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CRISPR/Cas9 mediated next generation gene therapy in chronic myeloid leukemia

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Present study, we aimed to manipulate the *BCR::ABL1* fusion gene, which is responsible for the etiopathogenesis of Chronic myeloid leukemia (CML), *in vitro*. Mechanism of this molecular pathogenesis is based on encoding the *BCR::ABL1*^{p210} oncoprotein with excessive and irregular tyrosine kinase activity and eventually causes the CML phenotype. On the other hand, patients developing drug resistance or the side effects of bone marrow transplantation on mortality and morbidity strengthened our hypothesis that CRISPR/Cas9 could be an advance in preclinical studies in this research. Cytogenetic and molecular genetic characterization of K562 cells has been performed. Intronic sequences were detected by sequencing in the translocation of the *BCR::ABL1* fusion gene. Genome editing in CML cells was done by transfection of lipofectamine and electroporation. The efficiency of CRISPR/Cas9 on the *BCR::ABL1*^{p210} was analyzed by qRT-PCR. Gene expression of the *BCR::ABL1*^{p210} fusion before and after CRISPR/Cas9 manipulation, which changed during the culture time, was compared logarithmic over the transcript values in the molecular response. We recorded that *BCR::ABL1*^{p210} manipulation showed an approximately 100-fold decrease in expression as (+1 log) before (-1 log) after CRISPR/Cas9 manipulation. Thus, the *BCR::ABL1*^{p210} fusion gene expression was significantly decreased by mediated CRISPR/Cas9 manipulation. As a result, the effect of the CRISPR/Cas9 genome editing was revealed *via* the knockdown of the *BCR::ABL1*^{p210} in our study. Thus, CRISPR/Cas9 can target the *BCR::ABL1p210* fusion gene due to the interference effect.

Keywords: *BCR::ABL1*^{p210} expression, Gene editing, Molecular response

Characterized by mutations in the genome, cancer is a genetic disorder at the cellular level with molecular etiopathogenesis. Chronic myeloid leukemia (CML) is the first neoplasm in which cancer was identified as a genetic disease. CML is a malignancy of hematopoiesis described by a single mutation developing in the progenitor stem cell in the differentiation of the myeloid cell line[1]. The mutation gives rise to *BCR::ABL1* fusion gene with upregulated tyrosine kinase activity, loss of proliferation control through the malignant transformation to apoptotic resistance, and CML. Tyrosine kinase inhibitor (TKI) molecules leading to inhibit the tyrosine kinase activity of the *BCR::ABL1* oncogene are in the first step of the CML therapy. However, in the long-term treatment of TKI, drug toxicity may develop; a complete cytogenetic response cannot be received by approximately 1/3 of the patients, or in some cases, resistance may develop due to mutations in the *BCR::ABL1* gene[2]. In those with resistance and cases where no permanent responses to TKI compounds are acquired, the cure is targeted with bone marrow transplantation (BMT). The treatment of BMT is preferable in those at an early age and with good general status in terms of organ functions[3]. However, the relationship between transplantation and mortality is highly correlated with overaged, especially in those with CML seen as an old age disease[4]. Therefore, more specific, cure-aiming, targeted treatment regimens where no morbidity and mortality correlations are witnessed are required.

Genome editing is a new generation gene therapy that can achieve modification in an organism

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triggered by DNA repair system. Cas9 enzyme led by guide ribonucleic acid (gRNA) for therapeutic use is targeted to induce double-strand break in DNA by recognizing the target sequences from the protospacer adjacent motif (PAM) regions[5]. Accordingly, non-homologous end-join (NHEJ) is mostly induced, and the DNA sequence is repaired despite the error in the target sequence. Since there is no homologous template, insertion-deletion mutations (indels) mostly develop in the repaired sequences, and the codons reading frame may change with the resulting indels[6]. If the direction of transcription shifts to a stop codon due to indel mutations, the target gene cannot be expressed, and thus, the gene due to indels can be silenced with error-prone NHEJ. Thus, the target gene regulation can be manipulated by CRISPR/Cas9-mediated induction of NHEJ[7].

In diseases with multistep molecular pathogenesis such as cancer, where the treatment is still insufficient, ineffective, or impossible, the reduction of the treatment to the genomic level and choosing gene-targeted therapy seem to be likely with genome-editing tools. For this purpose, we set out with the hypothesis of 'Can genome-editing tools dominate the *BCR::ABL1* gene in CML cells?' in this study.

Materials and Methods

Our study aimed to determine the Ph (Philedalphia) chromosome in cytogenetics, to analyze the *BCR::ABL1* fusion gene dependent on reciprocal translocation in molecular cytogenetics, and to quantify the *BCR::ABL1* transcript in molecular genetics since there is the CRISPR/Cas9-mediated genetic manipulation for the *BCR::ABL1* fusion gene in K562 cells. For this purpose, the genetic characterization of the K562 cell line was performed in three stages; I. the cytogenetic analyses; II. the molecular cytogenetic analysis; III. the molecular genetic analysis of the K562 cell line. For all these steps in the study, the optimization of the culture conditions of the K562 cells were achieved first.

Cell culture

K562 (ATCC CCL-243) cells were proliferated in long-term culture conditions under the ATCC criteria[8]. In order not to increase the variation depending on the passage number in the long-term cell culture, the cells were proliferated (~$3x10^6$ cells/mL) at the same time by dividing the T75 flask of the first culture into the T25 flask (~$1x10^6$ cells). The lymphoblast characterization of the K562 cell morphology was confirmed through the inverted microscope analyses, and the culture conditions were achieved. K562 cells were cultured with a mixture of 200 mM L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM). The proliferation of the cells was followed, reaching the growth phase at 37°C, 5% CO_2, and between 5-7 days[9]. Considering that each passaging would increase the genetic variation of the cells, the number of passages was minimized with as many culture flasks as possible.

Cytogenetic analysis

K562 chromosomes harvested for the K562 cytogenetic characterization were visualized *via* G (Giemsa) banding karyotypes. Through the image analysis (Lucia cytogenetics) system, K562 metaphase chromosomes were analyzed.

Molecular cytogenetic analysis

The fluorescence in situ hybridization (FISH) technique was used for the characterization of the molecular cytogenetic analysis of K562 cells. For the analysis, both the interphase K562 nuclei and the metaphase plaques from the cultured K562 cells were evaluated under a fluorescent microscope. In the analysis of FISH, the *BCR::ABL1* region was detected using a translocation dual fusion probe (LPH007A-cytocell)[10]. Additionally, K562 metaphase chromosomes were also evaluated based on the hybridization of the '2pter telomeric region probe' with the *BCR::ABL1* dual fusion translocation probe. Specifications of the probes were as follows[11]. The red signal covered the 9q34.11-q34.12 chromosome regions; these regions (from the distal 9q to the telomere respectively) are the gene of *ASS1* (D9S2057) located in the 173kb probe region and the genes of *FUBP3* (D9S1863), *PRDM12*, and *ABL1* labeled in the 346kb region. Even so, the green probe labels the chromosome regions of the 22q11.22- q11.23, and the probe covers *GNAZ* and *RAB36* (D22S1002E) located in the centromere of the *BCR* (D22S257-D22S1027) gene at 169 kb area and the gene of *IGLL1* located 148 kb further[12].

Molecular genetic analysis

Detection of *BCR::ABL1*^{p210} transcript

The *BCR::ABL1* molecular genetic characterization of the K562 cells were performed on two separate molecular platforms for the transcript quantification of the target fusion gene and the determination of the variant sequence[13]. While the high pure 'PCR

template preparation kit' (Roche diagnostics) was used for the isolation of DNA, RNA was isolated using the 'Hybrid-RTM blood kit' (315-150, GeneAll biotechnology, South Korea). The determination of *BCR::ABL1^{p210}* fusion gene transcript was also analyzed using the 'GeneMark (MBCR)IS-MMRv.2' kit on the Light cycler 480 RT-qPCR system. The study was performed on the Light cycler 480 RT-qPCR in a three-step program as one cycle of 50^0C for 10 min, one cycle of 95^0C for 15 min, and 45 cycles of 95^0C for 15 sec and of 62^0C for 60 sec.

Variant analysis of *BCR::ABL1^{p210}*

To detect the fusion breakpoints of the *BCR::ABL1^{p210}* variant, the *BCR::ABL1* fusion gene was amplified in two separate reactions for the b3a2 and b2a2 variants of RT-qPCR primers[13]. For the analyses, the *BCR::ABL1* RT-qPCR reaction was amplified for each sample at one cycle of 50°C for 10 min, one cycle of 95°C for 15 min, and 45 cycles of 95°C for 15 sec and 62°C for 60 sec, along with the 5µL of RNA kit reagents. The DNA sequence analysis of the fusion junctions for *BCR::ABL1* was also carried out. *BCR* forward: TGCAGGTGGA TCGAGTAATTGC and the *ABL1* reverse: CCAGC CTGATCAACACAGAGAAA primers and the coding regions of 5' and 3' DNA sequences of the target fusion gene by the sanger sequence analysis were determined with the 3130xl genetic analyzer. The e14a2, e13a2, e14a3, and e13a3 variants of the *BCR::ABL1* fusion gene were performed for the *M-BCR::ABL1^{p210}* oncogene by the Sanger sequencing of the 5' and 3' intronic regions and the PCR products were run on the electrophoresis of 1% agarose gel. The Sanger sequencing was performed in three separate PCR protocols. In the first reaction, the PCR products were obtained with 1×10^9nM of DNA, 6×10^9nM of master mix, 1×10^9nM of primer, 1×10^9nM of enhancer, and 1×10^9nM of distilled water volume for the target DNA sequence (GeneAmp PCR system 9700 and applied biosystems by life). Within the second step, 2×10^9nM of PCR product was reacted with $2x10^9$nM of exoSAP reagent, and in the last reaction, fusion junctions were determined with 4×10^9nM of 5× buffer, 1×10^9nM of primer, 1×10^9nM of big dye, and 2×10^9nM of distilled water.

CRISPR/Cas9 mediated genome editing

Design of sgRNA

The PAM sequences were determined at the fusion breakpoints of *BCR::ABL1^{p210}* oncogene. The complementary sequences of sgRNA to NGG-triplet repeats were designed through the program 'https://www.benchling.com/crispr/', and the sgRNA1 forward, sgRNA2 reverse, and sgRNA3 reverse were determined as the oligonucleotides of 'GACCC TGGCCGCTGTGGAGT', 'AACCCACTCCACAGC GGCCA', and 'TGATAAAACCCACTCCACAG', respectively.

Cloning

In the first step of the cloning, the plasmids of *TLCV2#79145 and pCAG-eCAS9-GFP-U6-gRNA# 87160 were* selected *via* the 'Addgene' program[14,15]. In the digestion reactions, the Esp3I (BsmBI) and the Bpil (BbSl) restriction endonucleases were used. The phosphate group was removed from the 5′ end of the plasmid DNA with the alkaline phosphatase (AP) enzyme in the phosphorylation reactions. The 5′end of sgRNA was phosphorylated by polynucleotide kinase (PNK). Digestion and dephosphorylation reactions of the plasmid DNA were performed at the same time. In the reaction, 3.5µg/mL of plasmid DNA was completed with the distilled water of 0.5µL fast digest Esp3I/Bpil, 3µL of fast digest buffer, 1µL of AP, and 0.3µL of DTT to hold a total volume of 30µL. The reaction mixture was incubated at 37ºC for 2 h in a double boiler. The plasmid DNA sequences were cut and analyzed in the transilluminator UV by running at 80-100 V for 30 min-60 min in agarose gel containing 1×TAE buffer. The purification of the gel was carried out with the 'Macharey-Nagel #740609,50' kit, and the sgRNA sequences were inserted into the purified plasmid DNA sequences of #79145 and #87160 at this stage; the enzyme catalyzing the reaction was the T4 ligation enzyme. On the other hand, the addition of phosphate groups to the 5'ends of sgRNA with the PNK enzyme was also catalyzed at this stage. In the ligation reactions, 0.5µL of ligase enzyme, 2µL of ligation buffer, 1:1µL (plasmid DNA: insert sgRNA), and a total volume of 20µL distilled water was used for the three different clonings of sgRNAs in each plasmid.

Transformation

The plasmids of #79145 and #87160 carrying the sequences of sgRNA were proliferated in *Escherichia coli (E.coli)* competent cells, and the antibiotic-resistant colonies were isolated from the bacteria. The mixture of the Luria Bertani (LB) medium and the Bacto Tryptone agar was transferred to the plates of 20 cm^2 with ampicillin for *E. coli* culture. The NEB-Stable C3040H culture was incubated for 2-4 days at 37ºC. The ligation products were transferred to the

competent cells by hot and cold shocking, and the plasmid purifications were completed with the 'NucleoBond® Xtra Midi prep' kit.

Transfection

In the K562 cells, the CRISPR/Cas9 products targeting the *BCR::ABL1* fusion gene were transfected with the lipofectamine^{TML}TX (Invitrogen, No: 15338-100) and the LipoFectMax (ABP Biosciences, No: FP310)[16]. The transfection control was achieved through green fluorescent protein (GFP) tracking and Fluorescence Activated Cell Sorting (FACS) analysis. RNA isolation from GFP+ selected cells was performed after FACS analyzing for the *BCR::ABL1* expression. On the other hand, the cytogenetic analysis and the Sanger sequencing were performed from the transfected K562 cell line. Besides, 16 individual flasks of the K562 culture were transfected with the CRISPR/Cas9 products by electroporation.

Flow Cytometry Cell Sorting (FACS)

The transfection efficiency of CRISPR/Cas9 products in the K562 cells was determined by the FACS analysis. Accordingly, after 5×10^5 cells/µLµL were transfected with lipofectamine on 6-well plates, the analysis of FACS-GFP was carried out. The cell viability and transfection efficiency after CRISPR/Cas9 were evaluated with the percentages of GFP values.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was isolated from the GFP+ selected K562 cells after the electroporation and lipofectamine transfections. The activity of CRISPR/Cas9 targeting *BCR::ABL1^{p210}* was evaluated by the *BCR::ABL1^{p210}* b3a2 variant expression analysis on the Light Cycler 480 RT-qPCR system. In the CML treatment response milestones, the amounts of transcriptions were compared before and after the manipulation of CRISPR/Cas9, as well as finding the effects of lipofectamine and electroporation-mediated CRISPR/Cas9 genome editing on the expression of fusion transcripts. The molecular response and deep molecular response in CML were provided by the evaluation of NCN-IS (%).

Sanger sequence

The Sanger sequence of PCR reactions were performed with the *3'ABL1* and *5'BCR* primers of the *BCR::ABL1^{p210}* fusion. The electrogram analysis depending on the amplification of *5'BCR* exon14 and *3'ABL1* exon2 was performed from the PCR products. Exons were sequenced with the primers of *BCR* forward: TGCAGGTGGATCGAGTAATTGC and *ABL1* reverse: CCAGCCTGATCAACACAGAG AAA and the introns were sequenced with the primers of *BCR* forward: GTCATCGTCCACTCAGCC ACTGG and *ABL1* reverse: CCTGGCTCTCTTA CCCCAGAGACTGAC using the 3130xl 'Genetic Analyzer' technology.

Western blot

Western blot assay was used for *BCR::ABL1^{p210}* expression analysis after CRISPR/Cas9 editing for *BCR::ABL1* fusion. 1 mL of 1×PBS was added onto the culture cell ($0.5\text{-}1\times10^6$) and 3 min at 2500 rpm was washed by centrifugation onto the cell pellet after removing PBS. Add 1mL ProtinEx total protein extraction solution (GeneAll, Cat No: 701-001) and pipet done. Iblot Gel Transfer System was used. For blocking; Diluent A:2mL, diluent B:3mL, distilled water, 5mL were added and mixed slowly for 90 min. It was kept in the shaker. Alternatively, 2% PBS-BSA prepared with PBS for blocking. Primary antibodies were diluted at the rate specified in the datasheet and kept overnight. After the membrane was washed with distilled water for 3×5 min in a fast shaker, 10 mL of secondary antibodies were added onto it. Antibody was added and kept on a slow shaker for 45 min[17]. The results obtained were analyzed with the Image J program.

Ethical statement

The present study was approved by the ethics committee of Meram Faculty of Medicine, Necmettin Erbakan University with the decision number: 2018/1417.

Results

Cell culture and FISH

The proliferation of the K562 cells was found to be compatible with ATCC criteria. In the cytogenetic analysis of the K562 cells, the hypotriploid (59-62) XX was revealed to have an atypical Ph chromosome in the complex karyotype and at each metaphase (Fig. 1). FISH results of 200 interphase nuclei were outside of the classical *BCR::ABL1* fusion signal patterns. Three red, two green, and one yellow signals and also amplification signals were detected approximately in size of G group acrocentric chromosome in all cells (Fig. 2). In the analyzed metaphase plaques, while two of the three red signals

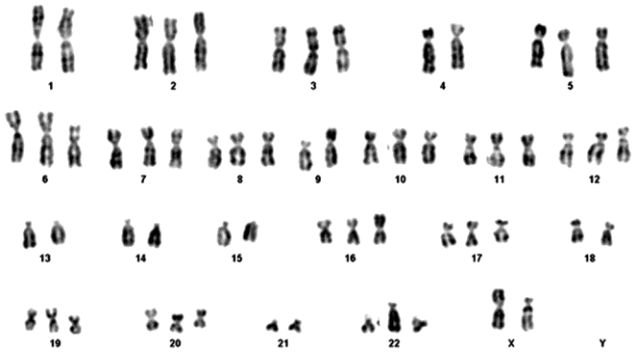


Fig. 1 — K562 karyotype, atipic Ph chromosome.

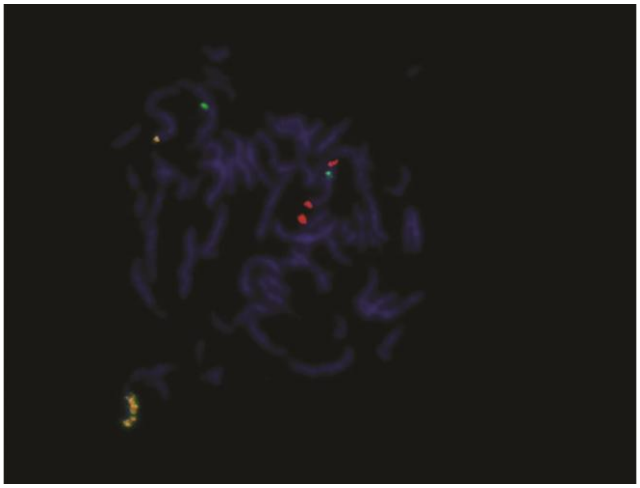


Fig. 2 — K562 cells, interphase FISH analysis before CRISPR/Cas9 manipulation; 3red; 2 green, 1 yellow signals and 1 amplification region are shown in two separate interphase nucleus. Red signals for the ABL1 gene on chromosome 9, and green signals for the BCR gene on chromosome 22. Two of the 3 red signals for dup(9)(q34) and der9q34. 2 green signals indicate normal 22q11 BCR gene. The single yellow signal indicates the single *BCR::ABL1* fusion translocated to the 2q33 region. The region with carring of yellow signals indicates BCR/ABL1 amplification.

were located in the telomeric regions of chromosome 9 as dup(9)(9q34::9p24→9qter), the third one was also analyzed in the other 9q34 band. The normal chromosomes of 22 were determined with two green signals. However, the single yellow signal *BCR::ABL1* fusion was analyzed on chromosome 2qter. In addition, the amplification region of the Ph+ chromosome consisting of yellow signals was also detected (**Fig. 3**).

Real-Time quantitative PCR (qRT-PCR) before CRISPR/Cas9 manipulation

Before the CRISPR/Cas9 manipulation, the expression analysis of the K562 cell line *BCR::ABL1*^{p210} fusion gene was detected by high

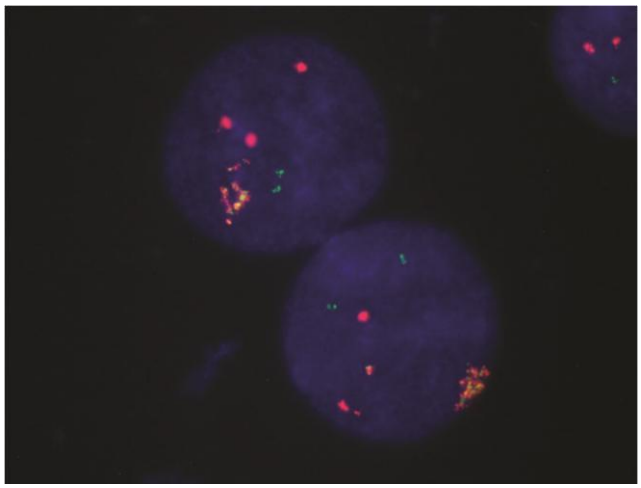


Fig. 3 — K562 metaphase cell for FISH analyses before CRISPR/Cas9 manipulation; the red signal for the *ABL1* on chromosome 9q34.11-q34.12, the green signal for the *BCR* on chromosome 22q11.22- q11.23,) one yellow signal for the *BCR::ABL1* fusion on 2qter, and the amplification region of *BCR::ABL1* fusion on atypical ph chromosome.

positive transcript quantification. Before the culture, the initial analyses for two separate measurements in the K562 cells of *BCR::ABL1*^{p210} were determined I-(ΔΔCt): 33,80 (log0/log-1) and II-(ΔΔCt): 48,05 (log0) . After the long-term culture, it was determined as *BCR::ABL1*^{p210}III-(ΔΔCt): 291,62 (log0/log+1) and for a week incubation IV: ΔΔCt): 17622,9 (Graphic 1).

Transfection and FACS

During the analysis of the cell viability and pre-transfection cell count, 3×10^6 viable cells were cultured in each T25 flask for complete optimization. At post-transfection, the vector of lipofectamin^{TML}TX revealed that the G3B2S culture contained the CRISPR/Cas9-transfected K562 cells with higher GFP ratios compared to the G1B1M and G3Q3M cultures. Findings of post-transfection FACS analysis detected with the lipofectamin^{TML}TX vector are as follows: 3,8% GFP(+) in the G1B1M culture transfected with #79145-sgRNA$_{1-3}$ CRISPR/Cas9 products (Fig. 4A), 2,8% GFP(+) in the G3Q3M culture transfected with #87360-sgRNA$_{1-3}$ CRISPR/Cas9 products (Fig. 4B), and 13% GFP(+) in the G3B2S culture transfected with two plasmids #79145 and #87360 were analyzed (Fig. 4C). Additionally, a second transfection was performed with Lipofectmax and the following ratios were determined: 25% GFP(+) in the mixed culture with together #79145 and #87360 (Fig. 4D), 38.4% GFP(+) in the P1G1 culture with #79145-sgRNA$_1$ (Fig. 4E), 38.0%

Fig. 4 — Findings post-transfection FACS analysis detected with the lipofectamin^{TML}TX vector are 3,8% GFP(+) in the G1B1M culture transfected with #79145-sgRNA$_{1\text{-}3}$ CRISPR/Cas9 products (A), 2,8% GFP(+) in the G3Q3M culture transfected with #87360-sgRNA$_{1\text{-}3}$ CRISPR/Cas9 products (B), and 13% GFP(+) in the G3B2S culture transfected with two plasmids #79145 and #87360 were analyzed (C), second transfection was performed with Lipofectmax and the following ratios were determined: 25% GFP(+) in the mixed culture with both plasmids #79145 and #87360 (D), but transfecting only plasmid as #79145; 38.4% GFP(+) in the P1G1 culture with sgRNA$_1$ (E), 38.0% GFP(+) in the P1G2 culture with sgRNA$_2$ (F), 38.0% GFP(+) in the P1G3 culture with sgRNA$_3$ (G), transfecting only plasmid as #87360; 37.9% GFP(+) in the P2G1 culture sgRNA$_1$ (H), 34.1% GFP(+) in the P2G2 culture transfected with sgRNA$_2$ (I), and 34.6% GFP(+) in the P2G3 culture transfected with sgRNA$_3$ (J), K562 interphase nucleus before CRISPR/Cas9 editing (K), K562 interphase nuclei after CRISPR/Cas9 editing (L-M).

GFP(+) in the P1G2 culture with #79145 $sgRNA_2$ (Fig. 4F), 38.0% GFP(+) in the P1G3 culture with #79145 and $sgRNA_3$ (Fig. **4G**), 37.9% GFP(+) in the P2G1 culture $sgRNA_1$#87360 (**Fig. 4H**), 34.1% GFP(+) in the P2G2 culture transfected with #87360 and $sgRNA_2$ (**Fig. 4I**), and 34.6% GFP(+) in the P2G3 culture transfected with $sgRNA_3$#87360 (**Fig. 4J**). The third transfection protocol was also carried out through electroporation. In the inverted microscope analysis after transfection, cell deaths were observed in 13 of the cultures. In the case of the proliferation, all cultures were incubated at 5% CO_2, 37°C. After a 72 h incubation period, the viability was determined in the P2, and P1G3 cultures. Although a strong GFP signal and a high cell proliferation were noted at the end of day 7 for the P1G3 culture transfected with #79145 $sgRNA_1$. After transfecting K562 cells, the inverted microscope analysis revealed remarkable findings. The morphological appearance of the cells were damaged, and the lysis was highly evident in the flask. For the negative control in the K562 cells, the untransfected and lipofectamine-mediated post-transfection cultures were compared for the manipulation efficiency of CRISPR/Cas9 in the FISH analysis, and any signal was observed in the FITC and Texas red probes within the transfected K562 cells. Even so, the increased variability of cell content was detected in the direction of granulocytosis in the DAPI filter (4k), compared to pre-transfection (**Fig. 4K-M**).

Real-Time quantitative PCR (qRT-PCR) after CRISPR/Cas9 manipulation

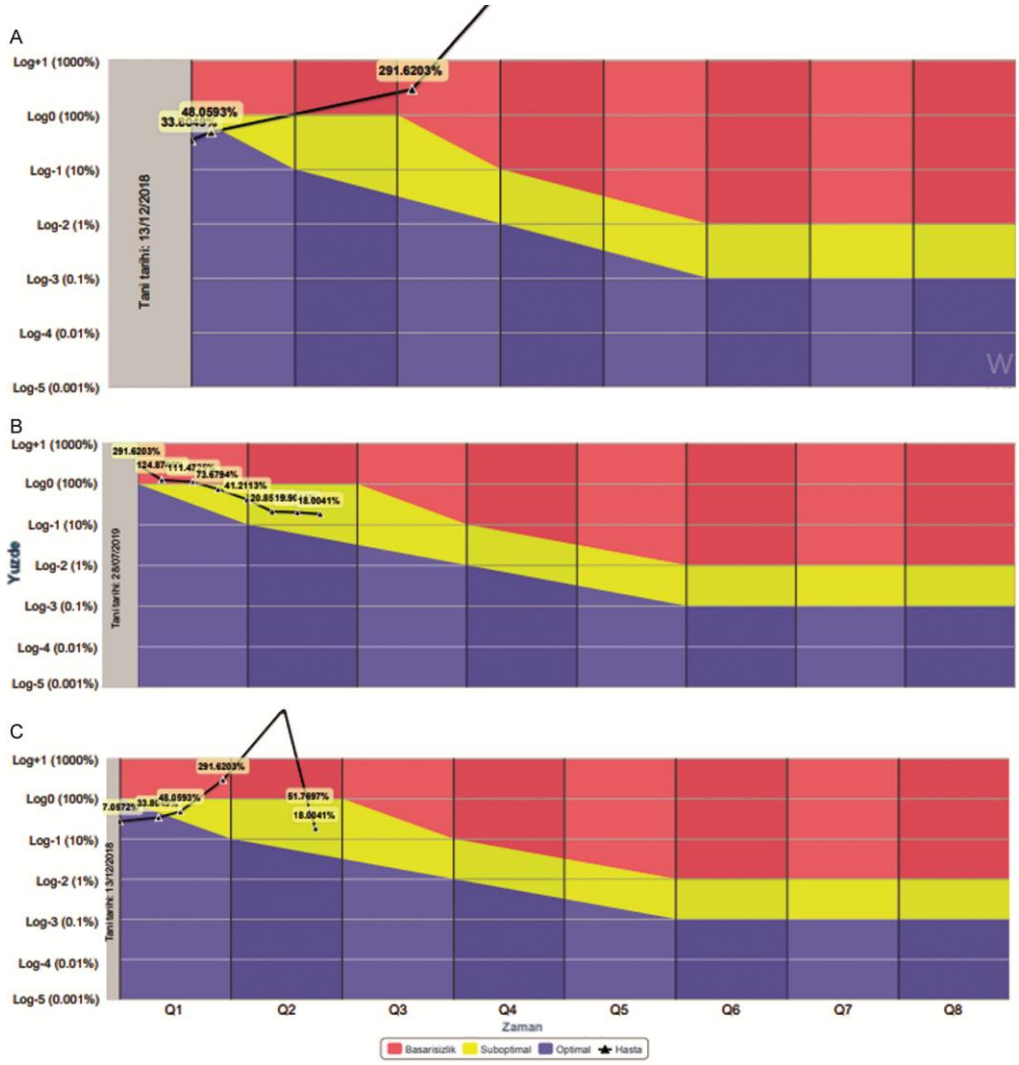
The manipulation efficiency of CRISPR/Cas9 after all transfections was compared by RT-qPCR *via* the *BCR::ABL1*^{p210} expression analysis. To determine the effect of the $sg_{1\text{-}3}$RNA#79145 CRISPR/Cas9 products transfected on the *BCR::ABL1*^{p210} fusion gene expression in the P1G3<$P_{lipo<}$P2<P1<P1G3*1<P1 G3*2<P1G3*3 were determined decreasely after manipulation as 291,62>124,87>111,47>73, 68>41, 21>20,85>19,9>18. The effect of the CRISPR/Cas9 manipulation on the *BCR::ABL1*^{p210} fusion gene expression was compared with the decreasing % NCN-IS values of in the K562 cells in the molecular treatment response (**Graphic 2**). To reveal the effect of the CRISPR/Cas9 manipulation on the *BCR::ABL1*^{p210} fusion gene expression in the K562 cells after the 7 day incubation period, the decreasing % NCN-IS values of all transfected K562 cells were compared in the molecular treatment response. To find out the effect of CRISPR/Cas9 within the 7 day cultures as 4966,37>269,44>229,53>178,09 >96,34>76,26> 57,22>51,77 (**Graphic 3**).

Sanger sequence

The effectiveness of the CRISPR/Cas9 manipulation in the K562 cells was analyzed by the post-transfected electroporation of the Sanger sequence, and the findings are as follows: For the K562 cells transfected with $sg_{1\text{-}3}$RNA#87360, no CRISPR/Cas9-mediated mutation was detected in the *BCR::ABL1*^{p210} fusion breakpoints. In the sequencing analysis of the P2C flask performed to investigate the P2 flask incubated for seven days in culture, no CRISPR/Cas9-mediated mutation was detected in the *BCR::ABL1*^{p210} fusion breakpoints (**Fig. 5A**). Besides, in the analysis performed for the K562 cells transfected with sg_3RNA#79145 in the P1G3 culture, no CRISPR/Cas9-mediated mutation was also detected in the *BCR::ABL1*^{p210} fusion breakpoints (**Fig. 5B**). In the Sanger sequencing analysis of the P1G3C flask incubated for seven days, no CRISPR/Cas9-mediated mutation was detected in the *BCR::ABL1*^{p210} fusion breakpoints. For the K562 cells transfected with sg_3RNA#79145 in the P1 culture, no CRISPR/Cas9-mediated mutation was detected in the *BCR::ABL1*^{p210} fusion breakpoint (**Fig. 5C**).

Western blot

To determine the efficiency of the CRISPR/Cas9 manipulation on the *BCR::ABL1*^{p210} fusion gene in the K562 cell line, the western blot analysis was performed at the protein level. Although the analysis was repeated twice, no band patterns of the CRISPR/Cas9-containing protein products were observed while the actin bands were recorded in the gel electrophoresis. It was extracted from the results of the fusion gene expression analysis, which depended on CRISPR/Cas9 manipulation. Although not precisely destroyed, the transcript decreased significantly in many different cultures. Therefore, what was expected from the western blot protein analysis was that the CRISPR-containing products compared with the pre-manipulation K562 cells demonstrated band patterns of less than 210 kD. However, the challenge arising from the *BCR::ABL1*^{p210} antibodies prevented such an expectation since the presence of actin bands suggested that the problem stemmed from the antibodies marking the *BCR::ABL1*^{p210} site while ruling out the study-originated challenges. Therefore, a western blot analysis was planned with the selection



Graphic (A). Expression analysis of *BCR::ABL1*^{p210} fusion gene by Rt-qPCR before CRISPR/Cas9 manipulation (Q1,Q2,Q3…showed that time, and log -5,log+1 values show that BCR:ABL1 expression levels); (B). The effect of the CRISPR/Cas9 manipulation on the *BCR::ABL1*^{p210} fusion gene expression comparing with different transfection method; (C) The comparasion of the CRISPR/Cas9 manipulation on the *BCR::ABL1*^{p210} fusion gene expression in the K562 cells after the 7-day incubation period with the previous times of editing.

of different antibodies labeling the *BCR::ABL1*^{p210} site (**Fig. 6**).

Discussion

As well as the first cancer type revealed its genetic basis, CML became the first genetic disease in which the targeted therapy was applied with the identification of the *BCR::ABL1* fusion gene. In diseases such as cancer with multistep molecular pathogenesis where the treatment is still insufficient, ineffective, or impossible, it seems possible to reduce the treatment to the genomic level and to choose the gene-targeted therapy with genome editing tools. In this study, for the purpose, the hypothesis of 'Can genome editing tools dominate the *BCR::ABL1* gene in CML cells?' has been established. So, our study aimed to knock out/down the *BCR::ABL1* oncogene *via* the CRISPR/Cas9 in *ex vivo* CML cells, thereby suppressing or destroying the over tyrosine kinase activity leading to malignant transformation. The K562 cells were derived from a 53 year old female patient′s pleural effusion with the terminal blastic stage CML phenotype in terminal blast crises and established by culturing in three years with a series of over 170 passages by Lozzio & Lozzio in 1970[18]. The researchers reported the K562 karyotype after the 24 h culture as 45,XX,-D,-E,+t(E;D) and also analyzed the Ph chromosome in each metaphase plate.

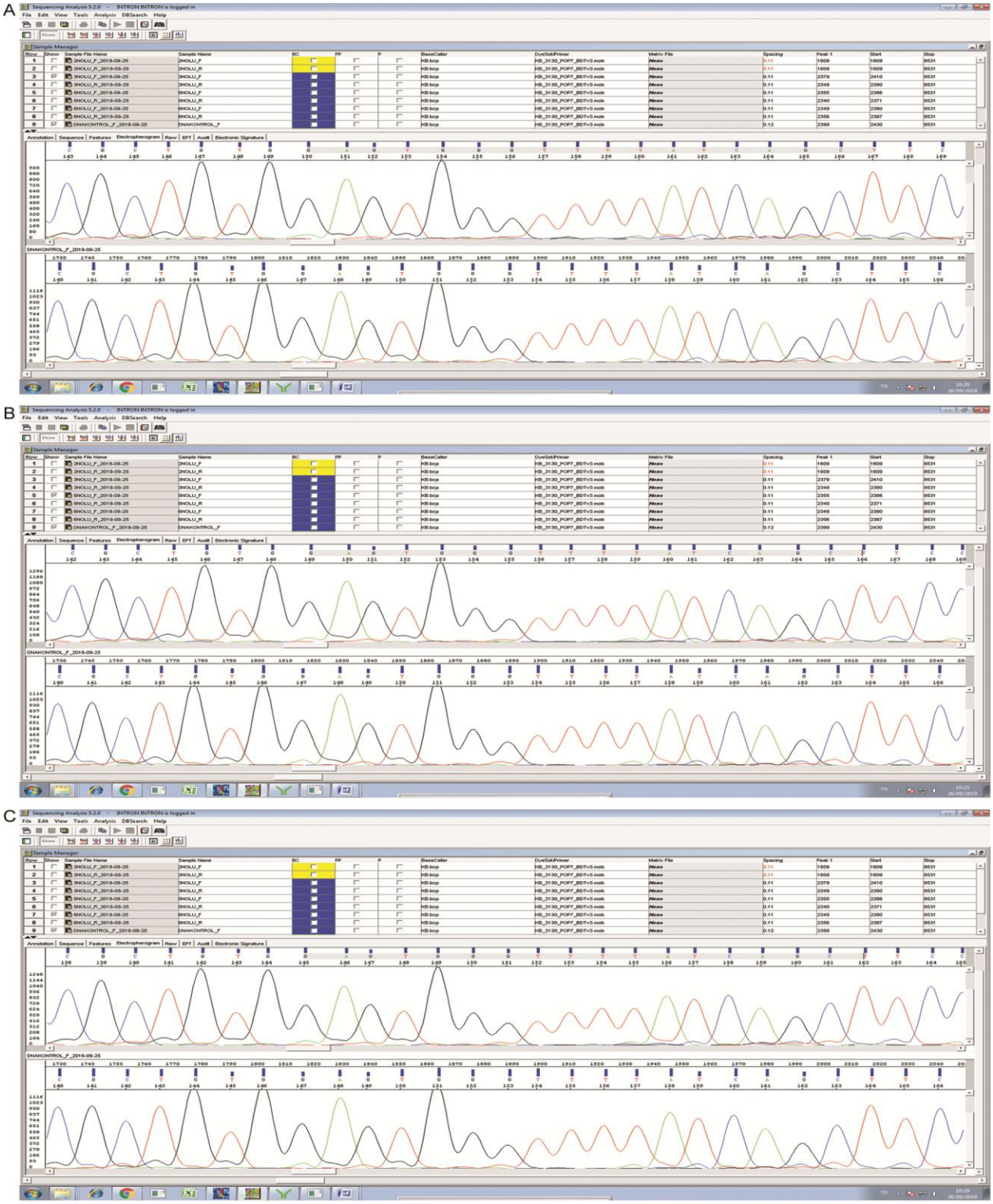


Fig. 5 — (A) Comparasion of the *BCR::ABL1*^{p210} fusion sequence (...GAGTGTTT...) before and after CRISPR/Cas9 editing in the P2C for seven days cultured transfecting with $sg_{1\text{-}3}$RNA#87360, (B) P1G3 flask for seven days cultured transfecting with sg_3RNA#79145, (C) P1 flask for seven days cultured transfecting with sg_3RNA#79145.

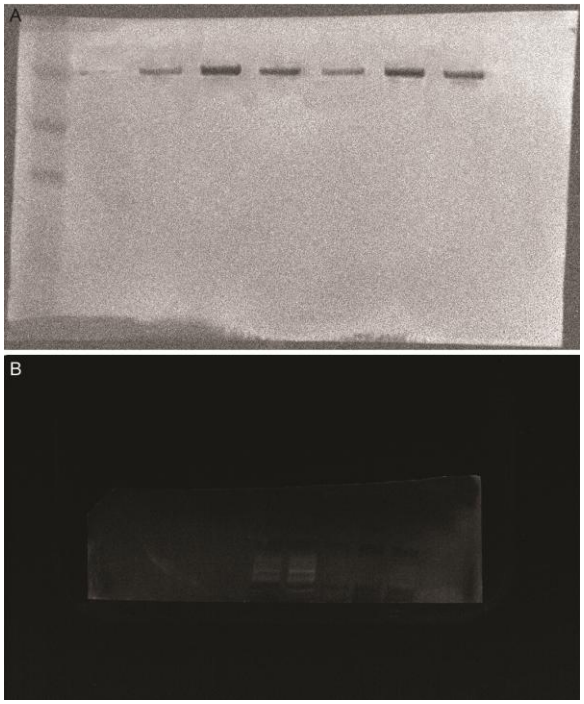


Fig. 6 — Western blot analysis detecting for the *BCR::ABL1*^{p210} fusion gene after CRISPR/Cas9 editing with actin band (A) and with K562 cell samples (B).

However, K562 cells have evolved into a complex karyotype as a result of both the clonal evolution and the variations in passage times. In our study, the K562 cells were analyzed to have the hypotriploid (59-62) XX, complex karyotype, and atypical Ph chromosome at each metaphase, and the molecular cytogenetic results supported our karyotype results. The presence of the amp(22)(q11.2) and one copy of the *BCR::ABL1* translocation at the 2qter localization by the FISH analysis and the detection of the *ABL1* gene through the dup(9)(9q34::9p249qter) were observed similarly in the literature[12]. On the other hand, the expression of the b3a2 variant through the K562 cells was also found to be compatible with the variant transcript reported by Sales *et al.* (2019) in CML patients and cell line[19]. It was also confirmed that the K562 origin was in the advanced-stage CML phenotype, with the K562 *BCR::ABL1*^{p210} RT-qPCR log0 expression profile without changing to the culture conditions. Differences were detected in the *BCR::ABL1*^{p210} fusion gene expression levels in the K562 cells after the 7 day culture period. Therefore, the alteration of the expression in such a short time caused us to research an answer to the question: Can the culture time lead to an increase in the *BCR::ABL1*^{p210} transcript level? Accordingly, we repeated the transcript quantitation for the *BCR::ABL1* fusion gene of the K562 cell line proliferated under the long-term post-modification culture conditions of the CRISPR/Cas9. Therefore, we determined the *BCR::ABL1*^{p210} fusion gene expression analysis in the K562 cell line long-term cultured. The findings supported the culture time increased the *BCR::ABL1*^{p210} fusion gene expression by approximately 2-logs. It was noteworthy that the targeted *BCR::ABL1*^{p210} fusion was at a +1log phase in the molecular response. In a study where the gene expression levels between fresh tissue, first passage, and long-term passages were compared, Januszyk *et al.* (2015) performed the expression profiling with the microarray for more than 40 genes, reporting the effect of culture period on the gene expression[20]. Among almost all of the investigated gene expressions showed a significant increase depending on the culture time. Therefore, the increase of the expression depending on the culture time appeared as a challenge for the target gene we tried to downregulate in our study. However, we consider that with our hypothesis, the decreases in *BCR::ABL1*^{p210} gene expression could pave the way for preclinical studies to undeniably reveal the knockdown effect of the CRISPR/Cas9 for the fusion genes. Since they are responsible for the main etiopathogenesis of cancer, they have high potential as therapeutic targets[21]. On the other hand, genome modification studies targeting fusion genes are much less common in the literature than single gene mutations.One of the leading reasons for such a situation is that apart from single or multiple gene mutations, the fusion sequences of chimeric genes have yet to be elucidated. In particular, the breaking of the translocation region from the introns containing hundreds of thousands of base pairs other than the exonic sequence such as *BCR::ABL1*^{p210}, in which the repeat sequences are present, accounts for the difficulty in the determination of fusion sequence. Therefore, the fusion sequence must be determined to perform the CRISPR-mediated genome editing in fusion genes. In the study performed by Shibata *et al.* (2010), the fusion sequence was reported in K562 and KU812 cell lines harboring the *BCR::ABL1* fusion[22]. Recently, due to the development of DNA sequencing technologies, fusion gene breakpoints can be sequenced with the new generation sequencing technique[23,24]. However, confirming fusion sites with Sanger sequencing is still the gold standard method.

In the CRISPR-mediated genome editing system, the PAM sequences existing in the target DNA region are demonstrated as the main determinant of the number of sgRNAs[25,26]. In a study, *GATA1* mutation, frequently encountered in myeloid leukemia cases, was modeled in the K562 cell line, and the genomic editing was achieved with the CRISPR/Cas9. The modification efficiency with the use of a single plasmid and a sgRNA for the target genomic sequence was exceeded by the transfection in 74 separate clones[27]. In another study, it was aimed to knock out the *ATG7* gene in the K562 cells with the CRISPR/Cas9, and the CRISPR/Cas9 modification was performed by targeting two different sgRNAs to the exon12 region of *ATG7* gene[28]. On the other hand, research on the effectiveness of genome modification tools and transfection methods with more effective methods has been increasing in recent years. In particular, multiple sgRNA directed to the target genome editing region results in more effective genetic manipulation and less off-target mutagenesis[29-31].

In this study, the success of the transfection was increased by cloning three different sgRNA sequences by which were targeted *BCR::ABL1* fusion breakpoints, into two plasmids. In addition, we aimed both to increase the efficiency of modification and to exclude off-target mutagenesis by using different transfection protocols with different vectors for transferring the CRISPR components into the cell. In the findings of the transfection, we revealed that the high sgRNA/Cas9 content significantly elevated the transfection efficiency by FACS/GFP analysis ($CRISPR/Cas9_{K562}$ %2,8<$CRISPR/Cas9_{K562}$ %38). The studies investigating CRISPR support that the sgRNA/plasmid increase elevates the transfection efficiency unless vector-induced cell deaths occur.

In the study investigating for the first time the CRISPR/Cas9 targeted *BCR::ABL1* fusion, genome editing was performed in the Boff-p210 hematopoietic cells through the lentiviral transfection[16]. The study also revealed that the CRISPR/Cas9 modification was achieved with two different sgRNAs targeting the *BCR::ABL1* sgRNA and *ABL1* complementary to the fusion sequence. In the CML xenograft animal modeling and Boff-p210 cell line, the level of CRISPR-mediated *BCR::* $ABL1^{p210}$ protein has been detected to decrease. In the study, although the *BCR::ABL1* fusion was knockout, the targeting of *the ABL1* gene via sgRNA was found to be controversial since *ABL1* is a protooncogene playing a key role in many phosphorylations involving molecular pathways in cell growth and maturation. Therefore, what the effects of the modifications performed in the *ABL1* gene are in the long term, and to what extent such modifications will affect the hematopoietic stem-cell population have yet to be elucidated; on the other hand, the fact that the breakpoints of the *BCR::ABL1* oncogene are in different variations in those with CML means sgRNA is specific to each fusion sequence. Accordingly, this and similar studies based on the CRISPR/Cas9-mediated *BCR::ABL1* manipulation will lead to preclinical research in personalized anticancer therapy. In designing our study, the expression of the *BCR* and *ABL1* gene expressions in the normal cell population, except for primary leukemia cells, was taken into consideration. Therefore, our study targeted only the *BCR::ABL1* fusion sequence with the CRISPR genome-editing tool, and the effect of the off-target mutagenesis was ruled out by keeping the wild-type *ABL1* and *BCR* genes.

In another study, it was reported that the manipulation of the *BCR::ABL1* fusion was achieved with polyethylene glycol-b-polylactic acid co-glycolic acid (PEG-PLGA)-based cationic lipid-assisted polymeric nanoparticles (CLANs)[32]. In the study, the *BCR::ABL1* gene in rats was modified with the CRISPR plasmids and the CLAN nanoparticles-carrying sgRNA, and the lipofectamine-transfected K562 cells were also included. In light of the study findings, the genome editing efficiency with *in vitro* lipofectamine is reported over the insertion/deletion (indel) ratios of the *BCR::ABL1* fusion sequence. In another study, BCR:ABL1 fusion is the driver mutation in chronic myeloid leukemia, was targeted via CRISPR/Cas9 not directly but through silencing miR-21 which was inhibit PI3K/Akt pathway and downregulating BCR::ABL1 fusion[33]. Leukemic stem cells in murine were manipulated through CRISPR/Cas9 and the results were showed arresting leukemia cell proliferation[34.]

In our study, we demonstrated the effect of CRISPR/Cas9 by the expression analysis of the *BCR::ABL1* fusion through lipofectamine^{TML}TX transfection. We also revealed the decrease in Log2 in the treatment response milestones of the CRISPR/Cas9-infected K562 cells. Our routine laboratory practice includes the alterations in the *BCR::ABL1* transcript amounts at the time of diagnosis and due to the treatment in CML patients.

Therefore, the aim of our *in vitro* study to evolve into patient profiling through *in vivo* studies also constitutes the basis of demonstrating the effectiveness of CRISPR-mediated genome editing through treatment response milestones. In a study carried out by Martinez-Lage *et al.* (2020), a deletion was shown in the *BCR::ABL1* fusion of 133.9 kb with four different sgRNA sequences complementary to the *BCR* intron8 and *ABL1* intron1 regions[35]. Accordingly, the manipulation sequence targeted by the researchers is quite far from the *BCR* intron14 and *ABL1* intron1 sequences, which are the *BCR::ABL1* fusion breakpoints. Although our study was based on the principle of targeting the different loci on two separate chromosomes with the LVCas9_dC3/4 control plasmid, the sgRNA sequences not targeting the fusion may have not been able to rule out wild-type *BCR* and *ABL1* sequences since the K562 cells we showed in the FISH analysis contained the two copies of the normal *BCR* gene and three copies of the *ABL1* gene, in which no fusion occurred. On the other hand, as consistent with the transfection method in the study by Martinez-Lage *et al*.We transfected CRISPR/Cas9 reagents to the cells *via* the electroporation, and as different from their study, we demonstrated the high transfection efficiency by comparing it with lipofectamine. In addition, while the apoptotic effect of the CRISPR/Cas9 on cancer cells was revealed by Lage *et al.*as 3-15% (P<0,001), we also showed the apoptosis effect of CRISPR in both *in vitro* K562 analysis and over the post-electroporation uncultured, degraded, agronucleocytotic cell lysis in the FACS and FISH[35-37].

The findings of a recent study performed for *in vitro* and *in vivo, ABL1* genome editing by Chen *et al.* (2020) contain various similarities and different features with those in our study[38]. In their study, two sgRNAs specific to the fusion-break sequence in K562 cells and an indel ratio of 41.2% in lentiviral-infected cells were detected. We also recorded that the three sgRNA combinations targeting the fusion sequence and the *BCR::ABL1* manipulation showed approximately a 2-log (~100 fold) decrease in the fusion gene expression as (+1 log) before and (-1 log) after CRISPR/Cas9 manipulation. Even so, we detected no indel or single nucleotide changes in the *BCR* and *ABL1* sequences in the fusion-break region. In addition, while reporting CRISPR/Cas9 transfection efficiency as 40% in the GFP+ K562 cell population, Chen *et al.* stated that the increase of viruses led to no increase in the transfection efficiency. In our study, the transfection efficiency was increased from 2.8% to 38% in the GFP+ K562 cells. We consider that the lipofectamine increase, different sgRNA contents, and electroporation transfection played a role in the increase. In addition, in the study by Chen *et al.*, it is predicted that the *ABL1* gene editing may have led to the clinical gene therapy modalities due to the diversity of fusion gene breaks. Accordingly, in the genome editing of *in vivo* CML cells, the *ABL1* gene was targeted instead of the fusion sequence and the $sgRNA_{ABL1}$ mediated 30,9% indel was reported in one patient. However, based on the study findings, the long-term effects of *ABL1* gene editing are noted to be unknown; in addition, how the *ABL1* manipulation will evolve in the hematopoietic stem cell population seems to be another issue in the study. In terms of the researchers′ concerns, it is necessary to show the anticancer effect in the primary culture of the samples obtained from CML patients to evaluate the *ABL1* manipulation because the lentiviral transfection may display differences between the primary CML cells and the K562 cell line[36]. On the other hand, the study by Chen *et al.* and the methods to be developed subsequently are crucial in that they can lead to the immortalization of primary cells, the systemic modeling of primary cultures, or the optimization or paving the way for the preclinical and clinical gene therapy studies[37]. We have not come across any studies reporting the effect of the CRISPR tool, which has been added to the recent literature[38-40], on BCR by molecular monitoring through chimeric gene expression of the fusion. The next step of our research in this direction is to strengthen our CRISPR-mediated targeted therapy hypothesis in the molecular follow-up of preclinical CML patients.

As revealed in our study, the *BCR::ABL1* junction includes the *5′BCR* and *3′ABL1* intronic sequences. Therefore, could the decrease in the fusion gene expression we detected after the modification have resulted from the effect of the CRISPR on the recombination or the alternative splicing mechanism? As a result, the effect of the CRISPR/Cas9 genome editing was revealed *via* the knockdown of the *BCR::ABL1* in our study. Thus, the CRISPR/Cas9 can target the *BCR::ABL1* fusion gene due to the interference effect. Accordingly, we concluded that novel studies including the Cas9 variants or modified Cas9 strains could evolve into clinical trials with the genome editing systems for CML.

Conclusion

The CRISPR/Cas9 genome editing system can knockout/down or lead to the deregulation of a gene through the indels it creates as a result of the cells-induced natural repair mechanism. Based on our study findings, no indels were detected in the targeted *BCR::ABL1* fusion region; however, the amount of the transcription decreasing between 10-100% in the *BCR::ABL1*^{p210} fusion gene was profiled on the treatment response milestones, reduced by approximately two logs. We also showed the efficiency of lipofectamine and electroporation-mediated transfection, and the genome editing role of sgRNA-$_{BCR::ABL1}$ ratio at different amounts in CML cells. With our study findings, we revealed the genetic characterization of the K562 cells through conventional diagnostic methods and the CRISPR/ Cas9 Gene Therapy as a new generation gene treatment modality.

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Conflict of Interest

The authors declare no conflict of interest.

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