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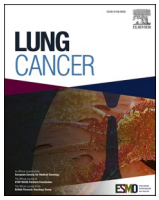

Research Paper

Genomic landscape of stage 0–IA lung adenocarcinoma identified by on-site reflex targeted NGS

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ABSTRACT

Introduction: Early molecular profiling in non-squamous non-small cell lung carcinoma (NSCLC), particularly lung adenocarcinoma (LUAD), is critical for guiding individualized treatment strategies. Limited data exist on the genomic landscape of Stage 0–IA LUAD. This study assessed the feasibility and clinical relevance of reflex targeted next-generation sequencing (NGS) performed on-site at diagnosis in resected early-stage LUAD.
Methods: We retrospectively analyzed 239 consecutive Stage 0–IA LUAD cases diagnosed between 2022 and 2024 at a single institution. Ultra-fast reflex DNA- and RNA-based NGS was performed on resected specimens using a 50-gene targeted panel. Alterations were classified according to the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT). Associations between genomic alterations, histologic subtypes, and tumor grades were evaluated.
Results: Stage IA1 was the most frequent diagnosis (46%). High-quality sequencing data were obtained in all cases, with a median turnaround time of 102 h. At least one genomic alteration was detected in 80% of tumors. *KRAS* mutations were most frequent (35.8%), including *KRAS* G12C in 16%. *EGFR* mutations were present in 27.2%, primarily classical sensitizing alterations. Other actionable findings included *ALK* fusions (3.3%), *RET* rearrangements (1.2%), *MET* exon 14 skipping (2.4%), *HER2* mutations (3.7%), and *BRAF* V600E (0.8%). ESCAT Level I alterations were found in 34% of tumors; 20% of these co-occurred with *TP53* mutations. Significant associations were observed between genomic alterations, histologic subtypes, and tumor grades.
Conclusions: Reflex NGS at diagnosis in resected Stage 0–IA LUAD is feasible, rapid, and reveals a high rate of actionable alterations, which may support its integration in the future into early-stage diagnostic workflows.

1. Introduction

The early detection of lung cancer has seen significant advancements in recent years, thanks to the adoption of screening programs using low-dose computed tomography.[1] These programs have led to an increasing number of patients being diagnosed at stage 0 or IA lung adenocarcinoma (LUAD), representing an opportunity for early intervention.[1] At this early stage, surgery is the standard treatment, offering the best chance of survival for most patients when complete tumor resection [2–4]. However, long-term outcomes reveal that not all stage 0–IA LUAD patients achieve durable remission, with a subset experiencing relapse or metastasis and death within five years [5]. In

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this context, there is a need for a more nuanced approach to managing stage 0–IA LUAD.

One of the challenges in stage 0–IA LUAD is identifying tumors with aggressive biological behavior that cannot be predicted by conventional histopathological assessments [6]. These high-risk tumors may harbor molecular alterations that drive early relapse and metastasis [7,8]. Neoadjuvant therapies, such as immunotherapy (IO) combined or not with chemotherapy, and adjuvant therapies, including chemotherapy and/or IO, and targeted agents (e.g., *EGFR* or *ALK* inhibitors), are strategies for reducing recurrence risk nowadays in early stages non-squamous non-small cell lung cancers (NS-NSCLC) [9–11]. While currently recommended primarily for stages IB-IIIA, these therapies could play a pivotal role in stage 0–IA LUAD in the future, notably, if reliable biomarkers or molecular signatures were available to stratify patients for targeted therapy or by risk [9,12,13].

Our understanding of genomic alterations in stage 0–IA LUAD remains limited [14–16]. Particularly, large-scale studies investigating the prevalence, diversity, and clinical impact of actionable mutations in stage 0–IA LUAD are scarce [17–19]. Additionally, the interplay between these genomic alterations and histological subtypes and tumor grading is poorly characterized [20]. This gap in knowledge restricts the ability to leverage molecular profiling fully in stage 0–IA LUAD where treatment decisions are based on histology.

Reflex targeted Next-Generation Sequencing (NGS) testing at diagnosis in stage 0–IA LUAD may offer important opportunities for a better patient care and may lead in the future to new therapeutic strategies. By performing comprehensive molecular profiling systematically on stage 0-I LUAD cases as part of the initial diagnostic workup, clinicians could establish a detailed "molecular portrait" of the tumor at diagnosis [7,8]. This information would not only guide decision for neoadjuvant and/or adjuvant therapies but could also provide insights into tumor biology, inform risk stratification, and identify patients who might benefit from emerging therapies [21]. Furthermore, integrating molecular data into routine clinical practice could facilitate broader translational research into the genomic landscape of very early-stage LUAD, ultimately leading to refined treatment algorithms.

This study aimed to characterize and analyze the genomic profiles of a large single-hospital center cohort of 239 consecutive stage 0–IA LUAD cases, using reflex targeted ultra-fast NGS in resected specimens.

2. Patients and methods

2.1. Patients and samples

Between 2022 and 2024, 239 patients with stage 0–IA LUAD were analyzed in resected specimen using reflex targeted NGS on site from a cohort of 1,217 NSCLC cases diagnosed by expert thoracic pathologists (MI, SG, SL, EL, VH, and PH) at the Laboratory of Clinical and Experimental Pathology, Nice, France. The diagnosis of pTNM stage and LUAD histological subtypes was determined, following TNM staging system proposed by International Association for the Study of Lung Cancer (IASLC) and the WHO classification [22].

All selected patients presented with single nodule detectable on CT-scan and after surgery. The complete clinical exams excluded other primary than lung.

All tumor specimens were used with the informed signed consent from the patients. The study was approved by the local ethics committee (Human Research Ethics Committee, Nice University Hospital Center/Hospital-related Biobank BB-0033–00025; http://www.biobank-cotedazur.fr/) and was performed in accordance with the guidelines of the Declaration of Helsinki.

2.2. Ultra-fast next-generation sequencing

The patients underwent fast DNA– and RNA–based NGS reflex testing at the Laboratory of Clinical and Experimental Pathology (LPCE), Nice University Hospital, France, as previously described [23]. The laboratory is accredited under the ISO 15189 standard for somatic genomic testing by NGS in routine clinical practice (www.cofrac.fr).

Briefly, nucleic acids were either extracted using the Maxwell RSC Instrument (Promega, catalog number AS4500) with the Maxwell RSC FFPE Plus DNA kit (catalog number AS1720) and Maxwell RSC RNA FFPE kit (catalog number AS1440), or using the Ion Torrent™Genexus™Purification System (Thermo Fisher Scientific, Waltham, MA, USA; catalog number A48148) with the Genexus™ FFPE DNA/RNA Purification Combo Kit (Thermo Fisher Scientific, catalog number A45539).

Following nucleic acids' extraction with the Maxwell RSC Instrument automaton, a Qubit Fluorometric quantification assay (Thermo Fisher Scientific, catalog number Q33327) was performed with the Qubit RNA HS Assay Kit (catalog number Q32852) and Qubit dsDNA HS Assay Kit (catalog number Q32851) to measure the concentration of extracted nucleic acid. The Ion Torrent™Genexus™ Purification System (Thermo Fisher Scientific) was equipped with a fluorometer and automatically assayed the extracted nucleic acid following the extraction step. Detection of genomic alterations was then performed using Ion semiconductor sequencing (Ion Torrent™ Technology, Thermo Fisher Scientific) on the Ion Torrent™Genexus™Integrated Sequencer. The panel used was the Oncomine™ Precision Assay GX (OPA, Thermo Fisher Scientific, catalog number A46291). This panel includes 50 key genes of which 45 were targeted for DNA mutation detection, 18 for fusion detection and 14 for Copy Number Variant (CNV) detection. The panel also incorporates a 5′/3′ expression imbalance caller for the detection of novel fusions. With this panel, the Genexus sequencer is able to sequence up to 16 samples ADN-ARN or 32 samples DNA or RNA on a single run.

Actionability for genomic variants was considered following the latest ESMO Clinical Practice Guidelines for advanced NSCLC [24,25].

2.3. Statistical analysis

Exploratory data analysis of clinicopathological and molecular features was carried out using R. For categorical variables, a Chi-Squared (χ^2) test of independence was performed.

3. Results

The mean age at diagnosis was 67 years (range: 26–85 years). The majority of patients were female (54%, n = 129) and either current smokers (48%, n = 115; mean pack-years = 42) or former smokers (43%, n = 103). Most cases were diagnosed as stage IA1 LUAD, with the following distribution: stage IA1 (46%, n = 111), stage IA2 (35%, n = 84), stage IA3 (14%, n = 33), and stage 0 (5%, n = 11).

High-quality DNA and RNA libraries were successfully generated for all patients, meeting the required quality metrics. NGS analysis revealed that 80% of cases (n = 198) harbored at least one genomic alteration (Fig. 1). Copy number variations (CNVs) were identified in only 2% of cases (n = 5), including *EGFR* amplifications in 3 cases, *MET* amplification in 1 case, and *ERBB2* amplification in 1 case.

The most frequent oncogenic alteration was a *KRAS* mutation, identified in 35.8% of tumor samples, with the *KRAS* G12C variant detected in 16% (n = 40) of patients. *EGFR* activating mutations were present in 27.2% (n = 65) of tumor samples, comprising common sensitizing mutations such as L858R (n = 31) and exon 19 deletions (n = 27) (Fig. 1). *TP53* mutations were found in 22% of cases, often co-occurring with other driver mutations. Less frequent but actionable alterations included: *BRAF* mutations in 4.5% (n = 11) of cases, including the actionable V600E variant in two cases (0.8%), *HER2* mutations in 3.7% (n = 9) of patients, *MET* exon 14 skipping alterations in 2.4% (n = 6) of cases, *ALK* fusions in 3% of cases, *RET* rearrangements in 2% of patients, and *ROS1* fusions in 0.4% of cases.

There was strong statistically significant evidence of an association between the histological subtype of LUAD and whether it harbors an *EGFR* mutation ($p < 0.0001$; Table 1). Specifically, tumors classified as

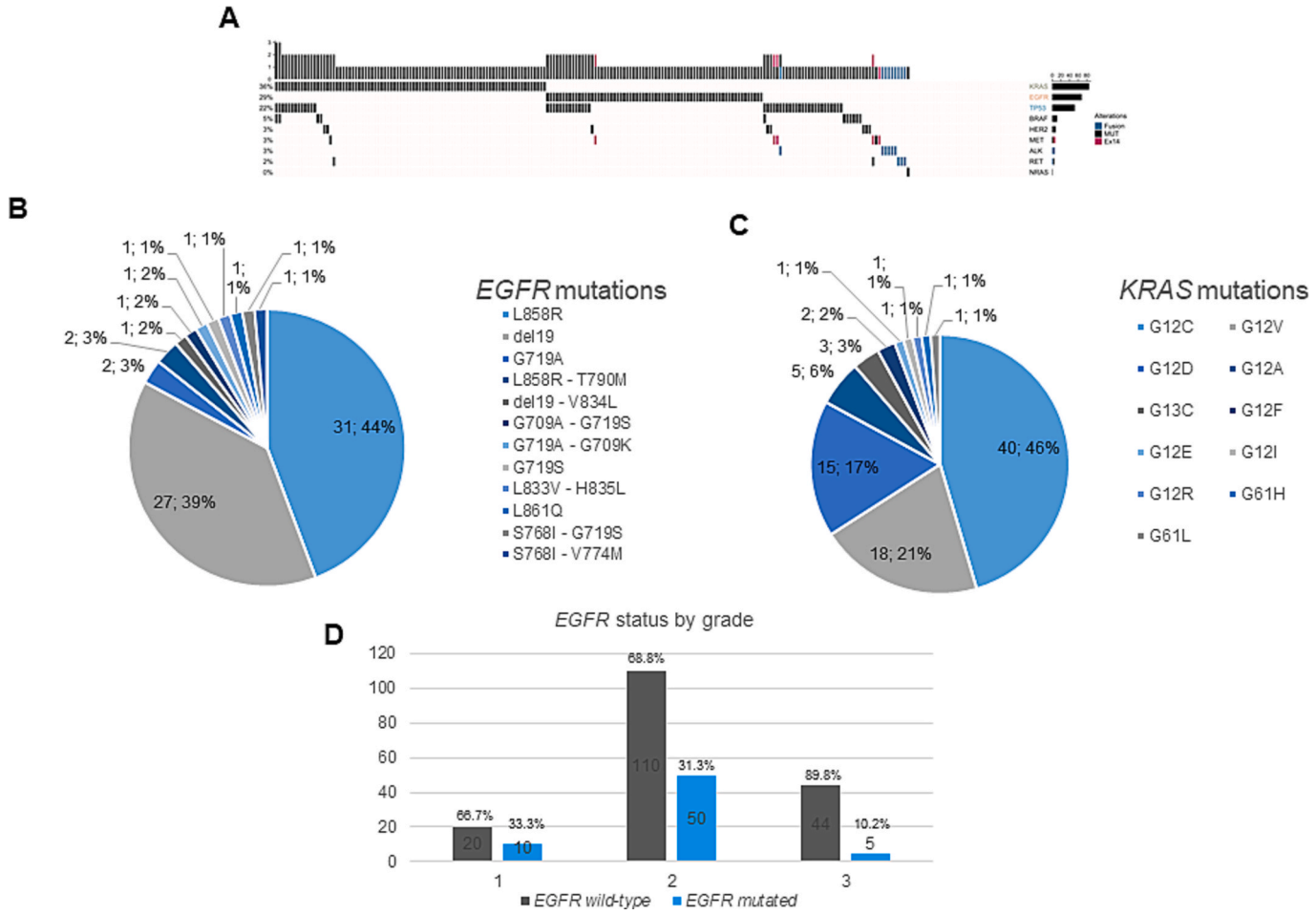

Fig. 1. *Upper panel:* Oncoprint summarizing the distribution of genomic alterations in our cohort. Each column represents an individual tumor sample; each row corresponds to a gene analyzed using reflex targeted NGS. The type of genomic alteration is indicated by color: point mutations or small indels (MUT), gene fusions (Fusion), and MET exon 14 skipping events (Ex14). Frequencies of alterations are shown to the right of each row. *Middle panel:* Insight on the *EGFR* and *KRAS* mutations as the most frequent mutations in our cohort. *Lower panel:* Frequency and percentage distribution of *EGFR* mutation status across LUAD histological grade.

Table 1
Distribution of predominant histologic growth patterns by *EGFR* mutation status in stage 0–IA LUAD. Counts are shown for *EGFR* wild-type and *EGFR* mutated cases, with Row % indicating the proportion of *EGFR* status within each histologic subtype, and % of *EGFR* WT/Mutated cohort indicating the proportion that each subtype contributes within the overall *EGFR* wild-type or *EGFR*-mutated cohorts, respectively. Abbreviations: ADC, adenocarcinoma; Minimally invasive ADC = MIA; In situ ADC = AIS; Mucinous invasive ADC = invasive mucinous adenocarcinoma (IMA).

Histological Subtype (predominant)	*EGFR* wild-type (n)	*EGFR* mutated (n)	Total (n)	Row % *EGFR* WT	Row % *EGFR* Mutated	% of *EGFR* WT cohort	% of *EGFR* Mut cohort
Acinar ADC	108	40	148	73.0	27.0	62.1	61.5
Fetal	2	0	2	100.0	0.0	1.1	0.0
Papillary ADC	25	13	38	65.8	34.2	14.4	20.0
Solid ADC	9	0	9	100.0	0.0	5.2	0.0
Minimally invasive ADC	3	3	6	50.0	50.0	1.7	4.6
In situ ADC	3	1	4	75.0	25.0	1.7	1.5
Lepidic ADC	11	8	19	57.9	42.1	6.3	12.3
Micropapillary	4	0	4	100.0	0.0	2.3	0.0
Mucinous invasive ADC	9	0	9	100.0	0.0	5.2	0.0

lepidic, papillary, or minimally invasive ADC are more likely to have an *EGFR* mutation compared to the average, while solid, micropapillary, mucinous, and fetal subtypes are significantly less likely, or were not observed with mutations in our cohort. Moreover, there is a statistically significant association between the tumor grade and the *EGFR* mutation status ($p = 0.0109$; Fig. 1). The significant association appears to be driven primarily by the significantly lower frequency of *EGFR* mutations in grade 3 tumors compared to lower-grade (grade 1 and grade 2) tumors.

In addition, there is strong statistically significant evidence of an association between the histological subtype of LUAD and the *KRAS* mutation status ($p < 0.0001$; Table 2). Specifically, invasive mucinous, solid, and micropapillary subtypes are significantly more likely to harbor *KRAS* mutations, while minimally invasive ADC and *in situ* ADC subtypes appear less likely. There were no other significant associations between histological subtypes or tumor grade and genomic status in our

Table 2
Distribution of predominant histologic growth patterns by *KRAS* mutation status in stage 0–IA LUAD. Counts are shown for *KRAS* wild-type and *KRAS* mutated cases, with Row % indicating the proportion of *KRAS* status within each histologic subtype, and % of *KRAS* WT/Mutated cohort indicating the proportion that each subtype contributes within the overall *KRAS* wild-type or *KRAS*-mutated cohorts, respectively. Abbreviations: ADC, adenocarcinoma; Minimally invasive ADC = MIA; In situ ADC = AIS; Mucinous invasive ADC = invasive mucinous adenocarcinoma (IMA).

Histological Subtype (predominant)	*KRAS* wild-type (n)	*KRAS* mutated (n)	Total (n)	Row % *KRAS* WT	Row % *KRAS* Mutated	% of *KRAS* WT cohort	% of *KRAS* Mutated cohort
Acinar ADC	103	45	148	69.6	30.4	67.3	52.3
Lepidic ADC	12	7	19	63.2	36.8	7.8	8.1
Papillary ADC	23	15	38	60.5	39.5	15	17.4
Solid ADC	4	5	9	44.4	55.6	2.6	5.8
Micropapillary ADC	1	3	4	25	75	0.7	3.5
In situ ADC	3	1	4	75	25	2	1.2
Mucinous invasive ADC	1	8	9	11.1	88.9	0.7	9.3
Minimally invasive ADC	5	1	6	83.3	16.7	3.3	1.2
Fetal ADC	1	1	2	50	50	0.7	1.2

cohort.

Actionable gene fusions were detected in 4.5% (n = 11) of patients, comprising *ALK* fusions in 3.3% (n = 8), and *RET* rearrangements in 1.2% (n = 3).

Overall, actionable genomic alterations classified as ESCAT Level I were identified in 34% (n = 84) of patients. Notably, 20% of these cases co-harbored *TP53* mutations alongside ESCAT level I alterations, highlighting the complexity of the genomic landscape in stage 0–IA LUAD.

Moreover, a significant association exists between *KRAS* mutation status and the PD-L1 TPS ($p = 0.0223$). Specifically, *KRAS* mutations are associated with an increased likelihood of high PD-L1 expression (TPS > 50), and a decreased likelihood of being PD-L1 negative, when compared to *KRAS* wild-type tumors. No other significant correlations were observed between the PD-L1 expression levels and the genomic alterations.

In addition, there was a significant association between the *TP53* status and the MET expression levels ($p = 0.049$). Specifically, *TP53* mutated tumors show a substantially higher observed proportion of MET high expression (32.1%), defined as 3 + intensity ≥ 90% tumor cells, compared to *TP53* wild-type tumors (13.1%). Conversely, *TP53* wild-type tumors have a higher proportion of MET low expression (75.4%) compared to mutated tumors (54.3%).

Median on-site NGS turnaround time was ~ 4 days (median, 102 h; range, 60 to 144 h) from FFPE receipt to signed report.

4. Discussion

This real-world, single-center experience shows that reflex targeted NGS at diagnosis in resected Stage 0–IA LUAD is both technically feasible and operationally rapid, returning high-quality profiles with a median TAT of ~ 4 days from FFPE receipt to signed report. By systematically profiling very early-stage tumors, we identified actionable alterations in a substantial proportion of patients, supporting the incorporation of reflex testing into routine diagnostic workflows, even in very early-stage disease.

4.1. A distinct genomic landscape in Stage 0–IA versus advanced NSCLC

One of the primary advantages of reflex NGS testing is its ability to identify actionable genomic alterations at an early disease stage [26]. A notable finding in our study was the high prevalence of oncogenic alterations, with 80% of cases harboring at least one genomic alteration. This aligns with prior studies suggesting that early-stage LUAD exhibits a heterogeneous and complex molecular landscape [27,28]. For instance, *EGFR* mutations, hypothesized to be early oncogenic events in lung cancer, were more frequently observed in early-stage tumors compared to advanced stages [29,30]. Studies have reported a higher prevalence of *EGFR* mutations in stage I LUAD *versus* stage III disease, with frequencies of 13% in early-stage (0–IIIA) tumors compared to 9% in late-stage (IIIB–IV) tumors. Similar findings have been documented in Asian and European cohorts, reinforcing the hypothesis that early-stage tumors harbor distinct molecular profiles that could influence treatment strategies [26,31,32]. Overall, these data suggest that early LUAD remains more "driver-dominant," whereas later stages reflect additional evolutionary events such as TP53 acquisition.

In addition to *EGFR* mutations, other actionable alterations such as *ALK* fusions, *MET* exon 14 skipping mutations, and *KRAS* G12C variants were identified in our cohort. The identification of these mutations underscores the clinical relevance of NGS panels over single-gene testing [33]. *KRAS* mutations were the most frequently detected alteration in our study, with the targetable G12C variant identified in a significant subset of patients. Recent evidence suggests that patients with resected stage I LUAD harboring a *KRAS* G12C mutation may have inferior survival outcomes [34]. This highlights the potential to guide future personalized treatment approaches, such as selecting these patients for neo(adjuvant) therapies with *KRAS* G12C inhibitors or implementing closer monitoring strategies to improve outcomes.

Similarly, detecting rare actionable alterations such as *HER2* mutations and *RET* rearrangements adds value to precision oncology in early-stage LUAD. Reflex NGS testing also enables the detection of co-mutations, such as *TP53* alterations, found in 20% of cases with actionable ESCAT level I mutations. The differences observed with the literature can be explained by several key factors, such as disease stage, tumor biology and clonal evolution, histological and molecular subtypes, patient selection and smoking status, technical and panel differences. Taken together, the pattern we observe is consistent with stage-dependent biology rather than technical artifact.

Most studies reporting high *TP53* mutation rates focus on advanced-stage NSCLC (stages III–IV), where genomic instability and tumor heterogeneity are more pronounced [35]. In contrast, our cohort, restricted to very early-stage LUAD (stage 0–IA), presented a distinct genomic profile that reflects its less advanced biological state (Table 3). The prevalence of *EGFR* mutations in our cohort (27.2%) was notably higher than in most Western studies, such as Muthusamy et al. (16.1%) [36] and Bruno et al [37] (20.3%), but remains lower than the rates commonly observed in East Asian populations, where *EGFR* alterations frequently exceed 45% (Table 3). Conversely, *KRAS* mutations were identified in 35.8% of cases, a frequency consistent with Western cohorts yet markedly higher than in Asian studies, where *KRAS* alterations are typically rare.

Notably, the overall prevalence of *TP53* mutations in our study was 22%, considerably lower than the 40–70% reported in broader NSCLC datasets such as the Cancer Genome Atlas (TCGA) and other studies that include more advanced stages [35]. This discrepancy likely reflects both the very early-stage nature of our cohort and the distinct biological characteristics of the tumors. *TP53* mutations are often considered secondary events that emerge during tumor progression or under therapeutic pressure [38,39]. Supporting this, we observed that among *TP53*-

Table 3
Summary of selected studies reporting genomic profiling in early-stage non-small cell lung cancer (NSCLC).

Study / Cohort	Country / Center	Stage Included	N (Patients)	EGFR (%)	KRAS (%)	TP53 (%)	Technique(s) Used	Key Notes
Our cohort	France (Nice, LPCE)	0–IA	239	27.2	35.8	21	Targeted NGS (Oncomine), ddPCR, IHC, FISH	High rate of actionable mutations; *TP53* co-mutations in 20% of *EGFR*/*KRAS*-mutated tumors
Muthusamy et al., 2022	USA (multi-center)	I–IIIA	1,177	16.1	41.7	Not reported	Hybrid-capture NGS	*KRAS* mutations most prevalent; *MET* exon 14 skipping higher in early-stage tumors
De Luca et al., 2023	Italy (single-center)	I–IIIA	486	20.3	42.5	Not reported	MassARRAY, amplicon-based NGS, FISH, IHC	*EGFR* mutations more frequent in early-stage; *MET* exon 14 skipping enriched in early-stage
TCGA LUAD Early-Stage Cohort	USA (multi-institutional)	I–IIA	470	10.5	29.4	Not reported	Whole-exome sequencing (WES)	Lower EGFR prevalence; data from frozen tissue samples
Yang et al., 2019	China (Zhejiang Cancer Hospital)	I–IIIA	640	48.8	2.0	Not reported	Real-time PCR (rtPCR)	High *EGFR* mutation rate; low *KRAS* mutation prevalence
Ye et al., 2014	China (Fudan University)	I–IIIA	123	52.8	8.3	72.2	rtPCR, FISH for ALK and RET	High *TP53* co-mutation rate; significant *EGFR* mutation prevalence
Suidan et al., 2019	Israel (Davidoff Cancer Center)	I–IIIA	186	23	Not reported	Not reported	rtPCR, NGS	Focus on young patients; notable *ALK* mutation prevalence
He et al., 2020*	Taiwan (Taipei Veterans General Hospital)	I–IIIA	5,051	22.6	Not reported	Not reported	rtPCR for EGFR, IHC for ALK	Large cohort; *EGFR* mutation prevalence varies with age

*Footnote: EGFR mutation rate of 22.6% reported only in patients < 40 years (n = 168) from the He et al. study; not representative of the entire cohort (n = 5,051).

mutated tumors harboring at least one additional genomic alteration, the prevalence of *TP53* co-mutations was 66%. In contrast, among tumors classified as otherwise wild-type (i.e., no additional mutations detected), the frequency of *TP53* mutations was substantially lower (38%). These observations reinforce a continuum from pure driver events toward driver-plus-TP53 architectures as disease advances.

4.2. Correlation of molecular markers with clinical/morphological findings

Our cohort is enriched for lepidic and minimally invasive adenocarcinomas—subtypes associated with low-grade histology, indolent clinical behavior, and reduced mutational burden. These histological patterns are less commonly associated with *TP53* mutations, which are more frequently observed in solid, poorly differentiated, or smoking-related LUAD [40]. This histo-molecular context, along with the relatively high proportion of never-smokers and light smokers in our population (as reflected in pack-year history), may further contribute to the lower *TP53* mutation rate observed. Tobacco exposure is known to correlate with a higher mutational burden, particularly in genes such as *TP53*.

Moreover, differences in technical approaches may also influence reported mutation frequencies. While some studies use whole-exome sequencing (WES) or broad targeted panels capable of detecting subclonal or non-hotspot *TP53* mutations, our study employed a focused clinical panel optimized for detecting actionable variants. This may have led to an underestimation of low-frequency or intronic *TP53* alterations.

Comparison with other studies on stage I–III NSCLC is further complicated by the underrepresentation of stage I cases in those cohorts. Our study, by focusing exclusively on stage 0–IA tumors, provides a clearer view of the genomic landscape in the earliest phases of lung cancer development. These early-stage tumors are often driven by a single dominant oncogenic alteration, most commonly *EGFR* or *KRAS*, and have had limited time to acquire additional mutations such as *TP53*, which are more characteristic of clonal evolution and therapeutic resistance in advanced disease [40].

Beyond a 50-gene panel, risk stratification in very early-stage disease will likely require broader orthogonal approaches. Transcriptomic or methylation classifiers and liquid biopsy for ctDNA-based minimal residual disease (MRD) may refine relapse prediction and guide adjuvant trials in stage 0–IA NSCLC [41–43]. However, blood assays sensitivity remains challenging in Stage IA and virtually absent in stage 0, but emerging ultrasensitive assays show promise [41–43].

Taken together, these observations underscore the biological distinctiveness of very early-stage LUAD and highlight the value of reflex NGS in capturing its molecular complexity [44]. A deeper understanding of the interplay between primary drivers and co-mutations like *TP53* could inform future risk stratification and guide personalized management strategies, including decisions about adjuvant therapies and the intensity of surveillance in early-stage patients.

Our findings reinforce and extend existing evidence that specific genomic alterations in LUAD are closely associated with distinct histological subtypes. In our cohort of very early-stage LUAD (Stage 0–IA), *EGFR* mutations were significantly enriched in tumors with lepidic, papillary, and acinar patterns—subtypes typically associated with non-mucinous and well-differentiated morphology. These results are consistent with previous studies, which, although largely focused on advanced-stage NSCLC, have also reported a higher prevalence of *EGFR* mutations in non-mucinous LUAD and a strong association with lepidic and papillary architecture [45].

Conversely, *EGFR* mutations were rare or absent in tumors with solid or mucinous features, particularly in invasive mucinous adenocarcinoma (IMA), where *KRAS* mutations were markedly enriched. This inverse relationship between *EGFR* and *KRAS* mutations supports the concept of mutually exclusive oncogenic pathways, each associated with a distinct morpho-molecular profile. In particular, *KRAS* mutations were significantly associated with mucinous, solid, and micropapillary subtypes in our study, consistent with prior reports demonstrating their predominance in mucinous and high-grade LUAD variants [46]. These findings highlight the biological relevance of genotype–phenotype correlations in LUAD and support the integration of histological patterns and molecular alterations into a more refined histo-molecular classification system [47–49]. Such integration not only enhances our understanding of LUAD pathogenesis but may also inform risk stratification and guide therapeutic decisions in early-stage disease.

In addition to the single-center design, absence of long-term outcomes, and lack of cost-effectiveness analysis, our panel did not include selected emerging biomarkers (e.g., *KEAP1*, *STK11*, *RB1*, *SMARCA4*) that may carry prognostic value [40,44]. Future prospective studies integrating imaging (radiomics), multi-omic profiling, and MRD will clarify how early molecular profiling should inform adjuvant strategies and surveillance [15,41,43,44]. Thus, histology and grade align with

genotype in a stage-appropriate manner, supporting integrated histo-molecular classification.

Reflex NGS testing has additional benefits beyond identifying actionable targets. It can distinguish between multiple primary tumors and intrapulmonary metastases in patients with multiple stage I tumors [50,51]. Genomic analyses can clarify whether distinct nodules represent independent tumors or metastatic spread, thus influencing immensely staging and treatment decisions. Moreover, reflex testing provides a molecular "portrait" that can be referenced in case of recurrence, enabling faster and more targeted treatment planning [52]. Of note, we excluded cases with more than one nodule.

4.3. Implementation and economic considerations

Given limited adjuvant indications in stage 0–IA, the economic value of reflex NGS warrants formal evaluation. Up-front profiling offers a baseline molecular blueprint that may expedite decisions at recurrence and avoid re-biopsy, whereas deferred testing could be sufficient for many patients; comparative modelling is needed and should consider smoking status and regional mutation prevalence [33,53,54]. Future health-economic analyses should compare reflex-at-resection *versus* test-at-recurrence within risk-defined strata.

Despite its promise, several challenges must be addressed for the widespread implementation of reflex NGS testing in stage 0–IA LUAD. Logistical and financial barriers, including the cost of testing and reimbursement and the need for infrastructure, remain significant hurdles [53,54]. Tissue sample limitations, in particular in *in situ* LUAD, could add another layer of complexity. High-quality DNA and RNA libraries were successfully generated in our study, demonstrating feasibility; however, ensuring reliable results requires meticulous attention to pre-analytic factors such as tissue preservation and extraction methods.

Another critical issue is the interpretation and clinical actionability of molecular findings in early-stage LUAD. While actionable alterations such as *EGFR* mutations or *ALK* fusions can guide therapies in advanced stages, their role in guiding adjuvant or neoadjuvant treatment in stage IA disease is under investigation and needs further clinical validation [55–58]. The clinical implications of early molecular profiling need validation in prospective clinical trials to determine its impact on recurrence rates and survival. Additionally, integrating NGS results into routine clinical workflows presents operational challenges, including turnaround time and multidisciplinary collaboration for decision-making.

Although our analyses used resection specimens, extending reflex NGS to pre-operative biopsies is clinically relevant where neoadjuvant/peri-operative strategies are considered. Feasibility has been reported with small specimens when pre-analytics and assay sensitivity are optimized [21]. Our ultra-fast workflow is adaptable to core biopsies and deserves prospective evaluation in this setting.

Emerging technologies such as artificial intelligence (AI) and radiomics offer new avenues to complement molecular testing. AI algorithms and radiomics tools could aid in differentiating malignant from benign nodules detected in stage IA1 disease, thereby improving diagnostic accuracy and treatment planning [15]. The integration of imaging and genomic data through these technologies holds promise for enhancing precision in early-stage lung cancer management.

4.4. The role of liquid biopsy

The role of liquid biopsy (LB) in early stage, including stage IA LUAD, warrants high attention. LB provides a non-invasive alternative for detecting circulating tumor DNA (ctDNA), and could facilitate longitudinal monitoring for minimal residual disease and early detection of recurrence [41]. However, sensitivity in Stage IA is very limited and virtually absent in Stage 0; even with ddPCR, *EGFR* often remains undetectable in early-stage LBs [42,59].

In addition, to the best of our knowledge, there is no evidence in the literature regarding the use of LB in routine practice for detecting ctDNA in stage 0 NSCLC [43]. The primary challenge lies in the minimal tumor burden at this very early stage, resulting in exceedingly low concentrations of ctDNA in the bloodstream. This scarcity makes reliable detection difficult with current technologies. Studies have demonstrated that ctDNA levels correlate with tumor stage, with lower concentrations observed in early-stage cancers, thereby reducing the sensitivity of LB in these cases [43]. Consequently, while LB shows promise in later stages of NSCLC, its application in Stage 0-IA remains constrained by technological limitations and the need for highly sensitive detection methods.

4.5. Limitations of the study

Our study has several limitations. First, the lack of long-term follow-up data precluded survival analyses and limited our ability to assess the prognostic impact of specific genomic alterations. Second, although the used NGS panel covered a broad range of actionable targets, it did not include certain emerging biomarkers, such as mutations in *KEAP1*, *STK11*, *RB1*, *MTAP*, and *SMARCA4* genes, which may hold prognostic or therapeutic significance in NSCLC. Third, the study was conducted at a single institution, which may limit the generalizability of the findings to other clinical settings with different patient populations or technical workflows. Fourth, our analysis did not include an assessment of cost-effectiveness or health-economic impact, both of which are essential to justify routine use of reflex NGS in stage 0-IA NSCLC.

Fifth, in our study, mature follow-up was not available at the time of analysis, precluding survival and recurrence correlations, nor did we include a stage IB–III comparator cohort. We have initiated prospective follow-up to capture outcomes and management impact, including whether molecular results influenced adjuvant enrollment or surveillance intensity. Finally, although we identified actionable mutations, the study was not designed to assess whether acting on these findings improves clinical outcomes, underscoring the need for prospective trials to validate the clinical utility of early molecular profiling.

5. Conclusions

In conclusion, reflex targeted NGS on site at diagnosis in stage 0–IA LUAD could represent a significant step forward in precision oncology. By enabling early and comprehensive molecular profiling, reflex testing has the potential to guide personalized treatment strategies, optimize therapeutic decision-making, and improve outcomes for patients with these very early-stage lung cancers. However, its implementation must address logistical, financial, and technical challenges. Future studies should focus on validating the clinical utility of reflex NGS in stage 0–IA −LUAD, exploring its role in guiding adjuvant therapies, and integrating emerging technologies like AI and liquid biopsy. Reflex NGS testing could transform the management of very early-stage LUAD and advance the field of precision oncology.

CRediT authorship contribution statement

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Virginie Lespinet-Fabre: Writing – review & editing, Writing – original draft, Resources, Methodology. **Olivier Bordone:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Virginie Tanga:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Caroline Lacoux:** Writing – review & editing, Writing – original draft, Resources, Methodology. **Christelle Bonnetaud:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Jonathan Benzaquen:** Writing – review & editing, Writing – original draft, Resources. **Jacques Boutros:** Writing – review & editing, Writing – original draft, Resources. **Charlotte Cohen:** Writing – review & editing, Writing – original draft, Resources. **Abel Gomez-Caro:** Writing – review & editing, Writing – original draft, Resources. **Charles Hugo Marquette:** Writing – review & editing, Writing – original draft, Resources. **Jean-Philippe Berthet:** Writing – review & editing, Writing – original draft, Resources. **Véronique Hofman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Paul Hofman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MI reports receiving speakers' bureau honoraria from MSD, Bristol-Myers Squibb and AstraZeneca, and Advisory Board honoraria from MilliporeSigma, outside the submitted work. SH reports receiving speakers' bureau honoraria from Qiagen and consulting fees from Boehringer Ingelheim, outside the submitted work. VH reports receiving speakers' bureau honoraria from Bristol-Myers Squibb, outside the submitted work. PH reports receiving financial research grants from BMS, Thermo Fisher Scientific, Amgen, Roche, Biocartis, AstraZeneca; Advisory Boards honoraria from Janssen, BMS, AstraZeneca, Amgen, Abbvie, Biodena, Roche, Pfizer, Ed Lilly, Biocartis, Guardant Health, Novartis, Qiagen, and speakers' honoraria from Janssen, BMS, AstraZeneca, Amgen, Abbvie, Guardant Health, Pierre Fabre, Illumina, Thermo Fisher Scientific, Ed Lilly, Biocartis, Roche, Pfizer, Bayer, MSD, Novartis, outside the submitted work. The remaining authors declare no conflict of interest.

Data availability

The datasets generated and analysed during the current study are not publicly available due to GDPR regulation in the EU, but are available from the corresponding author on reasonable request.

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