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ANATOMICAL PATHOLOGY

Molecular dissection of large cell carcinomas of the lung with null immunophenotype

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Summary

The aim of this study was to subcategorise large cell carcinoma (LCC) with null immunophenotype according to the World Health Organization (WHO) Classification of 2015 into the existing groups of adenocarcinoma and squamous cell carcinoma by further molecular genetic analysis. Lineage-specific molecular alterations of these tumours could depict additional therapeutic approaches. We analysed a cohort of 35 LCC diagnosed according to the 2004 WHO classification and reclassified them according to the criteria of the 2015 WHO classification. Subsequently, tumours with a null immunophenotype were analysed by targeted next generation sequencing (42 marker genes including *TP53*, *EGFR*, *KRAS*, *STK11* and *SMARC4A*) and fluorescence *in situ* hybridisation (*ROS1*, *ALK*). By applying the criteria of the 2015 WHO classification and subsequent molecular subtyping we could show that out of 35 previously diagnosed LCC, 16 cases could be reclassified into specific NSCLC subtypes using immunohistochemistry. Additionally, based on their mutational pattern, eight of the remaining 19 cases with null immunophenotype could be assigned as 'favour adenocarcinoma'. We demonstrate that molecular subtyping is helpful to further categorise LCC with null immunophenotype. Our findings argue for an algorithm including stratified molecular analysis of all respective cases.

Key words:: Large cell carcinoma; lung; next generation sequencing.

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INTRODUCTION

Due to the adoption of immunohistochemistry into the categorisation of resected large cell carcinoma (LCC), the frequency of this entity has declined over recent years. In the 1990s around 10% of lung cancers were diagnosed as LCC.[1] Over the past two decades, however, the rates have decreased from 9.4% to around 2.3%.[2,3] According to the 2004 and 2015 World Health Organization (WHO) classifications,[4] LCC can be diagnosed definitively only on resected tumours after thorough sampling to rule out keratinisation or glandular differentiation. According to the 2015 WHO Classification[4] and with the availability of lineage-specific immunomarkers,[5] some LCC can now be further classified as solid predominant adenocarcinoma or non-keratinising squamous cell carcinoma. However, the category of LCC still exists and contains so far undifferentiated non-small cell carcinomas with null or unclear immunophenotypes and, potentially, LCC with no stains available.[4] The officially recommended markers are TTF-1, napsin A, p40 (p63), cytokeratins 5 or 5/6 and a mucin stain.[4] Clinically, the diagnosis of LCC is a basket with no specified targeted therapies available and due to the lack of a specific lineage these cases are often not further analysed for druggable molecular alterations. Here, we aimed to further analyse LCC with null phenotype by means of targeted next generation sequencing (NGS) and fluorescence *in situ* hybridisation (FISH) in order to determine if some of these tumours can be categorised into the existing groups of adenocarcinoma (ADC) and squamous cell carcinoma (SqCC) even after all conventional analyses, i.e., if haematoxylin and eosin (H&E) in combination with histochemistry and immunohistochemistry have failed to subtype the tumour. We demonstrate that more LCC can be further classified by means of molecular characterisation; therefore, we suggest to include molecular pathology in future diagnostic approaches to LCC.

MATERIAL AND METHODS

Samples

Tumours included in this study were all cases diagnosed as LCC from 2002–2014 in the Institute of Pathology, Heidelberg University, based on the criteria of the 2004 WHO classification. Retrospectively, formalin fixed and paraffin embedded (FFPE) tissue blocks were collected from the archive.

All tumours were resected at the Thoraxklinik at Heidelberg University and were handled by the tissue bank of the National Center for Tumour Diseases (NCT; project # 1997, # 2379) in compliance with the ethical regulations of the NCT tissue bank established by the local ethics committee.

Immunohistochemical analysis

All LCC were stained for the following markers: p40, TTF-1, Ki-67 (MIB1), synaptophysin, CD56, and cytokeratin 5/6. Stainings were carried out using an autostainer (BenchMark Ultra, Ventana Medical Systems, USA) according to the manufacturer's instructions (Table 1).

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Table 1 Staining protocols used and antibody types with manufacturer

Antibody	Company	Catalogue no.	Clone	Pretreatment	Incubation time buffer	Antibody dilution	Incubation time antibody
CD 56	Ventana	760–4596	MRQ-54	Tris/Borat/EDTA, pH 8.4	40 min	RTU	24 min
p63	DCS Immunoline	PI0061001	SFI-6	Tris/Borat/EDTA, pH 8.4	40 min	1:100	24 min
Napsin	Novocastra	NCL-L-Napsin A	IP64	Tris/Borat/EDTA, pH 8.4	48 min	1:400	24 min
p40	Zytomed Systems	MSK097	Polyclonal	Tris/Borat/EDTA, pH 8.4	48 min	1:100	24 min
CK5/6	Dako	M7237	D5/16 B4	Tris/Borat/EDTA, pH 8.4	56 min	1:50	24 min
TTF-1	Novocastra	TTF-1-L-CE	SPT24	Tris/Borat/EDTA, pH 8.4	56 min	1:100	24 min
Synaptophysin	Ventana	760–4595	MRQ-40	Tris/Borat/EDTA, pH 8.4	32 min	RTU	24 min
Ki-67	Ventana	790–4286	30–9	Tris/Borat/EDTA, pH 8.4	32 min	RTU	24 min

RTU, ready to use.

DNA extraction and quantification

We used six consecutive unstained 10 µm tissue slides of each sample for DNA extraction. Following deparaffinisation and overnight proteinase K digestion, DNA was extracted automatically using a Maxwell 16 Research system with the Maxwell 16 FFPE Tissue LEV DNA Purification Kit (both Promega, USA). DNA concentrations were determined fluorometrically using the Qubit HS DNA system (ThermoFisher Scientific, USA) as well as with the RNaseP detection system on a StepOne qPCR system (both ThermoFisher Scientific). All assays were used according to the manufacturers' protocols.

Massive parallel sequencing

Hotspot regions for mutations in 42 marker genes linked to NSCLC pathogenesis including those relevant for targeted treatment and clinical trials were sequenced using a proprietary Lung Cancer Panel (LCPv2) with Ion Torrent AmpliSeq technology on an Ion Torrent PGM System (both ThermoFisher Scientific) as described previously.[6,7] The Ion PGM Hi-Q OT2 200 Kit was used for library preparation and the Ion Torrent Ion PGM Hi-Q sequencing chemistry was used for the sequencing.

For data analysis the Torrent Suite software (v. 5.2.1, ThermoFisher Scientific) was used to generate sequences from the raw data and for alignment to the human genome (hg19). Mutations were reported by the variant caller plug-in (v. 5.2.0.34) and manually confirmed in the IGV browser (v. 2.3.52) accepting an allele frequency greater than 5% and a minimum coverage of 100 reads as cut-offs. Variant annotation was performed using a custom-built pipeline in the CLC Genomics Workbench (v. 8.0.2).

FISH for *ALK* and *ROS1* rearrangements

All cases were further tested for *ALK* rearrangements on whole block slides of formalin fixed and paraffin embedded samples by dual colour break-apart FISH (Vysis/Abbott Laboratories, USA) according to the manufacturer's recommendations. For detection of *ROS1* rearrangements, the ZytoLight SPEC ROS1 Dual Color Break Apart Probe (Zytovision, Germany) was used as described previously,[8] according to the manufacturer's instructions. Briefly, 4 µm thick tissue sections were pretreated by deparaffinisation in xylene and dehydration in ethanol. FISH analysis and signal capture were performed using a fluorescence microscope (Axio; Zeiss, Germany) coupled with ISIS FISH Imaging System (Metasystems, Germany). At least 50 interphase nuclei from each tumour were scored and considered positive for *ALK* or *ROS1* rearrangement if >15% of tumour cells displayed broken-apart green/red signals and/or single red signals.

Molecular subtyping and assignment

The diagnostic algorithm used for morphological, immunohistochemical, and molecular classification of LCC is shown in Fig. 1. The final decision whether a case of LCC with null immunohistochemical features was categorised as 'favour ADC' or 'favour SqCC' based on molecular alterations was based on the Catalogue of Somatic Mutations in Cancer (COSMICv81) database and given that specific gene alterations have been only detected in ADC or SqCC so far. *STK11* and *SMARC4A* mutations are only prevalent in ADC. According to COSMIC, *KRAS* mutations occur with a frequency of 18% in ADC but only 4% in SqCC. Therefore, we decided to favour ADC over SqCC in these cases when only a *KRAS* mutation was detected.

RESULTS

Thirty-five cases with the diagnosis of LCC according to the 2004 WHO classification were retrieved from the archives and reconfirmed according to the 2004 criteria. After applying the criteria of the 2015 WHO classification[4] using additional immunohistochemistry the cases were categorised into three groups (Fig. 1). One group contained the cases that could be reclassified as solid predominant adenocarcinoma, as non-keratinising squamous cell carcinoma, and additionally, cases of large cell neuroendocrine carcinoma (LCNEC). The second group consisted of cases that had to be classified as LCC with null immunophenotype even according to the 2015 WHO classification (Fig. 1 and 2, Table 2). The last group, LCC with unclear immunophenotype, was not evident in this series.

Regarding the reclassified cases, 10 cases showed diffuse (n = 7) or focal (n = 3) expression of TTF-1 and eight cases showed diffuse (n = 4) or focal (n = 3) expression of napsin A. Ten cases were therefore reclassified as solid ADC; the remaining two cases showed additional positive

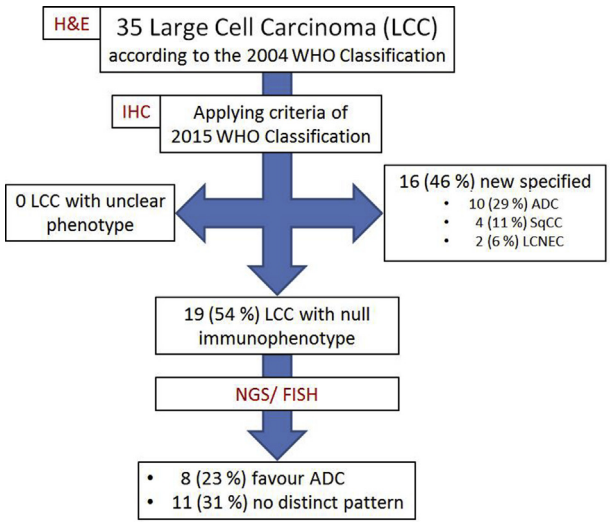

Fig. 1 Work flow applying the criteria of the 2015 WHO classification with subsequent molecular characterisation to LCC.

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