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MYC Amplification as a Potential Mechanism of Primary Resistance to Crizotinib in ALK-Rearranged Non-Small Cell Lung Cancer: A Brief Report[1,2]

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Abstract

INTRODUCTION: Translocations of the anaplastic lymphoma kinase (ALK) can be effectively targeted in advanced non-small cell lung cancer by ALK-TKI inhibitors including Crizotinib. However, the development of acquired resistance often limits the duration of these therapies. While several mechanisms of secondary resistance have been already identified, little is known about molecular determinants of primary resistance. In our brief report we investigated the tumor molecular profile of a patient who failed to respond to Crizotinib. *METHODS:* Fluorescence in situ hybridization (FISH) and next-generation sequencing (NGS) were run on tumor specimen as well as search and characterization of circulating tumor cells (CTCs) in the blood. Confirmation of clinical findings was achieved using a translational cell-line in vitro model. *RESULTS:* We identified the amplification of *MYC* as a potential new mechanism of primary resistance to ALK inhibition. Human EML4-ALK rearranged cells infected with a lentiviral vector carrying full-length human *MYC* cDNA were treated in vitro with crizotinib and alectinib. Overexpression of *MYC* overexpression was associated with a reduced sensitivity to both ALK-inhibitors. *MYC*-overexpressing clones displayed also increased levels of both cyclin D and E and their growth was reduced by using Cdk4/6 inhibitors such as Palbociclib. *CONCLUSIONS:* We postulate that the *MYC* gene may be implicated in the mechanism of primary resistance to ALK inhibitors. We also suggest potential *MYC*-directed inhibition strategies to overcome primary resistance in advanced ALK-rearranged NSCLC.

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Introduction

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related mortality worldwide, accounting for approximately 18% of all cancer [1]. About 2–8% of NSCLC carry molecular alteration of the anaplastic lymphoma kinase (*ALK*), most commonly a fusion between the *ALK* and echinoderm microtubule-associated protein-like 4 (*EML4*) gene. Such *EML4-ALK* translocation leads to a constitutively activated protein kinase that is essential for transfor-

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mation [2,3]. Preclinical evidence has confirmed the activation of several downstream oncogenic pathways caused by EML4-ALK fusion protein, including PI3K, JAK/STAT and RAS/MEK/ERK [4].

Crizotinib was the first approved ALK inhibitor, based on an overall response rate of 57% in ALK positive patients [5]. Although the majority of patients experience rapid impressive response to these targeted therapies, the development of drug resistance is inevitable. Moreover, intrinsic (or primary) resistance may explain why very few patients fail to respond already from the beginning to ALK-inhibitor therapy.

Several mechanisms of acquired resistance have been reported, including secondary mutations within the ALK tyrosine kinase domain, amplification of the *ALK* fusion gene and the activation of alternative signaling pathways, such as EGFR, KIT and insulin-like growth factor receptor 1 [6]. By contrast, molecular mechanisms underlying innate primary resistance to ALK-inhibitors have not been thoroughly elucidated yet.

Analyzing the case of a patient with primary resistance to Crizotinib, we first discovered an amplification of *MYC* gene and then studied it in vitro as potential new mechanism of primary resistance to ALK inhibition.

Clinical Case

A 48-year-old, never smoker, woman was referred to our clinic with a diagnosis of ALK-rearranged NSCLC with axillary, lateral-cervical and mediastinal lymphadenopathies and multiple bone, lung and liver metastases, plus pleural effusion. She was symptomatic for cough and dyspnea on exertion, which was related to both the disease and a concomitant pulmonary embolism. Since at that time ALK-inhibitors were not registered in Italy for first-line therapy, she was started on a first-line chemotherapy with cisplatin plus pemetrexed every 3 weeks. After an initial clinical benefit, the patient's overall conditions deteriorated: a CT scan performed after 3 cycles of chemotherapy, showed a progressive disease with worsening of lung metastases and the development of pulmonary lymphangitic carcinomatosis. The patient was therefore rapidly switched to a second-line treatment with Crizotinib 250 mg BID, achieving a quick, albeit transient, improvement of overall conditions. The following CT scan showed a mixed objective radiological response with a shrinkage of mediastinal lymphadenopathies, improvement of bilateral lung lesions but significant worsening of the pulmonary lymphangitic carcinomatosis and pleural effusion (Figure 1). Crizotinib was not interrupted but approval for off-label Ceritinib was sought. However, her overall conditions rapidly deteriorated leading to hospital admission due to a dyspnea on minimal exertion. CT pulmonary angiography showed recurrence of pulmonary embolism and a significant further worsening of the lymphangitic carcinomatosis and right pleural effusion was assessed for cytology. The patient then started third-line treatment with ceritinib 750 mg OD but deceased few days later due to acute respiratory failure. This work has been carried out in accordance with the Code of Ethics of the World

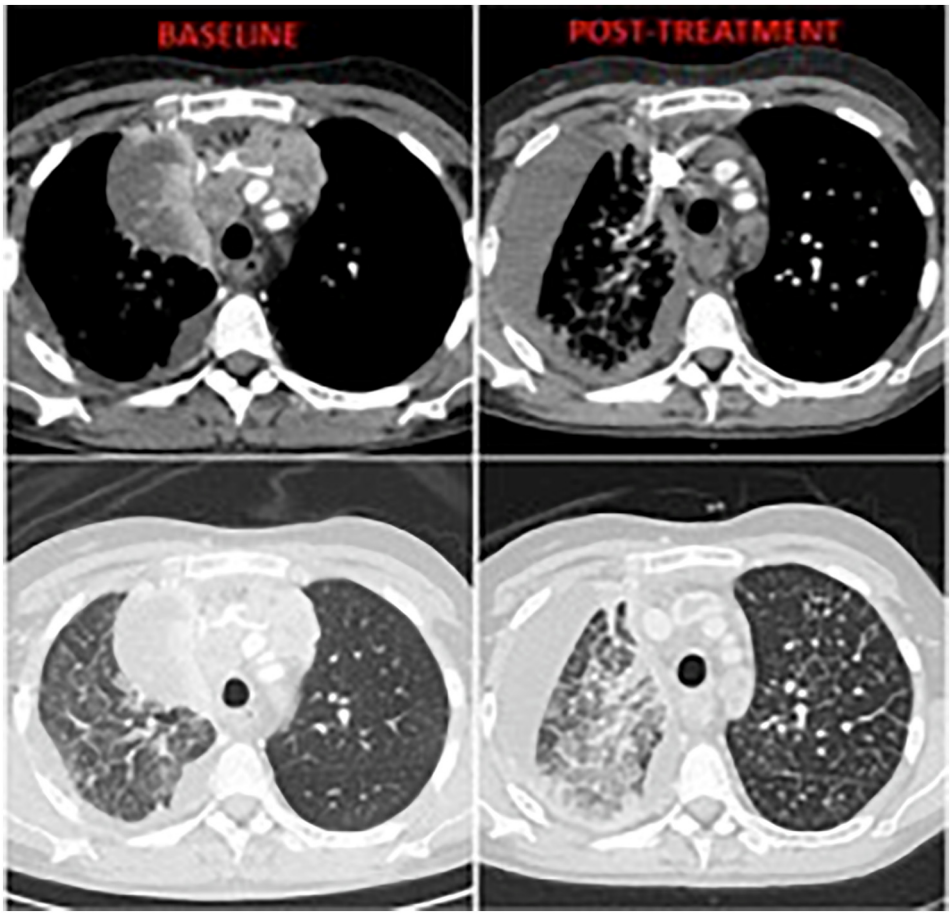

Figure 1. CT assessment before and after crizotinib treatment showing improvement in mediastinal lymphonodes enlargement with parallel worsening of pleural effusion and pulmonary interstitial disease.

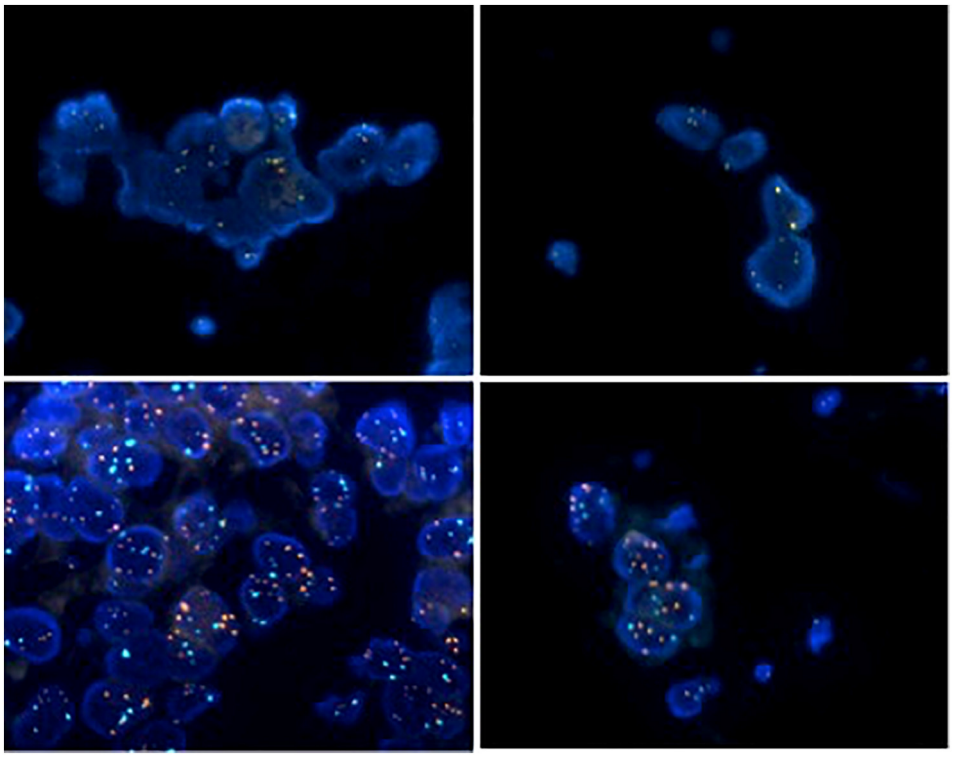

Figure 2. Break-Apart FISH for *ALK* and FISH for *MYC* carried out on pre-treatment diagnostic tumor biopsy and on pleural fluid tumor cells at the time of disease progression showing *ALK* rearrangement in >50% of tumor cells (upper panels) and *MYC* amplification (GCN 8) in both pre-treatment tumor biopsy and re-biopsy at disease progression (lower panels).

Medical Association (Declaration of Helsinki) for experiments involving humans. Informed consent was obtained from the patient.

Clinical Laboratory Workout

Diagnosis of ALK rearrangement was accomplished by fluorescence in situ hybridization (FISH) on paraffin tissue sections of cell pellets obtained after centrifugation of pleural effusion at the time of first diagnosis, using the Vysis ALK Break Apart FISH Probe Kit (Abbott Laboratories, IL). *ALK* was considered rearranged since 47% of the cells showed split signals.

In order to detect possible known mechanisms of primary resistance after Crizotinib, we run a wide spectrum mutational panel analysis on recurrent pleural effusion tumor cells using the Oncomine 318 Focus assay on an Ion Torrent PGM platform (ThermoFisher Scientific, Waltham, MA). This analysis confirmed the original *EML4-ALK* fusion, but none of the *ALK* mutations known to confer resistance to crizotinib were found. As a single additional information of the NGS analysis, we found an amplification of the *MYC* oncogene with a copy number gain of approximately 8 copies.

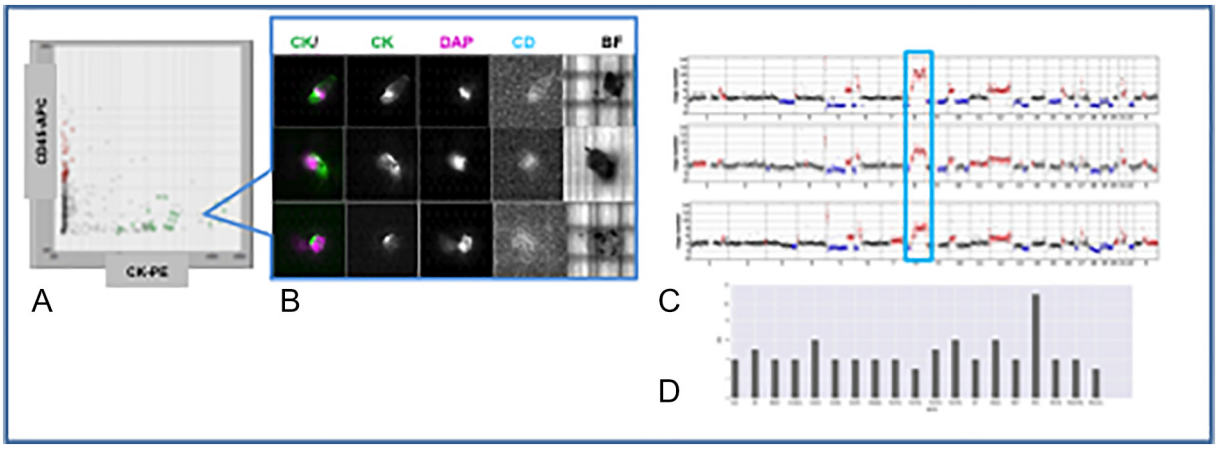

Figure 3. *MYC* amplification in single CTCs. Scatter plots of blood-derived Cell-Search enriched sample (A) visualized at DEPArray graphic interphase, and relative image galleries CTCs (B); LPCNA profiles of CTCs (C) highlight a gain on an area of the chromosome 8 corresponding to *MYC* locus (in blue). Histograms depicted in (D) quantify *MYC* amplification observed in CTCs.

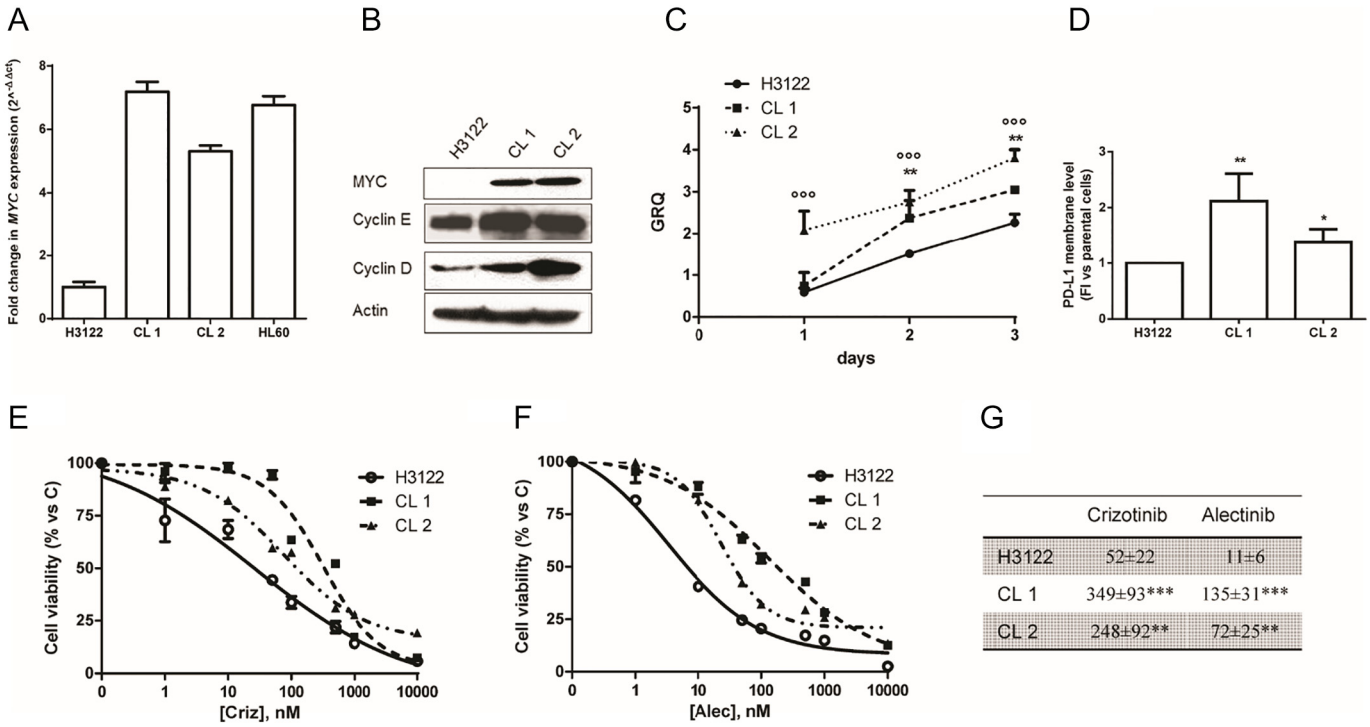

Figure 4. Myc overexpression caused resistance to both the ALK inhibitors crizotinib and alectinib. (A), H3122 parental cells (*EML4/ALK* fusion-positive cells), Myc-overexpressing clones (Cl1 and Cl2) and HL-60 cell line were analyzed for *MYC* mRNA expression by RT-PCR. Data are expressed as mRNA quantity normalized to H3122 cell line (=1). (B), Western blot analysis was performed on lysate proteins by using monoclonal antibodies directed against the indicated proteins. (C), H3122, Cl1 and Cl2 cells were plated and GRQ was determined after 1, 2 and 3 days. (**$P < .01$, ***$P < .001$ versus H3122 cells; Student's *t* test). (D), PD-L1 protein level on cell surface was evaluated by flow-cytometry, quantified as MEF, and expressed as fold increase versus H3122 cell line (=1). Mean values of three independent measurements (±SD) are shown ($P < .05$, versus H3122 cells; Student's *t* test). (E-F), H3122, Cl1, and Cl2 cells were treated with increasing doses of crizotinib or alectinib and after 72 h cell proliferation was assessed by CV assay. (G), IC_{50} values of crizotinib and alectinib. Mean values of three independent measurements (±SD) are shown (*$P < .05$, **$P < .01$, ***$P < .001$ versus H3122 cells; Student's *t* test). Data of A, B, C, E and F are representative of three independent experiments.

FISH using the Vysis LSI IGH/MYC/CEP 8 Tri-Color Dual Fusion Probe Kit (Abbott) on the pre-treatment pleural effusion specimen revealed a 7.8 mean copy number gain of *MYC*. We thus provided confirmation that this primary alteration was already present at the time of disease presentation and was not acquired during Crizotinib treatment (Figure 2).

Isolation of circulating tumor cells (CTCs) was run in parallel on a blood sample, taken at the time of radiological progression, using the CELLSEARCH instrument and single CTCs were sorted with the DEPArray platform followed by single cell whole genome amplification (*Ampli*1 WGA kit, all from Menarini-Silicon Biosystems, Firenze, Italy) and genome-wide copy-number analysis was performed by low-pass whole genome sequencing with Ampli1 LowPass kit (Menarini-Silicon Biosystems) on an IonTorrent PGM instrument. This additional test confirmed a common amplification of a genome region comprising the *MYC* gene (Figure 3).

Pre-Clinical Validation

In order to confirm the possible pathogenetic role of *MYC* amplification in conferring primary resistance to ALK inhibitors, human EML4-ALK rearranged H3122 cells were infected with the lentiviral transfer vector (pLenti-C-mGFP-P2A-Puro,Origene, Rockville, MD) carrying full length human *MYC* cDNA, as previously described [7]. The mRNA level of *MYC* was evaluated by RT-PCR and compared with the level expressed by HL-60, a human promyelocytic leukemia cell-line with *MYC* amplification (Figure 4*A*). Furthermore, Myc-overexpressing clones showed increased level of cyclin D and E, two key controllers of G_1-S checkpoint at western blot analysis (Figure 4*B*), with consequent overstimulation of cell growth compared to H3122 parental cells (Figure 4*C*).

Selected *MYC*-overexpressing clones were then analyzed for sensitivity to specific ALK inhibitors crizotinib and alectinib (Selleckchem, Munich, Germany). Reduction in sensitivity to both drugs with increase in IC_{50} values in *MYC*-overexpressing clones compared to H3122 parental cells was demonstrated (Figure 4, *E–G*). These results indicate *MYC* overexpression as a potential mechanism associated with resistance to specific ALK inhibitors.

In order to overcome the resistance caused by *MYC* overexpression, we evaluated the effects of the combination of crizotinib or alectinib with the specific inhibitor of the heterodimer Myc-MAX (KJ-Pyr-9 Merck-Millipore, MA), that synergistically reduced cell proliferation of Myc overexpressing clone 1, restoring sensitivity to ALK-inhibitors (Figure 5, *A* and *C*) [8]. Considering that Myc acts as a general transcriptional factor, able to up-regulate the transcription of many genes including programmed death-ligand 1 (PD-L1), we evaluated PD-L1 levels in Myc-overexpressing clones. *MYC* overexpression increased the level of PD-L1 as detected by flow cytometry compared to parental cells (Figure 4*D*) [9].

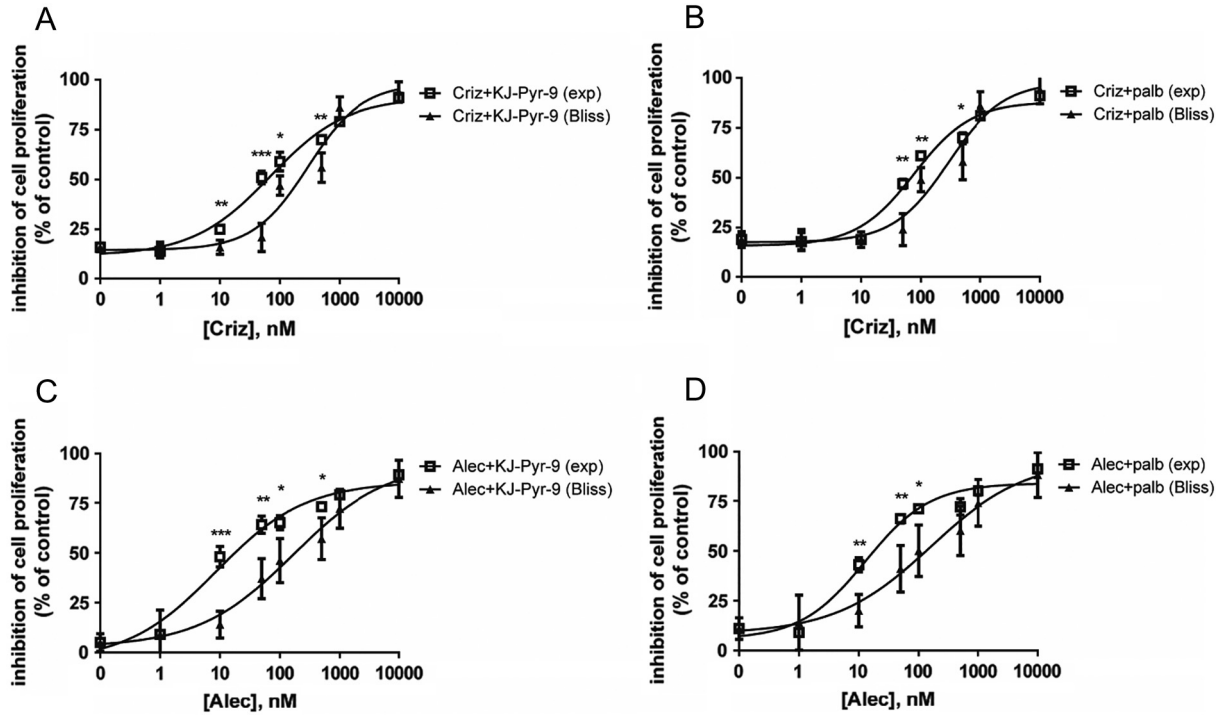

Figure 5. Effect of the combination of ALK inhibitors with Myc inhibitor or palbociclib in Myc- overexpressing cells. CI1 cells were treated with increasing concentrations of crizotinib (A, B) or alectinib (C, D) in absence or presence of 10 μM KJ-Pyr-9 (Myc inhibitor) or 100 nM palbociclib respectively. After 72 h cell proliferation was assessed by CV assay and the effect of drug combination was evaluated using the Bliss interaction model. Data are expressed as percent inhibition of cell proliferation versus control cells and are representative of three independent experiments. (*P < .05, **P < .01, ***P < .001 versus Bliss curve; Student's t test).

Moreover, considering the effect of MYC overexpression on increased level of cyclins D/E, we tested the effect of Palbociclib (Selleckchem), a highly selective inhibitor of cyclin dependent kinase CDK4/6. Like KJ-Pyr-9, also Palbociclib induced a synergistic effect on inhibition of cell proliferation when combined with Crizotinib or Alectinib (Figure 5, *B* and *D*).

Discussion

Diverse mechanisms of acquired (secondary) resistance to ALK-inhibitors in ALK-rearranged NSCLC have been described, including ALK-dependent ones, such as TK domain mutations or amplification of ALK gene, and ALK-independent ones, such as EGFR, MET and PI3K mutations or amplifications, which activate bypass signaling pathways [10]. In addition, few ALK-rearranged NSCLC patients fail to respond already at first exposure to TKI treatment suggesting the possibility, albeit rare (less than 5% in first-line Crizotinib trials), also of the presence of an innate (primary) resistance to ALK-inhibitors. Mechanisms of intrinsic resistance are poorly understood, representing an important unmet research need in the field of ALK TKI resistance [11].

In our case, the presence of ALK rearrangement together with *MYC* amplification has been confirmed with different techniques (FISH and NGS) in different tissue samples (baseline tumor cytology, recurrent tumor cytology and CTCs), suggesting a role for *MYC* in the innate resistance to Crizotinib. Our pre-clinical in-vitro work lead to hypothesize that MYC amplification could represent one of the possible causes of primary resistance to ALK-inhibitors. To our knowledge, this finding has not been previously described in ALK-rearranged NSCLC. Although our data are based on a single clinical case, given the rarity of this condition and the robustness of our preclinical work, we believe that they strongly support the role of *MYC* co-alteration in primary Crizotinib resistance and warrant further research in this area. Hence, our data are in keeping with the observation made in a recent study, carried out with a whole genome short-hairpin (shRNA) screen in ALK rearranged lung cancer cell lines, indicating MYC binding protein as a determinant of crizotinib sensitivity [12].

MYC is known to be a potent activator of oncogenic transcription programs and its role in promoting tumor growth in multiple tumor types is well known. The effect of copy number alterations on treatment resistance has already been shown in other tumors and emerging data seem to suggest that *MYC* overexpression is also associated with drug resistance in different tumor types [13].

In the study of Pilling et al. RNAi-mediated silencing of MYC increased sensitivity to ALK inhibition, providing further support to the hypothesis of a cooperative role of *MYC* and ALK in oncogenic signaling and therapy resistance [12]. Likewise, in our pre-clinical study the combination of the *MYC* inhibitor KJ-Pyr-9 with Crizotinib or Alectinib showed effectiveness in overcoming resistance in *MYC*-overexpressing cells.

We recently demonstrated that palbociclib, a highly selective inhibitor of CDK4/6, reduced *MYC* expression, as a result of E2F transcription factor inhibition, in both mesothelioma and breast cancer cell lines [14,15]. We assessed the effect of combined Palbociclib with Crizotinib or Alectinib, observing a synergistic effect on the inhibition of cell proliferation on ALK-rearranged NSCLC cells overexpressing *MYC*.

On technical grounds we demonstrated the robustness and the reliability of the Cell-Search/Dep-Array system for the isolation and the characterization of CTCs in NSCLC blood samples.

In conclusion, our data indicate that *MYC* amplification may be responsible for primary resistance to crizotinib and pave the way to exploration of *MYC*-directed inhibition strategies including CDK4/6 inhibition to improve clinical outcome of target therapies in advanced ALK-rearranged NSCLC.

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