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Retrospective multicentre study investigating the role of targeted next-generation sequencing of selected cancer genes in mucinous adenocarcinoma of the lung

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ABSTRACT

1
2 Mucin-rich lung adenocarcinomas (ADC), namely mucinous and colloid ADC, are classified as ADC
3
4 variants according to the WHO 2015 classification. A correlation between morphological patterns
5
6 and mutational status of these rare entities is not well established. We investigated the mutational
7
8 profile of mucin-rich lung ADC in correlation with histopathologic and morphological features with
9
10 the goal of identifying biological tumor characteristics of potential prognostic and therapeutic
11
12 interest. A series of 54 surgically resected primary mucinous lung ADC samples was retrospectively
13
14 analyzed for clinical-pathological characteristics and by targeted Next-Generation Sequencing.
15
16 Fifty cases were invasive mucinous (IMA, 32 pure and 18 mixed) and 4 were colloid predominant
17
18 ADC (CPA). IMA cases with pure mucinous pattern were associated to a lower risk of vascular
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20 invasion (p=0.01), absence of signet ring cell (p=0.03), negative nodal status (p=0.006) and early
21
22 clinical stage (p=0.02). The most prevalent mutations involved *KRAS* and *TP53* genes. The majority
23
24 of mutations clustered in the MAP-AKT kinase (*KRAS*, *PIK3CA*, *STK11*, *PTEN*, *AKT1*, *HRAS*, *BRAF*) and
25
26 the p53/DNA repair (*TP53*, *CDKN2A*, *ATM*, *RB1*) pathways. A few uncommon *EGFR* mutations were
27
28 found. A correlation between a higher number of mutations and favorable clinical outcome was
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30 seen (p<0.001). Our data showed that mucinous ADCs have peculiar pathological and molecular
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32 features that might imply a differentially tailored therapeutic approach compared to conventional
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34 lung ADC.
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49 **KEY WORDS:** lung, mucinous adenocarcinoma, next generation sequencing, mutation
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INTRODUCTION

1
2 Mucinous lung adenocarcinomas (ADC) account for 2-10% of all lung ADCs. Based on a proposal
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4 from the International Association for the Study on Lung Cancer¹, the WHO has recently classified
5
6 lung ADCs according to their invasive capacity, predominant architectural pattern and amount of
7
8 mucin production². Specifically, a distinction between invasive ADC of conventional type and its
9
10 variants, which include invasive mucinous ADC (IMA) and colloid ADC, was made. An alternative
11
12 classification of lung ADCs classifies these tumors into terminal-respiratory-unit (TRU) and non-
13
14 TRU types on the basis of distinct morphological features^{3,4}. Lung mucinous ADCs belong mostly to
15
16 the non-TRU type group, as they originate from the bronchial epithelium or submucosal glands,
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18 and exhibit a gastric-mucin phenotype and a poorer prognosis than TRU type ADCs⁵.

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Despite these efforts of re-classification, mucinous ADCs present a heterogeneous spectrum of morphological patterns^{6,7} and, potentially, a high degree of molecular heterogeneity could drive the observed histological differences, but few studies have specifically addressed this hypothesis. Indeed, while the prevalence and type of “druggable” molecular alterations in conventional ADC have been well characterized⁸, scant information is available about invasive mucinous ADC variants. Most of the available studies that included all types of ADC and employed low sensitive and *single-gene* technologies, have established that *KRAS* mutations occur in 61% and 15% of invasive mucinous and colloid ADCs, respectively^{9,10} and are associated with poor prognosis and lack of response to chemotherapy¹¹. *KRAS* mutations have been reported to often co-occur with *CDKN2A/B* inactivation and low expression of the Thyroid Transcription Factor 1 (TTF1)¹², although the clinical implications of these findings remain to be fully elucidated¹³. A recent study has identified a *CD74-NRG1* gene fusion as a novel driver in the carcinogenesis of invasive mucinous ADC. However, these fusions present at a low prevalence (<10%) and are confined to *KRAS* wild-type cases¹⁴. More recently, Shim and colleagues have performed targeted NGS for gene fusions

1
2
3 and mutations in 30 mucinous ADC samples previously found to be *KRAS* wild-type by Sanger
4 sequencing. They described a large number of gene fusions, unexpectedly infrequent *TP53*
5 mutations and an overall low number of mutations, without significant differences in terms of age,
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7 gender, smoking status and stage compared to *KRAS* mutated cases¹⁵.
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10 Based on the lack of complete and unanimous information on the clinical-pathological and
11
12 molecular characteristics of pulmonary mucin-rich ADC, we retrospectively performed a detailed
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14 morphological analysis of mucinous characteristics and targeted Next-Generation Sequencing (T-
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16 NGS) of 50 genes in 54 resected mucinous ADC samples (irrespective of their *KRAS* genotype) in
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18 order to investigate genotype-phenotype relationships and identify molecular alterations of
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20 potential prognostic and therapeutic interest.
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28 **MATERIALS AND METHODS**

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30 **Patients and tissue samples** – A consecutive series of surgically resected primary invasive lung
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32 ADC with mucinous features, diagnosed in Caucasian patients from April 2003 to December 2013,
33
34 was retrospectively collected at three Institutions (San Luigi Hospital, Orbassano, Torino;
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36 Policlinico Hospital, Modena; Santa Maria Nuova Hospital, Reggio Emilia). All cases were centrally
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38 reviewed by three pathologists (LR, MP and GR) according to the 2015 WHO lung adenocarcinoma
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40 classification². Study inclusion criteria were the presence of a mucinous pattern (i.e. IMA and
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42 colloid ADC, while enteric ADC cases were excluded), the lack of a prior non-lung related cancer
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44 diagnosis, the presence of accurate clinical follow-up data, as well as the availability of residual
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46 tissue for genetic analyses. Patients were staged according to the American Joint Committee on
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48 Cancer TNM Staging Manual¹⁶. The Institutional Review Board approved the study. All samples
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50 were de-identified and cases anonymized by a pathology staff member not involved in the study.
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60 Clinical parameters were compared and analyzed through coded data.
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1
2 **Morphological analysis** - Hematoxylin-eosin stained slides of each tumor were reviewed. The
3
4 following histological parameters were assessed (Figure 1): a) the presence of a non-mucinous
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6 component, that segregated the IMA group into *pure* (mucinous features $\geq 90\%$) or *mixed*; in the
7
8 mixed cases the predominant pattern of conventional ADC was also recorded; b) the presence of
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10 *extracellular mucin* that was recorded when observed in $\geq 10\%$ of the tumor; c) the extent of *tufts*
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12 (bunches or small clusters of neoplastic mucinous cells attached to the alveolar wall, closely at the
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14 base and loose at the upper ends) scored as percentage (using a cut-off $\geq 10\%$ to define the
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16 presence or absence of tufts); d) the presence of $\geq 5\%$ of signet ring cells (SRC) component,
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18 characterized by cells with large intracellular mucin drops and peripheral nuclei displaced toward
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20 one end of the cytoplasm.
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31 **Immunohistochemistry** - Formalin fixed paraffin embedded (FFPE) tumor tissue block and
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33 standard automated immunostainer (BenchMark, Ventana Medical System, Tucson, USA) were
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35 used. Specific primary antibodies against TTF1 (8G7G3/1, Dako/Agilent, Glostrup, Denmark), CDX2
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37 (DAK-CDX2, Dako), cytokeratin 7 (CK7, OV-TL 12/30, Dako), cytokeratin 20 (CK20, KS20.8,
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39 Neomarkers, Fremont, USA), p53 (DO-7, Neomarkers), ALK (D5F3, Roche-Ventana), c-
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41 erbB2/HER2/neu (HER2, SP3, Neomarkers) were used. Staining patterns were nuclear for TTF1 and
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43 CDX2 (scored positive even in the presence of a weak reactivity) and for p53 (in which a
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45 percentage higher than 5% of stained cells was considered positive); cytoplasmic for CK7 and
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47 CK20, and membranous for HER2. ALK was scored positive in the presence of granular cytoplasmic
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49 staining and positive cases were confirmed by FISH analysis using the FISH break apart probe from
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51 Vysis (Abbott Laboratories, Abbott Park, Illinois USA), as recommended¹⁷.
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Genomic DNA extraction, Targeted Next-Generation Sequencing and Variant Caller and

Annotation - Genomic DNA (gDNA) was obtained from FFPE tissues after manual micro-dissection (in mixed IMA cases, the mucinous component was selectively micro-dissected) with enrichment for neoplastic cellularity (at least 50% tumor cells), as previously reported¹⁸. T-NGS analyses were performed in 54 samples using the Ion Torrent Personal Genome Machine (PGM, Life Technologies, Grand Island, USA) and the 50-gene Ion Ampliseq Cancer Hotspot Panel v2 (Life Technologies), as previously reported¹⁸. This panel includes genes known to play a prominent role in carcinogenesis across several malignancies and/or that can be therapeutically viable (Supplementary Table S1). Alignment to hg19 human reference genome and Variant Calling included in Torrent Suite Software 4.0 was used to identify variations in target regions¹⁸. Target regions for mutational analysis and RefSeq sequences used for gene annotations are detailed in Supplementary Table S1. All genome coordinates are human genome build GRCh37/hg19.

Bioinformatics pipeline and experimental validation - A custom-designed bioinformatics pipeline, as illustrated in Supplementary Figure 1, was used to analyze genetic variants. To minimize the risk of calling alterations derived from sequencing errors or PCR-based enrichment artifacts, all reads with a QC<30 were excluded from further analyses. The genetic alterations were further annotated using the following databases: COSMIC (version 70), the Single Nucleotide Polymorphism (dbSNPs build 137), the 1000 Genomes Project (1000G), the 6500 exomes of the National Heart, Lung and Blood Institute Exome Sequencing Project and the Non-synonymous SNPs' Functional Predictions (dbNSFP light version 1.3). *In silico* analyses were carried out to predict both the amino acid changes potentially deleterious on protein function and clinical implications of genetic alterations not previously identified by means of SIFT (Sorting Intolerant From Tolerant) and PolyPhen-2 (Polymorphism Phenotyping) algorithms. Matched germ-line DNA

1 samples were not available for parallel sequencing analysis. Established tools can accurately call
2 somatic variants when matched normal and tumor samples are sequenced, but no gold standard
3 method is currently available in the absence of matching germ-line DNA data. Therefore, the
4 discrimination of likely germ-line or likely somatic variants was performed based on arbitrary
5 criteria. Known polymorphisms recorded in the above mentioned public polymorphisms databases
6 were removed from the analysis¹⁹. We then applied a stringent minimum variant read frequency
7 cutoff of 15% for all candidate alterations different from well-characterized known hotspots²⁰.
8 Lastly, we labeled single-nucleotide variants of likely germ-line origin those variants that followed
9 the Hardy-Weimberg equilibrium and displayed an allelic frequency either in the 45%-55% range
10 or nearly 100%. Whereas we considered likely somatic alterations those variants whose allelic
11 frequency violated the Hardy-Weimberg equilibrium, as previously described²¹. When feasible,
12 sequence variant confirmation of mutations was performed by conventional wet techniques
13 including pyro-sequencing or Sanger sequencing, allele-specific PCR and Real-Time PCR, as
14 previously described²².

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38 **Statistical analyses** - Chi-square or Fisher's exact test, as appropriate, were used to test the
39 association between mutations and clinical - histological variables. Cohen coefficient was used to
40 evaluate concordance between different tests. For statistical purposes patients were segregated
41 into groups based on the following criteria: a) presence of high or low number of likely somatic
42 non-synonymous mutations was defined by employing as a cut-off the statistical median value of
43 the dataset; b) overall wild type or mutated status, based on the presence of at least one mutation
44 in any given gene. Patients without evidence of progressive disease at the analysis were
45 considered censored at the time of the last follow-up. Median follow-up time was 61 months
46 (range 41-84). Median overall survival (OS) was defined as the time from diagnosis to death for
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disease or to date of the last follow up. Univariate analyses of survival were performed with Kaplan-Meier curves and the significance verified by log-rank test. In order to test the prognostic role of the number of mutations (and of other clinical-pathological variables) adjusted by stage, multivariate analyses were performed applying a Cox proportional hazards model. All analyses were performed using GraphPad software or the website <http://statpages.org/prophaz.html>. Multivariate analyses were performed using S-Plus (S-PLUS 6.0 Professional, release 1; Insightful Corporation, Seattle, WA, USA). *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Tissue samples and clinical-pathological data - Fifty-four cases of resected mucinous ADC (50 IMA, 32 pure and 18 mixed and 4 colloid predominant, CPA) were analyzed (Orbassano n=35; Modena n=21; Reggio Emilia n=15) (Table 1). Neither mucinous AIS nor MIA cases were found. Two patients had synchronous nodules: one patient had two different bilateral mucinous ADC nodes, while the other had two separate nodes (a mucinous ADC and a conventional acinar ADC) in the same lobe. No significant morphological differences were found between IMA and CPA, with the exception of extracellular mucin ($p=0.03$) and CDX2 expression ($p=0.02$) that were more represented in CPA, as expected. Conversely, in the mixed IMA subgroup, a higher prevalence of vascular invasion ($p=0.01$), predominant invasive growth pattern ($p<0.0001$), signet ring cells (SRC) ($p=0.02$), lymph node involvement ($p=0.006$) and higher stage ($p=0.03$) was found. The presence of tuft growth was significantly associated with the lack of SRC component ($p=0.04$), without correlation with any subtype.

The immunohistochemistry profile was characterized by a heterogeneous, not mutually exclusive expression of CK7 (100%) and CK20 (48%), TTF1 (48%) and CDX2 (13%). p53 was expressed in 24/54 (44%) cases, ranging from 5% to 40% of positive cells. All cases were negative for HER2,

1 while two (4%) mixed IMA samples were positive for ALK rearrangement in the SRC component,
2 (confirmed also by FISH, see materials and method section).
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7 **T-NGS of mucinous adenocarcinoma** - An adequate library was obtained in all but two of the 54
8 primary tumor samples analyzed by T-NGS. After read quality filtering, mapping and alignment to
9 reference genome (hg19), the median sequencing depth resulted 348x. A total of 837 genetic
10 variations (median=16, range 1-39) were identified either in exonic (namely non-sense,
11 synonymous, non synonymous likely-germinal and non synonymous likely-somatic alterations) or
12 regulatory regions (Figure 2A). The predominant nucleotide changes were C:G→T:A transitions,
13 consistent with previous ADC genomics data⁸, with no significant difference among individual
14 genes (Figure 2B).
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27 We elected to focus on 281 mutations leading to a protein change (i.e. non synonymous and
28 nonsense mutations), as these are more likely to play a causative role in tumorigenesis and
29 represent therapeutic vulnerabilities. As detailed in the Methods, we further categorized all non-
30 synonymous variants into likely somatic (e.g. tumor-specific) or likely germ-line based on prior
31 annotation in public polymorphism databases and their allelic frequency (Supplementary Tables S2
32 and S3). The number of non-synonymous mutations considered to be tumor-specific was 190
33 (median=2 per sample, range from 1 to 18). These variants were distributed across 26 different
34 genes and were found in 43 samples (83% with at least one mutation) (Figure 3). Nine cases (17%)
35 – which were named “all wild-type” – did not harbor any likely somatic mutations in the set of
36 sequenced genes, although one of these cases presented an ALK rearrangement by FISH analysis.
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38 Somatic mutated genes clustered mainly in four signaling pathways with the highest
39 prevalence of mutations in the MAP kinase (41%), tyrosine kinase (23%) and DNA repair (22%)
40 pathways.
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KRAS was the most commonly mutated gene and mutations were observed in 37/52 (71%) samples. Near all mutations affected glycine at position 12 and were confirmed by pyrosequencing. *TP53* was the second most mutated gene (24/52, 46%), mainly with missense or InDel changes with a consequent loss of protein function. In four cases, non-sense mutations with a premature stop-codon expected to down-regulate protein levels was detected. All *TP53* mutations were validated by Sanger sequencing.

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Six out of the seven *EGFR* single nucleotide variants were of uncertain biological and/or therapeutic relevance (G721S, E746K, D761N, V765M, R776C, A871T). However, a *de novo* T790M mutation, known to confer resistance to tyrosine kinase inhibitors, was detected in one sample. We found likely-somatic mutations of *PIK3CA* in seven tumors, most of which are known to be oncogenic (2 cases with E542K, 1 Q546H, 1 A1020V, 1 M1043I, 1 G1049S) and could have implications for targeted therapy. Two samples harbored the R132H hotspot mutation in *IDH1* that has been found to drive tumorigenesis in glioblastoma and certain hematological malignancies and for which clinical inhibitors are being developed²³. We also detected one case carrying a *PDGFRA* variant of unknown function but affecting the tyrosine kinase domain (G668S), as well as *KIT* mutations in six tumors (2 V530I, 2 L576F, 2 P577S) of uncertain functional relevance and not previously associated with clinical response to targeted therapy.

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TP53/p53 genotype/phenotype analysis - Immunohistochemistry for p53 was performed as a surrogate of validation of the *TP53* mutational status, since a strong positive nuclear staining is considered to be the phenotypic counterpart of genetic alterations²⁴. In our series, p53 was expressed in 24/52 cases (46%) with variable intensity and a heterogeneous pattern of distribution in tumor areas ranging from 5 to 40% of positive cells, while in 28/52 cases (54%) resulted completely negative or with few focal positive tumor cells. The observed intra-tumoral

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heterogeneity of p53 immunostaining may implicate that *TP53* mutations could be present in a fraction of tumor cells. To test this hypothesis, we assessed the predicted functional relevance of all *TP53* gene mutations occurring at an allelic frequency of at least 5%, indicating their possible subclonal origin. Overall, among p53 positive cases, 17 harbored missense mutations and 7 were WT in the sequenced regions. On the other hand in the p53 negative group, 5 cases harbored dominant negative mutations, 4 showed nonsense mutations and 19 cases were wild-type or with not-functional variants. A significant correlation ($p= 0.002$) and a moderate concordance (Cohen coefficient k value= 0.53) between the two tests were thus observed (Supplementary Figure 2).

Molecular to clinical-pathological correlations - Correlative analyses between molecular data and clinical-pathologic variables are summarized in Supplementary Table S4. Tuft growth was the only feature significantly associated with a higher somatic non-synonymous number of mutations ($p=0.01$). Notably, tuft growth was found in 27/42 samples displaying at least one mutation in any gene ($p=0.0002$) and none of the cases that were found to be 'all wild-type' displayed tuft growth. Rather, these samples lacking mutations presented with a higher pathological stage (67%) and all were IMA (100%), more frequently of the mixed subtype (67%). They also had lesser vascular invasion (78%) and TTF1 positivity (78%). Interestingly, cases with at least one mutation in any gene were most commonly associated with a lack of TTF1 expression (25/43, 58%). The same immunohistochemical pattern was also detected in *KRAS* mutated cases (21/37, 57%), even if the association was not statistically significant (Supplementary Table S4). Furthermore, *KRAS* mutated cases were associated with a low pathological stage (57%), absence of lymph node involvement (95%), low SRC component (89%), presence of tuft growth (68%) and absence of p53 protein expression (68%) (Supplementary Table S4).

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Survival analyses - At univariate analysis smoking status ($p=0.03$), pathological higher stage ($p=0.0006$), mixed IMA subtype ($p=0.03$), predominant invasive growth pattern ($p=0.02$), vascular invasion ($p=0.005$), and presence of SRC ($p=0.05$) were predictive of poor survival, together with *KRAS* and *TP53* wild type status. Since the median number of non-synonymous likely somatic alterations was 1, this was chosen as an arbitrary cut-off value to divide patients in two categories based on the number of mutations. The subset of patients displaying more than one somatic non-synonymous mutations in their tumors ($N= 29$) had a significantly better overall survival (median overall survival not reached) compared to cases showing 0-1 mutations (34 months, HR 0.21, 95% CI 0.08 to 0.54, $p<0.001$, Figure 4). When the prognostic role of the number of mutations was tested in a multivariate analysis including stage of disease, number of mutations remained independently predictive of overall survival (HR 0.37, 95% CI 0.14 – 0.99, $p=0.048$).

DISCUSSION

Mucinous lung ADCs have been associated with poor recurrence-free survival and adverse prognosis, as the disease often presents with minimal or misleading clinical symptoms and multifocal heterogeneous unresectable lesions^{25,26}. In this study, we explored the pathological characteristics and the genetic profile of 54 mucin-rich ADC of the lung with the aim of unveiling genotype-phenotype relations and identifying clinically valuable markers.

No significantly different clinical-pathological features or survival outcome were found between IMA and CPA subgroups with the exception of the presence (by definition) of extracellular mucin in 100% of CPA ($p=0.03$). Compared to conventional ADC, lung IMAs are associated with a worse prognosis²⁷ and are less sensitive to chemotherapy²⁸. In the present study, we failed to demonstrate significant differences in overall survival between IMA and CPA because of the extremely limited number of the latter, a very rare subtype¹⁰. On the other hand, the pure

1 mucinous IMA subgroup was significantly associated with better survival when compared to the
2 mixed one. Pure IMA samples presented favorable features such as the lepidic growth pattern, the
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4 absence of vascular invasion, SRC component, and lymph node involvement, as well as
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7 pathological stage I. In the absence of other high-grade morphological features, we could not
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9 stratify the current series of mucinous ADC into prognostically relevant grading-based subgroups.

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12 Our observations take into consideration the fact that lung mucinous ADCs were judged unsuitable
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14 for inclusion in the recently proposed grading systems based on architectural and cytological
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16 features^{29,30,31}. Nevertheless, the presence of tufts was distributed in all the histological categories
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18 analyzed (either CPA, pure IMA or mixed IMA) at very high percentage (75%, 94%, 72%,
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20 respectively), whereas the presence of signet ring cells occurred at low frequency, from 22% to
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22 28% in our series, possibly suggesting that the tufts could be a specific hallmark of this tumor type.
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25 Furthermore, tuft growth was also significantly associated with the presence of higher number of
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27 somatic mutations, thus confirming –for such tumor type- the importance of this specific
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29 cytomorphological feature, which is also detectable in small core biopsies³².

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32 A limitation of this study is the lack of matched germ-line DNA, which has prevented us from
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34 confirming the somatic nature of the genetic variants identified by T-NGS. However, we carefully
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36 reviewed all variants taking into consideration both their allelic frequency and annotation in
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38 polymorphism databases to minimize the risk of calling false-positive somatic mutations. This
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40 analysis has retrieved a list of 190 likely somatic tumor-specific mutations, with a median value of
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42 two mutations per sample, and *KRAS* mutations clearly dominated the mutational landscape of
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44 mucinous ADC. *KRAS* is the most frequently mutated oncogene in male, smoker and non-Asian
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46 lung ADC patients (5% to 40%) and associated to the mucinous ADC subtype with controversial
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48 clinical relevance^{9,33,34}. Consistently with earlier reports, mutations were detected in 71% cases
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50 (including 100% of CPA and 77% of IMAs)⁹. We found that *KRAS* mutations are significantly
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1 associated with lymph node negativity, absence of SRC and presence of tufts. Furthermore, in our
2 series, the presence of KRAS mutations was associated with better overall survival at univariate
3 analysis (HR 0.31, 95%CI 0.12-0.79, p=0.009). However, as mentioned in the results, KRAS
4 mutations were significantly more common in stage I patients compared to patients with more
5 advanced disease. In the Cox model adjusted by stage of disease, KRAS mutation was not an
6 independent prognostic factor (HR 0.57, 95%CI 0.21-1.53, p=0.27).
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10 TP53 was the second most commonly altered gene with a prevalence of 46%. Specifically, we
11 detected several TP53 mutations with a known dominant negative function and other less
12 common mutations of the truncating type, which are usually associated to absence of protein
13 expression. This finding could explain the relatively good concordance between p53
14 immunoreactivity and the TP53 genetic status. Our results are apparently conflicting with those of
15 Shim and colleagues, who reported a significantly lower prevalence of TP53 mutations¹⁵. A
16 potential explanation for this discrepancy could lie in the fact that they analyzed by NGS only
17 tumors known to be KRAS wild-type by Sanger sequencing, while we sequenced a larger cohort
18 that included all tumors irrespective of KRAS genotyping. Indeed, 18/24 TP53 mutant cases also
19 harbored KRAS mutations. Other differences between these studies include the specific genes
20 and/or the exons sequenced in the respective panels (Supplementary Table S5); as well as patient
21 ethnic origin, since Shi et al., analyzed a cohort composed of both Caucasian and East Asian
22 patients and our study only included Caucasian individuals. Finally, differently from the cohort
23 analyzed by Shim and colleagues, in this study the majority of patients were smokers, and some of
24 the detected genetic alterations are more commonly reported in smoking-related tumors.
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54 None of the examined tumor samples showed positive immunostaining of HER2, a gene highly
55 expressed in mucinous KRAS wild type cases displaying the CD74-NRG1 fusion product^{14,35}. This
56 might indirectly suggest the absence of CD74-NRG1 gene alterations in our dataset. Nevertheless,
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we acknowledge that gene rearrangements other than ALK were not investigated in this study.

Further analyses are therefore warranted in this direction, since a recent study has identified recurrent gene fusions implicated in the pathogenesis of *KRAS* wild-type mucinous lung ADC¹⁵.

Approximately half of the samples (27/52, 52%) were negative for TTF1 expression. This could indirectly point out at a pivotal role of *NKX2-1* (the gene encoding for TTF1) in tumorigenesis of mucinous pulmonary ADC, as suggested by previous studies^{36,37,38}.

Genotyping of mucinous ADC samples could reveal individual patients who might benefit from targeted therapeutic intervention. We found seven cases carrying *PIK3CA* oncogenic mutations with an overall prevalence of 13% that is higher than the 3% (1/30 cases) reported by Shim and colleagues earlier this year¹⁵. *PIK3CA* status might be used to select patients potentially responsive to PI3K inhibitors in clinical trials^{39,40}. However, all *PIK3CA* mutated tumors co-harbored *KRAS* mutations, which may be detrimental in showing single agent activity of PI3K/AKT/mTOR axis inhibitors⁴¹. Overall, in our study we investigated a highly morphologically selected series of microdissected mucinous ADC cases with a genetic profile which differs from that of conventional ADC: the mutation prevalence and the presence of *TP53* and *PI3KCA* genetic abnormalities could suggest a partial overlap with genetic profile of squamous carcinoma⁴².

Noteworthy, we detected the hotspot IDH1 R132H oncogenic mutation in two samples. IDH1 has been proposed as a plasma biomarker for the diagnosis of lung ADCs⁴³ and the pharmacological inhibition of the mutant enzyme has been shown to block the synthesis of the 'oncometabolite' R-2-hydroxyglutarate, inducing antitumor effects in glioma preclinical models²³. Although clinical studies are still in an early phase, a selective mutant IDH1 inhibitor called AG-120 has recently shown activity in patients affected by IDH1-mutant haematological malignancies⁴⁴. No data are available about the activity of AG-120 in IDH-1 mutant lung cancer patients, but IDH1 mutations have been reported to occur in 1.5% lung ADCs^{8,45}. Nevertheless, in our series, both IDH1 mutated

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tumours co-harbored KRAS mutations and showed a high number of mutations, raising caution on
the functional relevance of IDH1 as a driver of tumorigenesis in these particular cases. The
observed *EGFR* mutations occurred outside the “conventional” hot-spots (exons 19 and 21)⁹ that
confer *EGFR*-TK inhibitor sensitivity^{46,47}.

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We report that patients carrying tumors with a higher number of likely somatic non synonymous
alterations might have a better prognosis, even when adjusted by stage of disease. However, this
finding must be interpreted with caution, given the limited number of sequenced genes and the
small number of events available for prognostic analysis. The number of mutations in a cancer
sample can also inform treatment choice. Indeed, a recent study has shown that a higher
nonsynonymous mutation burden in NSCLC is associated with increased neo-antigen formation
and clinical benefit from immunotherapy⁴⁸.

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We acknowledge that our study has sequenced only a small portion of the cancer genome. In
order to draw a more detailed molecular landscape of lung ADC, future studies should incorporate
exome sequencing and transcriptome analysis of a large series of samples to unveil inter-tumoral
(and possibly intra-tumoral) genomic heterogeneity.

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In conclusion, novel somatic mutations were identified in mucinous lung adenocarcinoma and the
mutation frequency of altered genes were correlated with clinical-pathological data. Some of the
identified genes can be tackled by specific targeted therapies. While preclinical studies are needed
to assess the role of these gene alterations as oncogene drivers, the limited therapeutic
performance of cytotoxic chemotherapy in patients with mucinous or colloid ADC represents the
background for pivotal studies to be conducted in tumors harboring one or more of the currently
reported gene alterations.

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FIGURE LEGENDS

Figure 1. Representative morphological patterns of 54 mucinous ADC (hematoxylin and eosin stains). A) Pure IMA (mucinous features $\geq 90\%$); B) Mixed IMA with up to 50% of non-mucinous ADC component; C) mucinous ADC with abundant extracellular mucin ($\geq 10\%$ of the tumor); D) CPA showing mucous-pools lined by columnar tumor cells; E) Mucinous ADC with tuft growth (insert: high power field of bunches or small clusters of neoplastic mucinous cells attached to the alveolar wall); F) Signet ring cells component (cells with large intracellular mucin drops and peripheral nuclei displaced toward one end of the cytoplasm).

Figure 2. Total of genetic variations in 52 mucinous ADC. A) total genetic variations identified (namely non-sense, non synonymous likely-germinal, non synonymous likely-somatic and synonymous alterations) for each sequenced tumor in exonic or regulatory regions. B) Distribution of nucleotide changes. (Abbreviations: Syn: synonymous; LG: likely germinal; LS: likely somatic)

Figure 3. Distribution of tumor-specific somatic non-synonymous mutations. A) Heatmap of the tumor specific somatic mutations identified for 52 mucinous ADC samples in each gene, clustered according to specific signaling pathways (Abbreviations: *a sample was considered to harbor a complex mutation when an InDel was detected concomitant with point mutations; RTK: Receptor Tyrosine Kinases). B) Histogram of the number of sample for each mutated gene.

Figure 4. Kaplan-Meier survival curves for patients segregated according to the number of non-synonymous likely somatic alterations. Overall survival in tumors with ≥ 2 mutation count (N= 29) compared to tumors with 0-1 mutations (N= 23) (HR 0.21, 95% CI 0.08 to 0.54, log-rank $p < 0.001$).

TABLE 1. Clinical pathological characteristics of 54 primary lung mucinous adenocarcinoma tumor samples.

Parameter	Total #54 (%)	IMA#50 (%)	CPA #4 (%)	IMA #50		p-value
				Pure #32 (%)	Mixed #18 (%)	
Age, mean (range)	65 (41-84)	65 (41-84)	60 (47-78)	64 (41-84)	66 (42-82)	
Sex						
Male	31 (57)	29 (58)	2 (50)	19 (59)	10 (55)	<i>ns</i>
Female	23 (43)	21 (42)	2 (50)	13 (41)	8 (45)	
Smoking habit						
No	12 (22)	12 (24)	0	7 (22)	5 (28)	<i>ns</i>
Yes	42 (78)	38 (76)	4 (100)	25 (78)	13 (72)	
Vascular Invasion						
Yes	6 (11)	6 (12)	0	1 (3)	5 (28)	0.01
No	48 (88)	44 (88)	4 (100)	31 (97)	13 (72)	
Growth Pattern						
Predominant lepidic	45 (83)	41 (82)	4 (100)	32 (100)	9 (50)	< 0.0001
Predominant invasive	9 (17)	9 (18)	0	0	9 (50)	
Extracellular mucin						
Presence	27 (50)	23 (46)	4 (100)	14 (44)	9 (50)	<i>ns</i>
Absence	27 (50)	27 (54)	0	18 (56)	9 (50)	
Tufts						
Presence	46 (85)	43 (86)	3 (75)	30 (94)	13 (72)	<i>ns</i>
Absence	8 (15)	7 (14)	1 (25)	2 (6)	5 (28)	
Signet ring cell features						
Presence	12 (22)	12 (24)	0	7 (22)	5 (28)	0.03
Absence	42 (78)	38 (76)	4 (100)	25 (78)	13 (72)	
pT						
pT1	19 (35)	18 (36)	1 (25)	14 (44)	4 (22)	<i>ns</i>
pT2-3-4	35 (65)	32 (64)	3 (75)	18 (56)	14 (78)	
pN						
pN0	44 (82)	40 (80)	4 (100)	30 (94)	10 (55)	0.001
pN1-2-3	10 (18)	10 (20)	0	2 (6)	8 (45)	
Stage						
I	26 (48)	24 (48)	2 (50)	20 (63)	4 (22)	0.006
II-III-IV	28 (52)	26 (52)	2 (50)	12 (37)	14 (78)	
Status						
Alive	34 (63)	31 (62)	3 (75)	21 (65)	10 (56)	<i>ns</i>
Dead	20 (37)	19 (38)	1 (25)	11 (35)	8 (44)	
Mean OS (months)	61	61	NR	64	34	<i>ns</i>
TTF-1 IHC						
Positive	26 (48)	25 (50)	1 (25)	14 (44)	11 (61)	<i>ns</i>
Negative	28 (52)	25 (50)	3 (75)	18 (56)	7 (39)	

CK7						
<i>Positive</i>	54 (100)	50	4 (100)	32 (100)	18 (100)	-
<i>Negative</i>	0	0	0	0	0	
CDX-2						
<i>Positive</i>	7 (13)	5 (10)	2 (50)	3 (9)	2 (11)	<i>ns</i>
<i>Negative</i>	47 (87)	45 (90)	2 (50)	29 (91)	16 (89)	
CK20						
<i>Positive</i>	26 (48)	25 (50)	1 (25)	18 (56)	7 (39)	<i>ns</i>
<i>Negative</i>	28 (52)	25 (50)	3 (75)	14 (44)	11 (61)	
p53						
<i>Positive</i>	24 (44)	22 (44)	2 (50)	12	10	<i>ns</i>
<i>Negative</i>	30 (55)	28 (56)	2 (50)	20	8	

Abbreviations: IMA: Invasive Mucinous Adenocarcinoma; OS: overall survival, WT: wild type; MUT: mutated.







