The Generation of an EML4-ALK Fusion NSCLC Isogenic Cell Line Relevant for Drug Discovery and Development

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Abstract

Recent studies show that tumor cells derived from a subset of patients with non-small-cell lung cancer (NSCLC) harbor the echinoderm microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) fusion oncogene; the result of a Paracentric chromosomal inversion on the short arm of chromosome 2. The EML4-ALK oncogene, like other ALK fusion oncogenes, is a druggable target that is responsive to ALK inhibitors. However, there is a lack of EML4-ALK *in vitro* models for drug screening. Here we set out to generate an isogenic EML4-ALK fusion non-small cell lung cancer model in the A549 lung cancer cell line, which harbors other naturally occurring genomic aberrations inherent in non-small cell lung cancer. This model could serve as a clinically relevant drug screening cell model. In this study, we utilized the CRISPR/Cas9 genome editing platform to target endogenous loci in human cells and create the intended genomic translocation event. By employing sgRNAs-Cas9 constructs designed to cut precisely at relevant translocation breakpoints, we induced cancer-relevant genomic rearrangements that resulted in the expression of EML4–ALK fusions. Breakpoint junction analysis tested after sgRNA-CRISPR/Cas9 mediated genomic DNA cleavage in A549 cells showed the successful creation of the EML4-ALK fusion found in tumor cells from a subpopulation of NSCLC patients. Furthermore, single clonal isolation and functional screening demonstrated that the EML4-ALK isogenic cell line was sensitive to ALK inhibitors relative to the parental A549 cell line. This newly developed EML4-ALK isogenic lung cancer cell line could provide a very useful tool for oncology drug discovery and development.

I. Background information: ALK is a drug target and a diagnostic marker

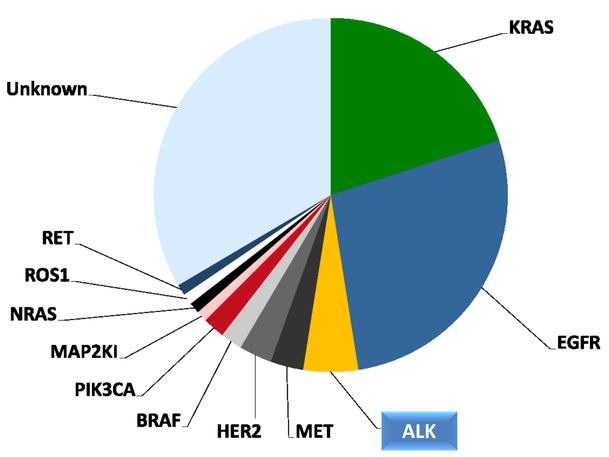


Figure 1. The Anaplastic Lymphoma Kinase (ALK) gene regulates cell growth and plays an essential role in the development of the brain by helping with the proliferation of nerve cells. When the ALK gene acquires gene specific mutations, forms a fusion gene with several other genes, or gains additional gene copies, it becomes oncogenic. This ALK genetic abnormality is a key oncogenic driver, especially in non-small cell lung cancer accounting for 3–7% of NSCLC cases observed in the United States. Recent studies of NSCLC patients have shown that a subset harbor the genetic variation involving EML4-ALK gene fusion.

II. Introduction to the EML4-ALK gene fusion: The EML4-ALK gene fusion is a gain of function mutation

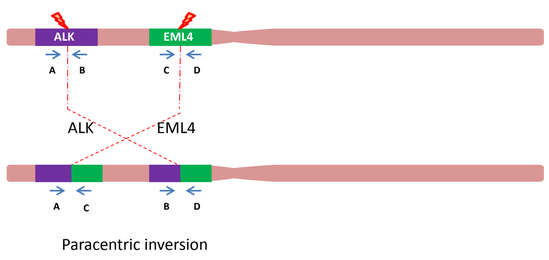
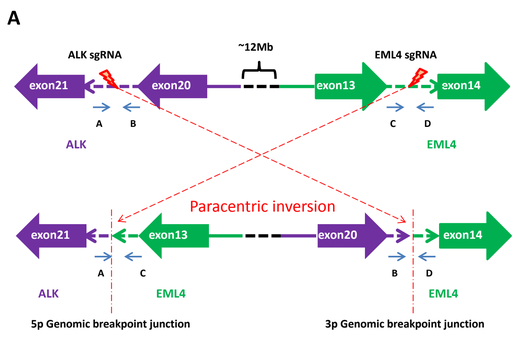
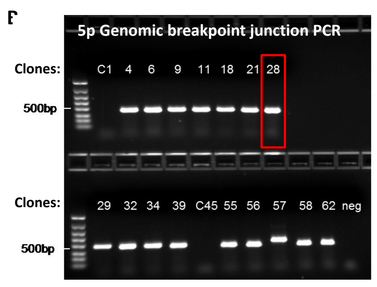


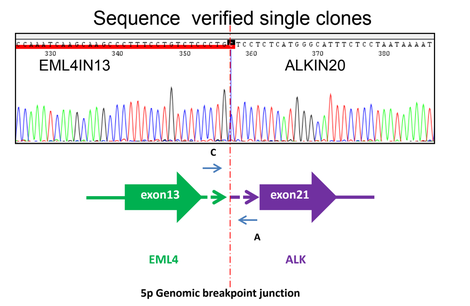
Figure 2. The EML4-ALK gene fusion caused by a chromosomal inversion, can produce constitutively active ALK tyrosine kinase protein, which leads to enhanced cell survival and cell proliferation. Particularly, the EML4-ALK fusion has been identified as important drug target and diagnostic biomarker. There are multiple EML4-ALK fusion variants, with the most prevalent being variant 1 (E13; A20) in which EML4 intron 13 is fused with ALK intron 20. A', 'B', 'C' and 'D' denote primer locations.

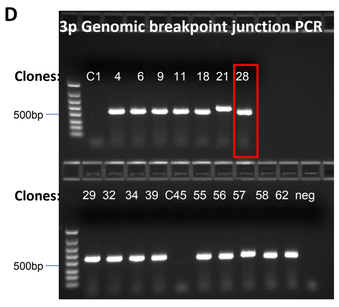
III. Project design and execution: Design and constructed CRISPR/Cas9 reagents to generate EML4-ALK gene fusion in A549 cells (ATCC - CCL-185)

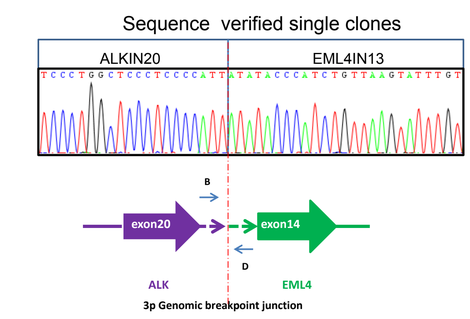
Figure 3. Identification of sgRNA target sites at EML4 and ALK genomic loci. **A**. SgRNAs designed and built to guide Cas9 to bind and cut desired intronic regions in the EML4 and ALK gene targets can trigger the paracentric genomic rearrangement event upon co-transfection. 'A', 'B', 'C' and 'D' denote primer locations. Transfected cells are sorted into single cells and expanded for screening of gene translocation events by junction PCR across the 5p & 3p genomic breakpoint junctions as shown. The occurrence of a positive ~500bp PCR band for any isoloated single cell clone using the primers 'A' and 'C' (**B**) as well as 'B' and 'D' (**D**) suggests the successful generation of an EML4-ALK fusion product. (**C, E**) A representative sequencing result for the 5p & 3p genomic junction PCR respectively, constitutes a confirmation of the accuracy of the generated EML4-ALK fusion product. Boxed in red is the ultimate selected clone 28 (ATCC® CCL-1851G™). In **B** & **D** clones C1 & C45 are negative for the EML4-ALK gene fusion product.



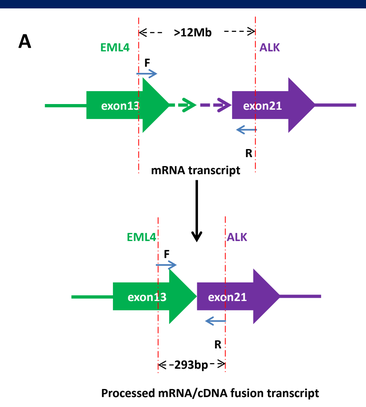


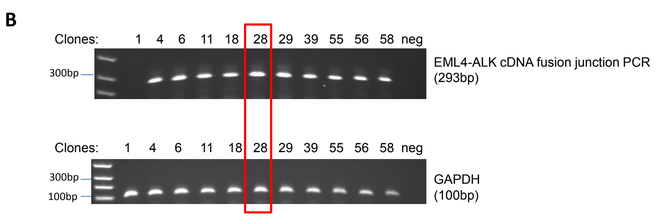






IV. Confirmation of EML4-ALK fusion mRNA expression in isolated single clones





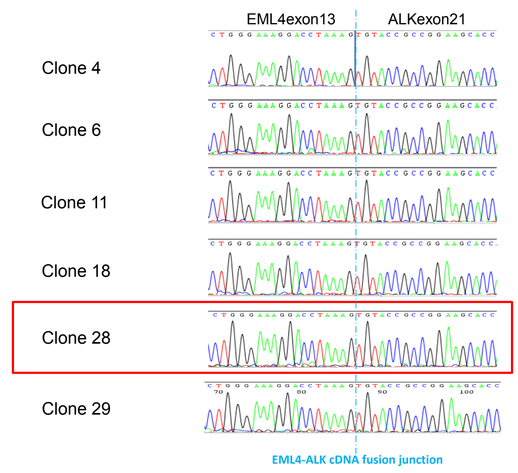


Figure 4. **A**. Transcription of the EML4-ALK fusion gene is expected to yield a nascent EML4-ALK mRNA, which is further processed into a relatively shorter mature mRNA transcript via splicing. The generation of cDNA from prepared mRNA isolated clones is expected to yield a PCR product of 293bp (with the F & R primer pair) only upon the occurrence of an EML4-ALK fusion transcript. **B.** EML4-ALK cDNA fusion junction PCR for isolated clones. A 100bp cDNA PCR product of GAPDH is used here as a PCR control. **C**. Sequence of EML4-ALK fusion transcript across cDNA breakpoint for genotyped isolated clones shown in 'B' above. Boxed in red is the ultimate selected clone 28 (ATCC® CCL-1851G™). The cyan dashed line represents the EML4-ALK cDNA fusion junction.

V. Confirmation of EML4-ALK fusion protein expression in isolated single clones

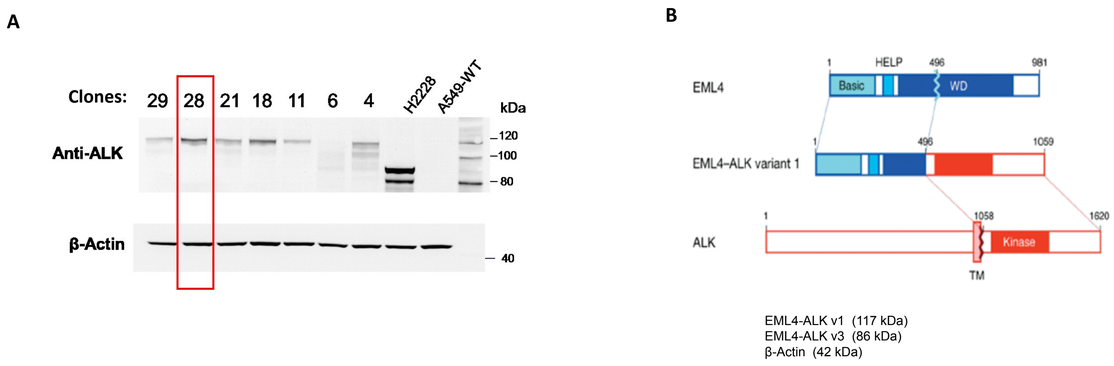
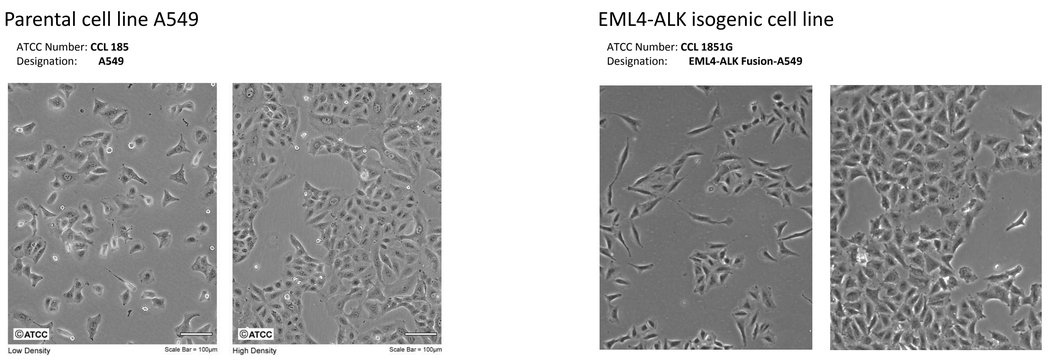
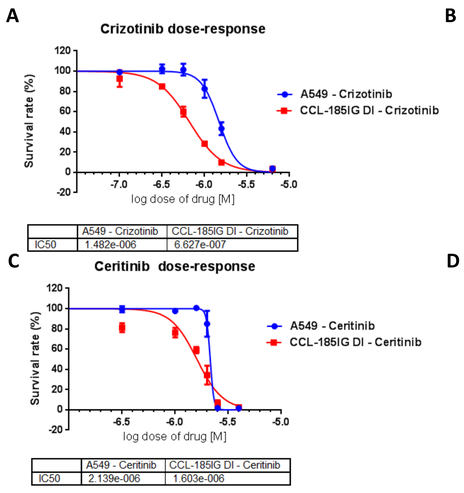


Figure 5. Verification of the expression of the EML4-ALK fusion protein by the EML4-ALK fusion gene. **A**. Western blot of isolated EML4-ALK v1 (117kDa) clones along with WT-parental **A549** cell line and **NCI-H2228** (positive EML4-ALK control) EML4-ALK v3 (86kDa) cell line; 40ug of protein from cell lysates were analyzed on a 4-12% Bis-Tris NuPage gel (Life Tech.) transferred to nitrocellulose and probed with anti-ALK (Cell Signaling Tech. #3633, 1:1500) or β-Actin (Sigma-Aldrich A5441) anti-bodies. Boxed in red is the ultimate selected clone 28 (ATCC® CCL-1851G™). **B** . A cartoon depiction of EML4 and ALK genes compared to the EML4-ALK fusion variant 1.

VI. Comparison of the morphology of isogenic clone and parental cell line: The morphology of isogenic clone 28 is similar to the parental line A549



VII. Relative drug response of isogenic clone: EML4-ALK A549 isogenic clone 28 is sensitive to ALK inhibitor drugs



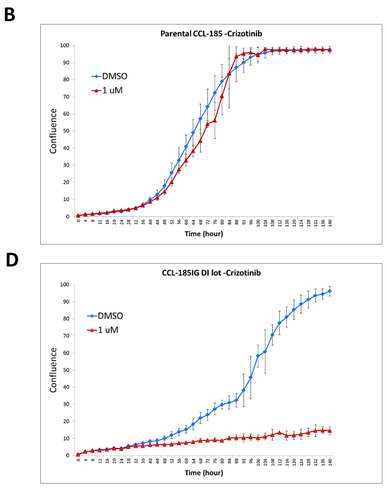


Figure 6. Dose response curves for cells treated with ALK inhibitors Crizotinib and Ceritinib. **A, B, D.** Isogenic clone 28 has selective drug sensitivity to ALK inhibitors Crizotinib and Ceritinib (**C, E**) relative to the parental A549 cell line. This trend is consistent irrespective of whether it is a dose-response based assay (**A, B, C, D**) the CellTiter-Glo lumniscent cell viability assay (**E, F**).

VIII. CONCLUSION

- The ATCC isogenic cell line CCL-185IG derived from the A549 NSCLC cell line (CRL-185), contains the EML4-ALK fusion variant 1 (E13; A20). It has been validated at the genomic, transcript and protein levels.
- Furthermore, CCL-1851G is more sensitive to ALK inhibitors crizotinib and ceritinib when compared to the A549 parental cell line. CCL-185IG can be a useful model to study the tyrosine kinase signaling pathway, and to screen for novel ALK inhibitors in anti-cancer drug discovery and development.