.7%) | 4 (50.0%) | 10 (71.4%) | |
| Unknown | 0 | 2 | 0 | |
| Tumor size (cm) |    |      |       | 0.503 |
| $\leq 5$ | 8 | 5 | 10 | |
| $>5$ | 1 | 1 | 4 | |
| Unknown | 0 | 2 | 0 | |
| Stage |    |      |       | 0.056 |
| UI | 7 | 2 | 7 | |
| IIUIV | 2 | 4 | 7 | |
| Unknown | 0 | 2 | 0 | |
| Symptom |    |      |       |           |
| Asymptomatic | 6 (66.7%) | 3 (37.5%) | 6 (42.9%) | |
| Cough | 2 | 3 | 7 | |
| Expectoration | 2 | 2 | 5 | |
| Hemoptysis | 0 | 1 | 2 | |
| Chest pain | 0 | 0 | 3 | |
| Expiratory dyspnea | 1 | 2 | 0 | |

**Table 2** The clinical information and preoperative imaging data of 31 samples

**Table 3** The postoperative treatment and prognosis of 31 samples

| Group | Samples | Postoperative treatment | Prognosis |
|-------|---------|-------------------------|-----------|
|       |         | A  | B  | C  | D  | Loss | Death | Alive |
| AC    | 9       |    |    |    |    | 0    | 0     | 9     |
| AC-h  | 8       |    |    |    |    | 2    | 2*    | 4     |
| LCNEC | 14      | 5  | 1  | 2  | 5  | 1    | 4     | 9     |

Abbreviations: Loss The contact information left was empty or out of service, Death Died of tumour recurrence or metastasis, *: death after lung transplantation
**Fig. 2** A Representative HE and IHC images of AC, AC-h and LCNEC under light microscope at ×100 magnification (inset ×400) for HE and at ×200 magnification for IHC; B IHC and mitosis results of the all 31 patients. Case: case number; Ki67: calculated on the hot spot area under the field of view ×400; Mitosis: counted on the 5th edition WHO diagnostic criteria and for these samples which the mitoses near the threshold of two or ten per 2mm², the average of counts in at least three hot sets of per 2mm² were taken as the result.

**Fig. 3** The 425-exon sequencing in 28 pure primary tumour samples revealing 113 altered genes. Abbreviations: X: AC; Y: AC-h; Z: LCNEC; x only: AKT1, PDK1; y only: CREBBP, SMARCA4, NCOA2, KMT2B, HPMEC, TEX, PIK3R1, RET, SETBP1, EPHA3, PRDM1, TERC, AKT2, TOP2A, CCNE1, CBL, NOTCH2, IDH1, CTCF, AXL; z only: RICTOR, MCL1, ATRX, MYC, ABCB1, FAT1, PALB2, FLT4, TERT, ARID1A, EPHA2, NTRK3, NOTCH1, PDGFRA, BRAF, JAK1, EPHA5, DAXX, ZNF217, ERBB4, TSC2, GATA2, PPARD, SDHC, SDHA, PLCN1, TUBB4A, PALLD, SRC, SMAD3, EZH2, BARD1, ATM, AKT3, TPMT, GRIN2A, MAP3K2, BTK, GATA4, MET, RUNX1, BIRC3, CHEK1, DENND1A, PTOR, AXIN2, TGFBR2, PMS2, ARID1B, EP300, POLH, DUSP9, MYCN, KSR1, DPYD, PRED2, CYSLTR2, CHEK2, EGF, DDR2, RADS4L, WDR, LTR1, LHCGR, BRCA1; x–z only overlap: ROS1, MEN1; y–z only overlap: TP53, PTEN, KEAP1, APC, RB1, PIK3C2, NTRK1, KIT, PIK3R3, PIK3CA, SOX2, GRM3, LRP1B, GNAS, Kras, IL-7R, POLE, NFI, CRKL, VEGFA, DLL3, CDKN2A, JAK3, STR11.
the gene mutation sites with the corresponding protein locations of the top ten genes, which showed that case 10 and 20 had the same mutation in TP53 (824G > T), corresponding to the protein location C275F. Moreover, several highly similar mutated sites existed between the AC-h and LCNEC groups (Table S1).

The KEGG web was used to search the pathways in which the top ten mutated genes are involved. As a result, the pathways which involved at least three of the top ten genes \( (n = 21) \) were collected (Table S2), within the six other common mutation pathways in LCNEC listed in Fig. 4C [20–23].

**Discussion**

Lung neuroendocrine tumours represent a group of heterogeneous malignancies, and according to the 5th edition of WHO, apparent differences exist between NET and NEC for NETs lack mutations in TP53, RB1, KRAS, and STK11/KEAP1, while, in 40% of cases they have mutations in chromatin-remodelling genes [13]. Genetic screening of 45 surgically resected pure-LCNEC by Rekhtman et al. found two cases of carcinoid-like molecular profiles. Moreover, these two cases displayed apparent carcinoid-like morphology, although the elevated proliferation rate above the cut-off value accepted of NET had led them to be classified as LCNEC [17]. Similar results were found in other studies [8, 24]. This type of neoplasm in the pancreas is classified as NET G3, which has a common mutation lineage with NET G1 and NET G2 and can evolve from G1/G2, and has nothing to do with the progress of NEC [15]. Although some researchers believe these tumours generally correspond to those regarded as NETs in the pancreas, however, in the lung, the WHO still classifies...
these neoplasms as LCNEC, although its prognosis has been suggested to be different from traditional LCNECs, which means more clinical, pathological, and genetic studies are needed to determine how to fit these rare tumours into the classification [1, 13]. Thus, it is essential to recognise these grey-zone AC-hs to improve disease classification and avoid incorrect clinical treatment choices.

Previous studies have pointed out that AC occurred in younger patients than LCNEC [15, 25–27], and comparison with the age is mentioned by WHO, the patients with AC or LCNEC in this study tended to be younger [13]. Unlike AC, which does not show a strong association with cigarette smoking [6, 15, 25, 28, 29], and is slightly more common in women [9, 11, 26, 27], AC-h primarily affects middle-aged men with a smoking history. Caplin ME et al. reported that well-differentiated lung NETs are usually located centrally in the main or lobar bronchi (up to 80% of tumours) [30–32], although some reports have opposed this view [4, 27, 33]. Results indicated all three groups have a trend of occurring in the periphery.

The WHO (4th edition) diagnostic criteria recommends the use of NE markers to confirm a diagnosis of neuroendocrine differentiation [1] the guidelines for the diagnosis and management of pNEN (2020) support this point [34], and this is reiterated in the 5th edition [13]. Our results suggest that compared with HE, IHC is more accurate in diagnosing lung carcinoids, in particular, Syn and CgA can be used to distinguish NET from NEC [35]. TTF-1, a putative regulator of neurogenesis expressed in pNEC at various sites [36, 37] was found to be mostly positive in peripheral NETs. However, for LCNEC, the positive expression rate of TTF-1 (50%) was slightly lower than that described by WHO (70%) [13].

The Ki67 antigen can identify proliferating cells and is important for distinguishing NETs and NECs, especially in small squeezed biopsy samples [38], and the value was now increased to 30% for AC [13]. Based on this change, some studies have used Ki67 to identify the proper cut-off value of these four pNENs, however, there was no conclusive result even in resected samples [6, 10, 38–40]. As shown in Fig. 2, malignant divergences existed in both the proliferation level and mitotic counts in these three neoplasms.

Global genomic studies have demonstrated that AC has a low mutation rate (0.3–0.4 mutations/Mb) [29, 41] and very few genetic changes [42]. High-frequency mutations include KRAS, and ERBB4, and MET [35, 43]. Unlike NECs, mutations in chromatin-remodelling genes are observed in approximately 40–50% of NET cases [3, 10, 41, 44]. For example, MEN1 (11–22%) is the most frequently mutated gene with somatic mutations in lung carcinoids [2, 41, 45]. Other statistically significant commonly mutated genes include EIF1AX and ARID1A [41]. However, except for the MEN1 mutation in case 8, NGS testing indicated no other commonly mutated genes in AC. Conversely, three other genes were found to be mutated in this cohort—ROS1 (a common driver gene), PDK1, and AKT1. These have never previously been reported for AC and should be investigated further.

Sazonova et al. recently applied IHC to surgical samples from 18 lung cancer patients, four of whom had defined borderline tumours (LCNEC with a low mitotic count and carcinoid-like morphology). They found that all AC and borderline tumours had preserved P53/RB expression [8]. Indeed, Meder et al. and Nakamura et al. reported that TP53 and RB1 gene inactivation are among the hallmarks of SCLC, existing in approximately 39.3% of SCLC cases. At the same time, for LCNEC, the rate was approximately 36.8% [41, 46]. Biallelic alterations of TP53 and RB1 are strikingly correlated with high-grade NECs, although uncommon in pNETs [2, 3, 21, 47]. The co-mutation of TP53/RB1, and common mutations in LCNECs like TP53, RB1, MEN1, STK11, KEAP1, and KRAS were commonly seen in AC-hs in our study. Furthermore, LCNEC is a heterogeneous tumour, which can be divided into three genotypes: (i) an SCLC-like subtype with biallelic inactivation of TP53 and RB1, (ii) a non-small-cell-like subtype with mutations in TP53 and STK11/KEAP1, (iii) a carcinoid-like subtype sharing the low TMB and MEN1 changes seen in lung carcinoids [2, 13, 17, 18, 21, 37, 48, 49]. These results, taken together, suggests a way to classify the AC-hs effectively. The extensive TMB fluctuation range of AC-hs also supports this. In addition, according to the summary results of the three neoplasms gene mutations provided in Fig. 3, there is no shared mutation gene type between AC-h and AC. This result reiterated that according to our data, in the lung, there exist a big difference between AC-h and AC, which is completely different from the existing research of NET G3 in pancreatic NEs.

Subsequently, the 27 pathways considered in this study showed a high degree of similarity in the level of involvement of AC-h and LCNEC, suggesting that differences do exist between these tumours and ACs.

Tumorigenesis results from multiple factors, and abnormal activation of the PI3K-Akt-mTOR pathway is a frequent event in the non-small cell lung cancer development [20, 22, 23, 50]. The mammalian target of rapamycin (mTOR) serves as a signal amplifier in this pathway [22, 51]. It is generally believed that mutated genes in the PI3K/AKT/mTOR pathway are significantly related to the occurrence of NEC [2, 3, 17, 52]. However, inconsistent findings have been reported in pNEN, with some reporting that most mutated genes in NETs are located in this pathway [53] or that these mutated genes exhibit a high degree of participation [3, 52]. Our data support
the conclusion that the mutated genes in AC are involved in these pathways. In addition, alterations in this pathway were far more common in LCNEC patients than previously reported [3, 17, 54].

Regarding survival and prognosis, several studies have suggested a similar prognosis between LCNEC and SCLC [55–57], which is significantly poorer than that of AC [55, 58]. The five-year survival rate of LCNEC patients is 15–57% [25, 59–63], while that of AC patients is 44–87% [6, 26, 59–61]. We attempted to enrol as many cases as possible but limited by the rare incidence and short DNA storage period, significantly different OS outcomes among these three tumour types were not obtained (P=0.123). However, a trend from the available information suggested that AC-hs seemed to have a better prognosis than LCNECs.

Conclusion
We present basic information on the clinical features and genomic changes in 31 tumour samples. Despite limitations in the number of cases and the lack of effective differential data for OS, we can still clearly see that AC-h and LCNEC patients are more similar to each other with respect to demographic characteristics, tumour size and location, clinical presentation, pathological data, and genomic changes. Thus, we believe that carcinoid morphology with increased mitotic index is more similar to LCNEC, but has a better survival prognosis. However, to test this hypothesis, a larger cohort study is needed and until these neoplasms are better classified, we endorse that it is necessary to add a diagnostic note stating the histological morphology, mitotic count and Ki67 index of this type of tumour in the clinical diagnosis process.

Abbreviations
WHO: World health organization; LCNEC: Large cell neuroendocrine carcinoma; pNENs: Pulmonary neuroendocrine neoplasms; NENs: Neuroendocrine neoplasms; NETs: Neuroendocrine tumours; NECs: Neuroendocrine carcinomas; TC: Typical carcinoid; AC: Atypical carcinoid; SCLC: Small cell (lung) carcinoma; pNENs: Pulmonary neuroendocrine neoplasms; NENs: Neuroendocrine neoplasms; NE: Neuroendocrine; TMB: Tumour mutational burden.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09391-w.

Additional file 1. Gene mutation sites and affected protein changes.
Additional file 2. The pathways in which the top ten mutated genes are involved.

Authors’ contributions
Y.Z. and W.W. analyzed the data and prepared the manuscript. Q.H., Z.L., and P.Z. performed the histopathological examinations. Y.T helped to carry out the NGS studies. Y.Z. and Z.L. carried out the IHC studies. L.J. was responsible for the diagnosis and revised the manuscript. Y.Z was responsible for submitting the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request. The KEGG datasets were obtained from:- Kanehisa, M. and Goto, S.; KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 28, 27–30 (2000). [PMID:10592173]- Kanehisa, M; Toward understanding the origin and evolution of cellular organisms. Protein Sci. 28, 1947–1951 (2019). [PMID:31441146]- Kanehisa, M., Furumichi, M., Sato, Y., Ikuguro-Watanabe, M., and Tanabe, M.; KEGG: integrating viruses and cellular organisms. Nucleic Acids Res. 49, DS45-DS51 (2021). [PMID:33125081].

Declarations
Ethics approval and consent to participate
This study was conducted in accordance with institutional ethics regulations. All pathological tissues used were obtained from the database of the Department of Pathology, West China Hospital of Sichuan University and were approved by the Ethics Committee of West China Hospital of Sichuan University (NO. 2020 (120)).

Consent for publication
N/A.

Competing interests
The authors declare that no conflicts of interest exist.

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