Clinical Trial Protocol

Title：Explore the primary resistance mechanisms of anaplastic lymphoma kinase inhibitor.

Principal Investigator and Co-Investigators Names

- Principal Investigator: Jin-Shing Chen, Title: Director,
  Department of Surgery, National Taiwan University Hospital
- Co-Investigator: Hsao-Hsun Hsu, Title: Director,
  Department of Oncology, National Taiwan University Cancer Center
- Co-Investigator: Pei-Ming Huang, Title: Director,
  Department of Thoracic Surgery, National Taiwan University Hospital
- Co-Investigator: Shang-Gin Wu, Title: Attending Physician,
  Department of Chest Medicine, National Taiwan University Hospital
- Co-Investigator: Chih-Hsin Yang, Title: Superintendent,
  National Taiwan University Cancer Center
- Co-Investigator: Jin-Yuan Shih, Title: Director,
  Department of Chest Medicine, National Taiwan University Hospital
- Co-Investigator: Chao-Chi Ho, Title: Associate Director,
  Department of Internal Medicine, National Taiwan University Hospital
- Co-Investigator: Tung-Ming Tsai, Title: Attending Physician,
  Department of Surgical Oncology, National Taiwan University Cancer Center
- Co-Investigator: Pei-Hsing Chen, Title: Attending Physician,
  Department of Thoracic Surgery, National Taiwan University Hospital
- Co-Investigator: Yen-Ting Lin, Title: Attending Physician,
  Department of Chest Medicine, National Taiwan University Hospital
- Co-Investigator: Pin-Chih Liao, Title: Attending Physician,
  Department of Oncology, National Taiwan University Hospital
- Co-Investigator: Yen-Lin Huang, Title: Attending Physician,
  Department of Pathology, National Taiwan University Hospital
- Co-Investigator: Chia-Lang Hsu, Title: Associate Research Fellow,
  Department of Medical Research, National Taiwan University Hospital

(1) Project Abstract:

Anaplastic lymphoma kinase (ALK) gene rearrangement is a known oncogenic driver in non-small cell lung cancer (NSCLC). ALK tyrosine kinase inhibitors (TKIs) have been clearly shown to produce excellent therapeutic effects and prolong survival in patients with this gene mutation. According to current treatment guidelines, ALK inhibitors are the first-line treatment of choice for ALK-positive advanced NSCLC patients. However, although ALK TKIs are very effective, there is still a small group of patients who do not achieve good treatment outcomes, developing resistance and tumor progression within 3 to 6 months of initial ALK TKI use. This is called primary resistance. Intrinsic resistance to ALK inhibition occurs when the best clinical response after first-generation and second/third-generation TKI treatment is disease progression. Approximately 5-7% of cases after crizotinib treatment, 9% after ceritinib treatment, and 25% after lorlatinib treatment show no response to treatment, and no specific ALK mutation has been found to explain the occurrence of primary resistance. Currently, many different resistance mechanisms are known, some of which are still ALK-related, while others are ALK-independent alternative survival pathways. However, most research focuses on acquired resistance, with very few studies on primary resistance, only a few case reports. Therefore, this study aims to explore the primary ALK TKI resistance mechanisms.

We plan to explore the incidence and mechanisms of primary ALK TKI resistance in ALK-positive advanced NSCLC patients who develop primary resistance or rapid progression (within 3-6 months) during ALK inhibitor treatment by re-obtaining tumor samples for genetic analysis.

Keywords: Lung cancer, Anaplastic lymphoma kinase (ALK) gene rearrangement, Gene mutation, Primary resistance, Next-generation sequencing, Targeted therapy.

(2) Background and Objectives of the Research Project:

Lung cancer is the most common cause of cancer death in Taiwan and worldwide [1]. The traditional chemotherapy, platinum-doublet chemotherapy, provided limited survival benefit. In the recent decades, the major evolution in lung cancer is the molecular subtyping of NSCLC, especially lung adenocarcinoma, which has resulted in a paradigm shift in the management of patients in advanced disease. The target therapy provided better treatment response and longer overall survival. For example, patients with NSCLC harboring Epidermal growth factor receptor (EGFR) mutations can get remarkable benefit and longer survival from EGFR tyrosine kinase inhibitors (TKIs) treatment comparing with the traditional platinum-base chemotherapy [2].

Anaplastic lymphoma kinase (ALK) gene rearrangements define a distinct molecular subset of NSCLC, typically occurring in younger, non-smoking individuals with adenocarcinoma histology [3]. The discovery of ALK fusions, particularly EML4-ALK, revolutionized the treatment of these patients with the development of ALK TKIs. First-generation ALK inhibitors, such as crizotinib, demonstrated remarkable initial responses, significantly improving progression-free survival (PFS) compared to standard chemotherapy [4]. However, the emergence of resistance, both primary and acquired, remains a major clinical challenge.

Primary resistance, defined as the lack of initial response or early progression within the first few months of ALK TKI therapy, occurs in a subset of patients and represents a significant obstacle to optimal treatment outcomes [5]. Primary resistance to ALK inhibition occurs, even with advanced TKI therapies, when the best clinical outcome is disease progression. Notably, approximately 5-7% of patients following crizotinib, 9% following ceritinib, and 25% following lorlatinib demonstrated a lack of response, and in these cases, no identifiable ALK mutations were detected [6]. Unlike acquired resistance, which typically arises from secondary mutations within the ALK kinase domain or activation of bypass signaling pathways, the mechanisms underlying primary resistance are less well understood.

Several potential factors contribute to primary resistance. One key aspect involves inherent tumor heterogeneity. NSCLC tumors are complex ecosystems with diverse subclones, some of which may harbor pre-existing genetic alterations that confer resistance to ALK inhibition [7]. These alterations might include co-occurring mutations in genes

involved in cell signaling pathways, such as KRAS, EGFR, or TP53, which can bypass ALK dependence [6]. Furthermore, variations in ALK fusion isoforms or copy number alterations may also influence initial drug sensitivity. For example, some less common ALK fusion variants might possess distinct structural properties that reduce their affinity for ALK TKIs.

Another potential mechanism involves the tumor microenvironment (TME). The TME, composed of stromal cells, immune cells, and extracellular matrix, plays a critical role in tumor growth and drug response [8]. Components of the TME, such as cancer-associated fibroblasts (CAFs) and immunosuppressive cells, can secrete growth factors and cytokines that activate alternative signaling pathways, thereby promoting resistance to ALK inhibition. Additionally, the TME can create a physical barrier that limits drug penetration and efficacy.

Furthermore, the complexity of ALK signaling networks and the potential for activation of bypass pathways contribute to primary resistance. For example, activation of the EGFR or MET signaling pathways can provide alternative growth signals, bypassing the need for ALK activation [9]. Understanding these bypass pathways is crucial for developing combination therapies that can overcome primary resistance.

Investigating primary resistance requires a comprehensive approach that integrates genomic, transcriptomic, and proteomic analyses. Next-generation sequencing (NGS) can identify pre-existing genetic alterations that confer resistance. Single-cell RNA sequencing can dissect tumor heterogeneity and identify resistant subclones. Proteomic profiling can reveal activation of bypass signaling pathways. Clinical studies analyzing pre-treatment tumor samples and correlating genomic and clinical data are essential for identifying predictive biomarkers of primary resistance.

In summary, primary resistance to ALK TKIs in NSCLC is a complex phenomenon driven by multiple factors, including tumor heterogeneity, TME interactions, pharmacokinetic variations, and activation of bypass signaling pathways. Further research is needed to elucidate the precise mechanisms underlying primary resistance and to develop strategies to overcome this clinical challenge, ultimately improving outcomes for patients with ALK-positive NSCLC.

(3) Research Methods and Procedures:

Number of Subjects, Selection and Exclusion Criteria:

- Trial Period and Progress: From July 1, 2026, to July 1, 2028. Approximately 20 participants are expected to be enrolled for testing.
- Enrollment Locations: Outpatient clinics and wards of Thoracic Surgery, Chest Medicine, and Oncology Departments at National Taiwan University Hospital and National Taiwan University Cancer Center.

Lung cancer patients who initially use ALK TKI as first-line treatment will be invited to enroll and sign the consent form. Only those who meet the criteria for primary resistance or rapid progression will proceed with tumor sample collection for genetic analysis. Alternatively, patients who develop primary resistance or rapid progression after using ALK TKI and are about to undergo tumor re-biopsy can also sign the subject consent form to be included in this study.

Expected number of enrolled cases is approximately 20 patients with primary resistance to ALK TKI.

<Inclusion Criteria>

Patient must meet the following criteria for study entry

1. Age ≥ 18 years old.
2. Histologically or cytologically diagnosed NSCLC
3. ALK gene fusion detected at initial diagnosis of NSCLC
4. Patients exhibiting primary resistance to ALK-TKI therapy, defined as the absence of initial response or progression within 3 to 6 months of treatment initiation, were included.
5. Able and willing to provide written informed consent prior to performing any study related procedures and to comply with the study protocol.

<Exclusion Criteria>

Patient who meet any of the following criteria will be excluded from study entry

1. Unable to provide written inform consent
2. The patients had received other systemic treatments except ALK TKI.
3. Unable to undergo tumor biopsy or venipuncture

This study is planned to be conducted at National Taiwan University Hospital, National Taiwan University Cancer Center, Tri-Service General Hospital, Linkou Chang Gung Memorial Hospital, and Taipei Veterans General Hospital, adopting a competitive enrollment approach.

3.1 Overall study design:

With appropriate written inform consent, we will collect all of the patients with advanced NSCLC harboring ALK rearrangement who received 2nd-or 3rd- generation ALK TKI as first-line treatment. A subtype of ALK TKI-treated patients suffered from primary resistance or rapid progression to ALK TKI treatment. Re-biopsy of the enlarged tumor will be arranged for NGS analysis. If tumor re-biopsy is not feasible, 20 ml peripheral blood samples form subjects who fulfill the inclusion/exclusion criteria of this study will be collected for NGS study, too. If clinically feasible, archival tissues at initial diagnosis of lung cancer will also be collected if the specimens were obtained when patients developed tumor progression on ALK inhibitor treatment. Both the blood and tissue samples will be sent for NGS analysis. Patients' clinical characteristics, tumors' pathological features, and the ALK inhibitor treatment response, will be reviewed. A correlation analysis between the clinicopathological characteristics and the genetic alterations in ALK and other associated genes will be performed.

3.2 Sample preparation

The collected tissue samples, including at initial diagnosis of lung cancer and re-biopsy after disease progression, will be collected. The formalin-fixed, paraffin-embedded tissue, if available, will also used for mutation testing by next generation analysis. One of the consecutive sections will be stained with hematoxylin and eosin and reviewed by pathologists to select tumor region(s) for genomic DNA extraction. The selected tumor region(s) will be marked on deparaffined tissue sections by the experienced pathologists.

If the patient will not have adequate tumor for re-biopsy, peripheral blood samples after disease progression will be collected into Cell-Free DNA BCT tubes (Streck, La Viintsta,

NE) and stored at room temperature (RT). Plasma will be separated from blood cells by centrifugation at 1,600 × g for 10 min at RT using a swing-out rotor. The supernatant will be transferred and centrifuged a second time at 6,000 × g for 10 min at RT. Plasma will then be gently mixed and aliquoted in cryogenic vials, and frozen at -80°C until DNA extraction.

3.3 DNA and RNA extraction

3.3.1 Genomic DNA Extraction

Genomic DNA will be extracted from three to five unstained, 5×5mm^2 with 5-µm thick FFPE sections using Cobas® DNA Sample Preparation Kit (Roche) according to the manufacturer's protocol. Cell-free DNA (cfDNA) will be isolated from peripheral blood using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Venlo, Netherlands). Isolated DNA and cfDNA will be quantified using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). DNF-474 High Sensitivity NGS Fragment Analysis Kit (AATI, Ankeny, IA, USA) along with Fragment Analyzer Automated CE System (AATI) will be used for quality control, including concentration and the extent of degradation of extracted genomic DNA.

3.3.2 Total RNA Extraction

The total RNA of the FFPE sections will be isolated using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX). Isolated RNA will be quantified using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNF-474 High Sensitivity NGS Fragment Analysis Kit (AATI, Ankeny, IA, USA) along with Fragment Analyzer Automated CE System (AATI) will be used for quality control, including RNA concentration and the extent of degradation of extracted RNA.

3.4 Next Generation Sequencing

Then, the DNA libraries and RNA samples will be sequenced by next generation sequence of Guardant 360. The panel, Guardant360 TissueNext™, will be used, and this panel can detect 742 genes whose structures are related to tumor message transmission pathways, analyze 741 single gene variation (SNV) or insertion/deletion fusion (InDel), 34 copy number

variation (CNV), 28 genes (fusion genes), and 47 promoter methylation genes, analyze the causes of tumor formation and characteristic fusion, and find suitable drugs.

(4) Expected Trial Results:

Different ALK fusion variants are also not created equal. In addition, *in vitro* data have suggested different secondary mutations at ALK kinase domain will result in different drug sensitivity to different kinds of ALK inhibitors [10]. In addition, the bypass survival pathway will contribute to treatment failure if it is not targeted appropriately. In this observational study, we will be able to comprehensively evaluate the primary resistance mechanisms of ALK TKIs. Meanwhile, we will also be able to evaluate the usefulness of liquid biopsy in ALK-positive NSCLC patients. We believe our study results will not only to provide the landscape of primary resistance mechanisms of ALK TKIs but also offer a potential solution to future clinical patients who are not able to undergo invasive tissue re-biopsy when their ALK-positive lung tumors become resistant to ALK inhibitors

(5) Data Collection, Processing, Evaluation, and Statistical Analysis Methods:

All categorical variables will be analyzed with Pearson's $\chi 2$ tests, except where a small size required the use of Fisher's exact test. The time to tumor recurrence, progression-free survival curve and overall survival will be plotted by the Kaplan–Meier method and compared by a log-rank test. Two-sided p-values of less than 0.05 will be considered significant. All analyses will be performed using the SPSS software (version 29.0 for Windows; SPSS Inc.)

(6) Clinical Adverse Reactions and Management Methods: This study does not involve treatment.

(7)Funding Source: This is an investigator-initiated study. The shipping and NGS testing of blood and tissue samples are sponsored by Taiwan Society of Thoracic Surgeons (TSTS). There is no consideration relationship. It has no impact nor responsibility on the prescription of clinical physicians.

(8) Potential Psychological and Physical Risks and Benefits:

There is no directly interested relationship between participating patients and sponsored company. Possible AEs related to study medication are mentioned above. Participating institutes and investigators will take the responsibility of patient follow-up and potential risk management of blood sampling.

(9) Potential Financial Risks and Benefits:

Although the NGS test is sponsored by Taiwan Society of Thoracic Surgeons (TSTS). There is no other conflict of interest related to sponsored TSTS. The research results will be published in academic literature, without any other intellectual property and substantial benefits related to TSTS. The National Taiwan University Hospital will be engaged in medical purposes such as disease diagnosis, prevention, treatment, and research.

(10) Conflicts of Interest:

Although the study is sponsored by Taiwan Society of Thoracic Surgeons (TSTS). There is no other conflict of interest related to sponsored TSTS. Investigators are responsible for study conception, design, protocol writing, IRB application/approval, operational execution, data handling, and data analysis, and interpretation, along with subsequent publication. Participating institutes and investigators will take the responsibility of patient follow-up.

(11) Research Personnel: The principal investigator and co-investigators will apply to the Department of Pathology to send samples to Guardant Health testing laboratory (Taiwan general agent: Welgene Biomedical Co., Ltd.) for testing according to the

general sample outsourcing procedures. No laboratory tests will be conducted in the hospital, so no research personnel are required.

(12) Research Funding Source: Taiwan Society of Thoracic Surgeons

(13) Subject Information and Consent Form Format: Please refer to the Subject Consent Form

(14) Required Drugs or Medical Devices and Quantity: None.

(15) Patient Data Confidentiality and Privacy:

Regarding patient data confidentiality, relevant clinical data records will be collected using a code instead of the patient's real medical record number and name. The relevant codes and corresponding patient list will be stored in Dr. Shang-Jun Wu's locked office. Relevant data will be stored electronically or filed for statistical analysis and will be kept in a dedicated computer with a password and appropriate anti-virus software (Dr. Shang-Jun Wu's in-hospital office). This data will be completely deleted after the publication of the thesis. In addition, it also includes:

(1) Only use hospital (NTU or NTUH) email to transmit data.

(2) Do not register for outpatient clinics in the name of patients without authorization.

(3) Follow the regulations that data should not be taken out of the hospital, and patient personal data will not be taken out of the hospital without reason.

(4) In principle, business will be conducted on public computers, and personal computers need to be encrypted when used.

(5) Patient data will be marked with codes instead of identifiable data.

Regarding patient privacy: To protect the patient's personal privacy, a trial number will be used instead of the name and related personal data to ensure that relevant retrospective data are fully confidential. At the same time, related protection methods are provided:

(1) Comply with the outpatient medical privacy maintenance regulations issued by the Department of Health.

(2) Do not use official duties to inquire about unrelated patient test reports.

(3) Implement the National Taiwan University Hospital's "Small Sealing Movement."

(16) Potential Psychological and Physical Risks and Benefits: This study retrospectively collects patients' clinical data and drug treatment effects, and there is no special interventional treatment, which does not affect patients' rights and does not subject subjects to any coercion or interference.

(17) Potential Financial Risks and Benefits: This study is not expected to generate commercial interests, nor will it generate patent rights or other commercial interests.

(18) Ownership and Use of Research Results: The research results belong to National Taiwan University Hospital and the Taiwan Society of Thoracic Surgeons, and the results may be published in academic papers in the future.

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