Dissecting Pulmonary Large-Cell Carcinoma by Targeted Next Generation Sequencing of Several Cancer Genes Pushes Genotypic–Phenotypic Correlations to Emerge

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Introduction: Little is known about genotypic and phenotypic correlations in undifferentiated large-cell carcinoma (LCC) of the lung.

Methods: Thirty LCC were dissected by unsupervised targeted next generation sequencing analysis for 50 cancer-associated oncogenes and tumor suppressor genes. Cell differentiation lineages were unveiled by using thyroid transcription factor-1 (TTF1) for adenocarcinoma (ADC) and p40 for squamous cell carcinoma (SQC), dichotomizing immunohistochemistry (IHC) results for TTF1 as negative or positive (whatever its extent) and for p40 as negative, positive, or focal (if <10% of reactive tumor cells).

Results: Three LCC were wild type (all TTF1+/p40−), whereas the remaining 27 (90%) tumors had at least one gene mutation. Twenty-four cases featuring TTF1+/p40−, TTF1+/p40±, TTF1−/p40±, or TTF1−/p40− phenotypes comprised *ATM, BRAF, CDKN2A, EGFR, ERBB4, FBXW7, FLT3, KRAS, NRAS, PIK3CA, PTPN11, RET, SMAD4, SMO, STK11*, or *TP53* mutations in keeping with ADC lineage, whereas three tumors showing TTF1−/p40+ phenotype harbored *TP53* only and no ADC-related mutations in keeping with SQC lineage. Single, double, triple, quadruple, and quintuple mutations occurred in 16, 6, 2, 2, and 1 patient, respectively. The occurrence of three mutations or more but not any immunohistochemistry categorization predicted shorter overall survival (OS, $p = 0.001$) and disease-free survival (DFS, $p = 0.007$), independent of age, sex, and tumor stage.

Conclusions: Albeit preliminary also because of the relatively small number of LCC under evaluation, this targeted next generation sequencing study, however, revealed gene mutation heterogeneity in LCC with some genotypic–phenotypic correlations. Negativity or focal occurrence of p40 made SQC diagnosis unlikely on molecular grounds, but suggested ADC confirming validity of the axiom "no p40, no squamous."

Key Words: Large-cell carcinoma, Lung, Immunohistochemistry, Targeted next generation sequencing, p40, TTF1.

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Large-cell carcinoma (LCC) of the lung is a poorly differentiated tumor lacking cytological, architectural, and immunohistochemistry (IHC) features of small-cell lung carcinoma (SCLC), adenocarcinoma (ADC), or squamous cell carcinoma (SQC).[1] The previous 2004 World Health Organization (WHO) classification included different histological variants under the umbrella term of LCC, namely undifferentiated LCC, large-cell neuroendocrine (NE) carcinoma, basaloid carcinoma, lymphoepithelioma-like carcinoma, LCC with rhabdoid phenotype, and clear cell carcinoma.[2] The new 2015 WHO classification has attributed a decisional role to IHC characterization of LCC based on a minimalist panel antibody approach,[3–5] which now are confined to three tumor categories only (LCC with null IHC features, LCC with unclear IHC features, and LCC with no additional stains).[1]

In LCC, IHC has been unveiling the same lineage heterogeneity,[6–13] as previously demonstrated by means of electron

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microscopy,[14,15] indicating that squamous, NE, and especially, glandular cell differentiation underlie most LCC, whereas completely uncommitted tumors are decidedly uncommon.[6,10,11,13,16,17] Among the many IHC biomarkers aimed at discovering the hidden face of LCC,[6,10,17–19] thyroid transcription factor-1 (TTF1)[20,21] and Δ(delta)Np63/p40 (henceforth, simply p40)[22–26] proved the best diagnostic biomarkers to highlight ADC and SQC lineages, respectively.[3,27–32] Although the duet TTF1/p40 remains the most reliable predictor of cell lineage and therefore of the ultimate diagnosis in lung cancer,[3,4,27,33,34] some uncertainty may arise in undifferentiated tumors when faced with complete absence of both biomarkers (null phenotype: TTF1−/p40−) and focal labeling for squamous differentiation biomarkers, such as p40, in otherwise TTF1-negative tumors (unclear phenotype: TTF1−/p40±).

A growing body of information is accumulating about the occurrence of nonrandom genetic alterations in defined subtypes of lung cancer.[4,35–41] New actionable driver genes are emerging from the molecular landscape of lung cancer, especially when using multiplexed or unbiased technologies of next generation sequencing (NGS).[42–47] NGS/targeted next generation sequencing (T-NGS) data or molecular investigations on LCC are relatively scant[16–18,48,49] for either novelty of these technologies or progressive disappearance of such histological type.[11,13] Recent surveys on the subject, however, have provided strong evidence that many, if not all, LCC display genetic profiles (including microRNA expression)[18] mostly aligned with ADC and/or SQC,[16,17,49] but the approach has been to correlate a priori-defined IHC diagnoses with underlying gene alterations rather than to interpret IHC profiles according to the relevant mutation portrait.

This study was aimed at establishing the relationship between genotype and phenotype in LCC also according to tumor categorization of the current 2015 WHO classification by comparing their molecular profile assessed by unsupervised T-NGS with stochastically defined IHC categories according to biomarkers of glandular and squamous cell lineages (TTF1 and p40) and then at a posteriori dissecting tumors on the basis of the preferential distribution of gene mutations between ADC and SQC.

PATIENTS AND METHODS

Design of the Study

We designed and conducted a two-phase investigation to test the relationship between genotype of LCC according to T-NGS analysis and phenotype according to TTF1 and p40 IHC, thereby providing a biological rationale to the diverse diagnostic algorithms with particular reference to null and unclear phenotypes. In this regard, in the first phase, we accomplished an unsupervised T-NGS analysis on 30 LCC by using a large panel of 50 oncogenes and tumor suppressor genes recurrently altered in human cancers and compared molecular results with clinicopathological characteristics of patients, including survival. In the second phase, we attributed the relevant gene mutations to the diverse IHC-prioritized diagnostic algorithms on the basis of their own preferential distribution in the two major categories of lung cancer, i.e., ADC and SQC, to molecularly validate the role of these biomarkers in constructing decisional algorithms even in undifferentiated tumors.

Patients and Tumors

A series of 30 consecutive surgical specimens of LCC from 24 males (range, 47–87 years; mean ± SD, 52.0 ± 14.8 years) and 6 females (range, 61–72 years; mean ± SD, 65.5 ± 10.9 years) were identified in the pathology archives of the participant Institutions (Milan, Turin, Modena, and Reggio Emilia). The lack of a previous history of cancer elsewhere in the body and the availability of complete clinical information were required for entering the study. Pertinent data regarding the 30 LCC patients as a function of gender, smoking status, and tumor stage are summarized in Table 1. Surgical specimens consisted of lobectomy or pneumonectomy along with radical mediastinal lymph node resection to ensure accurate staging. According to the 7th edition of the tumor, node, metastasis (TNM) staging system,[50] there were three tumors staged IA, nine IB, nine IIA, four IIB, three IIIA, one IIIB, and one IV (the latter featuring pT4pN0pM1a). Fifteen patients were current smokers and 15 former smokers.

All surgical specimens had been fixed in 4% buffered formaldehyde solution for 12 to 24 hours and embedded in paraffin according to standard histopathological methods. All the original hematoxylin and eosin (H&E)-stained sections were jointly reviewed by some authors (G.P., M.P., G.R., and A.C.) without the knowledge of the patients' identities or original tumor categorization. Solid ADC with mucin was excluded by mucin stain,[4,41] NE differentiation by morphology and NE biomarkers,[2,51-53] sarcomatoid carcinoma by morphology and vimentin IHC,[54,55] and unexpected metastases by clinical history and appropriate IHC work-up.[3,4,30,31,56] According to 2004 WHO classification on lung cancer primarily based on morphology,[2] the study comprised 20 undifferentiated LCC, six LCC–clear cell carcinoma, three LCC with rhabdoid phenotype, and one LCC–lymphoepithelioma-like carcinoma, which were considered poorly differentiated by definition. Out of eight patients with lymph node metastases, five were pN1 and three pN2, with involved lymph node percentage ranging from 5% to 67%. Vascular invasion was seen in 17 tumors and necrosis in 28, the latter ranging from 10% to 70% of the entire tumor mass. Pleural invasion, classified according to updated criteria,[57] was documented in 15 tumors, resulting in PL1, PL2, and PL3 in 11, 2, and 2 cases, respectively. Tumor size ranged from 10 to 120 mm, with a mean value of 41.8 ± 23.8 mm. Complete clinical follow-up (updated to July 2014) was available for all but one patient lost to follow-up, with OS averaging 47 ± 37 months (median, 32; range, 1–132). During this period, 13 (44.8%) patients had recurrent disease (nine systemic, three in the lungs, and one in the brain) and 12 (41.3%) died of disease, with mean DFS and OS being 43 ± 38 months (median, 31; range, 1–132) and 47 ± 37 months (median, 32; range, 1–132), respectively.

Ethics

The study was notified to and approved by the independent ethics committee of the "Fondazione IRCCS Istituto

