

T.C.
DOKUZ EYLÜL UNIVERSITY
İZMİR INTERNATIONAL
BIOMEDICINE AND GENOM
INSTITUTE

**INVESTIGATION OF THE EFFECTS OF 2-
HYDROXYGLUTARATE ONCOMETABOLITE ON
COLON CANCER METABOLISM ON CELL
MODELS GENERATED BY CRISPR/CAS9-BASED
GENE THERAPY AND IN VIVO MODELS**

ESRA BULUT ATALAY

MOLECULAR BIOLOGY AND GENETICS

PHD THESIS

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Supervisor: Prof. Dr. Hülya AYAR KAYALI

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DOKTORA TEZ SAVUNMA SINAVI TUTANAĞI

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı Moleküler Biyoloji ve Genetik Doktora Programı 2016850028 numaralı öğrencisi Esra BULUT ATALAY, “ 2-Hidroksiglutarat Onkometabolitinin Kolon Kanseri Metabolizması Üzerindeki Etkisinin CRISPR/Cas9-Tabanlı Gen Terapisi ile Oluşturulan Hücre Modelleri ve in vivo Modeller Üzerinde İncelenmesi” konulu Doktora tezini 29.08.2022 tarihinde yapılan savunma sınavı sonucunda başarılı olmuştur.

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ABBREVIATIONS

IDH1/2.....	socitrate dehydrogenase1/2
α -KG	α -Ketoglutarate
Arg... ..	arginine
D-2HG.....	D-2-hydroxyglutarate
HOT	hydroxyacid oxoacid transhydrogenase
GHB	γ -hydroxybutyrate
SSA	succinic semialdehyde
PHGDH... ..	phosphoglycerate dehydrogenase
3-PG	3-phosphoglycerate
3-PHP	3-phosphohydroxypyruvate
PSAT	phosphoserine aminotransferase
3-PS.....	phosphoserine
GLS	glutaminase
GDH	glutamate dehydrogenase
TCGA.....	The Cancer Genome Atlas
GTEEx... ..	Genotype-Tissue Expression
GEPIA.....	Gene Expression Profiling Interactive Analysis
GLUT	glucose transporters
SGLT.....	sodium-dependent glucose transporters
PGAL	phosphoglyceraldehyde
PFK	phosphofructokinase
MCT	monocarboxylate transporter
F6P.....	fructose-6-phosphate
F1,6BP	fructose-1,6-biphosphate
PFKFB.....	6-phosphofructo-2-kinase/fructose2,6-bisphosphatase
ENO	enolase
FH	fumarate hydratase
SDH.....	succinate dehydrogenase

EMT	epithelial to mesenchymal transition
ETC	electron transport chain
L2HGDH.....	L-2-hydroxyglutarate dehydrogenase
D2HGDH.....	D-2-hydroxyglutarate dehydrogenase
HIF1 α	hypoxia-inducible factor 1 α
GDH	glutamate dehydrogenase
GLS	glutaminase
wt	wild-type
MDH	malat dehydrogenase
LDH	lactate dehydrogenase
ADHFE1.....	alcohol dehydrogenase iron-dependent isoform 1
ZFN	zinc finger nucleases
TALENs	transcription activator-like effector nucleases
CRISPR	clustered regularly interspaced short palindromic repeats
pre-crRNA	precursor CRISPR RNA
crRNAs.....	CRISPR RNAs
Cas.....	CRISPR-associated
PAM	protospacer-adjacent motif
RNP	ribonucleoprotein
tra-craRNA	trans-acting crRNA
sgRNA.....	single-guide RNA
CARF	CRISPR-associated Rossmann fold
DSB.....	double-strand DNA
HDR	homology-directed repair
NHEJ	non-homologous end joining
PDX.....	patient-derived xenograft
NOD/SCID	nonobese diabetic severe combined immunodeficiency gamma
FBS	fetal bovine serum
DNS.....	dinitrosalicylic acid

DNPH..... 2,4-dinitrophenylhydrazine
IVT in vitro transcription
KCl..... potassium chloride
HCl..... hydrochloric acid
NaOH sodium hydroxide
H&E hematoxylin and eosin



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ABSTRACT

The normal concentration of D-2-hydroxyglutarate (D-2HG) is quite low but it is generated at a high level and role in colorectal carcinogenesis. This oncometabolite is generated by various cell-type-specific enzymes in addition to wild-type or mutant isocitrate dehydrogenase (IDH). This thesis aims to examine the D-2HG generation pathways and the impact of this oncometabolite on colon cancer metabolism.

Firstly, the level of some intermediates in the TCA and Cori cycles, glutaminolysis, and glycolysis were determined in cell lines and xenograft models. The D-2HG has significantly been generated in both colon adenocarcinoma cell lines (Caco-2 and SW620), but it could not be detected in the colon epithelial cells (CCD-18Co). The Sanger results showed that there was no mutation in the IDH1/2 gene in SW620. Then, D-2HG generation was almost 2.5-fold decreased in wild-type IDH1 knockout cells (IDH1 KO) compared to the control, but still it generated. The mRNA level of the malate dehydrogenase (MDH2) enzyme which role in the L/D-2HG generation was considerably increased in IDH1 KO. After treatment with MDH and lactate dehydrogenase (LDH) enzyme inhibitors, the D-2HG could not be detected. Similar findings were obtained in the patient-derived xenograft (PDX) models. Finally, the R132H mutation was generated using the CRISPR/Cas9 tool to examine the effect of mutant IDH1 enzyme on D-2HG generation, and it was revealed that the D-2HG level did not change.

In conclusion, the D-2HG generation pathway has been determined and a new therapeutic approach to colon cancer by inhibiting this oncometabolite generation is provided.

Key Words: D-2-hydroxyglutarate, colon cancer, metabolism, CRISPR/Cas9, PDX model.

2-HİDROKSİGLUTARAT ONKOMETABOLİTİNİN KOLON KANSERİ METABOLİZMASI ÜZERİNDEKİ ETKİSİNİN CRISPR/CAS9-TABANLI GEN TERAPİSİ İLE OLUŞTURULAN HÜCRE MODELLERİ VE IN VIVO MODELLER ÜZERİNDE İNCELENMESİ

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ÖZET

Normal konsantrasyonu oldukça düşük olan D-2-hidroksiglutarat (2-HG), yüksek seviyede üretilerek karsinogenez sürecine katılmaktadır. Bu oncometabolit yabancı tip veya mutant IDH enzimlerine ek olarak, hücre tipine özgü çeşitli enzimler tarafından üretilmektedir. Bu tezin amacı, 2-HG üretim yollarını ve bu oncometabolitin kolon kanseri metabolizmasındaki etkisini incelemektir.

İlk olarak, TCA ve Cori döngüsü, glutaminoliz ve Glikolizde rol alan bazı metabolitlerin konsantrasyonları *in vitro* ve *in vivo* olarak belirlenmiştir. D-2HG düzeyi kolon adenokarsinom hücre hatlarında (Caco-2 ve SW620) önemli ölçüde yükselirken, kolon epitel hücre hattında (CCD-18Co) tespit edilememiştir. SW620 hücrelerinde IDH1/2 genlerinde mutasyon olmadığı belirlenmiştir. Sonraki adımda, D-2HG üretimi yabancı tip IDH1 enziminin susturulduğu hücrelerde (IDH1 KO) kontrole kıyasla yaklaşık olarak 2.5 kat azalmıştır, fakat üretim devam etmiştir. L/D-2HG üretiminde rol alan MDH2 enziminin mRNA seviyesi IDH1 KO hücrelerde anlamlı seviyede artmıştır. MDH ve LDHA enzim inhibitörleri ayrı ayrı uygulandıktan sonra D-2HG seviyesi belirlenememiştir. Hasta kaynaklı ksenograft modeller (PDX) ile yapılan çalışmalarda da benzer bulgular elde edilmiştir. Son olarak, mutant IDH1 enziminin D-2HG üretimindeki etkisini incelemek için R132H mutasyonu CRISPR/Cas9 yöntemi kullanılarak oluşturulmuştur ve D-2HG seviyesinin değişmediği görülmüştür.

Sonuç olarak, D-2HG'nin üretim yolağı belirlenmiş ve bu oncometabolitin üretimi durdurularak kolon kanserine yönelik yeni bir terapötik yaklaşım sağlanmıştır.

Anahtar Sözcükler: D-2-hidroksiglutarat, kolon kanseri, metabolizma, CRISPR/Cas9, PDX modeli

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Cancer is a disease in which cells undergo metabolic and behavioral changes due to mutations in a multistage process. As a result, cancer cells exhibit atypical metabolic features in energy generation reactions (Hanahan and Weinberg, 2000). Colon cancer has a high incidence and mortality (Iacopetta, 2002). It is the third most common cancer type in both men and women (Xi and Xu, 2021). In addition to some protooncogenes and oncogenes, oncogenic mutations in the isocitrate dehydrogenase (IDH) enzyme affects the energy generation of the cell and play a role in the pathogenesis of colon cancer (Neitzel et al., 2020).

The TCA cycle is a vital energy generation step that constitutes a large part of aerobic respiration. The IDH enzymes perform decarboxylation of isocitrate to α -ketoglutarate (α -KG) (Dalziel, 1980). Tumor-based point mutations in arginine residues (Arg132, Arg172, and Arg140) in active sites of IDH enzymes affect its catalytic activity (Ward et al., 2010). Thus, mutant IDH enzymes gain neomorphic enzymatic activity and catalyze the conversion from α -KG to 2-hydroxyglutarate (2-HG) instead of isocitrate to α -KG conversion (Dang et al., 2009). 2-HG is a chiral molecule and exists in two different conformations; D- and L-enantiomer. While 2-HG is found at a very low level in healthy cells, it increases 10-100 times in cancer cells. 2-HG acts as an inhibitor of various α -KG-dependent dioxygenases, and this inhibition affects many pathways in the cell. Thus, 2-HG functions as an "oncometabolite" (Xu et al., 2011).

As mentioned above, mutant IDH enzymes acquire a new function result of 2-HG generation. However, this oncometabolite is not only generated by mutant IDH enzyme isoforms. There are various enzymes involved in the 2-HG generation:

- Wild-type (wt) IDH1 ve IDH2: IDH enzyme isoforms can also produce 2-HG without gaining a function with a mutation in arginine residues (Ježek, 2020).
- Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) enzymes in hypoxic conditions: Under normal conditions, LDH catalyzes the conversion of pyruvate to lactate, and MDH 1/2 enzymes catalyze the conversion of malate to oxaloacetate. However, under hypoxic

conditions, these enzymes convert α -KG to L-2-HG as their side functions (Intlekofer et al., 2017).

- Glutamine Anaplerosis: Glutaminolysis begins with glutamine reaching the mitochondria and converted into glutamate with glutaminase (GLS) enzyme. It continues with oxidative deamination of glutamate by glutamate dehydrogenase (GDH) enzymes to produce α -KG and ammonia. When glutamine is converted to α -KG, which is used for the 2-HG generation, it becomes the predominant source of 2-HG (Dang, 2010).

1.2. Aims of the Study

This thesis study consisted of three aims.

1. Firstly, it was investigated whether the D-2-hydroxyglutarate (D-2HG) oncometabolite is related to the metastatic property of colon cancer *in vitro* and *in vivo* models.
2. The second aim was to investigate the pathways of D-2HG generation in colon cancer cells.
3. The role of the IDH1 enzyme in the progression of colon carcinogenesis was investigated as the third aim.

1.3. Hypothesis of the Study

This thesis study examines the 2-HG generation pathways and the effects of this oncometabolite on colon cancer metabolism in diverse models. In addition, the role of R132H mutation in the IDH1 gene and the role of MDH2 and LDHA enzymes in 2-HG generation under normal conditions has been studied for the first time. Various *in vitro* models, such as colon adenocarcinoma cell lines at different stages and cell lines generated by CRISPR/Cas9-based gene therapy (IDH1 KO and IDH1 R132H), and *in vivo* models, such as xenograft and patient-derived xenograft (PDX) models were employed in this study.

2. GENERAL INFORMATION

2.1. Cancer

Cancer is a disease in which cells undergo metabolic and behavioral changes due to mutations in a multistage process. These changes occur as a result of modifications of the mechanisms that control cell proliferation and survival. Constantly dividing cancer cells do not use a single metabolic pathway. Instead, they survive and proliferate through different mechanisms. They adapted to have the ability to obtain the necessary nutrients from the environment and use these nutrients to build both bioenergetics and new biomass (Hanahan and Weinberg, 2000). When clinical cases are evaluated, it has been determined that cancer is the most common disease worldwide after significant developments in treating heart diseases. In addition, cancer, which causes so many deaths, has recently become the disease of our age (Twombly, 2005). According to GLOBOCAN estimations, 19.3 million new cancer cases were observed worldwide in 2020; unfortunately, 9.96 million patients died (Sung et al., 2021). In addition to being high in underdeveloped countries, cancer incidence is constantly increasing even in developing countries and developed countries such as Western Europe and America.

2.1.1. Colon cancer

In recent years, it has been determined that there are more than 200 types of cancer (Sung et al., 2021). Colon cancer develops in the colon through a multi-step process. It is known that the transformation of a benign tumor consisting of normal glandular epithelium cells into invasive adenocarcinoma proceeded as described in the classical adenoma-cancer progression model. In this model created by Fearon and Vogelstein, the first mutations that cause activation of oncogenes and inactivation of tumor suppressor genes cause the progression of colorectal cancer. Second, there must be mutations in at least four genes for malignant tumor development. Third, although genetic changes often occur in a preferred order, the aggregate accumulation of changes is responsible for determining the biological characteristics of the tumor. Fourth, tumor suppressor genes may have phenotypic effects even in heterozygous mutants in a specific situation (Fearon and Vogelstein, 1990).

Colon cancer has a high incidence and mortality (Iacopetta, 2002). It is the third most common cancer type in both men and women. Its incidence is increasing day by day in developing countries, including our country (Xi and Xu, 2021). According to GLOBOCAN

data, 1.93 million new colon cancer cases were observed in 2020; unfortunately, 935,100 patients died. With these values, colon cancer accounted for 10% of the total cancer incidence and 9.4% of all cancer-related deaths (Sung et al., 2021). Many of them are diagnosed in stages III and IV (Joachim et al., 2019). The most popular forms of treatment are surgery, chemotherapy, and radiotherapy (Kuipers et al., 2015). Although enhanced treatment choices and advanced metastatic surgery, the 5-year survival rate for patients in the late stages of the disease has not increased (Siegel et al., 2019). It brought attention to the need for novel therapeutic approaches. Recently, studies on rearrangement in the metabolism during cancer progression have been increasing (Neitzel et al., 2020).

The studies about the comprehensive molecular characterization of colon cancer have shown that various epigenetic changes (especially DNA methylation) in enzymes involved in metabolism have an essential role in the pathogenesis of cancer. Thus, it has been observed that the transformation of colonic epithelial cells into adenocarcinomas occurs as a result of multiple different molecular events, including genetic (chromosomal instability) and epigenetic changes (Muzny et al., 2012; Lao and Grady, 2011). Histone demethylase (KDM) enzymes are responsible for removing the methyl groups found in histone proteins that enable packaging DNA (He et al., 2022). When datasets of The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects are examined (Tang et al., 2017), it is seen that KDM enzymes are explicitly expressed according to the type of cancer (Figure 1). The levels of KDM 1A and 1B enzymes are novel prognostic markers for colon cancer (Uemura et al., 2010).

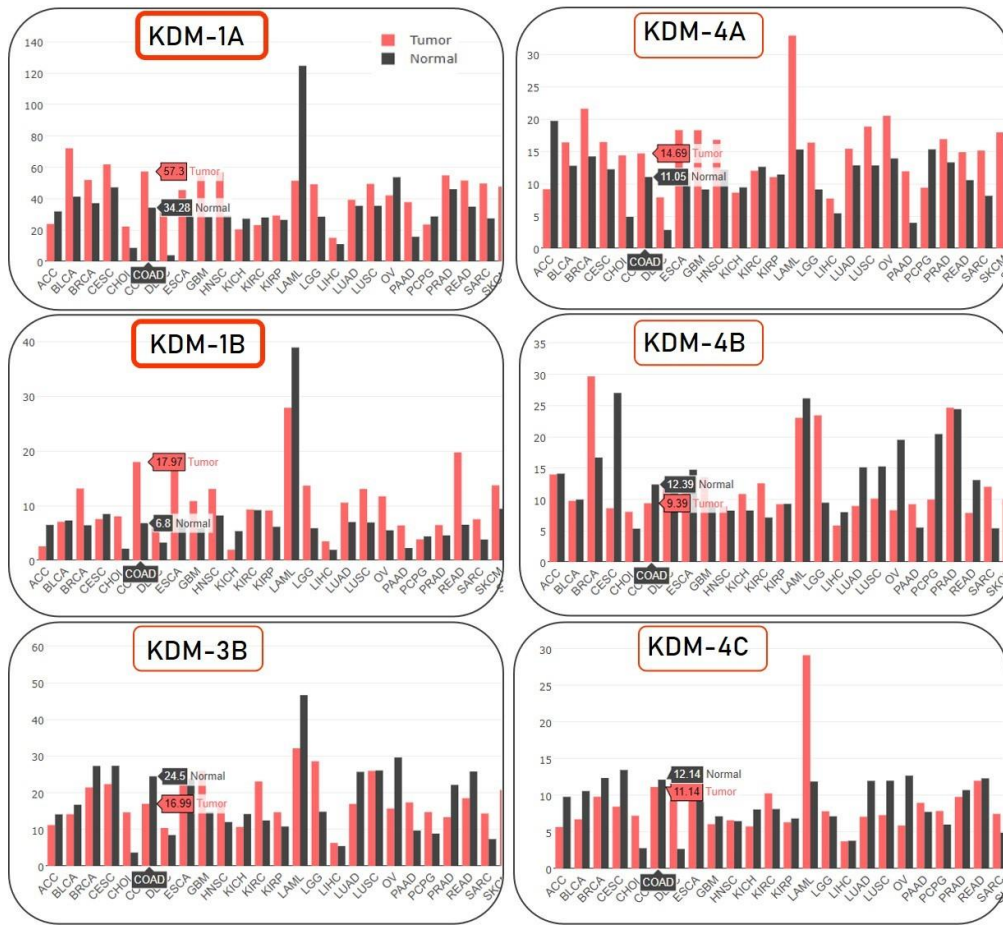


Figure 1: The mRNA levels of KDM1-4 enzymes in different cancer types.

2.2. Metabolism in Cancer Cells

Metabolism consists of biochemical reactions in which nutrients are converted into needed metabolites to maintain cell survival and regeneration. These transformations allow cellular hemostasis to be maintained, cells to produce biomass (amino acids, nucleotides, and fatty acids), and most importantly link metabolism to signaling pathways and epigenetic events. In particular, significantly increased or decreased metabolite concentration alters the activities of enzymes that modify DNA and histones (Lunt and Fendt, 2018; Sciacovelli and Frezza, 2016). Thus, metabolites emerge as essential communication components between the mitochondria and nucleus. In addition, these small molecules, which generation amount change due to genetic changes or environmental factors, act on epigenetic mechanisms that affect oncogenic signaling cascades (Sciacovelli and Frezza, 2016).

Cancer cells exhibit significant changes in many energy metabolisms. Metabolic changes in cancer are not only a result of oncogenic transformation but also occur in basic mechanisms that support and/or direct cancer initiation, progression, and treatment response. Some specific metabolites are synthesized in high amounts in cancer cells. There are significant differences in intracellular and extracellular levels compared to healthy cells. This situation is observed in glycolysis, TCA cycle, glutaminolysis, and oxidative phosphorylation (Lunt and Fendt, 2018). Significant changes in the synthesis of metabolites may play a role in cancer-related metabolic reprogramming. These metabolites majorly affect gene expression, cellular differentiation, and tumor microenvironment. There are three characteristic features of metabolism in cancer cells;

- (1) oncogene-based glucose and amino acid intake: influencing metabolite flux by increasing the ability to obtain essential nutrients,
- (2) rearrangement of cell metabolism: the use of TCA cycle and glycolysis pathway intermediates for biosynthesis and NADPH generation,
- (3) metabolites-driven changes in cell function: changes in metabolite-based gene expressions and changes in components of the tumor microenvironment (Pavlova and Thompson, 2016) (Figure 2).

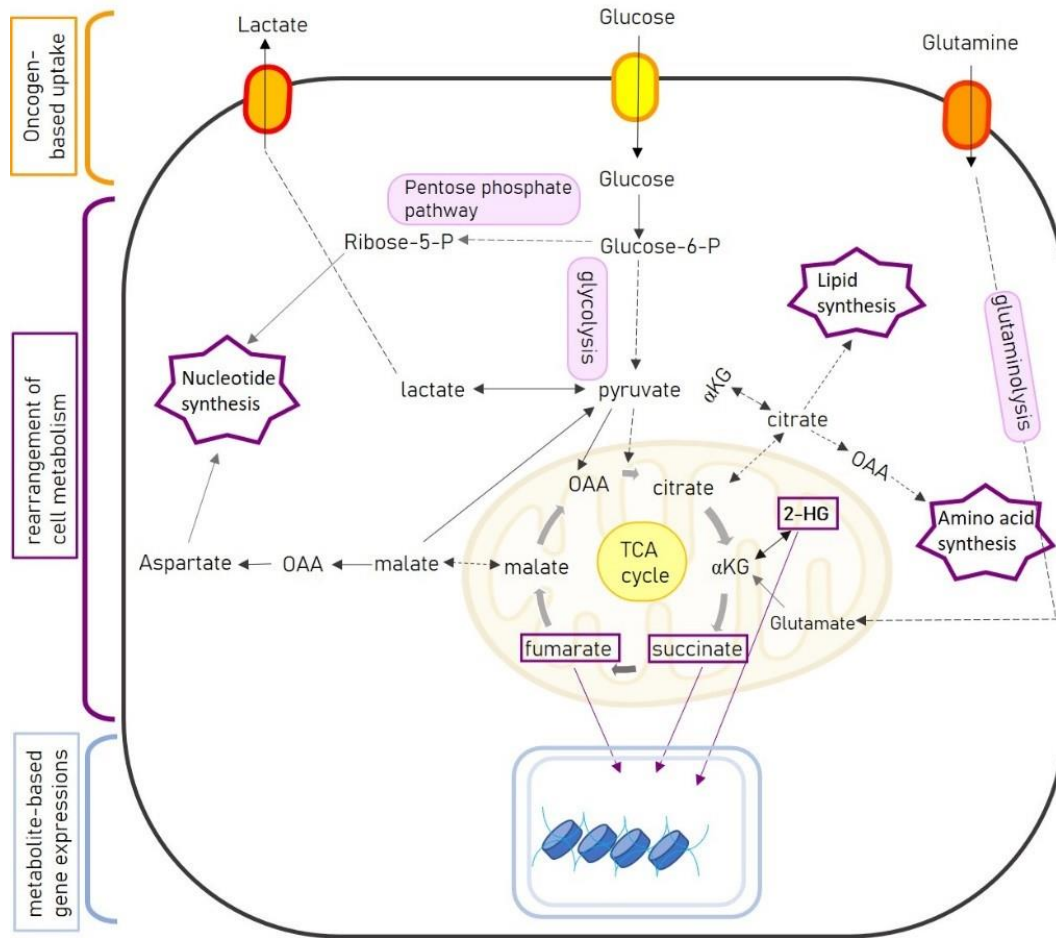


Figure 2: The schematic representation of the characteristic features of metabolism in cancer cells.

2.3. Glucose Uptake Mechanism

Overexpression of some proteins such as specific membrane transporters for glucose and the enzymes responsible for converting glucose to lactate, promote the cancer cells to redirect glucose metabolism. The uptake of glucose into the cell is known as the rate-limiting step. Two major protein families that mediate glucose uptake into the cell are glucose transporters (GLUT) and sodium-dependent glucose transporters (SGLT) (Burant & Bell, 1992; Hediger et al., 1987). The mechanism of glucose transport of GLUT and SGLT differ from each other regarding energy requirements. SGLT transports glucose into the cells against its concentration gradient using the $\text{Na}^+ - \text{K}^+$ ATPase pump to supply the Na^+ -electrochemical gradient. This type

of glucose transport occurs in the luminal membrane of cells in the small intestine (Hediger et al., 1987).

GLUT performs the task of glucose transport via diffusion across the cell membrane without energy dependence (Burant & Bell, 1992; Adekola et al., 2012). Studies show that there are 14 isoforms of GLUT genes and all isoforms exhibit different affinities for glucose. The expression of glucose transporter proteins is rearranged to acquire the increased glucose requirement in cancer cells. Increased expression levels of GLUT genes correlate with metastatic features and poor prognosis. Different GLUT genes are overexpressed in different cancer types, and GLUT1 and GLUT3 are overexpressed in malignant cells, and it is known that the increased level is a characteristic feature (Adekola et al., 2012; Farhadi et al., 2022). In addition, GLUT1 has been highly expressed in colon cancer patients (Shirvani et al., 2021).

2.4. Glycolysis and TCA Cycle

2.4.1. Glycolysis

Cells perform substrate-level phosphorylation to generate energy through glycolysis and the TCA cycle. In glycolysis, the first stage of energy generation, carbohydrate substrate (mainly glucose) is converted into pyruvate in aerobic and anaerobic environments. Normal cells metabolize glucose to pyruvate in the presence of oxygen and then oxidize the majority of the formed pyruvate to CO₂ in the mitochondria via oxidative phosphorylation. Under anaerobic conditions, however, they do not direct glycolytic pyruvate to mitochondrial oxidation but significantly reduce it to lactate (Lunt and Heiden, 2011). Cancer cells with atypical metabolic features raise glucose uptake and convert it to lactate in an aerobic environment, although only two molecules of ATP are generated. It is shown that cancer cells simulate anaerobic conditions. This phenomenon, observed only in cancer cells, is called the "Warburg Effect" (Warburg et al., 1927). It is also called aerobic glycolysis by Warburg. Because the amount of ATP to be obtained from one molecule of glucose is very low, cancer cells enhance the glucose uptake rate. In this way, glucose taken into the cell in large amounts contributes to the synthesis of amino acids and nucleotides and creates a fast way for energy generation. This provides an advantage for the survival of rapidly proliferating cancer cells by adapting to temporary or permanent hypoxic conditions (Lunt and Heiden, 2011). Moreover, lactate is not only a waste

product in this process, but also plays a role as an agent that leads to increased tumor invasion by providing cell migration, neovascularization, and immune escape.

Glucose uptake into the cell by specific glucose transporters (GLUT or SGLT) initiates glycolysis in the cytoplasm. In this pathway, the glucose molecule must be activated by receiving energy provided by the ATP molecule. 2 moles of phosphoglyceraldehyde (PGAL) are formed from glucose by consuming 2 moles of ATP, which are converted to pyruvate by subsequent reactions. Thus, most of the high energy contained in the glucose molecule is transferred to pyruvate. In addition, the protons (H^+) separated from PGAL are transferred to the NAD^+ molecule, resulting in 2 moles of NADH (Figure 3A). Besides energy generation, glycolysis is associated with many metabolic pathways (such as the pentose-phosphate pathway and TCA cycle) as it serves as a substrate for various intermediates. In addition, it supports biomass formation in dividing cells and maintains the redox balance (Lunt and Heiden, 2011).

The glycolysis pathway is controlled by three important enzymes, GLUT, phosphofructokinase (PFK), and monocarboxylate transporter (MCT). PFK-1 carries out the phosphorylation reaction of fructose-6-phosphate (F6P) to fructose-1,6-biphosphate (F1,6BP) (Ozcan et al., 2020). MCT transfers the lactate converted from pyruvate under anaerobic conditions. The increased MCT level in tumor cells causes a high lactate level in the extracellular medium. Since lactate is an agent that leads to raises in tumor invasion, its high level is critical for cancer cells (Végran et al., 2011). Recent studies have determined that glycolysis significantly affects the proliferation of various cancers due to changes in the expression of the enzymes, which control glycolysis. Ozcan et al., (2020) have investigated the role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFKFB) in pancreatic cancer cell proliferation. This irreversible reaction of F6P to F1,6BP is an important checkpoint in regulating glycolytic flow. F6P and F2,6BP are interconverted by the PFKFB enzyme family, which has both kinase and phosphatase activity. It was determined that PFKFB2 is overexpressed in various pancreatic cell lines and cancer patients (Ozcan et al., 2020).

As a result of the silencing of this enzyme, F2,6BP level, glycolytic activity, and colony formation capacity decreased. Conversely, when PFKFB2 expression is increased, proliferation and migration characteristics of cells are increased (Ozcan et al., 2020). In another study, the protein levels of MCT1, GLUT1, and PFK-1 were investigated in mouse models consisting of colon adenocarcinoma cell lines. The results show that MCT1, GLUT1, and PFK-1 protein

levels significantly increased, and p53 mRNA levels significantly decreased in tumor tissue compared to the control group. In this study, it was found that increased MCT1, GLUT1, and PFK1 levels were characteristic of colon cancer, and a decrease in the level of the p53 tumor suppressor gene was found to be inversely proportional to the glycolysis rate (Shirvani et al., 2021). Under normal conditions, p53 prevents excessive glucose uptake of the cell by decreasing the expression of some transporter proteins such as GLUT1-4 and MCT. However, the decrease in the level of the p53 gene during cancer development led to the formation affects glucose uptake rate and cell proliferation (Lago et al., 2011). In addition to the enzymes mentioned above (Chen et al., 2022) investigated the role of Enolase (ENO) in colon cancer cell proliferation and in vitro migration in various colon cancer cell models. ENO3, which is a glycolytic enzyme with three isoforms, catalyzes the 2-phosphoglycerate into phosphoenolpyruvate. In clinical studies, it has been determined that the ENO3 enzyme is overexpressed in colon cancer patients. After silencing the ENO3 gene, the proliferation and migration characteristics of colon cancer cells decreased, while proliferation and migration increased when this enzyme was overexpressed. Thus, it was concluded that ENO3 might be an essential biomarker in the development of colon cancer (Chen et al., 2022). Thus, it has been determined by studies that glycolytic enzymes have oncogenic properties.

2.4.2. TCA cycle

Under aerobic conditions, the pyruvate molecule synthesized in the cytoplasm is transported to the mitochondria and initiates the next phase, the TCA cycle. It consists of chemical reactions that oxidize pyruvate to carbon dioxide and water to produce energy under aerobic conditions. The TCA cycle begins with the formation of citrate by adding two carbons from Acetyl-CoA to oxaloacetate and continues with various reactions. First, citrate isomerizes to isocitrate by rearrangement of the molecule. Next, Isocitrate is oxidized to α -KG by the first oxidative decarboxylation reaction. One mole of CO_2 is released, and electrons pass to NAD^+ , yielding NADH and H^+ . α -KG is converted to succinyl CoA by a second oxidative decarboxylation reaction. GTP is generated from GDP by substrate-level phosphorylation during the reaction process where succinyl CoA is converted to succinate. Next, succinate is oxidized to fumarate, and the fumarate is converted to malate by adding water against the double bond. Finally, malate is oxidized to form oxaloacetate again, and thus, the cycle is

completed (Lehninger et al., 2005) (Figure 3B). In addition, one of the most critical features of the TCA cycle in dividing cells is its central location for the biosynthesis of biomass (Martínez-Reyes, and Chandel, 2020).

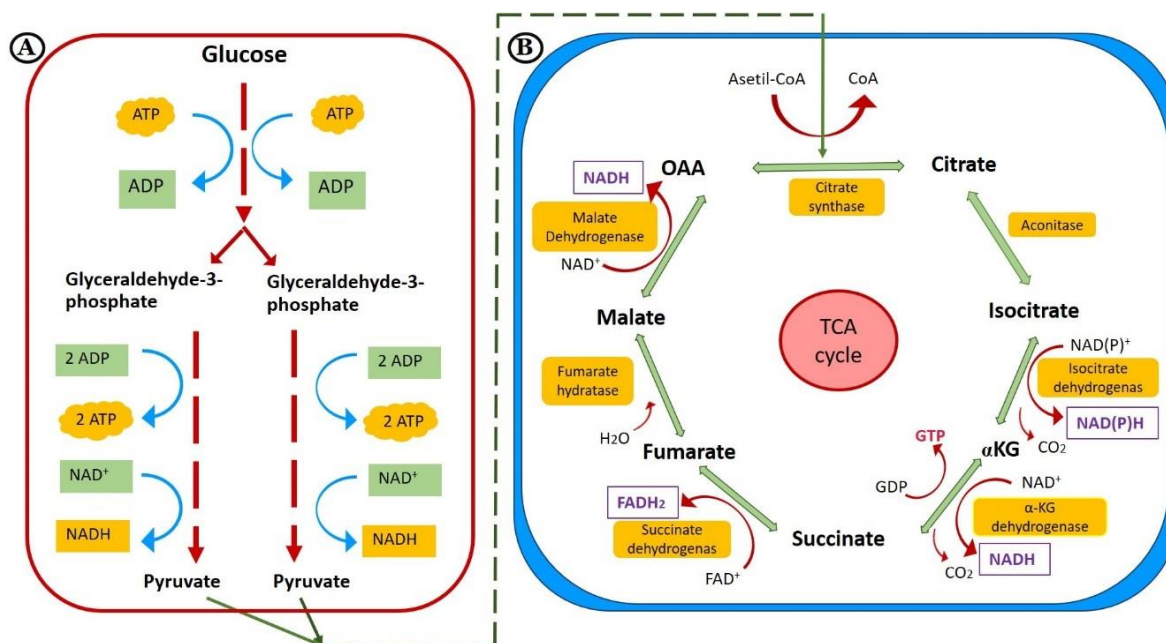


Figure 3: Glucose metabolizes into the pyruvate in glycolysis (A) and then participates in the TCA cycle (B).

2.5. The Role of Metabolic Enzymes Involved in The TCA Cycle during Cancer Progression

Some metabolites involved in the TCA cycle and glycolysis provide a connection between the mitochondria and the nucleus. As mentioned above, oncometabolite is synthesized as a result of oncogenic mutations in a metabolic enzyme that play an active role in the tumorigenesis process by inhibiting the function of enzymes involved in epigenetic mechanisms (Martínez-Reyes and Chandel, 2020). 2-HG (Dang et al., 2009), fumarate (Tomlinson et al., 2002), and succinate (Hao et al., 2009) oncometabolite are the most striking examples of this situation. The IDH, fumarate hydratase (FH), and succinate dehydrogenase (SDH) enzymes have been described as tumor suppressors, and 2-HG, fumarate, and succinate as oncometabolite (Dang et al., 2009; Tomlinson et al., 2002; Hao et al., 2009).

2.5.1. The role of SDH enzyme in the cancer progression

SDH is a hetero-tetrameric (consisting of SDHA, SDHB, SDHC, and SDHD) protein and carries out the conversion reaction of succinate to fumarate (Lehninger et al., 2005). The roles of succinate, the substrate of the SDH enzyme, in biological processes other than metabolism have been investigated, and tumorigenesis has been highlighted most remarkably (Dalla et al., 2020). It was found that the loss of function in the SDH complex induces metabolic stress, and genomic instability, and causes tumorigenesis (Slane et al., 2006). Aspuria et al. 2014 investigated the role of changes in SDH function in ovarian cancer biology in mouse ovarian cancer cell lines. Silencing of one of the SDH subunits (SDHD), resulted in a rise in both proliferative and mesenchymal properties. In addition, enhanced H3K27 methylation due to SDHB knockdown triggered the initiation of the EMT process. The effects of SDHB deficiency on metabolism were also examined, and it was found that glucose utilization was reprogrammed. In addition to the raise in succinate level and decrease in fumarate and malate levels in the TCA cycle, SDHB deficiency affected the intracellular metabolite level in glycolysis and pentose phosphate pathway (Aspuria et al., 2014).

2.5.2. The role of the FH enzyme in the cancer progression

FH converts fumarate to malate with an irreversible reaction (Lehninger et al., 2005). Tomlinson et al. (2002) determined that the FH enzyme plays a tumor suppressor role, and mutations in this gene cause fumarate accumulation, leading to renal cell cancer (Tomlinson et al., 2002). The metastasis of cells with fumarate hydratase deficiency leads to critical results in poor clinical outcomes. Sciacovelli et al. (2016) examined the link between epigenetic changes induced by fumarate accumulation and tumorigenesis. They showed that silencing of the FH enzyme led to the expression of epithelial to mesenchymal transition (EMT)-related transcription factors and enhanced migration characteristics. As a result, it was revealed that loss of FH and the consequent accumulation of fumarate contribute to the aggressive characteristics of FH-deficient tumor models (Sciacovelli et al., 2016).

Additionally, the high level of fumarate causes posttranslational modification of the protein by succination. Succinated glutathione forms proto-oncometabolite and activates

nuclear factors. This activates different biological processes that support cancer pathogenesis (Schmidt et al., 2020).

2.5.3. Mutations observed in the IDH enzyme

IDH catalyzes the conversion of isocitrate to α -KG by oxidative decarboxylation reaction (Lehninger et al., 2005). However, as a result of point mutations in arginine residues, mutant IDH enzymes gain neomorphic enzymatic activity and catalyze the conversion of α -KG to 2-HG (Dang et al., 2009). While the 2-HG oncometabolite is only generated at trace levels in healthy cells, its overexpression and accumulation in cancer cells impact the epigenetic pathways that lead the cell to metastasis. Thus, at the transcriptional level, the IDH mutation affects the function of several genes responsible for the formation and progression of cancer (Martínez-Reyes and Chandel, 2020).

2.6. The Role of 2-HG in Progression of Colon Carcinogenesis

Metabolic reprogramming contributes to malignant transformation because of the aberrant intermediate levels synthesized by the driver metabolic enzymes such as IDH1. The IDH, which has three isoforms, is a critical enzyme in the TCA cycle (Dalziel, 1980). NADP⁺-dependent IDH1 and IDH2 enzymes are homodimeric, and the reactions catalyzed by IDH1 and IDH2 are reversible. Their subcellular localization is different. IDH1 catalyzes the decarboxylation of isocitrate to α -KG in the cytoplasm, whereas IDH2 carries out the same reaction in the mitochondria (Pollard and Ratcliffe, 2009).

On the other hand, the NAD⁺-dependent IDH3 enzyme has a structurally different heterotetrameric chemistry, and it catalyzes the irreversible reaction in the mitochondrial matrix (Gabriel et al., 1986). IDH1 isoform has two crucial roles the generation of nonmitochondrial NADPH and acting a part in promoting the activity of several dioxygenases (cytoplasmic and nuclear) (Pollard and Ratcliffe, 2009). Furthermore, IDH3, which catalyzes one of the rate-limiting steps in the TCA cycle, acts as a part of the mitochondrial respiratory system (Barnes et al., 1971).

Recent studies show that mutations in IDH1 and IDH2 isoforms have been found in ~%70-80 glioma (grade II-III) and secondary glioblastoma multiforme (Parsons et al., 2008;

Yan et al., 2009). In addition, IDH1/2 mutations have been found less frequently in ~%20 of acute myeloid leukaemia (AML) (Mardis et al., 2009), in %10-20 of cholangiocarcinoma (Borger et al., 2012), and a few rare cases have shown in paraganglioma (Gaal et al., 2010), colon (Sjöblom et al., 2006.), lung (Sequist et al., 2011) and prostate cancers (Kang et al., 2009).

Three characteristics of mutations in IDH1 and IDH2 are prevalent in different cancer types.

1. These mutations are somatic, not germline.
2. Genes containing IDH1 / 2 mutations are heterozygous, one allele has gain-of-function, and the other allele remains wild-type.
3. Almost all IDH1 / 2 mutations occur at several points in the active sites of enzymes.

This showed that mutations had a direct effect on the catalytic properties of enzymes. These mutations share a common biochemical mechanism that targets the same pathway (Ward et al., 2010).

After the discovery of mutations, the roles of these enzymes in tumor formation were investigated, and it was found that tumor-based mutations in IDH1 and IDH2 disrupt normal catalytic activity. Mutant IDH enzymes acquire a new function, which catalyzes the reduction of α -KG to produce 2-HG instead of converting isocitrate to α -KG (forward reaction) (Dang et al., 2009; Ward et al., 2010). The chiral molecule 2-HG can exist in two different conformations; D- (R)-enantiomer and L- (S) -enantiomer (Xu et al. 2011). In healthy cells, the level of 2-HG oncometabolite, a minor by-product of metabolism, is kept low by the activity of L/D-2-hydroxyglutarate dehydrogenases (L2HGDH and D2HGDH), which catalyze its conversion back to α -KG (Achouri et al., 2004). In contrast, 2-HG accumulation at high molarity in cancer cells affects many metabolic and epigenetic pathways in cell metabolism. 2-HG is structurally similar to α -KG, but the oxygen atom bound to C2 in α -KG is attached to a hydroxyl group at 2-HG (differing only in the oxidation state of the C2 carbon) (Figure 4). Thanks to its chemical structure, 2-HG is a competitive inhibitor of α -KG-dependent dioxygenases, including histone demethylases (KDM), prolyl hydroxylases (HIF1 α), collagen prolyl-4-hydroxylase, mRNA demethylases (FTO and ALKBH5), and DNA hydroxylase (TET) (Xu et al., 2011) (Figure 5). This inhibition affects many pathways in the cell as α -KG-dependent dioxygenases are responsible for biosynthesis, post-translational modifications, and

epigenetic modifications that provide cellular differentiation (Lu et al., 2012). Thus, it has been suggested that 2-HG functions as an “oncometabolite” and mutant IDH is an oncogene (Xu et al., 2011).

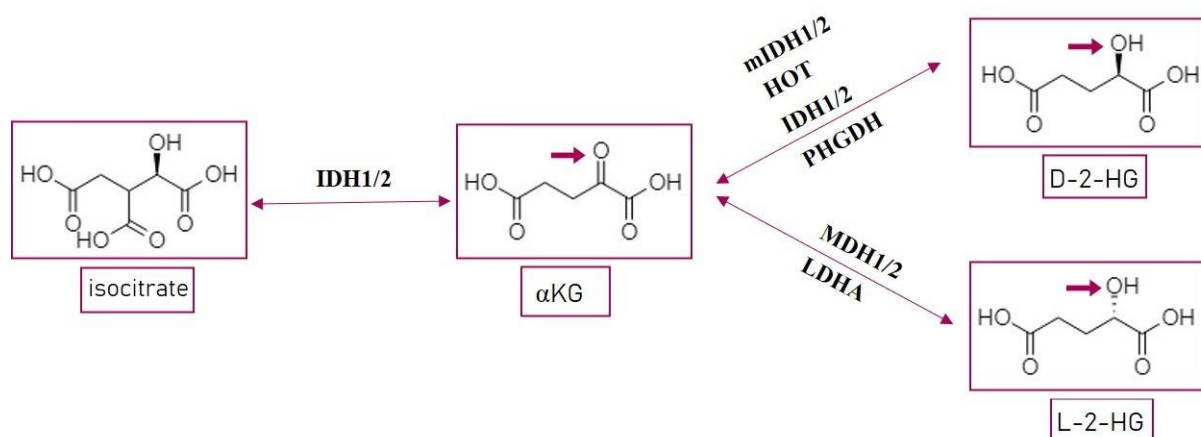


Figure 4: Chemical structures of isocitrate, α -KG, and D and L-2-HG.

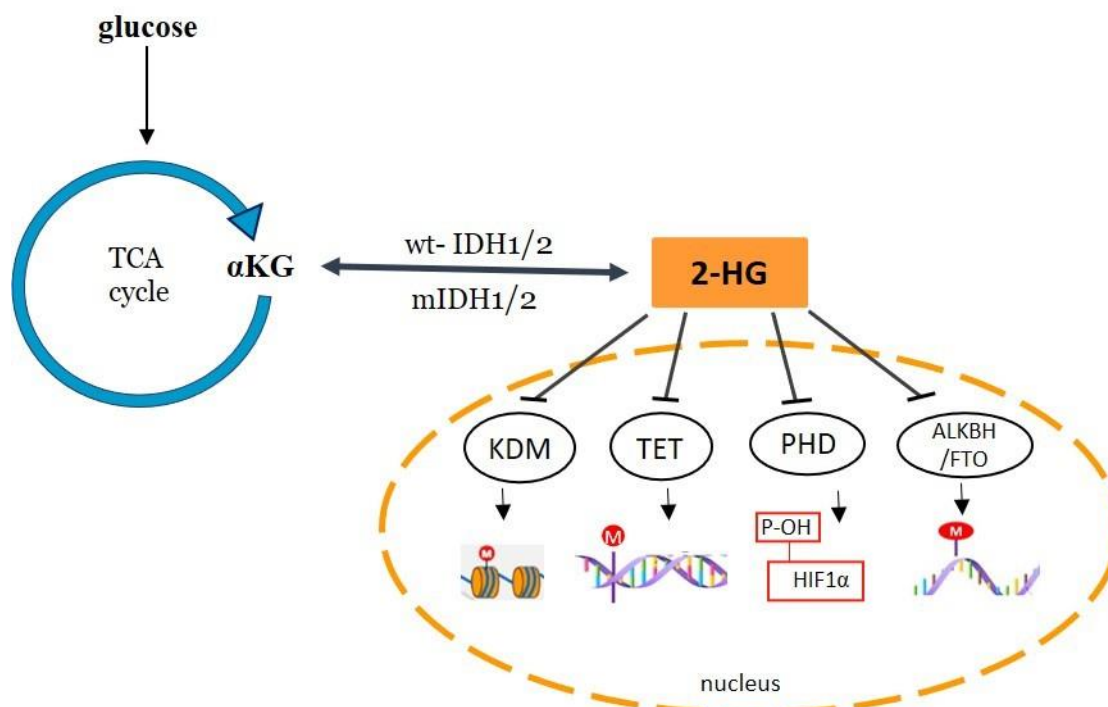


Figure 5: The accumulation of 2-HG oncometabolite results in inhibition of α -KG -dependent dioxygenases in the nucleus.

2.7. Metabolism of 2-HG

As mentioned above, mutant IDH enzymes gain a new function that performs 2-HG generation. However, this oncometabolite is not only generated by mutant IDH enzyme isoforms. The studies showed that tumor tissues and colon cancer cell lines have high levels of 2-HG oncometabolite without mutant IDH enzyme isoforms. Various enzymes, conditions, and pathways used in 2-HG metabolism are described below and shown schematically in Figure 6.

2.7.1. Glutamine anaplerosis

Glutamine, a non-essential amino acid, has become essential for the proliferation of cancer cells (Lacey et al., 1990). It is the most consumed carbon source in cancer cells after glucose to supply their energetic and biosynthetic requirements (Eagle et al., 1956). Studies have found that when a small amount of glucose is found in the plasma, the GDH enzyme has become an important way to provide carbon from glutamine to the TCA cycle. This pathway was found to be essential for the cancer cell to proliferate (Yang et al., 2009). Glutaminolysis begins with glutamine reaching the mitochondria and converting into glutamate with the GLS enzyme. Then, oxidative deamination of glutamate was performed by GDH enzymes to produce α -KG and ammonia (NADH occurs). In addition, glutamine transamination is the other way to produce α -KG (Dang, 2010).

When glutamine is converted to α -KG, which is then used for the 2-HG generation, it becomes the predominant source of 2-HG (Dang et al., 2009). Since 2-HG oncometabolite is involved in the tumorigenesis process in various types of cancer, it is hypothesized that GLS inhibitors will be the new therapy to decrease 2-HG generation. Therefore, Elhammali et al. (2014) chose Zaprinast as a GLS inhibitor and investigated whether it affects 2-HG generation in the pancreas, fibrosarcoma, and kidney cancer cell lines. HT1080 is a fibrosarcoma cell line and carries IDH1 mutation. Using mass spectrometry, cellular levels of glutamine, glutamate, α -KG, and 2-HG were measured in the Zaprinast-treated cell line (HT1080). It was concluded that the levels of 2-HG, α -KG, and glutamate were decreased, whereas glutamine level was unaffected after Zaprinast treatment. These results revealed that Zaprinast could inhibit the activity of GLS directly or indirectly, and it causes the reduction of the level of downstream metabolites.

Furthermore, when adding a cell-permeable form of α -KG to cells treated with Zaprinast, the baseline level of 2-HG was restored. They also found an extra effective GLS inhibitor, which is called BPTES. BPTES-treated HT1080 cells showed a similar pattern of metabolite modulation with Zaprinast (Elhammali et al., 2014). GLS inhibitors have been studied recently due to their therapeutic effects. CB-839, one of these inhibitors used for treating renal cancer, is currently in phase II trials. (Mullard, 2017).

2.7.2. *wt IDH1 and IDH2*

IDH enzyme isoforms can also produce 2-HG without gaining a function with a mutation in Arginine residues (Ježek, 2020). Until now, it has been studied within different cancer types such as breast (Terunuma et al., 2014), colon cancer (Han et al., 2018), and glioma (Wise et al., 2011), which generated a high level of D-2HG by wt-IDH1/2.

2-HG generation by wt-IDH1/2 takes place in hypoxic conditions in certain cell lines. Wise et al. (2011) studied a glioblastoma cell line (SF188) lacking IDH1/2 mutations. The incubation of SF188 in hypoxia resulted in higher 2-HG levels than in normoxia. This glutamine-derived 2-HG generation markedly diminished after the knockdown of IDH2. It is therefore concluded that the hypoxia-related 2-HG level raises in the cell line may be a biomarker for tissues undergoing chronic hypoxia and/or extreme glutamine metabolism (Wise et al., 2011).

Cells with wt-IDH enzymes produce 2-HG via hydroxyacid oxoacid transhydrogenase (HOT), Phosphoglycerate dehydrogenase (PHGDH), MDH, and LDH in different cell types (Han et al., 2018; Fan et al., 2015; Intlekofer et al., 2015).

2.7.3. *HOT*

The HOT enzyme called alcohol dehydrogenase iron-dependent isoform 1 (ADHFE1) catalyzes the oxidation of γ -hydroxybutyrate (GHB) in the presence of α -KG in rats and humans. Succinic semialdehyde (SSA) and D-2HG are generated as a result of this reaction. (Kaufman et al., 1988; Struys et al., 2005). The level γ -hydroxybutyric acid, which could cause D-2HG generation at a high level by HOT enzyme, is very high in human intestines (Tedeschi et al., 2003). Thus, the HOT enzyme with wt-IDH1/2 affects the 2-HG level (Struys et al., 2005). The level of D-2HG generated by HOT is kept low by the activity of D2HGDH, which

converts D-2HG to α -KG. D2HGDH is very active in some organelles such as the colon, liver, and kidney. These two enzymes (D2HGDH and HOT) affect the cellular levels of D-2HG oncometabolite (Achouri et al., 2004; Struys et al., 2005).

The elevated amount of D-2HG generated by the HOT enzyme has been observed in glioma and colon cancer. The raised D-2HG has been found in the progression of colitis to colon cancer. Han et al. (2018) investigated the role of HOT in the D-2HG generation in colon cancer progression. They concluded that D-2HG and D2HGDH expression in tissue could be a biomarker for identifying patients who have colitis (Han et al., 2018). In a different study on colorectal cancer, Tae et al. (2013) examined the function and expression of the HOT enzyme in 12 colorectal cancer cell lines, tumors, and adjacent normal mucosa of 124 patients and 59 adenoma tissues. It was found that the promoter of the HOT enzyme was highly methylated in cancer tissue compared to normal mucosa, compatible with the down-regulation of HOT mRNA and protein (Tae et al., 2013).

2.7.4. PHGDH

PHGDH couples the glycolysis and serine synthesis pathways by catalyzing the oxidation reaction of 3-phosphoglycerate (3-PG) to 3-phosphohydroxypyruvate (3-PHP) (Snell, 1986). Phosphoserine aminotransferase (PSAT) carries out transamination of 3-PHP to form phosphoserine (3-PS) and α -KG with glutamate as a nitrogen donor. Then, 3-PS is dephosphorylated, and serine is formed (Kit, 1955; Bismut et al., 1995).

PHGDH is a member of the 2-hydroxyacid dehydrogenase family, and 3-PG is the thermodynamically favoured substrate, similar to the α -KG structurally. Fan et al. (2015) found that PHGDH has a role in the 2-HG generation. The knockdown of PHGDH led to a decreased 2-HG level in breast cancer cells. Accordingly, the cellular level of 2-HG was raised over 2-fold by overexpression of PHGDH breast cancer cells, which express PHGDH at a very low level (Fan et al., 2015).

2.7.5. LDH and MDH $\frac{1}{2}$

In addition to the enzymes mentioned above, the cellular level of 2-HG raises under hypoxic conditions. Hypoxia is a significant environmental stimulus, and cells have evolved genetic and metabolic reprogramming to reduce the negative consequences of hypoxic

conditions. This programmed response first concerns the stabilization of hypoxia-inducible factor 1 α (HIF1 α). HIF1 α shifts cell metabolism to raise the expression of some proteins such as GLUT1, LDHA, and pyruvate dehydrogenase enzymes (Semenza, 2012). Although hypoxia limits the proliferation of many cell types, some cancer cells continue to grow and divide with metabolic adaptation. Since cells tend to glycolysis in hypoxic conditions, carbon entry into the TCA cycle is limited. It has been determined that the level of glucose-derived citrate decreases in macromolecular biosynthesis. The reductive carboxylation reaction of IDH and glutamine-derived α -KG has been raised to overcome this limitation. In addition to α -KG in hypoxic conditions, it was also observed that the L-2-HG isoform was specifically raised. It is known that L-2-HG is accumulated in response to raised α -KG and raised mitochondrial reduction potential. The accumulated L-2-HG role in the histone methylation level regulation and inhibiting electron transport and glycolysis leads to decreased reductive stress. Thus, L-2-HG integrates mitochondrial and cytoplasmic energy metabolism in a cellular redox regulation model (Oldham et al., 2015).

LDH catalyzes the interconversion of lactate and pyruvate, and MDH 1/2 catalyzes the conversion of oxaloacetate into malate in normal conditions. The side-function of LDH and MDH is interconverted α -KG to L-2-HG by expensing NADH under hypoxic conditions in both normal and malignant cells (Intlekofer et al., 2015; Intlekofer et al., 2017). Intlekofer et al. (2015) silenced the two enzymes separately and simultaneously with the CRISPR/Cas9 method to determine the role of LDH and MDH enzymes in L-2-HG generation. It was found that when the two enzymes were silenced simultaneously and the LDH enzyme was silenced alone, the L-2-HG level decreased significantly compared to the control group. In addition, the LDH enzyme had a more active role in the L-2-HG level (Intlekofer et al., 2015).

As mentioned above, 2-HG is generated by various enzymes that play a role in several pathways specific to cancer type. It was found that IDH1/2 mutations are very rare in colon cancer (Sjöblom et al., 2006), and wt IDH1/2 enzymes can be involved in 2-HG generation (Lv et al., 2012; Ježek, 2020). However, there is no detailed study on which enzymes are involved in the 2-HG generation in colon cancer. Determining the pathways in which this oncometabolite is generated is extremely important in offering new therapeutic options for colon cancer.

(Jinek et al., 2012). These methods have enormous potential for treating various human diseases, especially genetic ones. The use of cell line models generated by silencing or overexpressing target enzymes has provided information about signalling pathways in cancer formation or development. In addition, the generated cell line models have been actively used for studies such as testing anticancer drugs, developing new treatments, and identifying molecular markers (Wu et al., 2020; High and Roncarolo., 2019).

2.8.1. CRISPR/Cas9 method

The CRISPR-Cas9 tool is inspired by the natural adaptive immune system of bacteria and archaea. Prokaryotes have developed various RNA-mediated adaptive defence systems that detect and defend against invasive DNA elements using short CRISPR RNAs (crRNAs) and CRISPR-associated (Cas) proteins. There are two main elements of CRISPR/Cas systems, cas genes organized in operons and a CRISPR array composed of a series of repeats and genome-targeting sequences (called spacer) (Wiedenheft et al., 2012) (Figure 7A). As summarized in figure 7B, CRISPR adaptive immunity needs three steps. In the adaptation phase, the approximately 30 bp length of the sequence (proto-spacer) response to invasive elements (a virus or plasmid) is integrated into the host chromosome at the 5' end of the CRISPR array. Thus, each adaptation event results in creating a new unit. In the expression phase, the precursor CRISPR RNA (pre-crRNA) is transcribed from the spacer element and further processed into the mature crRNAs. In the interference phase, this short crRNA binds to protospacer sequences of viral or plasmid target near the protospacer-adjacent motif (PAM). After crRNA recognizes the target sequence, foreign DNA elements are cut with Cas enzymes to form a defence mechanism for prokaryotes (Makarova et al., 2020). There is a ribonucleoprotein (RNP) complex consisting of a single nuclease protein (Cas9) responsible for crRNA-guided silencing of foreign DNA, a mature crRNA, and a trans-acting crRNA (a tra-craRNA) complementary to the repeat sequences in pre-crRNA. The two-RNA structure is formed when the tra-craRNA binds to pre-crRNA and directs the Cas9 to cleave the target DNA. The crRNA sequence synthesized to guide the silencing of invading target DNA is called single-guide RNA (sgRNA) (Jinek et al., 2012). CRISPR/Cas systems are grouped into two classes depending on the organization of their effector modules. The effector complex comprises multiple Cas proteins in the class 1 systems. In contrast, it contains a single, multidomain crRNA-binding protein in the class 2 systems (such as Cas9 in type II systems). The involvement of Cas1 and Cas2

proteins in the adaptation process is only common to both classes 1 and 2. In the type II system found in class 1, the pre-rRNA is differently catalyzed by the ribonuclease RNase III (Makarova et al., 2020) (Figure 7A-B).

CRISPR/Cas9-based genome editing has recently been used for various purposes, such as engineering genetic models with specific point mutations, overexpressing a gene, repairing disease-causing mutations, or simply knocking out a target gene (Figure 7D). This method allows it to genetically edit any region of the genome *in vivo* or *in vitro* (in various cell types) models. Although there is more than one method for genome editing, the CRISPR/Cas9 method has become the most efficient method in a short time due to its advantages such as simplicity, low off-target effect, and adaptability (Wu et al., 2020). Until now, different cell models have been developed with the CRISPR-Cas9 method in glioma (Moure et al., 2019), AML (Brabetz et al., 2017), and chondrosarcoma (Li et al., 2019) cancer types in order to understand the roles of IDH enzymes on cancer pathogenesis. Špačková et al. (2021) knocked out the IDH2 enzyme to investigate the pathway by which 2-HG is generated in breast cancer cell lines and found that the IDH2 and HMT enzymes are related (Špačková et al., 2021).

In the CRISPR/Cas9 method, the Cas9 enzyme creates double-strand DNA (DSB) breaks in the target DNA. The cells use two different cellular DNA repair machinery to repair DSB, homology-directed repair (HDR) or non-homologous end joining (NHEJ). In the NHEJ pathway, the ligation of two DNA double-strand breaks take place, which leads to variable insertions and deletions at the end of DSB. In contrast to HDR, NHEJ does not require an undamaged partner DNA molecule as a template. In the HDR pathway, homologous chromosomes or template DNA are used to repair any insertion, deletion, or single base changes at the DSB. Nowadays, HDR pathways that allow for the precise repair of modifications are now being employed in genome engineering tools (Liang et al., 1998; Lieber et al., 2003).

The phase of the cell cycle is an important factor in determining the pathway for the DNA break. While the cell prefers the NHEJ pathway throughout the cell cycle, the HDR pathway is active in the late S and G2 phases when DNA is completely replicated (Heyer et al., 2010). Competition with NHEJ in the S and G2 phases prevents the use of the HDR pathway in genome editing tools (Orthwein et al., 2014). Treatment with a chemical agent or genetic disruption of the NHEJ pathway is preferred to raise the HDR pathway, even if it is difficult to employ or

harmful to the cell (Lin et al., 2014). Introducing the RNP complex formed by recombinant Cas9 enzyme and sgRNA and HDR-template into the cell is a powerful method that recently developed and raised the efficiency of genome editing. RNP delivery. Various studies have proven that RNP delivery reduces the off-target effect and raises HDR-based repair. Since this system can be applied to the embryo, it also enables the development of various animal models (Lin et al., 2014; Zhang et al., 2021; DeWitt et al., 2017).

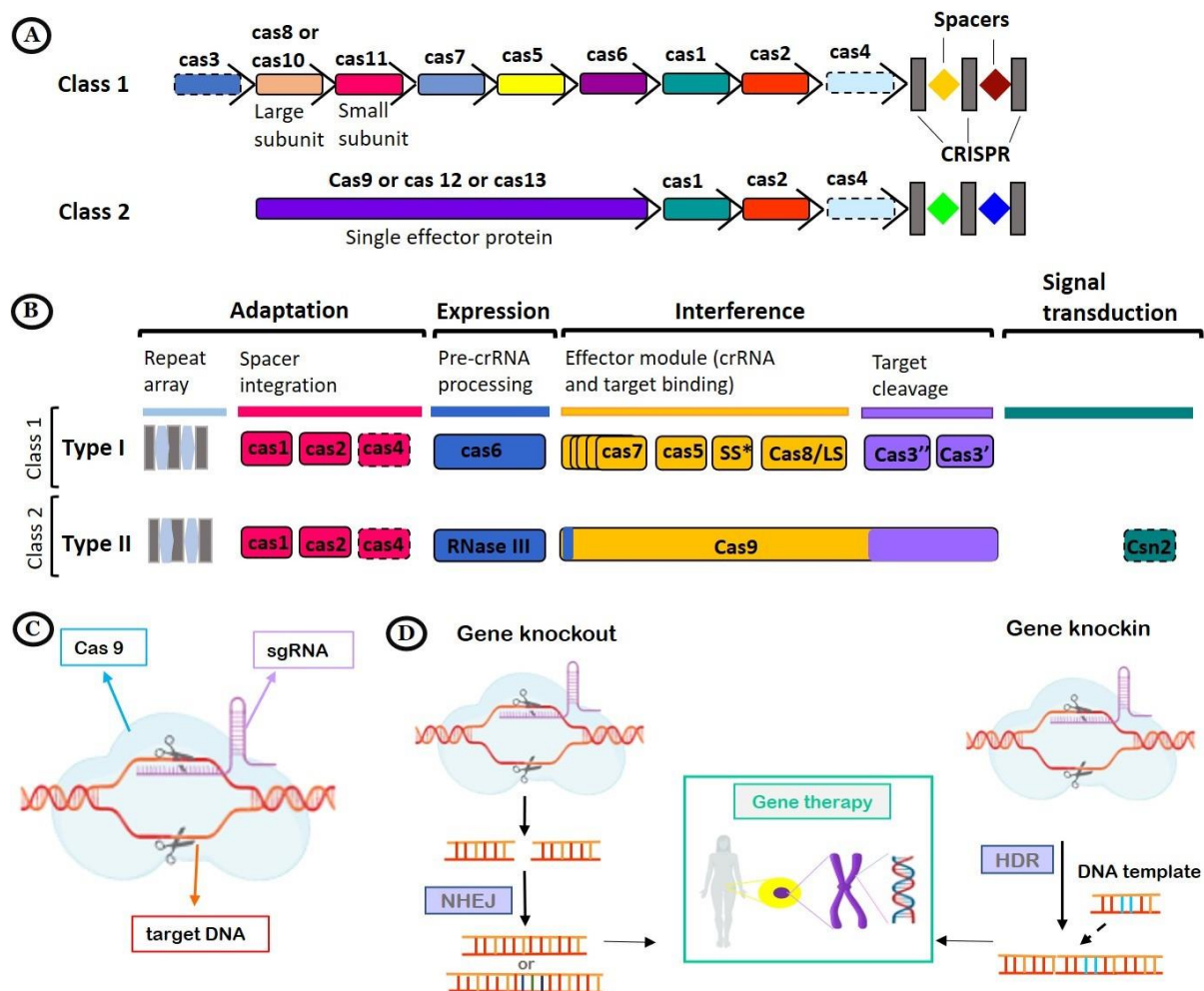


Figure 7: Schematic representation of the CRISPR-Cas system and CRISPR/Cas9-based genome editing.

Cas genes and a CRISPR array composed of a series of repeats and genome-targeting sequences (spacer) are two main elements of CRISPR-Cas systems (Wiedenheft et al., 2012) (A). CRISPR adaptive immunity needs three steps. First, the small sequence of invasive elements is integrated into the host chromosome in the adaptation phase. In the expression phase, pre-crRNA is

transcribed from the spacer element. In the interference phase, the crRNA binds to the protospacer sequence (B). After sgRNA recognizes the target sequence, foreign DNA is enzymatically cleaved with Cas enzymes to form a defense mechanism for prokaryotes (Makarova et al., 2020) (C). CRISPR/Cas9-based genome editing has been used for various purposes, such as specific point mutations or the knockout of a target gene (Wu et al., 2020) (D). pre-crRNA, precursor CRISPR RNA; SS, small subunit; sgRNA, single-guide RNA; LS, large subunit; HDR, homology-directed repair; NHEJ, non-homologous end joining.

2.9. Importance of *in vivo* Models in Cancer Studies

The most widely used treatments for colon cancer are chemotherapy and radiation, as in other types of cancer. In addition, the cancer diagnosis in the early stages has raised the treatment rate, and the five-year survival rate is expected to rise (Kuipers et al., 2015; Siegel et al., 2019). Investigation of new markers and newly synthesized or developed new generation chemotherapeutic agents have generally been tested on immortalized cell models. In vitro evolutions, systems consisting of immortalized cell lines have some advantages, such as being robust and tractable. However, changing phenotypic and molecular characteristics to environmental factors is a significant limitation (Pandita et al., 2004). It has been hypothesized that culture conditions do not fully reflect the tumor microenvironment, and cells must exhibit characteristic changes to survive in their new environment. In addition, the cells with the high capacity to overcome the adaptation with their changes will be chosen during passages. This causes the surviving cells to exhibit different genetic changes from the original tumor (Hamer et al., 2008). Since cell models have these limitations, *in vivo* models have been used to develop treatment strategies. Over the last decade, xenograft mouse strains have been generated by subcutaneous injection of cancer cells into immunodeficient mice. Xenograft models allow the response of the body as a result of drug treatment to be examined in different tissues. However, cancer cells that differentiate from the original tumor are still used (Pandita et al., 2004).

2.9.1. The use of PDX models in cancer studies

To overcome the limitations of immortalized cell lines, PDX models have been developed by transplanting human tumor tissues obtained through biopsy or surgical resection into NOD-SCID (non-congenital diabetic severe combined immunodeficiency) mice (Mattern

et al., 1985). Thus, small amounts of human tissue samples are augmented to a larger tumor mass. These tumor tissues were found to be more similar to the original tumor in terms of tumor architecture, microenvironment, and heterogeneity than the cell lines. PDX models are very suitable for cancer research, such as cancer metabolism, understanding the genetic and epigenetic changes of cancer, development of personalized cancer treatments, and determination of cancer-specific biomarkers. They have been frequently used in studies of colon cancer in recent years (Keil et al., 2021; Seol et al., 2014).

There are two different methods to generate the PDX models: heterotopic and orthotopic transplantation. In particular, PDX models created by the orthotopic transplantation method are quite similar to the original tumor structure. Therefore, heterotopic and orthotopic transplantation was performed to generate the colon PDX models. In the heterotopic method, tumor tissue is injected subcutaneously into the flank of mice (Lai et al., 2017).

3. MATERIALS AND METHODS

3.1. Type of the Study

The thesis work is an experimental study.

3.2. Place and Time of the Study

The experiments in the study were carried out between April 2018-April 2022 in the Ayar Kayalı Laboratory located in Izmir Biomedicine and Genome Center.

3.3. Research Population and Sample/Study Groups

The inclusion criteria of the volunteers participating in the study are listed below:

- Adult patients between the ages of 18-60
- Patients whose consent was obtained
- Patients at risk of colon cancer and diagnosed
- Patients who have not received chemotherapy and / or drug therapy

3.4. Material and Methods

3.4.1. Materials

Oxaloacetic acid, 2,4-dinitrophenylhydrazine (DNPH), lactic acid, succinic acid, α -ketoglutaric acid, fumaric acid, pyruvic acid, sodium hydroxide (NaOH), hydrochloric acid (HCl), D- α -hydroxyglutaric acid disodium, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, octyl- α KG, polyethylenimine (PEI), sulfuric acid (H₂SO₄) were obtained from Sigma-Aldrich. The properties of chemicals and consumables used in this study are given in Table 1.

Table 1. The properties of the consumables and chemicals used in this study.

Chemicals and consumables	Brand
FBS	Gibco

Penicillin-Streptomycin	Gibco
DMEM	Sigma-Aldrich
RPMI-1640	Sigma-Aldrich
MEM	Sigma-Aldrich
PBS	Gibco
Trypsin-EDTA	Gibco
3.5-dinitrosalicylic acid (DNS)	Aldrich
Potassium Sodium Tartrate Tetrahydrate	Merck
Corning® Collagen Matrix	Corning 354244
Primocin	InvivoGen
DNeasy Blood & Tissue Kit	Qiagen
OneTaq® Quick-Load® 2X Master Mix	New England BioLabs
TRIzol Reagent	Ambion
High-Capacity RNA-to-cDNA™ Kit	Applied biosystem
GoTaq® master mix	Promega
RIPA (Radioimmunoprecipitation assay) buffer	Santa Cruz (sc-364162)
Galloflavin	Biovision
K272c	Cayman
Lenti CRISPRv1	Addgene Plasmid 49535
T4 PNK enzyme	New England BioLabs
BsmBI enzyme	New England BioLabs
T4 ligase enzyme	New England BioLabs
LB Broth	Merck
NucleoSpin Plasmid QuickPure™ Kit	Macherey-NageI™
BbsI enzyme	New England BioLabs

Clean & Concentrator™ -5	Zymo
DNase I	Thermo Fisher
L-Broth	Merck
Ethanol	Isolab

3.4.2. Machines

The machines used in this thesis and their brands are given in Table 2.

Table 2. The machines used in this thesis.

Machines	Brands
Centrifuge 5810R	Eppendorf
Multiskan™ GO Microplate Spectrophotometer	Thermo Scientific
Centrifuge MicroCL 17R	Thermo Scientific
Balance - basic	Sartorius
HPLC System	Shimadzu
UPLC System	Agilent
Axio Vert.A1 Inverted Microscope	ZEISS
Vortex Mixer	Thermo Scientific
Dry Bath Incubator	Thermo Scientific
pH Meter	Hanna
Fume Hood	Köttermann
Laminar Flow Cabinet	Thermo Scientific
Varioscan Flash	Thermo Scientific
Water Bath and Lid	Nüve
Orbital Shaker	Thermo Scientific
PCR	SimpliAmp

Electrophoresis	Biorad
7500 Fast Real-Time PCR System	Applied Biosystems
Nucleofector	Lonza
NanoDrop 2000 Spectrophotometer	Thermo Scientific
Cell Metric™	Solentim

3.4.3. Methods

3.4.3.1. Cell lines and animals

CCD-18Co and HEK293T cell lines were grown in 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-streptomycin-containing DMEM. Caco-2 cell lines were grown in MEM (with 20% FBS and 1% Penicillin-streptomycin). SW480 and SW620 cell lines were grown in RPMI (with 10% FBS and 1% penicillin-streptomycin), respectively. All cell lines were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C (Subasi et al., 2020).

The athymic Nude mice (Foxn1^{nu}) and nonobese diabetic severe combined immunodeficiency gamma (NOD/SCID) mice were living in Vivarium Rodent Facility of İzmir Biomedicine and Genome Center. The mice were housed at 22 ± 2°C, with 12 hours of light/dark periods, and fed as ad libitum. The animal care conditions were approved by two different ethics committees. The experiment carried out using Nude mice was approved by IBG-AELEC, protocol number: 17/2019. The experiment carried out using NOD-SCID mice was approved by Dokuz Eylül University Clinical Research Ethics Committee, protocol number:2019/06-32.

3.4.3.2. Extraction of intracellular metabolites from cell

One million cells were seeded and then they were numbered using a Thoma lame 24-96 h later. 100 µl of cold 50% MeOH: water (v/v) solution for one million cells was added to the pellet and cells were lysed. The lysate was centrifuged at 17,000 g and +4 °C for 15 minutes (Figure 8A) (Dietmair et al., 2010; Bulut Atalay and Ayar Kayalı, 2022). Each sample was

performed three times independently, and the standard deviations were calculated using the average of the results.

3.4.3.3. Extracellular metabolites extraction

After 24, 48, 72, and 96 h, one ml of the growth medium was received from each Petri dish (initially containing one million cells), centrifuged at 17,000 g and +4 °C for 15 minutes, and kept at -80 °C until analysis (Figure 8B). The supernatant from 24-96th samples was only used for glucose determinations (Dietmair et al., 2010) (Figure 8B). The determination of extracellular metabolite levels corresponding to one million cells was described in previous publications (Jain et al., 2012; Bulut Atalay and Ayar Kayalı, 2022). Each sample was repeated in 3 replicates, and the standard deviations were calculated using the average of the results.

3.4.3.4. DNS method

DNS method was used to determine the reducing glucose content in the cell pellets, media, and tissues (Miller, 1959; Khuwijitjaru et al., 2017). In this method, the aldehyde group in glucose is oxidized under alkaline conditions and turns into a carboxyl group. The DNS reagent was made by dissolving one g of DNS in 20 ml of 2N NaOH and boiling at 95 °C. Potassium sodium tartrate tetrahydrate (30 g) dissolved in 50 ml distilled water was slowly added to the DNS reagent and the final volume was completed to 100 ml with distilled water. The sample (50 µl) and DNS reagent (1:1) were stirred at 140 rpm at 100 °C during 10 minutes. After incubation in ice during one minute, the results were obtained at 540 nm.

3.4.3.5. Colorimetric Determination of Pyruvate

The protocol was optimized for 100 µL (Yoo et al., 2011). This method is based on the formation of hydrazones by pyruvate under alkaline conditions. First, five mg of DNPH was dissolved in five ml of 2N HCl (37%), and then 10 µl was added to each well. Then, 30 µl of the samples were transferred to these wells and incubated for 5 minutes at room temperature. Finally, 60 µl of 2N NaOH was added and incubated again at room temperature for 10 minutes. The results were obtained at 520 nm absorbance.

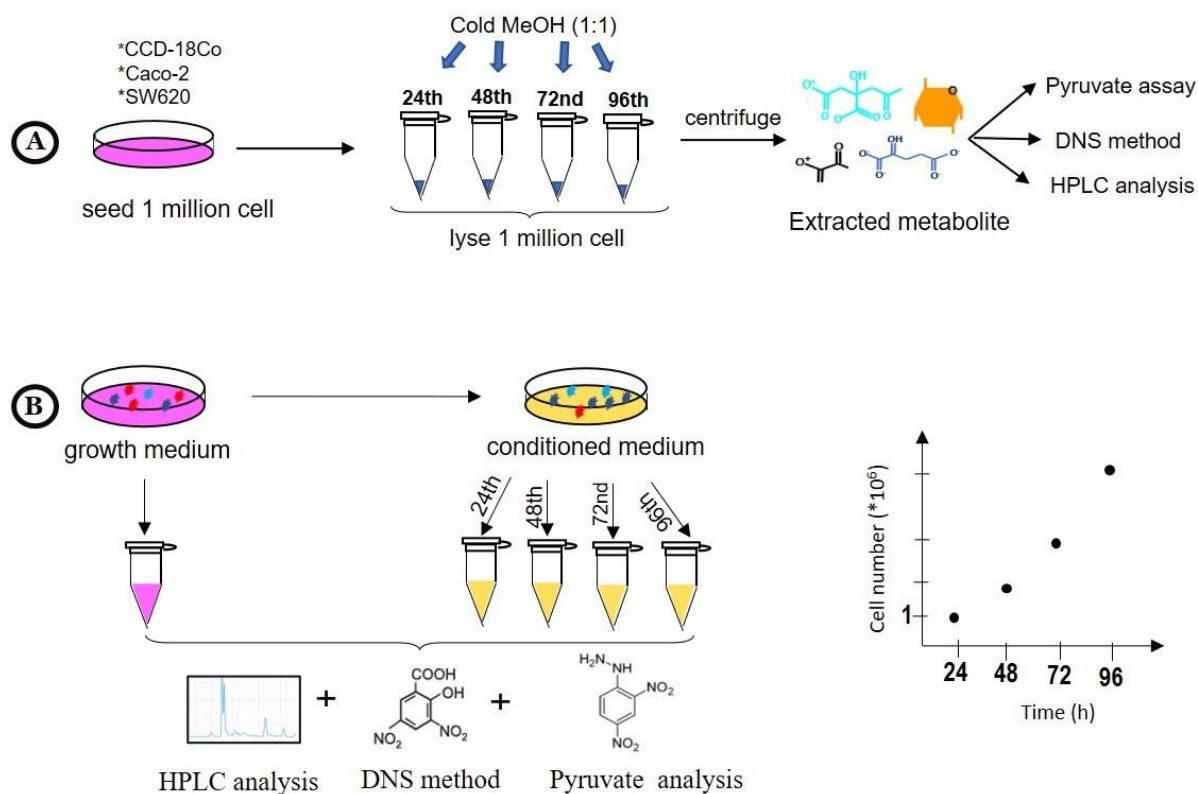


Figure 8: The intracellular and extracellular metabolite extraction procedure.

3.4.3.6. Development of xenograft model

Xenograft model development (Figure 9A), sacrifice, and tissue extraction were performed as described in our previous study (Bulut Atalay and Ayar Kayalı, 2022).

3.4.3.7. Development of PDX Models

PDX models were developed with biopsy samples from patients with colon cancer. Pr. Dr. Berat Ender Ellidokuz, who works in the Dokuz Eylul Research and Practice Hospital, Department of Internal Medicine, Gastroenterology Department, took 5-20 mm³ biopsy samples from tumor and healthy tissues of 10 patients. The healthy tissue was taken five cm away from the tumor tissue. Both tumor and healthy tissues were stored in RPMI medium with %0.1 P/S and 0.1 mg/ml primocin in falcon tube. Tumor tissues were divided into small pieces and centrifuged at 1500 rpm for one minute. The supernatant was removed until 100 µl of the medium remains. Tissues were mixed with matrigel at a ratio of 1:1 just before transplantation and injected subcutaneously into the flank of 6-8 week old female and male NOD-SCID mice.

The subcutaneous tumor development was monitored by measuring the tumor volume with a caliper every week. The tumor volume was measured with the formula given up, and when it reached one cm³, mice were sacrificed. The tumor, colon, and liver tissues were stored similarly to xenograft models (Park et al., 2016). The process was figured out in Figure 9B-C.

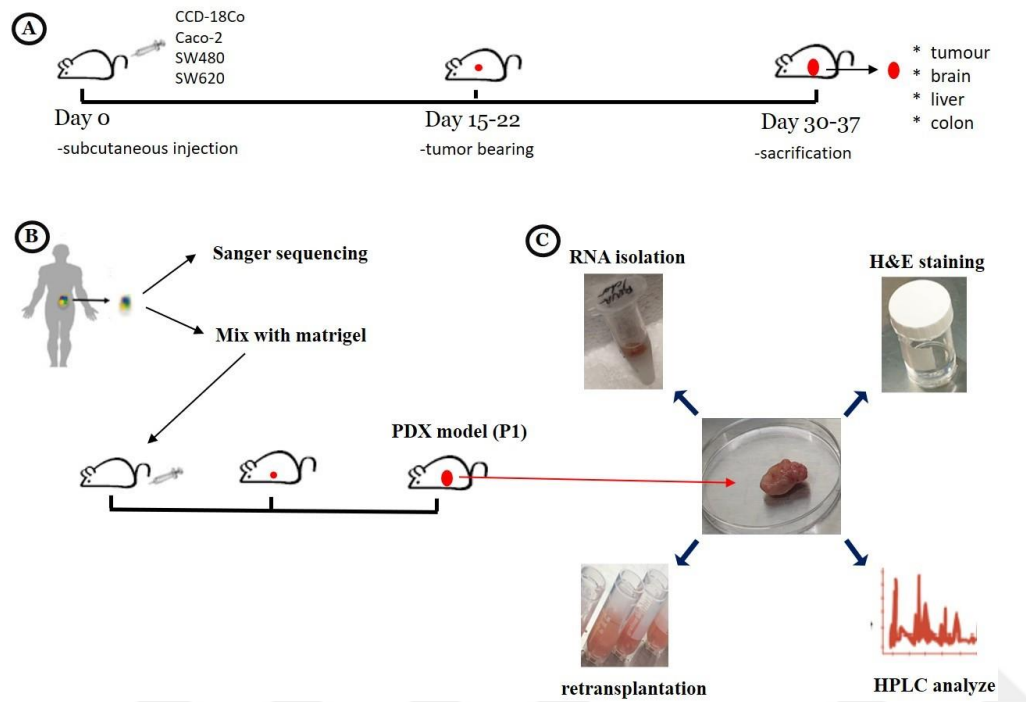


Figure 9: The procedures of xenograft (A) and PDX (B, C) model generation.

Five million cells were subcutaneously injected into Nude mice to generate xenograft models (A). Pieces of the tumor tissues were used for Sanger sequencing. The biopsy samples were mixed with matrigel (1:1) and injected subcutaneously into the flank of 6-8 week old female and male NOD-SCID mice (B). The tumor tissue extracted from the PDX model was used for further analysis (C).

3.4.3.8 Hematoxylin and Eosin (H&E staining)

Tissue embedding, tissue follow-up, sectioning, and H&E staining steps were performed by an expert at Izmir Biomedicine and Genome Center, Histopathology Unit. H&E staining protocol was carried out as described before (Fischer et al., 2008). H&E staining results were evaluated by veterinary pathologist Prof. Dr. Serap Birincioğlu.

3.4.3.9. Metabolite extraction from tissues

Firstly, the suitable solvent was optimized according to tissue type isolated from xenograft and PDX models. It was determined that methanol (MeOH) for liver and brain tissues and chloroform:MeOH: water (2:5:2) solutions for colon and tumor tissues were the most suitable solvents. Tissues were lysed in cold solvents at a ratio of 1:10 by a tissue grinder (NG010) for about five minutes on ice. After incubation on ice for 20 minutes, it was centrifuged at 13,000 rpm for 15 minutes at +4 °C. When using chloroform:MeOH: water (2:5:2) solution, two different phases occurred, and the upper phase was taken for metabolite extraction. (Want et al., 2013; Naz et al., 2014).

3.4.3.10. Analyzing metabolites with HPLC and UPLC systems

The HPLC (Shimadzu) and UPLC (Agilent) systems have an Alltech OA-1000 column, a UV detector, and a differential refractive index detector. In the HPLC system, only malate was detected in the RID detector (Ayar-Kayali and Tarhan, 2006). The HPLC conditions were detailedly explained in our previous study (Bulut Atalay and Ayar Kayalı, 2022).

3.4.3.11. DNA isolation

DNA isolation was performed from cells and tumor tissues taken from patients with the DNeasy Blood & Tissue Kit (Qiagen) as specified in the kit procedure. The purity of the DNA obtained using the kit was determined by the nanodrop (Thermo) device.

3.4.3.12. Polymerase chain reaction (PCR) and the Sanger Method

Target DNA sequences were amplified by PCR reaction for sequencing. Reverse and forward primers designed for IDH1 and IDH2 enzymes are given in Table 3. The IDH1 primer was designed for the region containing amino acids R132 and G97. IDH2 primers were designed for the region containing amino acids R140 and R172. PCR reaction was performed according to the OneTaq® Quick-Load® 2X Master Mix kit protocol (Table 4). T_m values of the primers were determined by gradient PCR. The PCR products were subjected to electrophoresis on 1.7 % agarose gel. It was run at 90 volts for 50 minutes. The PCR products were sequenced with the Sanger Method by Triogen Biotechnology Company.

Table 3. The primers were designed for PCR analysis.

Enzyme	Forward (5'- 3')	Reverse (5'- 3')
IDH1	GATGCCACCAACGACCAAGTC	GTGTTGAGATGGACGCCTATTTGTAAG
IDH2	ATTCTGGTTGAAAGATGGCGGC	AGTCTGTGGCCTTGTACTGC

Table 4. The PCR conditions.

Step	PCR				
	Initial Denaturation	30 cycles			Final Extention
		Denature	Anneal	Extention	
Time	30 sec	15 sec	30 sec	30 sec	5 min
Temp (°C)	94	94	62	68	68

3.4.3.13. Quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from 3×10^6 cells, and tissues were stored in RNAlater solutions using TRIzol Reagent with the kit procedure. The purity and amount of the obtained RNA were determined by Nanodrop. One μg RNA was used to convert cDNA with the High-Capacity RNA-to-cDNA™ Kit. GoTaq® master mix was used for quantitative RT-PCR (Table 5), and the relative quantification was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was the internal standard. Primer sequences for IDH1, IDH2, GAPDH, LDHA, MDH2, and GLS genes are listed in Table 6.

Table 5. The RT-qPCR conditions

Step	Enzyme Activation	PCR	
		40 cycles	
		Denature	Anneal/Extend
Time (sec)	120	15	30
Temp (°C)	95	95	60

Table 6. The primers were designed for RT-qPCR analysis

Enzyme	Forward (5'- 3')	Reverse (5'- 3')
IDH1	CGGTCTTCAGAGAAGCCATT	AGGCCCAGGAACAACAAAAT
IDH2	AGCTGGATGGGAACCAAGAC	CGTGGTGTTTCAGGAAGTGC
GAPDH	GGTGTGAACCATGAGAAGTATGA	GAGTCCTTCCACGATACCAAAG
GLS	CAGAAGGCACAGACATGGTTG	CACCAGTAATTGGGCAGAAACC
MDH2	GCCTGACCCTCTATGATATCGC	GGACTCCAGCCGGAATAACTAC
LDHA	TTGGTCCAGCGTAACGTGAAC	AATCCAGATTGCAACCGCTTCC

3.4.3.14. Western blotting

Cell pellets were subjected to physical lysis by sonication for 30 seconds in RIPA buffer and incubated on ice for 15 minutes. The supernatant was taken after centrifugation at 13,300 rpm for 15 minutes at 4°C. The cell lysates were mixed in a 3:1 ratio with an SDS-PAGE loading buffer and incubated at 95°C for 15 minutes. The samples were loaded with 30 micrograms of protein and run at 100 V for 2 hours on 12% SDS-PAGE gels. The samples were then transferred to a nitrocellulose membrane at 250 mA for 1.5 hours. Membranes cut according to the appropriate protein weight were blocked for 45 minutes in 1% BSA or milk powder according to the antibodies used. Then they were washed three times with Tris-buffered saline (TBS) solution containing 0.1% Tween-20. Then, it was incubated for two hours with IDH1 and GAPDH primary antibody solutions prepared in a blocking buffer under room conditions. Next, the membranes were rinsed three times with TBST solution before being incubated for one hour with a blocking buffer-prepared HRP-conjugated secondary antibody solution. Finally, the membranes were washed three times with TBST solution before being visualized using the ECL western blotting analysis technique (Subasi et al., 2020).

3.4.3.15. MTT assay

The determination of IC₅₀ values of inhibitors was evaluated by MTT assay. 7500 cells were seeded in a 96-well plate. After 24 hours, the growth medium was removed, and Galloflavin, K272c, and octyl- α -KG were applied. After 24 hours, the growth medium was

removed, and 100 µl of the 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent was added to each well. After four hours of incubation, the growth medium was removed, and 100 µl of DMSO was added. Formazan crystals were determined at 540 nm absorbance, and the IC₅₀ value was calculated. Three independent experiments were carried out (Subasi et al., 2020).

3.4.3.16. Silencing of the wt IDH1 enzyme using lentiviral vector by CRISPR/Cas9 method

3.4.3.16.1. Designing sgRNA

Two different sgRNAs listed in Table 7 were selected from the Brunello library and chop-chop website to match exon two of the IDH1. sgRNAs are selected to cut the PAM sequence at a distance of 4-6 nucleotides. Then, synthetic oligos of the selected sgRNA were synthesized by Triogen Biotechnology Company.

Table 7. sgRNAs selected for IDH1 gene

	Forward (5'- 3')	Reverse (5'- 3')
sgRNA1	CACCGCATAGGTCGTCATGCTTATGG	AAACCATAAGCATGACGACCTATGC
sgRNA2	CACCGTCATAGGTCGTCATGCTTATG	AAACATAAGCATGACGACCTATGAC

3.4.3.16.2. Transfection of sgRNAs

LentiCRISPRv2 vector was cut with BsmBI enzyme, and it was determined whether the cleavage was successful or not by running the samples in the gel. sgRNAs are phosphorylated and annealed by the T4 PNK enzyme (Table 8).

Table 8. The conditions of the phosphorylation reaction

	amount (µl)
10 X T4 Buffer	1
forward oligo (100 µM)	1
reverse oligo (100 µM)	1
T4 PNK enzyme	0,5
nuclease-free ddH ₂ O	6,5
total	10

PCR conditions:	
30 min	37°C
5 min	95°C
ramp down to 25°C at 5°C/min	
Hold at 4 °C	

Ligation of the phosphorylated oligos and 50 ng of vector cut with BsmBI enzyme was performed with T4 ligase enzyme (Table 9). Ligation was carried out in two hours at room temperature. In the ligation, there were two different negative control reactions. The first reaction contained the T4 ligase enzyme and did not contain an oligo duplex. The second reaction contained oligo duplex and did not contain the T4 ligase enzyme. In order to transform the ligation product into competent E.coli bacteria (Stbl3) by heat-shock method, 50 µl of bacteria and 50 ng of plasmid (all the ligation products) are mixed and kept on ice for 30 minutes. It is then incubated for 90 seconds at 42°C and two minutes on ice, respectively. After incubation, the bacteria were grown in one ml LB Broth for one hour at 37 °C. Then, it is centrifuged at 11000 g, and the pellet is spread on LB agar by pipetting. After approximately 16 hours, three clones formed on the agar are selected with a pipette tip, taken into LB Broth (containing 0.1 mg/ml Ampicillin), and incubated at 225 rpm at 37 °C for approximately 18 hours. At the end of the period, plasmid isolation was performed with NucleoSpin Plasmid QuickPure™ Kit, and sequence analysis was performed on the isolated plasmids using the Sanger method. According to the results, a plasmid without mutation was selected to use in the infection step.

Table 9. The conditions of ligation reaction

	amount (µl)
10X T4 ligase buffer	1
50 ng digested vector	-
phosphorylated and annealed oligo duplex	2
T4 ligase	0,5
nuclease-free ddH2O	-
total	10
incubation 2 h at room temperature	

3.4.3.16.3. Virus generation and transfection

HEK293T cells were infected with the plasmid mix containing sgRNA (lentiCRISPRv2), psPAX2, and pMD2G plasmids (4:2:1). PEI transfection agent was applied to the cell as five μ l to one μ g DNA. Plasmids were prepared in PBS in an eppendorf and incubated for five minutes after a short vortex. Then PEI agent was added to the eppendorf and incubated for 20-minute at room temperature. Finally, the plasmid and PEI mix was applied to the cell. Cells were grown in the incubator in the BSL2 laboratory. After 48 h, the medium was collected, and a new medium was applied. After 24 hours, the medium was collected again. Viruses were kept at -80 °C until infection. Viruses were filtered with a 0.22 μ m filter, and SW620 cells were infected at a ratio of 1:10. To raise the infection efficiency, polybrene was applied with a final concentration of 8 μ g.

3.4.3.16.4. Selection of single cells

Two days after infection, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) dye, and cells that express a green fluorescent protein (GFP)⁺ were differentiated by Fluorescence-Activated Cell Sorting (FACS) analysis to select the single-cell. FACS analysis was performed by an expert at Izmir Biomedicine and Genome Center, Flow Cytometry Support Unit. Single cells were first seeded in a 96-well plate. Then, colony formation from a single cell was imagined with Cell Metric™. Clones proliferating from a single cell were plated in 24-well, 12-well, 6-wells and then in a Petri dish, respectively (Giuliano et al., 2019). To demonstrate successful silencing of the IDH1 gene, the protein level was determined by the Western Blot method, and the mRNA level was determined by the RT-qPCR method (Figure 10). FlowJo program was used for flow cytometry data analysis and figure preparation.

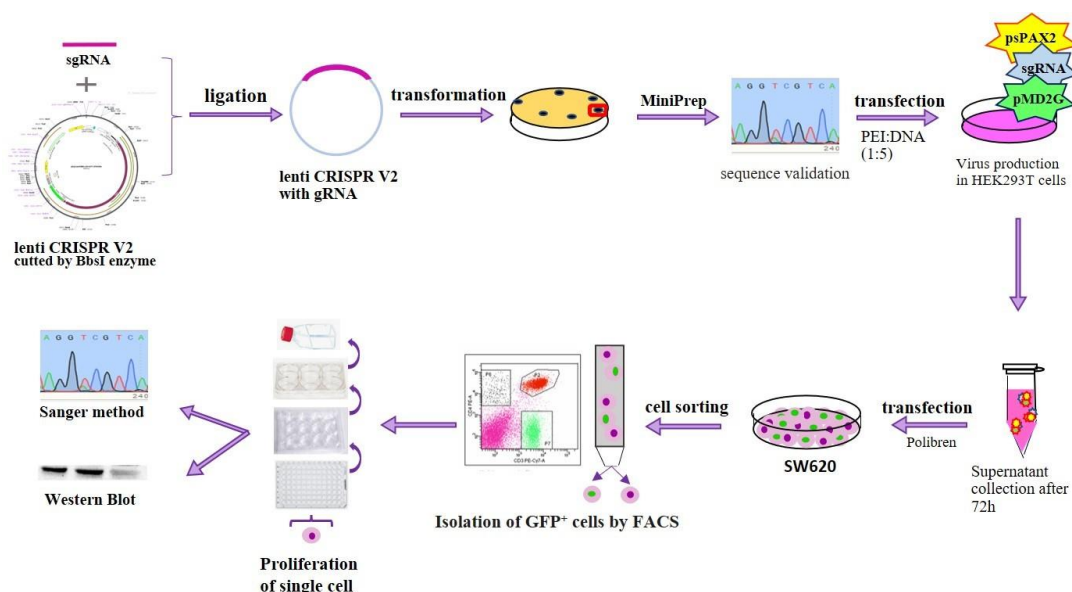


Figure 10: The schematic presentation of the knockout of the IDH1 gene by using the lentiviral vector by the CRISPR/Cas9 method.

3.4.3.17. Silencing of the wt IDH1 enzyme using mammalian expression vector CRISPR/Cas9

3.4.3.17.1. sgRNA design

Two different sgRNAs listed in Table 7 were used.

3.4.3.17.2. Transfection of sgRNAs

sgRNAs were cloned into a pX458 vector which can express Cas9 protein and contains GFP-tag. The vector was first cut with the BbsI enzyme. sgRNAs were phosphorylated by the T4 PNK enzyme (Table 8). Next, ligation of the obtained oligos and 50 ng of pX458 plasmid cut with the BbsI enzyme was carried out for two hours at room temperature using the T4 ligase enzyme. To transform the ligation product into competent *E. coli* bacteria (Stbl3) by heat-shock method, 50 μ l of bacteria and 50 ng of plasmid (all the ligation products) are mixed and kept in ice for 30 minutes. It is then incubated for 90 seconds at 42°C and 2 minutes on ice, respectively. After incubation, the bacteria were grown in 1 ml of LB Broth for one hour at 37 °C. Then, it is centrifuged at 11000 g, and the pellet is spread on LB agar by pipetting. After approximately 16 hours, three clones formed on the agar are selected with a pipette tip, taken into LB Broth (containing 0.1 mg/ml Ampicillin), and incubated at 225 rpm at 37 °C for approximately 18

hours. At the end of the period, plasmid isolation was performed following the kit procedure, and sequence analysis was performed on the isolated plasmids using the Sanger method. According to the Sanger results, a plasmid without mutation was selected to use in the infection step.

3.4.3.17.3. Selection of clones after FACS analysis

Cells were stained with DAPI two days after infection. GFP⁺ cells were differentiated by FACS analysis, and cells were selected individually. FACS analysis was performed by an expert at Izmir Biomedicine and Genome Center, Flow Cytometry Support Unit. Single cells were first seeded in a 96-well plate. Then, it was followed for 14 days with an inverted microscope in which only one cell was seeded in each well. Clones proliferating from a single cell were plated in 24-well, 12-well, 6-well, and then in a Petri dish, respectively (Giuliano et al., 2019). To demonstrate successful silencing of the IDH1 gene, the protein level was determined by the Western Blot method, and the mRNA level was determined by the RT-qPCR method (Figure 11). FlowJo program was used for flow cytometry data analysis and figure preparation.

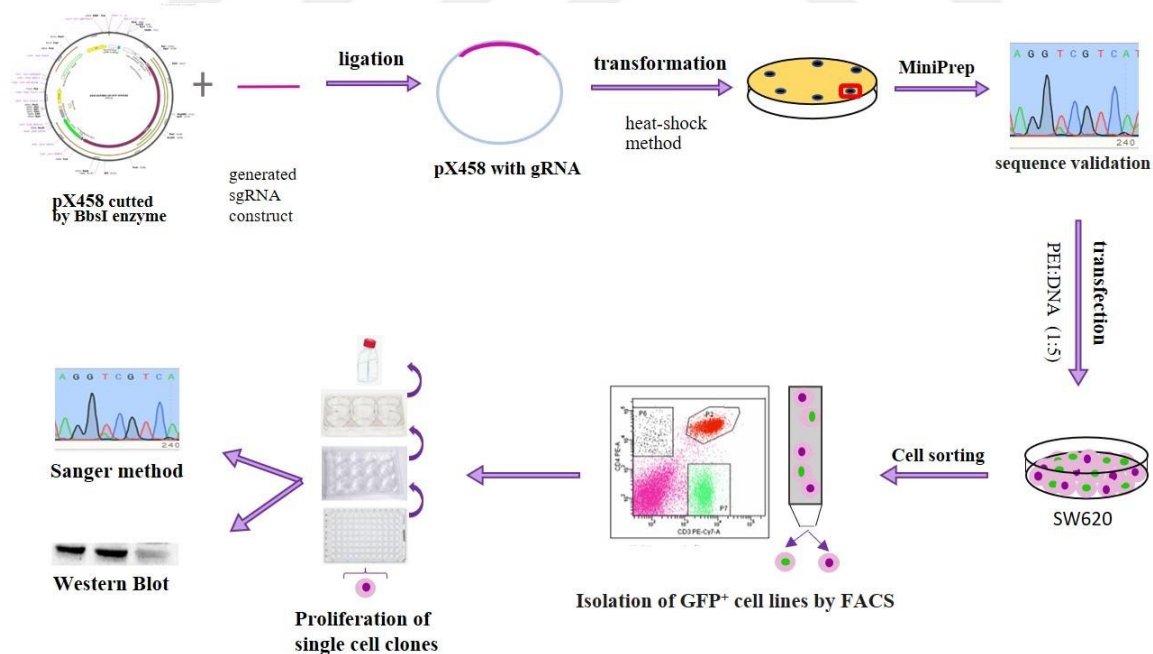


Figure 11: The schematic presentation of the knockout of the IDH1 gene by using the mammalian expression vector by the CRISPR/Cas9 method.

3.4.3.18. Generation of the R132H mutation in the SW620 cell line by CRISPR/Cas9 method

3.4.3.18.1. In vitro transcription of sgRNA

The selected sgRNAs were amplified by overlapping PCR containing (Table 10). The three primers are common to all reactions. The fourth primer containing the sgRNA sequence is gene-specific and contains a T7 promoter region (Li et al., 2017). IVT (in vitro transcription) reaction was performed with overlapping PCR product and T7 polymerase enzyme (Table 11). IVT reaction was stopped by DNase I treatment for 10 min at 37°C (Table 12). The synthesized RNA sequences were purified with the RNA Clean & Concentrator-5. The purity and amount of the obtained RNA were determined by Nanodrop.

Table 10. The conditions of overlapping PCR

	amount (μl)
5X Buffer	5
10 μM Reverse primer ML611	2,5
10 μM gene specific forward primer	2,5
100 μM Reverse primer ML 558	2,5
100 μM Forward primer ML 557	2,5
DMSO	0,75
2 mM dNTPs	2,5
Q5 polymerase	0,25
nuclease-free ddH ₂ O	6,5
total	25

conditions				
	35 cycle			
time	10 sn	30 sn	20 sn	2 min
temp	98°C	60°C	72°C	72°C

Table 11. The conditions of IVT reaction

	amount (μl)
10X Reaction Buffer	1,5
100 mM NTP each	1,5 x 4
T7 Polymerase	1,5
Template DNA	1000 ng

nuclease-free ddH ₂ O	
Total	20
incubation overnight at 37°C	

Table 12. The conditions of DNase I treatment

	amount (μl)
10X Buffer	5
RNA (1 μg)	20
DNase I	0,5
nuclease-free ddH ₂ O	24,5
total	50
incubation 10 min at 37°C	

3.4.3.18.2. ssDNA synthesis (HDR template)

The sequence containing the target point mutation (R132H) was designed on the Horizon Discovery website. This sequence was also designed to contain the cleavage site of the BclI enzyme. The extramers for each sgRNA were synthesized by the Triogen Biotechnology Company.

3.4.3.18.3. Preparation of RNP (Ribonucleoprotein) complex

RNP complexes were prepared in high salt buffer (580 mM potassium chloride (KCl), 40 mM Tris-HCl pH 7.5, 20% v/v glycerol, two mM TCEP-HCl pH 7.5, two mM MgCl₂) just before electroporation. 100 pmol of Cas9 protein and 130 pmol of sgRNA were added to the RNP buffer and incubated at 70°C for 5 min. Then 100 pmol of ssDNA was added and incubated at 37°C for 10 minutes to form the RNP complex. Water was added until the final volume reached 10 μL.

3.4.3.18.4. Nucleofection

200 ng/ml Nacodazole was applied to SW620 cells 24 hours before electroporation. At the end of the period, the RNP complex was given to 200 thousand cells by electroporation following the instructions of the 4D-Nucleofector (Lonza) kit. Nucleofection was performed on the Nucleofector instrument (Lonza). To calculate the transfection efficiency, the pmaxGFPTM

vector was given to the cells. After electroporation, cells were seeded into two wells in a 24-well plate.

3.4.3.18.5. Differentiation of cells

Cells containing GFP-tag in the first well were identified by flow analysis. FlowJo program was used for flow cytometry data analysis and figure preparation. Cells in the second well were seeded into a 96-well plate with 0.5 cells in each well. It was followed for 14 days with a cell display device (solentim cell metric), in which only one cell was seeded in each well. Five clones proliferating from a single cell were selected and proliferated in 24-well, 6-well plates, and then in a T25 flask, respectively. The cleavage and Sanger method were applied to identify the clones containing the R132H mutation. Firstly, DNA isolation was carried out with the DNeasy Blood & Tissue Kit (Qiagen) from five selected clones. The target region amplified by PCR was sequenced by the Sanger method. The primers used in the PCR were listed in Table 13. Secondly, the PCR products were cut by the BclI enzyme at 37 °C for three hours, and it was run at 90V for 50 minutes, and the image was taken on the Bio-Rad device (Li et al., 2017) (Figure 13).

Table 13. Primers were designed for the IDH1 gene.

Enzyme	Forward (5'- 3')	Reverse (5'- 3')
IDH1	GATGCCACCAACGACCAAGTC	GGGATCCTATTGTGCAGCCAG

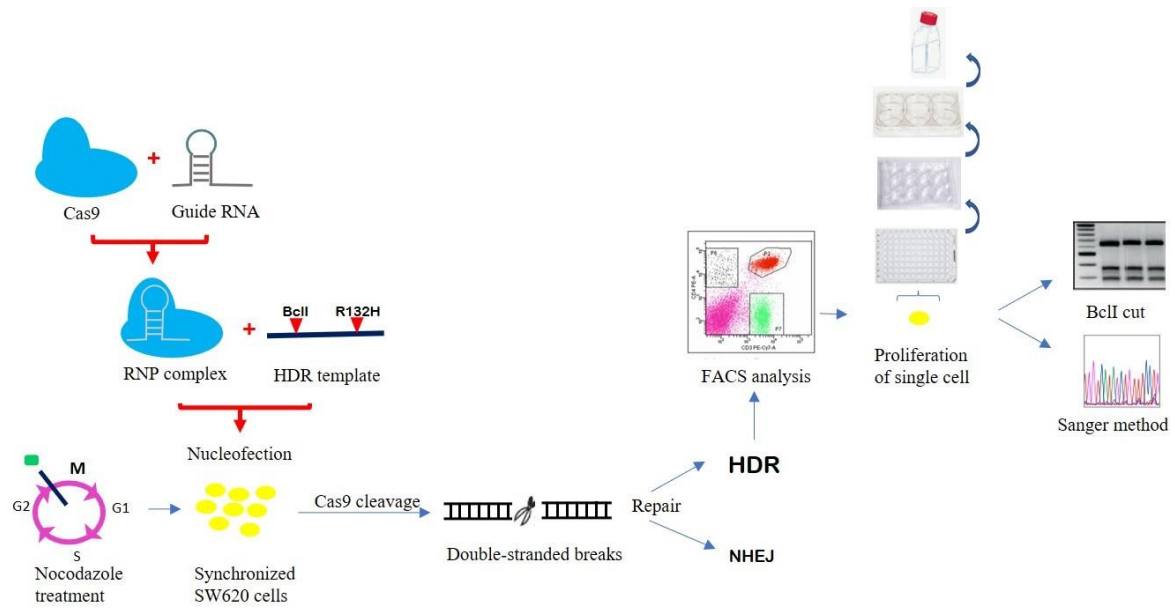


Figure 12: The schematic presentation of the generation of R132H mutation in the SW620 cell line by the CRISPR/Cas9 method.

3.4.3.19. Colony formation assay

Firstly, cell numbers were optimized for colony formation capacity. 5×10^4 , 10×10^4 , 5×10^5 , and 1×10^6 cells were seeded in a 6-well plate/per well and incubated for 14 days in RPMI with 10% FBS. Clones were fixed with 4% formaldehyde for 20 minutes. The PBS-washed clones were stained with 0.1% crystal violet for 30 minutes. After washing with distilled water, clones were counted using a stereomicroscope and analyzed by ImageJ software (Franken et al., 2006).

3.4.3.20. Cell Proliferation

For cell proliferation studies, 1×10^6 cells were seeded per 100mm diameter dish. After 24, 48, 72, and 96 h, cells in a Petri dish were collected and counted using Thoma lame under a light microscope.

3.4.3.21. Migration Assay (Wound Healing)

To analyze migration capacity, 3×10^5 cells were seeded in the 12-well plate. 24 hours later, a linear wound was created by scratching the surface of the per well by using a yellow

pipette tip and incubating in RPMI with % 1 FBS. The wounds were imaged, and their distance was measured using an inverted microscope.

3.4.3.22. Invasion Assay

The invasion abilities of cells were evaluated using a QCM™ 24-Well Cell Invasion Assay (Chemicon). 25×10^4 cells were seeded into the upper chamber (collagen-coated polycarbonate membrane-based) with media containing 0.1% FBS. The lower chamber (24-well plates, 8- μ m pore size, Corning) was filled with the growth medium with 10% FBS. After 24 hours, invasive cells that degrade the collagen and pass through the polycarbonate membrane and adhere to the lower surface of the membrane were incubated with the Cell Detachment Solution included in the kit for 30 minutes at 37°C and separated from the membrane. Cells were stained with CyQuant GR Dye diluted in 4X Lysis Buffer. Fluorometric measurement was performed with a 480/520 nm filter in 96-well black plates.

3.4.3.23. Cell Cycle

Cells (5×10^5) were seeded in each well in a 6-well plate. The medium was collected after 24 hours of incubation, cells were rinsed with one ml of cold PBS, and the PBS solution was collected in the same falcon. Cells are trypsinized and harvested into the same falcon. After 5 minutes of centrifugation at 800 rpm, the cell pellet is rinsed with five ml of cold PBS and centrifuged again. The cell pellet was resuspended in one ml PBS and four ml 100% ethanol, then incubated overnight at -20 °C. At the end of time, the pellet was thoroughly homogenized by vortexing at 1500 rpm for two minutes, followed by centrifugation at 1200 rpm for 10 minutes. The cell pellet is washed with five ml of ice-cold PBS and centrifuged at 800 rpm for five minutes. The cell pellet was resuspended in 200 μ l of PBS containing 1% Triton X100 and 10 μ l of RNAase A (10 ng/ml), and then 20 μ l of PI (one mg/ml in PBS) were added. After incubation for 45 minutes at room temperature, flow analysis was performed (Subasi et al., 2020).

3.4.3.24. Apoptosis analysis

The Alexa FluorR488 Annexin V/Dead Cell Apoptosis (Invitrogen) kit was used to identify cells that tended to apoptosis after silencing the IDH1 gene. 5×10^5 cells were seeded in a 6-well plate. The medium was collected after 24 hours of incubation; cells were rinsed with

one ml of PBS and collected in the same falcon. Cells are trypsinized and harvested into the same falcon. After centrifugation at 800 rpm for five minutes, the cell pellet is washed with one ml of cold PBS and centrifuged again at 800 rpm for five minutes. The cell pellet is resuspended with 500 µl of 1X Binding Buffer, and four samples are prepared. Dye was not added to the first tube. Only five µl of PI dye was added to the second tube. Only five µl of Annexin V to the third tube. Only five µl each of PI and Annexin V dye to the fourth tube. After five minutes of incubation at room temperature, flow analysis was performed.

3.5. Statistical Analysis

The results were collected from an experiment carried out at least three biological and three technical repeats. To conduct statistical analyses, GraphPad Prism 8.0 was used (GraphPad Software, CA, USA). GraphPad Prism 8.0 (GraphPad Software, CA, USA) was used for statistical analyzes. One-way or two-way ANOVA was used to compare the three groups' differences, and then Tukey's multiple comparisons test was performed. An asterisk (*) is shown on the graph to denote statistical significance ($p < 0.05$).

4. RESULT

4.1. Intracellular Metabolite Levels in the Cell Lines

The thesis study examined intracellular and extracellular levels of some essential metabolites in colon cancer cell lines and the results were compared with a colon epithelial cell line.

The first part of energy generation begins with reducing glucose to pyruvate. The intracellular glucose level was 1149.6 $\mu\text{mol/mg}$ in the healthy cell line, while it was determined as 620.6 and 476.3 $\mu\text{mol/mg}$ in the Caco-2 and SW620 colon adenocarcinoma cell lines, respectively. The pyruvate synthesized as the end product in the glycolysis pathway is converted to Acetyl CoA by the pyruvate dehydrogenase (PDC) enzyme complex and participates in the TCA cycle. Intracellular pyruvate levels were 22.9 and 17.2 $\mu\text{mol/mg}$ in Caco-2 and SW620, respectively ($p < 0.05$) (Figure 13A).

The TCA cycle begins by adding two carbon atoms from acetyl-CoA to oxaloacetate. The intracellular level of citrate was 46.8, 187.2, and 150.2 $\mu\text{mol/mg}$ in CCD-18Co, Caco-2, and SW620, respectively ($p < 0.05$). In the next step, isocitrate is oxidized to α -KG by the first oxidative decarboxylation reaction. As seen in Figure 13A, the intracellular α -KG level was 6.8 in CCD-18Co, 12.3 and 26.0 $\mu\text{mol/mg}$ in Caco-2 and SW620, respectively. Significant results were obtained for the level of D-2HG converted from α -KG by the wt or mutant IDH 1/2 enzymes. The D-2HG oncometabolite was not found in the control group (CCD-18Co). However, it was observed that 22.6 and 152.6 $\mu\text{mol/mg}$ of intracellular D-2HG metabolite were generated in Caco-2 and SW620, respectively. The intracellular level of succinate obtained by the second oxidative decarboxylation reaction of the α -KG metabolite was 39.6, 137.9, and 104.2 $\mu\text{mol/mg}$ in CCD-18Co, Caco-2, and SW620, respectively. Succinate is converted to fumarate by succinate dehydrogenase. Intracellular fumarate levels were 1.8 and 2.6 $\mu\text{mol/mg}$ in Caco-2 and SW620 cell lines, respectively. The level of intracellular malate synthesized from fumarate was 4.3 $\mu\text{mol/mg}$ in CCD-18Co, while it was 16.5 and 22.6 $\mu\text{mol/mg}$ in Caco-2 and SW620, respectively. Intracellular oxaloacetate levels obtained by the third oxidative decarboxylation reaction in the last step of the TCA cycle were 6.1, 13.0, and 38.9 $\mu\text{mol/mg}$ in CCD-18Co, Caco-2, and SW620, respectively (Figure 13A). The time-dependent glucose level is lower in Caco-2, and SW620 compared to CCD-18Co at 24-96 h (Figure 13B).

4.2. Extracellular Metabolite Levels in the Cell Lines

The time-dependent metabolic changes in the extracellular level were determined in the conditioned mediums after 24-96 hours.

Colon cancer cells consumed high glucose, whereas were released less pyruvate, D-2HG, and all TCA cycle metabolites into the medium compared to CCD-18Co at 24 h. As is seen in Figure 13C, the extracellular glucose level was similar in all the cell lines. The extracellular lactate was 224.8, and 173.7 nmol/ml Caco-2 and SW620, respectively. The extracellular pyruvate, citrate, α -KG, succinate, fumarate, malate, and oxaloacetate levels were 1.5, 2.0, 3.6, 1.4, 4.0, 1.7, 2.3 times decreased in primary colon cell lines, and 1.8, 3.0, 9.4, 2.0, 1.8, 1.6, 3.1 times decreased ($p < 0.05$) in metastatic colon cell lines according to colon epithelial cell lines, respectively (Figure 13C). Cancer cell lines have been shown to metabolize glucose to the TCA cycle. Unlike these metabolites, the D-2HG level was 3.2 and 11.4-times raised in Caco-2 and SW620 compared to CCD-18Co, respectively ($p < 0.01$) (Figure 13C).

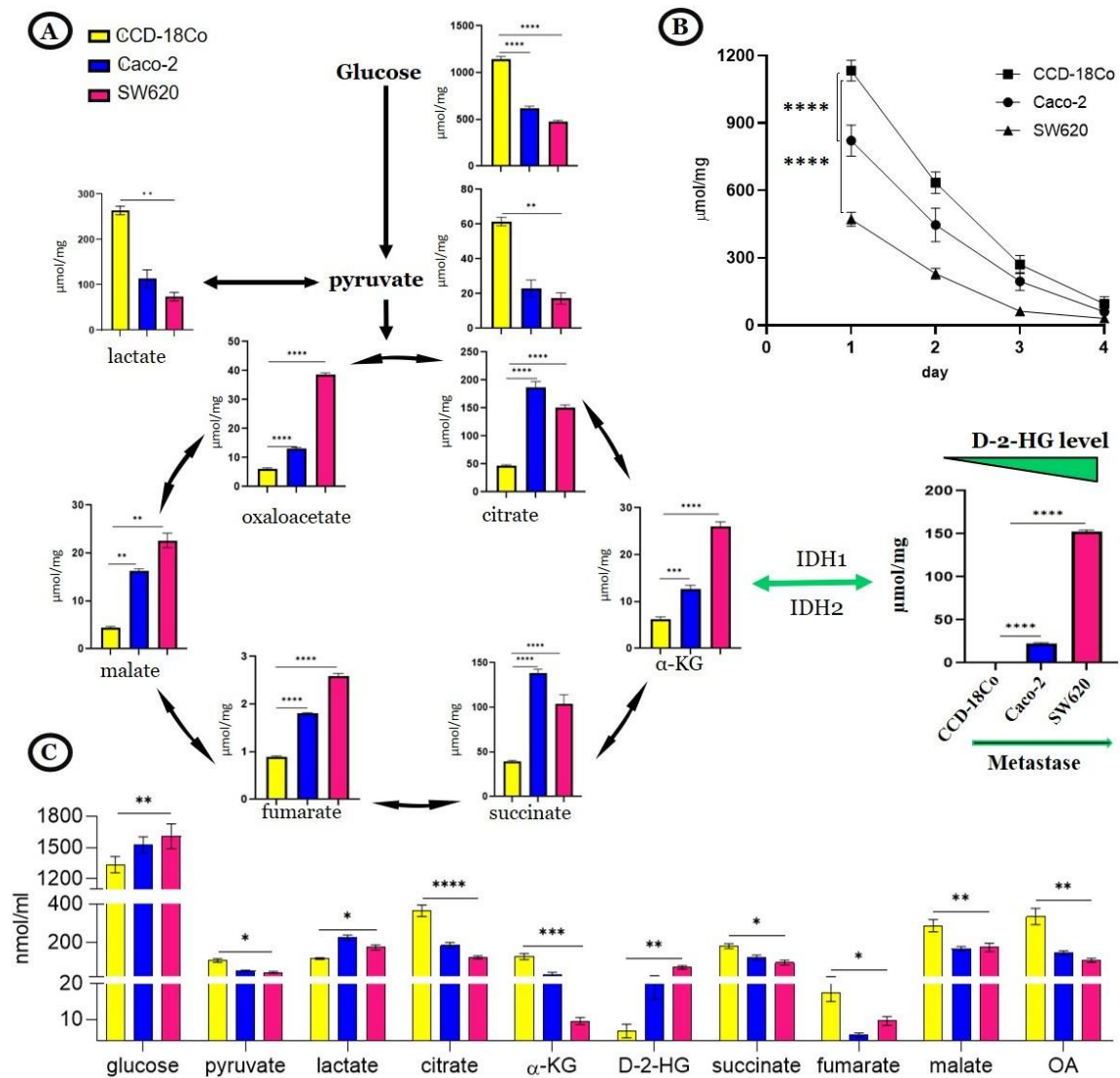


Figure 13: Intracellular (A) and extracellular (C) metabolite levels in colon cancer cell lines.

Time-dependent glucose levels (B). Abbreviations: ns, not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

In the time-dependent extracellular metabolite levels at 24-96th hours, glucose is consumed, and pyruvate and TCA cycle intermediates are released in decreasing amounts over time in all cell lines (Figure 14A-C). Concerning the intracellular level, pyruvate and TCA cycle intermediates were less released in adenocarcinoma cell lines than in healthy colon cells. It was also observed that SW620 consumed more glucose and released less TCA cycle intermediate than Caco-2 (Figure 14 B-C). In conclusion, glucose is used to synthesize the TCA cycle intermediates which are then gradually released into the medium.

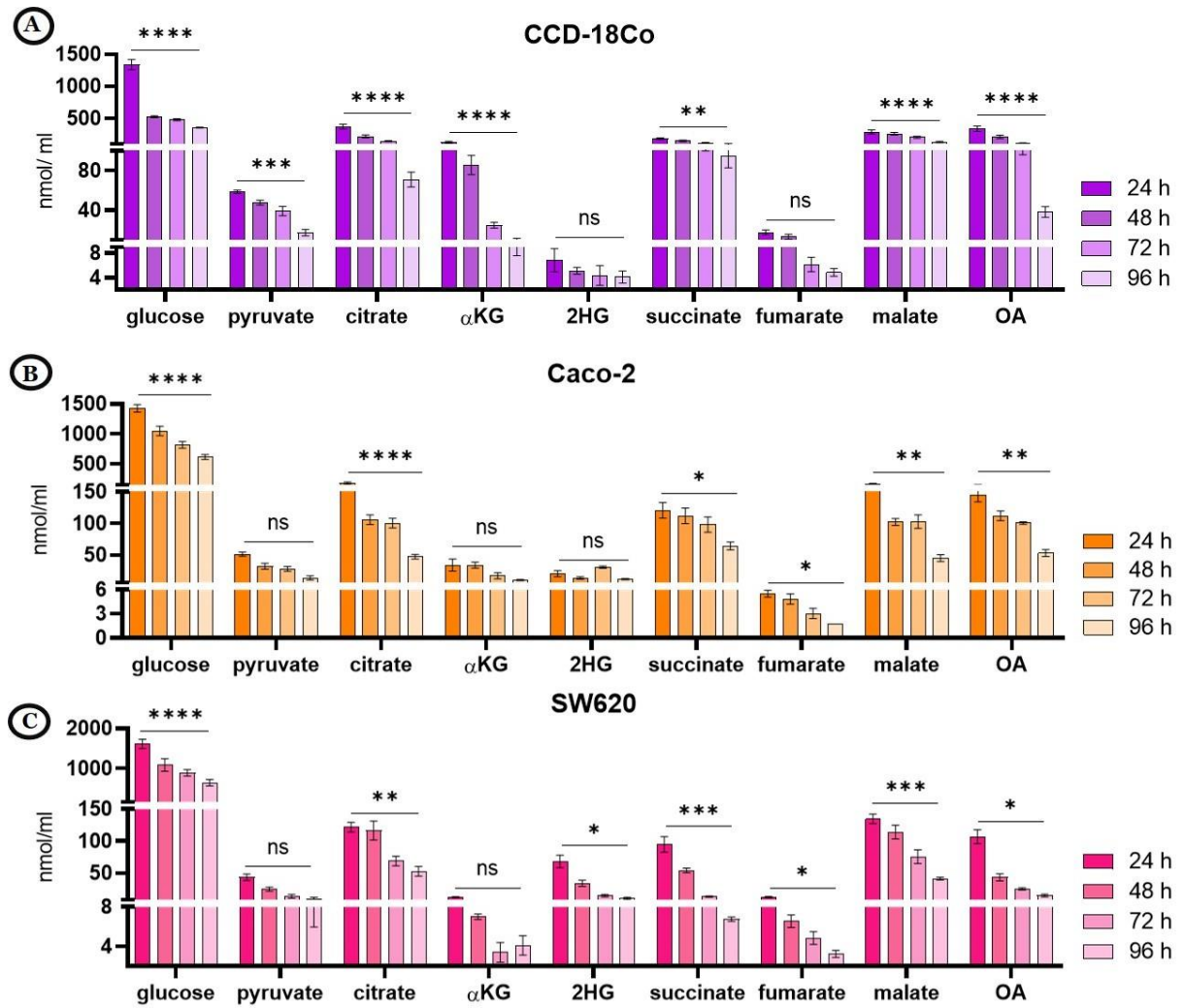


Figure 14: Time-dependent extracellular metabolite levels.

Extracellular metabolite levels of CCD-18Co (A), Caco-2 (B), and SW620 (C) at 24-96 h. Abbreviations: ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.3. Development of xenograft models and H&E staining

After important results were obtained on the cell lines, the intermediate levels were investigated in the xenograft models. The cells in the CCD-18Co tissue were found to be lobular-solitary. Very few cells go through mitosis, and very modest polymorphism has been seen. This tissue that does not have tumor cell features is called healthy tissue (Figure 15A). Caco-2 cells were multilobular localized in tumor tissues, and the lobules have borders around

them (shown in yellow arrow). Tumor cells were discovered to have a substantial cytoplasm–nucleus difference and have mitotic activity (mitotic cells are indicated by a white arrow) (Figure 15B-D). In SW480 and SW620 tumor tissues, tumor parenchyma in island forms was found around blood arteries. In these tissues anisocytosis, anisonucleosis, and moderately vigorous mitotic activity were observed (Figure 15C-D). The red arrow shows the capsule surrounding the tumor tissue (Figure 15D).

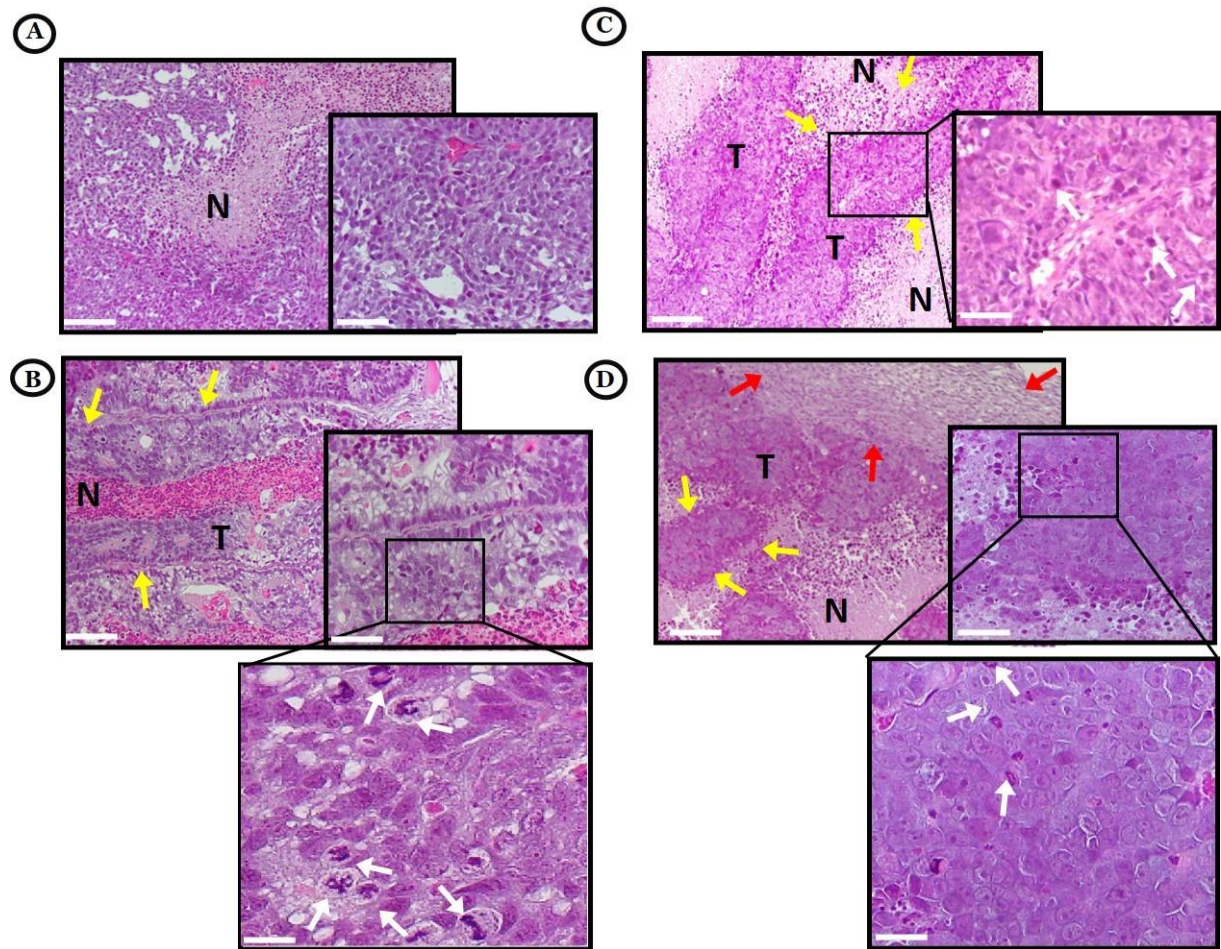


Figure 15: Histopathology results of tumor tissues.

H&E staining results of CCD-18Co (A), Caco-2 (B), SW480 (C), and SW620 (D) tumor tissues. In the upper panels some parts (marked boxes) were enlargement in the lower panels. Borders, mitotic cells, and the capsule were shown in yellow, white, and red arrows, respectively. The scale bar is 200 μ m. Abbreviations: N necrosis and T tumor part.

4.4. Investigation of Some Metabolite Concentrations of Energy Metabolism in Xenograft Models

The metabolite levels were examined in the tumor, brain, liver, and colon tissues of xenograft models (Figure 16A-B; Figure 17 A-B). The glucose levels were not changed in all brain tissues. In the colon, liver, and tumor tissues, glucose was highly consumed in all the xenograft models generated with colon cancer cells (Figure 16 and Figure 17). The D-2HG was not found in the CCD-18Co brain and colon tissues. Significantly it was generated in Caco-2, SW480, and SW620 brain tissue at 15.4, 32.1, and 57.4 ppm, respectively (Figure 16A). In the colon tissues, D-2HG is generated in Caco-2 SW480 and SW620 colon tissues at 7.7, 11.2, and 16.6 ppm, respectively (Figure 16 B). In the tumor tissues, D-2HG is generated in Caco-2, SW480, and SW620 at 54.8, 115.2, and 148.8 ppm, respectively (Figure 17A).

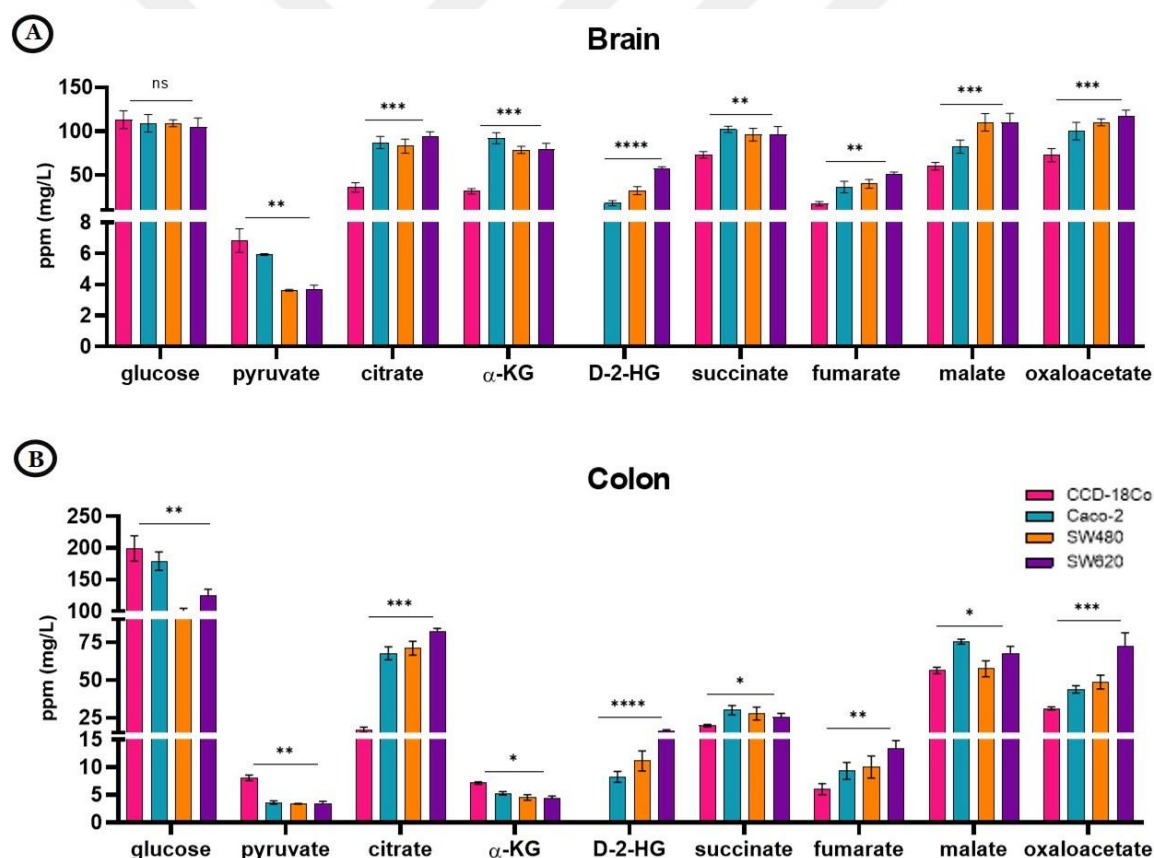


Figure 16: Metabolite levels in the brain (A) and colon (B) tissues.

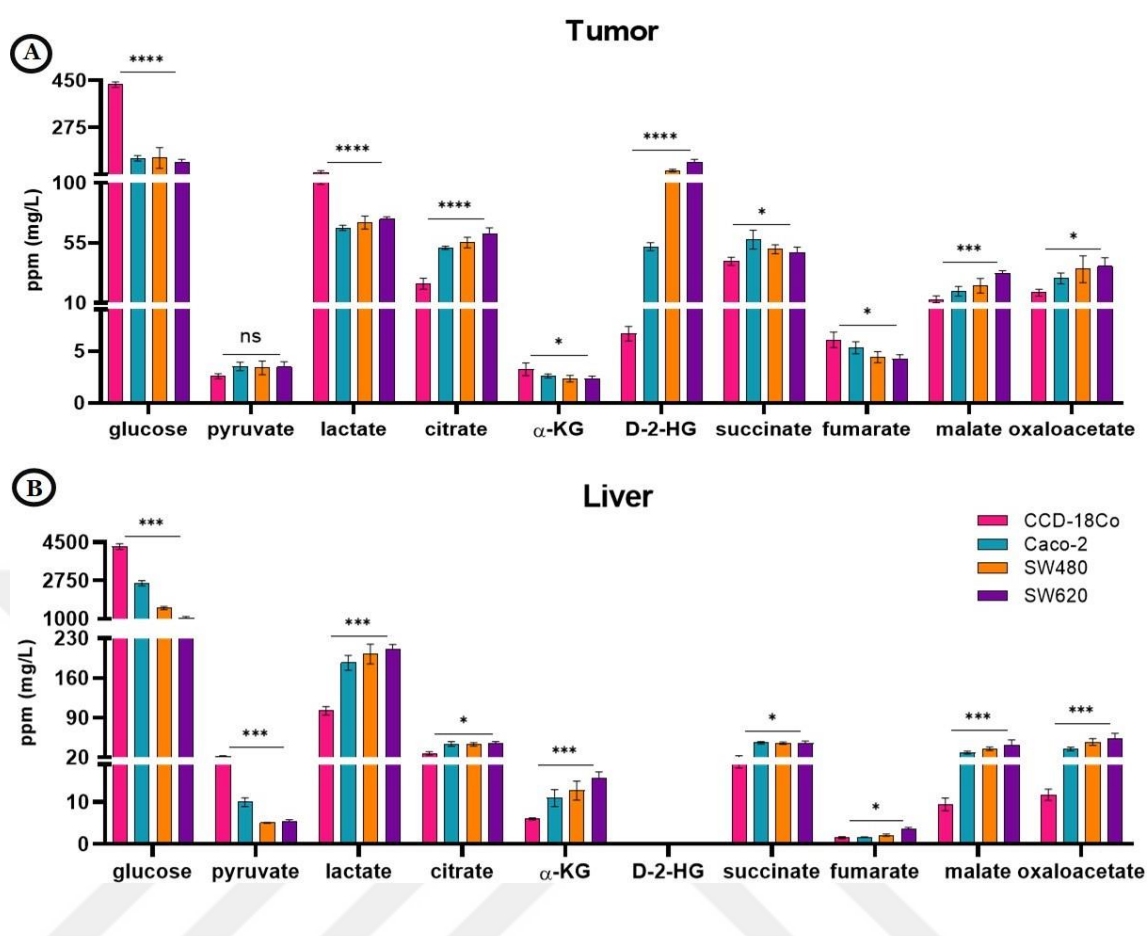


Figure 17: Metabolite levels in the liver (A) and tumor (B) from tissues.

The results of other metabolites were listed in Table 14 as fold changes relative to the control group.

Table 14. Metabolite results in the tissues of xenograft models as fold change relative to the control group

tissue	cell line	glucose	pyruvate	lactate	citrate	α-KG	succinate	fumarate	malate	OA
brain	Caco-2	1.0	1.2	-	2.3	2.8	1.4	2.1	1.3	1.3
	SW480	1.0	2.1	-	2.2	2.4	1.3	2.4	1.8	1.4
	SW620	1.1	1.9	-	2.5	2.4	1.3	3	2.2	1.6
colon	Caco-2	1.1	1.2	-	3.8	0.7	1.6	1.6	1.4	1.7
	SW480	2.0	1.1	-	3.9	0.7	1.4	1.5	1.1	3.5
	SW620	1.6	1.3	-	4.5	0.6	1.3	1.8	1.2	5.7
tumor	Caco-2	2.9	1.4	1.6	3.6	0.7	1.5	1.2	2.2	2.6
	SW480	2.9	1.3	1.5	3.9	0.7	1.9	1.4	2.1	2.1
	SW620	3.2	1.3	1.5	4.3	0.8	2.2	2.2	2.1	1.9

liver	Caco-2	1.7	2.4	1.9	1.5	1.9	2.8	2.7	3.0	3.0
	SW480	2.0	4.5	1.9	1.6	2.1	4.1	3.3	3.7	4.0
	SW620	4.6	3.9	2.1	1.7	2.5	4.1	5.2	4.4	4.5

4.5. Investigation of IDH1 and IDH2 Gene Sequences in SW620 Cell Line by Sanger Method

When the intracellular and extracellular metabolite levels were evaluated *in vitro* and *in vivo*, significant results were obtained at the D-2HG level. Although this oncometabolite could not be generated in the CCD-18Co, it was determined at a very high level in SW620. Therefore, the pathways in which the D-2HG level was generated in the SW620 cell line were investigated in the next step.

Mutant IDH enzymes catalyze α -KG reduction to D-2HG. Mutations occurring in the catalytic region of the IDH enzyme are specific to the cancer type. In colon cancer, mutations occur in the R132 and G97 regions of the IDH1 and the R140 and R170 regions of the IDH2 gene (Colvin et al., 2016; Grassian et al., 2012). In SW620 cells, these regions were examined by Sanger sequencing, and no mutations were detected in the IDH1 and IDH2 genes (Figure 18A-B).

4.6. Determination of mRNA Levels of the IDH1 and IDH2 Genes using *in vitro* and *in vivo* Models

The studies show that the wt IDH genes have a role in D-2HG generation independently of mutant IDH genes in some cancer types (Ježek, 2020). Furthermore, bioinformatics studies have determined that the imbalance of the expression of IDH1/2 genes in colon cancer plays a role in D-2HG generation (Koseki et al., 2015). Therefore, to determine whether the differences in expression levels of these genes affect D-2HG generation, the IDH1/2 mRNA levels were examined *in vitro* and *in vivo* models. CCD-18Co was the control group, and GAPDH was used as the control gene.

The IDH1 gene expression was raised in Caco2 and decreased in SW620 according to control. The mRNA level of IDH2 was reduced in the early stage of cancer and is highly expressed in the metastatic cells. The α -KG level was raised by 2.1-fold and the D-2HG level was approximately 7 times raised in the SW620 cell line according to Caco-2. Considering that α -KG is reduced to D-2HG, it is expected to decrease in SW620. However, highly expressed

IDH2 enzyme in SW620 can cause elevated α -KG levels (Figure 18C). Then, the mRNA levels of these enzymes were determined in mice models. As is seen in Figure 18D, IDH1 results were similar *in vitro*. The IDH2 gene expression was raised in Caco-2 and decreased in SW620 compared to CCD-18Co tissue. (Figure 18D).

When the *in vivo* and *in vitro* RT-qPCR findings were evaluated, it was concluded that significant differences in the mRNA level of IDH1 and IDH2 genes lead to D-2HG generation. Since the wt IDH1 gene plays a more active role in the D-2HG generation than the IDH2 gene in colon cancer, the thesis study was continued by silencing the IDH1 gene using the CRISPR/Cas9 method.

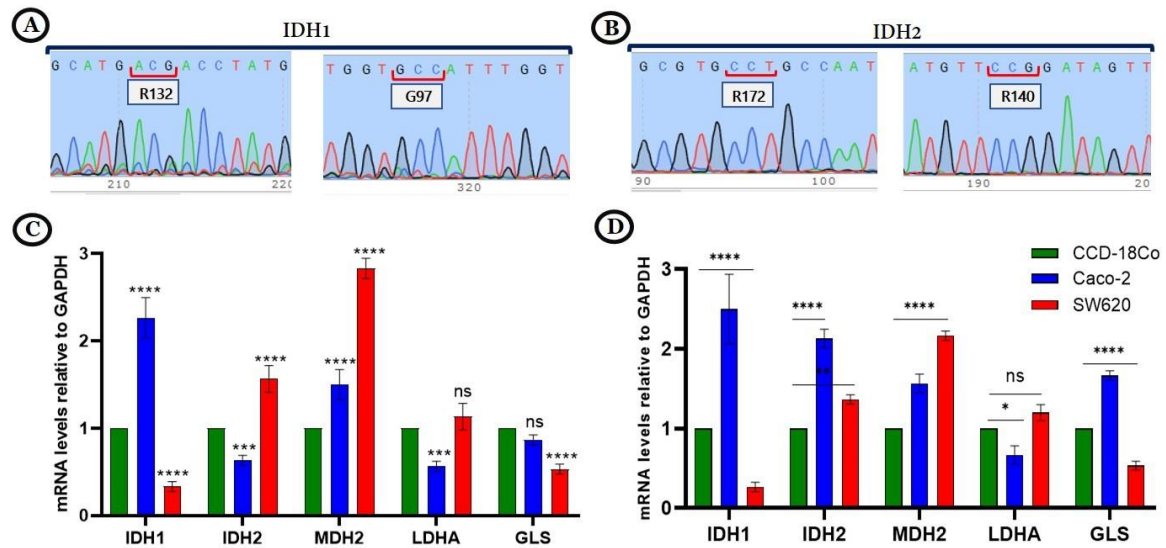


Figure 18: The results of the Sanger method and RT-qPCR analyses.

The Arginine residues in the IDH1 (A) and IDH2 (B) genes were sequenced. mRNA levels of genes were determined in cell lines (C) and xenograft model tissues (D). GAPDH was control.

Abbreviations: ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.7. Silencing of the IDH1 Gene by CRISPR/Cas9 Method

The CRISPR/Cas9 method was carried out with two different vectors, the mammalian (pX458) and the lentiviral (lentiCRISPRv2) vector. The two sgRNAs (sgRNA1 and sgRNA2) were selected from the Brunella library for the fourth exon of the IDH1 gene. After transfection of sgRNA-containing pX458 and lentiCRISPRv2 vectors into cells separately, colony

formation from a single cell was imaged for 14 days with Solentim Cell Metric™ (Figure 19 A; Figure 20A). The protein levels of IDH1 in eight different colonies in sgRNA1 and six different colonies in sgRNA2 were determined using the Western blot analysis. When the pX458 vector was used, the protein level of IDH1 was significantly reduced in two clones (Figure 19B). These clones were named IDH1 KO-1 and IDH1 KO-2. First, when the lentiCRISPRv2 vector was used, the bulk population was analyzed in both sgRNAs. The decreased IDH1 protein level was determined in the sgRNA2, and single-cell clones proliferated from this sgRNA. The protein level of IDH1 was significantly reduced in two clones, IDH1 KO-3 and IDH1 KO-4 (Figure 20B).

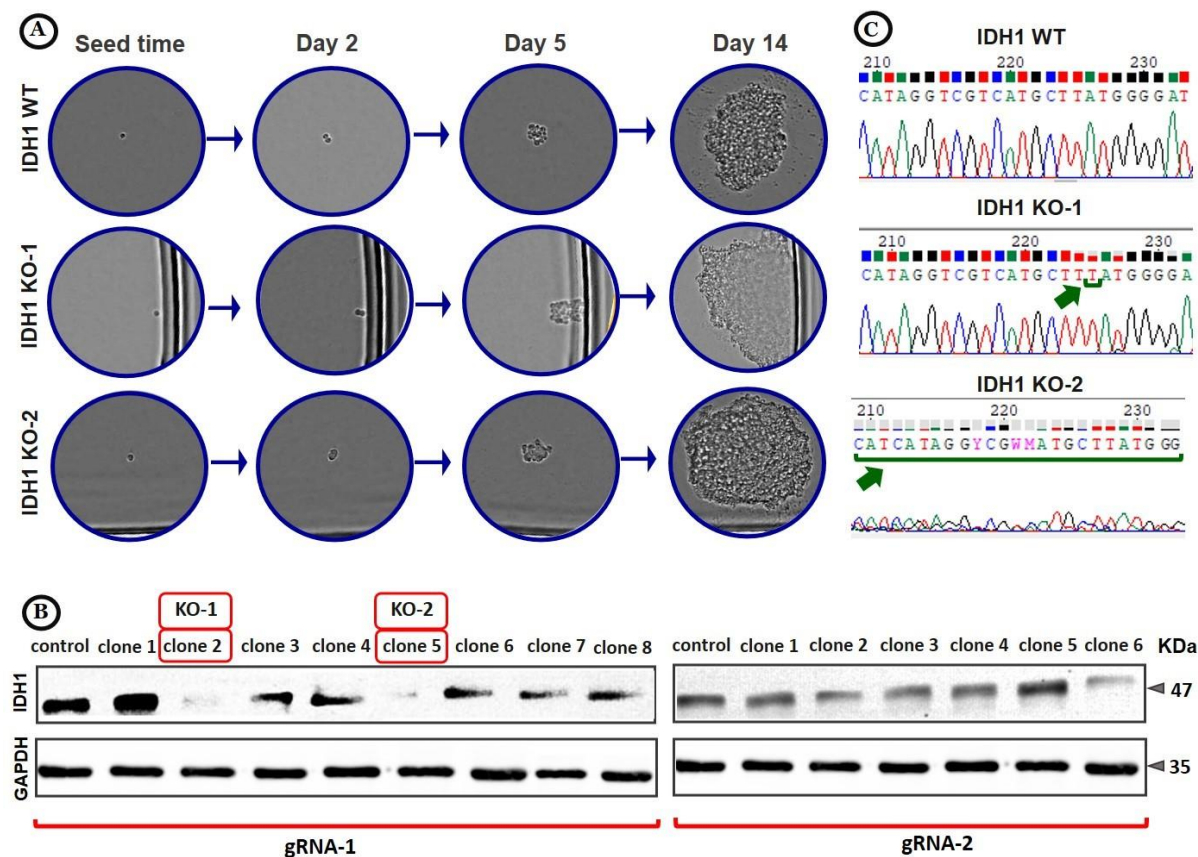


Figure 19: Silencing of the IDH1 gene by using the pX458 vector.

The IDH1 gene was sequenced in IDH1 KO1-4 clones by the Sanger method and the results were evaluated with Mutation Surveyor Software. Duplication T in IDH1 KO-1, heterozygous deletion (AAATATCCCCCGGCTTGTGAGTGGATGGGT) in IDH1 KO-2

(Figure 19C), heterozygous deletion (TCGTCA) in IDH1 KO-3, and heterozygous deletion (A) in IDH1 KO-4 were determined (Figure 20C).

In addition to Sanger sequencing, mRNA levels of IDH1 and IDH2 genes were examined after IDH1 silencing. While the mRNA level of the IDH1 gene was significantly decreased in IDH1 KO1-4 compared to the control, no significant difference was observed in the IDH2 gene in IDH1 KO1-2 (Figure 20D; Figure 23C).

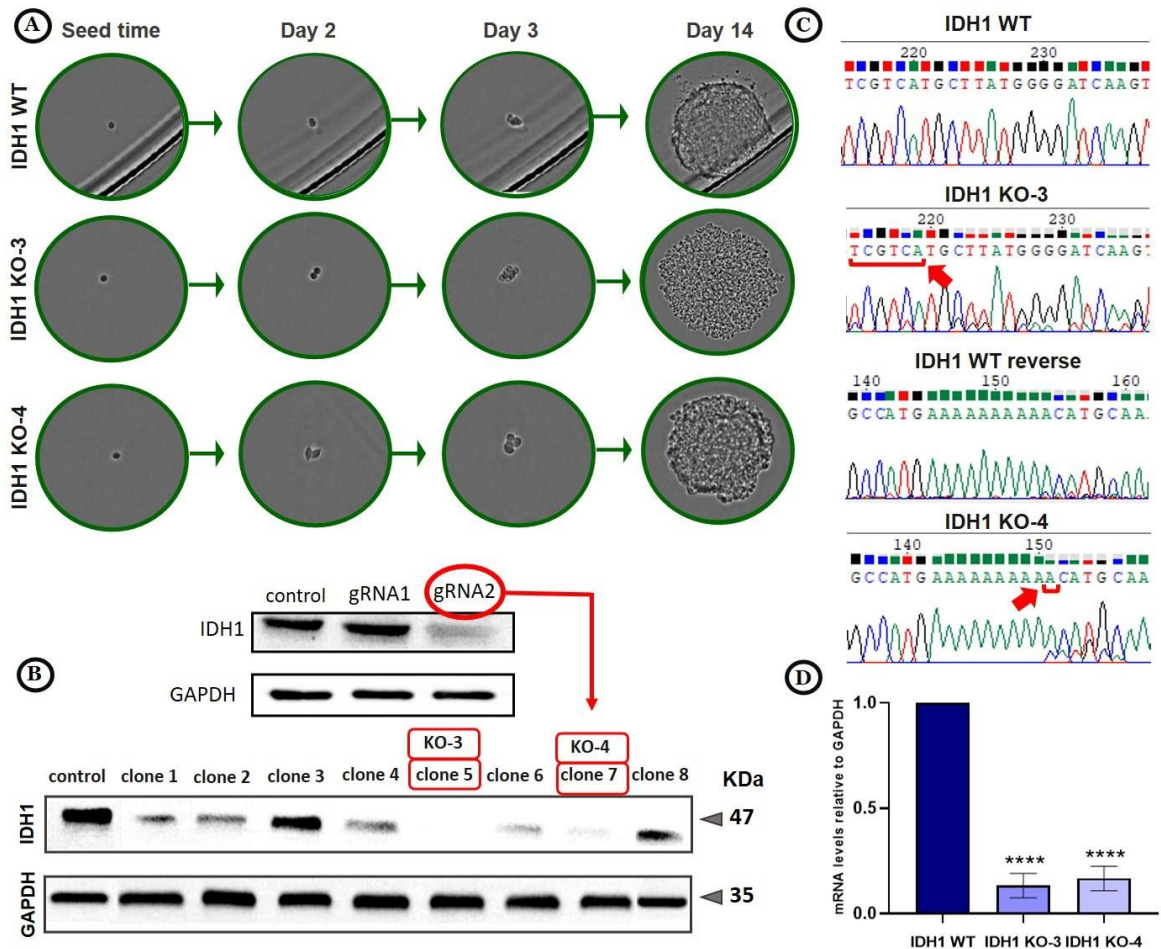


Figure 20: Silencing of the IDH1 gene by using the lentiCRISPRv2 vector.

After transfection, colony formation from a single cell was imaged for 14 days with Solentim Cell Metric™ (A). The protein levels of IDH1 in 8 different colonies were determined using the Western blot analysis in sgRNA2 (B). The IDH1 gene was sequenced in IDH1 KO3-4 (C). mRNA levels of IDH1 genes in IDH1 KO3-4 (D). GAPDH was control. Abbreviations: ns, not significant. *** $p < 0.001$, **** $p < 0.0001$

4.8. Investigation of the IDH1 Role in the Colon Cancer Progression

Various methods were used to investigate how IDH1 perturbing affects colon cancer progression.

4.8.1. The effect of IDH1 knockout in morphology, proliferation, colony formation, invasion, and migration of colon cancer cell line

To determine the changes in the cell morphology, 250 thousand cells were seeded in a 24-well plate, and after incubation, they were examined with an inverted microscope. The IDH1 WT cells have both rounded and fibroblast-like (red arrow) morphology. Important changes in cellular morphology were observed in IDH1 KO cells. They gained only rounded morphology (Figure 21A; Figure 22A).

In order to determine whether the IDH1 gene expression affects proliferation, one million cells were seeded in four different Petri dishes. At the end of 24-96 hours, cells were counted. The cell number was 1.4 and 1.5-fold decreased in IDH1 KO-1 and IDH1 KO-2 compared to the control at the end of the fourth day, respectively (Figure 21B). Similarly, the cell number in IDH1 KO-3 and IDH1 KO-4 were 1.5 and 1.6 times reduced according to the control group, respectively (Figure 22B). Silencing the IDH1 gene significantly inhibited cell proliferation.

To determine colony formation abilities, 100 thousand cells were seeded on a 6-well plate, and the colonies were counted after 14 days. The number of colonies formed by IDH1 KO1-2 cells decreased to 71.5% and 65.5%, respectively (Figure 21C). Similarly, the number of colonies formed by IDH1 KO 3-4 cells decreased to 55.8% and 60.8%, respectively (Figure 24C). As shown, knockout of the IDH1 gene reduces colony formation.

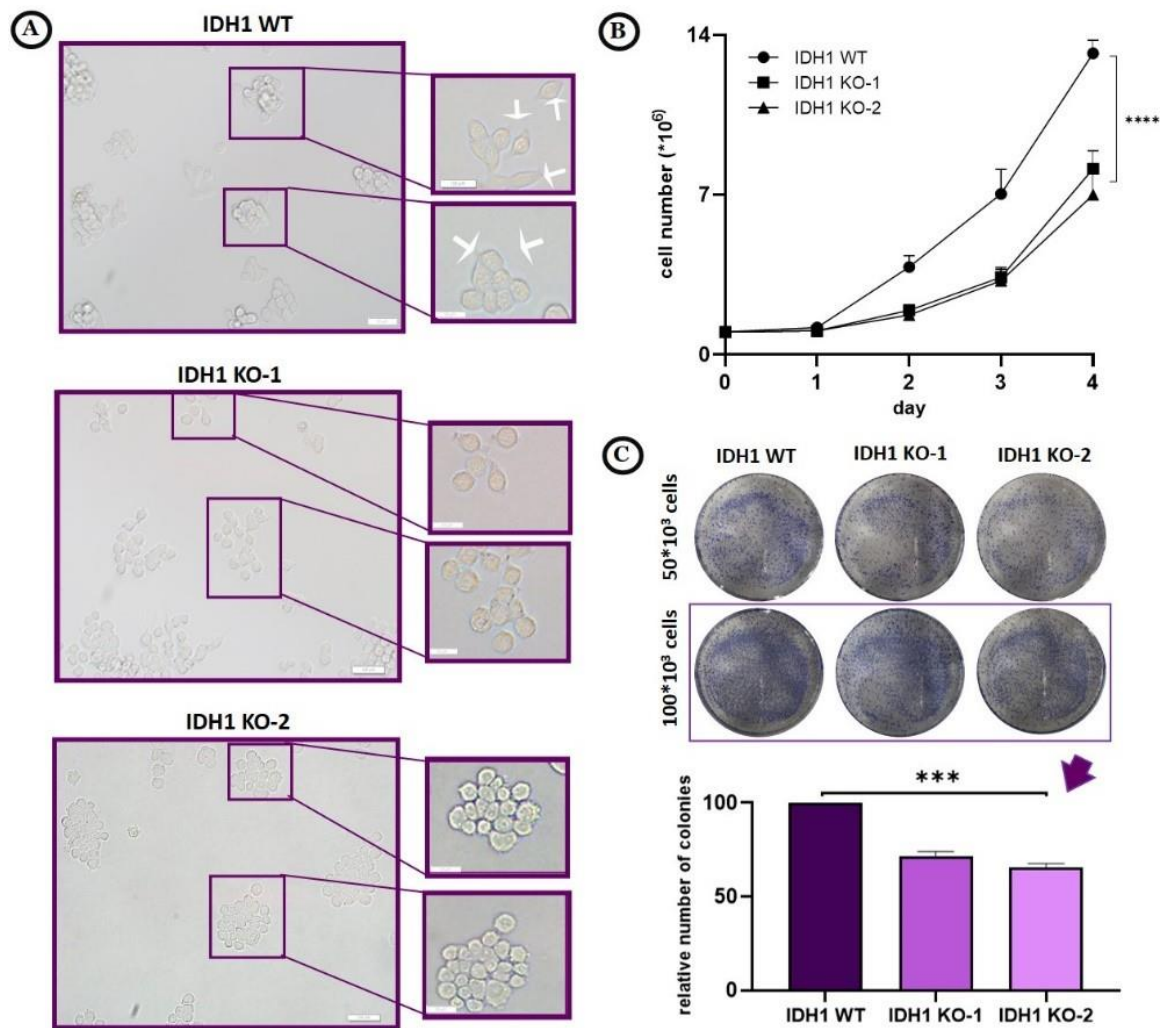


Figure 21: The results of morphological imaging, proliferation, and colony formation capacities of IDH1 WT and IDH1 KO 1-2 cells.

The morphological imaging of cells was analyzed with inverted microscopy with 10X and 20X (A). The effect of IDH1 knockout on cell proliferation during four days (B). Colony formation ability after IDH1 knockout was performed by counting colonies stained with crystal violet (C). The scale bar is 200 μm . *** $p < 0.001$, **** $p < 0.0001$

The invasion abilities of IDH1 WT and IDH1 KO 3-4 cells were evaluated using a transwell assay kit. Stained cells invaded from the top chamber in the lower chambers were detected with a fluorometric measurement, and invasion rates were determined relative to the control group. It was observed that the invasion abilities of IDH1 KO-3 and IDH1 KO-4 cells were 2.4 and 2.0-fold decreased compared to the control (Figure 22C).

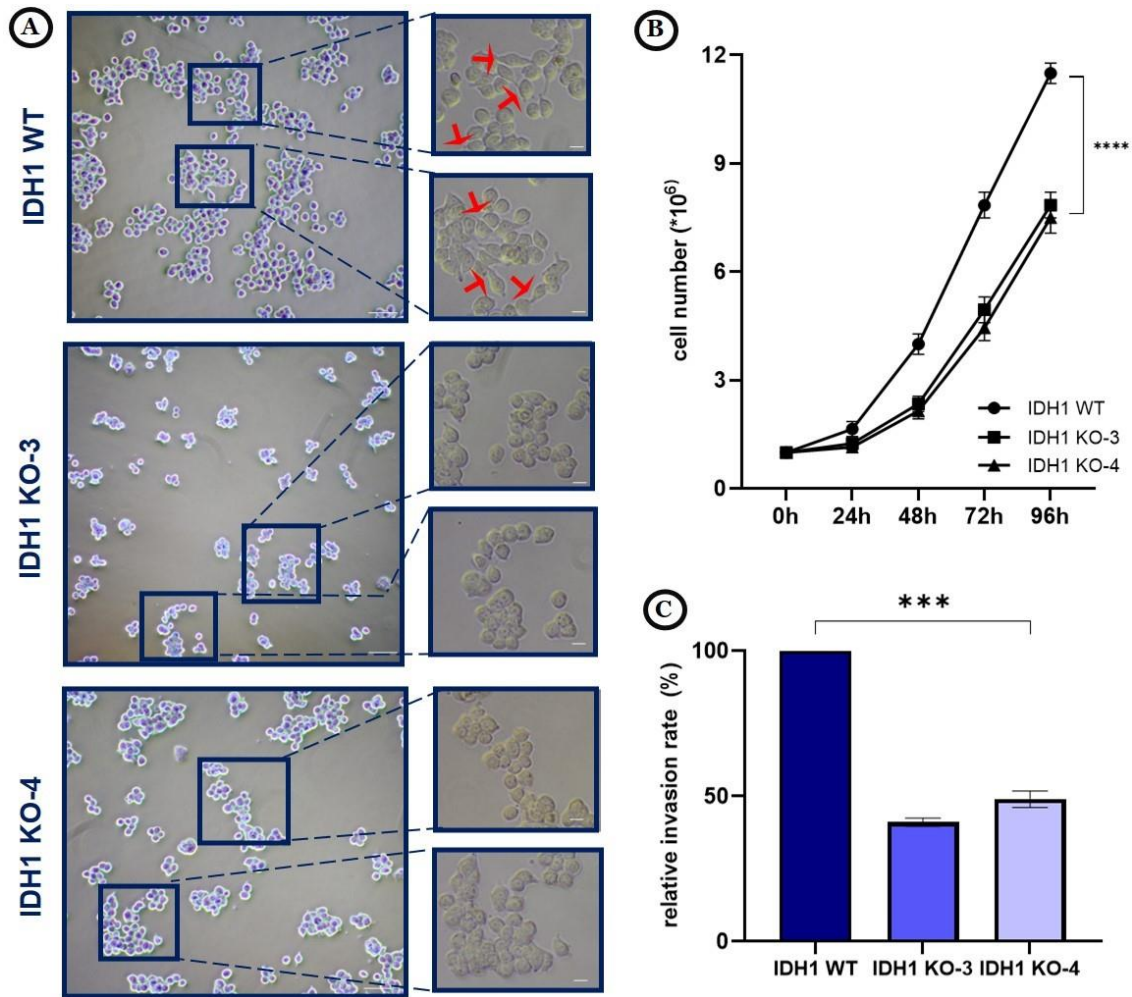


Figure 22: The results of morphological imaging, proliferation, and invasion abilities of IDH1 WT and IDH1 KO 3-4 cells.

The morphological imaging of cells was analyzed with inverted microscopy with 10X and 20X (A). The effect of IDH1 knockout on cell proliferation during four days (B). The invasion abilities of IDH1 WT and IDH1 KO 3-4 cells were evaluated using a transwell assay. At the end of 24 hours, the cells invaded from the top chamber in the lower chambers were detected, and invasion rates were determined relative to the control group (C). The scale bar is 200 μ m.

*** p<0.001, **** p<0.0001

To evaluate the migration abilities, a linear wound was created. The images were taken, and distances were measured with inverted microscopy for four days. Migration abilities of

IDH1 KO cells were decreased compared to control. At the end of the fourth day in IDH1 KO-1 and IDH1 KO-2 colonies, the distance was 21% and 20.2%, respectively, while the distance was 11.3% in the control cell. (Figure 23 A-B). Similarly, at the end of the fourth day, the distance in IDH1 KO-3 and IDH1 KO-4 colonies is 19.8% and 18.6%, respectively, while this distance is 11.2% in the control cell (Figure 24A-B).

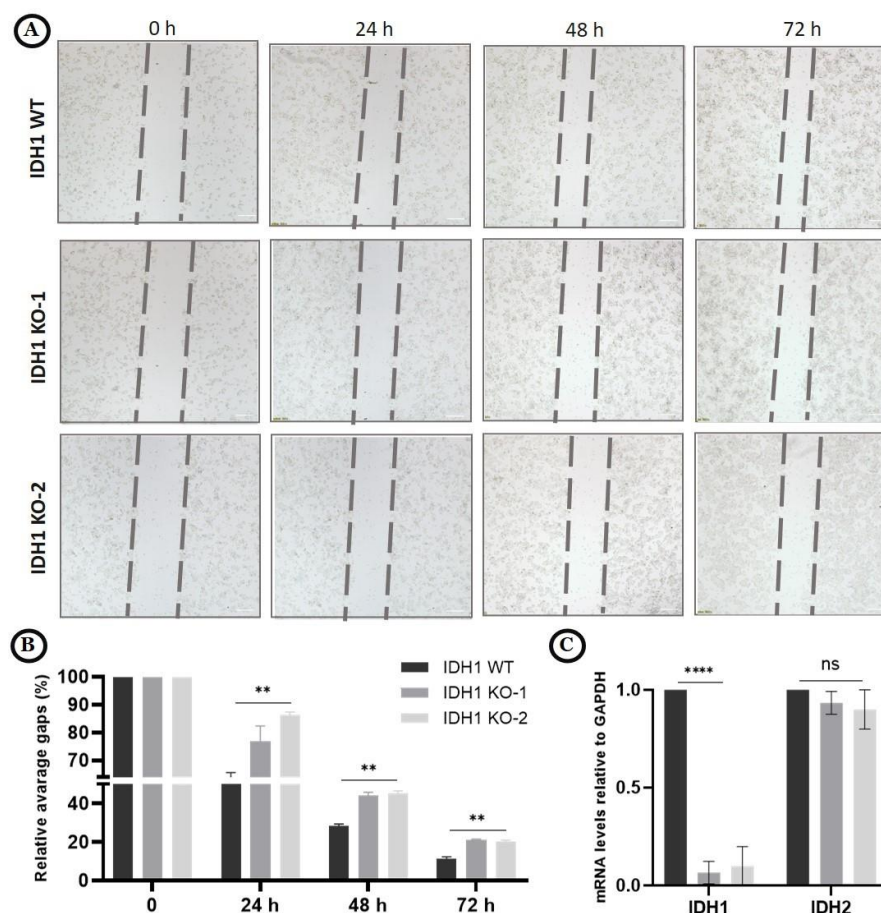


Figure 23: Cell migration analysis and mRNA levels of IDH1 and IDH2 genes after IDH1 knockout.

The wound-healing assay was used to determine cell migration ability in IDH1 WT and IDH1 KO 1-2 cells (A). The remaining gap was then measured using light microscopy and was quantified relative to the control group (B). mRNA levels of IDH1/2 genes in IDH1 KO1-2(C). GAPDH was control. Abbreviations: ns, not significant. **** $p < 0.0001$

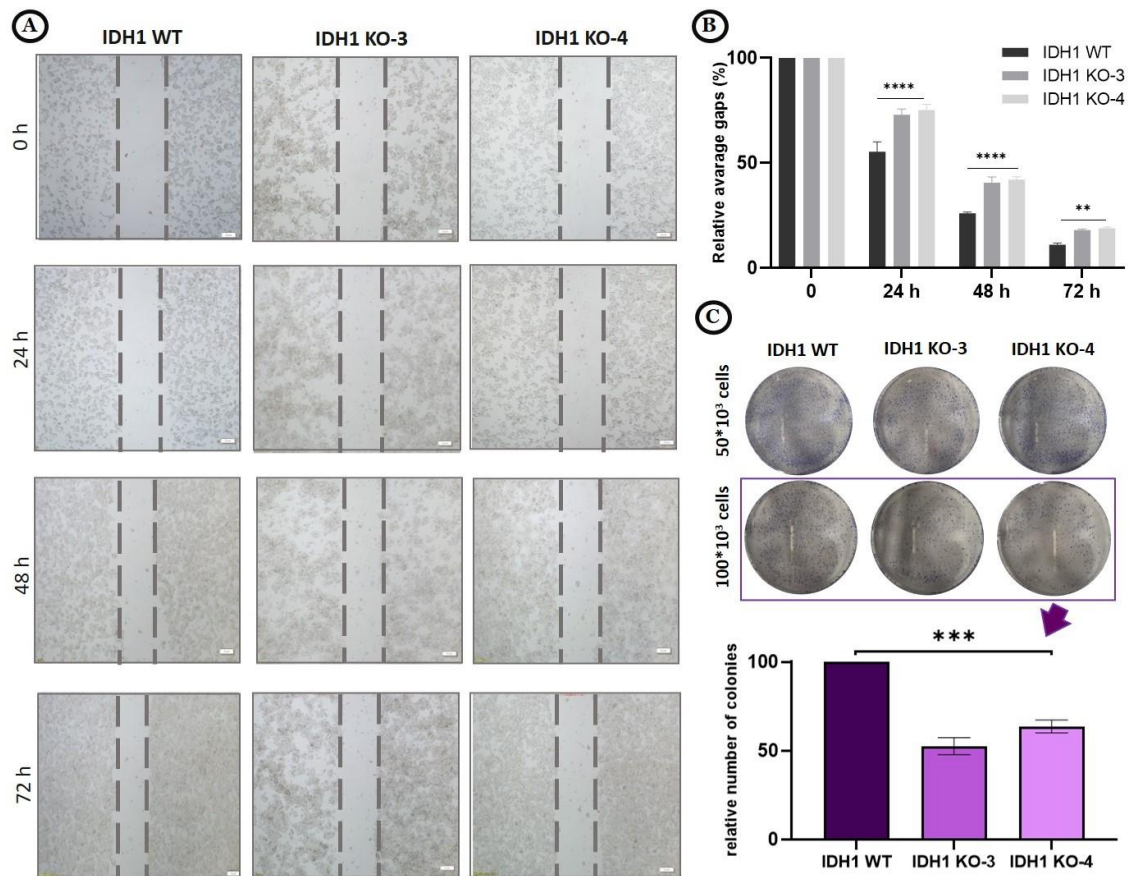


Figure 24: Cell migration and colony formation capacities of IDH1 WT and IDH1 KO 3-4 cells.

The wound-healing assay was used to analyze the cell migration abilities of cells after IDH1 knockout(A). The average gaps were determined relative to the control group (B). Colony formation ability after IDH1 knockout was evaluated by counting colonies stained with crystal violet (C).

To determine the effect of IDH1 knockout on apoptosis, cells were stained with Annexin V and PI dyes, and the results were analyzed with the FlowJo program. The Q1 and Q2 parts show the number of cells in necrosis or late apoptosis and early apoptosis, respectively. The number of cells undergoing apoptosis did not change in IDH1 KO3-4 colonies compared to the control (Figure 25 A-D).

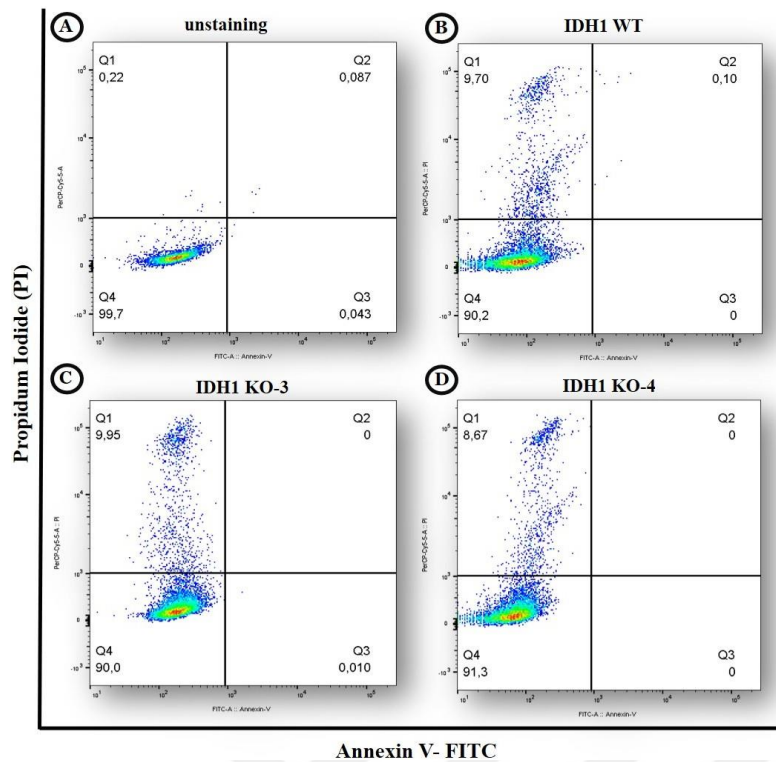


Figure 25: Determine the apoptotic cell number after IDH1 knockout.

The necrosis and apoptosis numbers of unstained (A), IDH1 WT (B), and IDH1 KO3-4 (C, D) cells were evaluated by using the Alexa FluorR488 Annexin V/Dead Cell Apoptosis kit. Q1 (late apoptosis): cells were stained with PI and Annexin V-FITC, and Q2 (early apoptosis): cells were stained with Annexin V-FITC.

To analyze the effect of IDH1 gene silencing in the cell cycle distributions, cells were stained with PI dyes. The number of cells in each phase of cell number was determined by flow analysis. Figure 26 shows that while the cell density kept in the G phase was 55% in the control group, it raised to 72% and 73% in IDH1 KO-3 and IDH1 KO-4 colonies, respectively. Concerning that, while the cell density in the S phase was 20% in the control group, it decreased to 9% and 11% in IDH1 KO-3 and IDH1 KO-4 colonies, respectively. Similarly, while the cell density in the G2/M phase was 25% in the control group, it decreased to 18% and 17%, respectively, in IDH1 KO-3 and IDH1 KO-4 colonies (Figure 26). It is shown that knockout of the IDH1 gene leads to G-phase arrest.

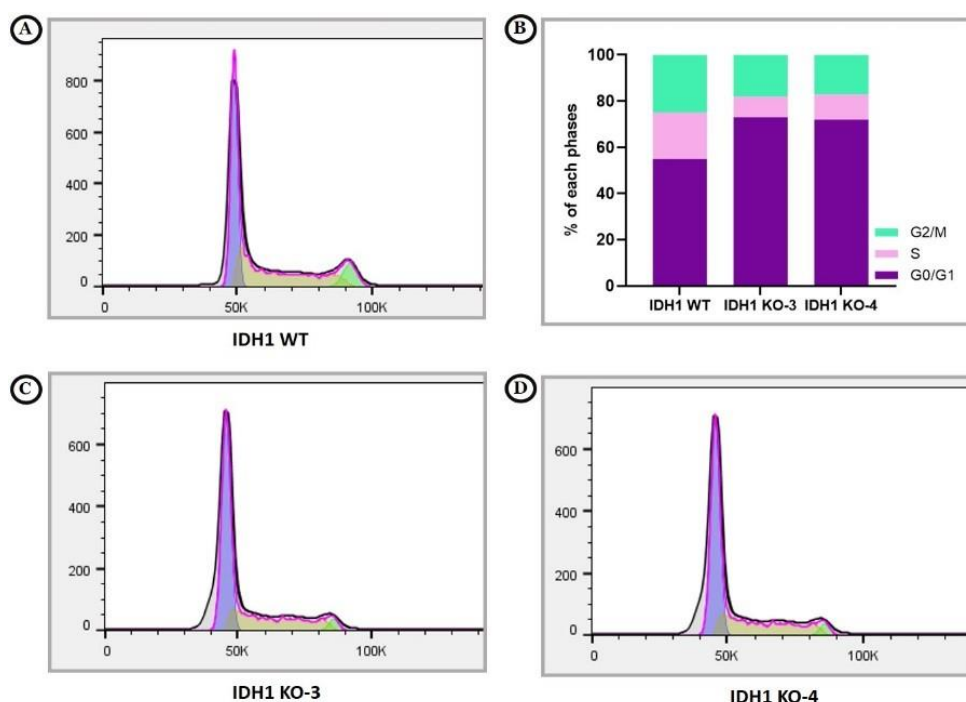


Figure 26: The analysis of cell cycle distributions after IDH1 knockout.

Cell cycle analysis was carried out in IDH1 WT (A) and IDH1 KO3-4 (C, D) cells after PI staining. The number of each phase (B).

4.8.2. The effect of IDH1 knockout on the colon cancer metabolism

The intracellular and extracellular concentrations of several metabolites of energy metabolism in 1 million cells were profiled to investigate the metabolic changes in the IDH1 KO clones. The intracellular glucose level was 538,4 and 571,7 $\mu\text{mol}/\text{mg}$ in the IDH1 KO-1 and IDH1 KO-2, while it was determined as 446,8 $\mu\text{mol}/\text{mg}$ at 24 h, respectively (Figure 27 A). In the time-dependent change, IDH1 KO clones used more glucose than IDH1 WT during the time (Figure 27 A). Intracellular pyruvate and malate levels were decreased and the intracellular citrate, α -KG, succinate, and oxaloacetate levels were raised in IDH1 KO clones compared to control (Figure 27 C). The intracellular fumarate levels were not significantly changed in IDH1 KO clones (Figure 27 C). Above all, the intracellular D-2HG level was 2.4 and 2.7 times reduced in IDH1 KO1 and IDH1 KO2 according to IDH1 WT (Figure 27C). The rise in intracellular α -KG and succinate levels after IDH1 silencing was consistent with a

decrease in D-2HG levels. The decrease in the intracellular malate and raise in the oxaloacetate level in IDH1 KO clones was related to the mRNA level of MDH2 enzyme (Figure 29 A).

The consumed glucose level was 1248,9 and 1198,9 nmol/ml in the IDH1 KO-1 and IDH1 KO-2, while it was determined as 1865,9 nmol/ml at 24 h, respectively (Figure 27 B). The extracellular glucose level was less consumed in IDH1 KO clones than in IDH1 WT along 24-96 h (Figure 27 B). The extracellular citrate, D-2HG, malate, and oxaloacetate levels were less released whereas the extracellular succinate levels were more released after IDH1 perturbing according to control (Figure 27 D). The extracellular pyruvate, α -KG, and fumarate levels were not significantly changed in IDH1 KO clones (Figure 27D).

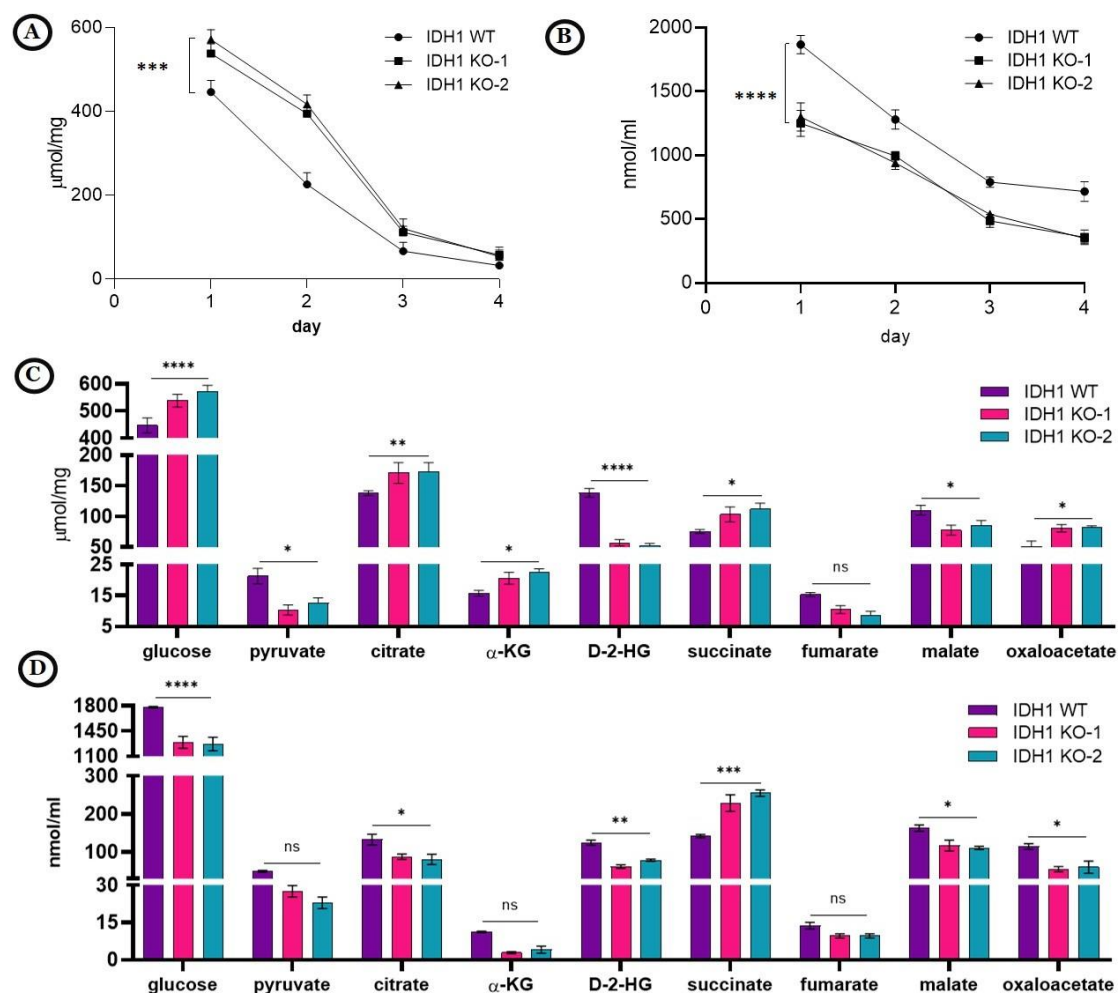


Figure 27: Effect of IDH1 on colon cancer metabolism after knocking out with the pX458 vector.

Time-dependent intracellular (A) and extracellular (B) glucose levels were investigated. In addition, intracellular (C) and extracellular (D) pyruvate levels were investigated after IDH1 silencing. Abbreviations: ns, not significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Similar results to IDH1 KO 1-2 were observed in clones (IDH1 KO 1-3) whose IDH1 gene was silenced using the lentiviral vector. The intracellular glucose level was 544,0 and 566,2 $\mu\text{mol/mg}$ in the IDH1 KO3 and IDH1 KO4, while it was determined as 438,5 $\mu\text{mol/mg}$ at 24 h, respectively (Figure 28 A). IDH1 KO clones used more glucose than IDH1 WT along 24-96 h (Figure 28 A). Intracellular pyruvate and malate levels were 1.7 and 1.5 times reduced in IDH1 KO3 and 1.8 and 1.3 times reduced ($p<0.05$) in IDH1 KO4 according to control, respectively (Figure 28 C). The intracellular citrate level was 2.0 and 1.8-fold raised ($p<0.05$) in IDH1 KO3 and IDH1 KO4 compared to IDH1 WT, respectively (Figure 28 C). Above all, the intracellular D-2HG level was 3 and 3.4 times reduced in IDH1 KO3 and IDH1 KO4 clones according to control (Figure 28 C).

The extracellular glucose was consumed more in the IDH1 WT than in IDH1 KO cells. The extracellular glucose level was less consumed in IDH1 KO clones than in IDH1 WT during the time (Figure 28 B). The extracellular citrate, D-2HG, αKG , and malate levels were 1.3, 2.1, 1.7, 2.4, and 1.3-fold less released in the IDH1 KO-1 and 1.4, 2.8, 1.6, 2.0, and 1.5-fold less released ($p<0.05$) in the IDH1 KO-2 according to control, respectively (Figure 28 D). According to the control, the extracellular pyruvate and fumarate levels were not significantly changed in IDH1 KO clones (Figure 28D).

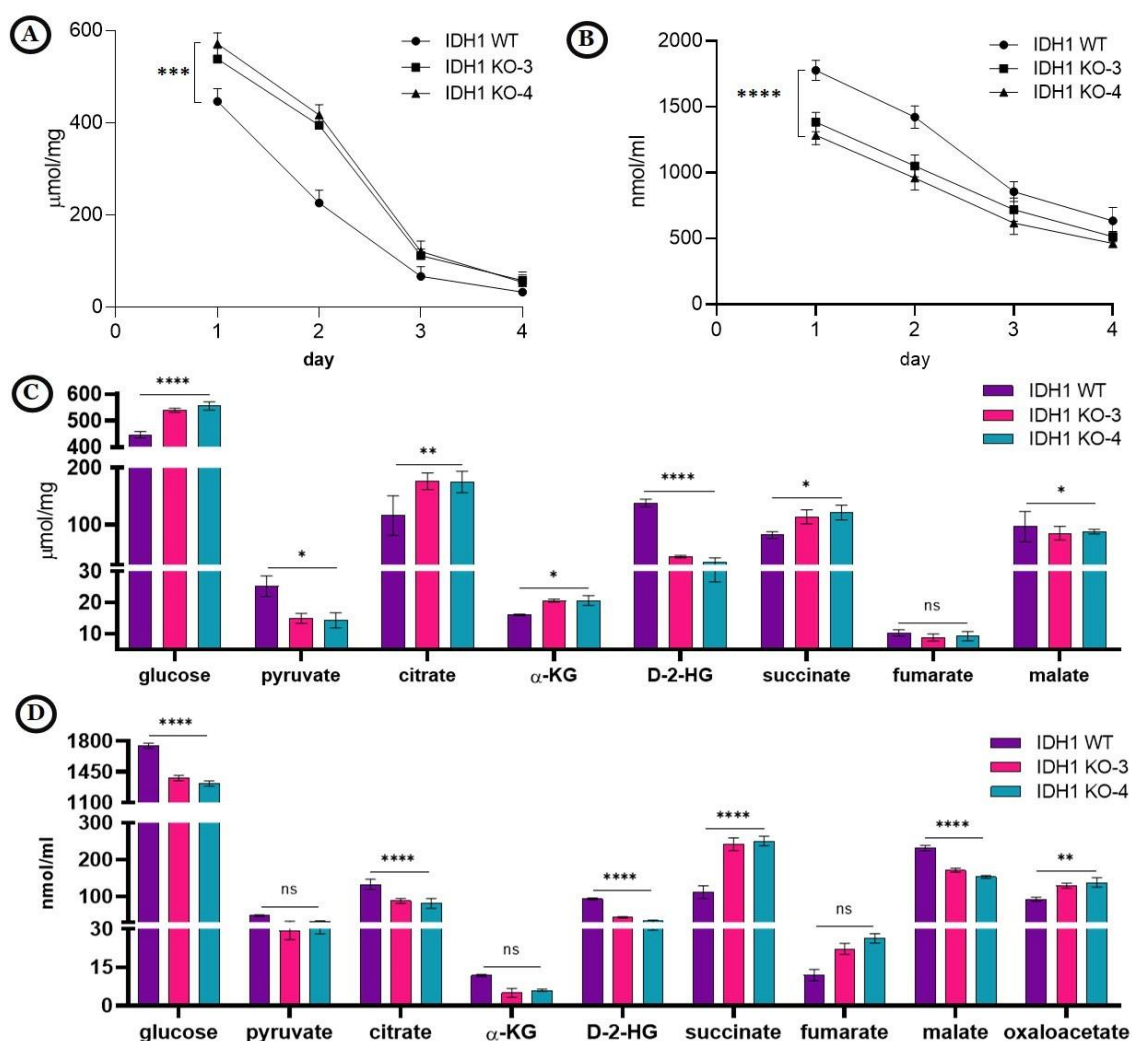


Figure 28: Effect of IDH1 on colon cancer metabolism after knocking out with the lentiCRISPRv2 vector.

Time-dependent intracellular (A) and extracellular (B) glucose levels were examined. In addition, intracellular (C) and extracellular (D) pyruvate level was examined after IDH1 silencing. Abbreviations: ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.9. Determination of mRNA Levels of MDH2, LDHA, and GLS Enzymes

After silencing the IDH1 gene, the D-2HG level was approximately 2-fold decreased in IDH1 KO1-4 compared to IDH1 WT (Figure 27C and Figure 28C). The continuation of the generation of this oncometabolite showed that additional enzymes are involved in the D-2HG

generation. It has been found that the GLS enzyme is involved in glutaminolysis (Dang, 2010), and MDH and LDH enzymes are responsible for 2-HG generation in hypoxic conditions in different cell types (Intlekofer et al., 2017). However, whether these enzymes contribute to the D-2HG generation in colon cancer is not known.

The mRNA levels of MDH2, LDHA, and GLS genes were determined. First, healthy colon epithelial and colon adenocarcinoma cells were examined. Figure 18B shows that the GLS gene expression is reduced in colon cancer cell lines. The mRNA level of LDHA was reduced in Caco-2 according to control. The MDH2 gene was 1.6 and 3 times highly expressed in Caco-2 and SW620 cell lines compared to the healthy cell, respectively. The rise in MDH2 mRNA level was associated with a 3.7-fold rise in the level of oxaloacetate (Figure 13A) reduced from malate in SW620 compared to Caco-2 ($p < 0.05$). Next, the levels of these enzymes were also investigated in healthy and tumor tissues from xenograft models. The mRNA level GLS was higher in Caco-2 but reduced in SW620. The mRNA level LDHA was reduced in Caco-2 and not changed in SW620 compared to healthy tissue. However, the MDH2 mRNA level was raised during metastasis (Figure 18D).

Similar results were observed in IDH1 KO cells. The mRNA level of the MDH2 enzyme was 1.9 and 1.8-fold raised in IDH1 KO-1 and IDH1 KO-2 compared to the control, respectively. No significant difference was observed in the expression of the LDHA enzyme. On the other hand, the GLS gene expression was 2 and 3.3-fold decreased in IDH1 KO-1 and IDH1 KO-2 clones compared to the control, respectively (Figure 29A). The significant rise in the mRNA level of the MDH2 enzyme both *in vitro* and *in vivo* showed that this enzyme might play a role in the generation of D-2HG.

The IDH1 KO and IDH1 WT cells were treated with different glutamine concentrations for 24 hours. Then, the intracellular D-2HG level was determined by the HPLC method. It was observed that there was no difference in D-2HG level as a result of the treatment of both 8- and 16-mM glutamine. When the GLS level decreased *in vitro* and *in vivo* models, and the glutamine treatment did not cause a rise in D-2HG generation, it was concluded that GLS does not have a role in this oncometabolite generation.

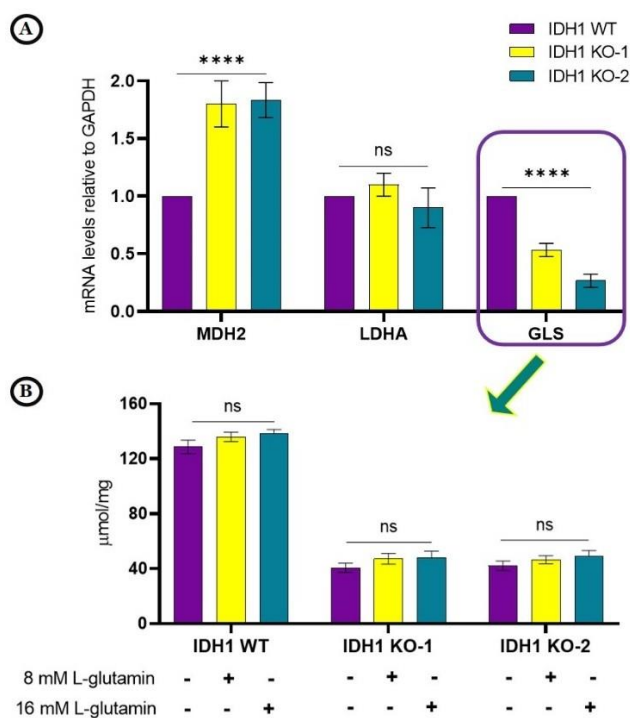


Figure 29: RT-qPCR and L-glutamine treatment results in the IDH1 KO1-2 cells.

mRNA levels of MDH2, LDHA, and GLS genes in IDH1 KO1-2 cells. GAPDH was used as a control (A). Determination of the D-2HG after L-glutamine (8 mM, 16 mM) treatment (B).

4.10. Determination of the Inhibitor's Effects on Cell Morphology and Cell Proliferation

According to the RT-qPCR results, it was observed that the mRNA level of the MDH significantly raised *in vitro* and *in vivo* models, and the mRNA level of the LDH enzyme did not change (Figure 18C; Figure 18D; Figure 29A). Therefore, in order to determine whether these enzymes contribute to the D-2HG generation, IDH1 KO and IDH1 WT cells were treated with inhibitors. K252c and Galloflavin are MDH and LDH enzyme inhibitors, respectively. Their IC₅₀ (μM) values determined by the MTT assay are shown in Table 15.

Table 15. IC₅₀ (μM) values of inhibitors against SW620 cell line.

	IC ₅₀ (μM)
K252c	4.2
Galloflavin	171.5

The morphological changes were examined using an inverted microscope after treatment of K252c, Galloflavin, and α -KG during 24 h incubation. The cells preserved their membrane structures and maintained their viability in the DMSO-treated control groups. However, after the treatment of Galloflavin (171.5 μ M) and K252c (4.2 μ M), some characteristic changes in cell morphology are determined, such as disruption of membrane integrity in both WT and KO cells. In addition, structures similar to apoptotic vesicles are observed and marked with a red arrow on the picture. It was observed that some cells reprotected their membrane structures after α -KG analog (50 μ M) treatment (Figure 30).

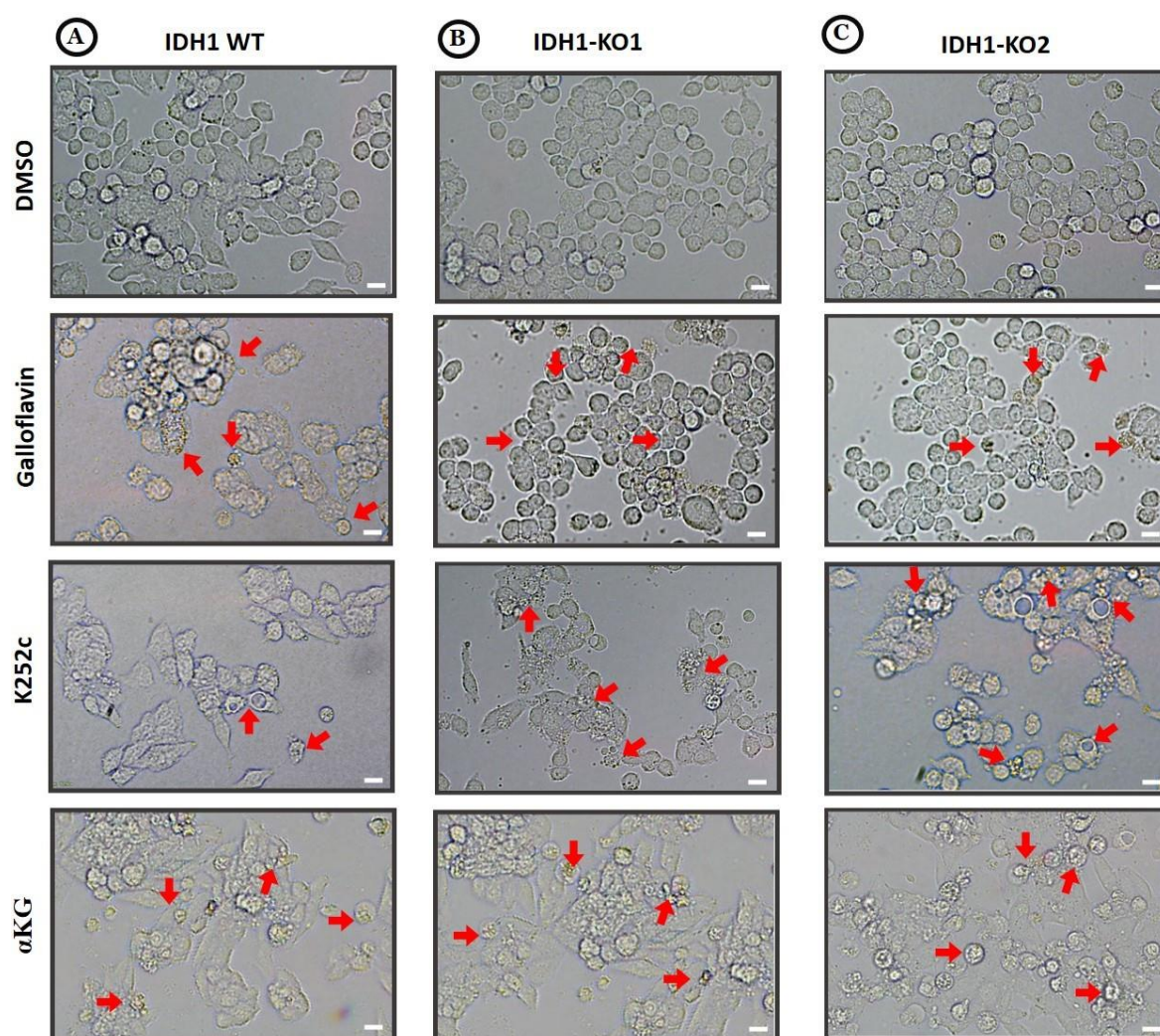


Figure 30: The morphological imaging after Galloflavin (171.5 μ M), K252c (4.2 μ M), and α -KG analog (50 μ M) treatment.

The morphologies of IDH1 WT (A) and IDH1 KO1-2 cells (B, C) were analyzed with inverted microscopy with 10X and 20X. Scale bar: 200 μ m.

Then, the effect of these inhibitors on cell proliferation was investigated. As seen in Figure 31, the proliferation of both WT and KO cells decreased significantly after K252c treatment. However, Galloflavin treatment reduced proliferation in the control group while not affecting the proliferation of KO cells. In addition, the cell number was significantly raised in the control group after α -KG analog treatment in both WT and KO cells compared to the control group.

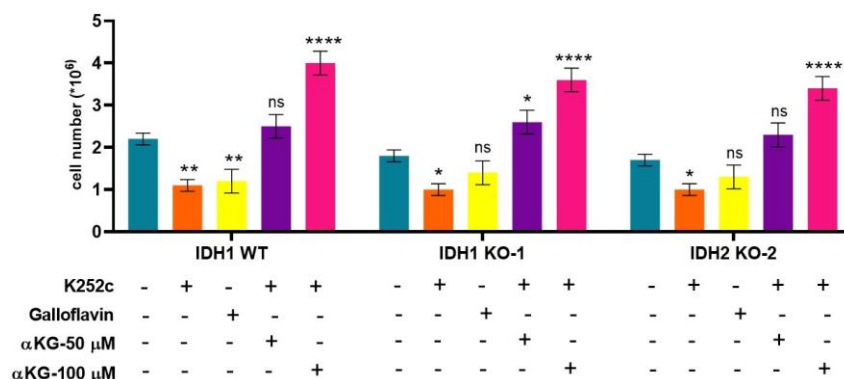


Figure 31: The effect of Galloflavin, K252c, and α -KG analog treatment on cell proliferation in IDH1 WT and IDH1 KO1-2 cells. Abbreviations: ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

4.11. Determination of the Inhibitor's Effect on D-2HG Generation

In order to determine whether MDH and LDH enzymes have a role in D-2HG generation, the D-2HG level was determined by HPLC analysis after the treatment with inhibitors for 24 hours. Treatment of K252c to IDH1 WT cells did not affect D-2HG generation, while Galloflavin treatment led to a 0.4-fold decrease in D-2HG level compared to control. Most importantly, after treatment of both K252c and Galloflavin in IDH1 KO 1-2 cells, the D-2HG level was not found. The D-2HG level raised again after α -KG analog treatment for 24 hours (Figure 32).

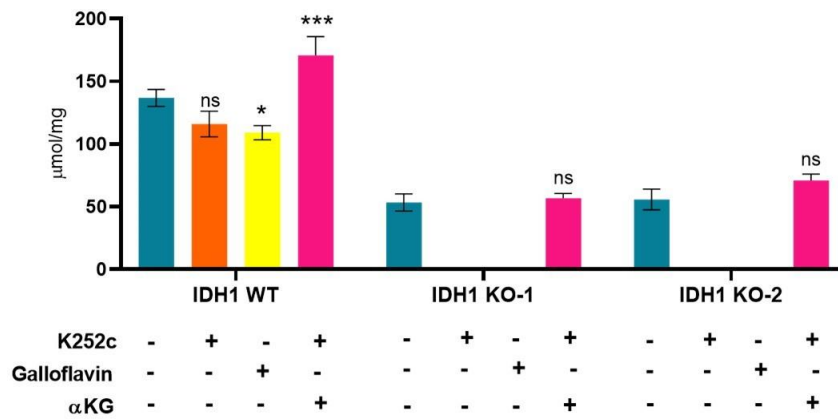


Figure 32: The effect of K252c (4.2 μ M), Galloflavin (171.5 μ M), and α -KG analog (50 μ M) treatment on D-2HG generation in IDH1 WT and IDH1 KO1-2 cells.

4.12. Generation of the R132H Mutation in the IDH1 Gene

When the IDH1 gene was silenced in the colon adenocarcinoma cell line, there was a significant decrease in the D-2HG level. These results show that the wt IDH1 enzyme plays a role in D-2HG generation. In addition, it is known that the R132H point mutation in the IDH1 gene leads to D-2HG generation in various cancer types (Dang et al., 2009). Therefore, to determine whether the mutant IDH1 enzyme affects the generation of D-2HG in colon cancer, the R132H mutation was generated with the CRISPR/Cas9 method.

In the first step, cells treated with nocodazole, an antineoplastic agent, block the cell in the M phase. Cells were treated with 100, 200, and 400 ng/ml nocodazole for 24 hours to determine the optimum concentration. After incubation, cells were stained with propidium iodide (PI) dye, and the phase in which the cell was retained in each phase was determined by flow cytometry. It is shown that the nocodazole treatment led to keeping the cells in the G2/M phase, and the best concentration was 200 ng/ml (Figure 33 A).

The CRISPR/Cas9 method generates site-specific double-strand DNA breaks using the Cas enzyme. The HDR template was designed to contain the target point mutation (R132H) and the BclI cleavage site. The RNP complex consisting of Cas9, sgRNA, and HDR template was given to synchronized cells to repair the generated DNA breaks by the HDR mechanism. The RNP complex given to the IDH1 WT cell did not include an HDR template. After

nucleofection, the cells are divided into two parts (Figure 12). In the first part, three days later, the nucleofection rate was determined by FACS analysis as %34,5 and %44 for IDH1 WT and IDH1 R132H, respectively (Figure 33 B).

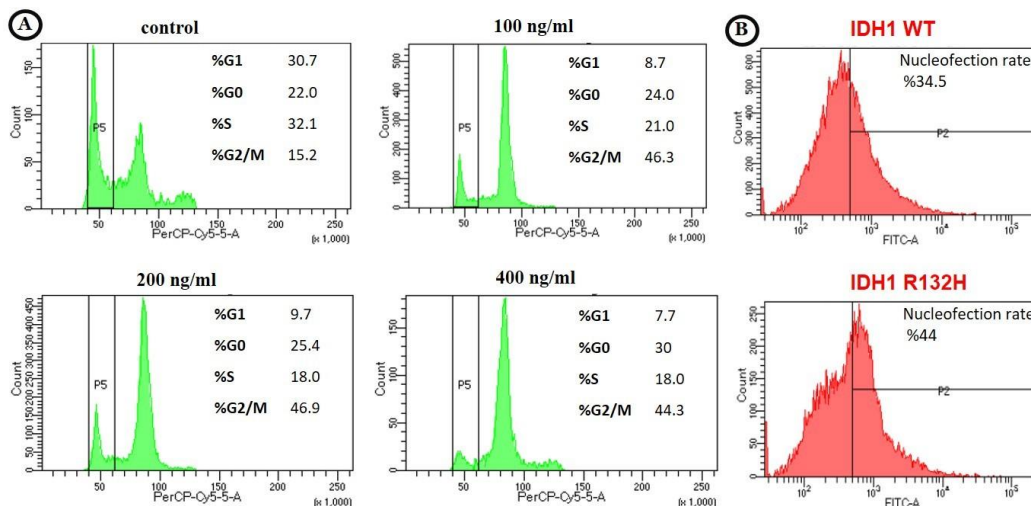


Figure 33: The analysis of cell cycle distributions after 100, 200, and 400 ng/ml Nacodazole treatment(A).

Nucleofection of the RNP complex and 100 pmol of DNA template was carried out with the kit, and the nucleofection rate was determined by FACS analysis in IDH1 WT and IDH1 R132H cells (B).

In the second part, the cells were monitored in the bright field and GFP filter for seven days (Figure 34 A). Then cell was seeded into a 96-well plate as one cell in each well. The colony formation from a single cell was imagined for 14 days with Solentim Cell Metric™ (Figure 34 B). 5 clones proliferating from a single cell were selected.

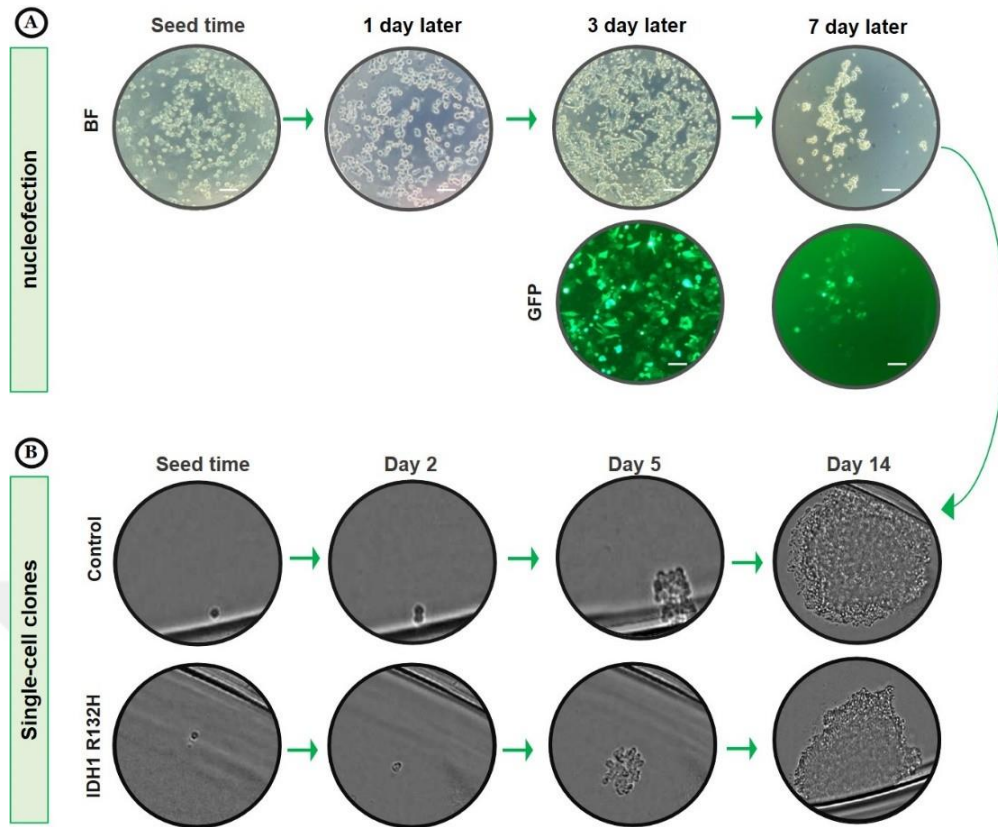


Figure 34: Imaging of morphology change and colony formation after transfection of SW620 cells.

The morphological changes after nucleofection were analyzed for seven days in both bright field and the GFP filter (A). Colony formation from a single cell was imaged for 14 days after nucleofection with Solentim Cell Metric™ (B).

Firstly, the PCR products (620 bp long) were cut by the BclI enzyme to determine the mutated clone. If the target enzyme was successfully mutated, two bands of 340 and 240 bp were expected after cleavage of the BclI enzyme. The gel images showed two bands were seen in clone 5. However, an extra band above 340 bp was observed due to the heterozygous mutation (Figure 35A). Secondly, the DNA sequences of clones were sequenced by the Sanger method. DNA was isolated as described above and the target region was amplified by PCR. The results show that clone 5 contains a heterogeneous R132H mutation (Figure 35B).



Figure 35: The validation of the generation of R132H mutation in SW620 cells.

The BclI enzyme digests the PCR product of every single cell after R132H mutation (A), and the single-cell clones were sequenced using the Sanger method, and R132H mutation was found in a clone (B).

4.13. Investigation of the R132H Mutation Effect on Cell Proliferation, Morphology, and Colony Formation

In the next step, it was investigated whether the R132H mutation affects cancer cell proliferation and colony formation capacity. The proliferation of IDH1 R132H cells was unchanged over four days compared to the control cells (Figure 36 A). Similarly, the R132H mutation did not affect colony formation capacity and cell morphology (Figure 36B and Figure 37C).

The effect of the R132H mutation on the mRNA levels of the enzymes contributing to the D-2HG generation was investigated. The expression level of IDH1, LDHA, and MDH2 were not changed in IDH1 R132H cells compared to the IDH1 WT cells. However, IDH2 expression was raised while GLS was decreased after R132H mutation according to the control (Figure 36D).

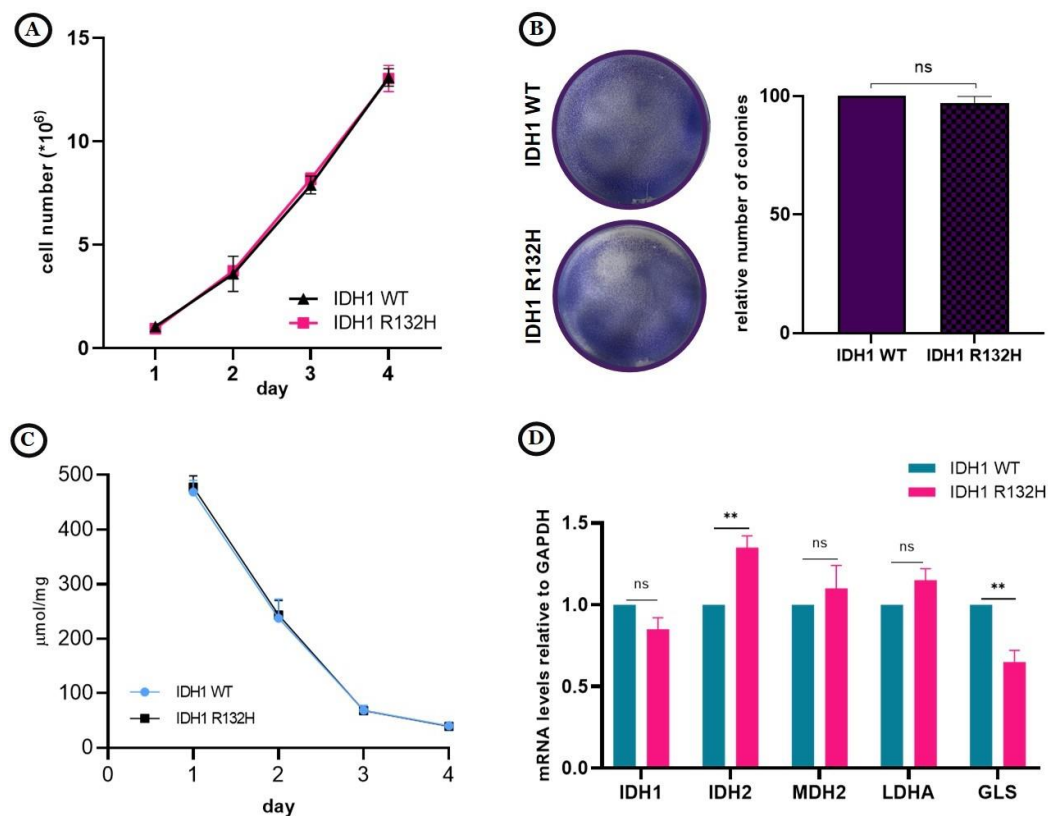


Figure 36: The investigation of the cytostatic effect of R132H mutation.

The cell proliferation was analyzed in IDH1 WT and IDH1 R132H (A). Colony formation ability was evaluated by counting colonies stained with crystal violet (B). Time-dependent intracellular glucose levels were investigated (C). mRNA levels of genes in IDH1 WT and IDH1 R132H cells. GAPDH was control. Abbreviations: ns, not significant. ** $p < 0.01$

4.14. Investigation of the Effect of R132H Mutation on Cell Metabolism

In order to examine how the R132H mutation affects cell metabolism, intracellular and extracellular metabolites in the energy metabolism were determined. There were no significant difference in time-dependent intracellular glucose levels in IDH mutated (R132H site) cells compared to the control group (Figure 34 C). The D-2HG level did not raise as much as expected. A 1.2-fold raise was seen in IDH1 R132H cells compared to control. Consistent with this, citrate, succinate, and fumarate levels were 1.17, 1.2, and 1.18-fold decreased in IDH1 R132H cells according to control (Figure 37A). There was no significant change in extracellular metabolite levels in IDH1 R132H cells compared to control (Figure 37B). When the results

were evaluated, it was observed that the generation of R132H mutation was not affected D-2HG generation.

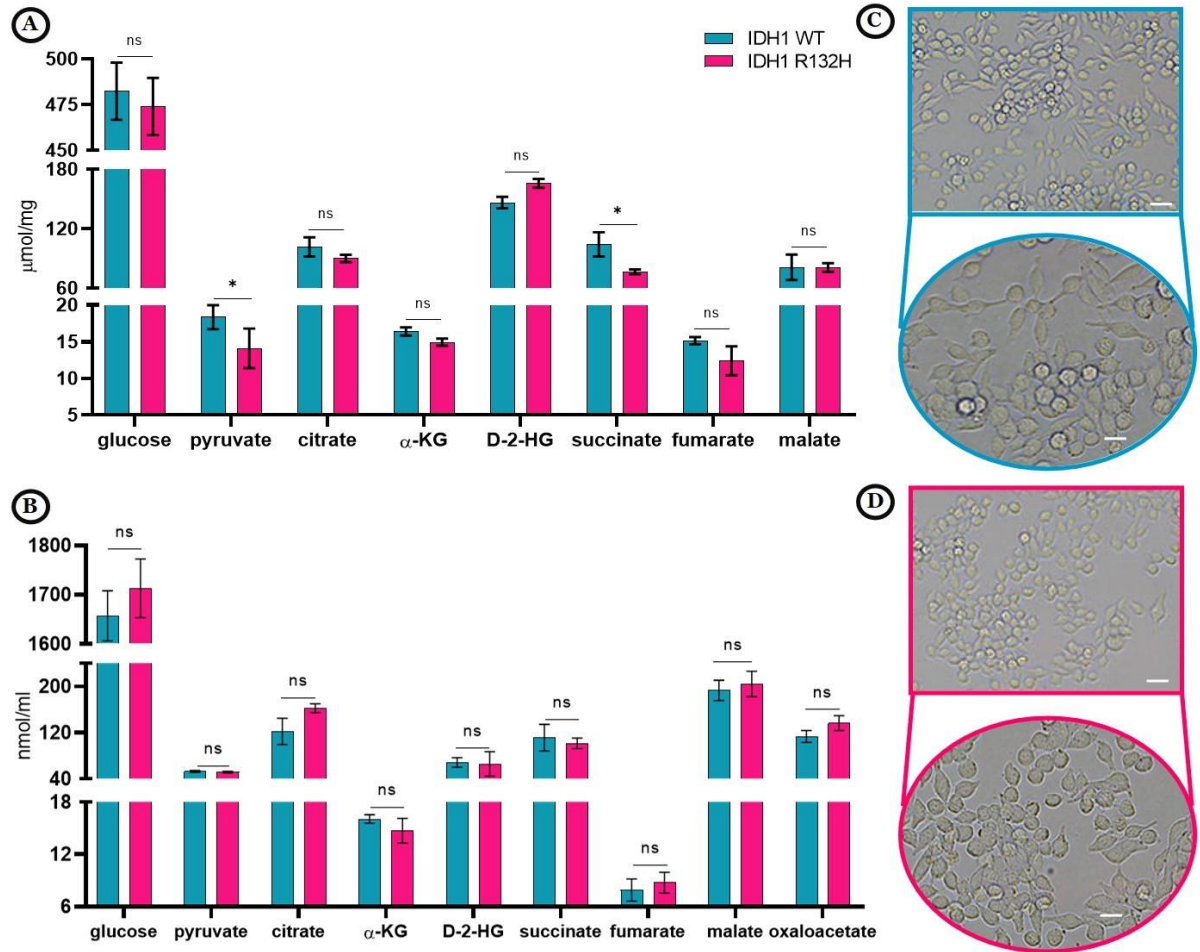


Figure 37: The effect of R132H mutation on both the cell metabolism and morphology.

Intracellular (A) and extracellular (B) metabolite levels. The morphologies of IDH1 WT (C) and IDH1 R132H (D) cells were analyzed with inverted microscopy at 10X and 20X. Scale bar: 200 μm. Abbreviations: ns, not significant. * $p < 0.05$

4.15. PDX Model Generation and H&E Staining

Tumor and healthy tissue samples were taken from 10 colon cancer patients during the operation. The tumor and healthy tissues taken from the first patient were named P1 and H1 respectively, and the tumor and healthy tissues taken from the second patient were named P2

and H2, respectively. Thus, they were named according to the order taken from the patients. The healthy tissues were frozen in nitrogen gas and stored at -80 °C until RNA extraction. Two different tumor tissues were taken from patients, which were used for the Sanger method and PDX model generation. Firstly, tumor tissues were used for the Sanger method to analyze whether mutations in IDH1 (Arg132 and G97) and IDH2 (Arg140 and Arg172) genes. DNA isolated from tumor tissues was amplified by PCR, and the PCR products were sequenced. The results showed that any mutation was not detected both in the IDH1 and IDH2 genes (Figure 38A-B).

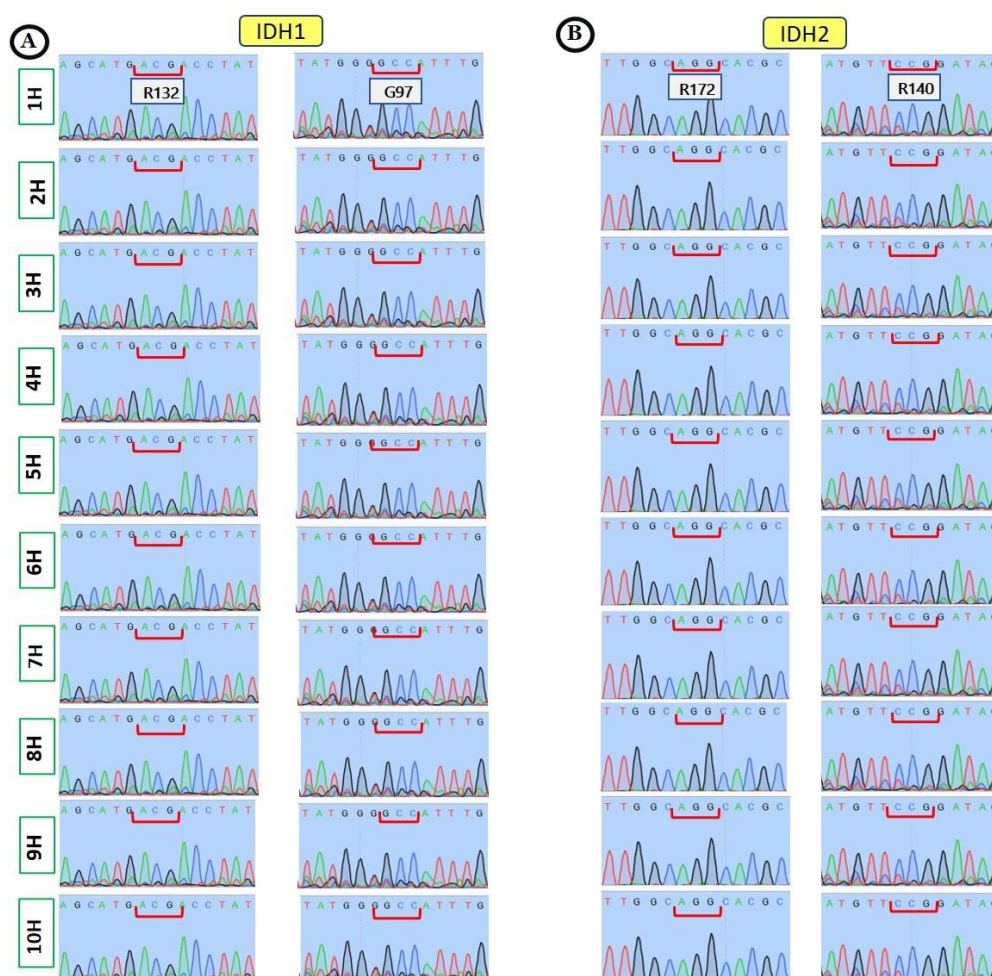


Figure 38: The Sanger sequencing results of all human tumor tissues.

The R132 and G97 amino acids in the IDH1 (A) and R140 and R172 amino acids in the IDH2 (B) gene of the biopsy samples taken from patients were sequenced using the Sanger method.

PDX models were developed with the freshly resected second part of tumor tissue in the NOD/SCID mice. Unfortunately, four out of 10 PDX models died before tumor development due to various health problems (Figure 39). PDX models were sacrificed when tumor volume reached 1 cm³, and the tumor, colon, and liver tissues were extracted and stored at -80 °C until HPLC analysis. Tumor tissue was divided into four pieces. Firstly, some of it was separated for HPLC analysis, like liver and colon tissues. Then, the remaining tumor tissue was grinded and mixed with the medium, which included %43 RPMI (with %1 P/S and 0.1 mg/ml primocin), %50 FBS, and %7 DMSO stored at -80 C° for retransplantation (Figure 9). The tumor tissue isolated from the first PDX model was named PDX1. Thus, tumor tissues were named according to the number of isolated PDX models.



Figure 39: The pictures of generated PDX models.

All H&E staining steps were performed by an expert at Izmir Biomedicine and Genome Center, Histopathology Unit. The staining results show that cancer tissues from different patients exhibit different phenotypic properties. The tumor cells were multilobular localized, and borders surrounded the lobules. Cells acted as intestinal crypts and went to gland formation. With the addition of multilobular localization, cells are solitary and diffusely distributed. In addition, it was observed that the cells tended to necrosis, a common feature of tumor cells, and necrosis areas were marked as N.

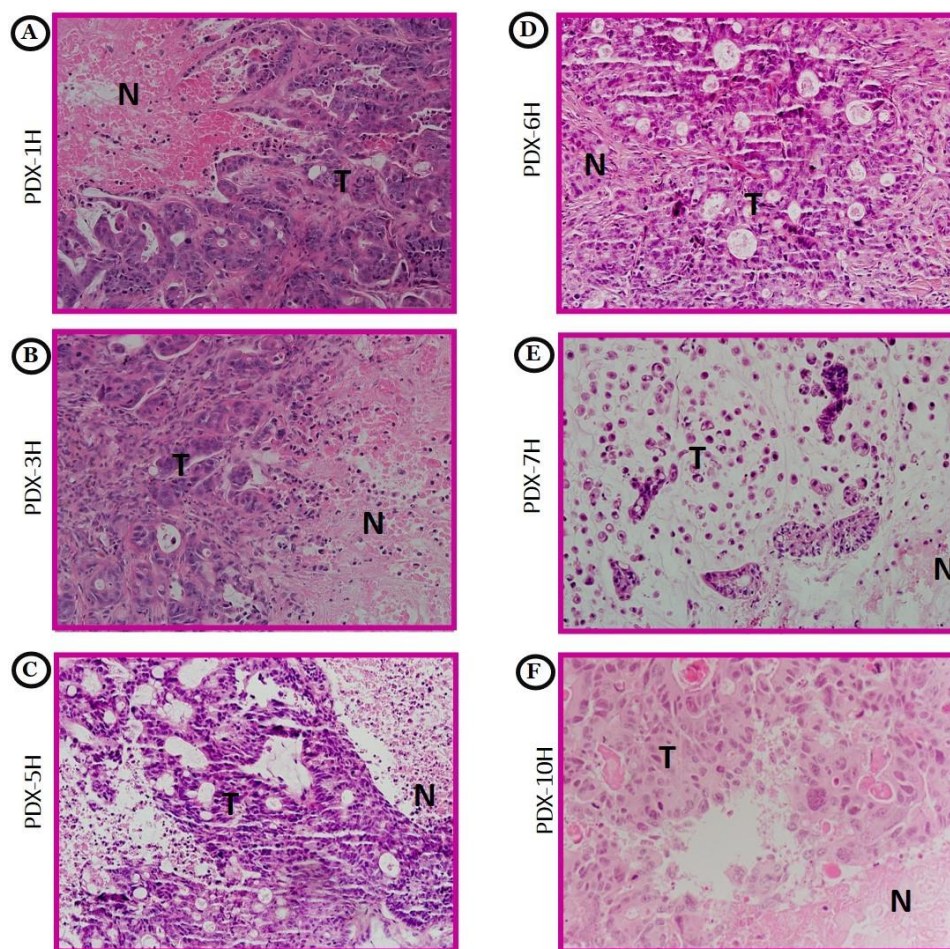


Figure 40: Histopathology of all the PDX tumor tissues.

The tumor tissues were stained with H&E dye. The scale bar is 200 μm (A-F). Abbreviations: N necrosis; T tumor part.

4.16. Investigation of Some Metabolite Concentrations of Energy Metabolism in PDX Models

The Sanger sequencing results showed that there was no mutation in the IDH1 and IDH1 genes in the colon cancer tissues. In the next level, the metabolism was investigated in tumor tissues of PDX models, which have the wt IDH1 or IDH2 genes.

The glucose, pyruvate, and fumarate levels were highest in the liver and were very similar in the colon and tumor tissues (Figure 41 A, B, G). The citrate, α -KG, succinate, and malate levels were highest in tumor tissues, followed by liver and colon tissues, respectively (Figure

41 C, D, F, H). The oxaloacetate levels were similar in all the tissues (Figure 41 I). Above all, the D-2HG was more highly generated in the tumor tissues than in the others (Figure 41 E).

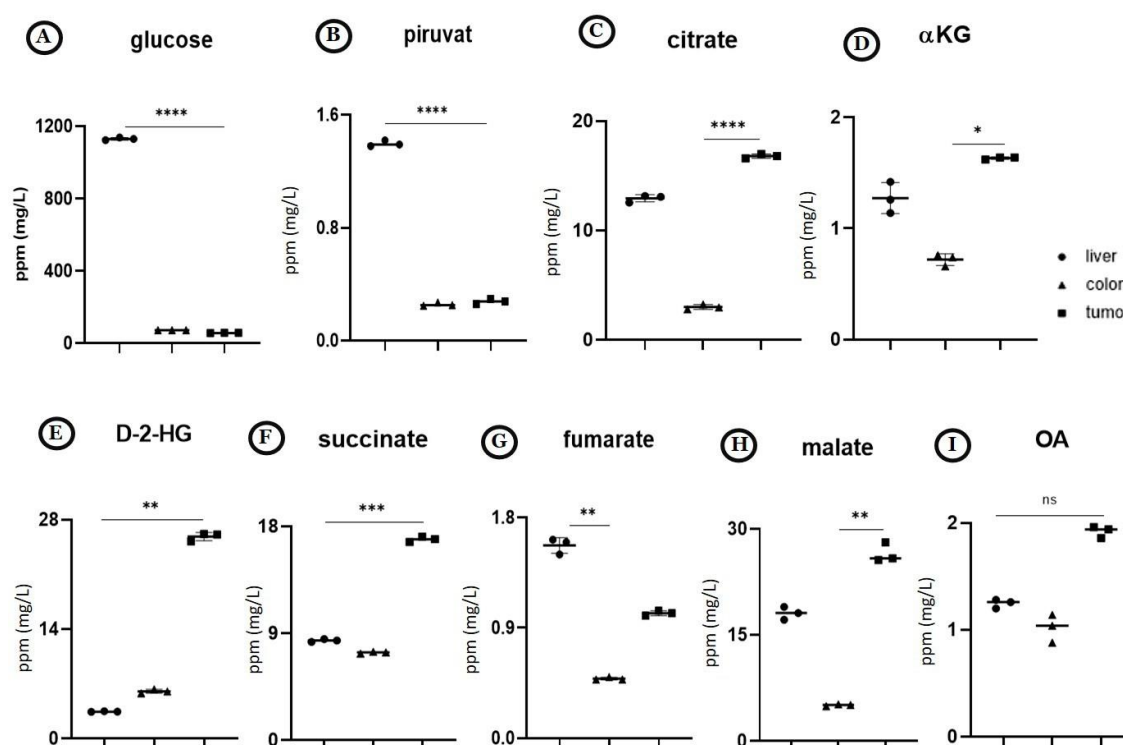


Figure 41: The metabolite levels in the tissues were extracted from PDX models.

The levels of glucose (A), pyruvate (B), citrate (C), α -KG (D), D-2HG (E), succinate (F), fumarate (G), malate (H), and oxaloacetate (I) were detected.

4.17. Determination of mRNA Levels of IDH1, IDH2, MDH2, LDHA, and GLS Enzymes in PDX Models

The wt IDH1/2 are involved in D-2HG generation independently of mutant IDH genes. The wt-IDH1 and IDH2 mRNA levels were imbalanced *in vitro* (Figure 18 B) and *in vivo* (Figure 15 C) models. Next, the effect of IDH1, IDH2, MDH2, LDHA, and GLS enzymes in the D-2HG generation was investigated. The isolated RNA from tumor tissue taken from PDX models and healthy tissues from patients was used to determine the mRNA levels of these enzymes. The healthy tissues were the control group, and GAPDH was control gene.

According to the control group, the mRNA level of IDH1 and GLS genes was quite low in all PDX models (Figure 42 A, D). The IDH2 level was decreased in PDX1-3, but it was

raised in PDX5-10 models compared to the healthy tissues (Figure 42 B). The mRNA level of MDH2 was not changed only in PDX1 but raised in PDX3-10 models than in the control group (Figure 42 C). The LDHA expression was reduced in PDX1-5 models according to the healthy tissues, but the gene expression level was not changed in PDX 6-10 models (Figure 42E).

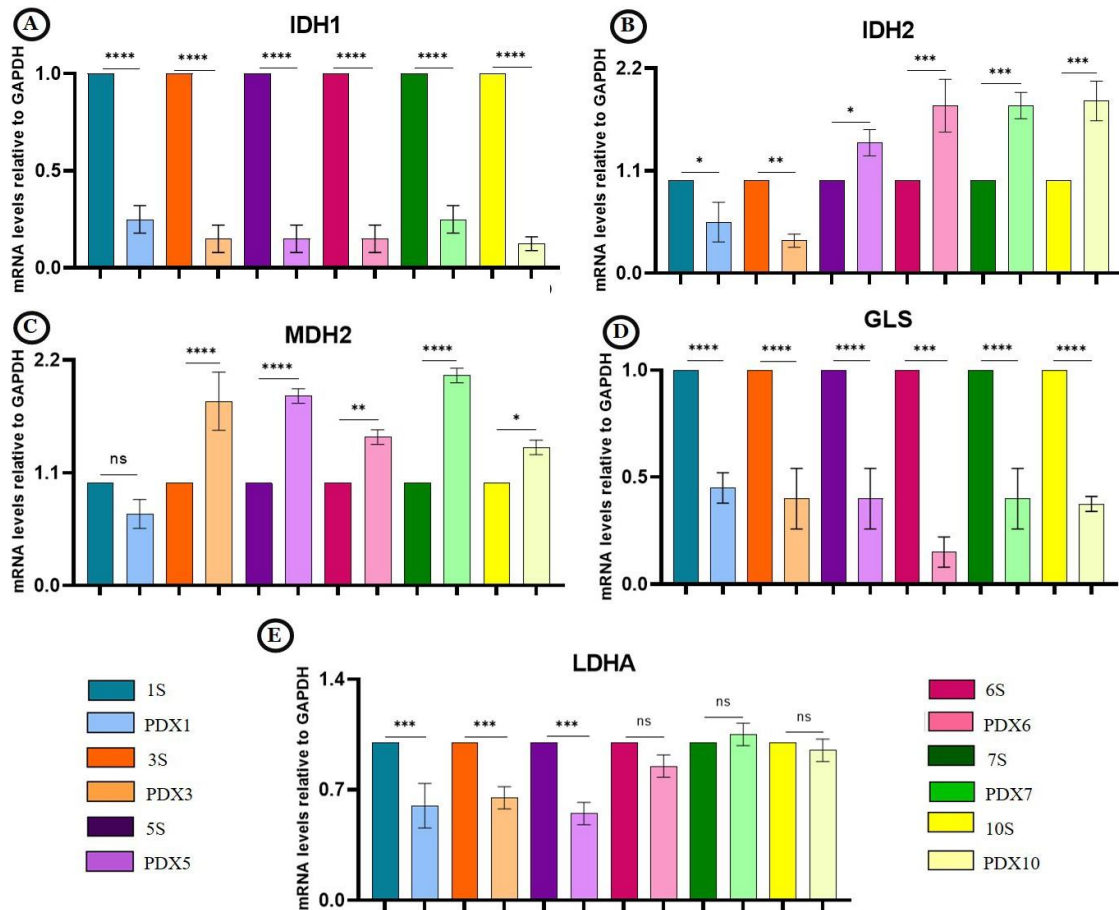


Figure 42: The mRNA levels of enzymes contribute to D-2HG generation in the tumor tissues of PDX models.

GAPDH was control. Abbreviations: ns, not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

5. DISCUSSION

The D-2HG generation pathways and the effects of this oncometabolite on colon cancer metabolism in various *in vitro* and *in vivo* models were examined in this thesis study. In this study, for the first time, the role of MDH2 and LDHA enzymes in D-2HG generation under normal conditions has been discussed in colon cancer. Furthermore, the impact of the high levels of D-2HG on colon cancer metabolism has been detailly investigated. Various *in vitro* models, such as colon adenocarcinoma cell lines at different stages and cell lines generated by CRISPR/Cas9-based gene therapy (IDH1 KO and IDH1 R132H), and *in vivo* models (xenograft and PDX models) were generated in this study.

The thesis studies started with the determination of intracellular and extracellular metabolite levels during colon cancer metastasis. Then the concentration changes in these metabolites were investigated in xenograft models consisting of CCD18-Co, Caco-2, and SW620, and significant results were achieved. Glucose is converted into pyruvate by glycolysis and participates in the TCA cycle (Montal et al., 2015). Our research found a small variation in the consumed glucose levels between healthy and Caco-2 and SW620 cell lines after 24 hours. On the other hand, the low intracellular glucose level in cancer cells shows that they metabolize glucose 2.6 and 3.9 times more than CCD-18Co, respectively (Figure 13A-B). Earlier studies showed that some cancer types consume more glucose (Dettmer et al., 2013; Scott et al., 2011).

In vivo results showed that tumor tissue generated with Caco-2 and SW620 consumed more glucose than healthy tissue (Figure 17), in agreement with *in vitro* results. Glucose levels were significantly different in all xenograft tissues. Although glucose levels were not significantly altered in all brain tissues, the liver and tumor tissues in the Caco-2 and SW620 xenograft models were more metabolically active than healthy tissues (Figure 16 and Figure 17). Genetic studies reveal that some oncogenes activity such as protein kinase B (AKT) and myelocytomatosis (MYC) and mutations in several glycolytic enzymes, including pyruvate kinase, alter cancer metabolism *in vivo* (Camarda et al., 2017; Israelsen et al., 2013). In colon cancer, MYC affects the expression of numerous metabolic and transporter genes (Satoh et al., 2017; Tang et al., 2019). As a result, the glycolytic flux is impacted in a tissue-specific way. We hypothesize that the differences in glucose metabolism in the different tissues can be related to the glycolysis-related enzyme equipment.

Lactate is one of the main key metabolites in metabolism. Cancer cells with metabolic rearrangement generate elevated lactate levels and are converted to TCA cycle intermediates in various cancer types (Montal et al., 2019). In our study, it was observed that the colon adenocarcinoma cells tended to the TCA cycle. In addition, intracellular lactate level was reduced in colon cancer cell lines according to colon epithelial cells (Figure 13 A), which is consistent with the literature (Montal et al., 2019). The decrease in intracellular lactate level was connected with the LDHA gene expression level (Figure 18). Furthermore, monocarboxylate transporters transfer elevated lactate from the cancer cells (Doherty and Cleveland, 2013). In our study, the low intracellular lactate (Figure 13 A) and the high extracellular lactate levels (Figure 13 B) in colon adenocarcinoma cells were similar to this metabolism.

Another crucial element in determining the lactate level is the Cori cycle. Part of the lactate is transported to the liver with alanine while some of it is used in tumor tissues. Lactate returns to the tumor tissue as a precursor to gluconeogenesis (Cori, 1931). The results showed that this lactate circulation was relevant to the *in vivo* data. Tumor tissue from Caco-2 (1.6-fold) and SW620 (1.5-fold) was shown to have significantly lower lactate levels than CCD-18Co tissue (Figure 17 A-B; Figure 43).

The TCA cycle is a main mitochondrial metabolic pathway that coordinates carbon metabolism, biosynthetic pathways, and energy generation (Krebs H and Johnson WA, 1937). Recently, there has been a rise in interest in researching the amount of TCA cycle metabolites in colon cancer (Neitzel et al., 2020). In our study, intracellular and extracellular levels of adenocarcinoma cancer cells significantly differ from healthy cells, owing to their high metabolic rate. This is demonstrated by the elevated amounts of intracellular metabolites D-2HG, malate, and oxaloacetate. Because α -KG was converted to D-2HG, the intracellular α -KG, citrate, and succinate levels were reduced, (Fig. 1B). Concerning these results, colon adenocarcinoma cell lines released more TCA cycle intermediate than the healthy cells (Figure 13 A-B). Colon cancer cells exhibit the Warburg effect because they consume more glucose. On the other hand, the raised level in the TCA cycle intermediates showed that metabolism does not proceed through glycolysis.

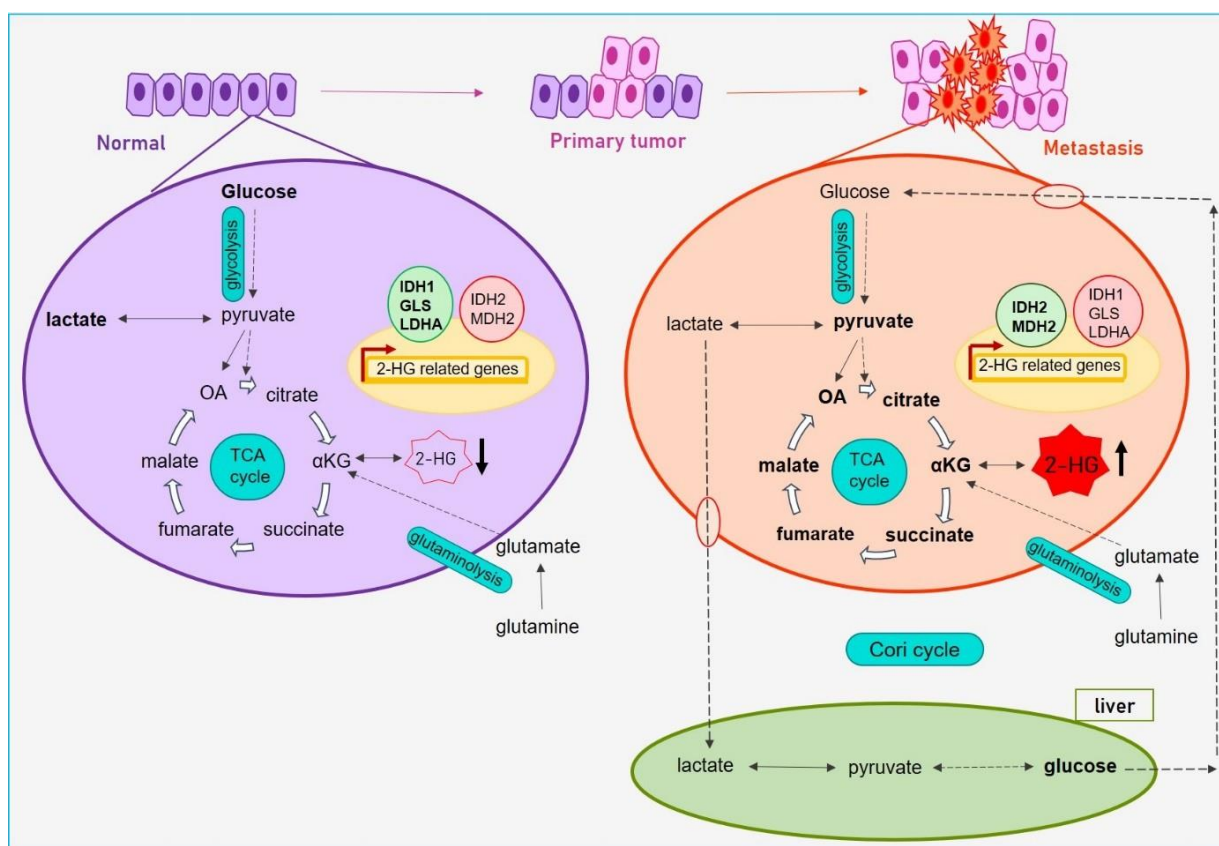


Figure 43: Schematic representation of metabolic rearrangement in the TCA and Cori cycles and glycolysis in colon cancer during metastasis.

The 2-HG metabolite can be 10-100-fold raised and role as an oncometabolite in the cancer cells (Dang et al., 2009). The oncometabolite level, which ranges from pmol to mol, depends on several factors such as the cancer type, the level of metastasis, its origin (tissue or cell culture), hypoxic conditions, and acidic pH (Dang et al., 2009; Colvin et al., 2016; Grassian et al., 2012; Du and Hu, 2021). The D-2HG level was found significantly high *in vitro* (Figure 13 A) and *in vivo* (Figure 16 and Figure 17) models. The intracellular D-2HG level has raised in Caco-2 (22.6 $\mu\text{mol/mg}$) and SW620 (152.6 $\mu\text{mol/mg}$) when cells go to metastasis, whereas it was not found in the colon epithelial cell line. According to previous studies, the metastasis feature has been found to correlate with the high levels of metabolites generated by various cancer cells, including the pancreas, stomach, and prostate (Lu et al., 2010; Mycielska et al., 2018). It was concluded that the elevated level of D-2HG might be connected to colon cancer metastasis.

When looking at all xenograft tissues, it has been found that D-2HG tissue-specifically metabolized. It was not generated in CCD-18Co colon and brain tissue (Figure 16). Additionally, it was not generated in all liver tissues (Figure 17). In the PDX models, while D-2HG was not highly generated in the colon (2-6 ppm) and liver (4-9 ppm) tissues, it was high in tumor tissue (20-45 ppm) (Figure 41). There are numerous studies on the metabolic analysis of tumor and healthy tissues derived from mouse models developed with various cancer cells (Montrose et al., 2012; Lau et al., 2020; Davidson et al., 2016). Malate, succinate, citrate, and 2-HG levels were raised, and glucose levels decreased in tumor tissue extracted from mouse models of azoxymethane (AOM)-induced colorectal carcinogenesis compared to the normal colonic mucosa (Montrose et al., 2012). In the mouse models created with pancreatic cells, it was observed that the levels of pyruvate and TCA cycle intermediates were more generated in pancreatic tumor tissues than in normal pancreas (Lau et al., 2020). In another study, they observed that glucose was more contributed to the TCA cycle tumors than lung tissue in xenograft models created with non-small lung cancer cells (Davidson et al., 2016), consistent with our results.

After the significant results were obtained at the D-2HG level, the pathways in which this oncometabolite was generated were investigated in the next step. There was no mutation in the IDH1/2 genes in the SW620 cell line (Figure 18A) and tumor tissues taken from colon cancer patients (Figure 38). We hypothesize that the imbalance in the mRNA levels of IDH1/2 enzymes can cause elevated D-2HG in both *in vitro* (Figure 18 B) and *in vivo* (Figure 15 B) models. The other pathways where 2-HG is generated were also analysed. After founding wt-IDH1/2 enzymes and elevated D-2HG levels in SW620 cells, it was hypothesized that the generation of this oncometabolite might occur via glutaminolysis. Glutamine is the second carbon source of 2-HG after glucose because it joins the TCA cycle through α -KG (Dang, 2010). The GLS gene was lowly expressed in SW620 than in the control group (Figure 18B), and it was decreased after IDH1 knockout (Figure 29A). Similar results were also obtained in both xenograft (Figure 15B) and PDX (Figure 42D) models, which are consistent with the literature (Xiang et al., 2019). After treatment with glutamine, the generation of D-2HG was not changed in IDH1 KO clones (Figure 29 B). The findings indicated that glucose would be the carbon source for D-2HG.

Since the wt IDH1 highly contributed to the D-2HG generation than the IDH2 in colon cancer, the thesis study continued with the silencing of the IDH1 gene by using the CRISPR/Cas9 method. This study presents an effective methodology for silencing the IDH1 gene (Figures 10 and 11). The effect of IDH1 on cancer metabolism was investigated. It was observed that the IDH1 KO 1-4 cell models consumed approximately two times less glucose than the control (Figure 27 A and Figure 28 A). These results were consistent with the 2.2 -fold decrease in the intracellular D-2HG level in IDH1 KO 1-4 compared to the control (Figure 27 C and Figure 28 C). In addition, the decreased level of intracellular pyruvate in IDH1 KO clones (Figure 27 C and Figure 28 C) may be related to the conversion of pyruvate to oxaloacetate and thus can participate in the TCA cycle. IDH1 KO cells had higher intracellular citrate levels and lower intracellular malate levels when compared to control cells (Figure 27 C and Figure 28 C). These results were associated with IDH1 silencing and are consistent with earlier research (Su et al., 2020). Contrary to this study, it was hypothesized that the raised α -KG level in IDH1 KO cells was due to the decreased D-2HG level. However, it is shown that the wt IDH1 gene is contributed to D-2HG generation in colon cancer. Similarly, Špačková et al. (2021) knocked out the IDH2 enzyme and found that the IDH2 and HMT enzymes are related to breast cancer. On the other hand, the continuation of D-2HG generation despite the IDH1 silencing showed that extra enzymes have a role in this oncometabolite generation.

Until now, different cell models using the CRISPR-Cas9 technique have been generated to examine the functions of IDH1 enzymes in the pathogenesis of various cancer types, including glioma (Moure et al., 2019), AML (Brabetz et al., 2017), chondrosarcoma (Li et al. 2019), and breast (Špačková et al. 2021) cancer. In our study, the IDH1 gene was successfully silenced, and the function of this gene was evaluated in colon adenocarcinoma cell lines. The significant hallmarks such as proliferation (Figure 21 B and Figure 22 B), invasion (Figure 22 C) and migration (Figure 23 A-B and Figure 24 A-B) abilities, and colony-forming capacity (Figure 21 C and Figure 24 C) were significantly decreased in IDH1 KO1-4 cells compared to the control. In addition, cell morphologies of IDH1 KO1-4 colonies were significantly changed (Figure 21 A and Figure 22 A), and the cells were retained in G0/G1 phases (Figure 26 A-D). It was concluded that the IDH1 gene had been shown to have a critical role in colon cancer progression (Figure 44). The literature found that the properties of proliferation, migration, and invasion of cholangiocarcinoma cell lines decreased after IDH1 perturbation (Su et al., 2020).

In another study on colon cancer cells, IDH1 knockdown led to G1-phase arrest and reduce cell proliferation and invasion ability (Yang et al., 2021).

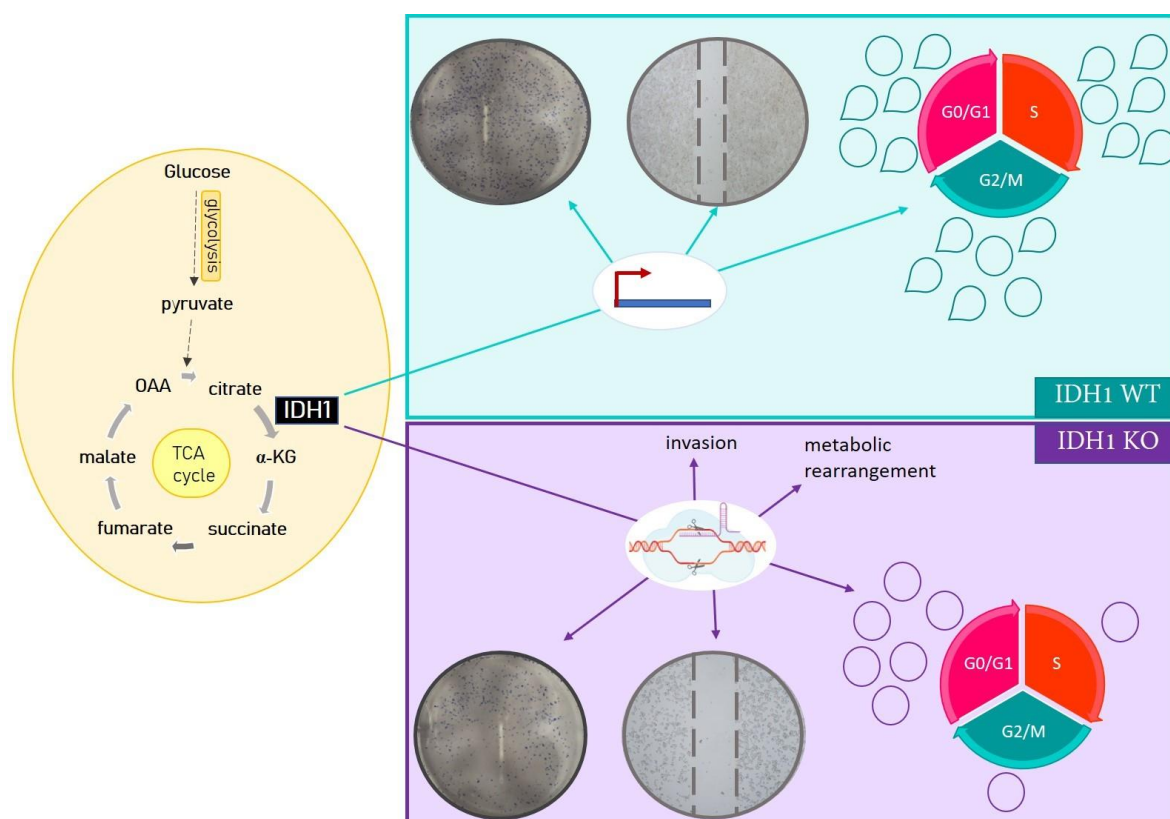


Figure 44: The effect of IDH1 knockout on some hallmarks of colon adenocarcinoma cells.

The upper and lower panels show the properties of IDH1 wild-type and knockout cells, respectively. The IDH1 KO cells were arrested in G0/G1-phase, and the invasion, migration, and colony formation abilities of IDH1 KO clones were significantly decreased, accompanied by metabolic changes. Abbreviations: α -KG, α -ketoglutarate; IDH1, isocitrate dehydrogenase 1; IDH1 KO, IDH1 knockout; IDH1 wt, IDH1 wild-type; OAA, oxaloacetate.

Cells have evolved genetic and metabolic reprogramming to reduce the negative consequences of hypoxic conditions. Since cells tend to glycolysis, carbon entry into the TCA cycle is limited. The reductive carboxylation reaction of IDH and glutamine-derived α -KG has been raised to overcome this limitation. LDH and MDH enzymes convert this α -KG to L-2-HG as a side function (Oldham et al., 2015). This study investigated whether these enzymes contributed to D-2HG generation in addition to wt IDH1/2 in colon cancer. The qPCR results

show that the MDH2 expression level is raised ($p < 0.05$), whereas the LDHA level was not changed in SW620 according to the control group in both *in vitro* (Figure 18B) and *in vivo* (Figure 15 B) models. Similarly, the MDH2 enzyme was highly expressed ($p < 0.05$) in IDH1 KO1-2 than in IDH1 WT (Figure 29A), and a high mRNA level was observed in tumor tissues taken from PDX models (Figure 42C). The significant rise in the mRNA level of the MDH2 showed that this enzyme may play a role in the generation of D-2HG. Both MDH and LDHA inhibitors showed significant results, D-2HG level was blocked (Figure 32), and proliferation of IDH1 KO cells was reduced (Figure 31). We found that in addition to wt-IDH1, LDHA and MDH2 enzymes contribute to the generation of D-2HG in colon cancer cells. The effect of some GLS inhibitors (Zaprinast, CB-839, and BPTES) on reducing D-2HG levels were found in different cell lines (Elhammali et al., 2014; Špačková et al., 2021). However, the effect of MDH and LDH inhibitors on D-2HG generation was firstly examined in this study.

In the next step of the thesis studies, the R132H mutation was created by CRISPR/Cas9 method to determine whether mutant IDH enzymes play a role in the 2-HG generation. The Cas9 enzyme mediates site-specific DSB in the interest location of the target DNA, and the DSBs should be repaired with an HDR template to make specific point mutations. Nowadays, CRISPR/Cas method has been widely used to generate different disease models (Wu et al., 2020). In this thesis study, site-specific and high-efficiency point mutations were successfully generated using this method, and it has shown great potential to generate new disease models in the future.

According to the Sanger sequencing results, it was observed that the R132H mutation was heterogeneously generated (Figure 35B). The mutant allele gained neomorphic enzymatic activity by reducing α -KG to 2-HG, but the wt allele continued the oxidative decarboxylation of citrate. The α -KG level must be present in the steady-state phase for the cell to survive. Therefore, one allele of the IDH1 gene can remain wild-type (Dexter et al., 2018). The generation of D-2HG was not changed in IDH1 R132H cells compared to the control (Figure 37A). Relatedly, the level of glucose was also unaffected by this mutation (Figure 37A). Similarly, the R132H mutation did not affect the cancer cell proliferation, colony formation capacity, or cell morphology (Figure 36B and Figure 37C-D). This mutation was generated in glioma cells to examine the role of the R132H mutation in the 2-HG generation. Intracellular TCA cycle metabolite levels were significantly altered in IDH1 R132H glioma cells compared

to the control. The intracellular 2-HG level in these cells raised approximately 3000-fold compared to the control (Biedermann et al., 2019). The findings reveal that D-2HG generation is independent of R132H mutation in SW620 colon cancer cells.

In the last part of this thesis, 2-HG metabolism was investigated using the colon cancer tissues in addition to *in vitro* and xenograft models. The biopsy samples from colon cancer patients were enlarged using PDX models, and subcutaneous tumor tissues taken from PDX models were used for different analyses (Figure 39). The most significant advantage of the PDX model is that it provides the large amount of tissue sample required for both the determination of D-2HG levels and the mRNA levels of significant enzymes role in the D-2HG generation. In addition, not only tumors but also colon and liver tissues taken from PDX models were used to determine some metabolite concentration changes in energy metabolism (Figure 41). D-2HG level was found to be high ($p < 0.05$) in tumor tissue compared to colon and liver tissues taken from PDX models (Figure 41). Relatedly, most of the TCA cycle intermediates were found at the highest levels in tumor tissue, while the levels of glucose and pyruvate molecules were highest in liver tissue. Shen et al. (2021) performed metabolomics analysis of cancerous and healthy colon tissues taken from colorectal cancer patients, and it was found that glucose and citrate levels were low and succinate and 2-HG levels were high in cancer than in healthy tissue (Shen et al., 2021), similar to our results. When the mRNA levels of enzymes that contributed to 2-HG generation were evaluated in tumor tissues taken from PDX models, the results were identical to those seen *in vitro* and *in vivo*. In several tumor tissues, the expression of IDH1, GLS, and LDHA genes decreased, but the expression of IDH2 and MDH2 genes raised compared to healthy tissues (Figure 42).

6. CONCLUSION AND RECOMMENDATIONS

This thesis study consisted of four different aims. Firstly, it was investigated whether the oncometabolite D-2HG has a role in colon cancer metastasis. The studies were carried out with colon adenocarcinoma cell lines in the primary and metastatic stages. When cells acquire metastatic properties, the intracellular D-2HG level was significantly raised in colon adenocarcinoma cell lines but not in colon epithelial cells. Similar results were achieved in the xenograft models. As a result, the first aim of thesis was successfully completed when the highly generated D-2HG oncometabolite was revealed to be closely related to colon cancer metastasis. In the future, the 2-HG level in other colon cancer cell lines at primary and metastatic stages can be examined to raise the reliability of this conclusion.

The second aim of this thesis research was to examine the pathways of D-2HG generation in colon cancer. The enzymes involved in the generation of 2-HG vary according to the cancer type. In this study, silencing the wt IDH1 enzyme led to reducing the D-2HG level by around half compared to the control. After treatment with MDH and LDH enzyme inhibitors, 2-HG generation was completely inhibited. Furthermore, the D-2HG level was not changed in cell models with the R132H mutation, indicating that 2-HG generation was unaffected by the R132H mutation. This study discovered for the first time that MDH2 and LDHA enzymes, in addition to the wt IDH1 enzyme, contributed to 2-HG generation under normal conditions. Thus, the second aim of the thesis study was successfully completed. In the future, the effect of MDH2 and LDHA enzyme inhibitors in 2-HG generation can be tested on xenograft models generated with IDH1 KO cells.

The third aim of this thesis was to study the role of the IDH1 enzyme in the progression of colon carcinogenesis. Several notable phenotypic changes, including G0/G1-arrest and reducing proliferation, invasion, and migration properties, were detected when the IDH1 enzyme was silenced using the CRISPR/Cas9 technique. This aim has been successfully accomplished thanks to these findings, which reveal that the IDH1 enzyme plays a crucial role in the development of colon cancer. In the future, the xenograft model can be generated using IDH1 KO cells to monitor subcutaneous tumor development. Thus, the role of the IDH1 gene in colon cancer proliferation will be demonstrated by *in vivo* models in addition to *in vitro* experiments.

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8. ADDENDA

8.1. Ethics Committee Reports

DOKUZ EYLÜL ÜNİVERSİTESİ
KLİNİK ARAŞTIRMALAR ETİK KURULU

Konu: Karar hk.

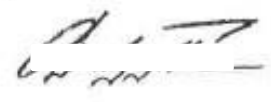
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28.03.2019

Prof.Dr.Ender ELLİDOKUZ,

Kurulumuz tarafından 28.03.2019 tarih ve 473-SBKA EK protokol numaralı 2019/06-32 karar ile onayı alınan “Kolon Kanseriinde 2-Hidroksiglutaratın Metabolizma ve Epigenetik Üzerine Etkisinin Aydınlatılması” konulu araştırmanıza ilişkin Kurulumuz kararı ekte sunulmuştur.

Bilgilerinizi ve gereğini rica ederim.


Prof.Dr.Ayşegül YILDIZ
Başkan

Ek: Etik Kurul Kararı

Dokuz Eylül Üniversitesi Sağlık Yerleşkesi İnciraltı 35340 İZMİR-TÜRKİYE
Tel:0 232 4122254 - 0 232 4122258 Faks: 0232 4122243 Elektronik posta:etikkurul@deu.edu.tr

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Kolon Kanseri ve 2-Hidroksiglutarat Metabolizması ve Epigenetik Üzerine Etkisinin Aydınlatılması
VARSA ARAŞTIRMANIN PROTOKOL KODU	-
ETİK KURUL PROTOKOL NUMARASI	473-SBKAEK

ETİK KURUL BİLGİLERİ	ETİK KURULUN ADI	Dokuz Eylül Üniversitesi Klinik Araştırmalar Etik Kurulu
	AÇIK ADRESİ	Dokuz Eylül Üniversitesi Sağlık Yerileşkesi Dekanlık Binası Kat:2 İçişleri 35340 İZMİR-TÜRKİYE
	TELEFON	0 232 4122254 - 0 232 4122258
	FAXS	0232 4122243
	E-POSTA	etikkurul@deu.edu.tr

BAŞVURU BİLGİLERİ	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADUSOYADI	Prof.Dr.Ender ELLİDOKUZ
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	İç Hastalıkları/Gastroenteroloji
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	Dokuz Eylül Üniversitesi Tıp Fakültesi İç Hastalıkları A.D, Gastroenteroloji B.D
	VARSA İDARI SORUMLU UNVANI/ADUSOYADI	-
	DESTEKLEYİCİ	-
	PROJE YÜRÜTÜCÜSÜ UNVANI/ADUSOYADI	Prof.Dr.Hülya AYAR KAYALI
	(TIBBİ VE/VEYA TIBBİ KAYNAKLARDAN DIŞI EKLER İÇİN) DESTEKLEYİCİNİN YASAL TEMSİLCİSİ	-
	ARAŞTIRMANIN FAZİ VE TÜRÜ	FAZ 1 ☐ FAZ 2 ☐ FAZ 3 ☐ FAZ 4 ☐ Güvenlik ilağı uygulanması ☐ Tıbbi etkiyi klinik araştırmaya ☐ In vivo tıbbi sonuçlarının in vitro yapılan performans değerlendirmeye çalışmaları ☐ İlaç dışı klinik araştırmaya ☒ Diğer ise belirtiniz
	ARAŞTIRMAYA KATILAN MERKEZLER	TÜK MERKEZ ☒ ÇOK MERKEZLİ ☐ ULUSAL ☒ ULUSLARARASI ☐

DEĞERLENDİRİLEN BELGELER	Belge Adı	Tarihi	Versiyon Numarası	Dil
	ARAŞTIRMA PROTOKOLÜ	20.03.2019	Versiyon 1	Türkçe ☒ İngilizce ☐ Diğer ☐
	Bilgilendirme Gerekli Olur Formu	20.03.2019	Versiyon 1	Türkçe ☒ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU	20.03.2019	Versiyon 1	Türkçe ☒ İngilizce ☐ Diğer ☐
	ARAŞTIRMA BROŞÜRÜ			Türkçe ☐ İngilizce ☐ Diğer ☐
DEĞERLENDİRİLEN DİĞER BELGELER	Belge Adı			Açıklama
	SIGORTA	☐		
	ARAŞTIRMA BÜTÇESİ	☐		
	BEYOLOJİK MATERYEL TRANSFER FORMU	☐		
	ILAN	☐		
	YILLIK BİLDİRİM	☐		
	SONUÇ RAPORU	☐		
	GÜVENLİLİK BİLDİRİMLERİ	☐		
	DİĞER:	☒		-Klinik Araştırmalar Başvuru Formu, -Araştırma Akış Şeması, 20.03.2019 -Araştırmacı Özgeçmişleri

Fakülte Kurul Başkanı

Unvanı/Adı/Soyadı: Prof.Dr.Ayşegül Yıldız

İmza:

Not: Etik kurul başkanı, imzalarını yer aldıkları her sayfaya imza atmalıdır.

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN ADI	Kolon Kanseri ve 2-Hidroksiglutarat Metabolizması ve Epigenetik Üzerine Etkisinin Aydınlatılması
VERİSİ ARAŞTIRMANIN PROTOKOL KODU	-
ETİK KURULU PROTOKOL NUMARASI	ETK-SBR/2019

KARAR BİLGİLERİ	Konferans No:201904-32 Tarih:29.03.2019 Yukarıda bilgileri verilen bağayarı dosyası ile ilgili belgeler araştırmanın/çalışmanın gerekçe, amaç, yaklaşımları ve yöntemleri dikkate alınarak incelenmiş ve uygun bulunmuş olup araştırmanın/çalışmanın bağayarı dosyasında belirtilen merkezlerde gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına ilişkin etik kurul üye tam sayısının salt çoğunluğu ile karar verilmiştir. İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik kapsamında yer alan araştırmalar/çalışmalar için Türkiye İlaç ve Tıbbi Cihaz Kurumu'ndan izin alınması gerekmektedir.
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KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA BAŞI	İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, İyi Klinik Uygulamalar Kılavuzu
BASKININ UNVANI / ADI / SOYAD	Prof. Dr. Aygün YILMAZ

Unvanı/Adı/Soyadı	Ünvanlık Alanı	Kuruma	Cinsiyet	Araştırma ile ilgili	Katılım *	İmza
Prof. Dr. Aygün YILMAZ	Psikiyatri	DEÜ Tıp Fakültesi Psikiyatri Anabilim Dalı	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Prof. Dr. Hilmi ELLİDOĞRU	Halk Sağlığı	DEÜ Onkoloji Enstitüsü Preventif Onkoloji A.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Prof. Dr. Nergiz DUMAN	Çocuk Sağlığı ve Hastalıkları (Yeni Doğan)	DEÜ Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	E ☐ K ☒	E ☐ H ☒	E ☐ H ☒	katipli
Prof. Dr. Aygün KABAL	Anesteziyoloji ve Reanimasyon	DEÜ Anesteziyoloji ve Reanimasyon A.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Prof. Dr. Feriye KESKİNOĞLU	Biyostatistik	DEÜ Tıp Fakültesi Biyostatistik ve Tıbbi Bilimler A.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Prof. Dr. Erdem YAKA	Nöroloji	DEÜ Tıp Fakültesi Nöroloji A.D.	E ☒ K ☐	E ☐ H ☒	E ☐ H ☒	katipli
Prof. Dr. M. Cenk ECEVET	KBB	DEÜ Tıp Fakültesi KBB A.D.	E ☒ K ☐	E ☐ H ☒	E ☐ H ☒	katipli
Prof. Dr. Uluc YETİ	Çocuk Sağlığı ve Hastalıkları (Çocuk Nöroloji)	DEÜ Tıp Fakültesi Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	E ☒ K ☐	E ☐ H ☒	E ☒ H ☐	
Prof. Dr. Tuğba YAVUZSEİN	Onkoloji	DEÜ Tıp Fakültesi İç Hastalıkları A.D. Tıbbi Onkoloji B.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Doç. Dr. Uğur ÖZEL TURK	Kardiyoloji	Kardiyoloji Kalp Hastalıkları Merkezi	E ☒ K ☐	E ☐ H ☒	E ☒ H ☐	
Doç. Dr. Yavuz ERAC	Farmakoloji	Ege Üniversitesi Eczacılık Fakültesi Farmakoloji Anabilim Dalı	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Doç. Dr. İlyas AKSU	Fizyoloji	DEÜ Tıp Fakültesi Fizyoloji A.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Öğr. Gör. Dr. Cevat Rıfat GÖVERCİN	Tıp Tarihi ve Etik	DEÜ Tıp Fakültesi Tıp Tarihi ve Etik A.D.	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Av. Nerve ÖZDEMİR TANIR	Hukuk	Hukuk	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	
Hayri ALBAYRAK	Sağlık Müşeri Müşaviri Olmayan Üye	Emekli	E ☐ K ☒	E ☐ H ☒	E ☒ H ☐	

* Toplamda Bulunan

Etik Kurul Başkanı

Unvanı/Adı/Soyadı: Prof. Dr. Aygün YILMAZ

İmza:

Not: Etik kurul başkanı, incelenen her sayfaya imza atmalıdır.



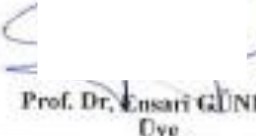
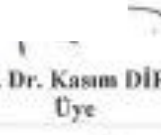
**İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(İBG-HADYEK)
KARARI**

TOPLANTI TARİHİ	06/03/2019	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	3	TOPLANTI SAATİ	15.30

Sayın Prof. Dr. Hülya Ayar Kayalı,

17/2019 Protokol No'lu; yürürlüğe konduğunuz "Kolon kanserinde 2-Hidroksiglutaratın metabolizma ve epigenetik üzerine etkisinin aydınlatılması" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

 Doç. Dr. Güneş ÖZHAN BAYKAN Başkan	 Vet. Hek. Kerem ESMEN Başkan Yardımcısı
 Prof. Dr. H. Alper BAKIRYANIK Üye	 Dr. Gülçin ÇAKAN AKDOĞAN Üye
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İZMİR BİYOTIP VE GENOM MERKEZİ
Hayvan Deneyleri Yerel Etik Kurulu

Prof. Dr. Hülya AYAR KAYALI

Kurulumuz tarafından onaylanmış olan 17/2019 protokol numaralı "Kolon Kanseriinde 2- Hidroksiglutaratın Metabolizma ve Epigenetik Üzerine Etkisinin Aydınlatılması" projenizin başlığınızın "2- Hidroksiglutarat Onkometabolitinin Kolon Kanseri Metabolizması Üzerindeki Etkisinin CRISPR/Cas9 Tabanlı Gen Terapisi ile Oluşturulan Hücre Modelleri ve İn vivo Modeller Üzerinde Araştırılması" olarak değiştirilmesi Kurulumuz tarafından oybirliği ile onaylanmıştır.

Bilgilerinizi arz ederim.

Dr. Gülçin ÇAKAN AKDOĞAN
İBG-HADYEK Kurul Başkanı

05/01/2022

8.2. Curriculum Vitae

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Scientific Technological Activity Fields

Scientific Technological Activity Field Information

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Kanser Moleküler Biyolojisi

Temel Bilimler -- Yaşam Bilimleri -- Biyokimya -- Metabolizma

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Kolon Kanseri

CRISPR

Kanser Metabolizması

Enerji Metabolizmaları

R&D Competency

Articles

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E. BULUT ATALAY & M. KAYA, Production of Three-Dimensional Chitin and It's Potential Application Areas, Sözlü Sunum, 2nd International congress on the world of technology and advanced materials (WITAM), 28 September 2016, 02 October 2017.

İ. SARGIN, M. KAYA, E. BULUT ATALAY, B. AKYÜZ & G. ARSLAN, Corn Silk And Sugar Beet Pulp For Removal Of Carcinogenic And Mutagenic Dye (congo Red) From Aqueous Solution?, Poster Sunumu, Ecology 2017 International Symposium, 11 May 2017, 13 May 2017.

İ. SARGIN, M. KAYA, B. AKYÜZ, E. BULUT ATALAY & G. ARSLAN, Macroalgal Bloom Biomass For Congo Red Removal: Equilibrium, Kinetic, thermodynamic And Competitor Ions Effects Studies, Sözlü Sunum, Ecology 2017 International Symposium, 11 May 2017, 13 May 2017.

İ. SARGIN, M. KAYA, B. YILMAZ, E. BULUT ATALAY & G. ARSLAN, Macroalgal Bloom Biomass for Congo Red Removal: Equilibrium, Kinetic, Thermodynamic and Competitor Ions Effects Studies, Sözlü Sunum, ECOLOGY 2017 INTERNATIONAL SYMPOSIUM, 11 May 2017, 13 May 2017.

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Awards

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, Sözlü Sunum Ödülü, En İyi Sözlü Sunum Üçüncülük Ödülü, Awarded Institution: 3rd International Cancer and Ion Channels Congress (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 18 September 2021.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, best oral presentation, best oral presentation, Awarded Institution: BIO Türkiye International Biotechnology Congress (Sistemde kayıtlı olmayan kuruluş), TÜRKİYE, 11 September 2021.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, best poster abstract, Bu ödül 2nd International Cancer and Ion Channels Kongresi tarafından en iyi 3. poster ödülü olarak verilmiştir, Awarded Institution: DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE, 24 September 2019.

TÜBİTAK Scholarships and Fundings

Project Info

114Z466, İstilacı Çekirge Türü *Doclostaurus Maroccanus* (Thunber, 1815)'ün Teknolojik Bir Ürün Olan Kitin Ve Kitosana Dönüştürülmesi, 1002 - Hızlı Destek, Burslu, Sonuçlandı, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 9/8/14 - 8/15/15, Project Start/End Dates: 8/15/14 - 8/15/15.

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BİDEB Supports

ESRA BULUT ATALAY, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-A Genel Yurt İçi Doktora Burs Programı, Destekleniyor, 2019 - 1, 3/1/19 - 9/30/22.

8.3. Publications from the Thesis

- **Bulut Atalay, E.,** Ayar Kayalı, H. (2022). Investigation of Energy Metabolism in The Different Tissues of Colon Cancer Xenograft Models Based on The TCA and Cori Cycles and Glycolysis. *International Journal of Engineering Research and Development*. In press.
- **Bulut Atalay, E.,** Ayar Kayalı, H. (2022). The elevated D-2-hydroxyglutarate level found as a characteristic metabolic change of colon cancer in both in vitro and in vivo models. *Biochemical and Biophysical Research Communications*. In press.
- **Bulut Atalay, E.,** Sentürk, S., Ayar Kayalı, H. (2022). Wild-type IDH1 knockout leads to G0/G1 arrest, impairs cancer cell proliferation, altering Glycolysis, and the TCA cycle in colon cancer. *Biochemical Genetics*. Under review.



The elevated D-2-hydroxyglutarate level found as a characteristic metabolic change of colon cancer in both *in vitro* and *in vivo* models

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ABSTRACT

The D-2-hydroxyglutarate (D-2-HG), whose normal cellular concentration is low, can be accumulated 10–100 times natural levels in some cancer types and participates in the carcinogenesis process. D-2-HG is produced by different pathways specific to cancer type. In this study, the level of significant metabolites produced in some metabolic pathways related to D-2-HG in the energy metabolism was determined in colon adenocarcinoma cell lines at different stages. Then, the differences in TCA and Cori cycle, glutaminolysis, and Glycolysis were investigated in the brain, colon, liver, and tumor tissues extracted from xenograft models. The levels of glucose, pyruvate, lactate, all TCA cycle intermediates, and D-2-HG were determined by the HPLC analysis, DNS method, and pyruvate assay. The intracellular D-2-HG level was found at 22.6 $\mu\text{mol}/\text{mg}$ in primary (Caco-2) and 152.6 $\mu\text{mol}/\text{mg}$ in metastatic (SW620) colon adenocarcinoma cells, whereas it could not be detected in colon epithelial cell line (CCD-18Co). In the xenograft models, D-2-HG could not be detected in CCD-18Co colon and brain tissues, whereas it was produced in Caco-2 and SW620 tissues. Most importantly, the level of D-2-HG was 7.4 and 19.9-fold increased in Caco-2 and SW620 tumor tissues compared to healthy tissue, respectively. In addition, the D-2-HG production pathways were investigated. The results revealed that the carbon source of D-2-HG is glucose, and the imbalance of wt-IDH1/2 enzymes plays a role in its production. Overall, the *in vitro* and *in vivo* results show that the enhanced production of endogenous D-2-HG is a characteristic change in the metabolism of colon cancer.

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1. Introduction

Deregulation of the cancer cell metabolism to deal with the need for energy, redox cofactors, and biomass is among the 10 hallmarks of cancer [1]. Depending on the phenotype of the cancer cell, different essential metabolic functions are rearranged to increase proliferation, invasion, motility, and other cellular phenotypes that lead to cancer initiation and progression. The function of many energy metabolisms such as Glycolysis, TCA cycle, and glutaminolysis was reprogrammed during the progression of cancer [2]. The TCA cycle is a fundamental turntable of cell metabolism [3]. In addition to energy production, produced TCA intermediates are used for the biosynthesis of amino acids, fatty acids, and nucleic acids, which are the building blocks of the cell. Due to it being crucial for basal metabolism, the level of oncometabolite produced

in mutant or wild-type (wt) enzymes of the TCA cycle may have a devastating effect on the cell such as tumor formation and cancer [2].

Colon cancer, the third most common type of cancer worldwide, is a clinically significant disease due to its mortality and high incidence, with approximately 1.9 million new cases in 2020 [4,5]. Unfortunately, more than half of new cases are diagnosed with advanced stages, stages III and IV [6]. Surgery, chemotherapy, and radiotherapy are the most commonly used methods in treatment. Although advanced metastatic surgery and improved options for treatment, the 5-year survival rate for late-stage patients has not been increased [7]. It has highlighted the requirement for new therapeutic methods, and metabolic reprogramming contributes to the malignant transformation has become a field of increasing interest [8]. Metabolites, known as intermediate, are small molecules produced by metabolic pathways and involved in cell growth and survival [9]. If the metabolite level rises to a point where it will cause metabolic disorder and progression of the cell into cancer, it is called an oncometabolite. While the oncometabolite level is low in healthy cells, their synthesis and accumulation in high amounts direct the cell to metastasis by activating the signaling pathways [10]. Consid-

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ering these properties, examining metabolite levels becomes very important in the early diagnosis of cancer. Therefore, scientists were focused on profiling and classifying metabolites in the plasma, serum, urine, or tumor tissue to determine if they could be a biomarker to detect pre-cancerous lesions and/or metastatic colon cancer [11–14].

In the TCA cycle, the potential impact of specific metabolites on tumorigenesis was demonstrated by the isocitrate dehydrogenase 1 and 2 (IDH1/2), which is considered a driver gene [15]. NAD⁺/NADP⁺-dependent IDH enzymes perform the reaction of oxidative decarboxylation of isocitrate. Catalytic mutation in the arginine residue results in the neomorphic activity that leads to the reduction of α -KG to the D-2-HG oncometabolite [10]. However, D-2-HG is also produced by wt-IDH enzymes, and recently many studies have focused on this subject [16]. In colon cancer, only bioinformatics studies have been conducted on the D-2-HG with wt-IDH enzymes [17]. In our study, the differences in the metabolite concentration produced in the TCA cycle and/or Glycolysis in different stages of colon cancer were profiled *in vitro* and *in vivo* models. Then, the D-2-HG production pathways were investigated in the metastatic colon adenocarcinoma cell lines. Xenograft models have been used to examine how the concentration of specific intermediates changes in the brain, colon, liver, and tumor tissues. It also allows examining the tissue type-specific metabolite levels.

2. Material and methods

2.1. Cell lines and animals

In the study, colon epithelial cell (CCD-18Co), primary colon cancer cell (Caco-2), and metastatic colon cancer cell (SW620) lines were used. Cell culture conditions were applied as previously described [18]. The 6–8 weeks old male or female athymic Nude mice (Foxn1^{nu}) were housed in IBG-Vivarium Rodent Facility. The animal care conditions were 22 ± 2 °C, with 12 h of light/dark periods and access to food and water *ad libitum*. All conditions were approved by Izmir International Biomedicine and Genome Institute Local Ethics Committee for Animal Experiments (IBG-AELEC), protocol number: 17/2019.

2.2. Intracellular metabolite extraction from cells

One million of each cell line were seeded into Petri dishes. Cells were trypsinized and counted using thoma lame 24, 48, 72, and 96 h later. Metabolites were extracted from the cell pellet containing 1 million cells with cold 50% MeOH: water (v/v). Cells were lysed in an ultrasonic bath and, after incubation on ice for 10 min centrifuged at 17,000 g and +4 °C for 15 min. The supernatant was used for HPLC analysis and glucose and pyruvate assays. The supernatant was removed using a vacuum centrifuge, and until analysis, the pellet was kept at –80 °C (Fig. 1A) [19]. Before analysis, the pellet was resuspended with double distilled water and spiked with internal standards such as (R)-2HG (16859, Sigma).

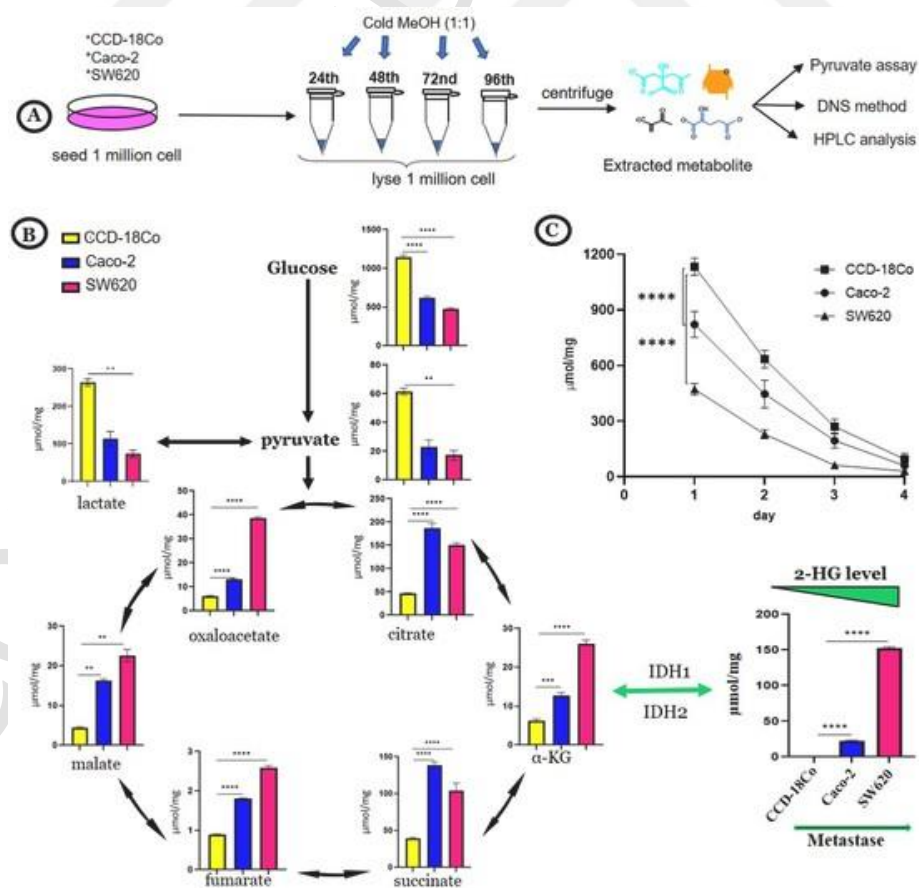


Fig. 1. Glucose metabolism to TCA cycle in colon epithelial and colon adenocarcinoma cell lines. (A) 1 million cells of each cell line were plated in a Petri dish. 24–96 h later cells were trypsinized and counted with Thoma lame. Cell pellets consisting of 1 million cells were lysed and after centrifuge extracted metabolites were taken from the upper phase. (B) Intracellular pyruvate level was determined by spectrophotometric method and lactate, citrate, α -KG, succinate, fumarate, malate, oxaloacetate, and D-2-HG levels were detected by the HPLC method at 24 h. (C) Time-dependent glucose levels were determined by the DNS method at 24–96 h **p < 0.01, ***p < 0.001, ****p < 0.0001.

2.3. Extraction of metabolites from the extracellular medium

One million cells were seeded into Petri dishes for each cell line. The conditioned medium was taken from the Petri dishes after 24, 48, 72, and 96 h and kept at -80°C until analysis. Before analysis, the conditioned medium was centrifuged at 17,000 g and $+4^{\circ}\text{C}$ for 15 min [19]. The metabolite concentration in the growth mediums (DMEM, MEM, and RPMI) was accepted as the baseline. The results of extracellular metabolite levels were calculated to give the metabolite level corresponding to 1 million cells (Fig. 2A). The released and consumed metabolite levels calculated using the formula were given below [20].

Extracellular metabolite level = $[X]_{\text{conditioned medium}} - [X]_{\text{growth medium}} / \text{cell number}$

2.4. Extraction of metabolites from the tissues isolated from xenograft models

50–100 mg of tissues were weighed and ground in cold solvents (1:5, mg/mL) in ice for approximately 5 min by tissue grinder (NGO10). The liver and brain tissues were homogenized in 100% MeOH, and the colon and tumor tissues were homogenized in chloroform:MeOH: water (2:5:2). After incubation on ice for 20 min, it was centrifuged at 16,000 g for 15 min at $+4^{\circ}\text{C}$. The supernatant was removed using a vacuum centrifuge, and the pellet was kept at -80°C until analysis. Before analysis, the pellet was resuspended with double distilled water [21].

2.5. Determination of reduced glucose by 3,5-dinitrosalicylic acid (DNS) method

The glucose content of cell pellets and growth media was determined by the DNS method. The DNS reagent was prepared as previously described [22]. Sample and DNS reagents were added at a ratio of

1:1 and mixed for 10 min at 100°C , and then incubated for 1 min in ice. Samples and standard glucose solutions (100–4500 mg/L) were measured at 540 nm using a spectrophotometer (Thermo, MULTISCAN GO).

2.6. Colourimetric determination of pyruvate

The previously used protocol was optimized to apply to 100 μL (96 well plates) [23]. Measurements were taken at 520 nm absorbance.

2.7. Analyzing metabolites by HPLC system

A Shimadzu HPLC system has an Alltech OA-1000 column, a UV and a RID detector were used. The mobile phase (0.4 mL/min) in 9.0 mM H_2SO_4 solution was applied to the column run at 42°C . Lactate, citrate, α -KG, oxaloacetate, succinate, fumarate, D-2-HG, and malate standards were detected in the UV detector (210 nm). All standards were HPLC-grade and purchased from Sigma.

2.8. DNA isolation and polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR)

Details of DNA and RNA isolation, PCR and RT-PCR conditions, and primer list are presented in the Supplementary Information.

2.9. In vivo studies

It was studied with 3 different groups of CCD-18Co, Caco-2, and SW620 cells, and each group included four mice. A suspension of 5 million CCD-18Co, Caco-2, and SW620 cells in the growth medium was mixed with matrigel (1:1) and subcutaneously injected into a flank of mice [24]. The volume of the subcutaneous tumor was measured with a caliper once a week for 4 weeks. The formula is given below. When the

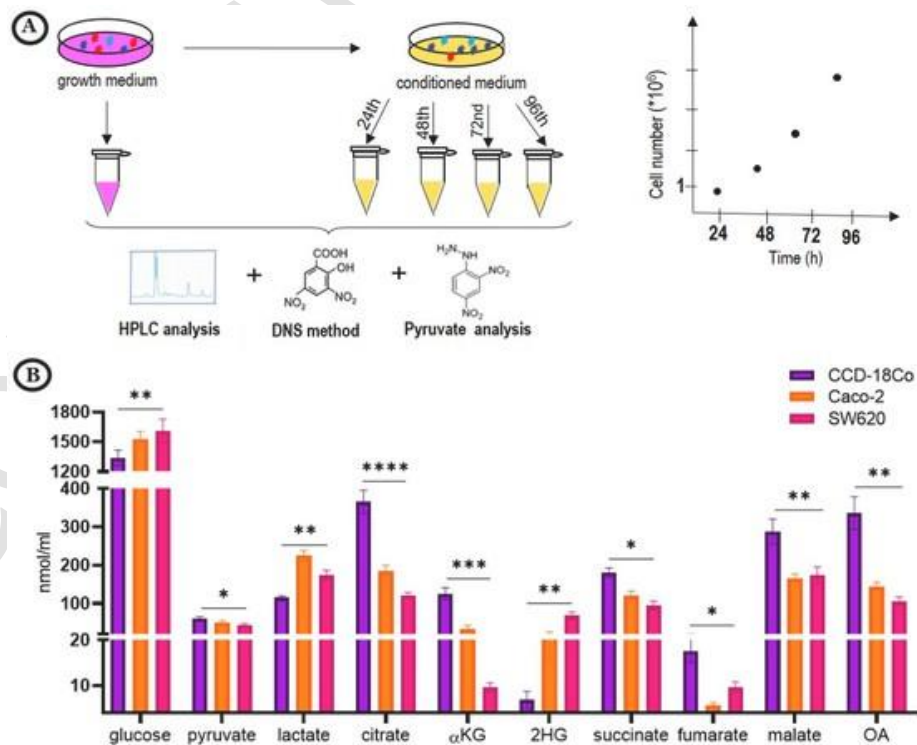


Fig. 2. Extracellular metabolite levels in 1 million cells in colon epithelial and colon adenocarcinoma cell lines in 24 h. (A) 1 million cells were plated in a Petri dish for each cell line. The conditioned medium was taken from the Petri dishes after 24–96 h, and cells were counted at these times. (B) Pyruvate and glucose levels were determined by pyruvate assay, and DNS methods, respectively. Lactate, citrate, α -KG, succinate, fumarate, malate, oxaloacetate, and D-2-HG levels were detected by the HPLC method at 24 h * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

diameter reached 1 cm³, the mice were sacrificed using cervical dislocation. The extracted tissues were quickly placed on ice, and then at least 3 different pieces of each tissue were taken and stored at –80 °C until metabolite extraction [19,24]. The remaining pieces of tumor tissue were taken up in the RNAlater solution and 10% formalin for RNA isolation and H&E staining, respectively.

$$\text{Tumor volume} = (\text{length} \times \text{width}^2) \times 0.5$$

2.10. H&E staining

When the xenograft models were sacrificed, a portion of the tumor tissue was taken in 10% formalin for H&E staining. As previously described, the H&E staining protocol was used [25].

2.11. Statistical analysis

The experiments were repeated at least three independent times, and the data were obtained separately in triplicate. Averages $\pm$ SEM are shown for three independent experiments. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, CA, USA). Differences between the three groups were analyzed by the one-way ANOVA followed by Tukey's multiple comparisons test. $p < 0.05$ was considered statistically significant and indicated by an asterisk (*) symbol on the graph.

3. Results

3.1. Investigation of intracellular concentration changes in several metabolites of energy metabolism

Significant differences are observed in the metabolites produced due to different phenotypes, such as proliferation and metastasis of cancer cells [2]. Therefore, metabolic changes in primary and metastatic colon cancer cell lines were studied by examining the intracellular and extracellular levels of glucose, pyruvate, lactate, citrate, α -KG, succinate, fumarate, malate, oxaloacetate, and D-2-HG. The results were compared with the colon epithelial cell (CCD-18Co). The intracellular metabolite levels are determined in 1 million cells at 24–96 h (Fig. 1A).

The intracellular glucose was 1.8 and 2.4-fold decreased ($p < 0.001$) in primary and metastatic cancer cells compared to healthy cells, respectively (Fig. 1B). The concentration of intracellular pyruvate was 2.7 and 3.6-fold decreased in Caco-2 and SW620 according to the healthy cell, respectively (Fig. 1B). Similarly, the intracellular lactate level was 2.3 and 3.5-fold decreased ($p < 0.05$) in the primary and metastatic cells than in colon epithelial cells, respectively (Fig. 1B). Intracellular citrate, α -KG, succinate, fumarate, malate, and oxaloacetate levels were interestingly 3.9, 1.8, 3.4, 2.0, 3.8, 2.1-fold increase in Caco-2, and 3.1, 3.8, 2.6, 2.8, 5.2, 6.8-fold increase ($p < 0.05$) in SW620 compare to the CCD-18Co, respectively. Most importantly, the intracellular D-2-HG level was detected at 22.6 $\mu\text{mol}/\text{mg}$ in Caco-2 and 152.6 $\mu\text{mol}/\text{mg}$ in SW620, while it could not be detected in the colon epithelial cell (Fig. 1B). The use of more glucose by colon adenocarcinoma cells indicates that these cells are under the Warburg effect. However, the high intermediate concentration reveals that the metabolism in these cells does not proceed through Glycolysis and the TCA cycle is active (Fig. 1B).

The time-dependent change of intracellular glucose was determined by its levels at 24–96 h (Fig. 1C). Intracellular glucose level was lower in cancer cells than in healthy cells during the time. The results indicate that metastatic cancer cell lines metabolize more glucose than their primary counterparts.

3.2. Investigation of time-dependent extracellular concentration changes in several metabolites of energy metabolism

The profiling of the extracellular metabolites in the consumed medium provides a systematic evaluation of the metabolic activity of cultured cells [20]. In this study, the time-dependent metabolic changes were profiled in primary and metastatic colon cancer and colon epithelial cell lines. The metabolite concentration in the growth mediums (DMEM, MEM, and RPMI) was accepted as the baseline. The results of extracellular metabolite levels were calculated to give the metabolite level corresponding to 1 million cells (Fig. 2A). As shown in Fig. 2B, glucose was more consumed, whereas pyruvate was less released into the medium in the colon adenocarcinoma cell lines at 24 h. The extracellular lactate levels were 1.8 and 1.4-fold ($p < 0.01$) increased in Caco-2 and SW620 cells than CCD-18Co, respectively. All TCA cycle intermediates released by colon adenocarcinoma were consistently decreased compared to the colon epithelial cell line. However, only the level of extracellular D-2-HG released into the medium increased in cancer cells compared to healthy cells that produce it at a very low level. Caco-2 and SW620 cells were 3.2 and 11.4-fold ($p < 0.01$) released D-2-HG into the environment more than the healthy cell (Fig. 2B). The incredible increase in the released D-2-HG level was associated with the extracellular α -KG level (Fig. 2B). The extracellular citrate, α -KG, succinate fumarate, malate, and oxaloacetate levels were 2.0, 3.6, 1.4, 4.0, 1.7, 2.3-fold decreased in Caco-2, and 3.0, 9.4, 2.0, 1.8, 1.6, 3.1-fold decreased ($p < 0.05$) in SW620 according to CCD-18Co, respectively (Fig. 2B). As a result, glucose was metabolized to synthesize intermediates of the TCA cycle, and these metabolites are released into the medium over time.

3.3. Wt-IDH enzymes involved in the production of D-2-HG in SW620 cell lines

After significant differences were observed in the intracellular D-2-HG level, it was investigated whether this oncometabolite was produced with mutant IDH1/2 enzyme in SW620. As known in the previous studies on colon cancer, the mutation of IDH1 in Arg132 and G97 and the mutation of IDH2 in Arg172 and Arg140 directly affect the catalytic properties of enzymes leading to the D-2-HG production [25]. In this study, all the possible mutations in the IDH1 and IDH2 were investigated by the Sanger sequencing method. However, none of the possible mutations in the IDH1 and IDH2 genes was observed in the SW620 (Fig. 3A).

3.4. The imbalance of wt-IDH enzymes may lead to high D-2-HG levels in metastatic colon cancer cells

The TCA cycle is like the junction point of essential pathways in cell metabolism, such as the Cori cycle, glutaminolysis, and Glycolysis. Different enzymes in different metabolic pathways can affect the concentration of a TCA cycle intermediate. This is exemplified by D-2-HG production. Some glioma and AML cell lines that carry mutant IDH enzymes produce high levels of D-2-HG [10]. However, the level of D-2-HG was high in the cell contain not only mutant but also wt-IDH enzymes [26]. In these cells, D-2-HG production has been though mostly associated with an imbalance in the wt-IDH1/2 mRNA levels [17]. The current study analyzed the mRNA levels of wt-IDH1/2, LDHA, and GLS enzymes using the RT-qPCR method. It has been found that the mRNA level of IDH1 was relatively high in Caco2 and quite low in SW620 compared to the healthy cell (Fig. 3B). IDH2 and LDHA gene expression decreased in the early stage of cancer, but IDH2 was expressed at high levels as cancer progressed (Fig. 3B). The levels of intracellular α -KG and D-2-HG were increased 2.1 and 7-fold in the SW620 compared to Caco-2, respectively (Fig. 1B). The α -KG level was expected to decrease in SW620 because D-2-HG reduced from α -KG. However, the 2.5-fold in-

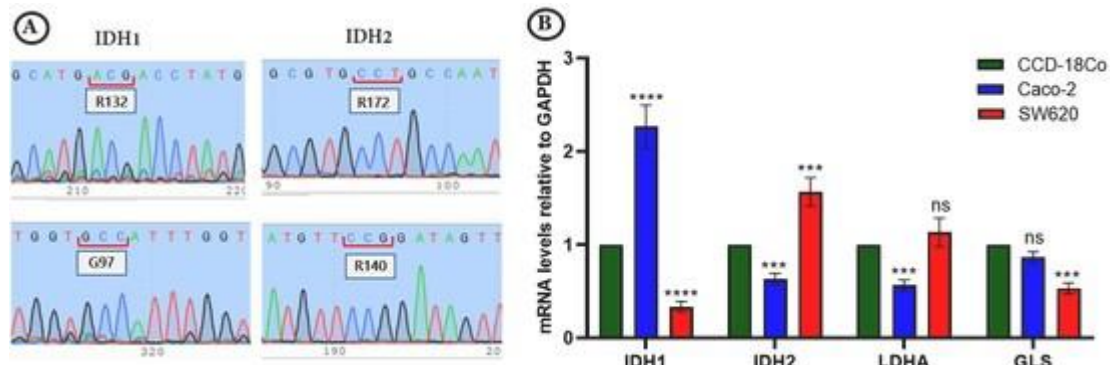


Fig. 3. Sanger method and RT-qPCR results. PCR products containing Arg132 and G97 residues in IDH1 and Arg140 and Arg170 residues in the IDH2 gene were sequenced by the Sanger method (A). mRNA levels of IDH1, IDH2, LDHA, and GLS genes. GAPDH was used as a control. *** $p < 0.001$, **** $p < 0.0001$.

creased mRNA level of the IDH2 enzyme in SW620 than Caco-2 can lead to an increase in α -KG level. According to the control group, the GLS level was decreased in both colon adenocarcinoma cell lines (Fig. 3B). We hypothesized that the α -KG level might be increased with glutamine anaplerosis, but the GLS level decreased slightly in the SW620 compared to the control ($p < 0.05$) (Fig. 3B). The result revealed that the carbon source of α -KG and D-2-HG is thought to be glucose. Significant differences in IDH1/2 levels show these enzymes play a role in D-2-HG production and cancer pathogenesis.

3.5. Histopathological analysis of xenograft models

Xenografts models were generated with CCD-18Co, Caco-2, and SW620 cells in the 6–8 weeks old male or female Nude mice (Fig. 4A). After sacrificing, a piece of tumor tissues was stored in %10 formalin for H&E staining. Finally, an expert veterinary pathologist evaluated the H&E staining results.

In the CCD-18Co tissue, cells were observed as lobular-solitary. The number of cells undergoing mitosis was very low, and mild polymorphism was observed. This tissue consists of healthy cells and is named healthy tissue because it does not exhibit tumor cell characteristics, as

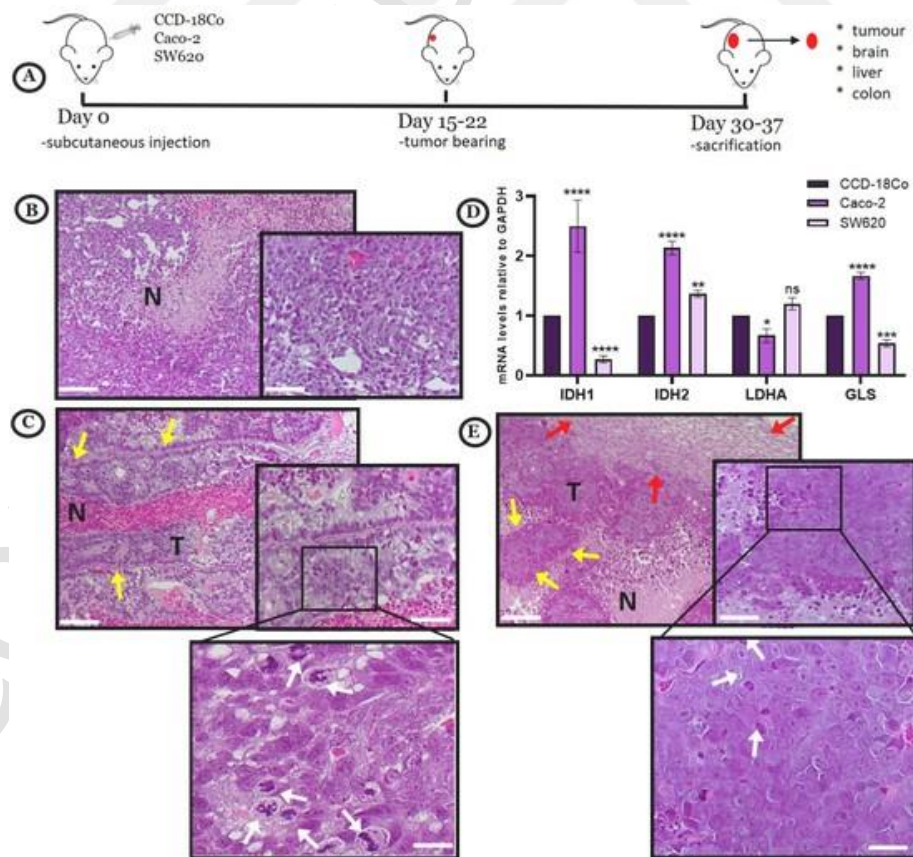


Fig. 4. Histopathology and RT-qPCR results of xenograft tumor tissues. Xenograft models were generated with subcutaneous injection of 5 million CCD-18Co, Caco-2, and SW620 cells into a flank of the 6–8 weeks old male or female Nude mice (A). Representative H&E staining images of CCD-18Co (B), Caco-2 (C), and SW620 (E). The lower panels illustrate enlargements apart from the upper panel (marked boxes). Borders and mitotic cells are indicated with yellow and white arrows, respectively. The capsule surrounding the tumor tissue was indicated with a red arrow. Scale bar is 200 μ m (B, C, E). mRNA levels of IDH1, IDH2, LDHA, and GLS genes of Caco-2 and SW620 tumor tissues and CCD-18Co healthy tissue. GAPDH was used as a control. Abbreviations: N necrosis and T tumor part. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

is seen in Fig. 4. The Caco-2 cells in the tumor tissues are multilobular localized, and the lobules are surrounded by borders (indicated with a yellow arrow). The difference in cytoplasm and nucleus in tumor cells was severe, and mitotic activity was intense (mitotic cells marked with white arrow) (Fig. 4C). Tumor parenchyma in the form of islands around blood vessels was observed in tumor tissues developed by SW620 cells. Both anisocytosis and anisonucleosis were clearly observed in tumor cells, and mitotic activity was moderately vigorous. The tumor tissue was surrounded by an external capsule (indicated with a red arrow) (Fig. 4E).

3.6. In vivo determination of mRNA levels of some enzymes involved in D-2-HG production

When the tumor and healthy tissues were removed from xenograft models, a piece of them was stored in RNAlater solution until RNA isolation. The mRNA levels of *IDH1*, *IDH2*, *LDHA*, and *GLS* were determined using the RT-qPCR method. *IDH1* was highly expressed in Caco-2 and lowly expressed in SW620 tumors compared to the CCD-18Co tissue (Fig. 4D). The gene expression level of *IDH2* and *GLS* was increased in the early stage of cancer but expressed at low levels as cancer progressed (Fig. 4D). The *LDHA* expression was decreased in the Caco-2, and it was not changed in SW620 according to the healthy tissue (Fig. 4D).

3.7. Investigating the levels of glucose, pyruvate, lactate, TCA cycle intermediates, and D-2-HG in xenografts models

Significant results have been obtained in intracellular and extracellular environments on cell lines. In the next step, we wanted to examine how the concentration of TCA and Cori cycle and Glycolysis intermediates changed in the xenograft models. It also allows examining the tissue type-specific metabolite levels. When the xenografts models were sacrificed, the tumor, brain, liver, and colon tissues were extracted and the level of glucose, pyruvate, lactate, TCA cycle intermediates, and D-2-HG in these tissues was determined (Fig. 5). There was very small tissue development in the models generated with CCD18Co healthy cells, and the tissue volume could not reach 1 cm³ in the expected time, and they were sacrificed.

In the brain tissue, glucose levels were determined as similar in all tissues. However, pyruvate was more consumed in xenograft models created with colon adenocarcinoma cell lines than CCD-18Co (Fig. 5A). Related to this, TCA cycle intermediates were more produced in cancer tissues. As is seen in Fig. 5A, citrate, α -KG, succinate, fumarate, malate, and oxaloacetate levels were 2.3, 2.8, 1.4, 2.1, 1.3, and 1.3-fold increase in Caco-2, and 2.5, 2.4, 1.3, 3, 2.2, and 1.6-fold increase in SW620 compared to the CCD-18Co tissues, respectively (Fig. 5A). The level of D-2-HG could not be detected in CCD-18Co brain tissue. However, D-2-HG was significantly produced in Caco-2 (15.4 ppm) and SW620 (57.4 ppm) brain tissues ($p < 0.05$) (Fig. 5A).

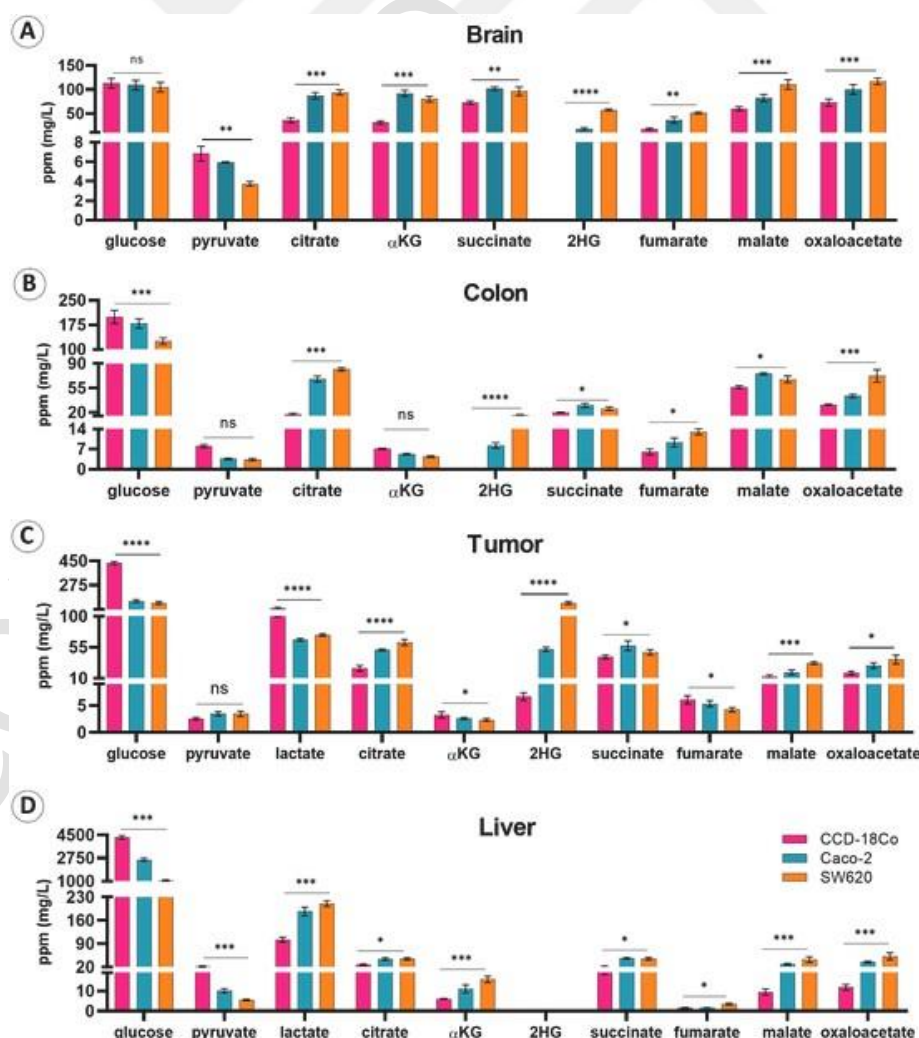


Fig. 5. Extraction of metabolites from tissues isolated from xenograft models. The glucose, pyruvate, lactate, citrate, α -KG, succinate, fumarate, malate, oxaloacetate, and, D-2-HG levels were determined in the brain (A), colon (B), tumor (C), and liver (D) tissues. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

A statistically significant decrease was observed in the glucose levels of Caco-2 and SW620 compared to CCD-18Co colon tissue (Fig. 5B). The high glucose metabolism led to the increased level of TCA cycle intermediates in Caco-2 and SW620 colon tissues. Especially, citrate level was 3.8 and 4.5-fold increased ($p < 0.05$) in Caco-2 and SW620 according to the CCD-18Co colon tissue, respectively (Fig. 5B). More importantly, the D-2-HG level was not produced in CCD-18Co, produced in Caco-2 (7.7 ppm) and SW620 (16.6 ppm) colon tissues. Correspondingly, the α -KG level was decreased in these tissues according to control (Fig. 5B).

Glucose contribution to the TCA cycle was increased in the tumor tissues compared to healthy tissue. The glucose levels were 2.9 and 3.2-fold decreased in Caco-2 and SW620 than in healthy tissue, respectively (Fig. 5C). Regarding this, pyruvate, citrate, succinate, fumarate, malate, and oxaloacetate levels were 1.4, 3.6, 1.5, 1.2, 2.2, and 2.6-fold increased in Caco-2 and 1.3, 4.3, 2.2, 2.2, 2.1, and 1.9-fold increase in SW620 compared to the CCD-18Co tissue, respectively (Fig. 5C). In addition, a significant decrease in lactate level was observed in Caco-2 (1.6-fold) and SW620 (1.5-fold) tumor tissues compared to healthy tissue (Fig. 5C). Above all, the D-2-HG level was overexpressed and accumulated during tumor progression. D-2-HG has produced 54.8 ppm in Caco-2 and 148.8 ppm in SW620 tumor tissues. Relatedly, α -KG levels were 0.7 and 0.8-fold decreased ($p < 0.05$) in Caco-2 and SW620 according to CCD-18Co tissue, respectively (Fig. 5C).

The glucose level in all the liver tissues was the highest in the study. The CCD-18Co liver had a very high glucose level (4451.0 g/L). Compared to healthy, glucose was highly metabolized in cancer tissues, and the TCA cycle intermediates were increased. The glucose and pyruvate levels were 1.7 and 2.4-fold decreased in Caco-2 and 4.6 and 3.9-fold decreased in SW620 than CCD-18Co tissue, respectively. (Fig. 5D). Lactate, citrate, α -KG, succinate, fumarate, malate, and oxaloacetate levels were 1.9, 1.5, 1.9, 2.8, 2.7, 3.0, and 3.0-fold increase in Caco-2, and 2.1, 1.7, 2.5, 4.1, 5.2, 4.4 and 4.5-fold increase ($p < 0.05$) in SW620 compared to the CCD-18Co tissue, respectively (Fig. 5D). More importantly, D-2-HG was undetectably low levels in all liver tissue, revealing that metabolites have produced tissue-specifically (Fig. 5D).

4. Discussion

Our results showed that there is a minor difference between healthy and cancer cell lines in the consumed glucose level at the end of 24 h. However, the decrease in intracellular glucose levels in Caco-2 and SW620 indicates that glucose metabolism was 2.6 and 3.9-fold ($p < 0.05$) increase in these cells according to CCD-18Co, respectively (Figs. 1B and 2B). In the literature, increased glucose consumption has been seen in different cancer cells [27,28]. As a result of the Warburg effect, cancer cells highly metabolize glucose and produce a high amount of lactate [1,29]. On the other hand, cells use lactate to fuel the TCA cycle by converting to pyruvate in some cancer types such as lung, breast, and colon [29]. Consistent with this, intracellular lactate level was decreased in colon adenocarcinoma cells compared to control (Fig. 1B), and it is associated with the mRNA level of *LDHA* (Fig. 3B).

In recent years, the level of TCA cycle metabolites in colon cancer has been investigated with increasing interest [8]. In our study, due to the high metabolic rate, significant differences in adenocarcinoma cancer cells were detected in intracellular and extracellular levels, exemplified by high amounts of intracellular D-2-HG, malate, and oxaloacetate metabolites. However, intracellular α -KG, citrate, and succinate levels were decreased in the SW620 according to Caco-2 because α -KG was converted to D-2-HG (Fig. 1B). In the literature, it has been found that the high amount of metabolite released by various cancer cells compared to healthy cells is associated with metastatic potential [30]. D-2-HG level was increased in metastatic (152.6 $\mu\text{mol/mg}$) compared to the primary (22.6 $\mu\text{mol/mg}$) colon adenocarcinoma cells, and it could not be detected in the colon epithelial cells (Fig. 1B). Therefore, we hypoth-

esize that the significantly high level of D-2-HG can be associated with the metastatic potential of colon cancer. In the literature, some studies found that increased D-2-HG as an oncometabolite and its association with colon cancer progression in cell models carry IDH mutation [25,31]. In our study, high D-2-HG metabolite levels were detected in the colon cancer cell lines carry the wt-IDH enzyme.

Previous studies showed that the D-2-HG oncometabolite level in cancer cells ranging from pM to mM depends on various factors [10,26]. In our study, the D-2-HG level reached μM level in both *in vitro* and *in vivo* models (Figs. 1B and 5C). After that, the pathways where D-2-HG is produced were investigated. Firstly, no mutation was observed in the IDH1/2 enzyme in SW620 (Fig. 3A). Then the imbalance of mRNA level of wt-IDH1/2 has found. To find the carbon source of the D-2-HG, the glutaminolysis pathway was investigated. As glutamine participates in the TCA cycle via α -KG, it occurs as a source of anaplerotic carbon [32]. As a result, the mRNA level of the *GLS* enzyme was decreased ($p < 0.05$) in SW620 compared to the control group (Fig. 3B). Following the discovery of wt-IDH1/2 enzymes and high levels of D-2-HG expression in SW620 cells, we hypothesized that the carbon source of D-2-HG is glucose and the wt-IDH1/2 enzymes play a role in its production. This hypothesis was also supported by increased glucose consumption in SW620 compared to CCD-18Co (Figs. 1B and 2B).

The concentration of TCA and Cori cycle and Glycolysis intermediates in the xenograft models were investigated (Fig. 6). In agreement with intracellular glucose levels, it was found that glucose was more consumed in Caco-2 (2.9-fold) and SW620 (3.2-fold) tumor tissue compared to healthy tissue (Fig. 5C). While glucose level in brain tissue was not significantly changed in all mouse models, it is more metabolized in the liver, tumor, and colon tissues of xenograft models created with colon cancer than colon epithelial cells (Fig. 5A, B, D; Fig. 6). The mutations in some glycolytic enzymes such as the pyruvate kinase, and activation of oncogenes such as AKT and MYC, affect cancer metabolism *in vivo* and lead to the dysregulation of glucose metabolism in different tissues [33]. MYC leads to a change in the expression of many metabolic enzymes and transporter genes in colon cancer and therefore affects the glycolytic flux in a tissue-specific manner [34].

The Cori cycle is another important factor in determining the lactate level. While some of the lactate is used for respiration in tumor tissues, some of it is sent to the liver with alanine. Lactate is used as a precursor for gluconeogenesis and returns to the tumor tissue [35]. This circulation seems relevant to the *in vivo* result. In our study, a significant decrease in lactate level was observed in tumors compared to healthy tissue (Figs. 5C and 6). In addition, while lactate levels decreased, glucose levels increased in both Caco-2 and SW620 than in the CCD-18Co liver (Figs. 5D and 6).

In our study, the metabolism of D-2-HG on the TCA and Cori cycle and Glycolysis were studied for the first time in the brain, colon, liver, and tumor tissues extracted from xenograft models (Fig. 6). The D-2-HG could not be detected in CCD-18Co colon and brain tissue, while this oncometabolite was produced in colon and brain tissues of xenograft models generated with colon cancer cell lines (Fig. 5A–B). Besides, this oncometabolite was not produced in liver tissue in all xenograft models (Fig. 5D), revealing that the metabolism of D-2-HG is tissue-specific. In the literature, they found that glucose contribution to the TCA cycle was increased and the levels of some TCA cycle intermediates and D-2-HG were also increased in tumors compared to normal tissues [36–38], consistent with our results.

In conclusion, our data from both *in vitro* and *in vivo* models demonstrated that the enhanced production of endogenous D-2-HG has been a characteristic metabolic change in colon cancer. Our findings provide an insight into the D-2-HG production pathway and promising therapeutic target for colon cancer. In the future, it is planned to work on both *in vitro* models developed by silencing the target enzymes with the CRISPR/Cas9 method and PDX models to inhibit the production of the D-2-HG.

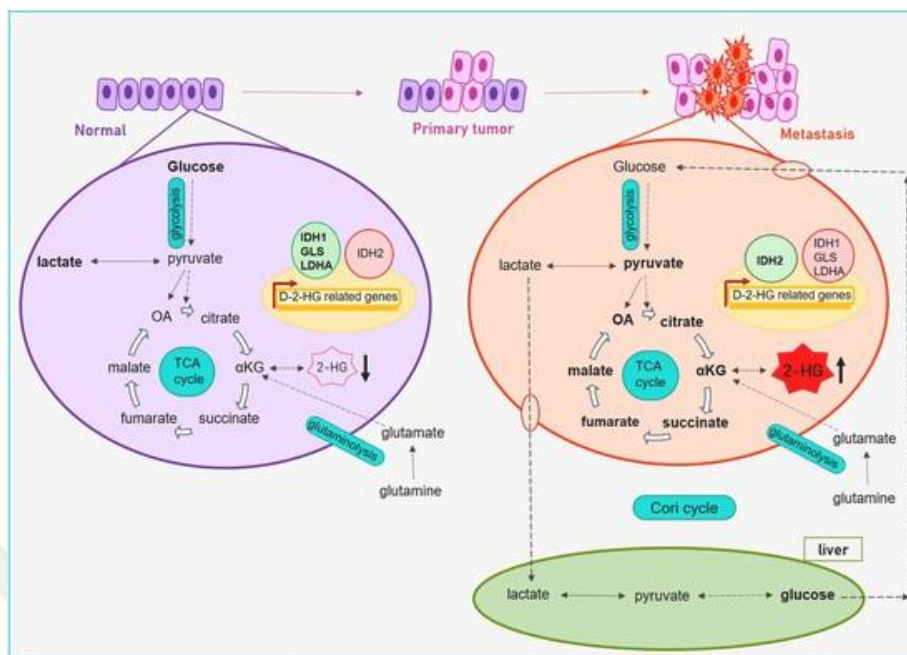


Fig. 6. Model of metabolic changes of the TCA and Cori cycle and Glycolysis in the different tissues of colon cancer xenograft models. Glucose coordinates the use of carbon required for intermediate synthesis. In the tumor tissue, glucose contribution to the TCA cycle was increased and the levels of some intermediates and D-2-HG were also increased. Intermediates in bold indicate that they are highly produced in the cell. While some of the lactate is used for respiration in tumor tissues, some of it is sent to the liver and used as a precursor for gluconeogenesis and then returns to the tumor tissue [35]. Glutaminolysis begins with the conversion of glutamine to glutamate. It continues with performing oxidative deamination of glutamate [32]. α -KG, α -ketoglutarate; D-2-HG, D-2-hydroxyglutarate; OA, oxaloacetate; IDH1/2, isocitrate dehydrogenase1/2; LDHA, lactate dehydrogenase A; GLS, glutaminase.

Declaration of competing interest

It is declared that there is not any conflict of interest in this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2022.08.019>.

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