Exploring the Therapeutic Potential of Using Alkylating Agent as a Novel Approach to Treat IDH1-Mutant Cholangiocarcinoma

Ji A Choi

The Graduate School
Yonsei University
Department of Medicine

Exploring the Therapeutic Potential of Using Alkylating Agent as a Novel Approach to Treat IDH1-Mutant Cholangiocarcinoma

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Thesis Supervisor Choong-kun Lee

Thesis Committee Member Hye Jin Choi

Thesis Committee Member Yong Joon Kim

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ABSTRACT

Exploring the Therapeutic Potential of Using Alkylating Agent as a Novel Approach to Treat IDH1-Mutant Cholangiocarcinoma

Mutation of the isocitrate dehydrogenase 1 (IDH1) gene is identified as a promising 'druggable target' in intrahepatic cholangiocarcinoma (CCA) with its high incidence (~20%). An IDH1 inhibitor, Ivosidenib, received FDA approval for treating IDH1 mutant (IDH1mt) CCA, yet the objective response rate was only 2% in the phase 3 ClarIDHy trial. Thus, novel approaches to target IDH1 mutation are necessary. Temozolomide (TMZ) is an alkylating agent widely used to treat IDH1mt glioma. However, its cytotoxic effects have not been previously investigated in CCA. Therefore, our study explored whether TMZ has therapeutic potential for treating IDH1mt CCA. We utilized human CCA cell lines harboring a wild-type (WT) IDH1 (SNU-1196, SNU-245) or IDH1 mutations (RBE: R132S; SNU-1079: R132C). Characterization of IDH1mt CCA was achieved using a D-2-hydroxyglutarate (D2HG) assay and metabolic profiling (Fuel Flex Test). The cytotoxic effects of TMZ in CCA were evaluated with CCK8 assay, cell cycle analysis, immunoblot analysis, and immunocytochemistry. We confirmed a significant elevation of oncometabolite D2HG levels in IDH1mt CCA cells compared to IDH1 WT ($p< 0.05$). D2HG competitively inhibits α-KG dependent enzymes, which cause epigenetic dysregulation and metabolic reprogramming. Metabolomic assays revealed that IDH1mt and WT CCA cell lines use distinct metabolic pathways with the highest dependency on glycolysis and fatty acid oxidation, respectively. Drug screening revealed that all CCA cells are sensitive to gemcitabine (IC50: ~0.005-0.01uM), and inhibition of cell proliferation was unachievable with Ivosidenib (IC50 > 100μM) regardless of the IDH1 mutation status. However, we found that TMZ selectively inhibits IDH1mt CCA cell proliferation (IC50: ~600μM) compared to IDH1 WT (IC50 > 1mM). TMZ induced more DNA damage in IDH1mt than WT CCA cells (~5 fold vs. ~1.2 fold in rH2AX expression following TMZ treatment). Furthermore, we noticed that Ivosidenib treatment induces G0-G1 arrest, while TMZ induces G2-M arrest in CCA. An increased TMZ-induced G2-M arrest was observable in IDH1mt (control vs. TMZ: 13.3% vs. 56%) than in IDH1 WT CCA cells (control vs. TMZ: 19.3% vs. 51.4%). Also, TMZ significantly reduced D2HG levels in IDH1mt CCA cells ($p< 0.05$), which resembles the mechanism of action of Ivosidenib. Our findings not only suggest that TMZ exhibits potent cytotoxic effects on IDH1mt CCA but also imply the synergistic effect of combining it with Ivosidenib, which exerts a cytostatic effect on CCA. We will validate these findings with ongoing studies involving CRISPR-Cas9-mediated genome editing and *in vivo* studies.

Key words: Cholangiocarcinoma, IDH1 Mutation, Temozolomide, Ivosidenib

I. INTRODUCTION

1.1. Cholangiocarcinoma and Current Treatment Options

Cholangiocarcinoma (CCA), also referred to as biliary tract cancer, is a heterogeneous group of malignant tumors that arise from the biliary tract[1]. Based on the anatomical site of origin, CCAs are classified into the following subtypes: Intrahepatic CCA (iCCA), Extrahepatic CCA (eCCA), and Gallbladder carcinoma (GBC)[2].

Though CCA is a relatively rare type of cancer, accounting for ~3% of gastrointestinal cancers, it is the second most common primary hepatic malignancy following hepatocellular carcinoma[3]. The incidence of CCA varies greatly between geographic regions, with a significantly higher incidence in Southeast Asia (incidence > 6 per 100,000 habitants in countries like South Korea) than in Western countries (0.3–6 per 100,000 inhabitants per year) [3]. Despite the advances in cancer diagnosis and treatment, the incidence and mortality rates of CCA have increased globally over the past decades. Moreover, patients with CCA have a poor prognosis with a 5-year survival ranging from 5% to 17%[4]. The main curative therapeutic options for CCA include surgical resection and adjuvant chemotherapy. However, surgery is only available in less than 20% of patients due to the anatomical location of the bile duct and late diagnosis at the advanced stage[5]. Moreover, the standard first-line regimen for CCA is Gemcitabine, combined with Cisplatin, following the phase III ABC-02 trial; Nonetheless, the median progression free-survival (PFS) and overall survival (OS) are very poor with 8 and 11.7 months, respectively[6]. Thus, therapeutic options for CCA not only remain limited but patients also experience inadequate responses to available therapies.

1.2. Isocitrate Dehydrogenase 1 (IDH1) and IDH1 Mutation

In recent years, next-generation sequencing (NGS) has revealed potentially actionable targets in CCA. The most prevalent ‘druggable’ targets in CCA include fibroblast growth receptor 2 (FGFR2) fusion, erb-b2 receptor tyrosine kinase 2 (ERBB2) amplification, and isocitrate dehydrogenase 1 (IDH1) mutations[7]. Following the discovery of such targets, considerable progress has been made in developing targeted therapies in CCA. For instance, Pemigatinib, which targets FGFR2 fusion, increased the median OS to 21.1 months[8]. Also, Ivosidenib recently received FDA approval for treating advanced CCA with IDH1 mutation following the ClarIDHy phase III trial[9]. Despite its clinical benefits, the overall response rate for Ivosidenib was solely 2 %, which accentuates the importance of developing more effective therapies for IDH1-mutant CCA[9].

In detail, the isocitrate dehydrogenase 1 (IDH1) gene is a key metabolic enzyme in the citric acid (TCA) cycle essential for cellular respiration. IDH1 mutations are frequently reported in iCCA with an incidence of 11.4% [10]. Specifically, IDH1 mutations result in an amino acid

substitution at residues located in the enzymatic active site at arginine 132 (R132)[11]. The mutational hotspots vary across cancer types with R132C and R132S being the most common IDH1 mutation variants in CCA[12]. Following IDH1 mutation, the normal enzymatic function of IDH1 becomes disrupted. Under normal circumstances, IDH1 converts isocitrate to α-ketoglutarate (α-KG) with the concomitant production of NADPH. However, IDH1 mutation converts α-KG into an oncometabolite, D-2-hydroxyglutarate (D2HG). It has been suggested that D2HG acts as a competitive inhibitor of α-KG, which inhibits various α-KG-dependent enzymes, including the Ten-Eleven Translocation (TET) family of 5-methylcytosine hydroxylases[13]. Moreover, mutant IDH1 genes consume NADPH to produce D2HG, which disrupts cellular redox homeostasis[14]. By this means, IDH1 mutation may not only result in various epigenetic abnormalities, such as DNA hypermethylation but may also lead to metabolic reprogramming in cancer.

However, most prior research on IDH1 mutation was done in glioma, which mainly harbor a R132H variant. In other words, the molecular mechanism and clinical impacts of IDH1 mutation in CCA have not been comprehensively studied and require further research.

1.3. Alkylating Agents in Treating IDH1-mutant (MT) CCA

Temozolomide (TMZ) is an alkylating agent widely used to treat IDH1 MT glioma due to its ability to penetrate the blood-brain barrier[15]. As a prodrug of the 5-(3-dimethyl-1-triazenyl) imidazole-4-carboxamide (MTIC), TMZ methylates the guanine group in DNA. The O^6 methyl guanine impairs with thymine upon DNA replication, which activates the DNA mismatch repair system (MMR) and induces apoptosis through direct DNA damage[15].

Prior research in glioma has demonstrated that patients with IDH1 mutation are more sensitive to TMZ treatment when compared to those with IDH1 wild type (WT) genes[16]. It has been proposed that IDH1 mutation hypermethylates the methylguanine-DNA methyltransferase (MGMT) promoter, which is responsible for removing the methyl DNA adducts from the O^6 position of guanine. Thereby, patients with IDH1 mutation have low expression of MGMT and become more susceptible to the cytotoxic effects of alkylating agents, such as TMZ[17]. Furthermore, recent studies have highlighted that the excessive accumulation of D2HG following IDH1 mutation sensitizes glioma to TMZ by downregulating the ITGB4/PI3K/ AKT pathway. Nevertheless, the cytotoxic effects of TMZ have not been previously investigated in CCA[18].

Herein, we conducted an original study to characterize IDH1 MT CCA and explore the potential of using an alkylating agent, TMZ, as a novel therapeutic option for treating IDH1 MT CCA. Subsequently, we aimed to investigate whether a combination of TMZ and Ivosidenib could exert a synergistic effect to improve the poor prognosis of patients with IDH1 MT CCA.

II. MATERIALS AND METHODS

2.1 Cell Lines and Reagents

The human CCA cell lines SNU-1079, SNU-1196, and SNU-245, were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). RBE cells were purchased from the Riken BRC Cell Bank (Koyadai, Japan). All CCA cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, supplemented with 100 unit/mL penicillin and 100mg/mL streptomycin (Lonza, Basel, Switzerland) and 10% heat-inactivated fetal bovine serum (FBS). Cells were grown at 37℃ in a humidified incubator containing 5% CO_2.

Ivosidenib and Temozolomide were purchased from Selleck Chemicals (Houston, TX, USA) and diluted in DMSO as 50 mM or 100 mM stock solutions, respectively.

2.2 Cell Viability Assay

Cells (8 x 10^3 / well) were seeded in 96-well plates and incubated at 37℃ for 24 h. Then, cells were treated with various concentrations of Ivosidenib or Temozolomide. After 72 h (Ivosidenib) or 96 h (Temozolomide) of incubation, 10 μl of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well, and cells were incubated for another 2 h at 37℃. The optical densities of the samples were analyzed at 450 nm using a microplate reader (BioTek) and IC50 values were determined using the GraphPad Prism8.

2.3 Cell Cycle Analysis

Cells were seeded in a 60 mm culture plate. After 48 h of incubation, the culture medium was completely removed, and the cells were either treated with fresh medium (control) or media containing Ivosidenib (100 μM) or Temozolomide (1mM) for 48 h. Then, the cells were harvested with 0.25 % trypsin/EDTA (Gibco, Carlsbad, CA, USA), washed with cold PBS, and 1 x 10^6 cells were fixed in 75% ethanol for 24h at – 20℃. The next day, cells were washed twice in cold PBS and stained with 500 μl of PI/RNase staining buffer (BD Pharmingen, Bedford, MA, USA) at room temperature for 20 min in the dark for cell cycle analysis. Cell cycle distribution was subjected to flow cytometric analysis using a BD FACSymphony A5 (BD Pharmingen, Bedford, MA, USA) and the FlowJo software (Tristar, CA, USA).

2.4 Apoptosis

Cells were seeded in a 60 mm culture plate. After 48 h of incubation, the culture medium was completely removed, and the cells were either treated with fresh medium (control) or media containing Ivosidenib (100 μM) or Temozolomide (1mM) for 96 h. Then, the cells were harvested with 0.25 % trypsin/EDTA (Gibco, Carlsbad, CA, USA), washed with 1x PBS, and 1 x 10^6 cells

were resuspended in 1X Binding Buffer. Then, 100 μl of the resuspended solution was transferred to a 5 ml round-bottom tube, stained with 5 μl of FITC Annexin V and 5 μl of PI at room temperature for 15 min in the dark (Cat# 556547, BD Biosciences, USA). 400 µl of 1X Binding Buffer was added to each tube and cell apoptosis was detected by flow cytometry using a BD FACSymphony A5 (BD Pharmingen, Bedford, MA, USA) and the FlowJo software (Tristar, CA, USA). Quantification of apoptotic cells involved counting cells in the upper right quadrant and the low right quadrant in the flow cytometry dot plots, which indicate late apoptotic and early apoptotic cells, respectively.

2.5 D2HG; NADP/NADPH; Cellular ROS Assays

Cells (5 x 10^5/ well) were seeded in a 60 mm culture plate. After 48 h of incubation, intracellular D2HG concentrations, NADP+/NAPDH ratio, and cellular ROS were measured using the D-2-Hydroxyglutarate colorimetric assay kit (Abcam), NADP/NADPH colorimetric assay kit (Abcam), and DCFDA/H2DCFDA assay kit (Abcam), respectively, according to the manufacturer's instructions.

2.6 Quantitative Real-Time PCR

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcription was performed from 500 ng of total RNA using Superscript II Reverse-Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed with SYBR® Green PCR Master Mix (Qiagen, Valencia, CA, USA) using a QuantStudio 3 Real-Time PCR Instrument (Applied Biosystems). All genes were normalized to GAPDH, and the delta-delta *Ct* method was used to calculate the fold changes in gene expression. The primer sequences were as follows: MGMT: (F): 5'CTCTTCACCATCCCGTTTTC3' (R): 5'CTCTCATTGCTCCTCCCACT3'; IDH1: (F) 5'GCACGGTCTTCAGAGAAGCCA3' (R): 5' CTACTTTTCCAGGCCCAGGAAC3'.

2.7 Western Blot Analysis

Cells were lysed in the M-PER™ mammalian protein extraction reagent solution, supplemented with 1x protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Sigma, St. Louis, MO, USA). The lysates were centrifuged for 10 min at 4'C, 13,000 rpm. The protein concentration of the supernatant was determined with the Bradford assay. Then, 15 ug of the protein extracts were separated by 10-15% SDS-PAGE, transferred to PVDF membranes and immunoblotted with the rH2AX (1:1000; Cell Signaling Technology) and IDH1 (1:2000; Cell Signaling Technology) primary antibodies at 4'C overnight.

Then, HRP-conjugated anti-rabbit was used as secondary antibodies and developed with an enhanced chemiluminescence reagent. Immunoreactive proteins were visualized using the

ChemiDoc XRS+ System (Bio-rad, Hercules, CA, USA) with Image Lab software (Bio-Rad, Hercules, CA). Data was normalized to either H2AX or GAPDH and blot intensity was quantified with the Image J software (NIH, Bethesda, MD, USA).

2.8 Immunofluorescence Staining and Confocal Imaging:

Cells grown on coverslips were washed with PBS, and fixed with 4% paraformaldehyde solution for 10 min. Then, the cells were washed three times for 5 min each with PBS and permeabilized with 0.1% Triton X-100 /PBS solution for 10 min. Blocking and staining were performed simultaneously by incubating cells with primary antibodies in a blocking medium (10% goat serum in 0.1% Triton X-100/ PBS) at room temperature for 1 h. This was followed by incubation with appropriate secondary antibodies (Alexa Fluor488/594) at room temperature for 1 h in the dark. Cells were stained with DAPI (Invitrogen) for 5 min and mounted in Fluorescence Mounting Medium (DAKO). Images were acquired using an LSM 710 confocal microscope (Carl Zeiss).

2.9 Seahorse XF Mito Stress Test & Fuel Flex Test

The Seahorse XF Cell Mito Stress Test and Fuel Flex Test were performed on XFe96 Bioanalyzer (Agilent). Data was processed with the Seahorse Wave Desktop software and GraphPad Prism 8 was utilized for data presentation and statistical presentation. Briefly, cells were seeded on XFe96 cell culture plates. After 48 h of incubation, the assays were performed following the manufacturer's instructions.

Mito Stress Test: This assay measures various parameters of mitochondrial function by measuring the oxygen consumption rate (OCR) of cells. Oligomycin (2μM), FCCP (0.5μM), and a mixture of Rotenone and Antimycin A (0.5μM) were sequentially treated to the XFe96 well plates.

Fuel Flex Test: This assay inhibits the import of three major metabolic substrates (glutamine, fatty acids, and glucose) to determine the cell's dependence on each metabolite to fuel mitochondrial metabolism. Inhibition of two metabolic substrates enables calculating the cell's capacity for utilizing a particular substrate when the others are blocked. Fuel pathway inhibitors included BPTES (3 uM), Etomoxir (4 uM), and UK5099 (2 M). BPTES is an inhibitor of the glutamine oxidation pathway, which allosterically inhibits glutaminase (GLS1). Etomoxir inhibits carnitine palmitoyl-transferase 1A (CPT1A), essential for mitochondrial oxidation. UK5099 inhibits the mitochondrial pyruvate carrier (MPC), which blocks the glucose oxidation pathway.

Fuel dependency was calculated as:
(baseline OCR - target inhibitor OCR)/(baseline OCR - all inhibitors OCR)

Fuel capacity was calculated as:

1 - [(baseline OCR - other 2 inhibitors OCR)/(baseline OCR - all inhibitors OCR)].

2.10 Establishment of genetically engineered CCA cell lines

Establishment of IDH1 Knockout (K/O) CCA cell lines:

IDH1 K/O CCA cell lines were generated by using the CRISPR-Cas9 method. Specifically, the following sequences of complementary oligos for each single guide RNA (sgRNA) were derived from the Human CRISPR Knockout pooled library (Brunello library): antisense (F): 5'CACCGCCCATCCACTCACAAGCCGG3'; (R): 5'AAACCCGGCTTGTGAGTGGATGGGC3'. Then, sgRNAs were cloned into the lentiCRISPR v2 (Addgene plasmid #52961) as described by GeCKO, and HEK293T cells were transfected with the lentiviral vectors along with the packaging (psPAX2; Addgene plasmid #12260) and envelop (pMD2.G; Addgene plasmid #12259) plasmids in a 2:1:1.5 ratio, respectively, following the manufacturer's instructions outlined by FuGENE HD Reagent (Promega, USA). The medium was replaced 24 h post-transfection, and the viral supernatant was collected every 24 h for 72 h following media change. The collected viral supernatant was filtered and used for the transduction of parental CCA cell lines with the help of polybrene (8μg/ml). Subsequently, Hygromycin B was utilized to select IDH1 K/O clones and kill curve titration was performed prior to selection to determine the optimal hygromycin B concentration in each CCA cell line. The Western blot analysis and qPCR were utilized to confirm the successful K/O of IDH1 in CCA cell lines.

Expression of IDH1 WT or IDH1 MT in IDH1 K/O CCA cell lines:

Once IDH1 K/O stable CCA cell lines were generated, the rescue of IDH1 WT or MT (R132C) genes into the IDH1 K/O cells was performed using the pHAGE-IDH1 (Addgene plasmid; #116751) and pHAGE-IDH1-R132C vectors (Addgene plasmid; #116410) containing a green fluorescent protein (GFP) expression cassette. Then, Lipofectamine 3000 (Thermo Fisher Scientific) reagents (2x) were used to transfect the HEK293T cells with the IDH1 vectors, packaging (psPAX2; Addgene plasmid #12260) and envelop (pMD2.G; Addgene plasmid #12259) plasmids in a 2:1:1.5 ratio, respectively, as outlined by the manufacturer's instructions. The medium was replaced 24 h post-transfection, and the viral supernatant was collected every 24 h for 72 h following media change. The IDH1 K/O CCA cell lines were transduced with the collected, filtered viral supernatant. The transduction efficiency was assessed through GFP expression and western blot analysis.

2.11 Statistical Analysis

Data were expressed as mean $\pm$ SEM (standard error of the mean) of at least three independent variables. GraphPad Prism 8 software was used for statistical analysis. Comparison between groups were determined using one-way ANOVA and unpaired Student *t*-test. p-value of less than 0.05 was considered statistically significant (*$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p \leq 0.0001$).

III. RESULTS

3.1. Characterization of IDH1 MT CCA Cell Lines

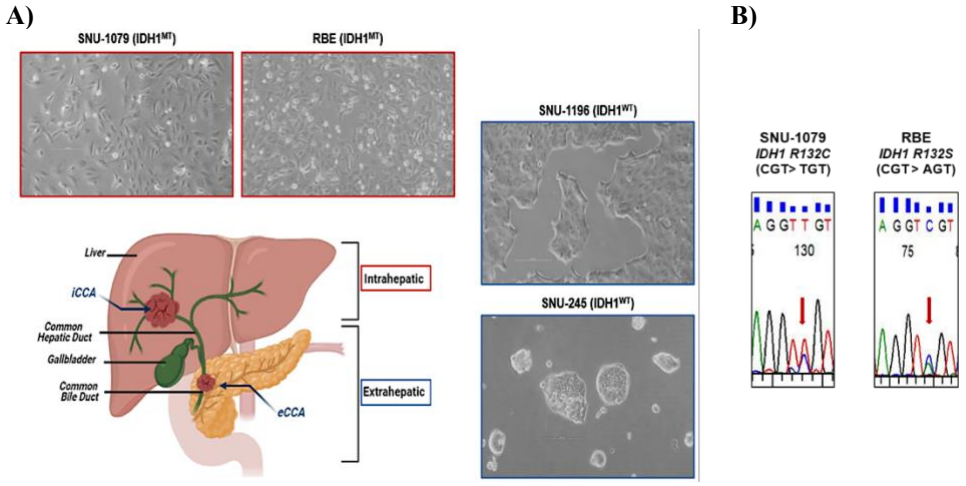


Figure 1. Human CCA Cell Lines (A) Morphology of CCA Cell Lines (*Created with Biorender*). (B) IDH1 mutation status in SNU-1079 and RBE was confirmed via PCR.

<Table 1> Cell Line Information

Cell Line	Organism	Tissue	Morphology	Disease	Age (yr.)	Gender	Ethnicity	Source
RBE	*Homo spaiens*	Liver	Epithelial-like	Intrahepatic Cholangiocarcinoma	64	Female	Japanese	RIKEN
SNU-1079	*Homo spaiens*	Liver	Mesenchymal cluster	Intrahepatic Cholangiocarcinoma	Unknown	Male	Korean	KCLB
SNU-245	*Homo spaiens*	Distal Common Bile Duct	Polygonal; Epithelial cluster	Adenocarcinoma of Common Bile Duct	Unknown	Unknown	Korean	KCLB
SNU-1196	*Homo spaiens*	Hepatic Duct Confluence	Epithelial cluster	Hilar Bile Duct Cancer	Unknown	Unknown	Korean	KCLB

Four human CCA cell lines were utilized in this study. IDH1 mutations are commonly found in iCCA. Interestingly, two cell lines (RBE, SNU-1079) that harbor an IDH1 mutation were both established from iCCA patients. Meanwhile, IDH1 WT CCA cell lines (SNU-1196, SNU-245) were established from eCCA patients (Figure 1B). IDH1 is frequently mutated at arginine at codon R132, and various hotspot mutation sites have been reported across different cancer types. For instance, the most predominant mutations in glioma and CCA are R132H and R132C, respectively. We first examined the IDH1 mutation status in four CCA cell lines via PCR. SNU-1079 and RBE harbored a R132C and R132S variant, respectively. These hotspot mutations feature a heterozygous missense mutation of either arginine to cysteine (CGT → TGT) or arginine to serine (CGT → AGT), respectively (Figure 1B).

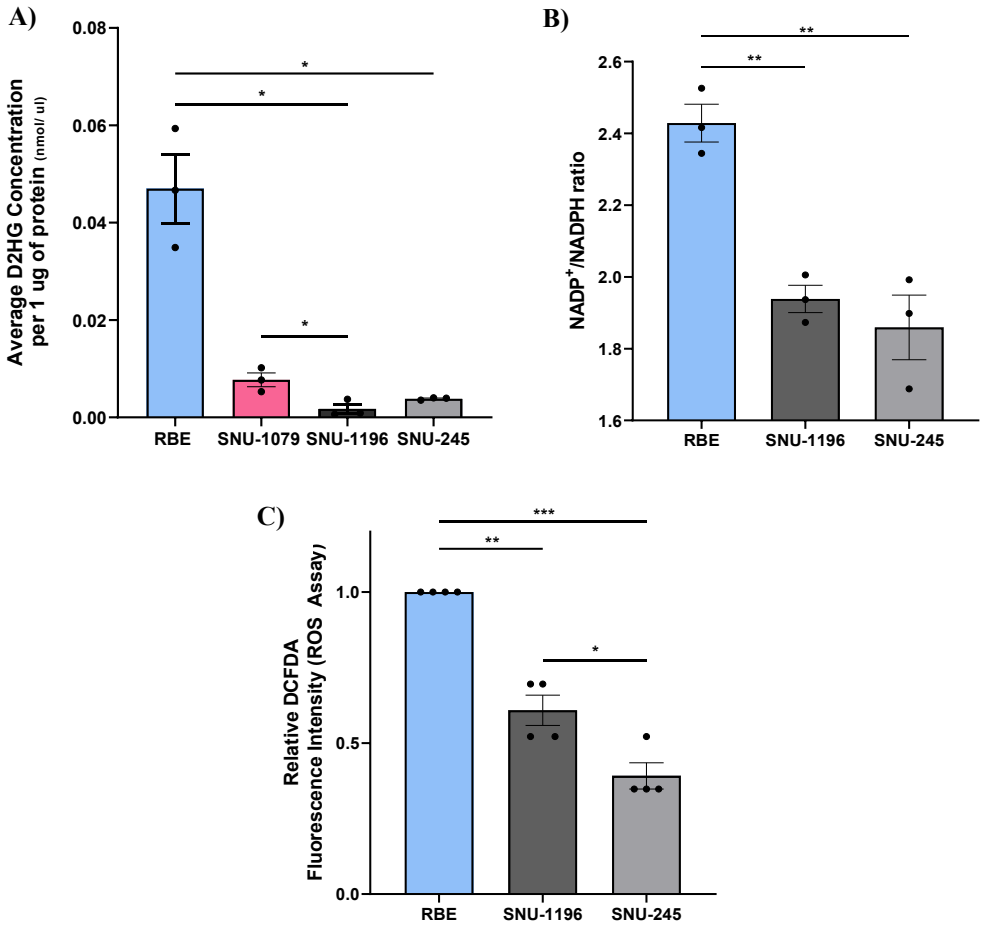


Figure 2. Relative intracellular abundance of D2HG, NADP+/NADPH ratio, and ROS levels are increased in IDH1 MT CCA cells in comparison to IDH1 WT CCA cell lines.
(A) Quantification of the average D2HG concentration per 1μg of protein in CCA cell lines. (B) NADP+/NADPH ratio in CCA cell lines. (C) Relative DCFDA Fluorescence Intensity (ROS) in CCA cell lines. Error bars represent SEM (*p≤0.05)

In various cancer types, IDH1 MT enzymes consume NADPH to convert α-KG into an oncometabolite, D2HG. As seen in Figure 2A, we observed a statistically significant elevation of intracellular D2HG levels in both IDH1 MT CCA cells (RBE and SNU-1079) in comparison to IDH1 wild-type cell lines (SNU-1196 and SNU-245) and further confirmed an increased NADP+/NADPH ratio following IDH1 MT CCA (RBE). Furthermore, disruption in NADPH homeostasis may lead to increased reactive oxygen species levels (ROS) and we noticed that the intracellular ROS levels are relatively higher in IDH1 MT (RBE) than in IDH1 WT CCA cell lines.

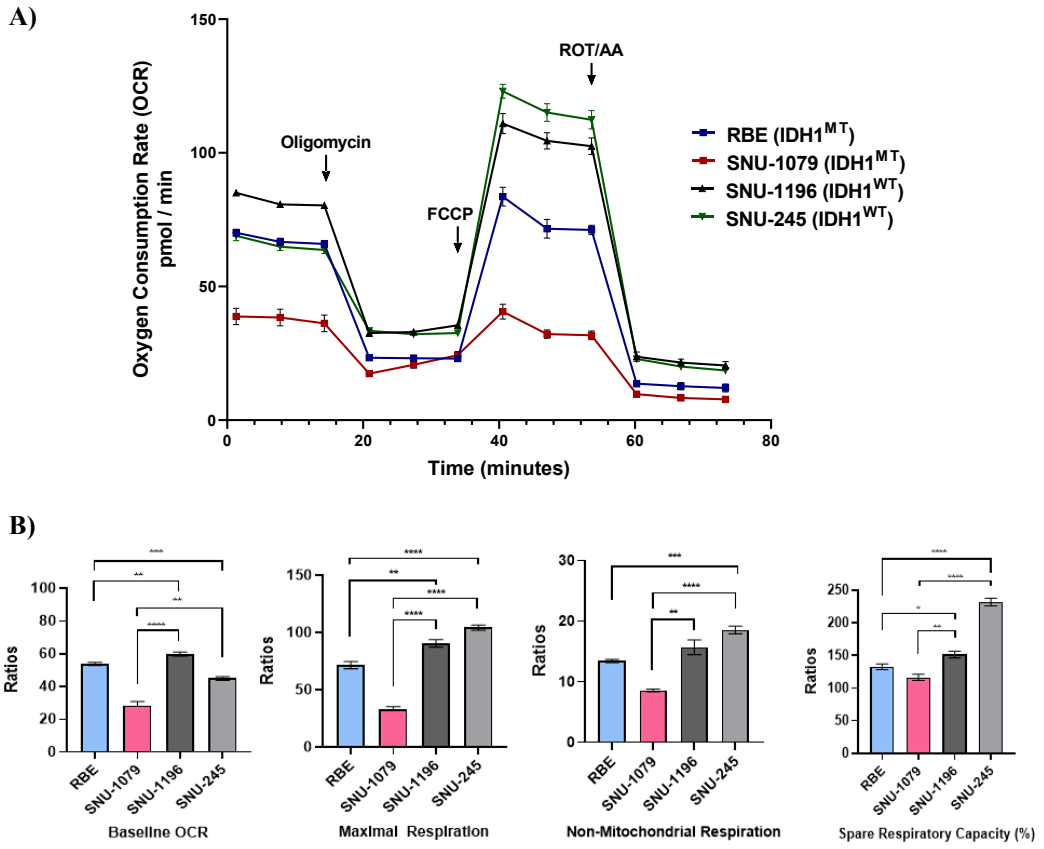


Figure 3. Mitochondrial functions were impaired in IDH1 mutant CCA when compared to IDH1 wild-type CCA (A) Oxygen Consumption Rate (OCR) curves presented as averages ± SEM of each measurement time point. (B) Summary of mitochondria functions. Error bars represent SEM (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001).

IDH1 is a metabolic enzyme in the tricarboxylic acid (TCA) cycle essential for cellular respiration. Thus, acquiring IDH1 mutations not only results in epigenetic abnormalities but also influences cellular metabolism. Nevertheless, only a few comprehensive studies have addressed the metabolic alterations in CCA following IDH1 mutations. Thus, we performed the Seahorse XF Cell Mito Stress Test to compare the key parameters of mitochondrial function in IDH1 MT and WT CCA cell lines. We observed a statistically significant decrease in basal mitochondrial oxygen consumption rate (OCR) of IDH1 MT CCA compared to WT cell lines (Figure 2B). Moreover, maximal respiration and spare respiratory capacity (%) were all reduced in IDH1 MT CCA, which suggests that mitochondrial functions are impaired in IDH1 MT CCA when compared to WT cells.

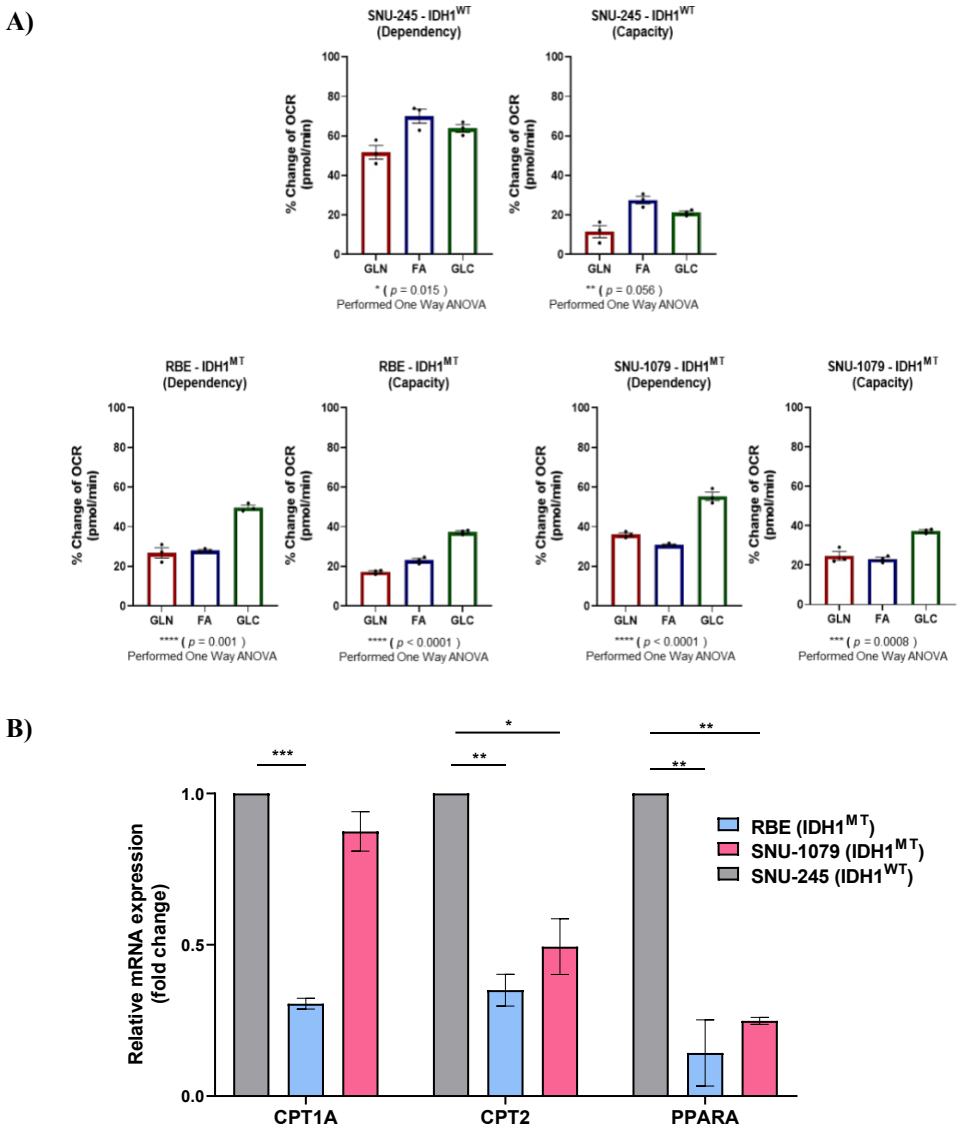


Figure 4. IDH1 mutant and WT CCA cell lines use distinct metabolic pathways with the highest dependency on glycolysis and fatty acid oxidation, respectively. (A) Seahorse XF Mito Fuel Flex Test was performed to analyze CCA cell's mitochondrial fuel (glutamine, fatty acids, glucose) oxidation. Statistical differences were calculated using one-way ANOVA tests. (B) Relative mRNA expression of genes essential for fatty acid oxidation in IDH1 MT CCA (relative to IDH1 WT CCA) determined via qPCR. Unpaired student t-test was used for statistical analysis (*p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001). Error bars represent STDEV.

In addition to assessing mitochondrial functions, we aimed to explore whether IDH1 mutation results in metabolic reprogramming in CCA. The Seahorse XF Mito Fuel Flex Test determines cellular dependence on each metabolite to fuel mitochondria and the capacity for utilizing each substrate when the others are blocked. As shown in Figure 4A, we noticed that while IDH1 WT CCA (SNU-245) had the highest dependency and capacity for fatty acid oxidation, IDH1 MT CCA cells (RBE, SNU-1079) were most dependent on glycolysis. Furthermore, we were able to observe a similar trend by using qPCR. mRNA expression of genes essential for fatty acid oxidation, such as CPT1A, CPT2, and PPARα, were significantly reduced in IDH1 MT CCA relative to IDH1 WT cells (Figure 4B).

3.2. Therapeutic Potential of TMZ in IDH1 MT CCA

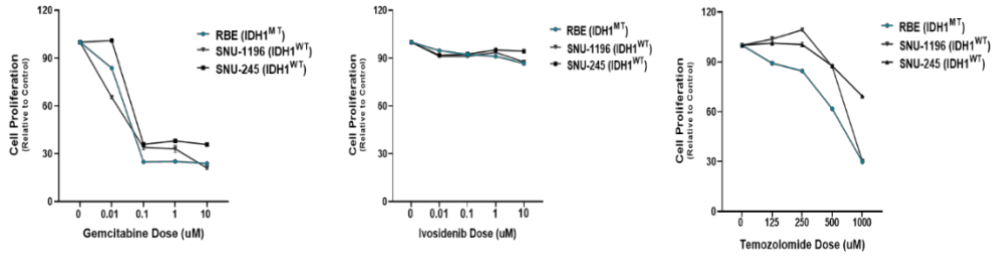


Figure 5. Drug Screening in CCA cells with Gemcitabine, Ivosidenib, and TMZ.
CCA cell lines were treated with varying concentrations of Gemcitabine, Ivosidenib, and Temozolomide for 72h. The dose response curves for IDH1 MT (RBE) and WT CCA (SNU-1196; SNU-245) are shown in blue and black lines, respectively. Error bars represent SEM.

Following the characterization of IDH1 MT CCA cell lines, we performed drug screening with CCA cells. Gemcitabine is one of the first-line treatments for treating advanced CCA, and Ivosidenib is the first IDH1 inhibitor that received FDA approval for treating IDH1 MT CCA. Temozolomide (TMZ) is an alkylating agent widely used to treat IDH1 MT glioma, yet its cytotoxic effects have not been previously investigated in CCA. When we performed cell viability assays, we noticed that all CCA cells were sensitive to Gemcitabine (IC_{50}: ~0.005 - 0.01 μM). In comparison, inhibition of cell proliferation was unachievable with Ivosidenib (IC_{50} >100 μM), regardless of the IDH1 mutation status. Interestingly, we found that TMZ selectively inhibits IDH1 MT CCA cell proliferation (IC_{50}: ~600 μM) compared to IDH1 WT (IC_{50} > 1mM).

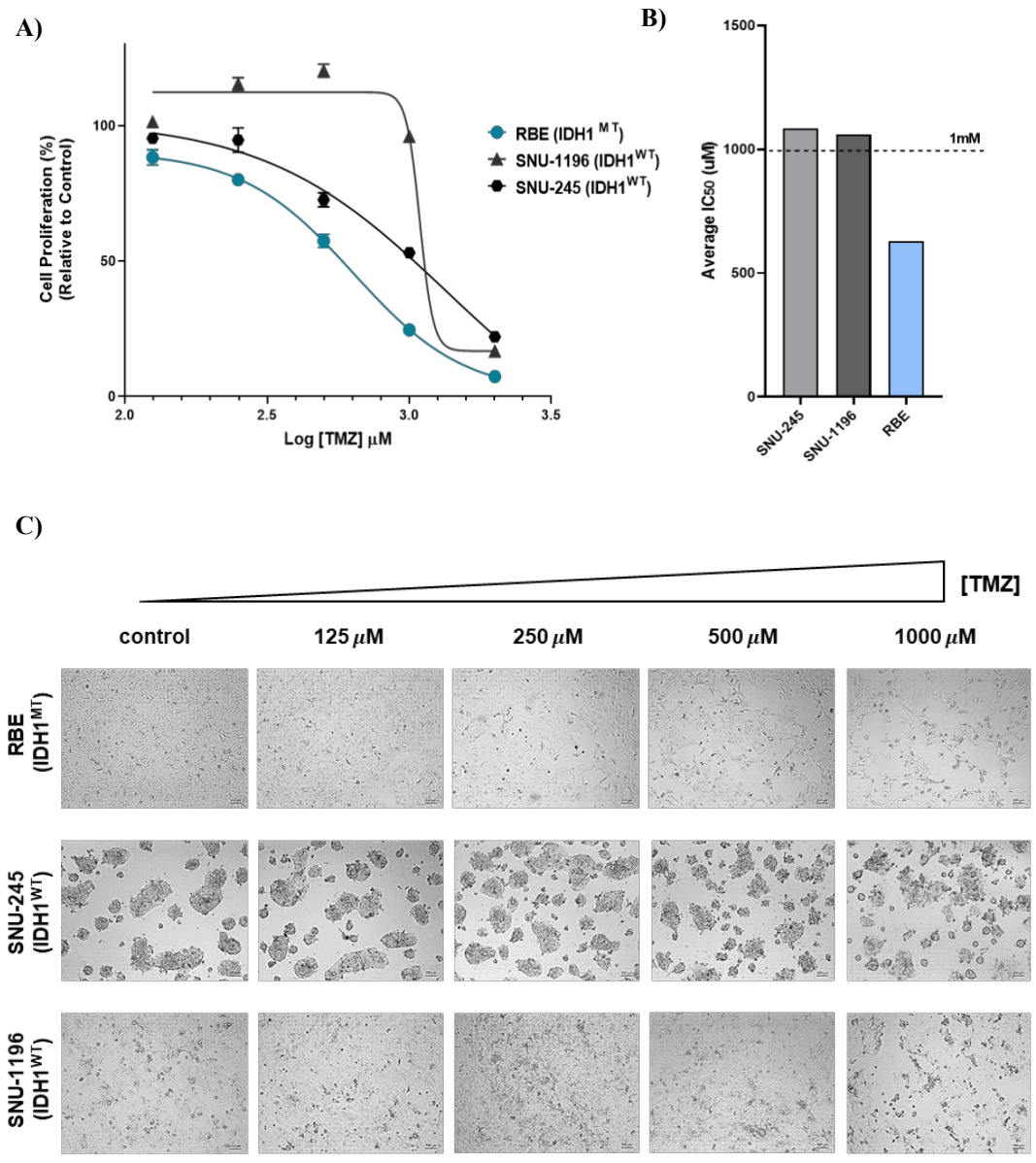


Figure 6. Increased sensitivity to TMZ in IDH1 mutant CCA cells (A) Dose-response curves for CCA cell lines: CCA cell lines were exposed to varying concentrations of TMZ for 96 h and CCK-8 assays were used to determine cell viability. (B) Waterfall plot representing the IC_{50} values of each CCA cell line. (C) Changes in cell morphology upon exposure to varying doses of TMZ. Error bars represent SEM.

Drug screening revealed that TMZ selectively inhibits IDH1 MT CCA cell proliferation. However, we noticed that 72 h incubation could not inhibit 50% of cell proliferation in IDH1 WT (SNU-1196) CCA cells (Figure 5). Thus, we have prolonged the incubation period from 72 h to 96 h for future CCK-8 cell viability assays. Specifically, we treated cells with different concentrations of TMZ (0, 125 μM, 250 μM, 500 M, 1mM, and 2mM) for 96h and determined the IC_{50} values using the GraphPad Prism 8 software.

As seen in Figure 6A, TMZ inhibited the proliferation and viability of CCA cells in a dose-dependent manner. IDH1 MT CCA cells were most sensitive to TMZ with an IC_{50} value of 629.1μM while IDH1 WT cells had an IC_{50} value greater than 1mM (Figure 6B). Moreover, the dose-dependent cytotoxic effects of TMZ on cell viability were in parallel with the cell morphology as more cells have undergone apoptosis in the highest dose (Figure 6C).

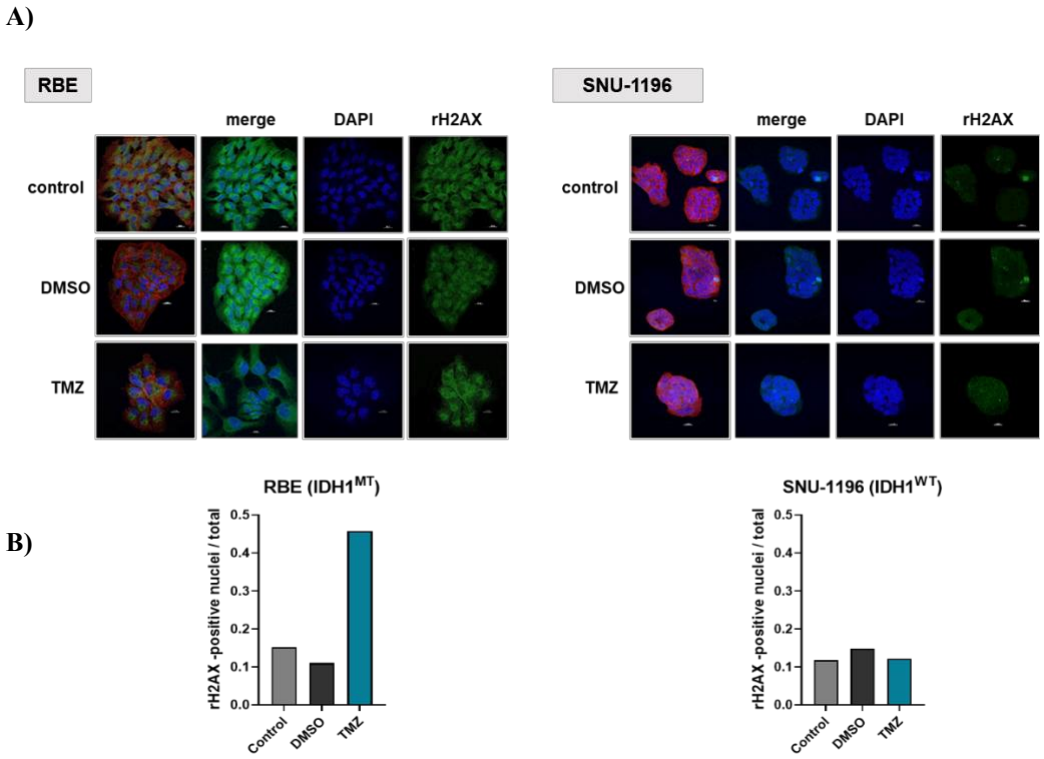
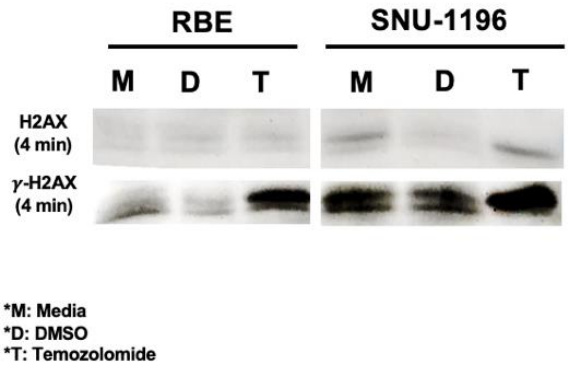


Figure 7. TMZ-induced more DNA Double Strand Breaks in IDH1 MT CCA than IDH1 WT cell lines (A) Representative images of confocal microscopy to demonstrate the induction of H2AX phosphorylation (γH2AX) in CCA cells following 48 h treatment with media (control), DMSO, or TMZ. (B) The ratio of γH2AX-positive nuclei / total in four randomly selected fields (Left: RBE, IDH1 MT; Right: SNU-1196, IDH1 WT).

TMZ is an alkylating agent, which acts by methylating the guanine group in DNA. Specifically, the O6-methylguanine mispairs with thymine upon DNA replication and further activates the DNA mismatch repair system. This results in DNA double-strand breaks (DSB) and induce cell death via apoptosis. Phosphorylated H2AX (γH2AX) is used as a biomarker of cellular response to DSB. To explore the cytotoxic effects of TMZ in CCA, we performed immunocytochemistry (ICC) staining with the rH2AX antibody, along with DAPI to stain the nucleus. We observed that rH2AX-positive nuclei noticeably increased following TMZ treatment for 48 h in IDH1 MT CCA (control vs. TMZ: 0.15 vs. 0.45). However, there was merely any difference between the control, DMSO, and TMZ group in IDH1 WT CCA (Figure 7B).

A)



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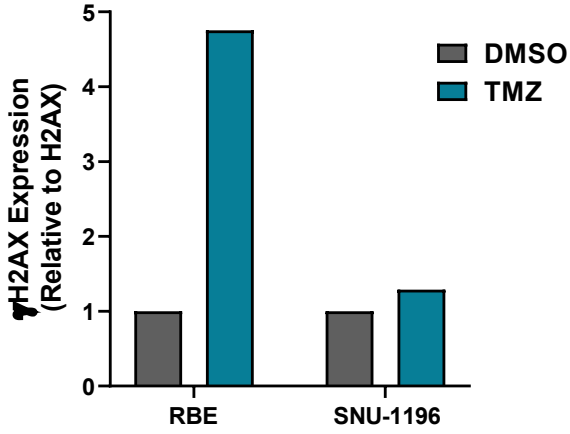


Figure 8. Western blot analysis of DNA damage following DMSO or TMZ treatment. (A) Western blotting analysis of γH2AX in CCA cells treated with media, DMSO, or TMZ (1mM) for 96 h. (B) Quantification of γH2AX expression relative to H2AX in each CCA cell line following DMSO or TMZ treatment.

Furthermore, γH2AX expressions following DMSO or TMZ treatments were investigated using western blot analysis. The γH2AX expression was outstandingly elevated in IDH1 MT CCA following TMZ treatment in comparison to IDH1 WT CCA (Figure 8A). Specifically, there was a ~5 fold and ~1.2-fold increase in γH2AX expression following TMZ treatment in IDH1 MT and WT CCA, respectively (Figure 8B). Therefore, both ICC and western blot results indicate that the cytotoxic effects of TMZ are noticeably enhanced in IDH1 MT CCA than in IDH1 WT CCA.

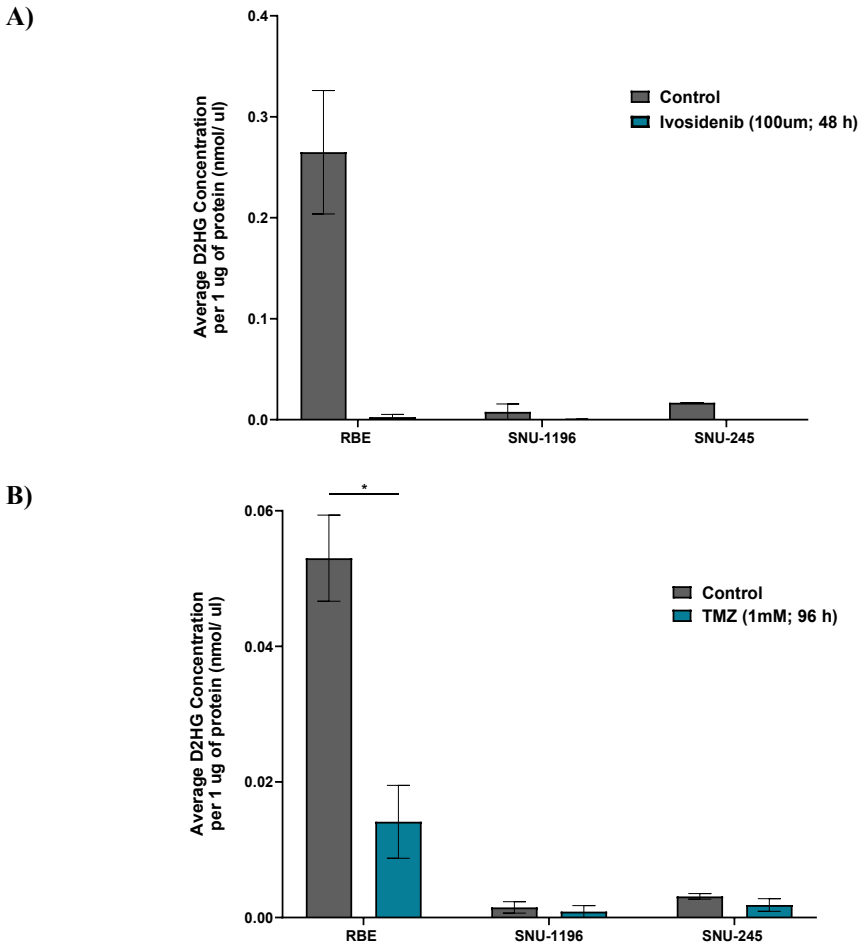


Figure 9. Exposure to TMZ significantly inhibited the accumulation of intracellular D2HG in IDH1 MT CCA. Average intracellular D2HG levels in CCA cells following (A) Ivosidenib (100 μM; 48h) and (B) TMZ treatment (1 mM; 96 h). Error bars represent SEM. (*p<0.05)

Ivosidenib is known to reduce the abnormal D2HG levels in IDH1 MT cancers. We confirmed that Ivosidenib treatment effectively reduces intracellular D2HG levels in CCA with a 98.5% reduction in IDH1 MT CCA (Figure 9A). Then, we examined whether TMZ also affects the abnormal D2HG concentration. Interestingly, exposure to TMZ (1mM) for 96 h significantly reduced the average D2HG levels across all CCA cell lines. Specifically, TMZ led to a 73.3% reduction in D2HG levels in IDH1 MT CCA, which was statistically significant (Figure 9B). By this means, we were able to deduce that TMZ not only induces DNA DSBs and apoptosis but also resembles the mechanism of action of Ivosidenib by reducing the intracellular D2HG levels in CCA.

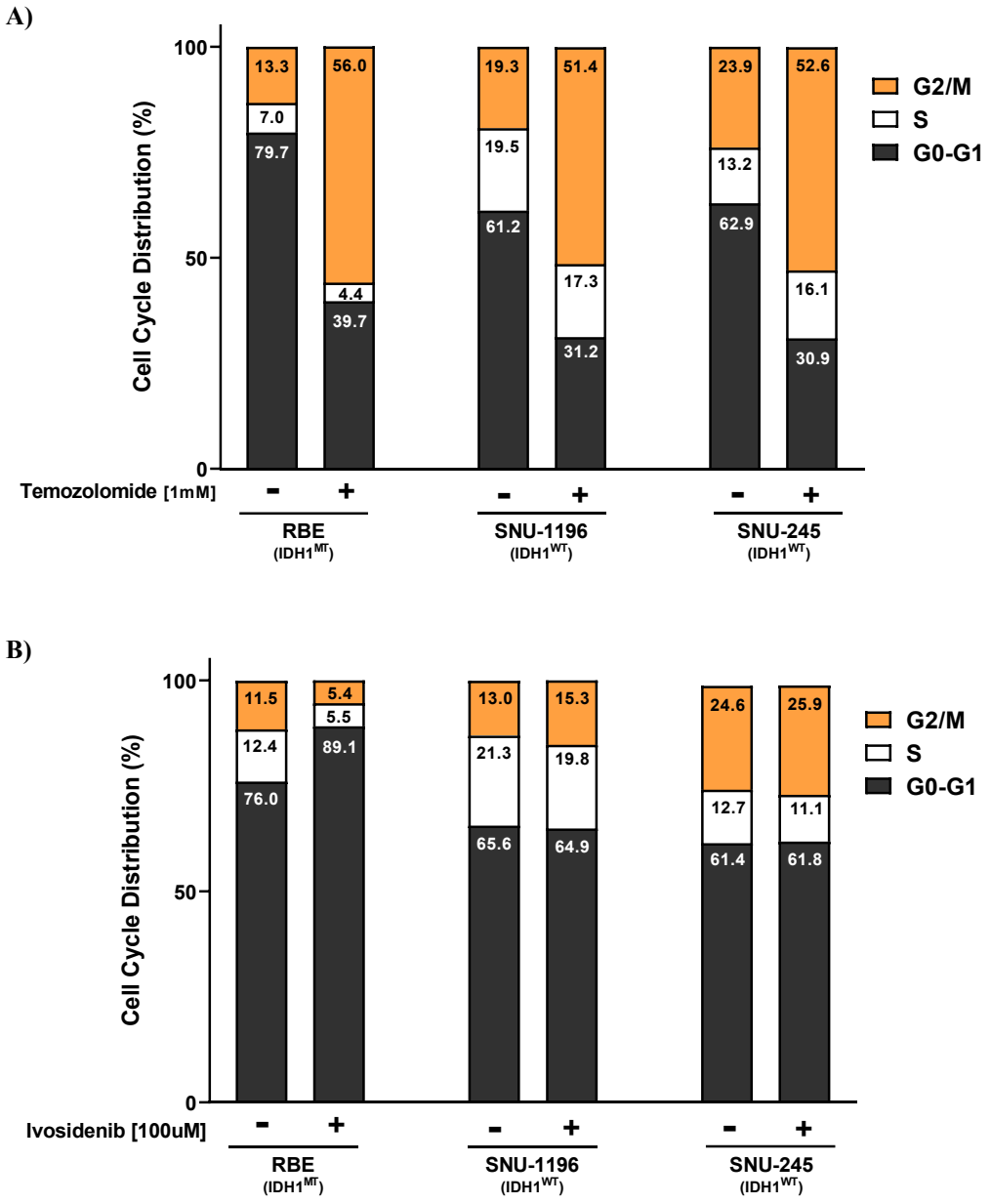
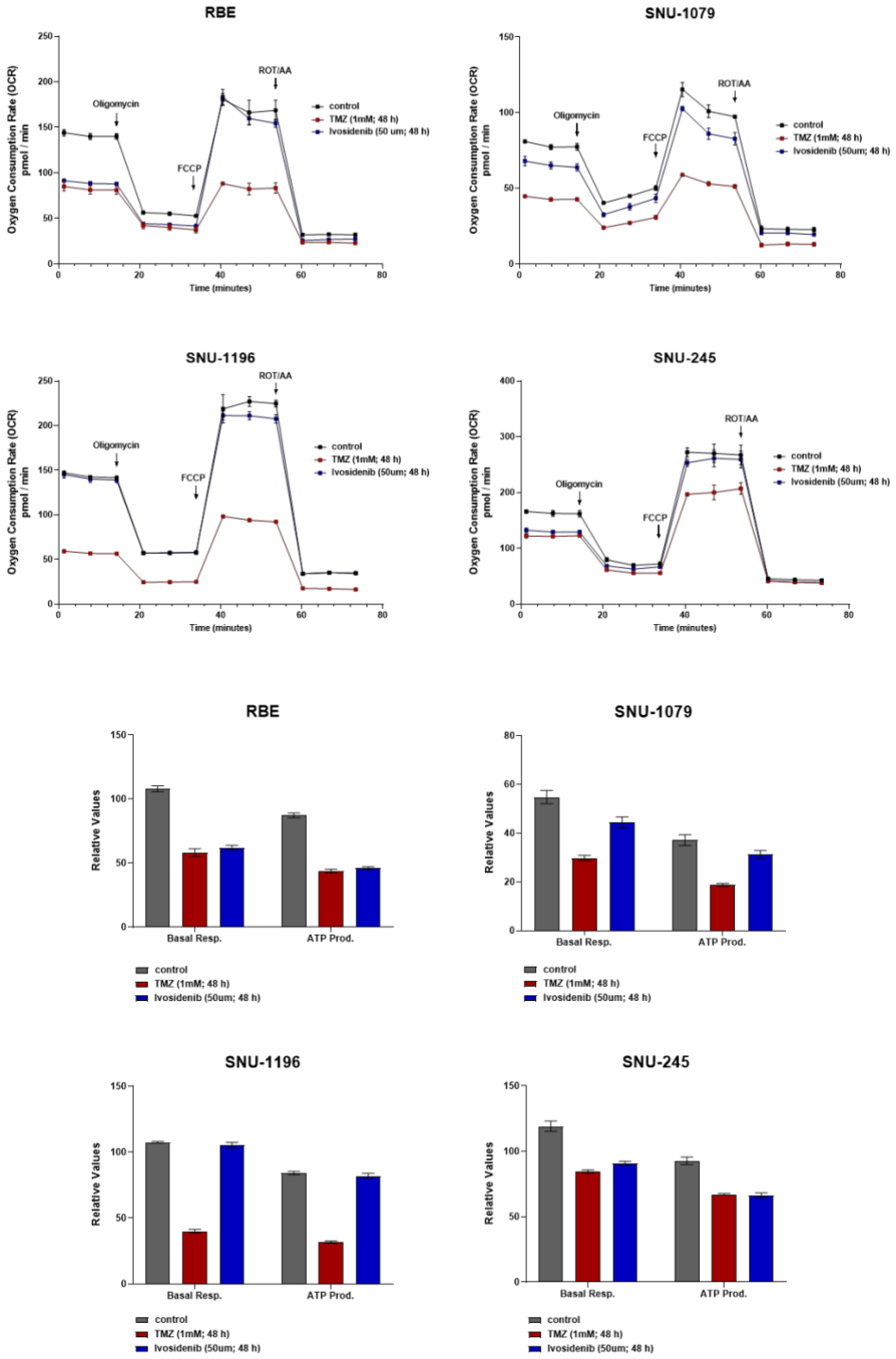


Figure 10. Increased TMZ-induced G2/M arrest and Ivosidenib-Induced G0-G1 arrest in IDH1 MT CCA (A) Changes in cell cycle distribution following TMZ treatment (1 mM) for 48 h in CCA cell lines. (B) Changes in cell cycle distribution following Ivosidenib treatment (100 μM) for 48 h in CCA cell lines.

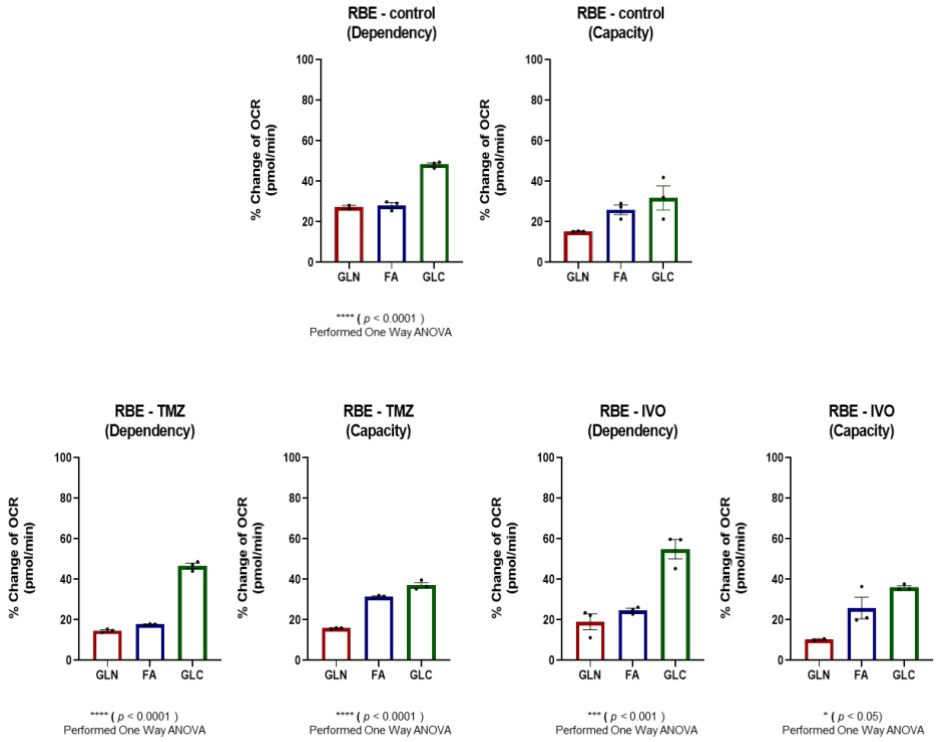
We performed cell cycle analysis to identify the effect of TMZ on cell cycle in CCA cells. As seen in figure 10A, TMZ induced G2/M cell cycle arrest across all CCA cell lines. Nevertheless, we observed an increased TMZ-induced G2/M arrest in IDH1 MT cells (control vs. TMZ: 13.3% vs. 56%) than in IDH1 WT CCA cells (control vs. TMZ: 19.3 vs. 51.4% or 23.9% vs. 53.9% for SNU-1196 and SNU-245, respectively).

In addition, we explored the effect of Ivosidenib on cell cycle in CCA cells (Figure 10B). Unlike TMZ, which induced a G2-M arrest, treatment with Ivosidenib induced a G0-G1 arrest in IDH1 MT cells (control vs. Ivosidenib: 76% vs. 89.1%). Nonetheless, there was little to no difference in cell cycle distribution in the G0-G1 phase following Ivosidenib treatment in IDH1 WT cells.

A)



C)



D)

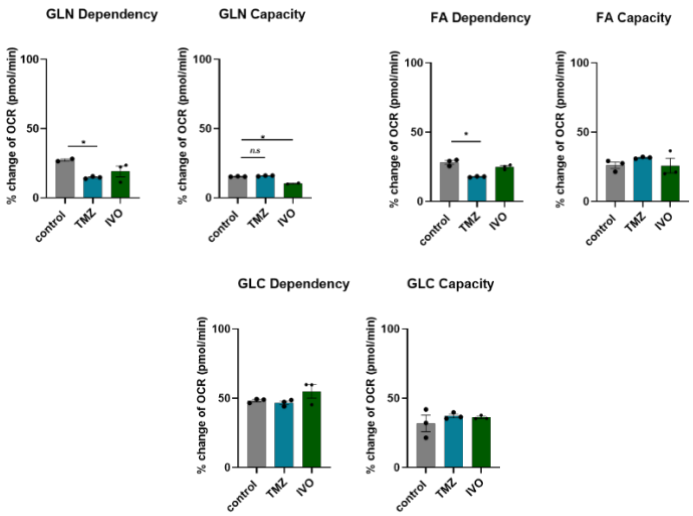


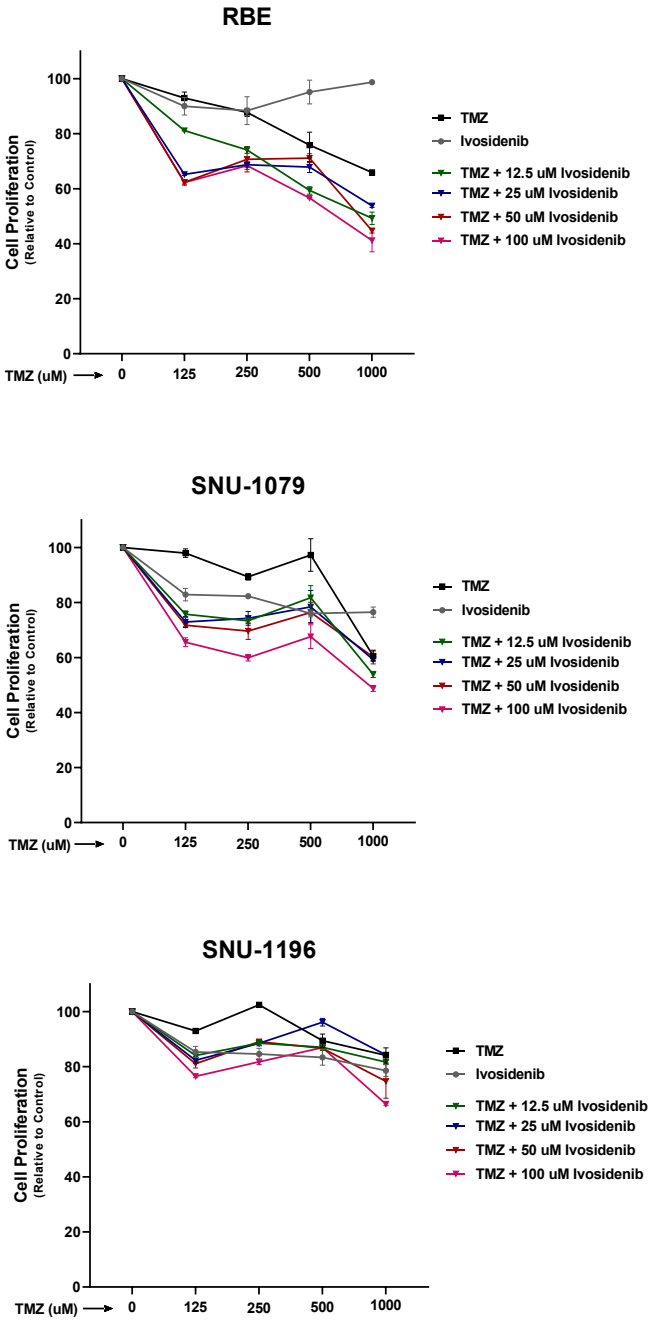
Figure 11. TMZ and Ivosidenib treatment impaired mitochondrial functions in all CCA cell lines yet did not result in metabolic reprogramming. (A) Seahorse XF Mito Stress test was performed to analyze the mitochondrial functions and OCR curves are presented as averages ± SEM of each measurement time point (B) Summary of mitochondrial functions. (C) Fuel Flex Test was performed to analyze CCA cell's mitochondrial fuel (glutamine, fatty acids, glucose) oxidation before and after TMZ/ Ivosidenib treatment. Statistical differences were calculated using one-way ANOVA tests. (D) Fuel Flex Test results were further analyzed to compare the dependency or capacity for each mitochondrial fuel among control, TMZ, and IVO groups. Unpaired student t-test was used for statistical analysis. (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$). Error bars represent SEM.

Then, we investigated whether the mitochondrial functions of CCA cells are also impaired following TMZ or Ivosidenib treatments. Seahorse XF Mito Stress Tests were performed after incubating cells with either media, TMZ (1mM), or Ivosidenib (100 μM) for 48 h. As seen in Figure 11A, basal OCR significantly decreased in the majority of CCA cell lines (RBE, SNU-1079, and SNU-1196) following TMZ and Ivosidenib treatments. Along with the basal OCR values, additional key mitochondrial parameter of mitochondrial function, ATP production, was also reduced following TMZ or Ivosidenib treatments.

Moreover, we performed Seahorse XF Fuel Flex tests to determine whether TMZ or Ivosidenib alters IDH1 MT CCA cells' dependency or capacity to oxidize a particular fuel. Despite drug treatments, there was no shift in the cells' dependency nor the capacity from one fuel to another. Specifically, IDH1 MT cells (RBE) remained to have the highest and lowest dependency for glucose and fatty acids, respectively (Figure 11D). Nevertheless, when we took a closer look into each fuel, there were slight changes in the % of OCR where the dependency for glutamine and fatty acid significantly decreased following TMZ treatment. Interestingly, we observed an increase in glucose dependency following Ivosidenib treatment, yet the trend was statistically insignificant (Figure 11D).

3.3. Synergistic Effect of TMZ & Ivosidenib Combination Therapy

A)



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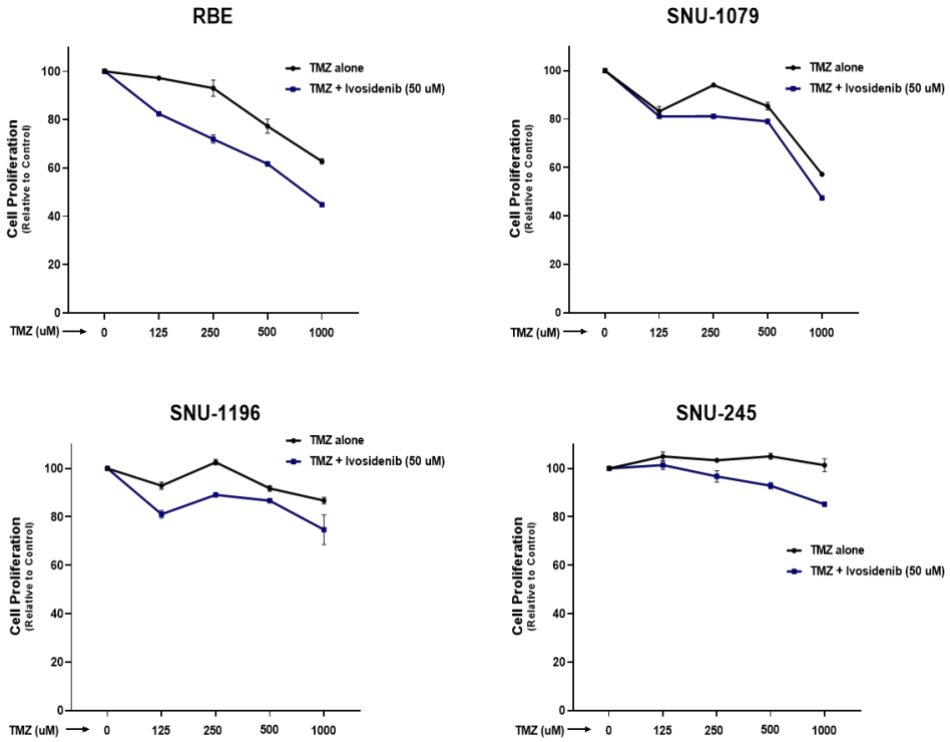


Figure 12. Synergistic Effect of TMZ & Ivosidenib Combination in CCA. CCA cell lines were exposed to varying concentrations of TMZ & Ivosidenib for 96 h and CCK-8 assays were performed to determine cell viability. (B) The dose response curves for IDH1 MT (RBE; SNU-1079) and WT (SNU-1196; SNU-245) following TMZ treatment given either as single or in combination with 50μM Ivosidenib as labelled in black and blue lines, respectively. Error bars represent SEM.

Our findings imply that TMZ exhibits potent cytotoxic effects on IDH1 MT CCA while Ivosidenib is known to exert a cytostatic effect on CCA. Thus, we aimed to explore the synergistic efficacy of combining TMZ with Ivosidenib in CCA cells. We treated CCA cells with serial dilutions of TMZ (0, 125 μM, 250 μM, 500 μM, 1 mM) and Ivosidenib (0, 12.5 μM, 25 μM, 50 μM, 100 μM) either alone or in combination for 96 h and performed CCK-8 cell viability assays. As shown in Figure 12A, the combination of TMZ and Ivosidenib more effectively inhibited the proliferation of CCA cells than single agent treatment, as labelled in black (TMZ) or grey (Ivosidenib) lines.

Table 2: Combination Index of Temozolomide and Ivosidenib in RBE cells

Cell Line	Drug 1	Drug 2	Dose of Drug 1 (μM)	Dose of Drug 2 (μM)	CI
	Temozolomide	Ivosidenib	0	12.5	
	Temozolomide	Ivosidenib	125	12.5	*0.31*
	Temozolomide	Ivosidenib	250	12.5	*0.4*
	Temozolomide	Ivosidenib	500	12.5	*0.38*
	Temozolomide	Ivosidenib	1000	12.5	*0.49*
	Temozolomide	Ivosidenib	0	25	
	Temozolomide	Ivosidenib	125	25	*0.13*
	Temozolomide	Ivosidenib	250	25	*0.3*
	Temozolomide	Ivosidenib	500	25	*0.57*
RBE	Temozolomide	Ivosidenib	1000	25	*0.59*
(IDH1 MT)	Temozolomide	Ivosidenib	0	50	
	Temozolomide	Ivosidenib	125	50	*0.11*
	Temozolomide	Ivosidenib	250	50	*0.33*
	Temozolomide	Ivosidenib	500	50	*0.68*
	Temozolomide	Ivosidenib	1000	50	*0.4*
	Temozolomide	Ivosidenib	0	100	
	Temozolomide	Ivosidenib	125	100	*0.11*
	Temozolomide	Ivosidenib	250	100	*0.3*
	Temozolomide	Ivosidenib	500	100	*0.34*
	Temozolomide	Ivosidenib	1000	100	*0.34*

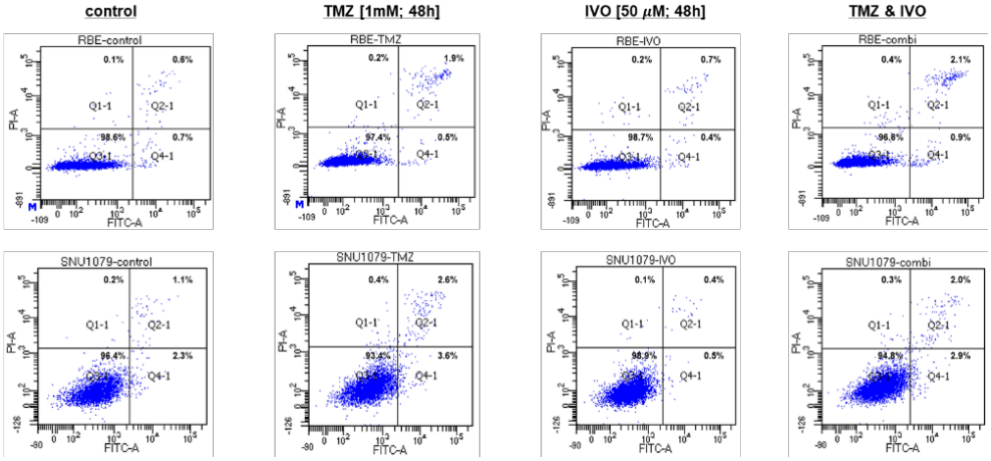
Table 3: Combination Index of Temozolomide and Ivosidenib in SNU-1079 cells

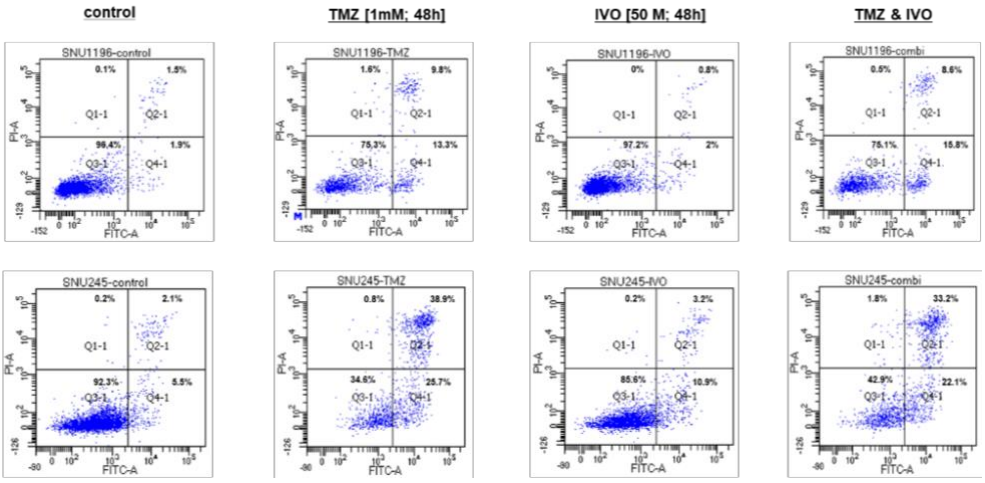
Cell Line	Drug 1	Drug 2	Dose of Drug 1 (μM)	Dose of Drug 2 (μM)	CI
	Temozolomide	Ivosidenib	0	12.5	
	Temozolomide	Ivosidenib	125	12.5	*0.21*
	Temozolomide	Ivosidenib	250	12.5	*0.16*
	Temozolomide	Ivosidenib	500	12.5	*0.47*
	Temozolomide	Ivosidenib	1000	12.5	*0.03*
	Temozolomide	Ivosidenib	0	25	
	Temozolomide	Ivosidenib	125	25	*0.3*
	Temozolomide	Ivosidenib	250	25	*0.35*
	Temozolomide	Ivosidenib	500	25	*0.58*
SNU-1079	Temozolomide	Ivosidenib	1000	25	*0.08*
(IDH1 MT)	Temozolomide	Ivosidenib	0	50	
	Temozolomide	Ivosidenib	125	50	*0.52*
	Temozolomide	Ivosidenib	250	50	*0.41*
	Temozolomide	Ivosidenib	500	50	*0.9*
	Temozolomide	Ivosidenib	1000	50	*0.17*
	Temozolomide	Ivosidenib	0	100	
	Temozolomide	Ivosidenib	125	100	*0.55*
	Temozolomide	Ivosidenib	250	100	*0.32*
	Temozolomide	Ivosidenib	500	100	*0.67*
	Temozolomide	Ivosidenib	1000	100	*0.12*

To determine whether the combined effects of TMZ and Ivosidenib are additive, synergistic, or antagonistic, the expected drug combination responses were calculated using an online tool: SynergyFinder. In detail, a combination index (CI) of 1, <1, or >1 reflects an additive, synergistic, and antagonistic effect, respectively. As shown in Tables 2 and 3, the CI of TMZ and Ivosidenib demonstrated a highly synergistic effect with a CI <1 across all doses in both IDH1 MT cell lines (RBE, SNU-1079).

In this matter, we performed future assays with three treatment groups: TMZ alone, Ivosidenib alone, and TMZ + Ivosidenib. For combination therapy, we utilized 1mM of TMZ and 50 μM of Ivosidenib as it demonstrated a reduced cell viability (%) than TMZ (1mM) alone across all CCA cell lines (Figure 12B).

A)





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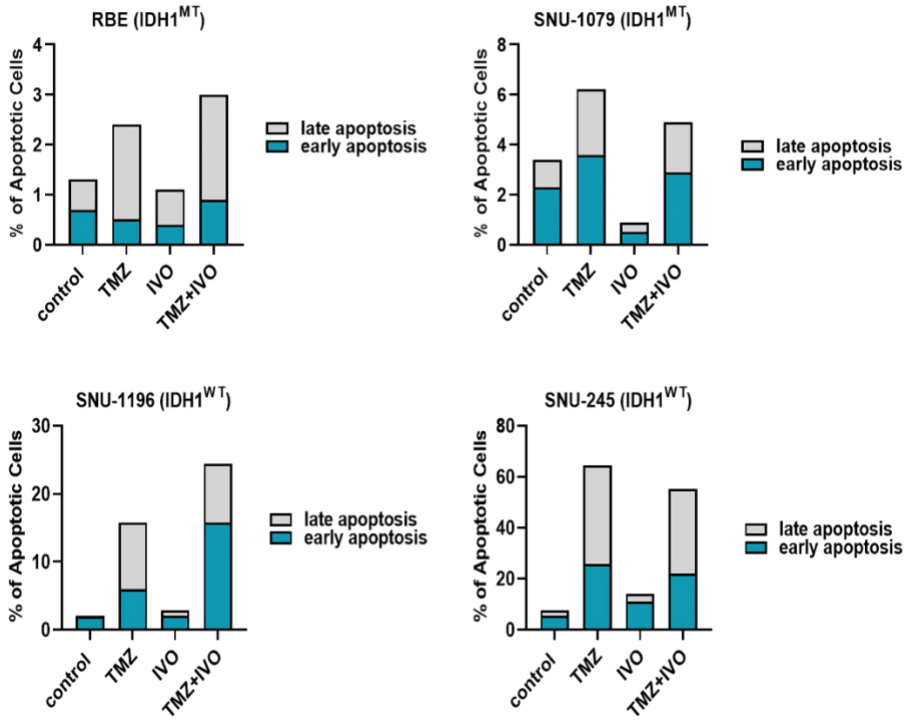

Figure 13. Cytotoxic Effects following TMZ and Ivosidenib as single agents or as combined therapy determined via % of apoptotic cells. (A) CCA cell lines were treated with TMZ (1mM), Ivosidenib (50 μM), or combination therapy (TMZ: 1mM; Ivosidenib: 50 μM). Cell apoptosis was measured by flow cytometry. (B) Quantification of % of total apoptotic cells (early + late) following each treatment conditions in each CCA cell lines.

To further investigate the cytotoxic effects of TMZ alone or in combination with Ivosidenib, we performed cellular apoptosis assay. Quantification of apoptotic cells (%) was done by counting cells in early apoptosis (low right quadrant; Q4-1) and late apoptosis (upper right quadrant; Q2-1). By doing so, we observed an increase in % of apoptotic cells across all CCA cell lines following TMZ treatment (1mM) for 96 h (Figure 13B). On the other hand, Ivosidenib did not result in a significant increase in the % of apoptotic cells. In fact, the % of apoptosis was higher in the control group than in the Ivosidenib group for SNU-1079 (IDH1 MT) and SNU-1196 (IDH1 WT). These data infer that while TMZ exerts a cytotoxic effect on CCA, Ivosidenib exerts a cytostatic effect as reported in pervious literature. As expected, we could observe an increased % of apoptotic cells following combined therapy in RBE and SNU-1196 cell lines. Nevertheless, this trend was not observable in remaining CCA cell lines (Figure 13B).

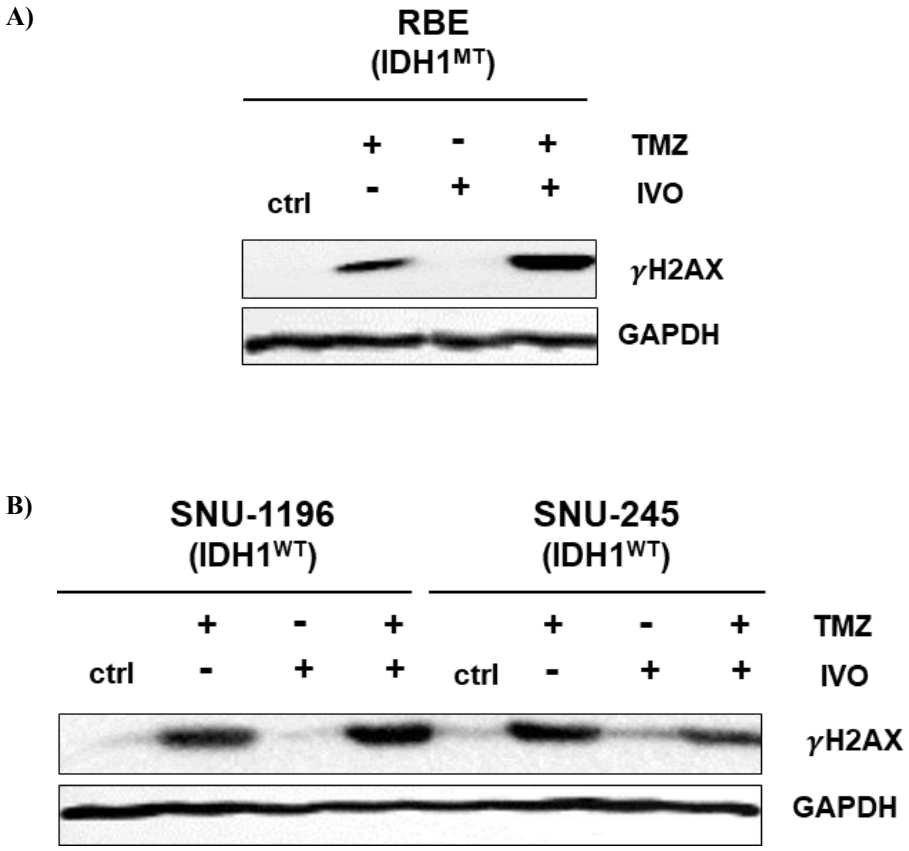

Figure 14. Western blot analysis of DNA damage following TMZ, IVO, or TMZ + IVO. Western blotting analysis of γH2AX following treatment with media, TMZ (1mM), IVO (50μM) or TMZ (1mM) + IVO (50μM) for 96 h in IDH1 MT (A) and WT (B) CCA cell lines.

Moreover, we performed western blot with γH2AX to explore whether combination therapy results in more DNA double strand breaks than single treatment. As seen in figure 14A, γH2AX expression noticeably increased following combination therapy to treating TMZ alone in IDH1 MT CCA (RBE) and this trend was not observable in IDH1 WT CCA (Figure 14 B). In addition, we observed that γH2AX expression following IVO treatment was similar to that of the control group across all CCA cell lines, which reinforces that IVO exerts a cytostatic effect on CCA.

3.4. Establishment of genetically engineered CCA cell lines

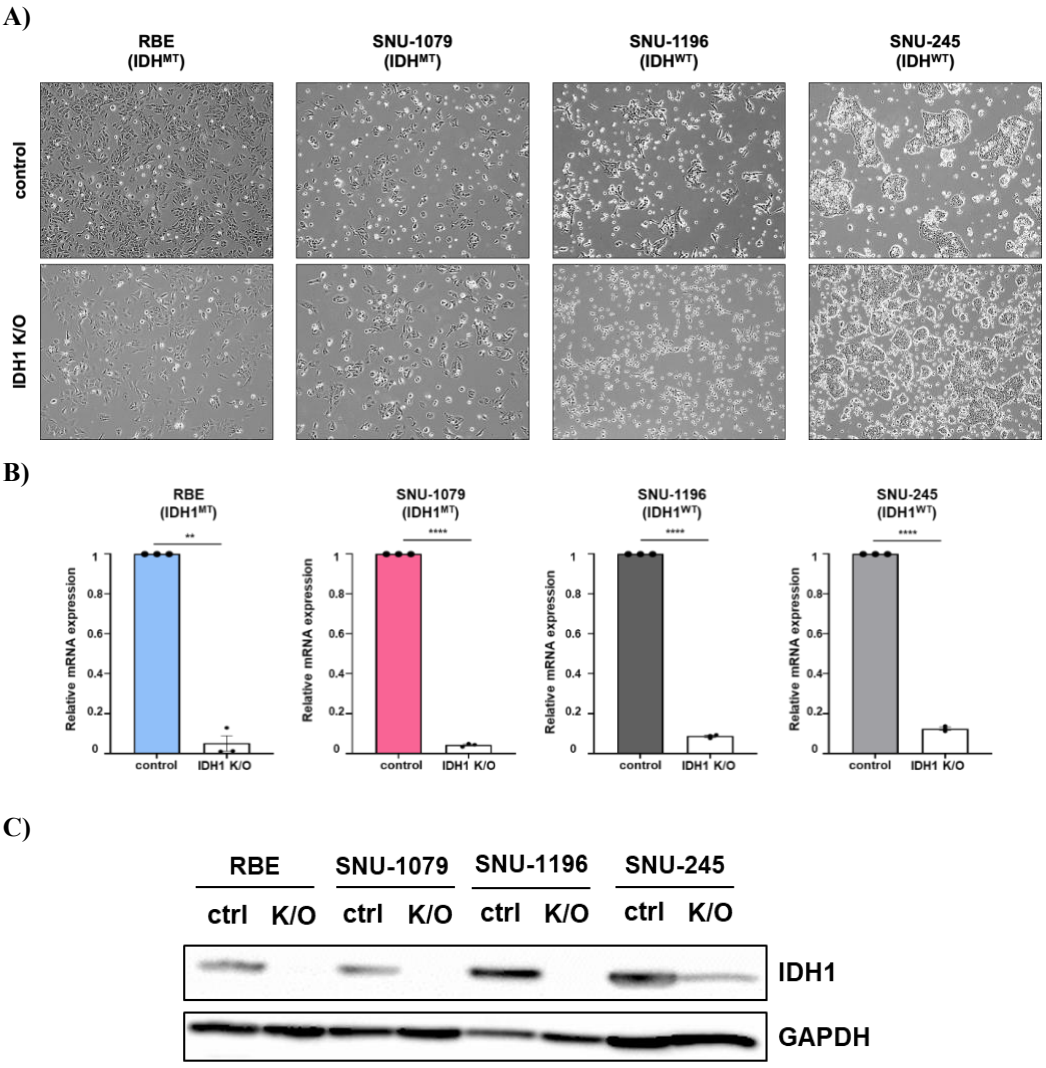

Figure 15. Establishment of IDH1 K/O CCA cell lines using CRISPR-Cas9 mediated genome editing (A) Cell morphology of parental and IDH1 K/O CCA cell lines (B) qPCR results revealed efficient K/O of IDH1 in CCA cell lines. (C) Western blotting analysis of IDH1 in parental and stable IDH1 K/O CCA cell lines.

Despite the positive results, we acknowledged that one limitation of our study is the use of previously established CCA cell lines with endogenous IDH1 mutation. Therefore, we have utilized CRISPR-Cas9 mediated genome editing to explore whether the enhanced sensitivity to TMZ in CCA

is directly due to IDH1 mutation and not influenced by other external factors. To do so, we have first established stable IDH1 K/O CCA cell lines and the efficiency of IDH1 K/O was determined via qPCR and western blotting analysis (Figure 15B and C). There were no noticeable changes in cell morphology despite IDH1 K/O in CCA cell lines as seen in figure 15A.

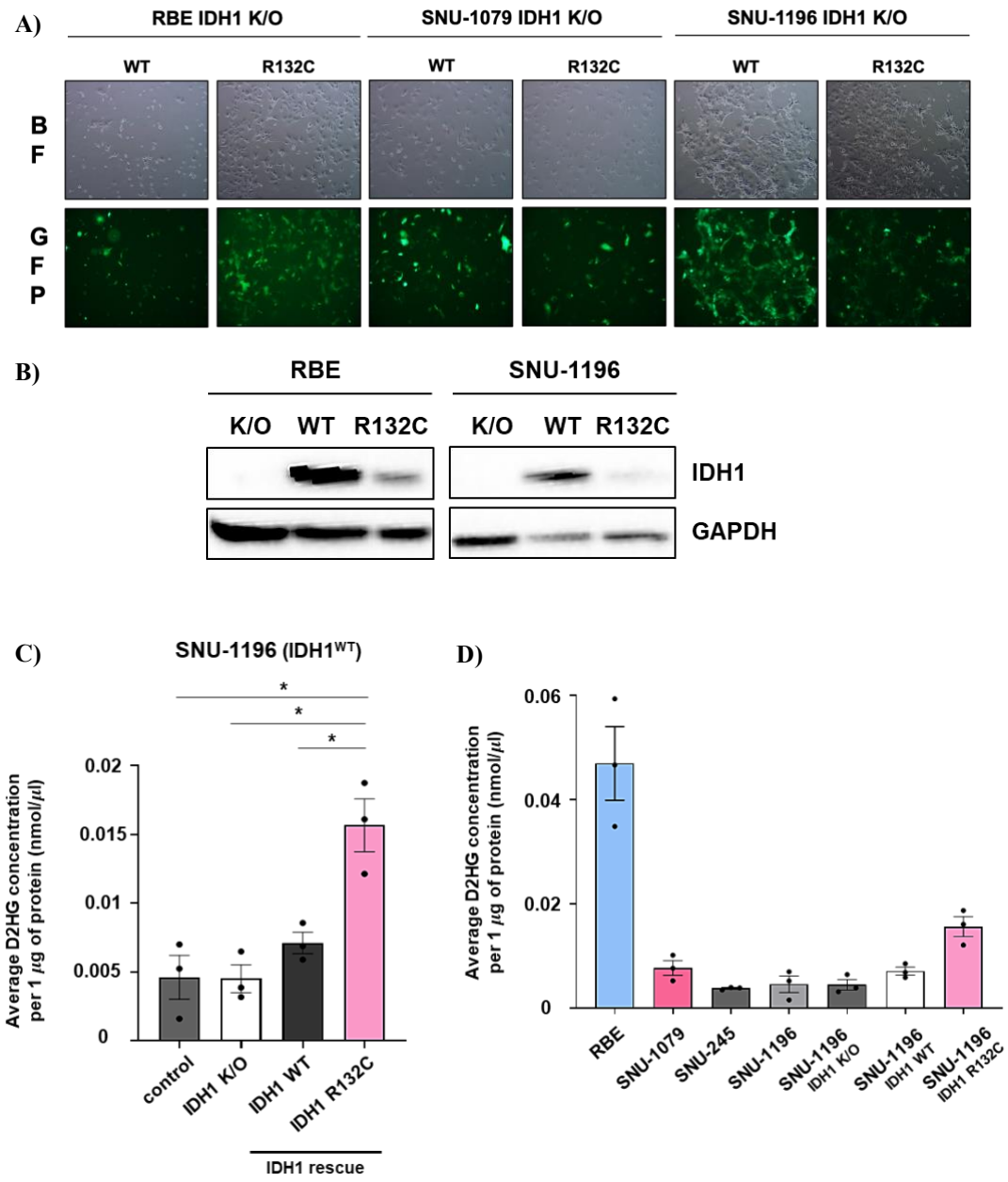

Figure 16. Rescue of IDH1 MT in IDH1 K/O CCA cell lines led to an increase in intracellular D2HG levels (A) GFP expression was used to monitor the efficiency of transduction for the rescue of either IDH1 WT or MT genes in IDH1 K/O CCA cell lines. (B) Average intracellular D2HG concentrations in SNU-1196 (parental, IDH1 K/O, IDH1 WT rescue, IDH1 R132C MT rescue). (D) Comparison of D2HG levels in parental CCA cell lines and genetically engineered SNU-1196 cells (parental, IDH1 K/O, IDH1 WT rescue, IDH1 R132C MT rescue).

Following the establishment of stable IDH1 K/O CCA cell lines, the rescue of IDH1 WT or MT (R132C) genes was performed using the pHAGE-IDH1 (WT) and pHAGE-IDH1-R132C vectors containing GFP. By doing so, we were able to monitor successful transduction of IDH1 vectors in IDH1 K/O CCA cell lines through GFP expression (Figure 16A) and western blotting analysis (Figure 16B).

As we have confirmed the generation of genetically engineered IDH1 WT or MT CCA cell lines, we questioned whether the intracellular D2HG levels would increase following the rescue of IDH1 MT genes in SNU-1196, which originally harbor IDH1 WT genes. As shown in figure 16C, the rescue of IDH1 WT genes increased the D2HG levels compared to parental or IDH1 K/O SNU-1196 cells. Interestingly, the rescue of IDH1 MT genes in SNU-1196 outstandingly increased the intracellular D2HG levels compared to parental, K/O, and IDH1 WT SNU-1196 cells, and these data were statistically significant. Moreover, the concentration of D2HG levels in genetically engineered SNU-1196 cells harboring a R132C mutation was higher than that of SNU-1079 cells, which endogenously harbor an IDH1 R132C mutation.

In this matter, we could phenotypically confirm the successful generation of genetically engineered IDH1 WT or MT CCA cell lines. In the near future, these cell lines would be further utilized to assess the drug sensitivity to TMZ and Ivosidenib. By doing so, it is expected that these cell lines would play a key role in understanding the characteristics of IDH1 mutation in CCA and unveiling the mechanism behind enhanced sensitivity to TMZ.

IV. DISCUSSION

CCA remains challenging to treat owing to the lack of treatment options and limited response to available therapies. Recently, NGS revealed various potential 'druggable' targets in CCA, including FGFR2 fusion, HER2 amplification, and IDH1 mutation. Following such discovery, targeted therapies have emerged as a pivotal constituent in CCA treatment by increasing the overall response rate of up to 20% and 40% for patients with FGFR2 fusion and HER2 amplification, respectively.

IDH1 mutation occurs in up to 20% of iCCA. Nevertheless, most prior research on IDH1 mutation was conducted in other cancer types, such as glioma or colorectal cancer. Thus, the pathogenic mechanism of IDH1 MT in CCA is still poorly understood. In this matter, our study aimed to characterize IDH1 MT CCA by utilizing previously established human CCA cell lines (RBE, SNU-1079) that harbor a hotspot mutation at either R132S or R132C, respectively. A well-known characteristic of IDH1 mutation is the excessive accumulation of the oncometabolite, D2HG. We confirmed that IDH1 MT also results in an elevation of intracellular D2HG levels in CCA cell lines. Moreover, IDH1 mutant genes consume NADPH to convert α-KG into D2HG and a disruption in NADPH homeostasis is often associated with elevated intracellular ROS levels. We observed that both NADP+/NAPDH ratio and ROS levels were elevated in IDH1 MT CCA in comparison to IDH1 WT CCA cell lines, which are findings consistent with previous research on different cancer types.

The IDH1 gene is a key metabolic enzyme in the TCA cycle, which not only converts isocitrate into α-KG but is also a major source of NADPH that is critical for maintaining cellular redox balance. Prior research has demonstrated that inhibition of the activity of various α-KG-dependent enzymes or alteration in the cellular NADPH/NADP+ ratio following IDH1 mutation, results in mitochondrial dysfunction and metabolic reprogramming. By performing metabolomics assays, we observed impaired mitochondrial functions in IDH1 MT CCA cell lines compared to IDH1 WT cells. Moreover, we discovered that IDH1 MT and WT CCA cell lines utilize distinct metabolic pathways with the highest dependency on glycolysis and fatty acid oxidation, respectively.

Ivosidenib is a small-molecule inhibitor of the IDH1 gene, which has received FDA approval for treating patients with advanced IDH1 MT iCCA. Despite its clinical benefits, the objective response rate was solely 2% in the phase III ClarIDHy trial. This was reflected in our study during drug screening as Ivosidenib failed to inhibit the proliferation of all CCA cell lines, with IC_{50} values greater than 100μM, regardless of the IDH1 mutation status.

Temozolomide is an alkylating agent widely used to treat patients with IDH1 MT glioma. However, its cytotoxic effects have not been previously explored in CCA. To determine whether TMZ could serve as a novel approach to treat IDH1 MT CCA, we treated all CCA cell lines with varying

concentrations of TMZ. By doing so, we noticed that TMZ selectively inhibits IDH1 MT CCA cell lines with an IC50 value of 629.1 μM and > 1mM for IDH1 MT and WT cell lines, respectively. Moreover, γH2AX expression, which serves as a biomarker for DNA DSBs, was outstandingly elevated in IDH1 MT cell lines in comparison to IDH1 WT (~5 fold vs. ~1.2 fold in rH2AX expression following TMZ treatment).

In addition to DNA DSBs following TMZ treatment, we observed that TMZ reduces the abnormal intracellular levels of D2HG in IDH1 MT CCA. This resembles the mechanism of action of Ivosidenib and other IDH1 MT-targeted therapies. Moreover, this trend was not reported in previous studies involving TMZ, and thus, may help propose a novel mechanism of increased Temozolomide sensitivity in IDH1 MT cancers.

Prior research has reported that TMZ induces G2/M arrest[19]. This was also reflected across all CCA cell lines. Nevertheless, TMZ-induced G2/M arrest was noticeably increased in IDH1 MT cell line (RBE) compared to IDH1 WT cells with a percentage increase of up to 321.1%. On the other hand, Ivosidenib induced G0-G1 arrest in CCA cell lines, which is in line with previous studies of non-small cell lung cancer[20]. Interestingly, IDH1 WT cell lines had little to no difference in the number of cells in the G0-G1 phase following Ivosidenib treatment.

Drug treatments often lead to metabolic reprogramming in cancer, which contributes to chemotherapy resistance. Therefore, we performed metabolomics assays to compare how the mitochondrial functions and cells' dependency or capacity for a particular fuel are disrupted following TMZ or Ivosidenib treatments. As expected, drug treatments significantly impaired key mitochondrial parameters, including basal respiration and ATP production. TMZ had a greater impact on such parameters than Ivosidenib. Though we confirmed impaired mitochondrial functions, we could not observe metabolic shifts following TMZ or Ivosidenib treatments in IDH1 MT cells.

With various assays, we noticed that TMZ exerts a cytotoxic effect on IDH1 MT CCA unlike Ivosidenib, which is known to exert a cytostatic effect on CCA. Moreover, we confirmed that TMZ and Ivosidenib induces cell cycle arrest in different phases. Therefore, we further explored whether the combination of TMZ and Ivosidenib could exert a synergistic effect on inhibiting cell proliferation of IDH1 MT CCA cell lines. Cell viability assays and the quantification of the CI index suggested that combining TMZ and Ivosidenib is highly synergistic with a CI <1 across all doses in both IDH1 MT cell lines. Subsequently, when we performed an apoptosis assay, we saw an increased % of apoptotic cells in one of the IDH1 MT cells (RBE) following combination therapy than TMZ or Ivosidenib alone. This trend was further supported with increased γH2AX expression following combination treatment than treating TMZ alone in IDH1 MT CCA (RBE). Furthermore, apoptosis results inferred that Ivosidenib exerts a cytostatic effect on all CCA cell lines as there was little to no difference in the % of apoptotic cells following Ivosidenib treatment.

Moreover, we acknowledge that one major limitation of our study is the use of previously established human CCA cell lines that endogenously harbor an IDH1 mutation. In other words, we could not exclude the notion that various external factors may have influenced the sensitivity of IDH1 MT or WT CCA cell lines to TMZ. Therefore, we have utilized CRISPR-Cas9 mediated genome editing to first generate IDH1 K/O CCA cell lines and later rescue either IDH1 WT or IDH1 MT (R132C) genes in the stable IDH1 K/O CCA cell lines. Specifically, IDH1 R132C variant was specifically chosen as it is one of the most common hotspot mutation in CCA. After successful transfection, transduction, and Hygromycin B selection, we were able to generate stable IDH1 K/O CCA cell lines. Then, the rescue of IDH1 genes were also confirmed with GFP expression and IDH1 expression using western blotting analysis. Though we have not yet explored the sensitivity of TMZ in these established CCA cell lines, we were able to notice a statistically significant increase in the intracellular D2HG levels in IDH1 K/O SNU-1196 cell lines expressing IDH1 MT (R132C) genes. This was particularly interesting as the rescue of IDH1 MT genes not only increased D2HG levels compared to IDH1 K/O SNU-1196 cells but were also greater compared to parental SNU-1079 cells, which endogenously harbor a IDH1 R132C mutation.

In addition, one limitation of previously established IDH1 MT CCA cell lines is that they are non-tumorigenic[21]. Thus, *in vivo* studies with IDH1 MT CCA cell lines were nearly impossible[21]. On the other hand, IDH1 WT CCA cell lines, such as SNU-1196 have been reported to form tumors in mice[22]. Thus, we look forward to assessing the effect of TMZ *in vivo* and unraveling the mechanism behind drug sensitivity with our genetically engineered IDH1 MT CCA cell lines.

V. CONCLUSION

The discovery and clinical development of therapies surrounding oncogenic IDH1 mutation have been a breakthrough in the translational research of CCA. Characterization of human CCA cell lines in our study provides insight into metabolic alterations following IDH1 mutation, which has only been addressed in a few comprehensive studies. Furthermore, our study provides a novel therapeutic approach to treat IDH1 MT CCA by demonstrating that an alkylating agent, TMZ, exhibits potent cytotoxic effects on IDH1 MT CCA by inducing apoptosis, G2-M cell cycle arrest and reducing the abnormal intracellular D2HG levels. Additionally, we have explored the synergistic effects of combining TMZ with Ivosidenib, which could be more effective in improving the prognosis of IDH1 MT CCA patients than treating TMZ or Ivosidenib treatment alone.

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Abstract in Korean

IDH1 돌연변이 담도암의 종양 특성 규명을 통한 새로운 치료 전략 수립 연구

담도암은 간에서 생성된 담즙이 십이지장으로 배출되는 경로를 이루는 담관에서 유래하는 종양으로 우리나라는 전 세계에서 남성 2위, 여성 3위로 발병률이 다른 국가 대비 월등히 높은 편에 속한다. 조기 진단의 어려움과 해부학적인 위치상 근치적 수술이 매우 어렵기 때문에 담도암 환자의 예후는 현재까지도 매우 불량하다. 최근 NGS를 통해 IDH1 돌연변이가 간내담도암에서 높은 빈도로 일어난다고 밝혀졌으며, 이를 타겟하는 표적치료제인 Ivosidenib이 2021년 미 FDA에서 담도암 치료제로 승인을 받았다. 단, 제 3상 임상시험에서 객관적 반응률이 2%에 그쳐 약물의 효과가 매우 미미하므로 더욱 효과적인 치료 전략이 절실한 상황이다. Temozolomide는 알킬화제에 속하는 항암제로써 IDH1 돌연변이를 갖는 뇌종양의 표준 치료제로 사용되고 있다. 그러나, 담도암에서 Temozolomide의 약물 효과를 연구한 전례는 없다. 따라서, 본 연구는 담도암의 불량한 예후를 개선하기 위해 IDH1 돌연변이를 보유한 담도암의 종양 특성을 규명하고 알킬화제 약물 재배치의 가능성을 제시함으로써 다각도로 새로운 담도암 치료 전략을 수립하고자 하였다. 4개의 담도암 세포주를 이용하여 IDH1 변이 유무에 따른 세포 특성을 확립하였고 대사체 분석을 통해 IDH1 변이에 따른 담도암의 대사적 변화도 관찰하였다. 담도암 세포주에서 Temozolomide 및 Ivosidenib의 약물효과를 분석한 결과 IDH1 변이를 보유한 담도암 세포주에서 야생형 대비 Temozolomide에 더욱 민감한 점을 확인했으며 약물에 의한 DNA 이중나선 절단도 증가하여 세포 사멸을 더욱 유도하는 것을 발견했다. 또한, Temozolomide에 의한 G2/M 기의 arrest 역시 IDH1 변이를 보유한 담도암 세포주에서 야생형 대비 증가하는 것을 관찰하였다. Ivosidenib의 경우 G0-G1 arrest를 유도하는 것을 확인하였다. 더 나아가, Ivosidenib의 약물 기전과 비슷하게 Temozolomide이 IDH1 변이에 의해 세포 내 비정상적으로 축적된

oncometabolite인 D2HG의 농도를 낮추는데 기여하는 것도 확인하였다. 본 연구에서는 세포 사멸을 유도하는 Temozolomide과 세포 성장을 억제하는 Ivosidenib을 병용했을 때 시너지 효과가 있으며 암세포 사멸이 더욱 증가하는 것을 in vitro 상에서 확인하였다. 이로써, Temozolomide이 IDH1 돌연변이를 보유한 담도암 치료에 있어 유망하며 향후 Ivosidenib 약물과의 병용 치료로써의 가능성이 있음을 제시하고자 한다.

핵심되는 말: 담도암, IDH1 변이, Temozolomide, Ivosidenib