CRISPR/Cas9-based manipulation of oncogenic chromosomal changes in vivo and drug impact on blood pressure

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***Abstract*---**Human malignancies develop in large part as a result of chromosomal alterations. These modifications might result in the production of gene fusions, which are aberrant combination of two distinct genes. Gene fusions are important cancer-causing factors because they can generate aberrant proteins that encourage unchecked cell proliferation and result in tumor development. Chromosome 2 inversions result in a specific gene fusion known as EML4-ALK, which is found in certain non-small cell lung cancer patients. The EML4-ALK gene fusion has clinical significance because it renders cancer cells susceptible to an exclusive class of medications known as ALK inhibitors. Targeted treatments called ALK inhibitors can selectively reduce the action of the aberrant ALK protein generated by the fusion gene. These drugs, which work by blocking ALK, can delay or stop the growth of cancer cells that have undergone the EML4-ALK fusion, providing a viable therapeutic option for individuals with this particular genetic change. The clinical significance of this gene fusion lies in its ability to render the cancer cells receptive to ALK inhibitors, enhancing their sensitivity to treatment. In this research, a novel approach for inducing targeted chromosomal changes in living organisms was introduced using the CRISPR/Cas9 system delivered by viruses to adult animal cells. A mouse model of EML4-ALK-driven lung cancer, a particular gene fusion seen in human non-small cell lung cancers (NSCLCs), was made using this technique. The EML4-ALK inversion and EML4-ALK fusion gene were persistently expressed in the mouse tumors, which also had certain molecular traits with human NSCLCs. Importantly, these tumor models responded to ALK inhibitor therapy, demonstrating their therapeutic applicability. While the effect of crizotinib on blood pressure was investigated in mice. Monitoring mice blood pressure enables comprehension and control of these effects. It also sheds light on the potential consequences of crizotinib on humans. This method greatly improves the

ability to study and mimic human malignancies in mice, making it a useful tool for cancer research and the development of new treatments.

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

1. Introduction

Scientific study frequently use genetically altered mice as disease models. Mice are well-suited for genetic alteration studies because they share a vast majority of their genes with humans and have similar tissues and organs. Mice are ideal for research due to their availability in a variety of genetically identical breeds. Their tiny size makes it possible to house and sustain them in huge numbers, which lowers the cost of tests and research. The most common kind of genetically altered mouse is called a knockout mouse, which has one or more genes turned off. Understanding the fundamental molecular underpinnings of carcinogenesis and researching medication responses and therapies requires the use of genetically modified mice that mirror human tumors. Despite the fact that current gene-targeting techniques may duplicate mutations that result in either enhanced or reduced function as seen in patient tumors, it has been difficult to duplicate chromosomal changes that result in cancer-causing gene fusions.[1-5]

It's crucial to research gene fusions to comprehend how they contribute to the growth of cancer. Using transgenes to express fusion oncoproteins is the most popular strategy, although it has limitations. The fusion protein is expressed at levels that are not physiological, which may not adequately reflect the body's normal physiological processes, and this is a significant problem. This method also restricts our ability to fully comprehend the impacts of the gene fusion since it prevents us from examining the effects of reduced amounts of wild-type alleles or the contribution of the reciprocal product of the translocation.[6-9]

The potential for gene fusions to make cancer cells susceptible to particular therapies, like ALK inhibitors, is what gives them clinical value. Nevertheless, it has proven difficult to develop mice models that precisely duplicate these genetic events, mostly because sophisticated germ line manipulation is required. Technically challenging and time-consuming, this procedure entails changing the genetic material in the reproductive cells of mice.[2, 3, 10-12]

The modelling issue is addressed in this paper with a promising approach that works well for employing a virus to introduce the CRISPR/Cas9 system into adult animals' somatic cells. The development of a model of lung cancer in mice triggered by the EML4-ALK gene fusion was made possible by this ground-breaking method, which enables precise chromosomal changes to be produced in vivo. By employing this technique, more thoroughly examine the processes, therapeutic potential, and response to ALK inhibitors of this gene fusion in a precise and pertinent context. This discovery creates new avenues for investigating different gene fusions and their function in the emergence of cancer, ultimately assisting in the creation of patient-specific targeted medicines. A drug called crizotinib may have an effect on the body's blood pressure levels. This medication is frequently given to treat specific malignancies, especially those brought on by genetic mutations. It's crucial to remember, though, that crizotinib may affect blood pressure, possibly causing it to rise or fall. Crizotinib can interact with specific biological processes and receptors that are involved in controlling blood pressure when take it. Blood vessel dilatation and constriction may change as a result of this interaction, which may have an impact on blood pressure levels.[4, 5, 13-15]

2. Materials and Methods

2.1. Ethical report: The Helsinki Declaration's principles were followed while obtaining verbal consent before the study began.

2.2. Crispr based assay: Sangon Biotech in Shanghai, China provided the CRISPR RNA (crRNA) and all of the primers utilized in this investigation. A number of chemicals, including Cas9, DNase I, and the HiScribe® T7 High Yield RNA Synthesis Kit, were bought from New England Biolabs (NEB). Taq Hot Start Version and RNase inhibitor were purchased from Takara Bio Inc. in Dalian, China. The Qiagen RT-PCR kit came from the United States. We bought RNA clean XP beads from Beckman Coulter Inc. in Indianapolis, Indiana, the United States. The study's clinical samples were kindly donated by a coworker. To ensure ethical standards and patient safety, the research was carried out in accordance with the WHO guidelines and the Helsinki declaration.

2.3. RT-PCR-CRISPR: A QIAGEN kit was used for the RT-PCR test, which contained all of the necessary parts for the experiment. Deoxyribonucleotide triphosphates (dNTPs),

forward and reverse primers (F and R), QIAGEN Enzyme Mix, EVATM Green Dye, and RNase-free water were among the ingredients used. In accordance with the procedure, either in vitro produced RNA template or RNA from clinical samples were added to the reaction along with the CRISPR/Cas9 test components. Multiple steps were taken during the thermal cycling process. The RNA was first transformed into complementary DNA (cDNA) by reverse transcription, which was carried out at 50 °C for 30 minutes. For the subsequent amplification, HotStar Taq DNA Polymerase was then initiated for 15 minutes at 94 °C. The amplification phase consisted of 40 cycles, involving denaturation at 92 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for one minute. Following amplification, the results were examined using electrophoresis, which makes it easier to see and classify the DNA fragments according to their sizes. Samples were also analysed with naked eyes by using the colorimetric method.

2.4. Primers: PCR: EML4 F-Primer: CGCAACTCCGCCGTTGCA, ALK R-Primer: GAGATGAGCACAAGCACT, single guided RNA: CGCCGCGCGGAGGAAAGATG, Sanger sequencing Primer (EML4 F-Primer: GGAGAACGCAACTCCGCCGT, ALK R-Primer: GTTGATGCTCTCACTCATCT

2.5. Vector: Starting with the pX330 vector encoding the Cas9 gene (a plasmid available from Addgene, labelled 42230), the required vectors for this study were created. An enzyme named BbsI was first used to break down the pX330 vector. Next, oligonucleotides with short guide RNA (sgRNA) specific to the Eml4-Alk gene were created. These sgRNA oligonucleotides underwent phosphorylation and annealing. The sgRNA oligonucleotides were then added to the digested pX330 vector and ligated together. The aim was to produce an Eml4-Alk-expressing recombinant adenovirus (Adeno-Eml4-Alk). Following the manufacturer's instructions, the study's cells were transfected with 2 mg of plasmid DNA total per well using the lipofectamine 2000 transfection reagent from Invitrogen. The created plasmids were introduced into the cells using transfection. Finally, Viraquest, a business that specializes in the creation of viral vectors, created the recombinant adenoviruses (Ad-EA and AdCas9). These adenoviruses would act as carriers for genetic material delivery into the target cells, in this case cells expressing the Eml4-Alk gene.

2.6. Mouse Embryonic Fibroblasts (MEFs) quantification and cell lines: The Eml4-Alk inversion was only present in one copy in a particular subset of NIH/3T3 cells, using a technique known as interphase FISH. The genomic DNA extracted from the isolated cell clone was mixed with increasing quantities of genomic DNA from the original parental NIH/3T3 cells. This created a series of reference standards with known proportions of Eml4-Alk alleles. The employed a method known as quantitative PCR (polymerase chain reaction) to determine the proportion of Eml4-Alk alleles in their test samples and certain primers that could amplify the DNA's Eml4-Alk junction. While mouse embryonic fibroblasts (MEFs) were produced using normal techniques from wild-type embryos. Typically, these processes entail dissecting embryos, extracting the necessary tissues, and cultivating the cells in an appropriate environment so that they can multiply and create a viable cell line.

2.7. Mice, adenoviral infection and FISH experiment: Mice were obtained for the study from a variety of sources. These mice were anaesthetized before the adenovirus was injected into their lungs by intratracheal instillation. For the infection, each mouse was given a well-known adenovirus. The Committee gave its blessing to all protocols and experiments involving mice, verifying that the research complied with ethical standards and animal welfare laws. While the cytogenetic core facility carried out and evaluated the FISH tests. To identify and distinguish the Eml4-Alk fusion. Standard methods were used to detect the fluorescence. Standard cytogenetics procedures were used to collect cell lines.

2.8. Preparation of samples: The lung tissue samples were first treated with a 4% solution of paraformaldehyde (PFA) by injecting it directly into the trachea to lungs. To fix the tissues, the samples were treated in a 4% PFA solution for 20–24 hours. The samples were then put into a 70% ethanol solution and allowed to sit for at least 24 hours before being processed further. The immunohistochemistry was employed in the investigation with a number of antibodies. Phospho-Erk1/2 (Thr202/Tyr204) from Cell Signalling Technology served as one of these antibodies. Western blot and CT scan were also performed.

2.9. Drug action: In this study, mice were given a medication called crizotinib (Tyrosine Kinase Inhibitor) at a daily dose of 50 mg per kg orally for a minimum of 14 days. The

mice were attentively watched every day for clinical trial purpose. Due to meticulous monitoring, collect important information.

3. **Results and Discussion**

The ability to precisely modify the genome has been revolutionized by the development of new genome-editing tools like CRISPR/Cas9. These technologies offer a more flexible and effective way to make particular genetic alterations, such as intricate chromosomal rearrangements that can promote the growth of cancer. As a result of the new opportunities that CRISPR/Cas9 has created for researching and simulating oncogenic changes, scientists are now better able to understand the molecular processes that underlie cancer, which may eventually result in the creation of tailored treatments.

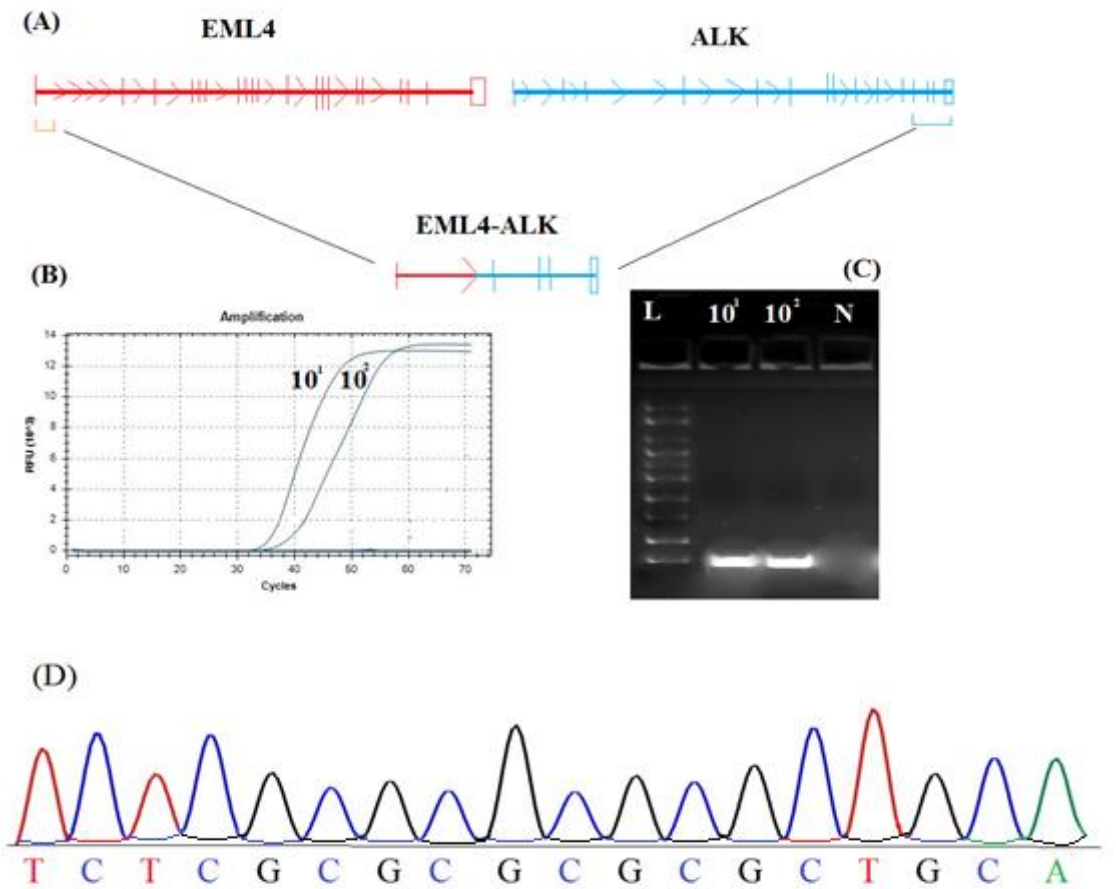

Figure 1: Eml4–Alk fusion. (A) The figure shows the successful induction of Eml4–Alk (Homo sapiens chromosome 1, GRCh38 reference primary assembly, GenBank: CM000663.2) rearrangement using the CRISPR-Cas9 system. In (B), there is a visual

representation of the serial PCR outcome. (C) Displays the gel bands derived from genomic DNA. In (D), the Sanger sequencing product reveals the accurate Eml4–Alk junction.

Method for detecting hydrogen ions (H^+): PCR was used to determine whether hydrogen ions (H^+) were present after amplification. The addition of dNTPs to the expanding DNA strand caused hydrogen ions (H^+) to be generated throughout the amplification process. A 20 µl PCR sample was combined with phenol red (Phenolsulfonphthalein), a pH indicator that is frequently used in laboratories (Sigma-Aldrich). When interacting with hydrogen ions (H^+), phenol red caused a color change in yellow. The visual distinction between positive and negative samples was made possible thanks to the addition of phenol red to the reaction mixture.

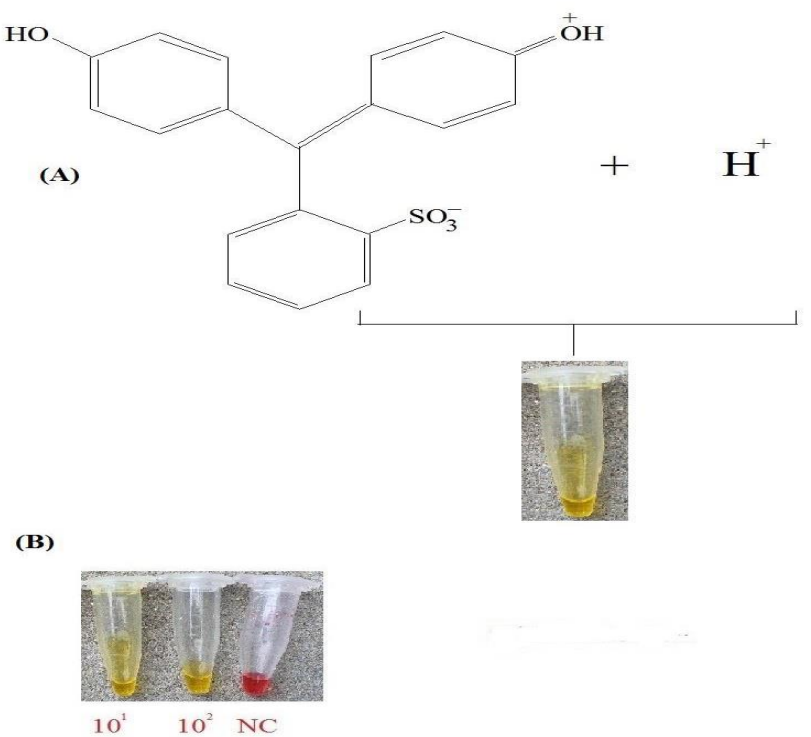

Figure 2: Chemical structure of phenol red (Phenolsulfonphthalein) with hydrogen ion (H^+). In (A), phenol red reacted with hydrogen ions (H^+) causing the solution to turn yellow. This color change occurred within the pH range of 6.8 to below, transitioning from red to yellow. In the presence of hydrogen ions (H^+) and a pH shift that exceeded the

phenol red threshold, positive samples underwent a color change to yellow, enabling easy visual differentiation. In (B), a serial dilution of positive colorimetric outcomes is displayed, with the negative control (NC) represented in red.

The research focused on a technique to produce genetically altered mice with particular chromosomal alterations that result in the production of oncogenic gene fusions. So EML4 and ALK genes, which are recognized to play a role in a selection of human non-small cell lung cancer. They created double-strand DNA breaks within the Eml4 and Alk genes using the potent CRISPR/Cas9 system, which caused the desired Eml4-Alk inversion in the mice's somatic cells. So created plasmids that simultaneously expressed guide RNA, Cas9, and other components to increase the technique' effectiveness for in vivo applications. This resulted in the creation of the Eml4-Alk inversion and efficient cleavage of the targeted sites, providing a useful tool for examining the genetic causes of lung cancer and perhaps even for the development of targeted treatments.

So used a sgRNA/Cas9 that was inserted into an adenoviral shuttle vector to perform targeted editing of particular regions in the lungs of adult mice. The Cas9 enzyme and the guide RNAs were then delivered to the targeted lung epithelial cells using recombinant adenoviruses known as Ad-EA. Because they can successfully infect the lung tissue of adult mice and because they can transport the editing components outside of the host genome, adenoviruses were chosen for this application. This method made it possible to precisely and carefully modify the targeted loci in lung cells, making it possible to explore gene functions and potential therapeutic uses in lung-related research.

With the exception of sporadic inflammation, the mice's lungs did not exhibit any notable changes two days or one week after contracting Ad-EA infection. There were also no clear indicators of damage. However, in the weeks that followed, the infected mice's lungs acquired numerous tiny lesions that, upon histological examination, turned out to be early, well-differentiated adenocarcinomas. Larger tumors started to become easily observable using micro-computed tomography (mCT) at 6-8 weeks after infection, and they were obvious to the unaided eye after necropsy. By 12-14 weeks after infection, the lungs of the Ad-EA-infected mice consistently contained a number of sizable lesions that were

histologically identified as lung adenocarcinomas. These results provide important insights into the carcinogenesis process and provide possible treatment targets for lung cancer. They show that Ad-EA infection caused the development and progression of lung adenocarcinomas in the mice. Further evidence that the CRISPR/Cas9 system in concert with adenoviral delivery is responsible for the observed lung cancer shows that this effect cannot be entirely attributable to adenoviral infection or the expression of Cas9 alone. These findings highlight the promise of this approach for investigating lung cancer and therapeutic approaches and offer compelling evidence for the role of the CRISPR/Cas9 system in tumor initiation.

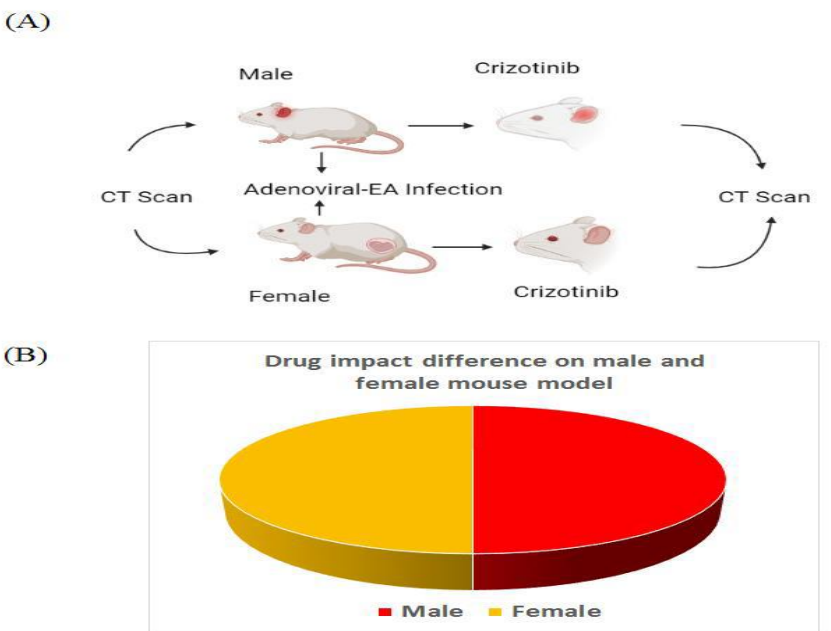

Figure 3: The experiment for lungs tumor treatment. Panel (A) shows how the experiment was set up. Mice with Ad-EA infections were given crizotinib medication and the status of their lungs was assessed using micro computed tomography (mCT) scans before and after the 2-week treatment period. In panel (B) drug impact difference on male and female mice tumor model was observed and not too much difference concluded after drug use. Crizotinib effects on blood pressure have been investigated in mice. Crizotinib drug targets certain genetic defects to treat a non-small cell lung cancer (NSCLC). Crizotinib

can affect mice's blood pressure levels when given to them, possibly causing them to rise or fall. Crizotinib interacts with a number of biological processes and receptors that control the constriction and dilation of blood vessels, which is thought to be the cause of its effects on blood pressure in mice. Changes in the mice's blood pressure may result from these interactions. After carefully tracking the blood pressure of mice receiving crizotinib, these side effects can be understood and controlled. It is possible to modify treatment plans and guarantee the welfare of the mice by keeping an eye out for any changes in blood pressure. This study contributes to a deeper understanding of crizotinib possible effects on human patients by offering insightful information about how the drug affects blood pressure regulation in a controlled experimental setting.

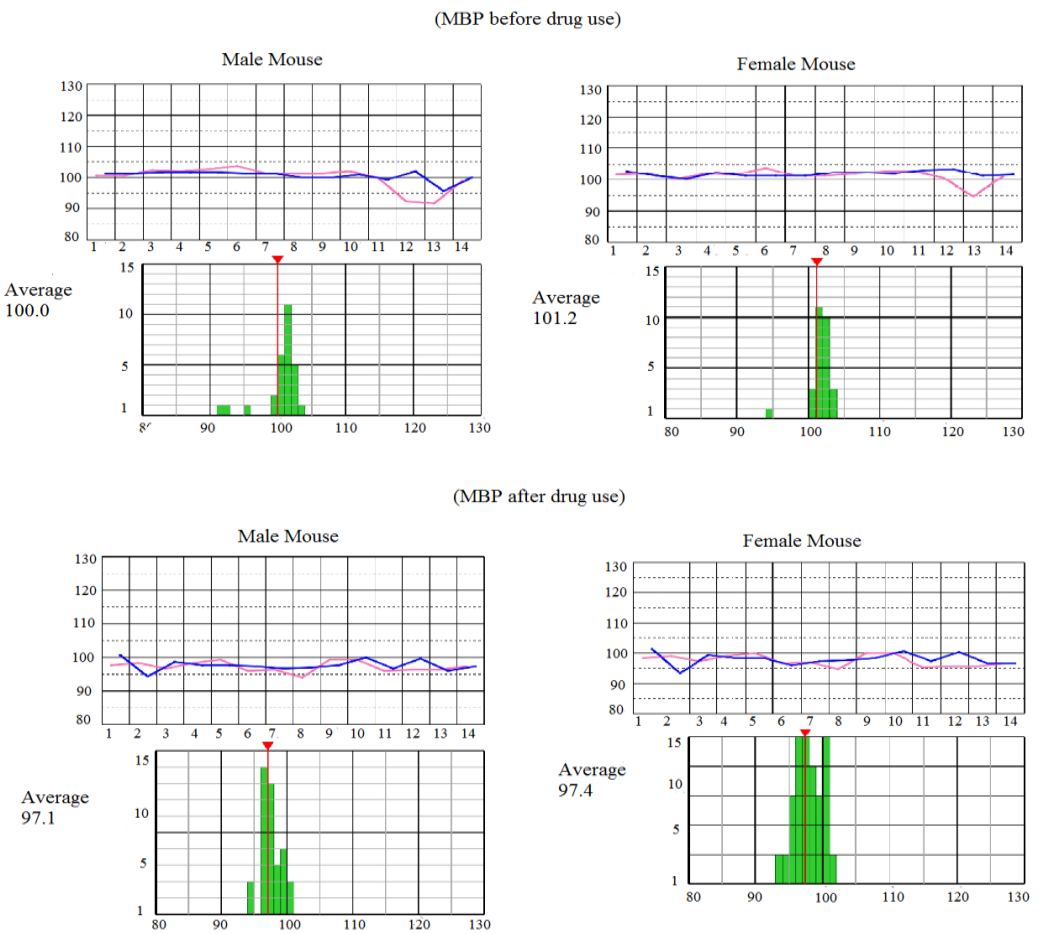

Figure 4: Mean blood pressure (MBP). MBP of mice (male and female) were taken day

(red line) and night (blue line) for two weeks to observe the crizotinib drug impact. For this purpose MBP was taken before and after drug use. Before drug use the MBP of male mouse was 100 and female mouse was 101.2 while after drug use male mouse was 97.1 and female mouse was 97.4 concluded. Before and after drug use the male mouse (100 to 97.1) and female mouse (101.2 to 97.4) data present that crizotinib impact on blood pressure was noticed and cause of decrease blood pressure according to the current study data.

A more accurate illustration of the random and stochastic nature of tumor formation observed in people is provided by the technique used to induce chromosomal reshuffles in a subset of somatic cells. This method is easily adaptable for use in non-human primates. However, it's critical to take into account the limited efficiency of inducing rearrangements as well as any potential difficulties that could arise from producing every possible allele combination. Despite these difficulties, this method has a substantial impact on the creation of pre-clinical models for the evaluation of innovative treatments and the investigation of drug in cancer resulting from chromosomal reshuffles.

Author declaration: The author declare that they have no competing interest.

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