

# Distinct profile of driver mutations and clinical features in immunomarker-defined subsets of pulmonary large-cell carcinoma

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**Pulmonary large-cell carcinoma—a diagnostically and clinically controversial entity—is defined as a non-small-cell carcinoma lacking morphologic differentiation of either adenocarcinoma or squamous cell carcinoma, but suspected to represent an end stage of poor differentiation of these tumor types. Given the recent advances in immunohistochemistry to distinguish adenocarcinoma and squamous cell carcinoma, and the recent insights that several therapeutically relevant genetic alterations are distributed differentially in these tumors, we hypothesized that immunophenotyping may stratify large-cell carcinomas into subsets with distinct profiles of targetable driver mutations. We therefore analyzed 102 large-cell carcinomas by immunohistochemistry for TTF-1 and  $\Delta$ Np63/p40 as classifiers for adenocarcinoma and squamous cell carcinoma, respectively, and correlated the resulting subtypes with nine therapeutically relevant genetic alterations characteristic of adenocarcinoma (*EGFR*, *KRAS*, *BRAF*, *MAP2K1/MEK1*, *NRAS*, *ERBB2/HER2* mutations and *ALK* rearrangements) or more common in squamous cell carcinoma (*PIK3CA* and *AKT1* mutations). The immunomarkers classified large-cell carcinomas as variants of adenocarcinoma ( $n=62$ ; 60%), squamous cell carcinoma ( $n=20$ ; 20%) or marker-null ( $n=20$ ; 20%). Genetic alterations were found in 38 cases (37%), including *EGFR* ( $n=1$ ), *KRAS* ( $n=30$ ), *BRAF* ( $n=2$ ), *MAP2K1* ( $n=1$ ), *ALK* ( $n=3$ ) and *PIK3CA* ( $n=1$ ). All molecular alterations characteristic of adenocarcinoma occurred in tumors with immunoprofiles of adenocarcinoma or marker-null, but not in tumors with squamous immunoprofiles (combined mutation rate 50% vs 30% vs 0%, respectively;  $P<0.001$ ), whereas the sole *PIK3CA* mutation occurred in a tumor with squamous profile (5%). Furthermore, marker-null large-cell carcinomas were associated with significantly inferior disease-free ( $P<0.001$ ) and overall ( $P=0.001$ ) survival. In conclusion, the majority (80%) of large-cell carcinomas can be classified by immunomarkers as variants of adenocarcinoma or squamous cell carcinoma, which stratifies these tumors into subsets with a distinct distribution of driver mutations and distinct prognoses. These findings have practical implications for diagnosis, predictive molecular testing and therapy selection.**

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Large-cell carcinoma is the third most common subtype of non-small-cell lung carcinoma after adenocarcinoma and squamous cell carcinoma, representing 3–9% of non-small-cell lung carcinomas.<sup>1,2</sup> It is defined in the 2004 World Health Organization classification of lung tumors as an ‘undifferentiated non-small-cell carcinoma that lacks the cytologic and architectural features of small-cell carcinoma, glandular or squamous differentiation’.<sup>1</sup> In essence, large-cell carcinoma is

a tumor in which the line of differentiation cannot be identified by light microscopy, as reflected by its alternative designation as 'large-cell undifferentiated carcinoma'. This designation is reserved for surgically resected tumors because the lack of morphologic differentiation in small biopsy or cytology samples is usually a reflection of incomplete sampling or poor cell preservation rather than a true lack of differentiating morphology in the entire tumor,<sup>1,3</sup> although this terminology has been applied inconsistently.

Large-cell carcinoma has inspired significant controversy over the years, with the main question centered on whether this tumor represents a truly distinct biological entity or an extreme in the poorly differentiated spectrum of the other major types of non-small-cell lung carcinoma, namely adeno- and/or squamous carcinomas. The latter concept is supported by the long-known observation that by electron microscopy, large-cell carcinomas commonly reveal ultrastructural features of either adeno- or squamous carcinoma.<sup>4-6</sup> Similarly, in more recent microarray-based expression profiling studies, these tumors were found to frequently display gene expression patterns resembling either adeno- or squamous carcinoma.<sup>7-9</sup> Likewise, a long-known observation from pre-immunohistochemistry era is that a subset of large-cell carcinomas elaborates cytoplasmic mucin as revealed by histochemical stains, leading to the recommendation to reclassify such tumors as variants of adenocarcinoma.<sup>1,10</sup> The limitation of mucin stains, however, is that their sensitivity for glandular differentiation is low (~30%),<sup>11</sup> and they are therefore variably utilized in routine practice. More recently, it has been noted that by immunohistochemistry, large-cell carcinomas commonly express markers typical of adeno- or squamous carcinoma,<sup>12-18</sup> raising the prospect that with increasing routine use of immunostains in current pathology practice, large-cell carcinoma could become an 'endangered species'.<sup>13</sup> However, some immunomarkers, previously utilized as 'markers of differentiation' in large-cell carcinomas, are now known to lack specificity (such as conventional p63 antibody (4A4) and 34 $\beta$ E12<sup>12,16</sup>—the squamous markers, which were recently shown to have a substantial cross-reactivity in lung adenocarcinomas<sup>19</sup>). Furthermore, no measure of biological accuracy or clinical value of marker-based stratification of large-cell carcinoma has been previously demonstrated.

Two recent advances—one in diagnostic pathology and the other in individualized therapy for non-small-cell lung carcinomas—make it timely to reassess the feasibility and utility of marker-based reclassification of large-cell carcinoma. First, there has been a significant progress in immunomarkers to determine the line of differentiation in non-small-cell lung carcinomas. In particular, a notable advance has been recent characterization of  $\Delta$ N isoform of p63 (p40) as a highly specific squamous

marker, unlike the conventional p63 antibody, which in combination with the glandular marker TTF-1 has been shown to reliably distinguish adeno- and squamous carcinomas.<sup>20-23</sup> Second, the treatment of patients with non-small-cell lung carcinomas has recently undergone a major paradigm shift to a highly individualized approach based on tumor histology and targetable molecular alterations.<sup>24</sup> In particular, the recent breakthroughs in targeted therapies have revealed fundamental molecular differences in therapeutically relevant genetic alterations between adenocarcinoma (eg *EGFR*,<sup>25</sup> *KRAS*,<sup>25</sup> *ALK*<sup>26</sup> and *BRAF*<sup>27</sup> mutations) and squamous cell carcinoma (eg *PIK3CA* mutations and several other recently described genetic alterations),<sup>28</sup> which forms the basis for a recommendation to employ predictive molecular tests differentially in patients with these tumors.<sup>29</sup> Given the uncertainty with the diagnostic approach and paucity of studies focused on large-cell carcinomas, the use of individualized therapies in patients with these tumors is not well established. In particular, there is little molecular data to inform a strategy for predictive molecular testing in patients with these tumors. While several studies did include a small number of large-cell carcinomas, and reported on the presence of *EGFR* (4%)<sup>30</sup> and *KRAS* (8–30%)<sup>31-34</sup> mutations in these tumors, a comprehensive screen for driver mutations in a large series of large-cell carcinomas has not been performed. Furthermore, it has not been explored whether the recent improvement in immunomarkers could translate into a more biologically precise classification of large-cell carcinomas, which could inform the selection of predictive molecular tests in patients with these tumors.

Given the above considerations, the goals of this study were to (1) establish the overall rate of targetable mutations in large-cell carcinoma, (2) determine whether the distribution of these mutations can be predicted by immunophenotyping and (3) explore whether immunomarker-defined subsets of large-cell carcinoma have distinct clinicopathologic characteristics. We therefore evaluated 102 large-cell carcinomas by immunohistochemistry for TTF-1 and  $\Delta$ Np63 as classifiers for adeno- and squamous carcinoma, respectively, and correlated the resulting subtypes with nine therapeutically relevant genetic alterations (*EGFR*, *KRAS*, *BRAF*, *MAP2K1*, *PIK3CA*, *NRAS*, *AKT1*, *ERBB2* and *ALK*) as well as various clinicopathologic parameters.

## Materials and methods

### Study Design

The study was performed with approval of the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center, New York. A total of 102 large-cell carcinomas were identified in the archives

during the period 1999–2011, after exclusion of 11 cases with unavailable or insufficient material for all assays in this study (this represents 2.2% of a total of 5267 resected non-small-cell lung carcinomas at our institution during that period). Large-cell carcinomas were defined as surgically resected non-small-cell lung carcinomas lacking the morphologic evidence of glandular, squamous or neuroendocrine differentiation by light microscopy. Mucin special stains were not used as part of inclusion or exclusion criteria in this study. Large-cell neuroendocrine carcinomas and sarcomatoid (entirely spindle or giant cell) carcinomas were not included. All cases were reviewed by two thoracic pathologists (NR, ALM) to confirm the absence of overt morphologic differentiation in all tumors. A representative formalin-fixed paraffin-embedded tumor block was selected for each case, and used for immunohistochemistry, molecular and cytogenetic studies, as described below.

### Immunohistochemistry

Immunohistochemistry was performed on a Ventana Discovery XT automated stainer (Ventana Medical Systems) as previously described.<sup>18,30</sup> Briefly, primary antibodies included  $\Delta$ Np63/p40 (CalBiochem, 1:2000 dilution) and TTF-1 (SPT24 clone, NovoCastra, 1:100 dilution). Percentage of immunoreactive tumor cells in each tumor was recorded. On the basis of prior studies, any reactivity for TTF-1 was considered as positive, whereas positivity for  $\Delta$ Np63 was defined as reactivity in >10% of tumor cells.<sup>20,21</sup> Additional immunostains were performed at the time of diagnosis or as part of this study, as needed, to exclude the possibility of unsuspected metastasis from extra-pulmonary sites, and/or other epithelioid neoplasms, such as melanoma, sarcoma or large-cell lymphoma.

### Mutation Analysis

**DNA extraction.** Tumor areas were macrodissected from 10 unstained 5- $\mu$ m thick sections of FFPE tissue to ensure >50% tumor cellularity. Genomic DNA was extracted using the DNeasy Tissue kit (QIAGEN). Extracted DNA was quantified on the NanoDrop 8000 (Thermo Scientific).

**Sequenom mass spectrometry genotyping and Sanger sequencing.** All cases were genotyped by Sequenom Mass ARRAY system (Sequenom) for 92 hot-spot point mutations in eight oncogenes: *EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *MAP2K1* (*MEK1*), *NRAS*, *AKT1* and *ERBB2* (*HER2*), as described in detail previously.<sup>25</sup> Samples were tested in duplicate using a series of six multiplexed reactions. Briefly, genomic DNA amplification and allele-specific single base extension reactions were performed using primers designed with the Sequenom Assay

Designer v3.1 software system (Sequenom). The extension products were quantitatively analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry on the Sequenom MassArray Spectrometer. Cases with equivocal Sequenom results upon manual review were retested in duplicate by standard sequencing with and without locked nucleic acid oligonucleotide for confirmation.<sup>35</sup>

***EGFR* exon 19 fragment analysis.** Cases lacking mutations other than *PIK3CA* by Sequenom were tested in duplicate for *EGFR* exon 19 deletions/insertions by fragment sizing assay, as previously described.<sup>25</sup> Briefly, a 207-bp genomic DNA fragment encompassing the entire exon 19 was amplified using fluorescently labeled primers, and PCR products were detected by capillary electrophoresis on an ABI 3730 Genetic Analyzer.

### Fluorescent in Situ Hybridization (FISH) for *ALK* Rearrangements

Cases lacking mutations other than *PIK3CA* by the above methods were further tested for *ALK* rearrangements by dual color break-apart FISH (Vysis/Abbott Molecular) according to the manufacturer's recommendations. Briefly, 4- $\mu$ m-thick tissue sections were pretreated by deparaffinization in xylene and dehydration in ethanol. FISH analysis and signal capture were performed on fluorescence microscope (AXIO, Zeiss) coupled with ISIS FISH Imaging System (Metasystems). At least 50 interphase nuclei from each tumor were scored, and a sample was considered positive for *ALK* rearrangement if >15% of tumor cells displayed broken-apart green/red signals and/or single red signals.

### Statistical Analysis

Mutation frequencies and clinicopathologic parameters were compared using Fisher exact or Kruskal–Wallis test. Disease-free and overall survival was estimated using Kaplan–Meier method with time origin at the time of surgery. Median (range) of available follow-up was 30 (1–120) months. Group comparisons were performed using log-rank test. Statistical analysis was conducted using SAS version 9.2 (SAS Institute Inc.) and the *clinfun* package in R (<http://www.r-project.org/>).

## Results

### Tumor and Patient Characteristics

Clinical characteristics of 102 patients with large-cell carcinomas were as follows: age, median (range) 63 (37–89), female  $n=51$  (50%), never smoker  $n=7$  (6%), and smoking pack-years, median (range) 40 (0–126). Stage distribution was as follows: stage

I  $n = 39$  (38%), stage II  $n = 35$  (34%), and stage III/IV  $n = 28$  (27%). Surgical procedures included wedge resection or segmentectomy ( $n = 25$ ), lobectomy or bilobectomy ( $n = 66$ ) and pneumonectomy ( $n = 11$ ). Morphologic review confirmed the lack of overt glandular, squamous or neuroendocrine differentiation in all tumors. Variant morphologies included basaloid ( $n = 7$ ; 1 focally, 6 diffusely), clear cell ( $n = 5$ ; 4 focally, 1 diffusely), rhabdoid ( $n = 3$ ; 2 focally, 1 diffusely) and with focal spindle and/or giant cells ( $n = 14$ ). The rest were classic large-cell carcinomas, not otherwise specified ( $n = 73$ ).

### Immunomarker-Defined Subsets of Large Cell Carcinoma

Immunohistochemistry for  $\Delta Np63$  and TTF-1 revealed the following immunoprofiles (Figure 1a): (1)  $\Delta Np63^-/TTF-1^+$  ( $n = 60$ ), (2)  $\Delta Np63^+/TTF-1^-$  ( $n = 20$ ), (3)  $\Delta Np63^+/TTF-1^+$  ( $n = 2$ ; each markers labeled a distinct cell subpopulation) and (4)  $\Delta Np63^-/TTF-1^-$  ( $n = 20$ ). On the basis of these immunoprofiles, tumors were classified as variants of (1) adenocarcinoma, (2) squamous cell carcinoma, (3) adenosquamous carcinoma and (4) marker null, respectively (Figure 1b). Expression of TTF-1 in group 1 and  $\Delta Np63$  in group 2 was typically seen in the majority of tumor cells: mean  $\pm$  s.d. for percentage of tumor cells immunoreactive for TTF-1 or  $\Delta Np63$  in those groups was  $90 \pm 25\%$  (range 10 – 100%) and  $92 \pm 14\%$  (range 50 – 100%), respectively. Examples of microscopic findings are illustrated in Figure 1c. Because of the previously shown similarity of adenosquamous carcinomas to adenocarcinomas in terms of driver mutations and clinicopathologic characteristics,<sup>36,37</sup> the former group was merged with the latter for further analysis.

### Distribution of Driver Mutations in Immunomarker-Defined Subsets of Large Cell Carcinoma

Molecular and cytogenetic analysis of 102 large-cell carcinomas revealed that 38 cases (37%) harbored non-overlapping mutations in *EGFR* ( $n = 1$ ), *KRAS* ( $n = 25$ ), *BRAF* ( $n = 2$ ), *MAP2K1* ( $n = 1$ ), *PIK3CA* ( $n = 1$ ) and *ALK* rearrangements ( $n = 3$ ) (Table 1). All mutations characteristic of adenocarcinoma (*EGFR*, *KRAS*, *BRAF*, *MEK1* and *ALK*) occurred in large-cell carcinomas with glandular or marker-null immunoprofiles but not in tumors with squamous profiles. The combined rate of adenocarcinoma-specific mutations in the above groups was 31/62 (50%) vs 6/20 (30%) vs 0/20 (0%), respectively ( $P < 0.001$ ). The sole *PIK3CA* mutation occurred in a tumor with a squamous immunoprofile (1/20; 5%). As illustrated in Figure 2, the combined frequency of mutations characteristic of adenocarcinoma was significantly different between large-cell carcinomas with adeno- vs squamous ( $P < 0.001$ ) and null vs squamous ( $P = 0.02$ ), but not between adeno- vs null

( $P = 0.13$ ) immunoprofile. *KRAS* mutations had a 5:1 ratio of smoking-related transversion mutations (G12V, G12C, G13R, Q61H) to transition mutations (G12D, G12S), respectively—a ratio similar to the one found in lung adenocarcinomas in our patient population.<sup>38</sup>

### Large Cell Carcinomas Harboring Genetic Alterations in *EGFR* or *ALK*

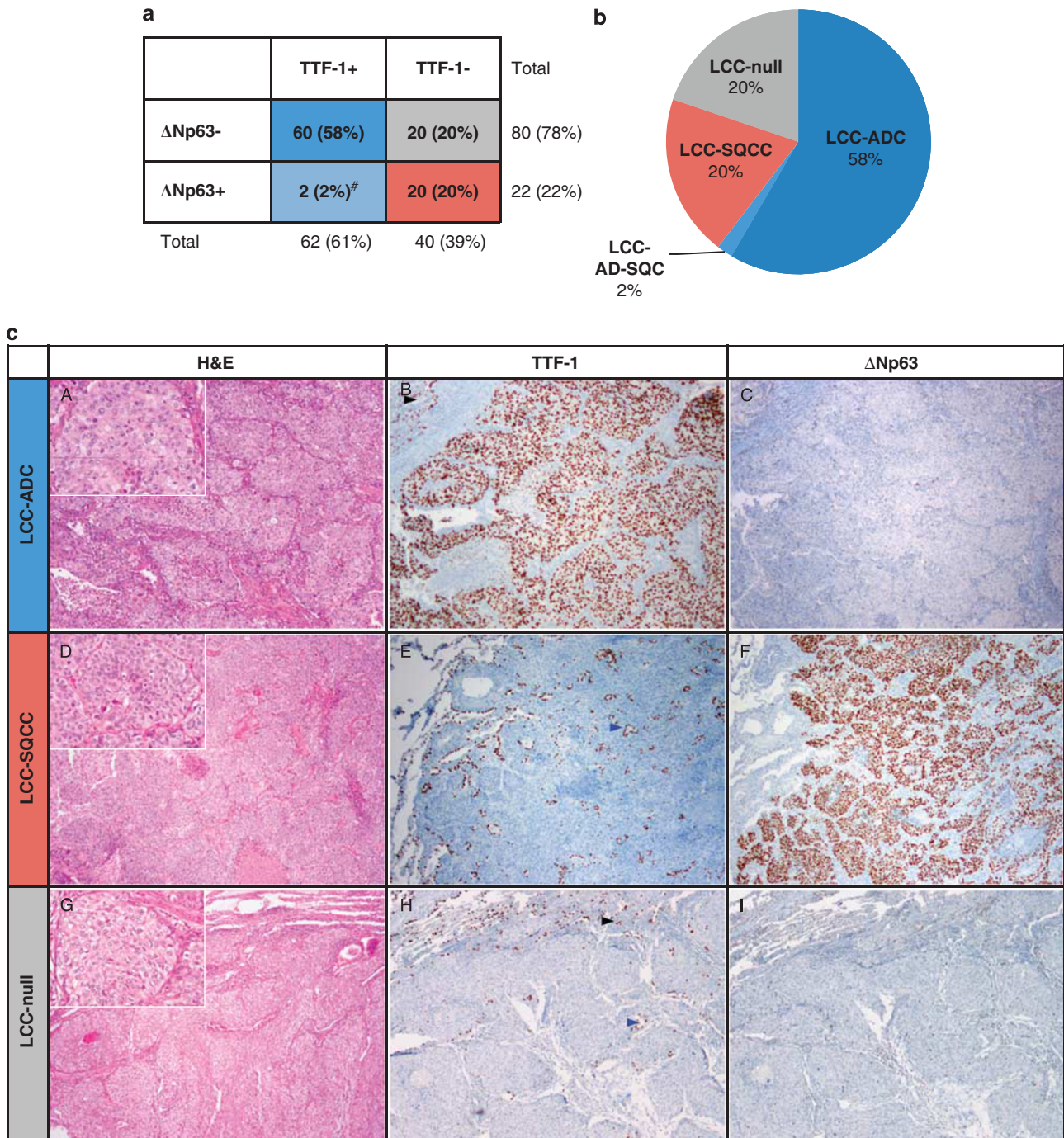
The sole patient with a tumor harboring an *EGFR* mutation was a 53-year-old woman, whose resected primary lung tumor was morphologically a classic large-cell carcinoma—an entirely solid/undifferentiated non-small-cell carcinoma with no microscopic evidence of glandular or squamous differentiation. Because of the resemblance of this solid morphology to squamous histology, this tumor was initially interpreted as squamous cell carcinoma, and this patient was therefore also included in our recent series on *EGFR* mutations in tumors mimicking squamous cell carcinomas (patient ID 12 in Rekhtman *et al*<sup>25</sup>). By immunohistochemistry, this tumor was TTF-1<sup>+</sup>/ $\Delta Np63$ <sup>-</sup>, supporting adenocarcinoma lineage. Nineteen months after surgery the patient developed brain metastases and was treated with erlotinib. She showed a marked radiologic response with near-complete regression of the brain lesions.

All three patients with *ALK* rearrangements were either never ( $n = 2$ ) or light ( $n = 1$ ; 0.7 pack-years) smokers and were younger (age 60, 52 and 48 years) than the median age of 63 years for patients in this series. The tumors were morphologically classic large-cell carcinomas, in which immunoprofiles similarly revealed glandular lineage (TTF-1<sup>+</sup>/ $\Delta Np63$ <sup>-</sup>). Crizotinib response data are not available for these patients. An example of *ALK*-rearranged large-cell carcinoma is illustrated in Figure 3.

### Clinicopathologic Characteristics of Immunomarker-Defined Subsets of Large-cell Carcinoma

As shown in Table 2, a comparison of clinicopathologic characteristics between large-cell carcinomas with adeno- vs squamous vs null immunoprofiles did not reveal significant differences in the analyzed parameters (age, gender, smoking, tumor size and stage), although patients with squamous profiles tended to be older, and had an invariable smoking history in contrast to the occurrence of rare never smokers in the other groups. Of morphologic variants, the only preferential association was between basaloid features and squamous immunoprofile, but this analysis is limited by a small number of cases in each subgroup with variant morphology.

As shown in Figure 4, survival analysis revealed that, remarkably, the marker-null group had a dismal 5-year disease-free and overall survival of 9% and 12%, respectively, compared to 55% and 56%,



**Figure 1** Immunohistochemistry-defined subtypes of large-cell carcinoma. (a) Coexpression profiles of TTF-1 and  $\Delta$ Np63 (p40). <sup>#</sup>TTF-1 and  $\Delta$ Np63 labeled distinct cell populations. (b) Pie chart showing TTF-1/ $\Delta$ Np63 -based subtypes of large-cell carcinoma. (c) Examples of microscopic findings. H&E shows morphologically indistinguishable non-small-cell carcinomas, all growing as entirely solid nests or sheets of tumor cells with no evidence of either glandular or squamous differentiation. Despite the lack of differentiating morphology, marker profiles provide evidence of submorphologic differentiation as adenocarcinoma (A – C) or squamous cell carcinoma (D – F); (G – I) illustrates a marker-null large-cell carcinoma. Benign pneumocytes (TTF-1<sup>+</sup>) are seen at the tumor periphery (black arrowheads) or entrapped within the tumor (blue arrowheads). Insets in a, d and g show higher-power images. Abbreviations: ADC, adenocarcinoma; AD-SQC, adenosquamous carcinoma; LCC, large-cell carcinoma; SQCC, squamous cell carcinoma.

respectively, for tumors with adenocarcinoma profiles, and 46% and 47%, respectively, for tumors with squamous profiles. The inferior survival of marker-null compared with marker-positive groups was statistically significant ( $P < 0.001$  for median

disease-free and  $P = 0.001$  for median overall survival), and remained significant after stage stratification. For stage I tumors with adenocarcinoma profiles, 5-year disease-free survival was 61% (95% confidence intervals 39–96%).

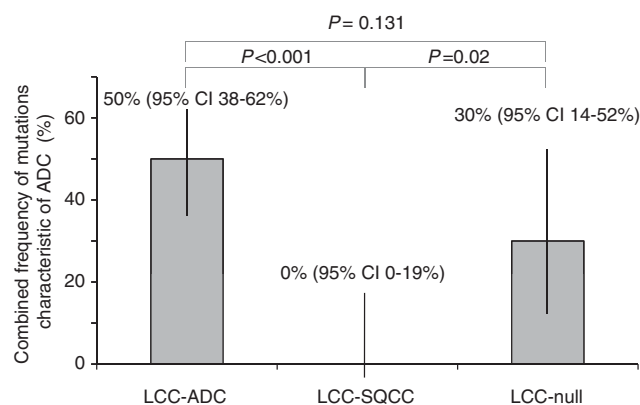
**Table 1** Distribution of mutations in immunomarker-defined subtypes of large-cell carcinoma

	Immunomarker-defined subtype of LCC			All cases (n = 102)
	LCC-ADC <sup>a</sup> (n = 62)	LCC-SQCC (n = 20)	LCC-null (n = 20)	
<i>EGFR</i>	1 (2%): Exon 19 18 bp Δ	0	0	1 (1%)
<i>KRAS</i>	25 (40%) G12V (n = 10) G12C (n = 9) G12D (n = 3) G13R (n = 1) Q61H (n = 2)	0	5 (25%) G12C (n = 3) G12D (n = 1) G12S (n = 1)	30 (29%)
<i>BRAF</i>	1 (2%): G469A	0	1 (5%): V600E	2 (2%)
<i>MEK1</i>	1 (2%): K57N	0	0	1 (1%)
<i>PIK3CA</i>	0	1 (5%): E542K	0	1 (1%)
<i>NRAS</i>	0	0	0	0
<i>AKT1</i>	0	0	0	0
<i>HER2</i>	0	0	0	0
<i>ALK</i> <sup>b</sup>	3 (5%)	0	0	3 (3%)
Any mutation	31 (50%)	1 (5%)	6 (30%)	38 (37%)

Abbreviations: ADC, adenocarcinoma; LCC, large-cell carcinoma; SQCC, squamous cell carcinoma.

<sup>a</sup>Includes two tumors with profiles of adeno-squamous carcinoma, of which one harbored a *KRAS* G12C mutation.

<sup>b</sup>No *ALK* gene rearrangement results are available for 4 cases owing to FISH failure.



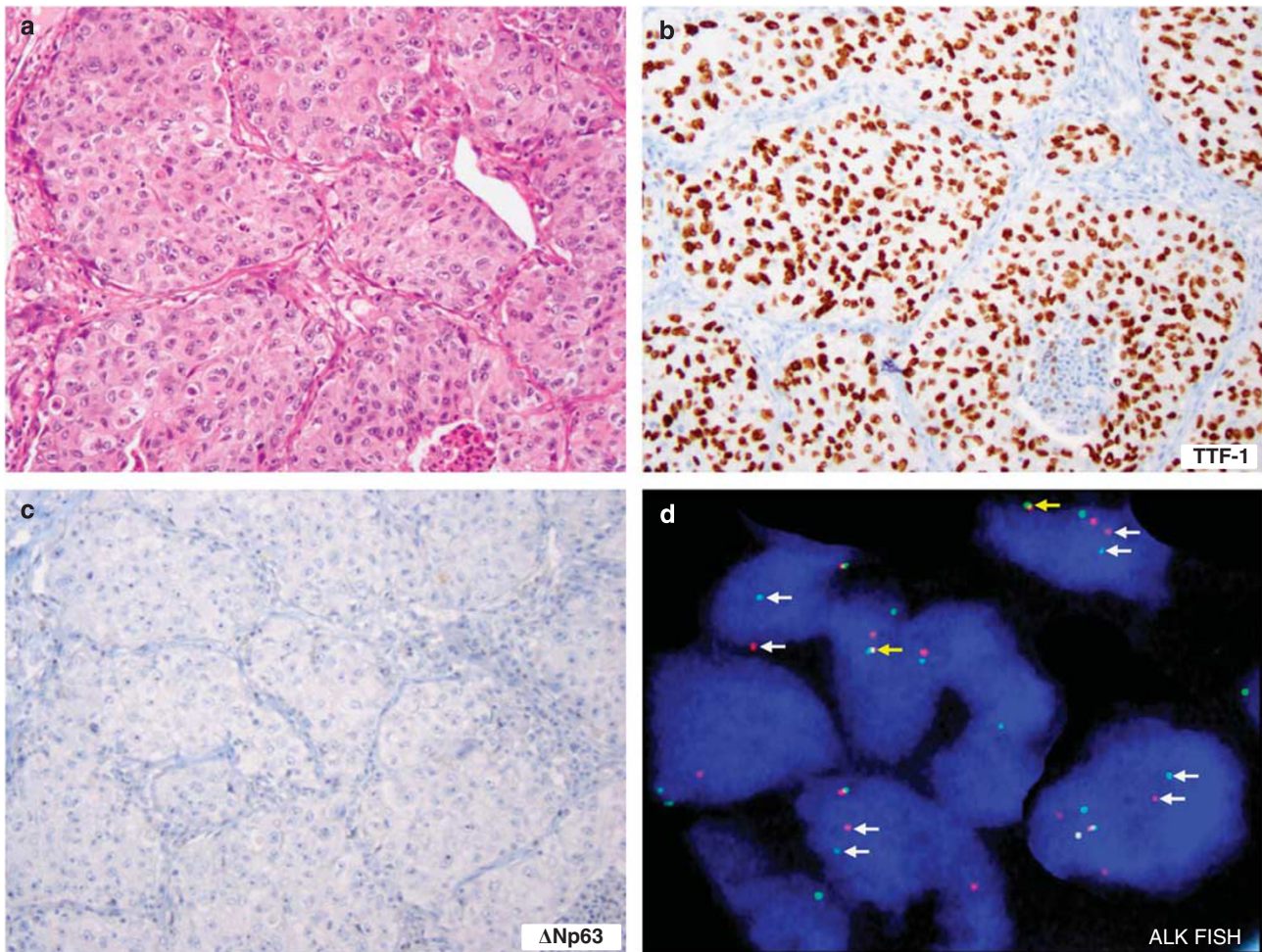
**Figure 2** Distinct distribution of driver mutations in immunomarker-defined subtypes of large-cell carcinoma. Shown is a combined frequency of genetic alterations characteristic of adenocarcinoma (*EGFR*, *KRAS*, *BRAF*, *MAP2K1* and *ALK*) ± 95% confidence intervals (CI).

## Discussion

The present data demonstrate that pulmonary large-cell carcinomas—non-small-cell carcinomas entirely lacking morphologic differentiation—retain the expression of differentiation markers, supporting their histogenetic relationship to poorly differentiated adeno- or squamous carcinomas in the majority of cases. We show that immunomarker-defined subsets of large-cell carcinoma have a distinct spectrum of therapeutically relevant driver mutations, including *EGFR*, *KRAS* and *ALK*, which parallels their distribution in tumors defined by traditional morphology. Finally, we identify marker-null large-cell carcinomas as tumors that have a mutation profile that is similar to adenocarcinoma and a particularly poor prognosis.

Our finding that the expression of TTF-1 and p63 (ΔN isoform) reveals marker profiles akin to adeno-

or squamous carcinoma, respectively, in 80% of large-cell carcinoma is comparable to the data from prior studies, showing the expression of glandular or squamous immunomarkers in 59–90% of large-cell carcinomas.<sup>12–18</sup> Similar to this series, prior studies also suggested a more frequent relationship of large-cell carcinomas to adeno- than squamous carcinomas, which may reflect the higher overall incidence of adenocarcinomas in the studied patient populations and/or the greater propensity of those tumors for complete loss of morphologic differentiation. However, a direct comparison across studies is limited by differences in the utilized markers, particularly in the studies performed prior to the recent advances in immunohistochemistry. Although the use of immunostains, particularly a combination of TTF-1 and p63/p40, is now widely recommended for subtyping of undifferentiated non-small-cell carcinomas in small biopsy/cytology samples,<sup>3,39</sup> applying this approach to entirely undifferentiated non-small-cell carcinomas in resections (ie large-cell carcinomas) has remained controversial. In small biopsy/cytology samples, morphologically unclassifiable non-small-cell carcinomas are in most cases derived from carcinomas with clear evidence of at least focal morphologic differentiation upon resection, and classification of those samples by ancillary studies has gained wide acceptance. Although a similar approach to large-cell carcinomas has been suggested by several investigators, the current recommendation is still to retain large-cell carcinoma as a morphologically defined entity,<sup>1,3</sup> at least in part because of the lack of data on accuracy of markers in classifying these tumors and the lack of evidence that such stratification has clinical utility. As discussed below, our data addresses these concerns by providing molecular corroboration for accuracy of



**Figure 3** An example of *ALK*-rearranged large-cell carcinoma. Although there is no evidence of morphologic differentiation by H&E (a), positive TTF-1 (b) and negative  $\Delta$ Np63 (c) immunostains support glandular lineage. (d) Split red and green signals (white arrows) indicate the presence of *ALK* rearrangement, whereas the native *ALK* allele is detected as merged red and green signals yielding a yellow color (yellow arrows).

immunomarker-based subtyping of large-cell carcinomas and by demonstrating a utility of this stratification for the current clinical practice.

The main novel observation in this study is that large-cell carcinomas, as a group, have a high frequency (37%) of therapeutically relevant driver mutations, and that specific mutations are distributed in specific immunomarker-defined tumor subsets, mirroring the mutation profiles expected for morphologically defined tumors. As such, *EGFR*, *KRAS*, *BRAF*, *MAP2K1* and *ALK* alterations, which are characteristic of adeno- but not squamous carcinomas, were found selectively in large-cell carcinomas with non-squamous immunoprofiles, whereas the only alteration in tumors with squamous profile was a *PIK3CA* mutation. It may appear aberrant, however, that the frequency of *EGFR* and *KRAS* mutations in large-cell carcinomas with glandular profiles is 2% and 40%, respectively, whereas the frequency of these mutations in unselected conventional adenocarcinomas in our patient population is ~20% and ~30%, respec-

tively.<sup>40</sup> In fact, this mutation frequency is entirely consistent with what is expected for a poorly differentiated subset of adenocarcinomas. Specifically, it is known that *EGFR* mutations occur preferentially in well-to-moderately differentiated adenocarcinomas with non-solid—bronchioloalveolar/lepidic and papillary—patterns,<sup>41,42</sup> while *KRAS* mutations are enriched in poorly differentiated adenocarcinomas with solid histology.<sup>34,43</sup> Thus, the lower *EGFR* and higher *KRAS* mutation frequency in large-cell carcinomas with glandular immunophenotype closely matches the expected frequency of these mutations for tumors in the spectrum of poorly differentiated adenocarcinomas. Similarly, the frequency of other genetic alterations (*ALK*, *BRAF*, *MAP2K1*, *PIK3CA*) is comparable to the expected rate of these mutations in conventional adeno- or squamous carcinomas. The lack of *NRAS*, *ERBB2* and *AKT1* mutations in large-cell carcinomas is in line with their overall low expected prevalence (<1%) in lung carcinomas. In addition to establishing the overall frequency of therapeutically

**Table 2** Clinicopathologic features by immunomarker-defined subtype of large-cell carcinoma

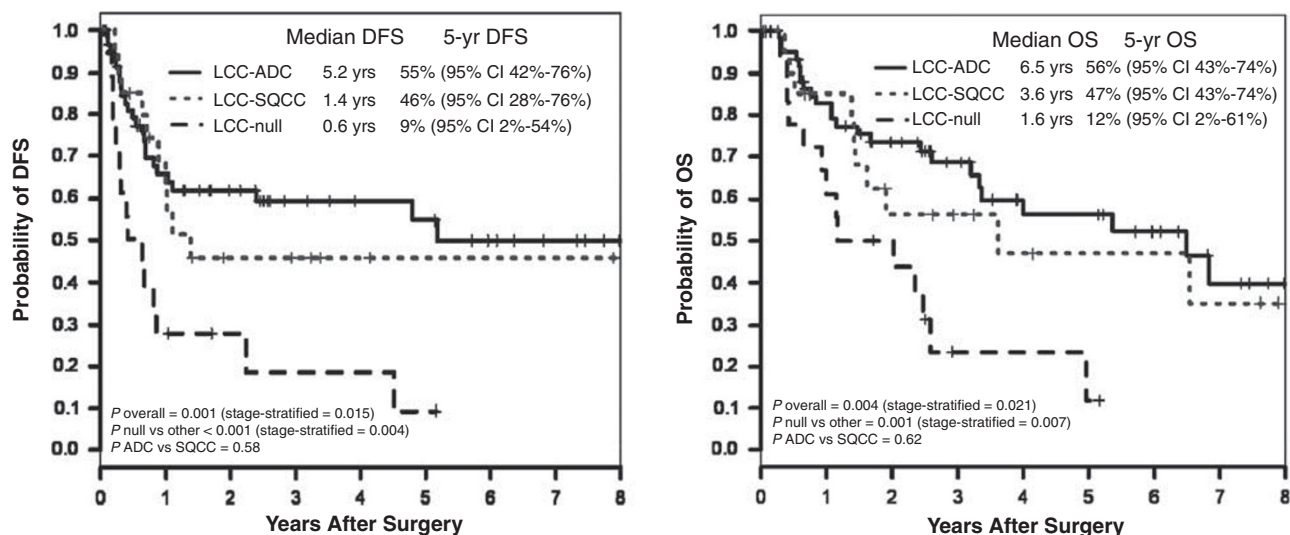
	LCC-ADC <sup>a</sup> (n = 62)	LCC-SQCC (n = 20)	LCC-null (n = 20)	P value
Age: median (range)	62 (41–86)	71 (37–89)	62 (45–81)	0.07
Gender: n (%)				
Female	32 (52)	10 (50)	9 (45)	0.96
Male	30 (48)	10 (50)	11 (55)	
Smoking status: n (%)				
Never	6 (10)	0	1 (5)	0.43
Current/former	56 (90)	20 (100)	19 (95)	
Smoking pack-years <sup>b</sup> : median (range)	45 (0–110) <sup>c</sup>	47 (1.5–90)	40 (0–130)	0.81
Tumor size, cm: median (range)	3.5 (0.4–12)	3.5 (1.1–9.5)	3.1 (0.9–9.2)	0.90
Stage: n (%)				
I	19 (15)	12 (60)	8 (40)	0.18
II	26 (42)	6 (30)	6 (30)	
III/IV	17 (27)	2 (10)	6 (30)	
Morphologic variants: n (%)				
Basaloid	1 (2)	4 (20)	2 (10)	0.08
Clear cell	3 (5)	1 (5)	1 (5)	
Rhabdoid	1 (2)	0	2 (10)	
Focal giant or spindle cells	9 (15)	3 (15)	2 (10)	
Not otherwise specified	48 (77)	12 (60)	13 (65)	

Abbreviations: ADC, adenocarcinoma; LCC, large-cell carcinoma; SQCC, squamous cell carcinoma.

<sup>a</sup>Includes two tumors with profiles of adeno-squamous carcinoma.

<sup>b</sup>Pack years = number of packs of cigarettes smoked per day × years of smoking.

<sup>c</sup>Number of pack-years not available for one smoker.



**Figure 4** Survival by immunomarker-defined subtype of large-cell carcinoma: an adverse prognosis associated with marker-null carcinomas. Abbreviations: CI, confidence intervals; DFS, disease-free survival; OS, overall survival.

relevant mutations, all of which are linked to either established or investigational targeted agents,<sup>44,45</sup> the mutation data in this study provide a measure of biological accuracy for immunophenotype-based classification of large-cell carcinoma by demonstrating a similarity of mutation profiles in immunomarker-defined and morphologically defined tumors.

To our knowledge, this is the first report of *ALK* rearrangement in large-cell carcinomas. This ex-

pands the previously recognized morphologic spectrum of *ALK*-positive lung carcinomas, although the propensity of these tumors for solid growth pattern (in addition to their classic association with signet ring cells) has been described.<sup>46</sup> Notably, the clinical characteristics of patients with *ALK*-rearranged large-cell carcinomas in this series (never/light smoker, younger age) are similar to what has been described for patient with *ALK*-positive adenocarcinomas.<sup>39,40</sup>



The identity of marker-null large-cell carcinomas, and whether these represent entirely undifferentiated carcinomas or whether the differentiation lineage can be identified in these tumors by other markers, needs further study. We speculate that at least some of these tumors represent variants of TTF-1-negative adenocarcinomas, as the absence of TTF-1 is known to occur in ~20% of adenocarcinomas, whereas complete absence of p63/p40 expression is unusual for squamous cell carcinomas.<sup>19,47–49</sup> The relationship to adenocarcinoma of at least a subset of marker-null large-cell carcinomas is further supported by our finding that they harbor a significant number of *KRAS* and *BRAF* mutations (combined rate 30%)—a mutation profile that is more similar to lung adenocarcinoma. Identification of a reliable pan-adenocarcinoma marker and more detailed molecular analysis would be needed to further clarify the nature of marker-null tumors.

Another novel observation in this study is that marker-null large-cell carcinomas are associated with a distinctly inferior prognosis compared with differentiation marker-positive tumors. Conversely, the prognosis associated with marker-positive large-cell carcinomas appears to fall in the lower range of what has been reported for poorly differentiated/high-grade subset of conventional adenocarcinomas and squamous carcinomas. In particular, several recent studies have demonstrated that the presence of solid growth pattern—a hallmark of poor differentiation—is a significant predictor of poor outcome in lung adenocarcinomas, conferring a 60–70% disease-free survival in stage I adenocarcinomas, compared with 80–>90% survival for better-differentiated adenocarcinomas.<sup>50–52</sup> Thus, 61% disease-free survival for stage I large-cell carcinomas with glandular immunoprofiles in this series appears to be comparable to what is expected for tumors in the spectrum of poorly differentiated adenocarcinomas. Although the survival data in this study are limited by a relatively small number of patients in each subgroup, these data are in keeping with the concept that large-cell carcinomas represent tumors in a continuum of solid growth/poor differentiation with usual types of non-small-cell carcinoma. The particularly poor prognosis associated with marker-null large-cell carcinomas may reflect the state of poorest differentiation—tumors undifferentiated at both morphologic and biomarker levels. We note that this observation parallels the known adverse prognostic effect of the lack of TTF-1 expression in adenocarcinomas.<sup>53–55</sup> The high risk of recurrence suggests that large-cell carcinomas overall and particularly marker-null subset could serve as a stage-independent indication for trials evaluating adjuvant chemotherapy, as has been recently suggested for poorly differentiated adenocarcinomas in general.<sup>52</sup>

A direct practical utility of the findings in this study is that immunomarker-based stratification of large-cell carcinomas could be used to guide the

selection of predictive molecular tests in clinical practice. Currently, the standard predictive testing of lung carcinomas includes screening of adenocarcinomas for *EGFR* mutations (and in some institutions *KRAS* mutations), as positive and negative predictors, respectively, of sensitivity to *EGFR* tyrosine kinase inhibitors—erlotinib and gefitinib, and for *ALK* rearrangements as a predictor of sensitivity to crizotinib.<sup>29</sup> The standard guidelines, including the National Comprehensive Cancer Network, recommend testing of all large-cell carcinomas for genetic alterations characteristic of adenocarcinoma,<sup>29</sup> although this recommendation is based on limited data. Our findings support this recommendation for large-cell carcinomas with non-squamous immunoprofiles. In contrast, our findings suggest that a biologically rational triage for large-cell carcinomas with squamous marker expression would include testing for genetic events characteristic of squamous rather than adenocarcinomas, which includes *PIK3CA* mutations,<sup>25</sup> as well as recently identified *DDR2* mutations<sup>56</sup> and *FGFR1* amplification<sup>57</sup>—the markers that are anticipated to become part of routine clinical testing for squamous cell carcinomas in the near future<sup>28,58</sup> (although the degree of tumor-type specificity for the latter alterations still needs further investigation). Identification of tumor lineage in large-cell carcinomas by immunoprofiling could thus direct the use of specific molecular and cytogenetic assays appropriate for that tumor type, thus optimizing the use of tissue and resources. The value of identifying targetable alterations in large-cell carcinomas is illustrated by a patient with an *EGFR* mutation in this series, whose metastatic tumor had a marked response to erlotinib.

The second potential practical utility of our findings is for the use of ‘histology-based’ agents—bevacizumab and pemetrexed—which are approved for patients with non-squamous non-small-cell carcinomas. In clinical studies, it was suggested that all large-cell carcinomas should be regarded as non-squamous on the basis of similarity of their response or adverse event profile to adenocarcinomas rather than squamous carcinomas,<sup>59,60</sup> although interpretation of these data is limited by the designation of tumors in small samples as large-cell carcinoma in those studies. Nevertheless, the data on immunoprofiling of large-cell carcinomas in this and other studies show that a subset of these tumors are variants of squamous cell carcinoma, and therefore lumping all large-cell carcinomas as ‘non-squamous’ is biologically imprecise, whereas clinical characteristics of the group overall could reflect the predominance of adenocarcinoma variants. Notably, Monica *et al.*<sup>18</sup> showed that squamous lineage identified in large-cell carcinomas by immunostains correlated with overexpression of thymidylate synthase—a putative target of pemetrexed—a profile that parallels thymidylate synthase expression in usual-type squamous cell carcinomas,<sup>18</sup> supporting the concept that

immunoprofiling of large-cell carcinomas could be useful for the exclusion of non-recommended therapies in patient with these tumors. The actual impact of marker-based stratification on treatment outcomes with 'histology-based' agents needs to be determined in clinical studies.

It is important to note that there are several potential limitations to TTF-1/ $\Delta$ Np63-based classification of large-cell carcinomas, despite these markers having been shown to be effective in distinguishing adeno- and squamous carcinomas in several recent studies.<sup>20–22</sup> The first limitation is that neither marker is restricted to these tumor types: TTF-1 is also expressed in thyroid carcinomas, high-grade neuroendocrine carcinomas, and occasionally in unexpected settings, such as carcinomas of gynecologic tract, whereas  $\Delta$ Np63 is expressed in squamous cell carcinomas of any site, urothelial, thymic, trophoblastic and basal cell/myoepithelial tumors (reviewed in Rekhtman *et al*<sup>19</sup> and Bishop *et al*<sup>20</sup>)—some of these tumors, when showing predominantly solid growth pattern, can enter in the differential diagnosis with large-cell carcinomas. Thus, the interpretation of these markers must be performed in the context of careful morphologic and clinicoradiologic correlation and, if needed, with the use of additional immunostains to exclude the possibility of tumor types other than non-small-cell lung carcinoma. This particularly applies to TTF-1/ $\Delta$ Np63-null tumors, which must also be distinguished from other epithelioid neoplasms, such as melanoma or sarcoma with epithelioid features. The second limitation, mentioned above, is that TTF-1 is not a pan-adenocarcinoma marker, as it recognizes only ~80% of lung adenocarcinomas. The third potential limitation is the uncertainty with the interpretation of focal (<50% but above isolated tumor cells)  $\Delta$ Np63 reactivity, which, on the basis of prior studies, is not characteristic of either squamous cell carcinoma or adenocarcinoma,<sup>20–22</sup> and has been suggested to indicate adenosquamous differentiation even in the absence of TTF-1 reactivity in  $\Delta$ Np63-negative cell population.<sup>22</sup> Such reactivity was not observed in this series, and the classification of large-cell carcinomas with this uncommon immunoprofile, if encountered, remains to be clarified. Although several other glandular and squamous markers are currently available, none are clearly superior in sensitivity and specificity to TTF-1 and  $\Delta$ Np63, and whether they add value to TTF-1/ $\Delta$ Np63 panel will be of interest to explore in future studies. Notably, we found that Napsin A - a recent marker of lung adenocarcinomas - labeled fewer large-cell carcinomas than TTF-1, and none of TTF-1-negative carcinomas were Napsin A-positive (data not shown). Nevertheless, coexpression of TTF-1 and Napsin A can be expected to be more specific for lung adenocarcinoma lineage than TTF-1 alone. Conversely, supplementing  $\Delta$ Np63 with lower-specificity squamous markers, such as p63 (4A4) or 34 $\beta$ E12, is unlikely to be of value.

Also of note is the distinction in the current classification scheme of lung carcinomas between large-cell carcinomas and the other class of undifferentiated non-small-cell carcinomas that exhibit features of dedifferentiation in the form of spindle or giant/anaplastic cells, which are classified as 'sarcomatoid carcinomas'.<sup>1</sup> Although there is some morphologic gray zone between giant cell/anaplastic carcinomas and large-cell carcinomas in the higher end of the spectrum of cytologic pleomorphism, the tumors composed entirely of frankly anaplastic or spindle cells were not included in this series, and the ability of immunomarkers to detect residual differentiation and predict mutations in this other class of undifferentiated/dedifferentiated carcinomas would be of interest to investigate in a focused study. Further study is also needed to evaluate the potential significance of variant morphologies in large-cell carcinomas.

In conclusion, our findings extend the concept that the majority of large-cell carcinomas exhibit immunophenotypic characteristics of either adeno- or squamous carcinomas, and further provide evidence that immunomarker-defined subsets of these tumors have distinct profiles of therapeutically relevant mutations and distinct prognosis. Although the current definition of large-cell carcinoma is based on the morphologic criteria (supplemented with low-sensitivity mucin stains),<sup>1,3</sup> this classification groups biologically heterogeneous tumors in a single category. Our data show that with currently available markers, stratification of large-cell carcinomas would have a utility for triage of tissue for *EGFR/KRAS/ALK* testing and for prognostication. Furthermore, marker-based stratification is also likely to be important for future clinical and molecular investigations where identification of biologically precise tumor lineages is increasingly important given a strong trend for tumor type specificity and molecular targeting of the emerging therapeutics. We therefore suggest that 'large-cell carcinomas' with the marker profiles of adeno- or squamous carcinomas should be classified as variants of these respective tumor types, and predictive molecular and cytogenetic tests selected accordingly, whereas the term 'large-cell (undifferentiated) carcinoma' be reserved for the minority of cases that lack differentiation at both morphologic and biomarker levels. Given the increasing use of immunohistochemistry to subtype non-small-cell carcinomas in small samples in the current practice, to what degree this approach may already be informally applied to large-cell carcinomas at individual institutions would be of interest to survey.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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