CRISPR/Cas9 based manipulating oncogenic chromosomal changes in vivo

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Abstract---Chromosomal changes are important factors in the development of human cancers, leading to the creation of gene fusions that can be targeted by therapeutic treatments. One such gene fusion is EML4-ALK, which results from an inversion on chromosome 1 and is found in a subset of non-small cell lung cancers. This gene fusion is clinically significant because it makes the cancer cells sensitive to ALK inhibitors. However, modeling these genetic events in mice has been difficult and requires complex manipulation of the germ line. This study presents an efficient method of inducing specific chromosomal changes in vivo using viral-mediated delivery of the CRISPR/Cas9 system to somatic cells of adult animals. The method was used to create a mouse model of EML4-ALK-driven lung cancer. The resulting tumors consistently exhibited EML4-ALK inversion, expressed the EML4-ALK fusion gene and had molecular features of human NSCLCs and react to handling with ALK inhibitors. The capacity to simulate human malignancies in mice is substantially improved by this method.

Keywords---EML4-ALK, NSCLC, RT-PCR, CT Scan, CRISPR/Cas9.

1 Introduction

Mouse that have been genetically altered are frequently utilized as disease models in scientific studies. Mouse make an excellent model for genetic alteration and research since they share nearly all of the same genes as humans and have similar tissues and organs. They are also more readily available in a large number of genetically homogeneous strains than other animals, which is advantageous for study. A further benefit of their size is that it is possible to house and keep them in huge numbers, which lowers the cost of experimentation and research. The most prevalent variety is the knockout mouse, in which one or more genes are made inactive. Mouse models that have been genetically engineered to mimic human cancers are essential for understanding the molecular mechanisms...
behind tumorigenesis and for studying drug sensitivity and fighting. While gain and loss of function mutations found in human cancers can be replicated using current gene-targeting technologies, recreating chromosomal rearrangements that result in oncogenic gene fusions has been challenging.\textsuperscript{1-6}

The fusion of the anaplastic lymphoma kinase (ALK) with the echinoderm microtubule-associated protein-like 4 (EML4) was identified in 2007 in Japanese non-small cell lung cancers (NSCLC). Additional studies, mostly involving East Asian patients, have reported that between 3\%–13\% of lung tumors harbor EML4-ALK fusions. By extrapolation this would suggest that approximately 5\% of all NSCLC cases contain an EML4–ALK translocation.\textsuperscript{7-15}

The most common approach to studying these gene fusions involves the expression of fusion oncoproteins from transgenes. However, this method results in the fusion protein being expressed at non-physiological levels, and neither the role of reduced dosage of the wild-type alleles nor the contribution of the reciprocal product of the translocation can be examined. The clinical significance of the gene fusion comes from the fact that it makes the cancer cells susceptible to ALK inhibitors. It has been challenging to model these genetic occurrences in mice and calls for intricate germ line manipulation. This paper describes an effective technique for delivering the CRISPR/Cas9 system to adult animals’ somatic cells using a virus to cause particular chromosomal modifications in vivo. Using this technique, a mouse model of lung cancer caused by EML4-ALK was produced.\textsuperscript{16-20}

![Mouse Model for Cancer](image)

Figure 1: Mouse Model

Cancer is a complex process that involve many parts of the human body. Murine model can be used to investigate the effect of drugs on cancer. Cancer cells are implanted into the mouse to check the genetic manipulation and therapeutic intervention.

2 Materials and Methods

2.1 Ethics report: Before the study began, verbal agreement was obtained in accordance with the Helsinki statement.
2.2 Crispr-mediated assay:
All of the primers and the CRISPR RNA (crRNA) from Sangon Biotech (Shanghai, China). Some reagents were purchased from New England Biolabs (NEB) include Cas9, DNase I, Hiscribe® T7 High Yield RNA Synthesis Kit. Takara Bio Inc. (Dalian, China) bought RNase inhibitor and Taq Hot Start Version. The Qiagen RT-PCR kit was purchased from USA. RNA clean XP beads was purchased from Beckman Coulter Inc. (Indianapolis, IN, USA). The clinical samples were gifted from one of the colleague. The study was observed according to the Helsinki declaration and WHO guidelines.

2.3 RT-PCR-CRISPR:
For the RT-PCR test, a QIAGEN kit was used. The assay contained 1 × QIAGEN Buffer (Mg²⁺ plus), 400 μM of each dNTP, 0.6 μM of each primer (F and R), 2μl of QIAGEN Enzyme Mix, 1 × EVA™Green Dye, 1μl of in vitro transcribed RNA template or 20μl of RNA from clinical samples and RNase-free water up to 50 μl with Crispr / Cas9 assay was added according to the protocol. Thermal cycling was performed by first performing reverse transcription for 30 minutes at 50 °C, activating HotStar Taq DNA Polymerase for 15 minutes at 95 °C, and then performing 40 cycles of denaturation at 92 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for one minute. Following amplification, 2μl of the amplified products were electrophoresed. Sample was also analysed with naked eyes by using the colorimetric method.

2.4 Primers: PCR: EML4 F-Primer: CGCAACTCCGCGGTGCA, ALK R-Primer: GAGATGAGCACAAGC, Synthetically guided RNA: CGCCGCGCGGAGGAAAG, Pyrosequencing Primer (EML4 F-Primer: GGAGAACGCAACTCCCGCCGT, ALK R-Primer: BIOTIN-GTTGATGCTCTCAGTCTCT)

2.5 Vector: To construct the necessary vectors for this study, the pX330 vector expressing Cas9 (Addgene plasmid 42230) was first digested with BbsI and then ligated to sgRNA oligonucleotides targeting Emf4-Alk, which had been annealed and phosphorylated. To create the Adeno-Emf4-Alk. The cells were transfected with a total of 2 mg of plasmid DNA per well using lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Recombinant adenoviruses were generated by Viraquest (Ad-EA and AdCas9).

2.6 MEF quantification: Initially, isolated a sub clone of NIH/3T3 cells that contained a mono-allelic Emf4-Alk inversion, which was confirmed using interphase FISH, then created a series of standards with known percentages of Emf4-Alk alleles by mixing the genomic DNA from this clone with increasing amounts of genomic DNA from the parental NIH/3T3 cells. To determine the fraction of Emf4-Alk alleles in test samples, performed quantitative PCR using primers amplifying the Emf4-Alk junction.

2.7 Cell lines: Standard procedures were used to create MEFs from wild-type embryos.
2.8 Mice and adenoviral infection: The obtained mice from different sources. To infect the mice with adenovirus, they were anesthetized and given the virus through intratracheal instillation. A known adenovirus was being used for each mouse. All procedures and studies involving the mice were approved by the Committee.

2.9 FISH experiment: FISH experiments were performed and interpreted by the cytogenetic core using a probe to detect and discriminate between Eml4-Alk fusion and other rearrangements of Alk. Probe fluorescence detection were done according to standard procedures. Cell line collection and metaphase spreads were prepared according to standard cytogenetics procedures.

2.10 Experimental analysis: The DNA sequence surrounding the target location of the CRISPR/Cas9 system was amplified using a technique called PCR. The resulting DNA fragment was then exposed to a process of heating and cooling to form a structure known as a heteroduplex. This heteroduplex DNA was then treated with an enzyme called evaluators nuclease, following the manufacturer’s instructions, at a temperature of 40 degrees Celsius for one hour. This enzyme specifically cuts the heteroduplex DNA at mismatched base pairs, creating fragments of various sizes. The resulting DNA fragments were then separated by size using a 2% agarose gel, allowing for the analysis and detection of potential mutations induced by the CRISPR/Cas9 system. After amplification pyrosequencing was also performed and achieved the targeted result.

2.11 Preparation of lung tissue samples and antibody based detection for immunohistochemistry: The lungs were expanded by intratracheal injection of a probably 4% solution of paraformaldehyde (PFA), and then incubated for 20-24 hours in a 4% PFA solution. Afterward, they were transferred to a 70% ethanol solution and left for at least 24 hours before being subjected to further processing. Several antibodies were employed for this study, phospho-Erk1/2 (Thr202/Tyr204) from Cell Signaling Technology at a concentration of 1 mg/ml etc.

2.12 Crizotinib (Tyrosine Kinase Inhibitor) treatment: The study involved randomizing mice to receive either a control vehicle (water) or crizotinib at a daily dosage of 50 mg per kg per os for a minimum of 14 days. The mice were observed daily for any indications of weight loss or clinical symptoms.

2.13 Western blot and CT scan analysis: RNA samples that were previously extracted using TRlzol (Life Technologies) were subjected to perform the western blotting. CT scan was performed to observe the lungs of mice.

3 Results and Discussion

Novel genome-editing technologies, such as CRISPR/Cas9, offer a more flexible approach to producing precise genomic changes, including oncogenic chromosomal rearrangements.
Figure 2: Eml4–Alk fusion. (A) The figure shows the successful induction of Eml4–Alk (Homo sapiens chromosome 1, GRCh38 reference primary assembly, GenBank: CM000663.2) rearrangement using the CRISPR-Cas9 system. (B) shows a schematic of the serial PCR result. Panel (C) is gel bands from genomic DNA. In panel (D), the pyrosequencing product showing the correct Eml4–Alk junction.

Hydrogen ions (H⁺) based detection: PCR was used to recognize the hydrogen ions (H⁺) after amplification. When dNTPs were added to the expanding strand during amplification, hydrogen ions (H⁺) were generated. A pH indicator that is often used in laboratories is phenol red (Phenolsulfonphthalein) (Sigma-Aldrich). Few drops of phenol red were used with 20µl PCR sample to detect the hydrogen ions (H⁺). In order to change the color of yellow, phenol red was utilized in a reaction with hydrogen ions (H⁺). The ability to distinguish between positive and negative samples visually were made possible by the reaction mix’s inclusion of phenol red.
Figure 3: Chemical structure of phenol red (Phenolsulfonphthalein) with hydrogen ion (H\(^+\)). (A) The phenol red was react with hydrogen ions (H\(^+\)) to change the solution in yellow form. The pH 6.8 to below pH range is where the phenol red color change from red to yellow begins. With the presence of hydrogen ions (H\(^+\)) in the positive sample and a pH shift that crossed the phenol red threshold in the original reaction mix, it was simpler to convert the sample solution's color to a yellow form for visual distinction. In panel (B) present the serial dilution of positive colorimetric result while NC is negative control in red color.

The research describe a method to create genetically engineered mice with specific chromosomal rearrangements that lead to the expression of oncogenic gene fusions. Specifically, targeted the EML4 and ALK genes, which are involved in a subset of human non-small cell lung cancers. By using the CRISPR/Cas9 system, they induced double-strand DNA breaks at intron 14 of Eml4 and intron 19 of Alk, leading to the desired Eml4-Alk inversion in mouse somatic cells. To optimize the method for in vivo use by engineering plasmids to simultaneously express Cas9 and two sgRNAs, allowing for efficient cleavage of the targeted sites and accumulation of the Eml4-Alk inversion.

To deliver Cas9 and sgRNAs to target specific loci in the lungs of adult mice, transferred the dual sgRNA/Cas9 cassette into an adenoviral shuttle vector and created recombinant adenoviruses, referred to as Ad-EA. Adenoviruses are ideal
for this purpose because they can efficiently infect the lung epithelium of adult mice and do not integrate into the host genome.

Two days and one week post-infection, the lungs appeared histologically normal with no obvious signs of cytotoxicity except for occasional inflammatory infiltrates. However, after Ad-EA infection, the lungs of mice of both strains presented multiple small lesions that upon histopathological examination appeared to be early well-differentiated adenocarcinomas. By 6-8 weeks post-infection, larger tumors were easily detectable by micro-computed tomography (mCT) and macroscopically visible at necropsy. At 12-14 weeks post-infection, the lungs of Ad-EA-infected mice invariably contained multiple large lesions histologically classified as lung adenocarcinomas.

In Ad-EA-infected animals, multiple bilateral lung tumors were frequently detected by 4-7 weeks post-infection, and invariably after 8 weeks post-infection. Whereas at the same time point, all Ad-EA infected mice had developed multiple tumors. These results indicate that intratracheal delivery of Ad-EA can initiate lung tumorigenesis with high penetrance and low latency, and that effect cannot be attributed to adenoviral infection or Cas9 expression alone.

The observed phosphorylation and nuclear localization in lung tumors derived from Ad-EA-injected mice. The tested sensitivity of Ad-EA-induced lung tumors to crizotinib and found that crizotinib caused complete or partial tumor regression. Overall, successfully engineered oncogenic chromosomal rearrangements in mice and created a mouse model of Eml4–Alk-driven lung cancer that accurately replicates the molecular and biological properties of human ALK1 NSCLCs. This model provides opportunities to study the mechanisms of Eml–Alk-driven tumor formation, test the efficacy of targeted therapies, and investigate drug resistance in vivo.
Figure 4: Shows the results of the experiment where Ad-EA-induced lung tumors were treated with crizotinib. The experiment was designed as shown in a schematic in panel (A). Mice (male and female) were treated with either crizotinib or vehicle, and their lungs were imaged using micro-Computed Tomography (mCT) before treatment and after 2 weeks of treatment. Representative images of lungs from mice treated with crizotinib or vehicle were shown in panel (B). The panel (C) magnification images of lung sections from two crizotinib-treated and two vehicle-treated were shown.

The method of inducing rearrangements in a subset of somatic cells allows for a more accurate representation of the stochastic nature of tumour formation in humans. Additionally, by modifying endogenous loci, the resulting fusion genes are subject to physiological transcriptional and post-transcriptional regulation, mimicking the reduced dosage of wild-type alleles and expression of reciprocal products seen in humans. This technique can be easily adapted for use in other species, including non-human primates, to study species-specific differences in tumour progression and therapy response in vivo. However, the low efficiency of inducing rearrangements and the potential complications arising from the induction of all possible allele combinations should be considered. Despite these caveats, our approach will greatly aid the development of pre-clinical models for testing novel therapies and studying mechanisms of drug resistance in cancers driven by chromosomal rearrangements.
Declaration of interest: Author declare no competing interest.

References


