A Novel Linc00308/D21S2088E Intergenic Region ALK Fusion and Its Enduring Clinical Responses to Crizotinib

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Approximately 5% of patients with NSCLC present ALK gene rearrangements, which define a distinct molecular subgroup of NSCLC.1 Nevertheless, in addition to the classic ALK fusion partner EML4, other emerging ALK fusion partners bring great challenges to the targeted crizotinib therapy in clinics.2 Herein we report a novel ALK fusion partner, the Linc00308/D21S2088E intergenic region, that conferred responsiveness to crizotinib in a patient with lung adenocarcinoma (LUAD).

A 61-year-old Chinese man with smoking history was found to have an irregular nodule, predominantly in the hilum of the lung, under computed tomography. Tissue biopsy (Fig. 1A) and subsequent imaging were carried out, resulting in a diagnosis of IIIB adenocarcinoma. In the absence of a standard treatment in this setting, the patient’s formaldehyde-fixed paraffin-embedded (FFPE) tissue was submitted for genomic testing by targeted next-generation sequencing to uncover genetic alterations in the initial tumor. The mutation profile revealed a novel rearrangement variant generated by a fusion of the Linc00308/D21S2088E intergenic region on 21q21.1 to the intron 19 of ALK on 2p23 (Fig. 1B). According to the DNA sequencing structure, the fusion transcript consisted of the intergenic region (novel exon) and the exons 20 to 29 of the ALK gene, the latter encoding the complete ALK intracellular kinase domain (Fig. 2A and B). This fusion product was further validated at the mRNA level by reverse-transcriptase polymerase chain reaction and Sanger sequencing of the tumor FFPE RNA sample (Fig. 2C and D). Before treatment with crizotinib, the FFPE sample was reconfirmed by conventional fluorescence in situ hybridization assay and immunohistochemistry (ALK D5F3 antibody) (Fig. 2E–G). Then, the patient was initiated on oral crizotinib therapy at a dosage of 250 mg twice daily. Computed tomography scans revealed considerable tumor shrinkage of the target lesions after 4 months, with the longest diameter of lung mass decreasing from 2.93 to 1.92 mm and sustained response after 6 months (Fig. 3A). Simultaneously, compared with the baseline circulating tumor...
cells level (13/mL) (Fig. 3B), the circulating tumor cell number significantly decreased at 4 months after therapy and remained at 1/mL for a period of 4 months (from April 2019 to August 2019). Repeated circulating tumor DNA analysis revealed that the mutant allele frequency of the intergenic region ALK fusion in the plasma was closely associated with the tumor burden, accurately reflecting the dynamic tumor response to crizotinib (Fig. 3C). Currently, crizotinib is being continued with good tolerance. In addition, as shown in Figure 4A and B, crizotinib treatment significantly inhibited ALK phosphorylation at position Y1507 and Y1604 in LUAD cells isolated from fresh tumor tissue. Moreover, the viability of intergenic region ALK-positive LUAD cells was highly suggestive of crizotinib treatment in vitro (Fig. 4C) and in vivo (Fig. 4D). To further assess the function of this novel ALK fusion protein in oncogenesis, plasmids expressing intergenic region ALK fusion protein were transfected into Ba/F3 cells whose growth is dependent on interleukin-3. In the absence of interleukin-3, Ba/F3 cells expressing intergenic region ALK grew at an exponential rate, confirming the kinase-dependent oncogenic activity of intergenic region ALK (Fig. 5A). Simultaneously, self-phosphorylation of ALK at position Y1507 and Y1604 could be observed in the intergenic region ALK-expressing 293T cells (Fig. 5B), suggesting the constitutive activation of the intergenic region ALK fusion protein. In 293T cells overexpressing the intergenic region ALK, we observed the significantly increased phosphorylation levels of MEK1/2 in the MAPK pathway, AKT in the PI3K-AKT pathway, and STAT3 in the JAK-STAT pathway (Fig. 5B and C). Meanwhile, the addition of crizotinib inhibited ALK phosphorylation and the activation of its downstream effector molecules, MEK1/2, AKT, and STAT3 in the intergenic region ALK-expressed 293T cells (Fig. 5B and C).

A vast array of oncogenic ALK variants are formed by the fusion of 3’-half of the ALK gene which retains its kinase catalytic domain, and the 5’-part of a partner gene that provides its promoter. Structural studies have revealed that fusion with multiple 5’ partners facilitates multimerization and autophosphorylation of ALK kinase and increases the oncogenic potential of ALK, as evidenced by EML4-ALK in NSCLC. In this study, we identified a new fusion partner of ALK, the Linco00308/Linc00308/D21S2088E intergenic region ALK fusion was discovered in a patient with lung adenocarcinoma. (A) Immunohistochemistry results revealed that the tumor cells were positive for TTF-1, Napsin A, CK7, and negative for P40. (B) Schematic of intergenic region ALK fusion mRNA and protein expression. The intergenic region fusion breakpoint of chr21 is located in the region between Linc00308 and D21S2088E. In this region, an exon was confirmed through transcription expression by RNA-Seq. CK7, cytokeratin 7; RNA-Seq, RNA sequencing; TTF-1, thyroid transcription factor-1.
D21S2088E (two noncoding genes\textsuperscript{5,6}) intergenic region, in LUAD. To our knowledge, an intergenic region between CENPA and DPYSL5-ALK fusion was reported previously.\textsuperscript{7} Nevertheless, they did not clarify whether the rearrangement affected the expression or function of the ALK gene. Here, we not only detected the presence of intergenic region ALK fusion transcripts, but also found that intergenic region ALK fusion could enhance ALK kinase activity and activate the phosphorylation of downstream AKT, MEK1/2, and STAT3 in 293T cells. Thus, we have provided proof that patients with advanced NSCLC harboring a Linc00308/D21S2088E intergenic region ALK may benefit from crizotinib, expanding the spectrum of ALK fusion variants to optimize treated strategy.

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Figure 3. Dynamic monitoring of the response of the patient with lung adenocarcinoma to crizotinib. (A) Chest CT scans on October 15, 2018 revealed the presence of an irregular nodule predominantly in the hilum of the lung. Significant (February 15, 2019) and (June 15, 2019) consistent reduction of the tumor volume was observed by the follow-up CT scans after the first-line therapy with crizotinib. (B) Noninvasive detection and monitoring of CTC number and CT scan during the patient’s clinical course. (C) Dynamic change of intergenic region ALK mutant allele frequency in ctDNA during the treatment course. CT, computed tomography; CTC, circulating tumor cells; ctDNA, circulating tumor DNA; NGS, next-generation sequencing; po bid, orally twice a day.

Figure 4. Evaluation of drug sensitivity of Linc00308/D21S2088E intergenic region ALK fusion to crizotinib. (A) LUADCs were isolated from fresh tumor tissues of the patient with intergenic region ALK fusion using the Thermoresponsive NanoVelcro system. Cell passage of LUADCs in vitro did not affect ALK status. (B) ALK protein was detected in LUADCs and its phosphorylation level was decreased by crizotinib. (C) A line graph of MTT assay revealed that the proliferation of LUADCs was significantly inhibited by crizotinib compared with DMSO control. (D) Crizotinib suppressed tumor formation of LUADCs in mice. *p < 0.01. LUADCs, Lung adenocarcinoma cells.
References


Figure 5. The oncogenic activity of Linc00308/D21S2088E intergenic region ALK fusion and its effect on ALK self-phosphorylation and the phosphorylation of other key proteins located in its downstream signaling pathways, including MEK1/2 in the MAPK pathway, AKT in the PI3K-AKT pathway, and STAT3 in the JAK-STAT pathway. (A) Intergenic region ALK-overexpressed Ba/F3 cells grew in an interleukin-3-independent manner, which could be inhibited by crizotinib; whereas Ba/F3 cells with empty vector did not. (B, C) The phosphorylation levels of ALK, MEK1/2, AKT, and STAT3 in 293 T cells could be inhibited by crizotinib. 293 T cells were transfected with pcDNA3.0-intergenic region ALK and empty vector, respectively. Representative results are from three independent experiments. *p < 0.01. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; po bid, orally twice a day.