Regulation of glucose and glutamine metabolism to overcome cisplatin resistance in intrahepatic cholangiocarcinoma

So Mi Yang1,*, Jueun Kim1,2,#, Ji-Yeon Lee3,#, Jung-Shin Lee1,* & Ji Min Lee3,*

1Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon 24341, 2Kangwon Institute of Inclusive Technology, Kangwon National University, Chuncheon 24341, 3Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 34141, Korea

Intrahepatic cholangiocarcinoma (ICC) is a bile duct cancer and a rare malignant tumor with a poor prognosis owing to the lack of an early diagnosis and resistance to conventional chemotherapy. A combination of gemcitabine and cisplatin is the typically attempted first-line treatment approach. However, the underlying mechanism of resistance to chemotherapy is poorly understood. We addressed this by studying dynamics in the human ICC SCK cell line. Here, we report that the regulation of glucose and glutamine metabolism was a key factor in overcoming cisplatin resistance in SCK cells. RNA sequencing analysis revealed a high enrichment cell cycle-related gene set score in cisplatin-resistant SCK (SCK-R) cells compared to parental SCK (SCK WT) cells. Cell cycle progression correlates with increased nutrient requirement and cancer proliferation or metastasis. Commonly, cancer cells are dependent upon glucose and glutamine availability for survival and proliferation. Indeed, we observed the increased expression of GLUT (glucose transporter), ASCT2 (glutamine transporter), and cancer progression markers in SCK-R cells. Thus, we inhibited enhanced metabolic reprogramming in SCK-R cells through nutrient starvation. SCK-R cells were sensitized to cisplatin, especially under glucose starvation. Glutaminase-1 (GLS1), which is a mitochondrial enzyme involved in tumorigenesis and progression in cancer cells, was upregulated in SCK-R cells. Targeting GLS1 with the GLS1 inhibitor CB-839 (telaglenastat) effectively reduced the expression of cancer progression markers. Taken together, our study results suggest that a combination of GLUT inhibition, which mimics glucose starvation, and GLS1 inhibition could be a therapeutic strategy to increase the chemosensitivity of ICC. [BMB Reports 2023; 56(11): 600-605]

INTRODUCTION

Cholangiocarcinoma (CCA) is a tumor that develops in the epithelial cells of the bile duct (biliary tract), which serves as a passage for transporting bile produced in the liver to the duodenum. It is largely classified into intrahepatic cholangiocarcinoma (ICC), which occurs in the bile duct located inside the liver, and extrahepatic cholangiocarcinoma (ECC), which occurs in the bile duct located outside the liver, and where it connects to the duodenum (1, 2). ECC is divided into perihilar CCA and distal CCA based on the location of occurrence (3). According to the anatomical location, approximately 10-20% of tumors occur in the intrahepatic bile duct, 50-60% in the perihilar bile duct, and 20-30% in the distal bile duct (4). The early detection of CCA is challenging because the majority of early-stage CCA patients as asymptomatic, making it difficult to recognize, and by the time symptoms appear, the cancer is already at an advanced stage (5). Although combination therapy with gemcitabine and cisplatin is widely recognized as the most effective approach for treating CCA, it is often not feasible due to limited access to clinical trials. As a result, monotherapy with gemcitabine, 5-fluorouracil, cisplatin, or oxaliplatin is commonly used in clinical practice (6). The treatment of ICC is becoming increasingly diverse, with the ongoing research and development of various approaches, such as immunotherapy and targeted therapy. However, these approaches are not universally applicable to all ICC patients. Furthermore, chemoresistance leads to cancer recurrence and metastasis, and it is necessary to elucidate the mechanisms of resistance to enhance sensitivity to anticancer drugs (7, 8).

Autophagy, which is regulated by various autophagy-related genes (ATGs), plays a crucial role in conserving cell energy by breaking down damaged cell components and reusing nutrients. Specifically, autophagy is induced in response to stressful conditions (9, 10). Moreover, to maintain cellular homeostasis, it is upregulated in response to stressors, such as nutrient deficiency, hypoxia, the accumulation of misfolded proteins, the
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Inhibition of protein synthesis, and the presence of pathogens (9). Cancer cells exhibit an elevated demand for nutritional requirements because of increased cell proliferation, and they undergo metabolic reprogramming as an adaptation to stress caused by the reduced supply of essential nutrients (11). Some of the metabolic products derived from metabolic reprogramming increase cell survival by activating autophagy and increasing nutrient recycling (11). In contrast, autophagy can suppress cancer and improve sensitivity to radiotherapy or chemotherapy, functioning as a double-edged sword (12).

Cancer cells restrict the entry of pyruvate into the tricarboxylic acid (TCA) cycle via the Warburg effect (aerobic glycolysis), a characteristic of cancer metabolic reprogramming, which helps cancer cells to absorb glucose faster than normal cells and produce lactate despite the abundance of O2 (13). In this process, glucose-derived pyruvate is converted to lactate and secreted outside the cells. Then, glutamine serves as a conditionally essential amino acid (14). Primarily, glutamine transported inside the cells by ASC12 is converted to glutamate by glutaminase 1 (GLS1), a mitochondrial enzyme. Next, glutamate is converted to α-ketoglutarate (α-KG), which enters the TCA cycle. The overall process by which glutamine is converted to α-KG is referred to as glutaminolysis, which functions as a source of metabolic intermediates for the TCA cycle and serves as a precursor for amino acid and nucleotide biosynthesis (15). Therefore, the upregulation of glutaminolysis represents a significant characteristic of metabolic reprogramming in cancer cells. An increased influx of glutamine into the mitochondrial matrix upregulates glutaminolysis, also facilitating aerobic glycolysis (16).

Most cancers, including ICC, require a consistent supply of glutamine for tumor progression and cell proliferation (14). Increasing evidence indicates that an abnormal expression of GLS1 is associated with tumor invasion and metastasis in liver cancer, breast cancer, and colon cancer (17-19). GLS1 is involved in epithelial-mesenchymal transition (EMT) induction in breast cancer and colon cancer (20). EMT, in which epithelial cells lose their adhesive ability and acquire mesenchymal characteristics, is an important cause of infiltration and migration in various cancers of epithelial origin (21, 22). GLS1 plays a significant role in promoting the progression of cancer cells in ICC (23). Therefore, targeting GLS1 could be a potential therapeutic approach for regulating the infiltration and migration of ICC cells induced by EMT.

In this study, we aimed to elucidate the mechanism of cisplatin resistance by comparing the human ICC cell line SCK (SCK-WT) to cisplatin-resistant SCK (SCK-R) cells that had attained maximum cisplatin resistance by treatment with cisplatin for 12 months. We examined the increased expression of cell cycle-related factors and cancer progression-related genes, focusing on the characteristics of cancer cell metabolic reprogramming. We also investigated the impact of glucose starvation on cisplatin re-sensitization and assessed the effectiveness of treatment with CB-839 (telaglenastat), a GLS1-specific inhibitor, based on the changes in the expression levels of GLS1. The findings suggest that simultaneously blocking glutamine and glucose uptake could help to overcome cisplatin resistance.

RESULTS

Acquired resistance of SCK-R cells to cisplatin is associated with increased cell proliferation and metastasis, driven by accelerated cell cycling

We first tested whether SCK-R cells were more resistant to cisplatin than SCK WT cells. Cisplatin was administered to both cell lines at 10-50 μM for 24 h, and cell viability was evaluated. The survival rate of SCK-R cells was higher than that of SCK WT cells, indicating that SCK-R cells are more resistant to cisplatin (Fig. 1A).

Next, total gene expression was compared between SCK WT and SCK-R cells by RNA sequencing (RNA-seq) analysis. Raw counts were normalized using the Deseq2 R package and scaled to Z-scores (standard scores), and a heatmap was generated using the Pheatmap R package. The expression patterns in duplicate samples of SCK WT and SCK-R cells were similar (Supplementary Fig. 1A). Based on Gene Set Enrichment Analysis (GSEA) of total gene expression, using 50 hallmark gene sets, SCK-R cells exhibited an elevated expression of E2F transcription factor target genes associated with the cell cycle, G2/M checkpoint, and mitotic spindle assembly (Fig. 1B). Following GSEA using 1636 Reactome gene sets, we used gene sets exhibiting positive correlations after applying cutoff values (P < 0.05 and FDR-q < 0.1) to generate an enrichment map using Cytoscape. This revealed that the expression of genes...
associated with the cell cycle was increased (Supplementary Fig. 1B). The volcano plot showed differences in gene expression between SCK WT and SCK-R cells (Fig. 1C). The up-regulated genes in SCK-R cells included L1CAM, AXL, and ZEB2, which are cancer progression-related genes (24-26). Elevated cancer progression marker expression indicates cancer metastasis and invasion. The increased expression of L1CAM, AXL, and ZEB2 in SCK-R cells compared to SCK WT cells was confirmed using real-time quantitative reverse transcription PCR (qRT-PCR) (Fig. 1D). These findings suggest that cisplatin resistance in SCK-R cells was induced by accelerated cell cycling and was associated with cancer cell proliferation and metastasis.

Increased cell cycling leads cancer cells to have a higher demand for nutrients, including glucose and glutamine, and can alter the metabolic pattern of cancer cells. Therefore, we investigated the metabolic patterns of SCK WT and SCK-R cells using the Seahorse XF analyzer. We measured the function of the glycolytic pathway following glucose starvation. According to the results obtained by the Seahorse XF analyzer, SCK-R cells exhibited higher metabolic activity compared to SCK WT cells (Supplementary Fig. 2A, B). SCK-R cells demonstrated significantly higher glycolytic reserves compared to SCK WT cells, indicating their capability to respond to energy demands and closer proximity to the cell’s theoretical maximum glycolytic function (27). These findings indicate that the altered metabolism could be attributed to the rapid cell cycling and proliferation characteristics observed in SCK-R cells.

Glucose starvation re-sensitizes SCK-R cells to cisplatin and suppresses their metastasis

We hypothesized that the SCK-R cells obtain cisplatin resistance via upregulation of their cell cycle rate and metastatic ability. If the cell cycle progresses rapidly, it constantly requires nutrients, whereas nutrient starvation leads to cell cycle arrest (28). Indeed, we observed the increased expression of glucose and glutamine uptake-related genes, as indicated by RNA-seq (Supplementary Fig. 1C, D). The results imply that SCK-R cells require a continuous supply of glucose and glutamine for rapid cell cycle progression. Thus, we tested whether nutrient deficiency could inhibit the proliferation of SCK-R cells to overcome cisplatin resistance.

We applied various nutrient starvation treatments to SCK WT and SCK-R cells to investigate whether nutrient deficiency suppressed SCK-R cell proliferation and restored sensitivity to cisplatin. The transcription levels of cancer progression-related genes in the SCK-R cells, which were substantially higher than in SCK WT cells, were markedly reduced under glucose starvation (Fig. 2A). In contrast, in glutamine and amino acid starvation conditions, there was no correlation between mRNA transcription levels and the duration of starvation, and the levels were not greater than those observed in glucose starvation (Supplementary Fig. 3A).

Glucose starvation was induced in SCK WT and SCK-R cells for 6 and 12 h, respectively, and the changes in protein levels were evaluated. With increasing durations of glucose starvation, we observed a proportional decrease in L1CAM and AXL protein levels, which were initially higher compared to SCK WT cells (Fig. 2B).

We further investigated the correlation between glucose starvation and the sensitivity of SCK-R cells to cisplatin. Cell viability assays were conducted following cisplatin treatment only (10 μM for 24 h), glucose starvation only (12 h), and combined application in SCK-R cells. As expected, the treatment of SCK-R cells with cisplatin only had no effect relative to the control, whereas glucose starvation only reduced cell survival (P < 0.001). Furthermore, SCK-R cell survival was further reduced by treatment with cisplatin and glucose starvation (P < 0.0001), suggesting the possibility of overcoming cisplatin resistance in SCK-R cells (Fig. 2C).

To test whether glucose starvation also affected migration, a wound healing assay was performed at 24 h intervals for a total of 48 h. The SCK-R cells had faster wound closure than the SCK WT cells, whereas their wounded area was larger under glucose starvation, eventually leading to cell death. These results suggest that the SCK-R cells migrated faster than SCK WT cells, and glucose starvation effectively interfered with this process (Fig. 2D). These results also indicate that among various nutrient deficiency treatments, glucose starvation re-sensitized SCK-R cells to cisplatin, which exhibited a higher rate of proliferation compared to SCK WT cells.

Inhibition of upregulated glutaminolysis in SCK-R cells compared to SCK WT cells is effective in suppressing cancer progression

Based on the importance of glutaminolysis in cancer metabolic reprogramming, we predicted that it would also be associated

Fig. 2. SCK-R cells exhibit upregulated glucose and glutamine uptake, and the impact of glucose starvation on cancer progression, metastasis, and cell survival in SCK-R cells is investigated. (A) qRT-PCR analysis of L1CAM, AXL, and ZEB2 after glucose starvation. (B) Western blot analysis showing the expression of L1CAM and AXL under glucose starvation. β-actin was used as a loading control. (C) Growth rate of SCK-R cells under glucose starvation and cisplatin treatment only and the combination was determined using the cell viability assay. (D) Wound healing assay was performed after glucose starvation to determine migration ability. **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are expressed as mean ± SEM.
and SCK-R cells. The expression of GLUT family members has been proposed as a treatment to overcome cisplatin resistance. Furthermore, inhibiting GLUT family members could help to elucidate the association between glucose transport, starvation, and GLS1 suppression could effectively overcome cisplatin resistance. Indeed, we observed the increased expression of GLS1, which was previously effective, and the inhibition of GLUT, which mimics glucose starvation, would be even more effective. To test this, we treated the GLUT inhibitor DRB18 (10 μM for 48 h) and CB-839 (1 μM for 48 h) alone and in combination, examined cell viability. Our results show that the treatment with CB-839 alone led to a decrease in cell viability compared to the control. However, the treatment with DRB18 alone resulted in a more pronounced reduction in cell viability compared to CB-839 alone. Additionally, GLUT inhibition was more effective than GLS1 inhibition, once again confirming that the inhibition of glucose uptake, mimicking glucose starvation, was the most effective treatment. Combined treatment caused a marked reduction in the SCK-R cell survival rate relative to that in cells treated with each inhibitor alone (Fig. 4B). This suggests that the simultaneous suppression of GLS1 and GLUT in SCK-R cells could be a new combination therapy to overcome cisplatin resistance.

**DISCUSSION**

Current cancer treatments encompass a range of approaches, including hormone therapy, surgery, bone marrow transplantation, chemotherapy, radiotherapy, targeted therapy, and immunotherapy. Recent advances in targeted therapy and immunotherapy have brought about significant progress, but with cisplatin resistance. Indeed, we observed the increased expression of GLS1, which was previously effective, and the inhibition of GLUT, which mimics glucose starvation, would be even more effective. To test this, we treated the GLUT inhibitor DRB18 (1 μM, 24 h) only and DRB18 (10 μM, 48 h) only or in combination. (C) A schematic illustration showing the cause of cisplatin resistance in SCK-R cells compared to SCK WT cells with an effective method to overcome cisplatin resistance. **P < 0.01, ****P < 0.0001. Data are expressed as mean ± SEM.

**Fig. 4.** Simultaneous inhibition of GLS1 and GLUT most effectively re-sensitizes SCK-R cells to cisplatin. (A) GLUT1 mRNA expression in SCK WT and SCK-R cells measured using qRT-PCR. The expression of GLUT1 was normalized to that of GAPDH. (B) Cell viability assay was performed by treating SCK-R cells with CB-839 (1 μM, 24 h) only and DRB18 (10 μM, 48 h) only or in combination. (C) A schematic illustration showing the cause of cisplatin resistance in SCK-R cells compared to SCK WT cells with an effective method to overcome cisplatin resistance. **P < 0.01, ****P < 0.0001. Data are expressed as mean ± SEM.
not all patients can receive these treatments, which poses a challenge (33, 34). In contrast, chemotherapy is a less novel approach, yet it remains the most widely used adjuvant cancer therapy. Platinum-based drugs, including cisplatin, carboplatin, and oxaliplatin, are widely used to treat most types of tumors. However, despite the initial positive anticancer effects of platinum-based drug administration, some patients develop resistance over time, reducing their long-term effectiveness (35).

Our study aimed to examine the mechanisms behind cisplatin resistance and develop a strategy to overcome it. In this study, RNA-seq analysis and GSEA revealed that the expression of cell cycle-associated genes was markedly elevated in cisplatin-resistant SCK-R cells. Specifically, target genes associated with the E2F transcription factor, the G2/M checkpoint, and mitotic spindle assembly had high enrichment scores.

Cancer cells rapidly adapt to hypoxia and nutrient deficiencies, enhancing their survival via metabolic reprogramming, which is one of the 10 characteristics of cancer (14). Specifically, the reprogramming of glucose, amino acid, and lipid metabolism is critical in promoting tumor development. In addition to epigenetic regulation, metabolic reprogramming is central in processes such as tumor formation, metastasis, and drug resistance, as it satisfies the energy and nutrient requirements of cancer cells (36). Previous studies revealed metabolic changes during each phase of the cell cycle in cancer cells. Based on prior findings, cisplatin resistance in SCK cells is likely to be caused by cell cycle acceleration. Therefore, we predicted that this metabolic change is associated with tumor formation and metastasis and that regulating it will help to overcome cisplatin resistance.

First, we confirmed the enhanced proliferation and migration of SCK-R cells based on elevated levels of L1CAM, AXL, and ZEB2 with rapid wound closure. The RNA-seq results revealed that the expression of glucose transporter, glutamine transporter, and GLS1 increased in SCK-R cells compared to SCK WT cells. As expected, the activation of metabolic reprogramming occurred with the activation of the cell cycle. We expected that suppressing metabolic reprogramming would overcome cisplatin resistance in SCK-R cells. Notably, glucose starvation more effectively than glutamine or amino acid starvation condition. The increased expression of GLUT1, a glucose transporter, reflected elevated glucose uptake in SCK-R cells, and we confirmed that glucose starvation re-sensitized SCK-R cells to cisplatin. GLUT1 is also known to act as a transporter of vitamin C into mitochondria (37). The experimental results showed no significant difference in vitamin C concentration between SCK WT and SCK-R cells (Supplementary Fig. 4A). This finding provides supporting evidence for the logical emphasis on glucose, rather than vitamin C, by targeting GLUT1 as the shared transporter for both molecules.

The observed elevation in GLS1 expression, which is important in glutaminolysis, and intracellular glutamine and glutamate levels in SCK-R cells, revealed that glutaminolysis was associated with metabolic reprogramming represented by cisplatin resistance in SCK-R cells. Indeed, treatment with CB-839 led to reduced L1CAM, AXL, and ZEB2 expression. Combination treatment with DRB18 and CB-839 reduced SCK-R cell proliferation. Based on these results, we speculate that inhibiting glucose and glutamine metabolism re-sensitized SCK-R cells to cisplatin.

In prior studies, it has been demonstrated that inhibiting glucose transporter and glutaminase leads to a synergistic effect in anticancer therapy (38). However, we aimed to focus on overcoming cisplatin resistance in SCK-R cells, which leads to enhanced therapeutic effects in patients with cisplatin resistance.

Our study had a limitation in that we did not conduct clinical verification using patient samples to examine whether normal cells around the SCK cells were affected. Therefore, additional studies are required to investigate the specificity of cisplatin-resistant cells. Glucose starvation represented by GLUT inhibition was more effective, but no correlation with glutaminolysis was identified. The expression of GLS1 protein was still confirmed after glucose starvation. The result that GLS1 expression levels, which were higher in SCK-R cells compared to SCK WT cells, were not significantly different under glucose starvation suggests that glutaminolysis was activated to compensate for glucose starvation.

In summary, SCK-R cells, which acquired maximal cisplatin resistance, exhibited accelerated proliferation and metastasis characteristics, as well as active cell cycle progression, requiring more nutrients. Blocking glucose and glutamine uptake re-sensitized these cells to cisplatin, suggesting that simultaneous inhibition was more effective than inhibiting just one (Fig. 4C). Our data demonstrate that metabolic reprogramming by reducing the activity of metabolic enzymes could be a novel therapeutic strategy to overcome chemotherapeutic resistance in ICC.

MATERIALS AND METHODS

Cell culture, Western blot, RNA sequencing (RNA-seq) analysis, Measurement of glutamine and glutamate levels and data processing are described in the supplementary information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

2. Rizvi S and Gores GJ (2013) Pathogenesis, diagnosis, and
and drug resistance of hepatocellular carcinoma. World J Gastroenterol 24, 4643
to epithelial-to-mesenchymal transition and cancer complexity. J Cell Physiol 234, 14783-14799
27. Mookerjee SA, Nicholls DG and Brand MD (2016) Determining maximum glycolytic capacity using extracell-
ular flux measurements. PLoS One 11, e0152016
port inhibit cell proliferation and induce apoptosis in cancer cells via glucose-deprivation-like mechanisms. Cancer letters 298, 176-185
30. Zhang TB, Zhao Y, Tong ZX and Guan YF (2015) Inhibition of glucose-transporter 1 (GLUT-1) expression re-
31. Oh S, Kim H, Nam K and Shin I (2017) Glut1 promotes cell proliferation, migration and invasion by regulating epider-
mal growth factor receptor and integrin signaling in triple-negative breast cancer cells. BMB Rep 50, 132-137
1624
guage for tumor response to immunotherapy: immune-related response criteria using unidimensional measure-
mentsunidimensional irRC as a common language for immunotherapy. Clin Cancer Res 19, 3936-3943
37. Klepper J, Vera JC and De Vivo DC (1998) Deficient transport of dehydroascorbic acid in the glucose trans-
porter protein syndrome. Ann Neurol 44, 286-287
38. Reckzeh ES, Karageorgis G, Schwalfenberg M et al (2019) Inhibition of glucose transporters and glutaminase synergis-
tically impairs tumor cell growth. Cell Chem Biol 26, 1214-1228 e1225