A Retrospective Study of DNA+RNA NGS Testing in Non-Small Cell Lung Cancer Patients With Non-Pathologic Complete Response Following Neoadjuvant Immunotherapy

Version: V1.0

Date: July 3, 2025

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1. Background

1.1 Overview of Non-Small Cell Lung Cancer (NSCLC)

NSCLC accounts for 80%-85% of lung cancers and is a leading cause of cancer-related mortality globally. In 2020, lung cancer caused ~2.2 million new cases and ~1.8 million deaths worldwide, with NSCLC as the predominant subtype [1]. In China, the incidence of NSCLC is 61.4 per 100,000, with distinct gender and regional differences [2]. NSCLC also carries a high mortality rate. Approximately 75% of patients are diagnosed at stage III–IV, and the 5-year survival rate is <15% [3]. Molecular targeted therapy has improved outcomes for patients with oncogenic driver mutations. Patients with EGFR mutations are sensitive to EGFR tyrosine kinase inhibitors (TKIs), which significantly prolong survival—as evidenced by a median progression-free survival of 18.9 months with first-line osimertinib [4]. Similarly, those with *ALK* fusions achieve a median overall survival exceeding 7 years following ALK-TKI treatment. Therefore, precise identification of actionable genetic alterations is critical for NSCLC management.

1.2. Current Status and Challenges of Perioperative Immunotherapy in NSCLC

Traditional neoadjuvant chemotherapy achieves a pathologic complete response (pCR) rate of only ~5% in resectable stage II-III NSCLC, with limited survival benefit [5]. The introduction of immune checkpoint inhibitors (ICIs), including nivolumab, pembrolizumab, and tislelizumab, combined with chemotherapy has transformed this landscape, becoming the standard neoadjuvant options for NSCLC. In the CheckMate-816 trial, nivolumab plus chemotherapy resulted in a pCR rate of 24%, compared to 2.2% with chemotherapy alone, and extended median event-free survival by nearly 11 months (31.6 months vs. 20.8 months), without affecting resection rates (83% vs. 75%) [6]. The KEYNOTE-671 trial further demonstrated a pCR rate of 18.1% (vs. 4.0% with control) and a 42% reduction in EFS risk. Collectively, immunotherapy-based combinations achieve major pathologic response (MPR; ≤10% residual tumor) rates of 45%–65% and pCR rates of 20%–40%, significantly higher than chemotherapy (MPR 15-25%, pCR <5%) [7].

Real-world evidence from the NEOSTAR trial indicate that patients receiving neoadjuvant immunotherapy exhibit 1-year disease-free survival (DFS) of 80.6% and overall survival (OS) exceeding 90%, markedly superior to historical controls [8]. Meanwhile, the incidence of grade 3–4 treatment-related adverse events (TRAEs) associated with immunotherapy was approximately 13–25%, without increasing surgical complications [8]. Given these advantages, NMPA has approved pembrolizumab, nivolumab, and durvalumab for neoadjuvant treatment in NSCLC, making it a common clinical practice. However, approximately 40% of patients

exhibit insufficient response (non-MPR) to neoadjuvant immunotherapy [8]. About 20% of NSCLC patients receiving neoadjuvant immunotherapy experience postoperative recurrence, with significantly higher risk in non-pCR patients. The 2-year recurrence-free survival rate is only 55% for non-pCR patients vs. 92% for pCR patients (P=0.005) [8]. The optimal postoperative adjuvant therapy for patients with insufficient response to neoadjuvant immunotherapy remains uncertain. Therefore, determining rational adjuvant strategies to improve long-term survival for patients failing to achieve pCR, particularly MPR after neoadjuvant immunotherapy is a crucial clinical challenge.

1.3 Role of Driver Genes in Perioperative Immunotherapy

In advanced NSCLC, patients with oncogenic driver mutations response poorly to immune monotherapy or combinations, a major cause of primary resistance. Most NSCLC patients with gene fusions exhibit primary resistance to first-line immunotherapy-containing regimens. A meta-analysis showed a pooled ORR of 0% in three clinical trials and 3% in eight retrospective studies for ALK+ NSCLC receiving immunotherapy; pooled ORRs of 6% and 8% for RET and ROS1 rearrangements respectively [9]. Data from advanced studies suggest oncogenic driver mutations may be a potential reason for suboptimal perioperative immunotherapy outcomes. For patients who detect oncogenic driver mutations and fail to achieve pCR after neoadjuvant immunotherapy, seeking adjuvant treatment strategies beyond immunotherapy is necessary.

Most neoadjuvant immunotherapy studies primarily excluded EGFR+ and ALK+ patients. NCCN guidelines and some consensuses recommend excluding EGFR+ and ALK+ from neoadjuvant immunotherapy, but requirements for other drivers are less clear. CSCO guidelines recommend EGFR and ALK testing postoperatively in early-stage patients to guide adjuvant therapy. Beyond established adjuvant targeted therapy for EGFR and ALK, the feasibility of adjuvant targeted therapy for other driver genes remains undetermined and under exploration.

1.4. Limitations of DNA-NGS and PCR in Detecting Driver Genes

Traditionally, molecular diagnosis of NSCLC relied on IHC, FISH, and single-gene PCR. With the prevalence of next-generation sequencing (NGS), DNA-based NGS (DNA-NGS) has become mainstream. It simultaneously detects point mutations, indels, and copy number variations across hundreds of genes, covering recommended biomarkers (e.g., EGFR, KRAS, BRAF). However, DNA-NGS has significant technical limitations in detecting structural variants (SVs), especially gene fusions and splice variants [10]. Complex rearrangements, long intronic deletions/inversions, and low-frequency fusions may be missed due to inadequate probe coverage or difficulties in breakpoint localization. One study used DNA-NGS for mutations and IHC for ALK in lung tissue samples. RNA-based NGS on EGFR/KRAS/HER2/MET/ALK negative samples detected fusions in 42 out of 148 cases (28%); 20 patients receiving targeted therapy showed significantly improved OS compared to

19 receiving chemo/immunotherapy (p=0.033) [11]. Another study of 5570 advanced NSCLC patients found that DNA-NGS alone missed 13.2% out of 8.8% clinically targetable SVs (aSVs), with missed detections rates of 25.4% for ROS1 fusions and 18.6% for MET exon 14 skipping [10]. Moreover, DNA-NGS exhibits even more limited capacity to detect emerging structural variations (eSVs), with only 47.5% of detections rates for such variants—including NRG1 and BRAF fusions. But the addition of RNA-NGS increased eSV detection to 100% [10].

In real-world, PCR is often used for driver gene testing before neoadjuvant immunotherapy in operable NSCLC. However, PCR (e.g., ARMS-PCR, ddPCR, real-time PCR) for detecting EGFR mutations can leading to false negatives, with lower detection rates than tissue samples or high-sensitivity methods. Primer design can limit specificity for certain mutation types. For example, the cobas EGFR Mutation Test (a type of real-time PCR) performs poorly in covering all variants, particularly for some allele variations, potentially missing rare or complex mutations due to primer design limitations [12]. ARMS-PCR in another case misdiagnosed EGFR L747P (c.2239_2240TT>CC) as an exon 19 deletion due to primer misguidance, thus leading to ineffective treatment [13]. PCR sensitivity is typically lower than NGS or digital PCR (dPCR). One study reported PCR detected only 58.8% of EGFR mutations [14], while NGS identified PCR-missed variants (e.g., rare insertions/deletions).This discrepancy are often attributed to PCR's single-gene focus, insufficient coverage, especially in poor sample quality. PCR often misses some EGFR subtypes, such as exon 20 insertions (≤12% of EGFR mutations) due to primer design constraints, whereas NGS detects them more comprehensively [15, 16].

Similarly, PCR Rare variants may produce false-negative reports in detecting rare variants（e.g.，T790M or S768I） due to low sensitivity, and thus require verification with high-sensitivity methods (ddPCR/NGS).

1.5. RNA NGS Effectively Avoids Missing Driver Genes

Unlike DNA-NGS, RNA-NGS directly captures transcripts, enabling efficient detection of gene fusions and splice variants. Its essential advantages include: (1) independence from genomic breakpoints to detect mature mRNA; (2) capability to identify splicing products from complex rearrangements or intronic variants (e.g., MET exon 14 skipping); (3) Better tolerance for low tumor content. Multiple cohorts show higher fusion or rearrangement detection rates with RNA-NGS compared to DNA-NGS. An MSKCC real-world study found 36 additional targetable fusions/rearrangements by RNA-NGS in 232 patients negative by MSK-IMPACT (a 468-gene DNA-NGS panel), indicating a 14.2% miss rate. Subsequent targeted therapy benefits 80% of these patients [17]. A Chinese study parallel-tested 1253 NSCLC samples with DNA-NGS and RNA-NGS; RNA-NGS confirmed all 110 fusions detected by DNA-NGS and found 14 additional fusions in DNA-NGS negative samples [18]. Another Chinese study of 1171 resected I-III NSCLC patients found that while ARMS/IHC/DNA-NGS detected 88%

oncogenic driver mutations, RNA-NGS revealed additional targetable fusions (including in-frame fusions or MET splice site mutations) in 10% (14/140) of negative samples [19].

Furthermore, RNA-NGS can exclude DNA-level fusion false positives by verifying functional transcript products. Some intergenic or exon breakpoint fusions detected at the DNA level may not produce functional transcripts or oncogenic proteins [19]. This suggests a significant portion of intergenic fusion variants detected by DNA-NGS may not be viable therapeutic targets, and relying on them could lead to clinical confusion.

1.6. Scientific Significance of This Study

This study aims to address key questions using large-scale, multicenter observational data: (1) The proportion of NSCLC patients positive for EGFR/ALK by DNA+RNA NGS, but negative in previous DNA-NGS/PCR detection before neoadjuvant immunotherapy and not achieving pCR after surgery. (2) The proportion of above patients positive for other driver genes. (3) The efficacy of adjuvant immunotherapy between driver-positive and driver-negative patients (by DNA+RNA NGS). The results are expected to provide critical evidence for guideline updates, diagnostic optimization, and health economic evaluation, ultimately advancing precision medicine in NSCLC.

2. Objectives

To investigate the positive rate of driver genes (by DNA+RNA NGS) in postoperative samples from real-world lung adenocarcinoma patients with non-pCR after neoadjuvant immunotherapy and negetive for GFR/ALK by previous PCR or DNA-NGS.

To analyze the characteristics of driver-positive patients and compare the efficacy of adjuvant immunotherapy between driver-positive and driver-negative patients.

3. Inclusion and Exclusion Criteria

Inclusion Criteria:

- Age ≥ 18 years, male or female.
- Pathologically resectable NSCLC treated with neoadjuvant therapy containing an immune checkpoint inhibitor, with postoperative pathological assessment confirming non-pCR.
- Molecular characteristics: Preoperative biopsy sample tested by DNA-NGS or PCR , showing no EGFR mutation or ALK fusion.
- Sample requirement: 5-10 FFPE slides from surgical specimen, with ≥5% tumor cell content confirmed by HE staining.

Exclusion Criteria:

- Failure to meet any inclusion criteria.
- History of malignancy at other sites.

- Patients who did not receive the planned cycles of neoadjuvant immunotherapy due to toxicity.
- Other conditions judged by the investigator as unsuitable for study participation.

4. Procedures

4.1. Sample Screening and Grouping

This multicenter retrospective cohort study plans to enroll NSCLC patients. Cases meeting inclusion criteria between January 2022 and December 2024 will be identified through hospital pathology management systems. Screening involves two stages: Initial screening: Extract information from electronic medical records for patients aged ≥18, pathologically diagnosed with NSCLC, and who underwent preoperative genetic testing. Neoadjuvant immunotherapy data collection: a. Received preoperative neoadjuvant immunotherapy (ICI mono, ICI+chemo, ICI+anti-angiogenic, etc.) with postoperative pathological assessment; b. Preoperative biopsy sample excluded EGFR and ALK positivity; c. Availability of qualifying surgical sample.

Sample size calculation: Calculated using PASS software (α=0.05, β=0.2), estimating a requirement of 300 samples.

4.2. NGS Sequencing and Analysis

DNA/RNA NGS co-detection will use the 3DMed OncoTM Core Tissue Detection Kit.

Sample processing:

Co-extraction of nucleic acids: Simultaneous isolation of DNA and RNA from the same tumor tissue sample (FFPE slides).

Quantification and cDNA synthesis: DNA and RNA are quantified. If minimum requirements are met (DNA≥10 ng, RNA≥20 ng), RNA is reverse transcribed to cDNA.

Automated library preparation: Using the ANDiS 500 automated NGS library preparation system with the 3DMed OncoTM Core kit. Extracted DNA and synthesized cDNA are mixed and added to the provided Library Cartridge, loaded into the ANDiS 500 for amplicon library preparation. Includes two PCR amplifications and purifications:

- 1st PCR: Amplifying interest region by target-specific rhAmp primer.
- Magnetic Bead Purification: Purify the amplified products to remove impurities.
- 2nd PCR: Add Primers containing sample indices (Index) and P5/P7 sequencing adapters for library labeling.

- Positive and negative control libraries prepared similarly.

Library quantification and sequencing: Quantify indexed libraries, normalize, pool, and sequence on an Illumina platform.

Bioinformatics analysis:

3DMed's proprietary TiNAiLab software analyzes data, generating reports including variants, MSI status, fusions. QC standard: House-keeping gene (e.g., CCDC6, BBS7) expression >40 reads.

4.3. Clinical Data Collection and Quality Control

Data will be collected via a unified electronic Case Report Form (eCRF):

Baseline characteristics:Demographics (gender, age), comorbidities, smoking history, BMI, AJCC stage, tumor size, histopathology, immunotherapy regimen.

Treatment and follow-up: Neoadjuvant regimen type and cycles. Imaging assessment frequency (every 8-12 weeks, RECIST 1.1), ORR, postoperative pathological response, receipt and type of adjuvant therapy, recurrence.

Data quality control: A central monitor will verify data completeness and logical consistency (e.g., pathology date before treatment start). All data stored in REDCap with role-based access control.

4.4. Statistical Analysis

Primary endpoint analysis: Proportion of patients with driver gene positivity (especially fusions) detected by DNA+RNA co-testing.

Secondary endpoint analysis: Comparison of adjuvant immunotherapy efficacy between driver-positive (especially fusions) and driver-negative populations; stratified analysis for EGFR/ALK positive vs. other drivers positive.

Exploratory analysis: Characteristics of driver-positive non-pCR population. Preoperative driver positivity rate in pCR vs. non-pCR cohorts.

R 4.3.1 was used in all analyses. Significance level set at two-sided $p<0.05$. Multiple testing correction using Benjamini-Hochberg method.

5. Reference

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