T.C.

BIRUNI UNIVERSITY

INSTITUTE OF GRADUATE EDUCATION

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MOLECULAR AND MEDICAL GENETICS GRADUATE PROGRAM

KLK GENE EDITING USING CRISPR TECHNOLOGY IN PROSTATE CANCER CELL LINES FOR CANCER THERAPY

Nasim KHERAD

ADVISOR

Asst. Prof. Dr. Meryem ALAGÖZ

June, 2020

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THESIS APPROVAL PAGE

DECLARATION

I declare that I have designed and performed all experiments in the current study entitled “Klk Gene Editing Using Crispr Technology In Prostate Cancer Cell Lines For Cancer Therapy” according to good scientific practices. I obtained all the information contained in this thesis under academic and ethical rules. All the information I have used from secondary literature has been respectively referenced. I also declare that, I have not violated any patents and copyrights during the preparation and writing of this thesis.

Nasim Kherad

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Nasim Kherad

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ABBREVIATIONS

Abbreviation/Symbols	Definition/Full form
PC	Prostate Cancer
KLK	Kallikrein
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/Cas9
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
PARP	Poly (ADP-ribose) polymerase
TA	transit amplifying
CB	committed basal
PAP	prostatic acid phosphate
BPH	benign prostatic hyperplasia
RNASEL	ribonuclease L
BRCA	breast cancer susceptibility gene
AR	androgen receptor
E.coli	Escherichia coli
LB	Lucia-Bertani
SOB	super optimal broth
$MgCl_2$	Magnesium chloride
CaCl2	Calcium chloride
DNA	Deoxyribonucleic Acid
SOC	Super Optimal broth
MgSO4	Magnesium sulphate
EDTA	Ethylenediaminetetraacetic acid
TBE	Tris-borate-EDTA
UV	ultraviolet
EMEM	Eagle's minimal essential medium
FBS	Fetal Bovine Serum
DMSO	Dimethyl sulfoxide
CO2	Carbon dioxide
PBS	Phosphate-buffered saline

GC%	guanine-cytosine content
PCR	Polymerase chain reaction
ddH_2O	Double distilled water
Dntp	deoxyribonucleotide triphosphate
SAP	shrimp alkaline phosphatase
gRNA	Guide Ribonucleic Acid
PAM	protospacer adjacent motif
sgRNA	Short Guide Ribonucleic Acid
CFA	Colony formation assay
Kb	Kilobyte
DSB	Double strand break
NHEJ	Non-homologous end-joining
^oC	Degree of Celcius
MW	Molecular weight
OD	Optical density
µG	Microgram
ug/umole	Microgram per micromole
uM or µM	Micromolar
3D	Three dimension
2D	Two dimension

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TURKISH ABSTRACT and KEYWORDS

Kherad, N. (2020). Kanser terapisi için Klk Gen Düzenlenmesini Crispr Teknolojisi Kullanilarak Prostat Kanseri Hücre Hatlarinda Yapilmasi. Yüksek Lisans Tezi, Biruni Üniversitesi Lisansüstü Eğitim Enstitüsü, İstanbul.

Erkeklerde, kansere bağlı ölümlerde prostat kanseri ikinci sırada yer almaktadır. Genetik mutasyon, etnik köken, yaş, diyet vb. gibi iç ve dış faktörlerin, kanserin ortaya çıkmasında ki nedensel faktörlerden olduğu gözlemlenmiştir. Kallikrein (KLK) gen ailesi tarafından kodlanan serin proteazlar PC kanseri üzerinde dikkate değer etkiler gösterirler ve KLK3,KLK2 gibi bazı çeşitleri PC varlığında androjene bağlı kanda tespit edildiği için biyo marker olarak tanınmaktadır. Bununla birlikte, androjen bağımlılığı ve düşük özgüllük nedeniyle, tam olarak güvenilir değillerdir. Bu çalışmada, mutasyonun PC de tümör oluşumuna etkisini gözlemlemek için androjenden bağımsız PC3 ve DU145 PC hücre hatlarında KLK5 gen mutasyonuna odaklandık. Gen düzenleme teknolojisi, uzun vadede bilim adamlarının en az yan etki ile kanser tedavisi araştırmalarına faydalı olabilecek yeni bir yol sağladı.Bu çalışmada KLK geninde tespit edilen ve Sanger dizilemeyle doğrulanan mutasyon c.245C>A (p.A82D) CRISPR/Cas9 teknolojisi kullanılarak nakavt edilmiştir.Bu çalışmada, gelecekte kanser tedavisinde CRISPR sisteminin umut veren sonuçlarını göstemek için, yapılan gen düzenlemesi Doxorubicin ve PARP inhibitörü kullanılarak tripan mavisi ile hücre canlılığı, MTT testi ve koloni oluşumu gibi çeşitli sitotoksisite deneyleri ile değerlendirilmiştir.

Anahtar kelimeler: prostat kanseri; klk gen ailesi; CRISPR sistemi; klk 5 mutasyonu

ABSTRACT and KEYWORDS

Kherad, N. (2020). Klk Gene Editing Using Crispr Technology In Prostate Cancer Cell Lines For Cancer Therapy. Master Thesis, Biruni University, Institute of Graduate Education, İstanbul.

Prostate cancer (PC) has been accounted for as the second leading cause of death by cancer in men. Internal and external factors, such as genetic mutation, ethnicity, age, diet, etc., are observed to have been the causative elements for cancer to occur. Serine proteases, that are encoded by Kallikrein (KLK) gene family, show a remarkable impact on PC and some are introduced as PC biomarkers, such as KLK3 and KLK2, as both androgen-dependent are detected in the bloodstream when PC occurs. However, due to androgen dependence and low specificity, they are not quite reliable. In this study, we focused on KLK5 gene mutation in different PC androgen-independent cell lines, PC3, and DU145 cell lines to observe the effect of mutation of PC tumorigenesis. To monitor the effect of the mutation, the KLK5 gene's detected mutation point was knocked out using a genome-editing tool. Gene-editing technology has opened a new path to the scientists' investigation for designing the method that can benefit cancer treatment with the lowest side effects in the long term. In this study, CRISPR/Cas9 gene-editing technology was used to knock out c.245C>A (p.A82D) mutation in the KLK gene which was detected and confirmed by Sanger sequencing. To evaluate the performed gene editing, various cytotoxicity assays, such as cell viability with trypan blue, MTT assay, and colony formation was carried using Doxorubicin and PARP inhibitor which showed a promising result of CRISPR system as a future prostate cancer treatment.

Keywords: Prostate Cancer, KLK gene family, CRISPR system, KLK5 mutation

1. INTRODUCTION AND PURPOSE

Prostate cancer (PC) has been posing a huge threat to men because of heterogeneous factors and carcinogenic chemicals (N.J. Maitland 2013). The First report of Prostate cancer occurrence belongs to ancient times. However, the first treatment method that developed more than a century, is the development of prostatectomy, a surgical procedure with which prostate glands are removed (Capasso LL. 2005). PC has been reported as a slow and fast life-threatening tumour. Therefore, scientists focused on finding a better solution to detect PC at early stages to avoid the surgical approach.

Prostate-specific antigen (PSA) blood test has been commonly used to determine the manifestation of PC by the presence of kallikrein-related serine proteases, which are found in prostate secretion and the case of prostate glands' functional abnormality, in the bloodstream. And therefore, the test facilitates early stages of prostate cancer's diagnosis (Lilja H. Eet.al. 2008). However PSA dropdown in a mild and acute case of prostatitis and other prostate-related problems makes the antigen unspecific and difficult to consider as a PC biomarker (Guess HA. et.al. 1993).

Human serine proteases are members of human tissue kallikrein (KLK) family with 15 homologues serine proteases (KLK1-KLK15) that are encoded by the largest clustered human genome located on chromosome 19 (Borgoño CA.et.al. 2004). KLK2, KLK4, KLK5, KLK14, and KLK15 have shown to be the key element in KLK3 activation whose function includes digestion of seminogelins I and II, and fibronectin (Michael IP et.al. 2006, Emami N et.al. 2008)

Over the past few decades, various therapeutic methods have been developed to reduce the mortality rate of PC; drug treatment such as bicalutamide (Gravina GL et.al 2010), application short interfering RNA (siRNA) with chemotherapy (Izquierdo M. 2005), transfecting LNCaP and PC3 cell lines with RNA-aptamer (Marangoni K et.al. 2015), and many other methods has been used. Nevertheless, drawbacks, toxicity, cost, and PC recurrence due to short-term effects were observed.

Genome editing technologies are considered to be the most efficient tools to assist therapeutic approaches. Many studies are being carried out to design new methods that can improve gene editing function, their effectiveness, with minimum off-target effects. Clustered regularly interspaced palindromic repeats associated protein Cas9 (CRISPR/CAS9) provide has shaken up the genome editing in disease treatment, including cancers. The great capability of CRISPR/CAS9 in the treatment of broad ranges of rare and neurodegenerative diseases has been demonstrated. Use of CRISPR-Cas9 editing system is performed in ex-vivo models of somatic and pluripotent stem cells as wells as in-vivo animal models that can shed light on developing an approach that can be used in gene alteration and correction.

Before the discovery of gene editing tools, prostate cancer therapy complications such as drug resistance eluded physicians and scientists as they could not identify the causes of drug effects. The CRISPR-Cas9 ability of insertion and deletion of particular gene sequence on target DNA has given scientists and medical professions a remarkable solution in identifying drug-resistance genes by exposing drug-resistant cells to CRISPR/Cas9 gRNAs, which individually knockout single gene at the time in each cell (Scott, A. 2018). Therefore, the result of the experiment can improve drug therapy by introducing other more effective drugs for successful therapy (Scott, A. 2018).

This study aimed to introduce the effect of CRISPR knockout on the KLK5 gene in PC cell lines which is reported to be down-regulated in PC. Moreover, the experiments focused on monitoring the effect of KLK5 knockout on PC cells mutagenesis, proliferation, and viability rate under drug combination therapy which is hoped to be the positive approach for a better and less-aggressive therapy in a long term.

2. GENERAL INFORMATION

Prostate cancer (PC)is considered the second most common cancer among the male population and it has been posing a huge threat to men due to its high prevalence as 3.8% of death in men was caused by PC in 2018 (Ferlay J EM et.al 2019). Cancer begins in the prostate gland, located inferior to the bladder, which is a part of the male reproductive system and responsible for semen production in the male body (Robinson EJ et.al. 1998). Almost 98% of prostate cancers are reported to have been originated in glandular origin (Greenberg R. 2003). This organ is made of epithelial cell clusters which are arranged in the form of the basal layer and contains stem cells, transit-amplifying (TA) cells, and committed basal (CB) cells as the most dominant cell population in this structure (Robinson EJ et.al. 1998). Based on histological analysis of prostate organ, stem cells undergo division and form TA cells which are further divided by mitosis and differentiate into CB cells, the only cell type in prostate capable of DNA synthesis and proliferation, which are finally transformed into differentiating luminal cells in human (Greenberg R. 2003). Prostate cancer showed lack, deformation, and abnormal cell number of the basal layer in the prostate gland (M.M. Shen. et.al 2010).

Varieties of internal and external factors including family history, hormonal dysregulation, age, ethnicity, and infections (Patel, A.R.et.al 2009) has made the design of particular treatment difficult and ineffective. Age is of significance in occurrence and death by PC as the average age is calculated to be 66 at the time of diagnosis (Ferlay J EM et.al 2019). Ethnic difference is another element as the rate of PC in African-American men shows twice the rate of death caused by PC compared to white men (Ferlay J EM et.al 2019). Moreover, external factors such as social, environmental, and dietary factors are significantly effective in the cause of PC (Chan JM.et.al 2005, Willis MS.et.al 2003). Prostate cancer has existed since ancient times. However, the development of prostatectomy, a surgical mean for prostate cancer treatment, has been achieved for more than a century (Capasso LL. 2005). PC has been categorized as heterogeneous and it is observed as a slow-growing and fatal fast-growing form. Many studies have been researched investigating genes and genetic impairments involving in PC, which include the epidemiology of the disease while evaluating all the risk factors causing the disease.

2.1. Epidemiology of Prostate Cancer

Recent statistics show that the rate of PC is 7.1% of all cancers in males. However, the percentage correlates with the geographical location (Bray F.et.al 2018). The incident rate of PC elevates as the age of individuals is higher and it is 60%. Various methods were designed to identify the most convenient methods to diagnose PC at an early age. Stem cells characterization, collected by biopsy of patients, were carried out using CD44+ α2β1hi CD133+ markers as the phenotype which unfortunately was not specific for only cancer stem cells (CSCs) (G.D. Richardson.et.al 2004). Additionally, PAP (prostatic acid phosphate) was found to not be fully special for prostate cancer diagnosis since it was found to be elevated in benign prostatic hyperplasia (BPH) and other organ's malfunctions such as liver (Demichelis F.et.al 2015). Years of studies and analysis have introduced a more convenient way for diagnosis of the disease. Prostate-specific antigen (PSA) blood test is the test performed to determine the presence of kallikrein-related serine protease, an enzyme which is normally found in prostate secretion, but found in the bloodstream due to functional and histological anomaly of the prostate, and helps to diagnose prostate cancer in early stages for further treatment (Lilja H.et.al 2008).

Genetic factors are considered to have approximately a 5% contribution to the occurrence of the disease (Ferris-i-Tortajada J.et.al. 2011, Sridhar G.et.al. 2010). Still, the percentage elevates when causative alleles are passed on to the individuals. Added to the genetic factors, the loci and their rate of susceptibility has a slight contribution to the cause of the PC. Linkage study of PC introduces 7 loci that are estimated to be the most sensitive loci whose distortion or damage can lead to PC as these loci harbor the genes that maintain the sustainability of immune system mechanism and cell cycle that can eliminate abnormally-grown cells (Malathi K.et.al. 2007, Eeles RA .et.al. 2008, Schlaberg R.et.al 2009). Cell-death programming and antiviral activity are regulated by the enzyme known as ribonuclease L (RNASEL) which is encoded by the HPC1 gene on chromosome 1q 24-25 (Chen H.et.al. 2003, Malathi K.et.al. 2007). Impairment of some genes has shown to be the causative factor in retrovirus activities that is likely to cause PC (Schlaberg R.et.al 2009). An aggressive and familial form of PC was demonstrated to be closely associated with BRCA1, BRCA2, and PLAB2 genes (Gallagher DJ.et.al 2010). Besides generic mutations, distortion of the X

chromosome in Xq26.3-q27.3 causes hereditary prostate cancer, as the X chromosome carries a gene that encodes androgen receptor (AR) (Xu J.et.al. 2000).

2.1.1. microRNAs in Prostate Cancer

microRNAs, also known as miRNAs, are small, non-coding endogenous RNA molecules that are found in eukaryotes as well as some viruses and function in RNA regulations and diagnosis of various diseases because of their circulation in body fluids (Wahid F.et.al. 2010). Recent studies on miRNA transcripts have demonstrated their significance in prostate cancer as biomarkers. MicroRNA-21, also known as miR-21, mammalian microRNA encoded by *MIR21* gene located on chromosome 17, has been observed to suppress *PTEN* (tumor suppressor gene) in various cancers such as colorectal and breast cancer (Sheng WZ.et.al and Fragni M.et.al. 2016) but no indication of its direct effect on prostate cancer. Experiment on the effect of miR-21 in prostate cancer cells (PC3 cell lines) by transfecting them miR-21 to show its effect on prostate cancer cells. In the presence of matured miR-21, which is generally conserved in mammalian cells, prostate cancer cells evade immune defense due to suppression of *PTEN* by miR-21 and cause cell proliferation and metastasis (Yang Y.et.al. 2017). Therefore, miR-21 has been introduced as a prostate cancer biomarker, however, needs further studies are required to detect possible epigenetic influence on cancer cells.

Up-regulation of miR-21 led to the down-regulation of miR-15 and mirR-16 as well as overexpression of TGF-β signaling due to suppression of SMAD7 (Mothers against decapentaplegic homolog 7 leads to degradation of TGF-β and inhibits hedgehog signaling). which leads to AR hyper-activation and generation of the bone lesion in prostate cancer (Bonci D.et.al. 2016). Moreover, the impact of miR-221 and miR-222 on tumorgenesis and cancer progression in prostate cancer was seen (Yang, X.et.al. 2014). Furthermore, when PC3 cells are induced with miR-221 and miR-222 inhibitors in-vivo and in-vitro, their proliferation dropped down. This result indicates miR-221/222 increasing expression in prostate cancer and its metastasis (Yang, X.et.al. 2014). Other studies revealed elevation of other different miRNAs by examining prostate cancer patients against controls which could be advantageous in the diagnosis of prostate cancer.

Other miRNAs expressed a rather significant change in their level in response to tumorigenesis. To demonstrate, researchers analyzed miR-152-3p in LNCaP and PC3 in vitro compared to the bloodstream. The result indicated a low miR-152-3p level of tumour cells compared to healthy control cells. However, the impact of high-level miR-152-3p caused elevated proliferation and metastasis which shows presenting the external effect of miR-152-3p on tumorigenesis (Matin F.at.al 2018).

2.1.2. KLKs in Prostate Cancer

To this date, 15 members of tissue kallikrein (KLK1) and the kallikrein-related peptidases (KLK2–KLK15) with either trypsin or chymotrypsin-like activities have been identified and categorized (Lawrence MG.et.al. 2010, Emami N.et.al, 2007). KLK2 and KLK3 are found only in prostate and KLK3 (Prostate-specific antigen (PSA) is used as the biomarker of prostate cancer diagnosis (Borgoño CA.et.al. 2004, Shaw JL.et.al. 2007). The discovery of PSA dates back to 1979 when it was found in prostatic tissues (Wang MC.et.al. 1979). Apart from contributing to cancer diagnosis, KLK3 is the main modulator of sperm status in terms of semen liquefaction and coagulation (Lilja H.et.al. 1987). KLK3 also contributes to male infertility, as a degrading factor of semenogelin (Lilja H.et.al. 1987). Increased expression of KLK2, KLK3/PSA, and KLK4 in prostate cancer has been correlated with over-expression of KLK mediated proteolytic cascades that increases the rate of mutagenesis (Avgeris M.et.al. 2011, Herrala AM.et.al. 2001, Pollak M. 2008, Lawrence MG.at.al. 2010). Additionally, a study on DU145 cell lines shows their increased proliferation rate which is caused by KLK2- and KLK4 protease-activated receptors (PARs) (Mize GJ.et.al. 2008).

Moreover, the epithelial-mesenchymal transition (EMT) of prostate cancer cells have been observed to be strengthened by KLK3, KLK4, and KLK7 which could be a potential indicator of KLK3, KLK4, and KLK7 role in prostate cancer mutagenesis (Veveris-Lowe TL.et.al. 2005, Whitbread AK.et.al. 2006)

2.2. External Factors

Apart from genetic factors, external factors play a crucial role in PC occurrence. Many studies refer to the role of geographical location to be a significant element in the development of prostate cancer. Studies on Chinese and African immigrants in the USA showed a higher prevalence of PC compared to the ones living in their homeland, which could indicate the importance of lifestyle (Chu LW.et.al. 2011). Furthermore, dietary intake has shown to impart the prevalence rate of PC. Multiple studies found the effect of hight percentage of fat intake (meat, beaf, etc) to increase the rate of PC development and this could be due to effect of the saturated fat on insulin growth factor and higher proliferation rate, higher leukotrienes and prostaglandins, and increasing carcinogenicity of prostate cancer cells by androgen stimulations (Aronson WJ.et.al. 2010, Pauwels EK. 2011). The investigations on people of less fatty diets indicated that high-fat levels increase oxidative stress which can cause pre-oxidation leading to DNA damage (Lloyd JC.et.al. 2013). Another study shows that food and oils that are rich in omega 6 can increase proliferation by producing pro-inflammatory prostaglandins which enhances the malignancy of PC. On the other hand, a rich amount of Omega 3 can reduce the chance of the cancer cells' proliferation and eventually cause malignancy (Berquin IM.et.al. 2007). The amount of meat in a man's diet presents remarkable effect on the occurrence of PC as men with lower intake of meat shower lower rate of PC compared to the ones with higher intake of meat especially if the meat is cooked at great temperature (Major JM.et.al. 2011, Rohrmann S.et.al. 2007). This could be due to DNA damage which happens by the free radicals that are formed with lipid peroxidation caused by high temperature (Sinha R.et.al. 2009). The type of the consumed has shown to have a significant effect on the prevalence of PC. Consumption of calcium and calcium-rich products, such as dairy food increases the risk of PC (Wilson KM.et.al. 2015). Additionally, men with a rich diet of crucifers e.g. Broccoli have a low risk of PC over their lifespan which could be due to their antioxidants (Stram DO.et.al. 2006). Other things such as green tea, soy, and tomato have shown to decrease the chance of PC in males (103-119). The level of exposure to sunlight was shown to have a direct relationship with the risk of prostate cancer, as lack of vitamin D can lead to a higher risk of PC (Erdman JW.et.al. 2009). Further studies on race presented the effect of high melanin concentration on vitamin D deficiency, as African-American males showed a higher prevalence of PC which

remarks on inhibitory role of vitamin D on cell proliferation and invasion as well as apoptosis stimulator (Bhatia V .et.al. 2009, Chen TC.et.al. 2003).

2.3. CRISPR

Since the discovery of the CRISPR system, there have been remarkable achievements in the field of gene therapy. CRISPR system, an adaptive prokaryotic immune system acts as a bacterial defense mechanism against the foreign genome invasion and prevents destructive impacts of mobile genetic elements (MGE) which are delivered by phages and plasmids. CRISPR/Cas9 has shown to boost the host immune system using the invader's genetic material to protect the host from further invasion. The protection mechanism is completed with the acquisition of "spacer" sequences' by Cas (CRISPR-associated CAS) proteins. Cas proteins are guided to the exogenous spacer sequences of foreign nucleic acids by crRNA (CRISPR-associated RNA). The mechanism involves spacers' identification and anchoring by Cas proteins which is to provide a vindicative shield against further invasion. The CRISPR existence was found in 1987 by Yoshizumi Ishino et al., whose initial target, which was the iap gene, was cloned with a portion of CRISPR array (Y Ishino.et.al. 1987). The discovery incepted a new path in the gene treatment of challenging disorders. Following the discovery, further analysis of prokaryotes, such as Archaeoglobus fulgidus, showed other constituents of the CRISPR system including non-messenger RNA sequencing, transcription of DNA repeats loci to small RNAs (DNA repeats are the target DNA sequences that are acquired and preserved in CRISPR loci), (Barrangou R.et.al. 2007) and cas gene family (cas gene family are associated with CRISPR loci during immunity mechanism) (Tang TH et al..2002). Moreover, identification of specific spacer sequences from the viral genome described how bacterial systems show phenotypic resistance against the phage (Hefferin ML.et.al. 2002).

Classification of CRISPR/CAS systems **are** in 2 classes, with each class comprising various types (Type I-V) based on their flanking cas genes and location of the target on foreign DNAs (Sergey Shmakov et al. 2017) has given valuable information to design phage-resistance strains and phylogenetic classification of bacteria. During the phage/plasmid invasion, CRISPR-Cas functions in three phases-

adaptation, expression, and interference. Each stage expresses particular characteristics that result in anti-plasmid or antiviral immunity (Sergey Shmakov et al. 2016). The adaptation stage consists of an integration process by which the Invader-derived spacers (known as spacer sequence) merge with CRISPR array. In the next step, the CRISPR loci are transcribed into trans-activating RNA (crRNA) that contains spacer sequences. Consequently, cas endonuclease manifests itself and uses the spacer sequences as a guide to cleave the invader genome (Jeffry D.et.al. 2014)

The functional characteristics CRISPR-CAS system is defined by cas1 and cas2 genes. Recent taxonomic studies have classified the CRISPR-CAS system into three types based on the particular marker proteins; cas 3 (type I), cas 9 (type II), and Csm/Cmr (type III) (Makarova KS et al. 2011). Recent studies have introduced class IV and V CRISPR editing system (Makarova KS et al. 2015). A general classification of the CRISPR system is based on genes that express the functional proteins and factors (Cascade, Csm, Cmr complex, or Cas 9).

To accomplish drug design, animal models and human cell lines are the most ideal elements to test specific drug toxicity and efficacy before its applications on humans. However, animal models, as well as in-vitro analysis of human cell lines, may not provide a conclusive result on the effectiveness of a particular drug. CRISPR-Cas9 has provided means to modify cells that can represent human models for different types of cancers. ID8 (mouse ovarian surface epithelium) cells, that were obtained from ID8 mouse with ovarian cancer and the TP53 and BRCA2 genes knocked out by CRISPR/Cas9, demonstrated characteristics of human ovarian cancer cell lines (; high-grade serous carcinoma) (Walton, J.et.al. 2016). HGSCs show inhibited the action of TP53 and BRCA genes, loss of ability to form Rad51 foci (help in DSB repair), and sensitivity to PARP inhibition (Cruz, C.et.al. 2018). CRISPR-Cas9 has assisted in designing strong models that are likely to be used in measuring drug safety and eliminating drug resistance in human diseases.

In addition to drug design, CRISPR-Cas9 has been indicated as a promising gene-editing tool in cancer therapy. The dCas9 (mutated Cas9 without endonuclease activity, with added transcriptional activators on dCas9 or gRNA) is used to target specific genes by either activating or knocking them out when it is fused to

transcriptional activation or inhibition (Chen, B.et.al. 2013). Epigenome editing is another approach against cancer, with which dCas9 is tethered to histone modifiers involved in DNA methylation to disturb cancer mechanism, as DNA methylation is observed to have been affected in many cancers (Klann, T.S..et.al. 2017).

To find out the effect of the CRISPR-Cas9 system on cancers, such as Leukaemia, the human myeloid leukemia cell line, K562, without mutation on Isocitrate dehydrogenase (IDH2) gene underwent DSB and point mutation on IDH2 R140Q locus after being transfected by a plasmid that carries sgRNA and CRISPR-Cas9. Following such mutation, the gene repair was carried out by another transfection using pBS-SK(+) vector with CRISPR-Cas9 and fluorescent-template DNA followed by sgRNA to check the gene correction rate on point-mutated cells (Cruz, C.et.al. 2018). The result showed a high level of H3K9me2, H3K27me2, and H3K4me3 that indicated hypermethylation of chromatin in mutated cells129. In other studies, CRISPR-Cas9 was used as a tracking device to find out an effect of IL4 (Interleukin 4)-induced Stat6 activation on the elimination of leukemia cells by using lentiviral vectors that carry Cas9 and sgRNA for Stat6 (Signal transducer and activator of transcription) (Chen, B.et.al. 2013). The following experiment presented that IL4 and its antileukemic effect depended on AML cells to activate stat6 (Chen, B.et.al. 2013) which restate the efficiency of the CRISPR-Cas9 system as a therapeutic and diagnostic tool in various diseases. Several researchers have been carried out in cancer therapies using the CRISPR system. The 2CT-CRISPR assay is used to identify the genes that cause resistance to immune cells (Klann, T.S..et.al. 2017). The test consists of 2 types of target cells and effector cells such as melanoma cells and CD8+ T cells respectively. The assay aims to find out the factors mediating the growth of melanoma cells in response to an immune system and to design the efficient therapeutic ways in immunotherapy against cancer cells which were performed in-vivo on C57BL/6J mice (Klann, T.S..et.al. 2017).

3. MATERIALS and METHOD

3.1. Competent Cell Preparation

3.1.1. Materials

1. Single colony of E.coli cells
2. LB (Lucia-Bertani) medium (1000 ml volume); 10gr tryptone, 5g yeast extract, 10 gr sodium chloride. The medium was sterilized by autoclaving
3. SOB (super optimal broth) medium (1000 ml volume); 20 gr tryptone, 5 gr yeast extract, 0.5 gr sodium chloride. Sterilization was performed by autoclaving and 5 ml of 2M $MgCl_2$ was added.
4. 0.1M $CaCl_2$
5. LB plates with ampicillin; 10 gr tryptone, 5 gr yeast extract, 10 gr sodium chloride, 15 gr bacteriological agar. The medium was sterilized by incubating and after cooling down, 50 mg/ml ampicillin was added to avoid contamination.
6. plasmid DNA
7. 50-ml polypropylene tubes
8.15-ml polypropylene tubes

3.1.2. Method

E.coli was used as candidate bacteria to prepare competent cells before plasmid transformation. First, E.coli cells were grown on LB (Lucia-Bertani) agar medium and were harvested after 48-72 hours growth while they were in the logarithmic phase of growth. To make sure the growth phase is adequate, we checked cell viability not to be more than 1×10^8 viable cells/ml.

3.2. Bacterial Transformation

To transfer the CRISPR system and yield plasmid DNA, E.coli bacteria were transformed with the plasmids that contained the CRISPR product (vector). After incubating E.coli cells for 24-48 hours at 37°C, a single colony of 2-3 mm diameter was picked and transferred into 50-ml of LB medium into a sterile 300-ml flask. Then,

the flask was incubated at 37ºC overnight in a rotary shaking incubator with optimum shaking of 50-60 cycles/min. The next day, cells were transferred into 50-ml prechilled, sterile polypropylene tubes and left on ice for 10 minutes. Next, the suspension was centrifuged at 2400g for 10 minutes at 4ºC. After centrifugation, the supernatant was discarded and the pellet was suspended in 2.5 ml of an ice-cold solution of 80 mM $CaCl_2$ 50mM $MgCl_2$ and stored in ice for 10 minutes. The procedure was repeated twice to increase the recovery yield of cells.

Next, repeat the above procedure and resuspend the palette in 2 ml of the ice-cold 0.1M $CaCl_2$ an equal volume f 50% glycerol into polypropylene tube. Next, the cells were frozen at -70ºC. To increase the transformation efficiency, we used the stored cells no later than 24 hours.

1-5 µl of DNA (usually 10pg-100 ng) was transferred in 10-ml test tubes and was put in ice. Following this, the competent cells were thawed by warming them with hand and 20-50 µl of the suspension was added to the DNA tubes and mixed gently by flicking the bottom of the tube with your finger a few times. Next, the mixture was placed on ice for 30 minutes. Without shaking the tubes, they were given a heat shock by putting them into a water bath at 42ºC for 2 minutes. Then the tubes were transferred to ice immediately and held for 1-2 minutes. Then, 1 ml of LB or SOB medium was added and placed in a rotary shaker for 45 mins-1 hour for allowing the bacteria to acquire antibiotic resistance marker from the plasmid. The suspension was transferred on LB ampicillin medium with 20mM $MgSO_4$ and spread over the surface of the medium and left in room temperature until the suspension is adsorbed to the medium. Then, the plates were inverted and incubated at 37 ºC for 12-20 hours until the colonies appeared. To ensure DNA transformation was successful, E.coli colonies were collected from the plates and DNA purification was carried out.

3.3. Plasmid DNA Extraction (MiniPrep)

3.3.1. Material

1. *E.coli* cells
2. Zymo Research Quick-DNA Universal Kit

3. Freezer
4. Micropipette
5. Microcentrifuge tube
6. Water bath
7. Collection tube
8. Centrifuge machine

3.3.2. Method

To find out whether DNA transformation was successful in the plasmid, the DNA of *E.coli* cells were purified. To extract the DNA, Zymo Research Quick-DNA Universal Kit was used. The pellet was removed from the freezer and washed with 200 µl PBS. Next, 20 µl proteinase K was added to 200 µl of DNA sample and mixed well by pipetting up and down. The mixture was transferred into a microcentrifuge tube and the tubes were incubated in a water bath set at 55 ºC for 10 minutes until a clear mixture was observed. Next, 1 volume (420 µl) of genomic binding buffer was added to the digested sample and were transferred into Zymo-Spin IIC-XL Column in a collection tube. Then, the columns were centrifuged at 12000 x g for a minute and flow through in a collection tube was discarded. Then, 400 µl of DNA pre-wash buffer was added into a column and centrifuged at 12000 x g for another minute. The flow-through in the collection tube was discarded and 700 µl of g-DNA wash buffer was added into a column and centrifuged at 12000 x g for another minute. Next, 200 µl g-DNA wash buffer was added to each column and centrifuged at 12000 x g for another minute. The collection tube was discarded and the columns were put in a clean sterile microcentrifuge tube. Then 40-50 µl of elution buffer was added into the columns and they were incubated for 5 minutes at room temperature and then centrifuged at 12000 x g for 1 minute. The columns were discarded and the microcentrifuge tubes containing extracted DNA were stored at -20 oC for further analysis.

3.4. Gel Electrophoresis

3.4.1. Material

1. E.coli cells
2. Gel electrophoresis chamber
3. Agarose gel
4. TBE buffer; Tris base, Boric acid, EDTA, Distilled water
5. Microwave
6. 500 microwave flask
7. Ethidium bromide
8. Casting tray
9. Comb
10 DNA ladder
11. Loading dye
12. Paraffin paper
13. Micropipette
14. UV light

3.4.2. Method

To confirm the DNA purification of E.coli cells, gel electrophoresis was run.0.7 % gel was prepared by mixing 0.35 gr of agarose gell in 50 ml of 1X TBE buffer in microwave flask and then the mixture was put in microwave for about 2 minutes until a clear solution was seen. (To prepare 10X TBE buffer, 11.6 gr Tris base, 5.5 gr Boric acid, and 0.93 gr EDTA was added in 100 ml distilled water and mixed properly until a clear solution was observed). The solution was cooled down at room temperature until it reached 50 ^{o}C and then 2 µl of ethidium bromide (is a dye that binds to DNA and helps DNA to be observed under ultraviolet (UV) light was added to the solution. The solution was transferred into a casting tray with a comb put in before pouring the gel. The tray was settled until the gel was solidified. Then the gel-containing tray was moved into the electrophoresis chamber and 1xTBE buffer was poured in the chamber until it covered over the gel. Then, in the first hole, 2 µl of 1 kb DNA ladder was loaded as control. In the next holes, the sample that was mixed with

loading dye (5 µl DNA sample+ 1 µl of loading dye on paraffin paper) was loaded into the holes using a micropipette. Finally, the gel was run at 70 V for 45-60 minutes. The gel was carefully removed and moved to a tray and observed under UV light.

3.5. Cell culture: Growing Cells

For further cell culturing protocol, the growth medium component percentage was adjusted based on the cell line type as follows;

Table 3.1 : Cell Culture Conditions

Components (ml)	PC3	DU145
EMEM medium	87 ml	88 ml
FBS	10 ml	10 ml
L-Glutamine	2 ml	1 ml
penicillin	1 ml	1 ml

3.6. Cell Passaging (Subculturing)

3.6.1. Material

1. Cell Lines (PC3 and DU145)
2. CO2 incubator
3. PBS
4. Trypsin-EDTA
5. EMEM/DMEM medium containing FBS, Glutamine, penicillin
6. Culturing flask
7. Centrifuge tube
8. Centrifuge Machine

3.6.2. Method

To enhance cell growth, cells were passaged when they were 70-80% confluent. For sub-culturing, the flasks containing grown cells were removed from the incubator and the medium was discarded by pipetting out. Then, cells were washed with 1 ml of PBS to remove disturbing material that can cause contamination. Then cells went through trypsinization by adding 1 ml trypsin-EDTA to each flask and then the flasks were incubated at 37 ºC for about 2-3 minutes. The time of incubation can be adjusted by checking the detachment of the cells from the flask bottom observing under a microscope. Trypsinized cells start floating into trypsin-EDTA. 4 ml of fresh growth medium was added to each flask to deactivate the trypsin function to avoid cell death. Then, the whole medium containing cells was pipetted out and transferred into 10-ml centrifuge tubes and centrifuged at 1000-1200 rpm for 5 minutes. The supernatant was gently discarded and the pellet was transferred into a new growth medium (EMEM+10 FBS+ 1-2% L-Glutamine+ 1% penicillin) and incubated at 37 º C.

3.7. Preparation of Cell Pellet

3.7.1. Material

1. Cell Lines culture
2. PBS
3. Trypsin-EDTA
4. Culturing flask
5. EMEM/DMEM medium containing FBS, Glutamine, penicillin
6. Centrifuge tube
7. Centrifuge machine
8. CO2 incubator

3.7.2. Method

For further experiments, cells were preserved at a low temperature by making pellets. To make pellets, the growth medium was removed and cells were washed by

1 ml PBS to remove harmful substances. 1 ml of trypsin-EDTA was added for trypsinization step and the flasks were incubated at 37 °C. For 2-3 minutes. Then fresh growth medium was added to deactivate trypsin reaction and the whole culture was transferred into a 10-ml centrifuge tube and then, centrifuged at 1000-1200 rpm for minutes, the supernatant was discarded.

3.8. Cryopreservation of Cell Culture

3.8.1. Materials

1. Cell pellet
2. 10% DMSO
3. Cryotube
4. Cryogenic freezer
5. Freezing container (Mr.Frosty)
6. Liquid nitrogen tank

3.8.2. Method

Prepared cell pellets in the above technique were preserved by freezing technique in which cells were suspended in 10% DMSO growth medium and were transferred into cryotube. Then, the tubes were kept in a cryogenic freezer where the temperature reduced gradually to -30℃ followed by lowering the temperature to -60℃ overnight. Then, for a longer preservation period, cryotubes were transferred to a freezing container to be kept in a liquid nitrogen tank where the temperature drops to -130 ºC.

3.9. Thawing Frozen Cell Lines

3.9.1. Material

1. PC3 and DU145 cell lines
2. Water bath
3. EMEM medium containing 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% penicillin

4. CO2 incubator

3.9.2. Method

PC3 and DU145 cell lines that were obtained in a frozen form, were warm in a water bath which was set at 37ºC for about 3-5 mins and then cultured to the growth EMEM medium containing 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% penicillin and were grown in a CO2 incubator at 37 ºC.

3.10. Genomic DNA Purification from Mammalian Cells

3.10.1. Material

1. PC3 and DU145 cells lines
2. Zymo Research Quick-DNA mini-prep Kit
3. Freezer
4. Micropipette
5. Microcentrifuge tube
6. Water bath
7. Collection tube

3.10.2. Method

To carry out Sanger sequencing, PC3 and DU145 cell lines underwent DNA isolation to extract the DNA for further analysis. For the DNA isolation method, Zymo Research Quick-DNA Universal Kit was used. The pellet was removed from the freezer and washed with 200 µl PBS. Next, 20 µl proteinase K was added to 200 µl of DNA sample and mixed well by pipetting up and down. The mixture was transferred into a microcentrifuge tube and the tubes were incubated in a water bath set at 55 ºC for 10 minutes until a clear mixture was observed. Next, 1 volume (420 µl) of genomic binding buffer was added to the digested sample and were transferred into Zymo-Spin IIC-XL Column in a collection tube. Then, the columns were centrifuged at 12000 x g for a minute and flow through in a collection tube was discarded. Then, 400 µl of DNA pre-wash buffer was added into a column and centrifuged at 12000 x g for another minute. The flow-through in the collection tube was discarded and 700 µl of g-DNA

wash buffer was added into a column and centrifuged at 12000 x g for another minute. Next, 200 µl g-DNA wash buffer was added to each column and centrifuged at 12000 x g for another minute. The collection tube was discarded and the columns were put in a clean sterile microcentrifuge tube. Then 40-50 µl of elution buffer was added into the columns and they were incubated for 5 minutes at room temperature and then centrifuged at 12000 x g for 1 minute. The columns were discarded and the microcentrifuge tubes containing extracted DNA were stored at -20 ^{o} C for further analysis.

3.11. Primer Design For Pcr and Sanger Sequencing

To design the desired primers for Sanger sequencing and other analysis, Primer3 software was used and the following criteria were taken into account while selecting the primers; length should be 20 bps, and GC% should be between 50-6-%, and to increase primer efficiency, the primers with G or C 3' end was selected.

3.12. PCR Amplification

3.12.1 Material

1. ddH_2O
2. Master Mix; Taq polymerase enzyme, dNTPs, 1.5 5mM $MgCl_2$, and reaction buffer
3. Vortex
4. Eppendorf reaction tube
5. Thermal Cycler

3.12.2. Method

All components were mixed and centrifuged for 30 sec-1 min before use. 22 µl ddH_2O and 25 µl of the master mix were added to 200 µl PCR eppendorf reaction tube. The components were mixed and vortexed gently. Next, 1 µl of the DNA template, 1 µl of forward primer, and 1 µl of reverse primer were added and gently mixed.

Table 3.2. PCR Amplification Components

Component	50 µl reaction
Water, nuclease-free (ddH_2O)	22 µl
Invitrogen Platinum II Taq Hot-Start PCR master mix (taq polymerase enzyme, dNTPs, 1.5 5mM $MgCl_2$, and reaction buffer)	25 µl
10 µM forward primer	1 µl
10 µM reverse primer	1 µl
template DNA	1 µl

The tubes were then incubated in a thermal cycler (3-step protocol) according to the table below.

Table 3.3: PCR Conditions

step		3-step protocol		2-step protocol	
		Temp	time	Temp	time
Initial denaturation		94ºC	2 min	94ºC	2 min
25035 PCR cycles	Daneutre	Anneal	15 sec	98 ºC	5 sec
	Anneal	60 ºC	15 sec	60 ºC	15 sec
		68 ºC		60 ºC	15 sec
hold		4 ºC	hold	4 ºC	hold

3.13. Sanger Sequencing Workflow

To ensure KLK5 gene mutation in cell lines, Sanger Sequencing was carried out by the Genetiks company. Primer design for PCR (Polymerase Chain Reaction)

and Sanger sequencing was carried out using ThermoFisher primer design tool (Invitrogen™ Primer Designer™ Tool) by uploading KLK5 gene sequence and the following criteria were taken into account while selecting the desired primer; primer length (between 18-22 bps), primer's GC content (50-55%), GC-lock on the 3' end of the primer, melting temperature (50-55^{o}C), no poly base regions in the primer's sequence, no 4 or bases complement either direction of primer.

After selecting the primers, the sequences were sent to the company for designing, where they were checked by mass spectroscopy to check the success rate to more than 95%. Invitrogen Platinum II Taq Hot-Start DNA Polymerase was used in the PCR protocol.

3.14. PCR clean-up

3.14.1. Material

1. ExoSAP-IT Express Reagent
2. Freezer
3. Ice
4. Vortex
5. Eppendorf reaction tube
6. Centrifuge machine
7. Thermal cycler

3.14.2. Method

PCR reactions were cleaned up using ExoSAP-IT Express Reagent (a mixture of exonuclease I and shrimp alkaline phosphatase (SAP) that removes excess primers and dNTPs after a PCR reaction). The method was as follows; the ExoSAP-IT Express Reagent was removed from -20^{o}C freezer and transferred on the ice during the whole procedure. 5 µl of the above PCR product was mixed with 2 µl of the reagent and vortexed gently to mix them properly. Next, the tubes had a quick spin to help the content precipitate to the bottom of the tube. Then the tubes were incubated in a thermal cycler at 37^{o}C for 4 minutes (to degrade the primers nucleotides that are still left in the PCR product) and 80^{o}C for 1 minute to inactivate ExoSAP-IT Express

Reagent. Then the tubes were held at 4 °C and transferred to ice.

3.15. Cycle Sequencing

3.15.1 Material

1. Genomic DNA
2. Forward and reverse primers
3. BigDye Direct PCR master mix
4. ddH_2O
5. Eppendorf reaction tube
6. Thermal cycler
7. Micropipette

3.15.2. Method

The sequencing was carried out by Applied Biosystems BigDye Direct Cycle Sequencing Kit. To prepare the reaction for PCR, the following components were added as it is shown in the table below;

Table 3.4. Components of Cycle Sequencing in Sanger Sequencing

Component	Volume
Genomic DNA (4ng/ µl)	1 µl
M13-tailed PCR primers (0.8 µ each) (it included both forward and reverse primers)	1.5 µl
BigDye Direct PCR master mix	5 µl
ddH_2O	2.5 µl
Total volume for each PCR reaction	10 µl

All the components were mixed by pipetting up and down and were sealed and

span briefly. Then, the tubes were run in a thermal cycler with the following time and temperature panel;

Table 3.5. Cycle Sequencing Condition

Stage	Verity thermal cyclers		9700 thermal cycler	
	Temp	time	Temp	time
Hold	95 ºC	10 min	96 ºC	5 min
Cycle (35 cycles)	96 ºC	3 sec	94 ºC	30 sec
	62 ºC	15 sec	62 ºC	45 sec
	68 ºC	30 sec	68 ºC	45 sec
hold	72 ºC	2 min	72 ºC	2 min
hold	4 ºC	∞	4 ºC	∞

In the next step, the cycle sequencing reactions were prepared according to the company's manual based on the table below;

Table 3.6. Cycle Sequencing Reaction Components

Component	Volume
BigDye Direct sequencing master mix	2 µl
One sequencing primer BigDye Direct M13 forward primer or BigDye Direct M13 forward primer	1 µl
The total volume to add to each sequencing reaction	3 µl

The above reaction was added to the appropriate well of amplified DNA for each reaction. The plates were sealed and span briefly. And then incubated in a thermal cycler according to the table below;

Table 3.7. Cycle Sequencing Incubation Condition

stage	Verity thermal cyclers		9700 thermal cycler	
	Temp	time	Temp	time
hold	37 °C	15 min	37 °C	15 min
hold	80 °C	2 min	80 °C	2 min
hold	96 °C	1 min	96 °C	1 min
Cycle (25 cycles0	96 °C	10sec	96 °C	10sec
	50 °C	5 sec	50 °C	5 sec
	60 °C	75 sec	60 °C	4 min
hold	4 °C	∞	4 °C	∞

3.16. Purification of sequencing products

3.16.1. Material

1. Eppendorf reaction tube
2. SAM solution
3. Centrifuge machine
4. Vortex

3.16.2. Method

After the reaction was completed, the tubes were span briefly and the seals were removed and 55 µl of SAM solution were added to each well. The preparation o SAM solution was as follows;

Table 3.8. Components for Sequencing Products' Purification

Component	Volume for 1 well
SAM ™ solution	45 µl
XTerminator Solution	10 µl
Total volume	55 µl

Before adding the solution, it was vortexed vigorously for 10 seconds to read to a homogeneous solution. Then, the well was sealed properly and vortexed at 1800 rpm for 20 minutes. Then the wells were centrifuged at 1000 × g for 2 minutes.

3.17. Capillary Electrophoresis and Data Analysis

As a next step, capillary electrophoresis was carried out using 3500 Series Genetic Analyzers and then the sample was run on minor Variant Finder Software that detects minor variants as low as 5%.

3.18. Computational Analysis of Protein Structure and Severity of Damage Caused by Mutation

To determine structural changes of KLK5 protein, protein structure analysis was performed using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). In addition, damage sensitivity of the mutation was analyzed using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). The mutation taster program (www.mutationtaster.org/) was performed to check the effects of the mutation on the protein

3.19. Construction of the CRISPR system

A total of 3 target sequences (gRNAs) were selected in KLK5 based on the position of the mutation detected by Sanger sequencing. To design the CRISPR system, sgRNA was designed using chop-chop Harward software, where the KLK5 gene sequence was entered to analyze the gRNA sequence of 20 bps that are followed

by the PAM sequence (5'NGG). The program selected the ideal possible gRNAs with the optimum GC percentage (53-56%) and off-target sites which are 17-24 bps between the gRNA and KLK5 sequence due to mismatching. After loading the gene into chop-chop Harward software, we selected desired sgRNAs based on the location upstream of the PAM sequence of the target sequence (without counting the PAM sequence)

3.20. Mammalian cell Transfection with DNAfectin Reagent

3.21.1. Material

1. DU145 cell line
2. DNAfectin reagent
3. CRISPR-sgRNA
4. EMEM medium
5. 6-well plates
6. Vortex
7. Micropipette
8. Culture dish
9. CO2 incubator

3.22.2 Method

After completion of the plasmid replication and confirmation of KLK5 mutation by Sanger sequencing, DU145 cell lines were subjected to transfection by 3 designed plasmids using DNAfectin reagent. The reagent and protocol were obtained from abm pharmaceutical company (www.abmGood.com). DNAfectin is a nanoparticle. Nonliposomal reagent that transfects the mammalian cells with DNA plasmid with the minimum cytotoxicity. The reagent was selected due to its advantage of not affecting cell viability and low risk of contamination as the medium is not required to be pipetted out during the procedure. The highly efficient transfection (according to the company's protocol) was accomplished by optimizing the cell density against DNAfectin which are mentioned in the table below;

Table 3.9. Reagent Quantities for Different Culture Vessels in Mammalian Cell Transfection

Culture vessel	The volume of plating medium per well	DNA (µg)	DNAfectin (µl)	Transfection medium volume (µl)
24-well	500 µl	0.2-0.4	0.6-1.2	50
12-well	1ml	0.5-0.8	1.5-2.5	100
6-well	2 ml	1-2	3-6	200
35mm	2 ml	1-2	3-6	200
60mm	5ml	3-6	10-20	300
10cm	10ml	8-16	25-50	500

As we used 3 different sgRNA for 3 different target sequences on DNA, 3 samples (2 for each) were prepared. 6-well plates were selected to carry out the transfection. The 24-hour pre-cultured cells in EMEM medium was used while the cells were 70-90% confluent. as the number of the wells were 6, 6 DNAfectin-DNA complex were made. 6 sterile eppendorf tubes were taken and in each, 200 µl of EMEM medium (without antibiotic and serum) and 20µg of DNA were added. DNAfectin was warmed at room temperature and vortexed gently before use. After that, 6µl of DNAfectin was added to each tube and pipetted up and down for mixing the complex properly. Then, the mixture was incubated at room temperature for 20 minutes to form the DNAfectin-DNA complex (the following complex is stable for 3-5 hours at room temperature). Then, the solution was added drop by drop to different areas on cultures dish of DU145 cell lines. To distribute the solution, the dish was gently moved side to side and back-and-forth to evenly spread the solution over the whole culture. The plates were incubated overnight (12-16 hours) and culture medium was changed by pipetting out the medium and adding the fresh medium to the plates.

3.23. Antibiotic Selection

3.23.1. Material

1. DU145 cells
2. Plasmid DNA
3. Puromycin
4. EMEM medium
5. Incubator
6. Micropipette
7. Culture plate
8. Agarose gel electrophoresis
9. Gel electrophoresis chamber
10. Casting tray
11. Comb
12. Ethidium bromide
13. DNA ladder
14. Loading dye
15. UV light

3.23.2. Method

To select DU145 cells, which were successfully transfected by plasmid DNA, puromycin was used for screening purposes. After optimizing the concentration for DU145 cell lines, we determined 5µg/ml is an ideal concentration to kill the cells not carrying the pac gene. EMEM medium was prepared with 5 µg/ml of puromycin. DU145 cells, which were previously transfected with the plasmids containing pac genes 48 hours before the following experiment, were cultured in the medium+ puromycin in a sterilized condition. To enhance the result of selection, we diluted the cell to have no more than 25% confluence. The following plates were then incubated at 37ºC for 3-4 days. Next, the medium was pipette out and a fresh selection medium was added. The procedure was repeated after 3-4 days. After 7 days of selection, the cells were evaluated for foci formation. Finally, 5-10 resistant clones were picked and transferred to a 35mm culture plate with a selection medium for another 7 days. To

confirm the presence of DNA, DNA extraction, and gel electrophoresis were carried out to check whether DNA is available.

3.24. Drug Resistance Assay

Wild type DU145, knock out DU145, and PC3 cell lines were subjected to drug resistance assay using Doxorubicin and PARP (poly ADP ribose polymerase) inhibitor. Doxorubicin has been commonly used in cancer treatment, including prostate cancer. It is a member of tetracycline family and damages cell's DNA and drives them towards apoptosis by halting the cells in G2/M phase PARP are the catalyst enzymes that facilitate ADP-ribose transfer to the target proteins that are involved in DNA synthesis and repair mechanism PARPs are shown to have a significant role in maintaining and moderating chromatin structure, cell proliferation, and apoptosis.

3.25. Cell Counting

3.25.1. Material

1. Cell lines
2. EMEM medium with 10% FBS, 1% L-Glutamine and 1% penicillin
3. CO2 incubator
4. Micropipette
5. PBS
6. Trypsin-EDTA
7. Microscope
8. Culturing flask
9. Centrifuge machine
10. Haemocytometer

3.25.2. Method

To estimate cells' viability, cell lines were cultured in EMEM medium with 10% FBS, 1% L-Glutamine, and 1% penicillin. The plates were incubated in a CO2 incubator at 32^{o}C. After adequate growth with 70-80% confluence, cells were

collected by the following procedure:

The medium was pipetted out and the cells were washed with 1ml PBS (Phosphate-Buffered Saline) to get rid of all disturbing substances. After a slight moving of the flask, PBS was pipetted out and discarded. Next, trypsinization was carried out where 1 ml of trypsin-EDTA (trypsin is a proteolytic enzyme that degrades proteins to stop cell adhesion to the bottom of the flask) was added to the flasks and they were incubated at 32 ° C incubator for 2-3 minutes until cells dissociated themselves from the flasks (dissociation was confirmed by mounting cells under a microscope when the cells were floating in trypsin). 4 ml fresh serum contained-EMEM medium was added to deactivate the trypsin effect and to avoid cell death. And the whole culture was centrifuged for 5 minutes at 1000-1200 rpm. The supernatant was discarded and fresh medium was added to platelet and mixed properly by pipetting in and out to let the cells spread evenly in the medium. 20 µl of the culture medium was pipetted and transferred on a hemocytometer for total cell counting (dead+ live cells). To calculate the total cell number, the following formula was used;

Average cell count per square: total number of cells in 4 squares/4

Total cell count: average cell count per square×dilution factor (5 ml medium) $\times 10^4$ (number of squares)

3.26. Cell Viability Assay

3.26.1. Material

1. PC3, DU145 wild type, and DU145 knock-out cell lines
2. 35-mm plates
3. EMEM medium
4. Incubator
5. Doxorubicin
6. PARP inhibitor
7. DMSO
8. Freezer

9. Trypsin
10. Methylene Blue
11. Haemocytometer
12. 100-mm Petri dish

3.26.2. Method

PC3, DU145 wild type, and DU145 knock-out were cultured in 35-mm plates with 5 ml serum-contained EMEM medium and incubated for 48 hours until reaching to appropriate confluence. 2 sets of plates were prepared for each cell line to test drug resistance against Doxorubicin as well as Doxorubicin+PARP inhibitor. The drugs were obtained from a pharmacy in the form of 5mg-powder in a vial and they were made in the form of solution using DMSO according to the following calculation below;

Table 3.10. Preparation of Drug Solution

Drug information	PARP inhibitor	Doxorubicin
Molecular weight (g/ml)	438.08	543.52
The volume of powder in vial	5mg	5mg
DMSO (dimethyl sulfoxide) (ml)	1.149	0.920
Final volume (mM/ml)	10	10

After the drugs were made into a solution, there were kept at -80^{o}C to preserve them. For the desired concentration, a serum-contained EMEM medium was used to dilute the drugs. After 48 hours of incubation, the plate for the drug combination test (Doxorubicin+PARP inhibitor) was removed from the incubator, and 10μM of PARP inhibitor was added to each well. Next, the plate was incubated at 37^{o}C for 1 hour. The plates were removed and the following doxorubicin concentrations were added to each

well and incubated for 48 hours. Then the cells underwent through trypsinization process to collect the cells. After centrifugation, cells were mixed with Methylene blue (1:4 volume) to calculate cell viability using a haemocytometer. Methylene Blue can pass dead cells' cell walls and turn blue under a microscope, while live cells remain intact without a change in their colour.

Table 3.11. Cell Numbers and Single Drug Concentration

Plate number	Cell number/ml	Doxorubicin/ml
1	10^4	Control
2	10^4	500 nM = 0.5 µl
3	10^4	1µM = 1 µl
4	10^4	2 µM = 2 µl
5	10^4	4 µM = 4 µl
6	10^4	8 µM = 8 µl

Table 3.12. Cell Numbers and Single Drug Concentration

Plate number	Cell number/ml	PARP inhibitor	Doxorubicin/ml
1	10^4	control	control
2	10^4	10 µM= 10 µl	none
3	10^4	10 µM= 10 µl	500 nM = 0.5 µl
4	10^4	10 µM= 10 µl	1µM = 1 µl
5	10^4	10 µM= 10 µl	2 µM = 2 µl
6	10^4	10 µM= 10 µl	4 µM = 4 µl
7	10^4	10 µM= 10 µl	8 µM = 8 µl

3.27. MTT Assay

3.27.1. Material

1. PC3, DU145 wild type, and DU145 knockout cell lines
2. 96-well tissue culture plates
3. EMEM medium
4. CO2 incubator
5. Doxorubicin
6. PARP inhibitor
7. MTT reagent
8. DMSO
9. Micropipette
10. FLUOstar Omega microplate reader

3.27.2. Method

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay was performed to measure the cell viability under certain drug concentrations.

10^4 cells/well of PC3, DU145 wild type, and DU145 knockout were plated into 96-well tissue culture plates with a serum-contained EMEM medium. The plate was incubated for 48 hours at 37°C. For each cell line, two sets of treatments were prepared for doxorubicin and doxorubicin+ PARP inhibitor. And the concentration of the following drug was added to the wells;

Table 3.13. MTT Assay Doxorubicin Concentration Per Well

Plate number	Cell number/ml	Doxorubicin/100µl
1	10^4	Control
2	10^4	500 nM = 0.5 µl
3	10^4	1µM = 1 µl
4	10^4	2 µM = 2 µl
5	10^4	4 µM = 4 µl
6	10^4	8 µM = 8 µl

Table 3.14. MTT Assay Doxorubicin and PARP Inhibitor Concentration Per Well

Well number	Cell number/ml	PARP inhibitor/100 µl	Doxorubicin/100 µl
1	10^4	control	control
2	10^4	10 µM= 10 µl	none
3	10^4	10 µM= 10 µl	500 nM = 0.5 µl
4	10^4	10 µM= 10 µl	1µM = 1 µl
5	10^4	10 µM= 10 µl	2 µM = 2 µl
6	10^4	10 µM= 10 µl	4 µM = 4 µl
7	10^4	10 µM= 10 µl	8 µM = 8 µl

After 48 hours, the plate was removed from the incubator and 10 µl of MTT solution was added to each well mixed gently. The plate was then incubated at 37ºC for 4 hours. Then, the plates were removed again from the incubator and 200 µl of DMSO was added to each well and pipette up and down to dissolve formazan salt. The final volume was each well is 300 µl. The absorbance was measured using a spectrophotometer (FLUOstar Omega microplate reader)at 570 nm. In the resulted chart (obtained in the form of an excel sheet), the background absorbance was subtracted from signal absorbance to obtain normalized absorbance values. And the

graphs were drawn to create a comparison of drug resistance among the cell lines.

3.28. Colony Formation Assay

3.28.1. Material

1. PC3, DU145 wild type and DU145 knock out cell lines
2. Doxorubicin
3. PARP inhibitor
4. EMEM medium
5. CO2 incubator
6. PBS
7. Trypsin-EDTA
8. Centrifuge Machine
9. Trypan blue
10. Haemocytometer
11. Microscope
12. Tab water
13. Tissue paper

3.28.2. Method

Colony formation assay (CFA) or colonogenic assay is a 3D in vitro survival assay to determine the ability of the cell to form colonies and measuring cell death rate under specific drug treatment. In this method, colorimetric dye such as trypan blue was used to measure the number of colonies formed by the single cells. This method is beneficial to observe tumorigenic characteristics of specific cancer cells when they are treated with specific drugs.

In our experiment, PC3, DU145 wild type, and DU145 knock out were subjected to Doxorubicin and PARP inhibitor to compare the tumorigenic rate of the specific cell lines after the incubation period. To optimize drug concentration to the desired one, a serum-contained EMEM medium was used. Cell lines were cultured in EMEM medium with 10% FBS, 1% L-Glutamine, and 1% penicillin. The plates were

incubated in a CO2 incubator at 32ºC. After adequate growth with 70-80% confluence, cells were collected by the following procedure:

The medium was pipette out and the cells were washed with 1ml PBS to get rid of all disturbing substances. After a slight moving of the flask, PBS was pipetted out and discarded. Next, trypsinization was carried out where 1 ml of trypsin-EDTA was added to the flasks and they were incubated at 32 º C incubator for 2-3 minutes until cells dissociated themselves from the flasks 4 ml fresh serum contained- EMEM medium was added to deactivate trypsin effect and to avoid cell death. And the whole culture was centrifuged for 5 minutes at 1000-1200 rpm. The supernatant was discarded and fresh medium was added to the palette and mixed properly by pipetting in and out to let the cells spread evenly in the medium. 20 µl of the culture medium was pipette and transferred on a haemocytometer for total cell counting (dead+ live cells). The number of the cells was optimized and calculated to reach the desired cell numbers as mentioned in table 15. After optimization, the number of the cells in plates was calculated as follows;

Table 3.15. Cell Number and Single Drug Concentration in CFA

Plate number	Cell number/ml	Doxorubicin/ml
1	250	control
2	250	500 nM = 0.5 µl
3	500	1µM = 1 µl
4	1000	2 µM = 2 µl
5	2000	4 µM = 4 µl
6	4000	8 µM = 8 µl

Table 3.16. Cell Number and Double Drug Concentration in CFA

Plate number	Cell number/ml	PARP inhibitor	Doxorubicin/ml
1	250	control	control
2	250	10 µM= 10 µl	500 nM = 0.5 µl
3	500	10 µM= 10 µl	1µM = 1 µl
4	1000	10 µM= 10 µl	2 µM = 2 µl
5	2000	10 µM= 10 µl	4 µM = 4 µl
6	5000	10 µM= 10 µl	8 µM = 8 µl
7	10000	10 µM= 10 µl	8 µM = 8 µl

The 5 ml of the growth medium and the desired cell number were transferred into 35-mm plates and incubated for 48 hours at 32ºC. Then the plates that were designed for drug combination therapy were removed from the incubator and diluted 10 µl/ml of PARP inhibitor was added to each plate. The plates were incubated for an hour and all plates were removed and prepared doxorubicin was added into each plate and kept at 32ºC for another hour (the time was maintained based on doxorubicin half-life which is 20-48 hours. Finally, the plates were removed, the medium was pipetted out and discarded, and the cells were washed twice with 1 ml cold PBS to ensure no drugs remained in the plates. Finally, a 5 ml fresh growth medium was added to each plate and incubated for 5-7 days to observe colony formation of each cell line. After the appropriate numbers of colonies were observed under a microscope, the staining was carried out to count the colonies. For this procedure, the medium was discarded and the plates were washed with 2 ml PBS (in each). Then, a trypan blue solution was added to each plate until the whole bottom of the plate was covered blue. The plates were incubated at room temperature for 1-2 hours until all colonies were stained. Finally, the plates were gently washed with tab water (without disturbing the colonies) and were inverted over tissue papers and left overnight until they are completely dried. Then the colonies were counted and noted with each cell line and drug concentration.

4. RESULTS

4.1. Bacterial Transformation

Bacterial transformation is used to replicate the plasmid and the resulted cells contain both bacterial resistance gene (puromycine resistant) and replication gene of bacteria. To maintain the plasmids without rearranging the plasmid DNA and to increase transformation efficiency by making bacteria more sensitive towards the chemical or electrical shock, the competent cells were generated. Generation of competent cells, *Escherichia coli* (*E.coli*) was used. E.coli are gram-negative aerobic bacteria that are used for bacterial transformation purposes as they grow faster and cells do not create clump. Although many strains of E.coli are pathogenic, some are non-pathogenic which are used to design the desired plasmid to transform the CRISPR and replicate CRISPR-containing plasmids. In this experiment, DH5-α *E.coli* was gifted by Dr. Meryem Alagoz and used in the bacterial transformation. DH5-α is a non-pathogenic lab-engineered strain of *E.coli* for cloning purposes. This strain contains a plasmid that facilitates cloning and transformation of genes. The strain's genome is a circular chromosome that has 4,686,137 nucleotides, 4359 genes, and 4128 protein-encoding genes. The mutations in this strain are as follows; dlacZ Delta M15 Delta (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-mK+) supE44 thi-1 gyrA96 relA1. endA1 mutation allows higher efficiency of transformation as it decreases the endonucleases degradation.

After the bacterial transformation was completed, the LB agar medium containing ampicillin as an antibiotic allowed the selection of the colonies that acquired the antibiotic resistance marker from the plasmid (figure 1). In this technique, ampicillin was used as an antibiotic to select DH5-α E.coli plasmids that successfully acquired the ampicillin resistance gene from pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro (our lentiviral vector containing designed CRISPR). And the colonies that appeared showed the ones that were successfully transformed by the plasmid.

4.2. Gel Electrophoresis

Agarose gel electrophoresis is shown to be one of the most efficient laboratory tools to separate DNA fragments based on their sizes which can vary from 100 bp-25kb. Agarose is a repeated L- and D-galactose subunits that form a network that acts as a sieve. The sieve is a bundle containing pore of different sizes that are arranged in a gel matrix. And the pore size is inversely related to Agarose concentration i.e. as the Agarose concentration increases, the pore sizes reduce. DNA has a phosphate backbone that makes DNA molecule negatively charged. And therefore, DNA migrates towards a positive anode. Ethidium Bromide is a most applied DNA stain for gel electrophoresis and it binds to DNA which helps DNA to be visualized under ultraviolet (UV) light. Loading dye, which is combined with the DNA sample, helps the DNA sample to sink into the gel and helps to identify DNA migration through the gel during the run. As we had 3 vectors containing 3 different gRNA sequences that targeted 3 specific sequences on the KLK5 gene, we had 3 DH5-α plasmids each containing the following sgRNA sequence;

sgRNA164; ATCCGACCGGGCGTCTTCCC
sgRNA218; GCGATATGCACACCCAGCCG
sgRNA265; GCCCCGCAGTAGAGCTGGTT

To confirm the presence of DNA in all plasmids, DNA was purified and was run by gel electrophoresis in 0.7% Agarose gel and set at 70 V for about an hour.

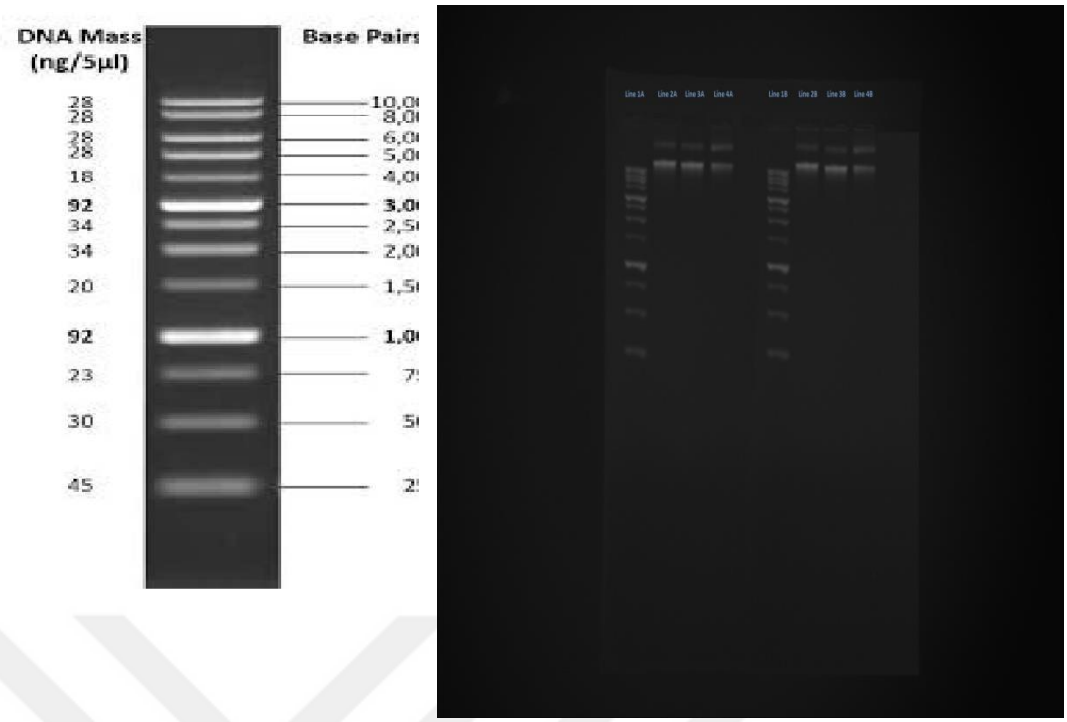


Figure 4.1. Gel Electrophoresis of Competent Cells

As figure 2 explains, the plasmid containing the vector DNA sequence was successful, as one band was observed for all the plasmids that indicated no contamination as well as no homologous recombination. Line 1A and 1B is the DNA ladder with 1 Kb size. The electrophoresis for plasmid DNAs was performed twice Line 2A and Line 2B are the first plasmid DNA. Line 3A and 3B are second plasmid DNA. Line 4A and Line 4B are third plasmid DNA

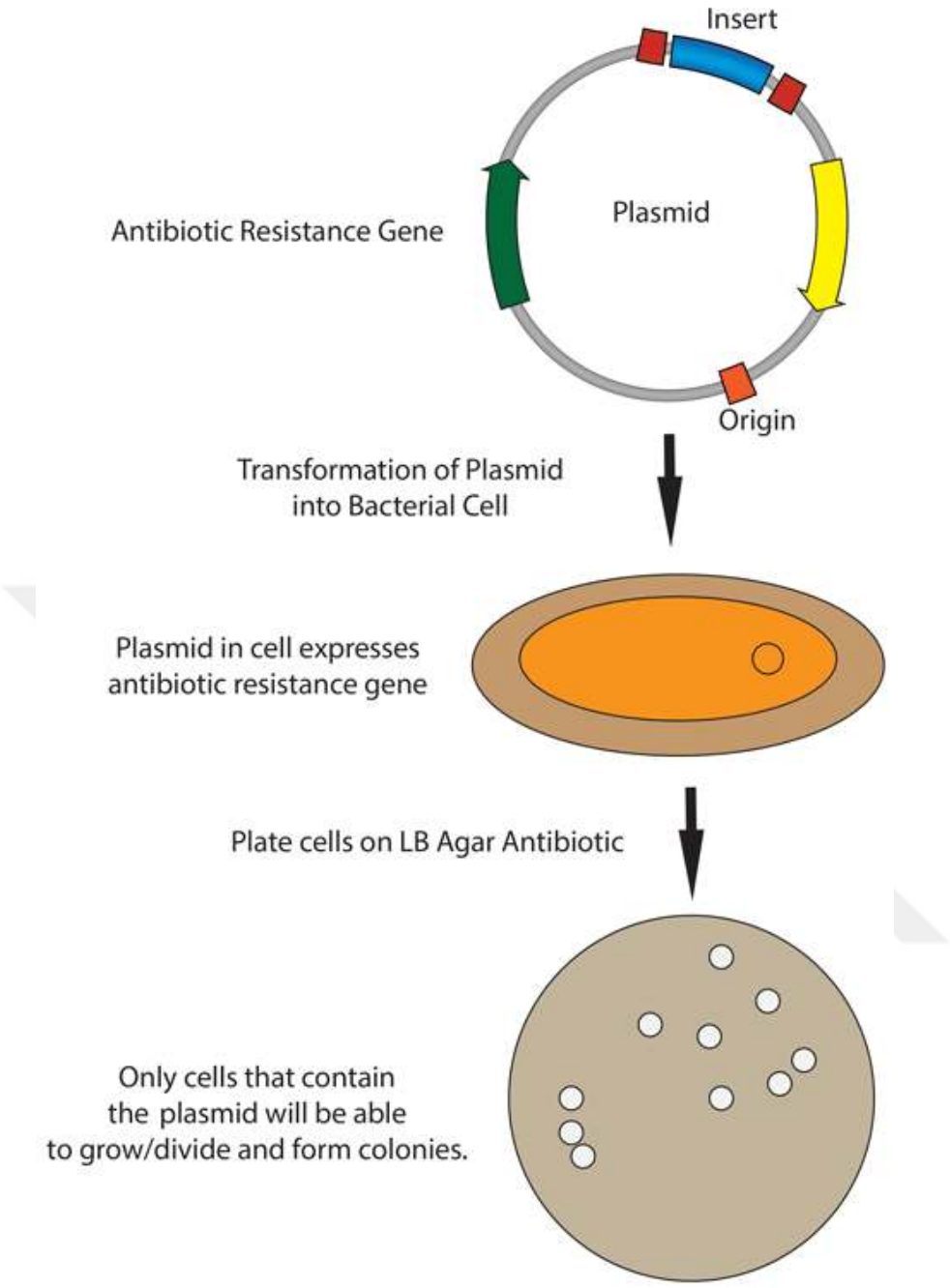


Figure 4.2. Antibiotic Resistance Screening on Plasmids.

4.3. CRISPR Construct

CRISPR has been used to knockin and knockout the target short sequence of a gene and has been applied for treatment purposes in many diseases. The CRISPR editing tool is an adaptive immune system in prokaryotes that protects host cells from

mobile genetic elements that are entered the host cells by phage or plasmids. In this system, after the introduction of double-strand break (DSB) by cas nuclease. The DSB position happens by specific gRNA that guides cas nuclease to the target sequence of nucleotides of the interest gene. After introduction DSB, DNA repair mechanism, most likely non-homologous end joining (NHEJ), initiates to repair the cut DNA.

After designing sgRNAs that were selected based on the position of the mutation on the KLK5 gene confirmed by Sanger sequencing, the sequence was sent to ABM company (www.abmgood.com) where they constructed sgRNA and cas protein. The products were packaged into lentivector to form pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro with the following map below

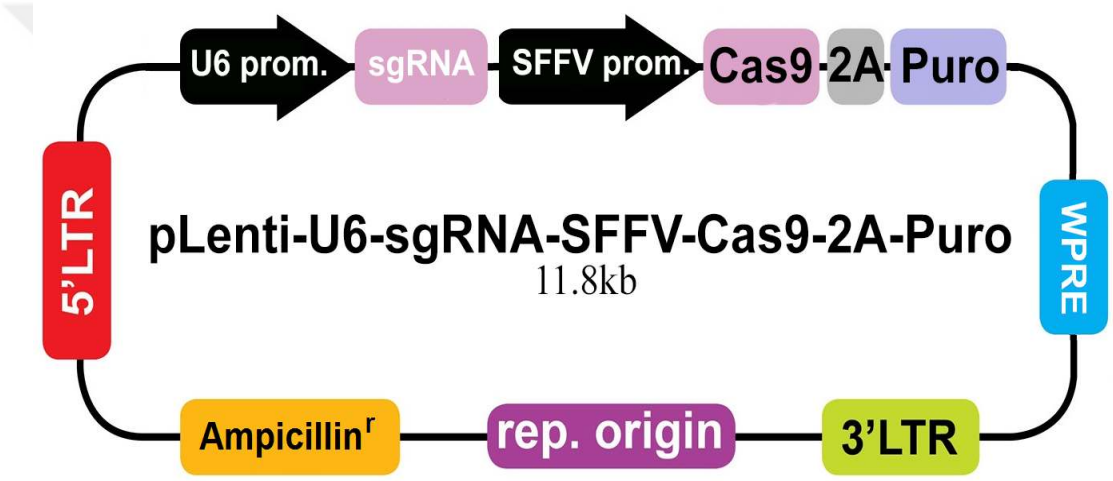


Figure 4.3. Lentiviral Vector Map

As the map shows, our designed vector contained ampicillin (bacterial) as well as puromycin (mammalian) resistance genes with 11.8 kb DNA, was received in the liquid form as a plasmid in the amount of 1 µg and was stored in -20ºC to preserve the product for a year.

4.4. Sanger Sequencing for KLK5 Mutation Analysis;

Based on the previously mentioned criteria, the forward and reverse primers were selected using a primer designer tool (by Genetiks) that added M13 tails with following sequence and characteristics;

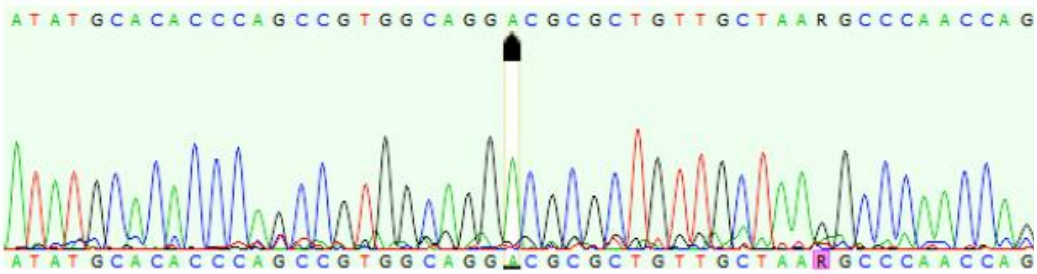
Table 4.1. Primers Characteristics

Characteristics	Forward Primer	Reverse Primer
Sequence	TCAgACACCCACCCTCTAgC	TATCCCCAACTCgACCTCCT
Tm (melting temperature)	59.16	57.49
OD'S	2	2
µG per OD	33.04	34.02
nmoles per OD	5.5	5.7
Purification	Desalt	Desalt
Primer length	20	20
MW (ug/umole)	5966.95	5932.92
%GC	60	55
µg's	66.08	68.04
Nmoles	11.1	11.5
Aggregate MW	5966.95	5932.92
Buffer volume to make 100uM stock solution	111 µl	115 µl

Sanger sequencing is used to find the desired sequence of a DNA molecule and synthesize it using forward and reverse primers that act as an initiation point for DNA synthesis on the DNA template. The method was introduced by Frederick Sanger in 1975 and includes 4 separate reactions for DNA synthesis; PCR amplification, PCR clean-up, cycle sequencing, and Purification of sequencing products. After the 4 steps, the data is analyzed by capillary electrophoresis and data analyzer software that detects the minor variants.

Based on the data collected and confirmed using variant finder software in Sanger sequencing, KLK5 mutation at c.245C>A (p.A82D) in exon 3 was detected in DU145 human prostate cancer cell lines (Figure A) However, the result of Sanger sequencing on PC3 human prostate cancer cell lines does not indicate mutations in KLK5 genes (Figure B). Therefore, PC3 cell lines were considered as wild type cells for KLK5 gene.

A



B

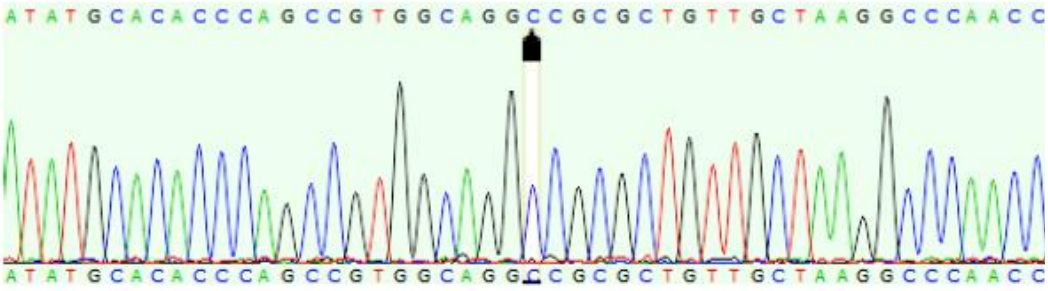


Figure 4.4. Schematic Representation of Sanger Sequencing Analysis for KLK5 Gene

4.5. Computational Analysis of Protein Structure and Severity of Damage Caused by Mutation

4.5.1. KLK5 protein domain structure

KLK5 gene is one of the members of kallikrein subgroup of serine proteases and it is located on chromosome 19. Serine proteases have been known to involve in DNA synthesis and maintenance.

Figure 4.5. Schematic Representation of KLK5 Protein Domain. The figure was taken from ensembl.org

As the image indicates, the c.245C>A (p.A82D) is located on the trypsin domain of serine proteases of KLK family genes which are located in exon 3.

4.5.2. 3D protein structure conformation

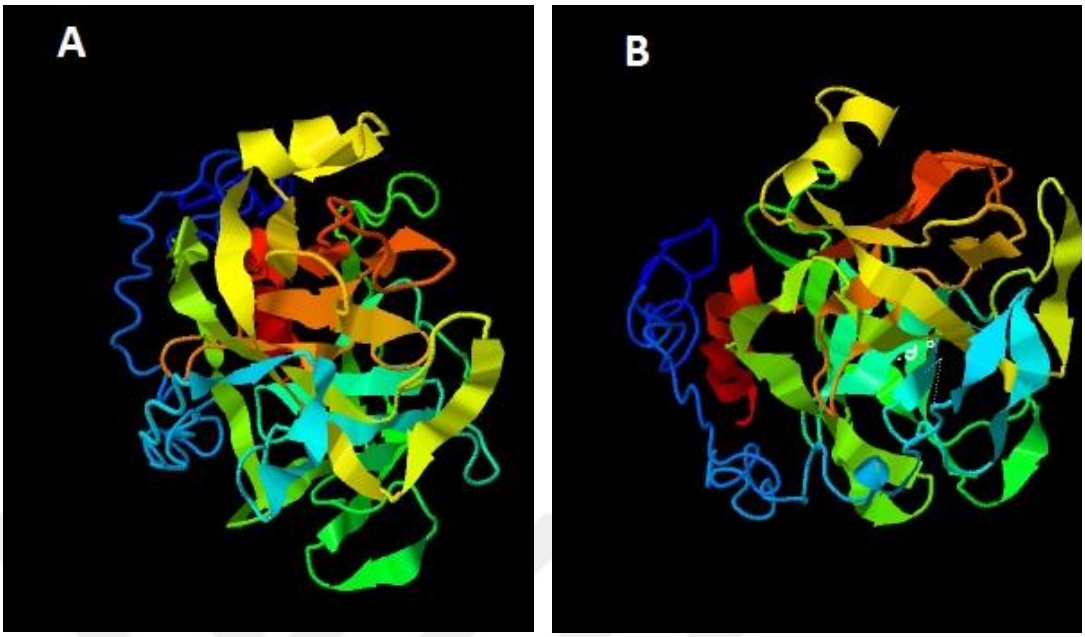


Figure 4.6. Schematic Representation of 3D Protein Structure of KLK5

The c.245C>A (p.A82D) mutation DNA sequence led to a change in the 3D protein structure (figure 6). The figures were designed by iTassar 3D protein software. The substitution of Alanine (Figure 6A) by Aspartate (Figure 6B) changed protein configuration. The effect of the nucleotide mutation predicted mutation to cause a change in the amino acid sequence that can affect protein features. Following the analysis of the mutation on protein function, Polyphen2 analysis of the c.245C>A (p.A82D) on KLK5 gene showed that the mutation is probably damaging with the score of 1 out of 1 as the Figure 7 presents;

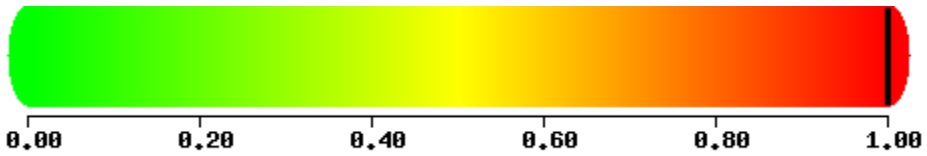


Figure 4.7. Polyphen2 analysis KLK5 Amino Acid Substitution

4.6. Antibiotic Selection

Puromycin is an antibiotic that is extracted from Streptomyces

alboniger bacteria and it inhibits protein synthesis by interfering with the translation process. It is also used a selection of the cells that carry puromycin resistance gene (pac gene encodes Puromycin N-acetyl-transferase (PAC). As our plasmid contained a puromycin resistance gene, we used this antibiotic to select DU145 cells that successfully harbored our designed plasmid genes containing pac genes. The applied concentration of puromycin on mammalian cells is between 1-10 µg/ml.

After DU145 were transfected by DH5-α plasmid and selected by puromycin to confirm the successful transfection, extracted DNA by DNA purification was run in Agarose gel electrophoresis against the DNA ladder and wild type DU145 to ensure adequate transfection without a change in DNA size.

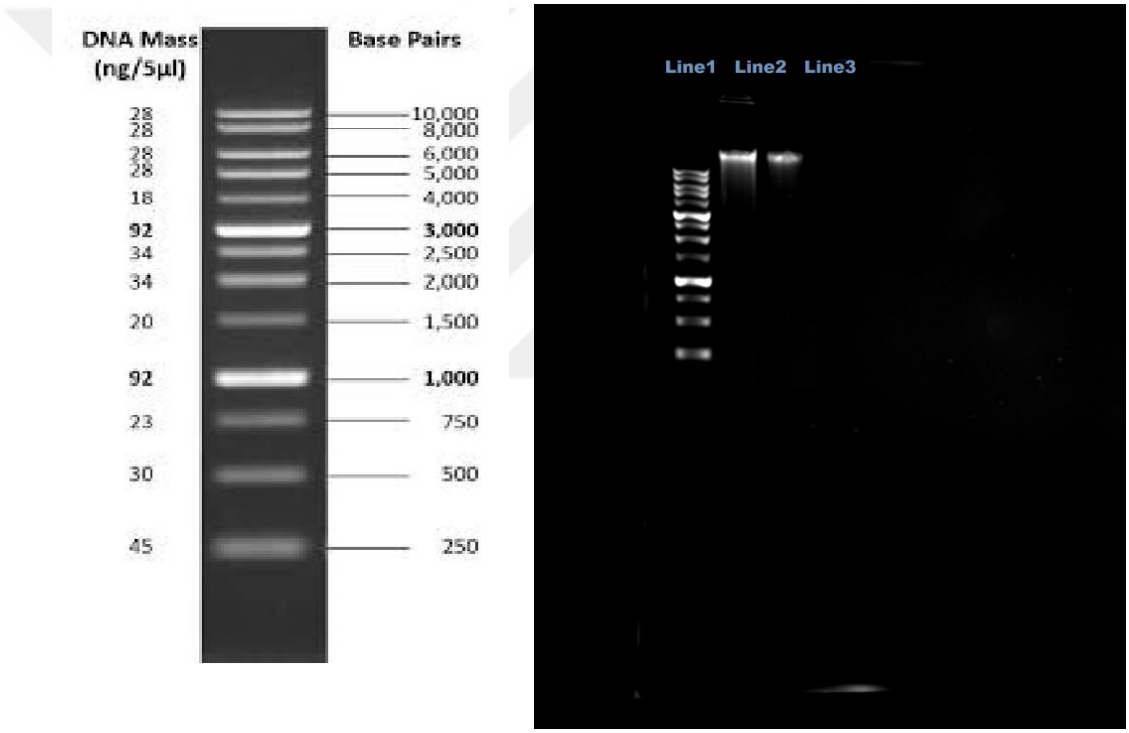


Figure 4.8 . Agarose Gel Electrophoresis of Transfected DU145 Cell Line and Wild DU145 Cell Line

As figure 8 shows, Line 1 represents the DNA ladder of 1 kb size. Line 2 represents the DNA of wild DU145 and Line 3 represents DU145 knockout after transfection. The size of DNA in line 2 (DU145 wild type) and line 3 (DU145 knockout) DNA bands were visualized on the same line that indicated successful DU145 transfection with the absence of any homologous recombination between bacterial plasmid DNA and DU145 DNA.

4.7. Cytotoxicity Assay

Doxorubicin, known as anthracycline, has been commonly used as chemotherapeutic medicine in cancer treatment, including prostate cancer. It is a member of the tetracycline family and damages the cell's DNA and drives them towards apoptosis by halting the cells in the G2/M phase. PARP is the catalyst enzyme that facilitates ADP-ribose transfer to the target proteins that are involved in DNA synthesis and repair mechanism. PARPs are shown to have a significant role in maintaining and moderating chromatin structure, cell proliferation, and apoptosis (2). After treating PC3, DU145 wild, and DU145 knockout with doxorubicin as well as doxorubicin+PARP inhibitor, the following results were observed;

4.7.1. Cell viability Assay Using Trypan Blue

Cell viability technique is a 2D technique that is used to observed the viability percentage of the cells under specific drug treatment conditions. Trypan blue dye is an azo dye that is used for industrial as well as medical purposes. The dye is applied to differentiate between dead and viable cells by staining dead cells in blue, while viable cells remain unstained.after staining and counting dead and viable cells separately, the viability percentage was calculated and a graph was made to illustrate the effect of a single drug (doxorubicin) and combination drug (doxorubicin+PARP inhibitor).

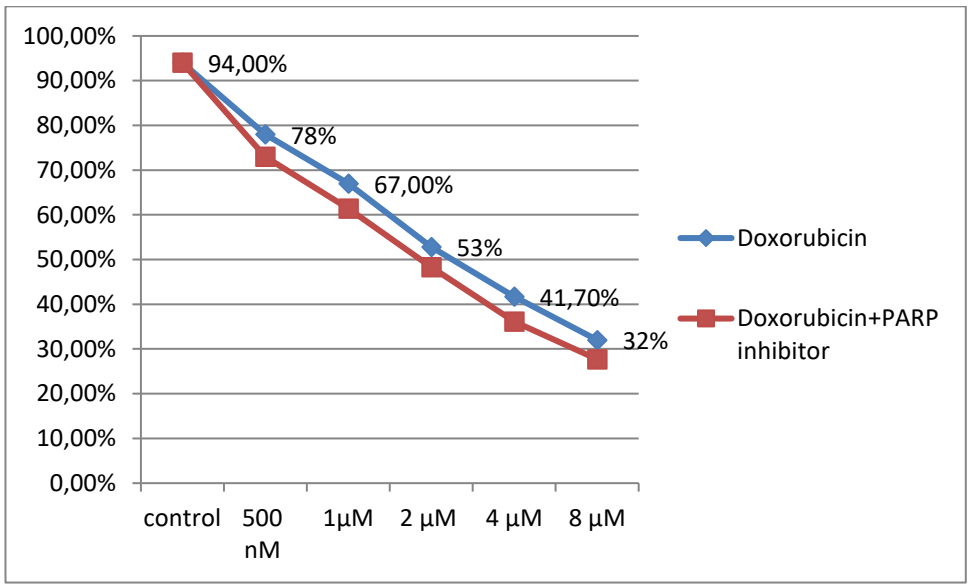


Figure 4.9. Schematic Representation of Cell Viability Percentage in PC3 Cell Line

As the graph shows, with the control that showed 94% viability in both single and double drug treatments, the effect of both was shown when the concentration of doxorubicin was at the highest (8µM). Moreover, the addition of the PARP inhibitor increased the effect of Doxorubicin with approximately 8-10% difference in viability.

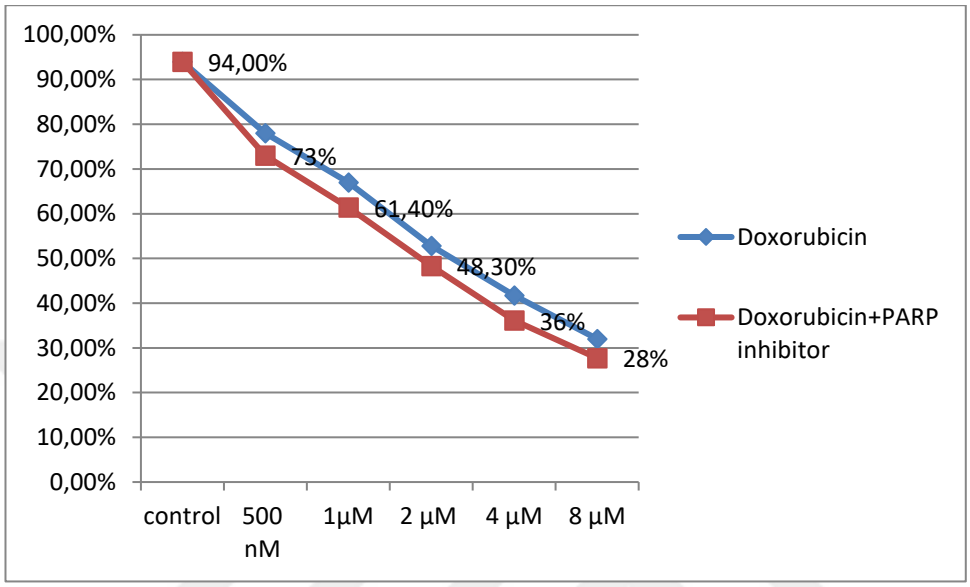


Figure 4.10. Schematic Representation of Cell Viability Percentage in DU145 Wild Cell Lines

Furthermore, the same drug concentration on the DU145 wild type showed almost the same effect with 27% viability with 8 µM of doxorubicin+PARP inhibitor.

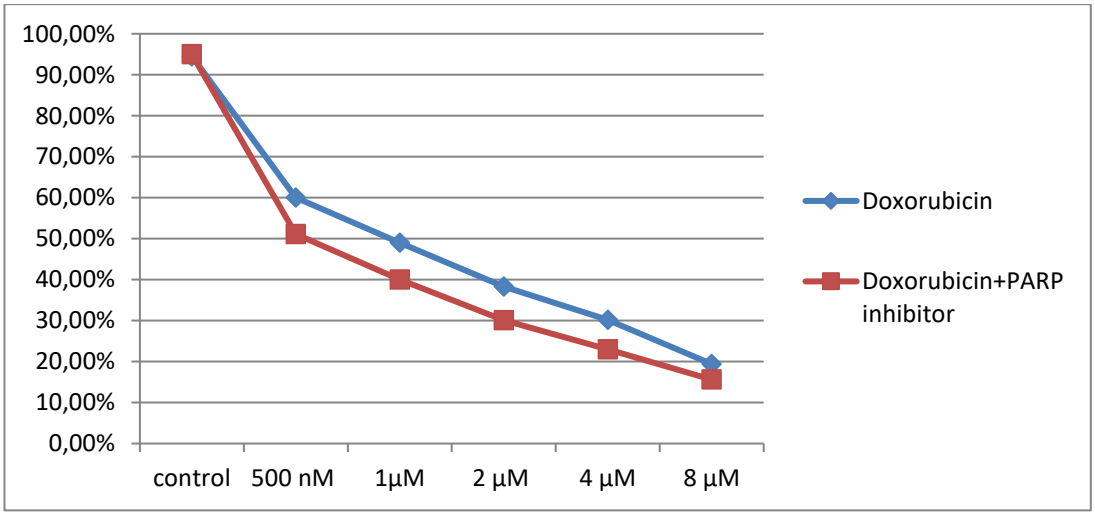


Figure 4.11. Schematic Representation of Cell Viability Percentage in Knockout

DU145 Cell Lines

However, with the DU145 cell lines that were knocked out by CRISPR, the viability percentage reduced in half compared to wild type, as the viability reached almost 15% with 8 µM of doxorubicin+PARP inhibitor

4.7.2. MTT Analysis

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric 2D assay that is designed to assess cell mitochondrial activity by measuring the viability of cells. MTT assay is widely used for measuring drug Cytotoxicity on cells/cell lines. And the viability of the cells is measured formazan concentration which is the result of tetrazolium salt MTT conversion by the cells' mitochondrial activity. The formazan concentration is shown by optical density (OD) using a microplate reader at 570nm or 720 nm. In this experiment, the cells' OD was checked at 720 nm using FLUOstar Omega microplate reader. To calculated the cell viability (%), the following formula was applied to each well's reading;
Cell viability (%) = OD of treated cells/OD of control cells × 100

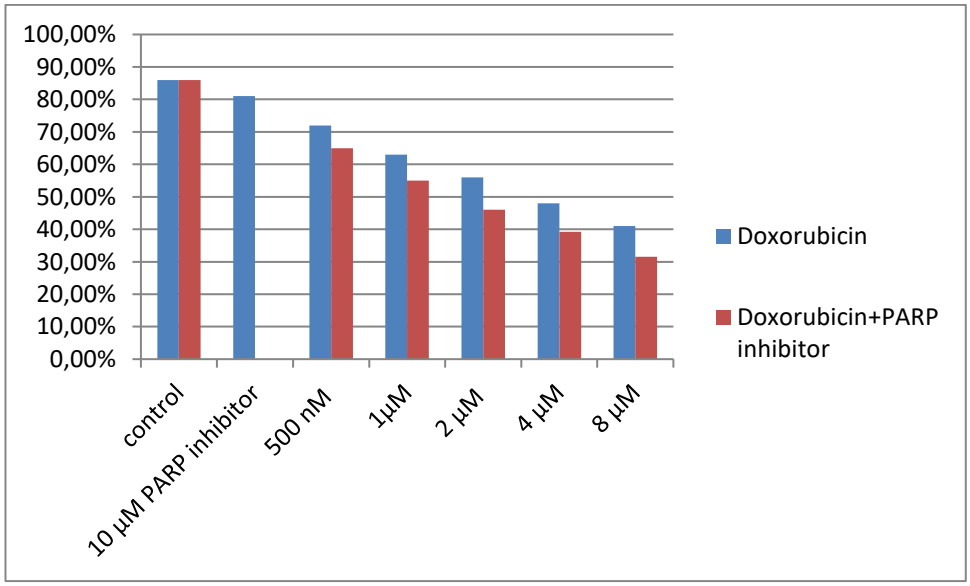


Figure 4.12. Schematic Representation of MTT Analysis in PC3 Cell Lines

Based on the OD data received from the analysis, PC3 cell lines showed the lowest viability with the double drug (doxorubicin+PARP inhibitor) with the rate of almost 32% Even though, the effect of PARP inhibitor, in comparison with a single drug (doxorubicin), is not high, but considerable with viability decline between 6-9%

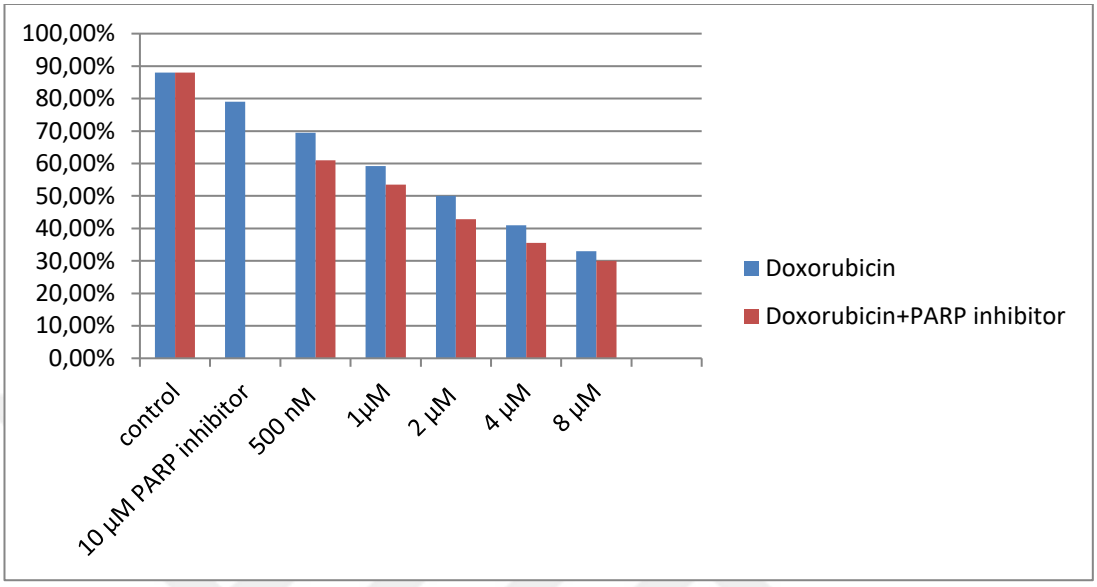


Figure 4.13. Schematic Representation of MTT Analysis in Wild Type DU145

Furthermore, the same drug concentration on the DU145 wild type showed almost the same effect with 27% viability with 8 µM of doxorubicin+PARP inhibitor.

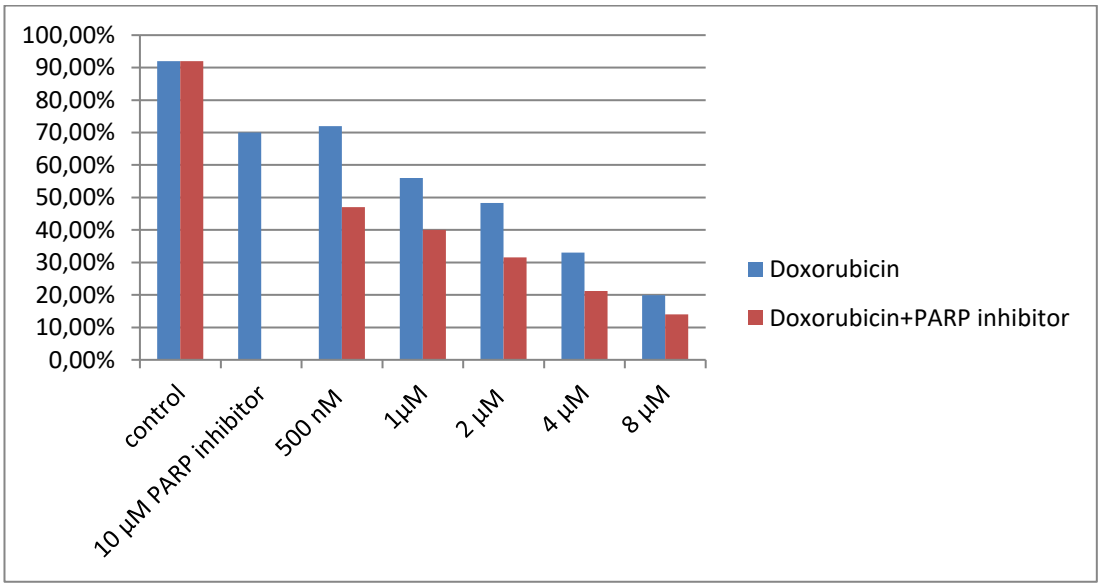


Figure 4.14. Schematic Representation of MTT Analysis in Knockout DU145

However, OD data received from the MTT plate reader, DU145 knockout cell lines showed the lowest viability when the double drug (doxorubicin+PARP inhibitor) and it was 14%. Although, the effect of PARP inhibitor, in comparison with a single drug (doxorubicin), is not high but noticeable. As it can be seen in the graph, PARP inhibitor combined with doxorubicin dropped cell viability from 6-12%.

4.7.3. Colony Formation Assay (CFA)

Colony formation assay known as CFA is a 3D in vitro survival cell culture assay that can determine tumorigenic characteristics of specific cells or cell lines under drug treatment conditions. The method is used for all cell lines including genetically modified cells to create tumorigenic comparison with the cell lines' wild type.

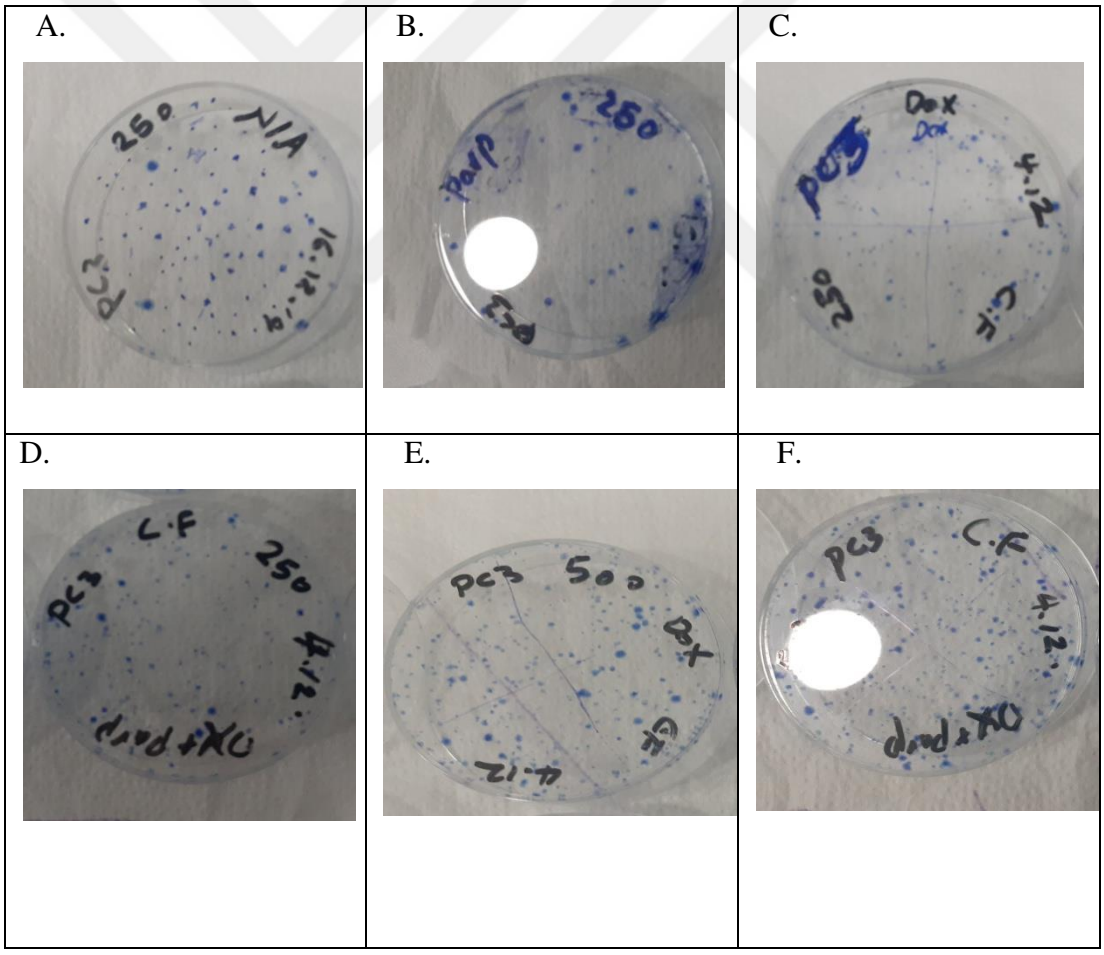


Figure 4.15. CFA for PC3 at Different Single and Double Drug Concentration.

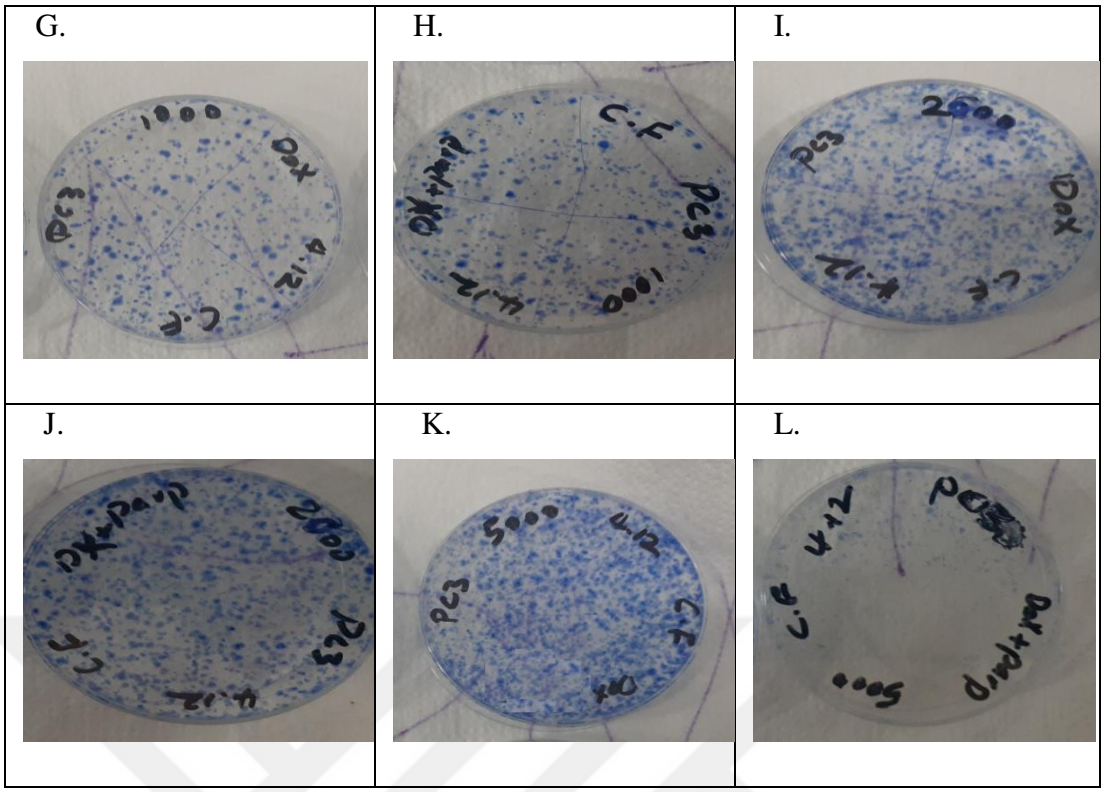


Figure 4.15. CFA for PC3 at Different Single and Double Drug Concentration. (continued)

Change in drug concentration for both single and double treatment reduced colony formation in PC3 cell lines (Figure 15) which affected tumorgenesis of the cell line as it is illustrated (Figure 16).

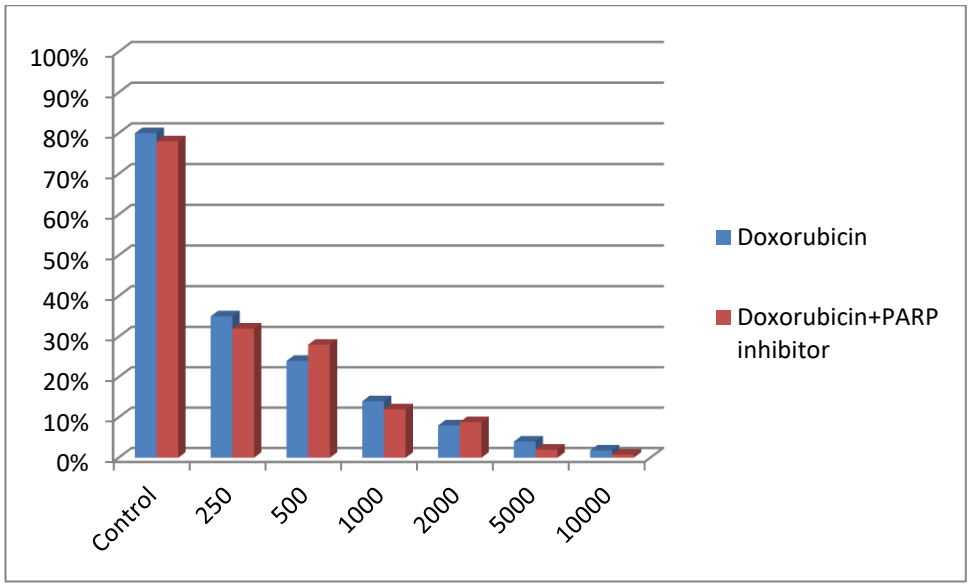


Figure 4.16. Schematic Representation of CFA Effect on PC3 Tumorgenesis

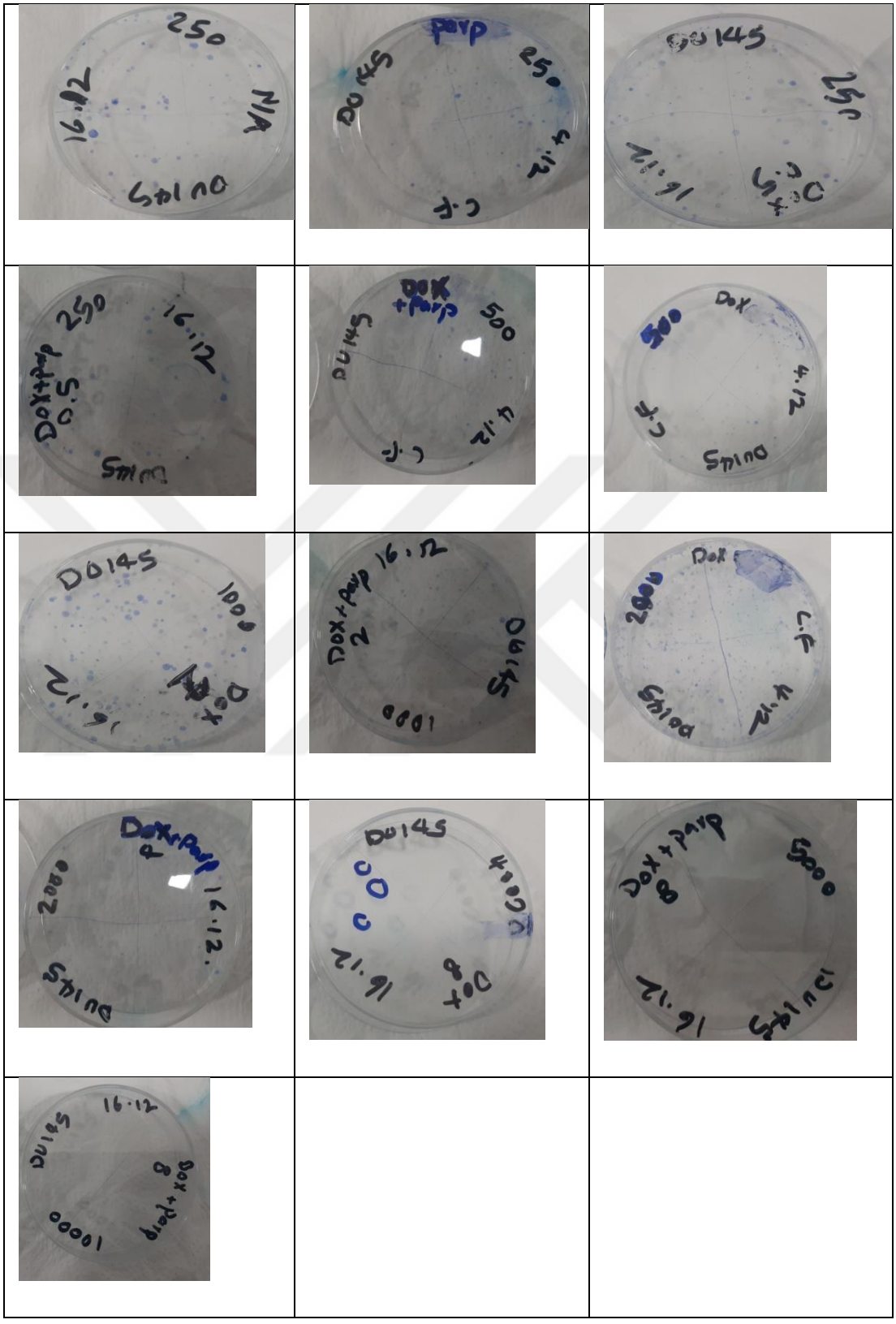


Figure 4.17. Colony Formation Assay for Wild Type DU145

However, DU145 wild type shows a lower number of the colonies compared to PC3 under the same drug concentration and condition (Figure 17). And as it is shown, the tumorgenesis percentage dropped down as the drug concentration increased, however more significant the PC3 (Figure 18).

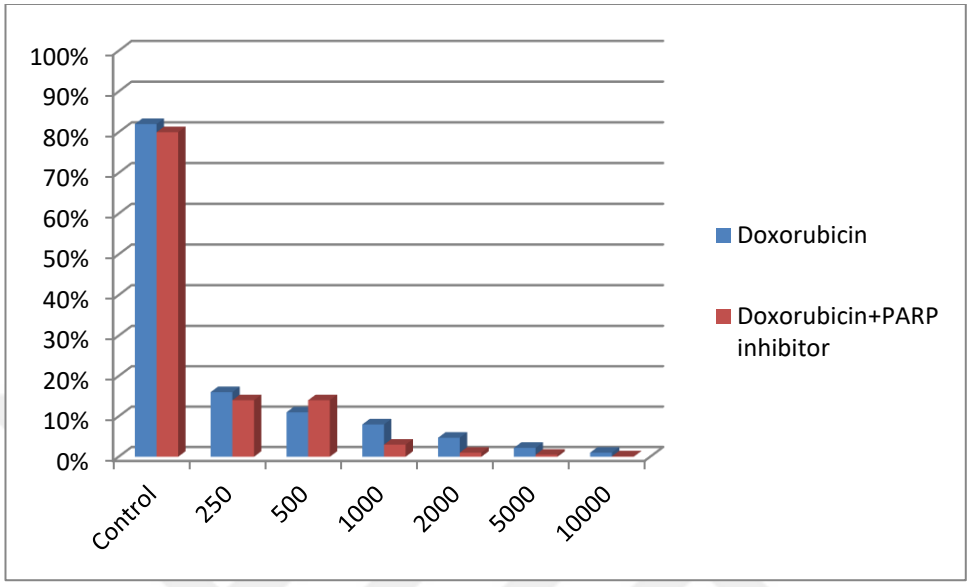


Figure 4.18. Schematic Representation of CFA Effect on Wild Type DU145 Tumorgenesis

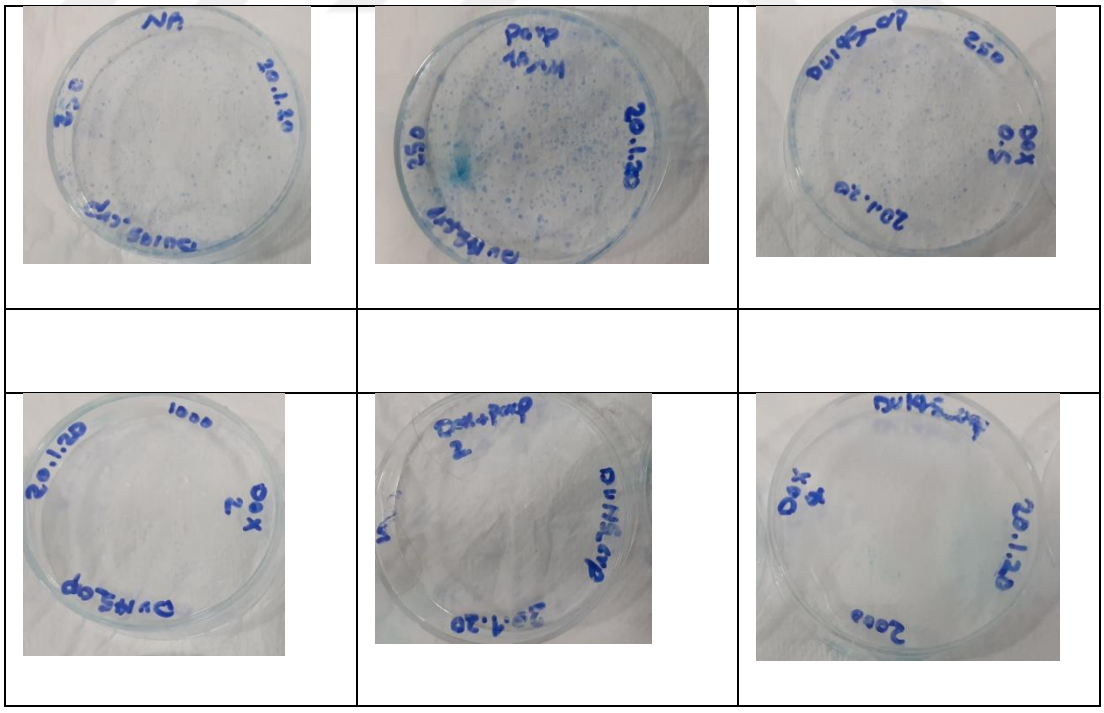


Figure 4.19. CFA for Knockout DU145

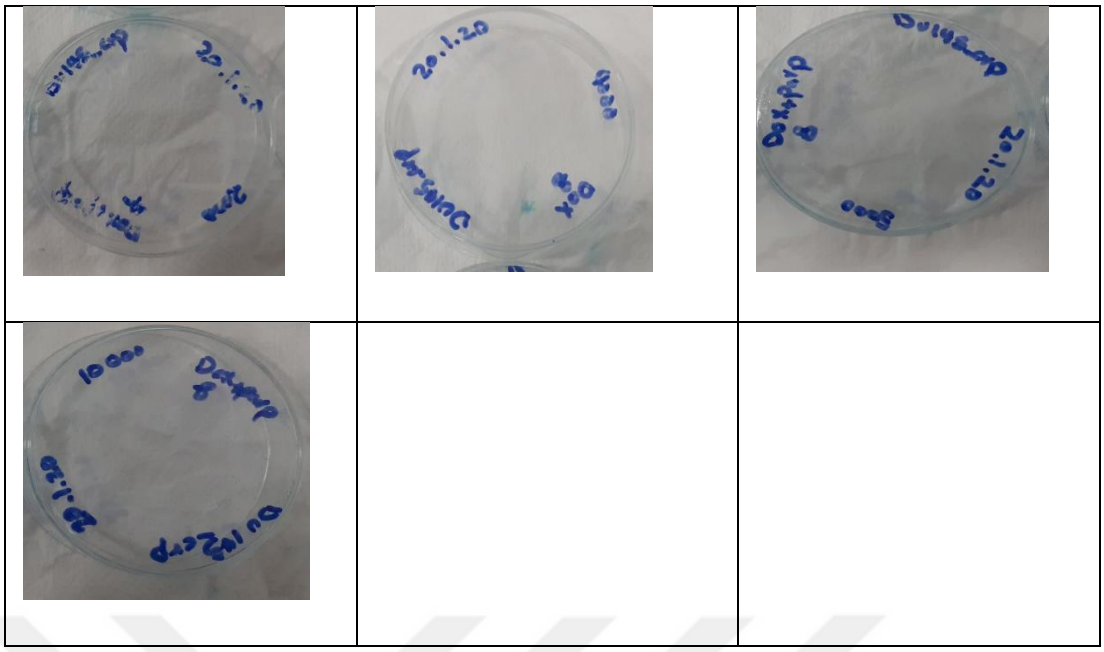


Figure 4.19. CFA for Knockout DU145 (continued)

Following, knockout DU145 shows smaller and fewer colonies as the colony formation is almost negligible at the highest drug treatment concentration (Figure 19). Compared to wild type DU145, knockout DU145 cell lines showed significant loss of tumorgenesis under drug treatment in CFA (Figure 20).

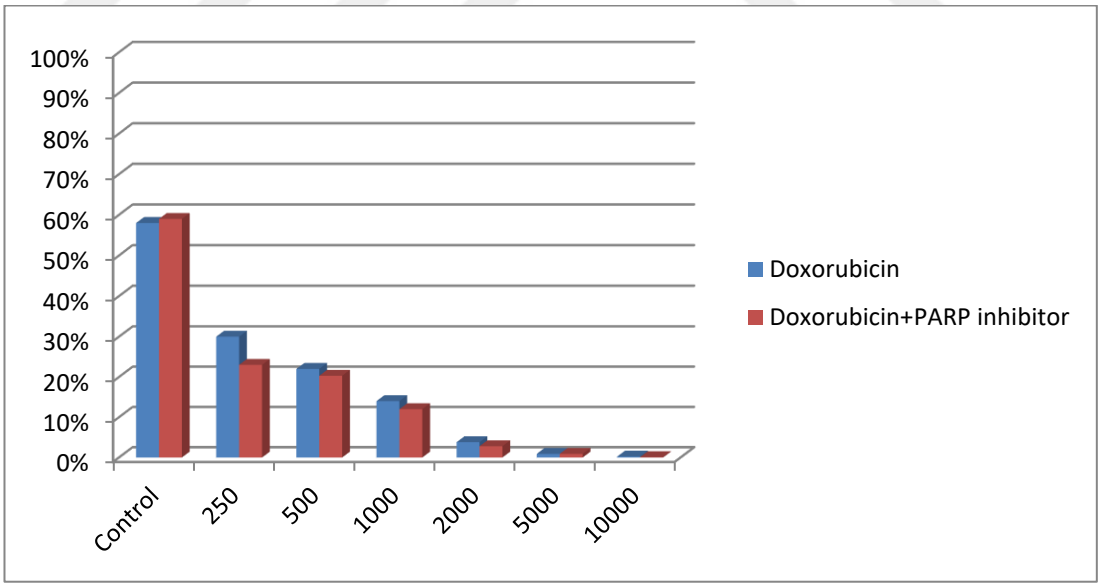


Figure 4.20. Schematic Representation of CFA Effect on Wild Type DU145 Tumorgenesis

5. DISCUSSION, CONCLUSION AND RECOMMENDATION

Cancer is one of the predominant diseases worldwide and gene/genes mutations that affect cell growth, function, proliferation, and apoptosis. Prostate cancer (PC) cells' growth is androgen-dependent and therefore, many therapies use hormones that weaken androgen activity to stop the malignancy (Centenera,M.M.et.al., Mateo,J.et.al 2018, Rathkopf,D.E.et.al. 2013). However, due to recurrence of PC into castrate resistance PC (CRPC) which happens as a result of hormone-therapy resistance and expression of spliced-form of androgens (AR-Vs) (ntonarakis,E.S.et.al. 2014, Nuhn,P.et.al. 2018, Rodrigues,D.N.et.al. 2017), scientists have been trying to design and develop the most authentic approaches for the treatment with minimum side-effects, inexpensive and reachable methods that can be customized depending on the patients' condition, cancer type, mutation type and location, and patient's sensitivity towards specific drug treatments.

In this study, we focused on two androgen-independent prostate epithelial cell lines, PC3 (derived from the brain as a metastatic site) and DU145 (derived from bone as a metastatic site), that detected to have increased transcript level compared to other prostate cancer cell lines. Proteomic profiling of DU145 and PC3 cell lines detected 1974 and 1448 proteins, respectively (Saraon, P.et.al. 2012). Although many mutations have been detected on cell lines, we decided to focus on KLK5 gene mutations in these cell lines, as not many studies have been carried out to confirm its effect on prostate cancer mutagenesis and progress. Furthermore, some research studies have shown the relationship between expression of the KLK5 gene and benign/ lower-stage tumours (Paliouras M.et.al. 2007). On the other hand, KLK5 gene was shown to have been down-regulated in higher-stage prostate cancer (Paliouras M.et.al. 2007), which is contrary to its expression level in other types of cancer, such as lung cancer (Paliouras M.et.al. 2008). Sanger sequencing result revealed KLK5 gene c.245C>A (p.A82D) in exon 3 of the gene in DU145 cell lines which is located in the serine-protease domain. Nevertheless, PC3 cell lines did not show any mutation on KLK5 which led us to perform CRISPR gene editing on DU145 cell lines and creating a comparison between DU145 wildtype (with KLK5 mutation), PC3 (with no mutation on KLK5) and DU145 knockout (KLK5 edited cell line using CRISPR tool). The substitution of Alanine by Aspartate in KLK5 protein has shown to be probably

damaging (with a damage score of 1/1 by poylyphen2) in this position. Although both amino acids are non-essential amino acids and help to boost the immune system and maintain cell cycle, it is predicted that the substitution of alanine by aspartate in KLK5 protein promotes prostate cancer progression. Alanine concentration have shown to reduce prostate cancer, as this amino acid is found to exist in prostate gland liquid and acts as an anti-inflammatory factor in damaged protects cells and in prostate hyperplasia (M. Kratochvilova.et.al. 2017). Also, an increase in the concentration of aspartate has been reported in prostate cancer tumors (M. Kratochvilova.et.al. 2017). Therefore, it is expected that change in the amino acid sequence of KLK5 protein from analine to aspartate can elevate tumorgenesis in prostate cancer. This is expected to have resulted due to the chemical structure of aspartate as containing more available binding pockets that can increase metabolic activities of prostate cancer cells and enhance their proliferation and division. As it was explained previously (figure 6), the reported mutation (c.245C>A (p.A82D)) caused alteration of the 3D structure of KLK5 protein which is predicted to affect protein interaction and functioning of KLK5 in cleavage of proteins' peptide bonds to provide the active sites (serine) in enzymes. Further analysis of the KLK5 protein after Sanger sequencing confirmation showed that position of mutation to be located in exon 3 and in serine-protease trypsin domain which is an active-site containing domain in serine proteases trypsin family members including plasma kallikreins (such as KLK5) and mutation in this domain can lead to affect KLK5 function and subsequently lead to increased metastasis of prostate cancer cells.

Various genome editing tools have been developed by scientists to facilitate the treatment process in various diseases including cancers. Although many of them developed remarkable side effects or long-term effects were not observed due to cancer repression, with the development of the CRISPR system, many previously applied editing tools have been obsolete or used for limited purposes. In this study, the CRISPR system was selected to edit mutation on the KLK5 gene in DU145 cell lines as this system performs high-efficiency of gene delivery with minimum side effects including off-target effects that are considered as one of the causes of mutation rates to increase. Besides, permanent insertion or deletion by CRISPR, compared to RNA-interference (RNAi) that down-regulates the target gene by incomplete gene silencing it is more effective as the impact of the target gene after particular knockin or knockout

by CRISPR can be observed and evaluated in long-term(Barrangou, R.et.al 2015, Unniyampurath, U.et.al. 2016). In addition to RNAi, ZFNs (Zinc Finger Nucleases) and TALENs (transcription activator-like effector nucleases) have shown to cause the undesirable rate of off-target effects as their gene recognition function is not quite specific and it is based on an interaction between the proteins and target DNA (Ratan, Z. A., Son.et.al. 2018, Wyman C and Kanaar R. 2006). While, off-target effects of the CRISPR system can be minimized and controlled by optimizing Cas9 half-life (Maji, B. et al. 2017). For designing the most effective CRISPR system, various factors are considered including the vector/plasmid size to enhance gene delivery. In this experiment, lentiviral vectors were selected as a vector for CRISPR gene editing system and compared to Adeno-associated viral vectors (AAV), they have shown to present the highest efficiency in a delivery system, low cytotoxicity and low rate of immunogenicity (Kay, M.A.et.al. 2001, Vigna, E.et.al. 2000). Lentiviral vectors have been used in gene treatment of other purposes such as haematological phenotype correction (Garcia-Gomez, M.et.al. 2016), maintaining and regulating neural activity in neurodegenerative diseases (Horvath, L.et.al. 2011, Singer, O.et.al. 2005), and inhibition of HIV infection (Peterson, C.W.et.al. 2016). In this study, plenti-u6-sgrna-sffv-cas9-2a-puro vector was selected as it has successfully experimented in transfecting other mammalian cells (9 methods) and down-regulating/suppression of CD133 in melanoma, one of the deadly form of cancer, where they knocked out CD133 that down-regulated ABCBG2 and elevated melanoma cells' sensitivity towards drug resistance (Simbulan-Rosenthal.et.al. 2019).

Gel electrophoresis result of transfected DU145 cell lines showed successful transfection with no homologous recombination occurrence between DH5-α plasmid DNA and DU145 DNA, as the clear band appeared with the same DNA size as wild type DU145 showed.

The result of DU145 transfection by the CRISPR system revealed significant differences compared to wild DU145. Cell viability result of all applied cells lines, which are PC3 cell lines (with no KLK5 mutation), wild DU145 (with KLK5 mutation), and knockout DU145 cell line (KLK5 gene was knocked out by CRISPR system) showed the great difference when cells were exposed to single-drug treatment (Doxorubicin) and double drug treatment (doxorubicin+PARP inhibitor). A

comparison between PC3 and wild DU145 showed different sensitivity and survival rates when they were exposed to the same drug concentration. Therefore, it is believed that the following difference could be due to other mutations in DU145 that might have sensitized the cells against drug therapy. However, PC3 cell lines' viability dropped as the drug concentration increased, it is understood that the mutations on this cell line could be the cause of sensitivity. Past studies on using Doxorubicin and PARP inhibitor on androgen-dependent (LnCap cell lines) and androgen-independent cell lines (DU145 and PC3 cell lines) showed higher cell lines resistance of cells compared to the current study (Anil K .et.al., Kelly, M. M.et.al 2002, Schwarze, S. R.et.al. 2005). However, the applied dosage of the drug was found to be lower (in nM) which could be the reason for lower drug influence on low apoptosis and higher survival rate compared to the current study. Furthermore, double drug treatment with the inclusion of PARP inhibitor lowered the percentage of the viability which is likely to indicate the synergetic effect of doxorubicin with a PARP inhibitor. PARP inhibitor suppresses PARP enzymes that are the main key enzyme of DNA damage response (DDR) mechanism by modifying target protein with ADP-ribose, known as PARylation, that activates when DNA of cells undergo constant tension and damage, specifically PARP1 and PARP2 (Masutani, M.et.al. 1999, Murcia, J.M.D.et.al. 2003, Virtanen.et.al. 2019). Therefore, suppression of PARP enzymes provides the condition for Doxorubicin to invade DU145 and PC3 DNAs and cause lethal DNA damage which drives the cells towards apoptosis. In a recent study, fatal sensitivity of androgen-dependent cells with to PARP inhibitor has been observed, particularly androgen receptor splice variants in CRPC (Kounatidou, E.et.al. 2019) which once again put forth the importance of PARP inhibitor in drug therapy against prostate cancer as our study show more significant effect of doxorubicin when it was applied with a PARP inhibitor. The result of previous studies and our current study together shows the importance of PARP inhibitor in both androgen-dependent and androgen-independent cells of prostate cancer that further analysis in designing a therapeutic way. Doxorubicin interferes with topoisomerase II function and creates radicals that cause DNA damage and cell membrane disruption leading to cell death (Gewirtz DA. 1999). Other studies presented a higher effect of doxorubicin with other techniques, such as UltraSound targeted nanobubbles (NBs), and showed a decrease in PC3 cells' viability (Fan, X.et.al. 2016). Nevertheless, our study showed a higher rate of apoptosis and lower viability percentage specifically at a rate of 8µM/ml for

Doxorubicin and 10 μM/ml of PARP inhibitor. Therefore, it is anticipated that combination drug therapy in this study could be considered for future therapeutic purposes. comparison of the above result with knockout DU145 cell lines has shown a much greater effect of viability percentage, which indicates the importance of KLK5 expression which is found to have been down-regulated in DU145 cell lines due to KLK5 gene impairment which is not been reported before. With the maximum double drug therapy (8μM doxorubicin+10 μM PARP inhibitor), knockout DU145 cell line showed almost 15% viability. The data demonstrated in this study shows a promising result of CRISPR gene therapy to enhance the apoptosis rate of prostate cancer cells. To this date, no study has focused on KLK5 deletion or insertion by CRISPR gene therapy. Although the study focused on single gene therapy and did not analyze the CRISPR gene editing effect on PC3 cell lines, the calculated data provides a reason for future work on other cell lines in prostate cancer and other cancers.

For thorough quantization of Doxorubicin and PARP inhibitor's inhibitory effect on PC3, wild DU145, and knockout DU145 cell lines, MTT analysis was carried out to observe death rate of cells based on OD received from viable cells. Although viability using trypan blue is a useful assay, the possibility of error exists as the counting is performed manually. Our study shows similar (with minor difference) result of MTT assay with cell viability assay and the lowest viability was observed with the highest single and double drug concentration in knockout DU145 (14%) that once again indicates the significance of KLK5 down-regulation in prostate cancer progression, cancer cells' proliferation and metastasis. Other studies applied MTT assay to quantify various cancer cells' survival under different drugs' stimulation/inhibition (Kamalidehghan, B.et.al. 2018, Romijn, J. C.et.al. 1988, Singh, R.et.al. 2015, Zhang, X.et.al. 2019). However, no studies showed knockout DU145 by CRISPR and their MTT assay analysis under drug therapy. A recent study on Proto-oncogene serine/threonine-protein kinase pim-1 encoded by pim-1 gene showed that when the gene was knocked down by pim-1-specific short hairpin RNA (shRNA), DU145 and PC3 cells' proliferation was inhibited when they were exposed to specific doxorubicin concentration and MTT assay reduced viability (Zhang, X.et.al. 2019). Although the result is promising, we failed to make a comparison as gene-editing tools and the target genes are different from one another. However, the study reveals the efficiency of MTT analysis to calculate cell survival and apoptosis rate on prostate

cancer cell lines. In another study, the impact of koenimbin on PC3 cell viability (Kamalidehghan, B.et.al. 2018). Koenimbin is a natural dietary compound of Murraya koenigii (L) Spreng that have shown some chemotherapeutic effects by inhibiting various cancer cells' proliferation, including breast cancer (Kamalidehghan, B.et.al. 2015). The effect of Koenimbin showed a lower percentage of PC3 cells' viability (almost 2%) compared to our study that applied doxorubicin and PARP inhibitor. The study suggests considering Koenimbin as a chemotherapy drug for future treatment of prostate cancer cells.

Finally, colony formation assay (CFA) on 3 sets of cell lines (PC3, wild DU145, and knockout DU145) showed PC3 cells to form the highest number of colonies compared to wild DU145 and knockout DU145. Although all cell lines were grown and incubated in the same condition and time, wild DU145 formed a less and smaller number of colonies under single and double drug treatment. Nevertheless, knockout DU145 showed a remarkable change in colony formation including the colony size and numbers. The result of colony formation on the cell lines showed that the CRISPR editing technique on KLK5 gene mutation is believed to be successful as knockout DU145 presented a negligible number of colonies that shows the effect of knockout to have affected the tumorigenesis and carcinogenic characters of DU145. However, the use of other drugs, such as quercetin showed a contradictory influence on PC3 cell lines and DU145 cell lines colony formation. Quercetin is a flavonoid with antioxidant characters that is used to boost the body against various diseases including various cancers. A study on PC3 and DU145 cell lines showed that quercetin significantly reduced PC3 colony formation in comparison with its average effect on DU145 cell lines (Nair, H. K.et.al. 2004).

5.1. Conclusion

Many other mutations involving prostate cancer cell lines that are to be studied and CRISPR editing to be carried out to find out the effect of it. Although our in vitro study presents some promising features of CRISPR editing technology on cancer cells (DU145 cell lines in this study), further in vivo investigations must be carried out to observe side effects of gene editing, such as off-target effects due to indel formation, and drug resistance therapy (single or double treatment), such as toxicity and

immunosuppressive effects in physiological condition. Moreover, further analysis of KLK genes in other prostate cancer cell lines requires further studies as this study was limited to the KLK5 gene and to develop the most valid and effective therapy in prostate cancer, synergetic effects, and interdependence of KLK genes and their function must be taken into account. furthermore, other engineered CRISPR tools such as modified human Chimeric Antigen Receptor T cells (huCAR19 T cells) with CRISPR/Cas9 can be examined to find the most efficient system for tumor eradication in various types of cancer. Finally, recent studies on anti-tumorgenesis of other drugs, such as quercetin and Koenimbin, need to be further considered in drug treatment of prostate cancer.

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7. CURRICULUM VITAE

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4. **Education status** : Master Degree
5. **current workpplace** : JustEnglish Language Center

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Bachelor Degree	Science (Microbiology)	Bharati vidiyapeeth University	2009
Master Degree	Microbiology	Pune University	2011
Master Degree	Business Administration	Bahcesehir University	2015
Master Degree	Molecular Biology and Genetics	Biruni University	2020

6. Publications

Articles published in international refereed journals (SCI, SSCI, Arts and Humanities)

6.1. Meryem Alagoz, **Nasim Kherad**, Sureyya Bozkurt, Adnan Yuksel (2020).New mutations in KCNT2 gene causing early infantile epileptic encephalopathy type 57: Case Study and Literature Review. Acta Biochim Pol

6.2. Alagoz, M., **Kherad, N**., Gunger, E., Kaymaz, S., & Yuksel, A. (2020). The New CIC Mutation Associates with Mental Retardation and Severity of Seizure in Turkish Child with a Rare Class I Glucose-6-Phosphate Dehydrogenase Deficiency. Journal of Molecular Neuroscience. doi:10.1007/s12031-020-01614-8

6.3. Meryem Alagoz and **Nasim Kherad**. (2020). Advance genome editing technologies in the treatment of human diseases: CRISPR therapy (Review), Int J Mol Med. 2020 Aug; 46(2): 521–534.Published online 2020 May 19. doi: 10.3892/ijmm.2020.4609

6.4. Meryem Alagoz, **Nasim Kherad**, Selda Turkmen, Hatice Bulut, and Adnan Yuksel. (2020). A novel mutation in the SERAC1 gene correlates with the severe manifestation of the MEGDEL phenotype, as revealed by whole-exome sequencing. Exp Ther Med. 2020 Jun; 19(6): 3505–3512.

6.5. Meryem Alagoz, **Nasim Kherad**, M. Gavaz, Adnan Yuksel (2019). New Genetic Approaches for Early Diagnosis and Treatment of Autism Spectrum Disorders, *Review Journal of Autism and Developmental Disorders*, Volume 6, Issue 4, 1 January 2019, https://doi-org.eres.qnl.qa/10.1007/s40489-019-00167-w

8. PLAGIARISM REPORT

KLK GENE EDITING USING CRISPR TECHNOLOGY IN PROSTATE CANCER CELL LINES FOR CANCER THERAPY

ORIJINALLIK RAPORU

%14	%6	%4	%12
BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	Submitted to The Scientific & Technological Research Council of Turkey (TUBITAK) Öğrenci Ödevi	%2
2	Submitted to University of Colorado, Denver Öğrenci Ödevi	%1
3	Submitted to University of Ulster Öğrenci Ödevi	%1
4	www.rj-robbins.com İnternet Kaynağı	<%1
5	Submitted to Yeditepe University Öğrenci Ödevi	<%1
6	Submitted to Indonesia International Institute for Life Sciences Öğrenci Ödevi	<%1
7	Submitted to Bilkent University Öğrenci Ödevi	<%1
8	Wan-Yi Wu, Baley A. Fong, Allison G. Gilles,	