ABEMACICLIB, A CDK4/6 INHIBITOR, INHIBITS PROLIFERATION AND INDUCES CELLULAR SENESCENCE IN CHOLANGIOCARCINOMA CELLS

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ABSTRACT

Cholangiocarcinoma (CCA) is an aggressive bile duct cancer with a poor prognosis and a high mortality rate, primarily due to late diagnosis which often results in standard treatment failure. Therefore, targeted therapies offer a promising approach for CCA treatment. Our preliminary bioinformatics analyses have shown that Cyclin D and CDK4/6, key regulators of cell cycle progression, are significantly overexpressed in CCA patients, suggesting these as potential therapeutic targets. In this study, we demonstrated that Abemaciclib, an FDA-approved CDK4/6 inhibitor, effectively inhibits CCA cell proliferation. In addition to inhibiting cell proliferation, CDK4/6 inhibitors have also been demonstrated to induce senescence, a condition in which persistent senescent cancer cells may lead to unfavorable treatment outcomes, including the potential for cancer relapse. Since there is no publicly available database in CCA to analyze the association between Abemaciclib and senescence induction, we utilized a database of breast cancer cells treated with Abemaciclib. The results showed that the cellular senescence processes are associated with Abemaciclib treatment. This was further confirmed by a RT-qPCR and β-galactosidase staining assay, which are markers of senescence. The results indicated an upregulation of senescence marker genes, *p16* and *p21*, along with a significantly higher number of positively blue-stained cells in Abemaciclib-treated CCA cells compared to untreated cells. Collectively, this is the first study to evaluate the effects of Abemaciclib on cell proliferation and cellular senescence in CCA cells. These findings could contribute to the development of strategic combination therapies by combining CDK4/6 inhibitors with senolytic drugs to effectively target persistent senescent cells in a sequential manner.

Keywords: Cholangiocarcinoma, Targeted Therapy, CDK4/6 Inhibitor, Abemaciclib, Cellular Senescence

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INTRODUCTION

Cholangiocarcinoma (CCA) is a highly heterogeneous biliary malignancy, arising from abnormal division of the biliary duct epithelial cells (Banales et al., 2022). The global incidence of CCA is currently increasing, with a particularly high prevalence in northeastern Thailand. The most contributing risk factors of CCA are *Opisthorchis viverrini* (foodborne liver fluke) infection, primary sclerosing cholangitis (PSC), hepatolithiasis, and hepatitis B and C infection (Plentz & Malek, 2015). CCA is a highly aggressive cancer that has a poor prognosis and a high mortality rate. The majority of early stage CCA patients are asymptomatic; thus, patients often present clinically symptoms at an advanced stage, leading to high mortality due to late diagnosis and limited treatment options (Treeprasertsuk et al., 2017). For patients with advanced CCA, therapeutic options are extremely limited, with chemotherapy often proving ineffective and frequently resulting in chemoresistance (Ilyas et al., 2023; Ilyas et al., 2018). Therefore, the development of more effective therapeutic strategies, particularly targeted therapies, is urgently needed to improve survival rates for CCA patients

Targeted therapy offers a new hope in the CCA treatment by exploiting the distinct characteristics of cancer cells, known as the 'Hallmarks of cancer'. These characteristics include the ability to sustain proliferative signaling and evade growth suppressors (Hanahan, 2022; Hanahan & Weinberg, 2011). Dysregulated cell division and cell death mechanisms are fundamental drivers of cancer, driving sustained proliferation and cancer progression. Therefore, targeting the hallmarks of cancer may more effectively inhibit cancer progression. The cell cycle is a sequence of events in which cellular components are duplicated and then precisely separated into two genetically identical daughter cells (Johnson et al., 2002). The transition from one phase to another phase occurs sequentially in unidirectional and is strictly controlled by the cell cycle checkpoint (Barnum & O'Connell, 2014; Matthews et al., 2022; Visconti et al., 2016). The G1/S checkpoint is a critical checkpoint in determining whether a cell proceeds to the S phase or remains in the G0 phase. Once a cell passes this checkpoint, it is irreversibly committed to completing the cell cycle. Cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, are key regulators of cell cycle progression. In the G1/S checkpoint, Cyclin D associates with CDK4/6 to phosphorylate the Rb protein, leading to its release from E2F transcription factors, and then transcriptions the required genes for entering the S phase (Malumbres, 2014; Vermeulen et al., 2003). Aberrant regulation of the cell cycle and mutations in the key cell-cycle regulatory proteins result in uncontrolled cell proliferation, leading to cancer formation and progression. Previous studies and bioinformatics analysis have shown that CCA has overexpressed cyclin D and CDK4/6, and patients with highly expressed cyclin D and CDK4/6 have a short overall survival and disease-free survival (Dong et al., 2022; Du et al., 2020; Sia et al., 2013). Accordingly, the targeted therapeutic agents for CDK4/6 activity, CDK4/6 inhibitors, might be a promising strategy for the treatment of cancer with overexpressed Cyclin D and CDK4/6. Currently, CDK4/6 inhibitors have entered clinical trials and been approved by the U.S. FDA. Abemaciclib, one of the FDA-approved CDK4/6 inhibitors, effectively inhibits both CDK4 and CDK6 kinase activity. Abemaciclib induces G1-phase cell cycle arrest by inhibiting CDK4/6 activity, thereby preventing Rb phosphorylation (Torres-Guzmán et al., 2017). Recently, the U.S. FDA approved Abemaciclib for the treatment of advanced-stage HR+/HER2- breast cancer (Raheem et al., 2022). However, using Abemaciclib as a single treatment remains a limitation due to the main mechanism is to inhibit cell proliferation but induce minimal cell death, resulting in a reduced drug response. Furthermore, cancer cells can develop acquired drug resistance and enter a state of cellular senescence, contributing to cancer relapse (Wang et al., 2022). Therefore, combining CDK4/6 inhibitors with other agents that promote cell death and eliminate senescent cells may improve therapeutic efficacy.

Cellular senescence is a stable cell cycle arrest that occurs in response to endogenous and exogenous stimuli, including DNA damage, oxidative stress, oncogene activation (OIS), and therapeutic-induced senescence (Kumari & Jat, 2021). The main mechanism of action of the CDK4/6 inhibitor is to inhibit CDK4/6 activity and then halt cell cycle progression, leading to a senescence-like state. This arrest can result in either quiescence or senescence, depending on various factors. Quiescent cells can resume growth when stimulated by mitogenic signals, while senescent cells exhibit a stable growth arrest along with distinct phenotypic changes (Wagner & Gil, 2020). Previous studies have reported that the CDK4/6 inhibitor induces senescence in breast cancer cells (Vijayaraghavan et al., 2017), hepatocellular carcinoma (Bollard et al., 2017), liposarcoma (Kovatcheva et al., 2015) and melanoma (Yoshida et al., 2016). Cellular senescence in cancer is a double-edged sword. On one hand, senescent cells can cause irreversible cell growth arrest and enhance innate immune surveillance, inhibiting tumor development. On the other hand, they secrete various senescence-associated secretory phenotype (SASP) factors that can promote tumor proliferation, migration, invasiveness, angiogenesis, and epithelial-mesenchymal transition (EMT), which are major contributors to cancer relapse and mortality (Di Micco et al., 2021; Guan et al., 2017; Qiu et al., 2021; Xiao et al., 2023). Therefore, combination therapy involving CDK4/6 inhibition, and the elimination of senescent cells may improve efficacy and reduce relapse.

In our study, we aim to evaluate the efficacy of monotherapy CDK4/6 inhibitor Abemaciclib and to identify biological pathways involved in Abemaciclib response, with a particular focus on cellular senescence. Our finding provides valuable preclinical data for developing novel combination strategies utilizing Abemaciclib and senolytic agents. This approach demonstrates potential for clinical application and may enhance the therapeutic Abemaciclib monotherapy in CCA patients.

MATERIALS & METHODS

CCA cell model selection and cell culture

KKU-213B was selected as an *in vitro* model of CDK4/6 inhibitor-responsiveness, due to the high expression of key regulatory proteins in the G1 cell cycle checkpoint, including CDK4/6, cyclin D, and phosphorylated RB (Sittithumcharee et al., 2019). MMNK-1 was chosen as a comparative human non-tumor cholangiocyte. KKU-213B and MMNK-1 cell lines were grown in HAM's F-12 medium supplement with 10% fetal bovine serum and 1% penicillin streptomycin under the standard protocol at 37℃ in a 5% CO_2 humidified atmosphere. All cultures were tested for mycoplasma contamination and were mycoplasma-free.

Drug treatment and cell viability assay

For the primary screening drug response in KKU-213B and MMNK-1, the cells were seeded into a 96-well plate, then treated with various concentrations of Abemaciclib (0.1, 0.5, 1, 5, 7.5, and 10 μM) and incubated for 72 hours, using DMSO as a vehicle control. The MTT assay was used to measure cellular metabolic activity, which indicates cell viability. In brief, 10 μL of 5 mg/mL MTT solution was added to each well and incubated at 37℃ in a 5% CO_2 humidified atmosphere for 2 h, during which purple crystals were observed in the cytoplasm under a light microscope. Then the medium was carefully removed and 100 μL of DMSO was added to solubilize formazan crystal. The absorbance of this solution was measured at 540 nm using a microplate reader. The percentage of cell viability and IC_{50} was calculated (Inc. AB, 2014).

$$\%cell\ proliferation = \frac{Absorbance\ of\ each\ Abemaciclib\ treatment}{Absorbance\ of\ vehicle\ control} x100$$

Bioinformatics analysis for the identification of differential expressed genes (DEGs)

Differentially expressed genes (DEGs) were identified by using RNA sequencing data from the GSE222984 cohort (Li et al., 2023), which included MCF-7 and MDA-MB-231 breast

cancer cells treated with Abemaciclib and untreated controls. The data were analyzed to identify differentially expressed genes (DEGs) with a *p*-value < 0.05 and log2 fold change >1 or <-1. The DEGs were mapped to the STRING database to construct protein-protein interaction (PPI) networks with a confidence score >0.4.

Investigation of Abemaciclib-induced cellular senescence by Reverse transcription-quantitative PCR

KKU-213B cells were treated with Abemaciclib 5μM for 24, 48, and 72 hours. RNA was then extracted from the treated cells using GENEzol Reagent (Geneaid Biotech, Taiwan). Subsequently, 1 μg of RNA was reverse-transcribed using a Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Republic of Korea). RT-qPCR was performed using iTaq universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocols. Relative expression in Abemaciclib-treated cell was normalized to that of untreated cell using the $2^{-\Delta\Delta Ct}$ method with β-actin (*ACTB*) as an internal control.

Investigation of Abemaciclib-induced cellular senescence by SA-β Galactosidase staining

Senescence-associated-β-galactosidase (SA-β-gal) was accessed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technologies, 9860) according to the manufacturer's instructions. The vehicle control-treated and Abemaciclib-treated KKU-213B cells were washed twice with PBS, and then fixed with 4% paraformaldehyde for 5 min at room temperature. The cells were then washed three times with PBS and incubated with SA-β-gal staining solution overnight at 37 °C without CO_2 atmosphere. After incubation, the positive cells were observed and counted under a bright field microscope.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 28.0.0.0, IBM Corp; Armonk, NY, USA). All results were present as the mean ± standard deviation (S.D.) of at least three independent experiments. Comparisons between two groups were made using the Student's t-test. Statistical significance was defined as *p*-value <0.05 (*), *p*-value < 0.01 (**), and *p*-value < 0.001 (***).

RESULTS

Effect of CDK4/6 inhibitor Abemaciclib on cell proliferation in CCA cells

To investigate the efficacy of Abemaciclib on cell proliferation inhibition, the response of KKU-213B to Abemaciclib was evaluated using the MTT assay and the IC_{50} (half-maximal inhibitory concentration) was calculated. MMNK-1 cell line was used as a non-tumor cholangiocyte control. KKU-213B and MMNK-1 cells were seeded in a 96-well plate and treated with Abemaciclib at various concentrations, starting from 0.1, 0.5, 1, 5, 7.5, to 10 μM, with DMSO used as a vehicle control for 72 hours. The cell morphology was observed under a microscope, showing a decrease in cell number, enlargement, and a more flattened appearance in some cells, with minimal cell death (Figure 1A). The results show that the IC_{50} values of KKU-213B and MMNK-1 are 3.24 and 7.45 μM, respectively (Figure 1B). When compared to the response of CCA cells and non-tumor cholangiocytes to Abemaciclib, the IC_{50} value of MMNK-1 is higher than that of KKU-213B. The results indicate that the CDK4/6 inhibitor Abemaciclib can inhibit cell proliferation with a slight increase in cell death, with KKU-213B showing a greater response than MMNK-1.

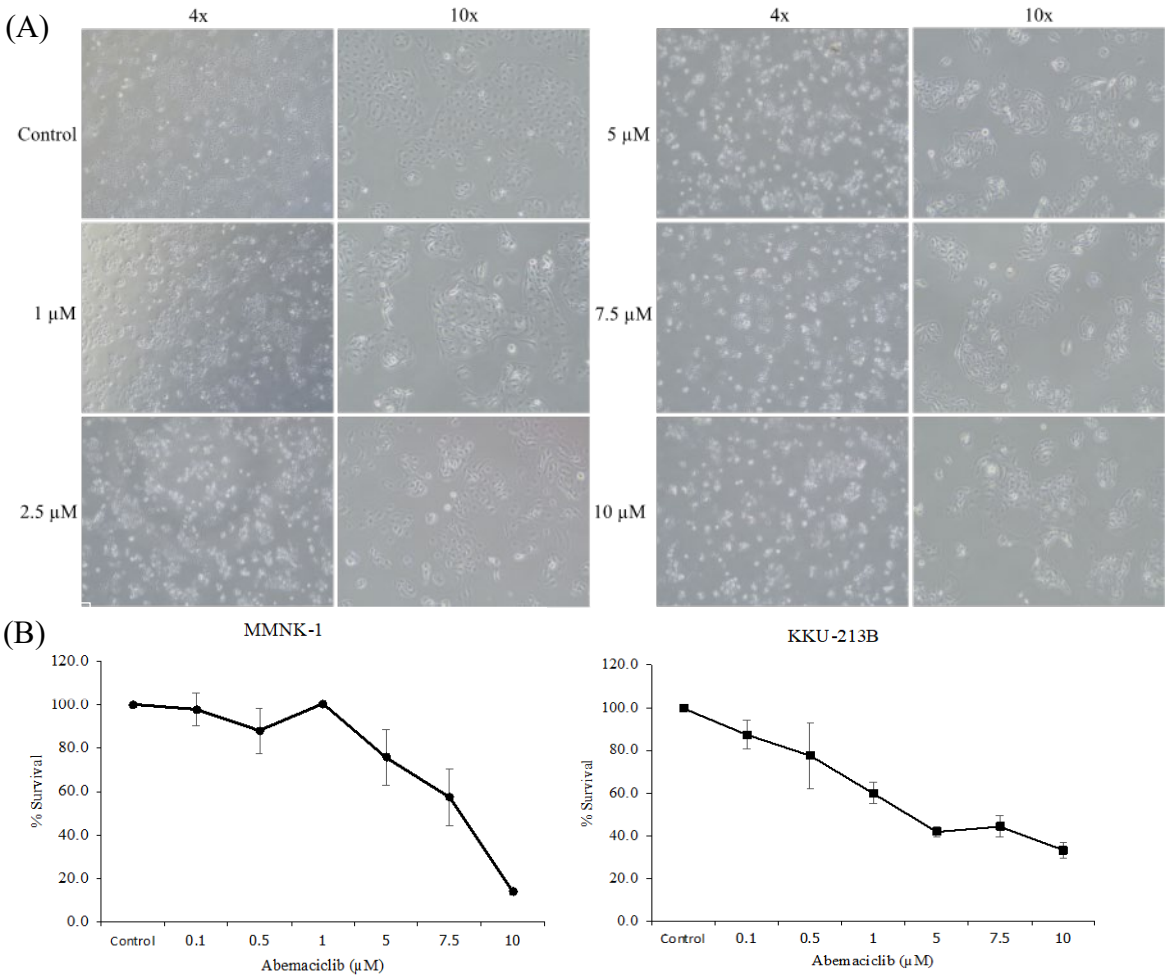


Figure 1: Effects of Abemaciclib on cell proliferation. KKU-213B and MMNK-1 cells were treated with Abemaciclib at different concentrations for 72 hours. (A) Representative images of KKU-213B cells treated with Abemaciclib for 72 hours. (B) Cell proliferation was determined by the MTT assay and calculated as % cell survival normalized to the DMSO vehicle control.

Differential Expressed Genes (DEGs) in Abemaciclib-treated cells

As shown under the microscope, the cell morphology resembled that of senescent cells, with enlargement and a more flattened appearance. To investigate the association of genes and pathways in Abemaciclib-treated cells with senescence-associated pathways, we analyzed Differentially Expressed Genes (DEGs) using the GSE222984 breast cancer cohort, as there is no publicly available database for CCA cells. The RNA-seq in the GSE222984 cohort were derived from therapy-challenged breast cancer cells (MCF-7 and MDA-MB231) treated with 500 nM Abemaciclib for 96 hours, compared with control cells. The volcano plot shows gene expression levels and differentially expressed genes (DEGs), with 700 genes significantly downregulated and 361 genes upregulated after breast cancer cells were exposed to Abemaciclib. Then, a Network Functional Enrichment Analysis of the DEGs was performed and is shown in the bubble plot, which indicated that cellular senescence processes are associated with Abemaciclib treatment (Figure 2B).

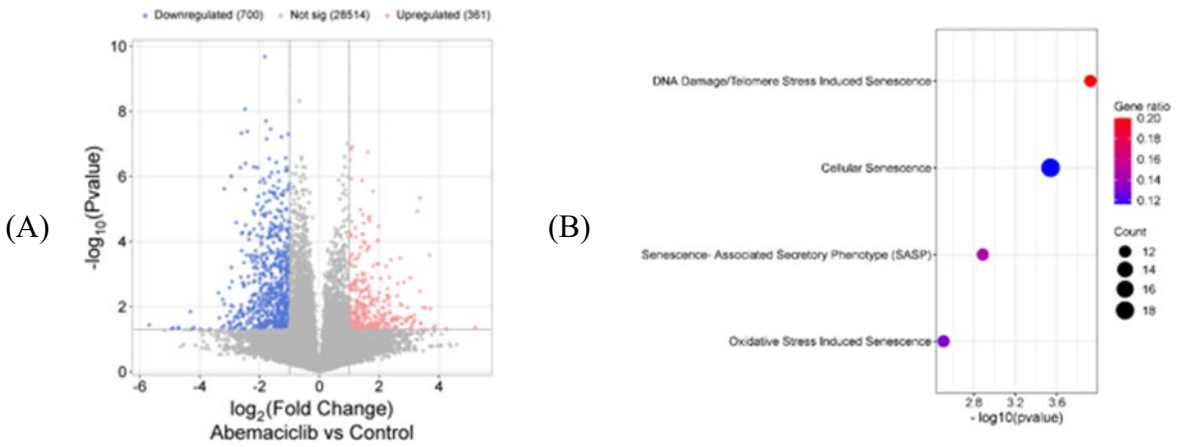
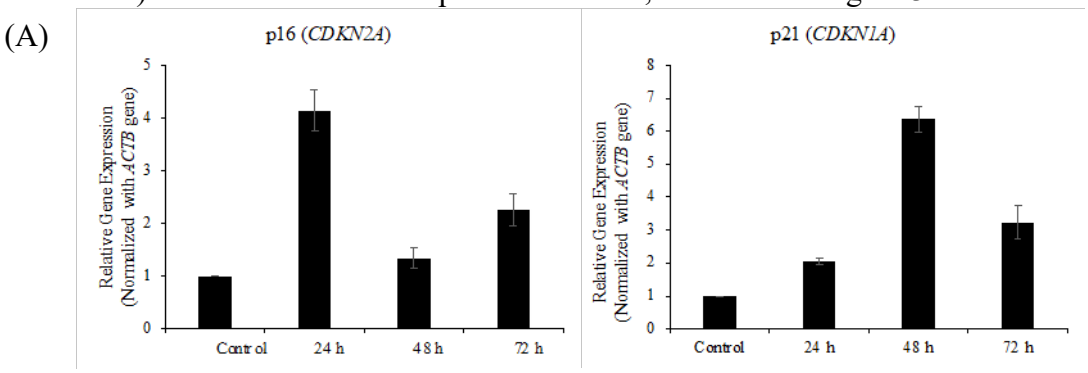


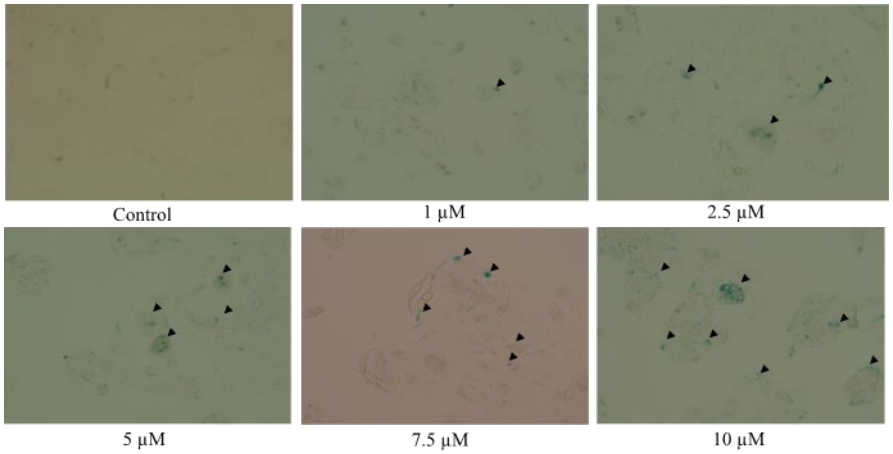
Figure 2: Bioinformatics analysis of Differential expressed genes (DEGs) in Abemaciclib-treated cells compared to control untreated cells form the GSE222984 cohort. (A) Volcano plot representing gene expression level and differentially expressed genes (DEGs). The blue dots represent downregulated genes, while the red dots represent upregulated genes with *p*-values < 0.05. (B) The bubble plot shows the results of the Network Functional Enrichment Analysis of DEGs, illustrating the biological pathways associated with Abemaciclib.

Induction of Cellular Senescence by Abemaciclib in CCA Cells

To study cellular senescence induction in Abemaciclib-treated CCA cells, the senescence marker genes and activity of β-galactosidase under acidic conditions at pH 6.0 were investigated for cellular senescence induction. KKU-213B was treated with 5 µM Abemaciclib for 24, 48, and 72 hours, then RT-qPCR was performed for p16 and p21 relative expression compared to untreated cells. The result shows that p16 and p21 were upregulated in Abemaciclib-treated cell (Figure 3A). To confirm cellular senescence induction, KKU-213B cells were treated with varying concentrations of Abemaciclib for 72 hours, then stained with X-gal staining solution overnight, and the blue-stained (positive) cells were observed under a bright-field microscope. The result indicated that the number of positive cells (black arrowhead) increased in a dose-dependent manner, as shown in Figure 3B-C.



(B)



(C)

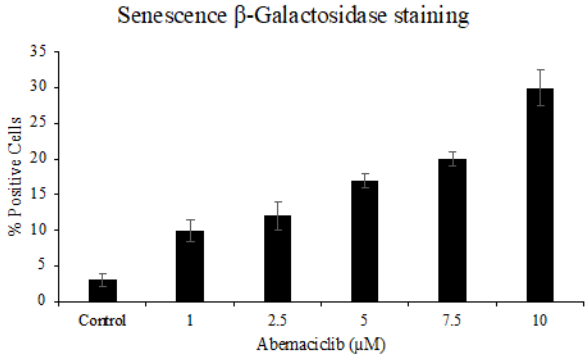


Figure 3: Induction of cellular senescence by Abemaciclib in CCA Cells. KKU213B CCA cells were exposed to Abemaciclib. (A) The relative expression of p16 (CDKN2A) and p21 (CDKN1A) of Abemaciclib-treated KKU-213B cells. (B)Representative images of Abemaciclib-treated KKU-213B cells positive for Senescence-associated β-Galactosidase (SA-β-Gal), displayed as blue staining, compared to control cells. (C) Quantification of SA-β-Gal+ cells as shown in A. SA-β-Gal+ cells were counted in three fields, with a minimum of 100 total cells assessed. Results are expressed as the percentage of stained cells.

DISCUSSION

Cholangiocarcinoma is a highly aggressive malignancy associated with poor prognosis and high mortality rates. Currently, therapeutic options for CCA are still a major challenge due to late diagnosis and standard treatment failure. To address these limitations and improve clinical outcomes, this study aims to evaluate novel targeted therapies that enhance therapeutic efficacy for CCA patients. In our previous study, we demonstrated that the Cyclin D1 and CDK4/6 were overexpressed and associated with shorter overall survival of CCA patients. Therefore, Cyclin D1 and CDK4/6 are potential targets for CCA targeted therapy. First, we investigated the effectiveness of Abemaciclib on inhibiting cell proliferation and inducing cell death in CCA cells. The results show that Abemaciclib alone can inhibit cell proliferation and minimally induce cell death in CCA cells compared to non-tumor cholangiocytes. To enhance the effectiveness of CDK4/6 inhibitor treatment in CCA patients, we identify the biological pathway associated with the CDK4/6 inhibitor response using bioinformatics analysis in a breast cancer cohort. The results indicate the association between the cellular senescence-associated genes and Abemaciclib treatment. We further confirmed that Abemaciclib can induce cellular senescence using RT-qPCR for senescence gene marker expression and Senescence-associated β-Galactosidase (SA-β-Gal) staining in CCA cells. Our findings provide preliminary insights into the effectiveness of CDK4/6 inhibitor Abemaciclib in CCA cells and the induction of senescence following Abemaciclib exposure.

The most prominent hallmark of cancer is sustaining proliferative signaling. Cyclin D and CDK4/6 are key regulatory proteins involved in the cell cycle progression. Dysregulation of the cell cycle process and mutations in key cell cycle regulatory genes or proteins, particularly involved in G1 phrase regulators (RB, CCND1, CDK4, CDK6, and CDKN2A), have been reported in CCA (Sittithumcharee et al., 2019; Churi et al., 2014; Farshidfar et al., 2017). Therefore, CDK4/6 might be a promising target for CCA-targeted therapy. Presently, CDK4/6 inhibitors are used in therapeutic approaches for treatment of various cancers (Sheikh & Satti, 2021). For instance, the U.S, FDA has approved CDK4/6 inhibitor Abemaciclib for treating HR+/HER2- advanced stage breast cancer (Raheem et al., 2022). In this study, we investigated the impact of CDK4/6 inhibitor Abemaciclib on inhibiting cell proliferation and its ability to induce cell death in CCA cell lines. Our results show that CDK4/6 inhibitor Abemaciclib single treatment can specifically inhibit cell proliferation and minimally induce cell death in CCA cells compared to non-tumor cholangiocytes. The results illustrate the specificity of the CDK4/6 inhibitor as a targeted therapeutic for CCA. However, as its primary mechanism involves inducing cell cycle arrest, the efficacy of Abemaciclib as a monotherapy in promoting cell death is limited (Wang et al., 2022). Recent studies suggest that CDK4/6 inhibitors may also induce a senescence-like phenotype through non-canonical pathways (Bonelli et al., 2019). The CDK4/6 inhibitor-mediated senescence-like phenotype might be reversible, allowing cells to resume proliferation after the treatment is discontinued (Nehme et al., 2024). The cytostatic effect and the senescence-like state induction are contributed to the limited efficacy of CDK4/6 inhibitors and reduce their clinical benefits. Since the effect of CDK4/6 inhibitors is primarily to induce cell cycle arrest without triggering cell death, intrinsic and acquired resistance frequently emerge in clinical practice. As a result, combining CDK4/6 inhibitors with other therapeutic agents is a necessary strategy to enhance their efficacy and prevent resistance. Currently, CDK4/6 inhibitors are being evaluate in combination with aromatase inhibitors, immunotherapy, and mTOR inhibitors in ongoing clinical trials (Bonelli et al., 2019; Rampioni Vinciguerra et al., 2022). In this study, we aimed to identify biological pathways that could influence the effectiveness of CDK4/6 inhibitors and to develop a rational combination treatment strategy. Bioinformatic analysis results showed that cellular senescence-related pathways were upregulated after breast cancer cells were exposed to Abemaciclib. The induction of cellular senescence by Abemaciclib was then confirmed in the CCA cell lines using RT-qPCR to measure p16 and p21 expression, along with SA-β-Gal staining, which are markers of senescence. The results align with other studies that demonstrated Abemaciclib induces senescence in pancreatic cancer (Dhir et al., 2019) and dedifferentiated liposarcoma (Gleason et al., 2024). As mentioned above, CDK4/6 inhibitors primarily exert cytostatic effects and induce a senescence-like phenotype, which can lead to cancer recurrence. Taken together, the results suggest that the CDK4/6 inhibitor Abemaciclib should be combined with a senolytic agent, to selectively eliminate senescent cells. This strategy might enhance the response of CDK4/6 inhibitor in CCA treatment. However, this is the preliminary study investigating the impact of CDK4/6 inhibitors in CCA. Further studies are needed, including those with additional CCA cell lines, evaluation of the efficacy of CDK4/6 inhibitor combined with senolytics, and *in vivo* study, before translating these into clinical practice for treating CCA patients.

CONCLUSION

This is the first study evaluating the impact of Abemaciclib, a CDK4/6 inhibitor, on CCA cells, highlighting its inhibitory effects on cell proliferation and its capacity to induce senescence in these cells. These findings provide a strong rationale for further exploration of Abemaciclib as a potential treatment option for CCA patients. Additional studies are required to maximize the efficiency of Abemaciclib and minimize possible consequences of therapy-induced senescence

by employing strategic combination treatments for targeted therapy, ultimately advancing precision medicine in cancer treatment.

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