Blocking glutamine and one-carbon metabolism promotes ROS accumulation and enhances chemotherapy efficacy in anaplastic thyroid cancer

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A Dissertation Submitted
to the Department of Medicine
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy in Medical Science

Hyeok Jun Yun

December 2024

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The Graduate School
Yonsei University
December 2024

ACKNOWLEDGEMENTS

First and foremost, I wish to express my profound and heartfelt gratitude to my esteemed advisor, Prof. Hang-Seok Chang, for his unwavering support and guidance throughout my Ph.D. studies and related research. His patience, motivation, and extensive knowledge have been a source of inspiration and have greatly contributed to the successful completion of this thesis. His scientific insight and profound expertise have consistently steered me in the right direction, making this journey in cancer research both rewarding and enlightening. I deeply hope to continue this fruitful collaboration in the future.

My sincere thanks go to Prof. Young Hoon Ryu, Beom Jin Lim, Shinae Kang (Yonsei University College of Medicine), and Young Joo Park (Seoul National University College of Medicine) for their insightful comments and kind encouragement, which greatly enriched my research.

I am also deeply grateful to Prof. Seok Mo Kim and Prof. Sungsoon Fang for providing crucial anaplastic thyroid cancer samples and their invaluable support in this research.

Lastly, I wish to extend my sincerest thanks to my beloved family—my wife, Da Eun Sung, and my parents, Han Jin Yun and Sun Yeon Kim—whose boundless love, encouragement, and unwavering support have been my constant source of strength and purpose throughout this journey and beyond.

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ABSTRACT

Blocking glutamine and one-carbon metabolism promotes ROS accumulation and enhances chemotherapy efficacy in aplastic thyroid cancer

Anaplastic thyroid cancer (ATC) is one of the most aggressive and lethal malignancies, characterized by rapid tumor growth, high metastatic potential, and poor response to conventional therapies. Despite ongoing efforts to improve therapeutic options, survival rates remain dismally low, largely due to the limited efficacy of current chemotherapeutic agents and the unclear molecular mechanisms driving ATC progression. This study investigates the therapeutic potential of simultaneously targeting glutamine metabolism and one-carbon metabolism in ATC. Our findings demonstrate that ATC cells rely heavily on glutamine for proliferation, and inhibiting glutaminolysis using the glutaminase inhibitor BPTES significantly reduced cell growth. However, ATC cells adapted to glutamine deprivation by upregulating one-carbon metabolism, driven by the transcription factor ATF4, which promotes the expression of key enzymes such as PHGDH and SHMT2 involved in serine biosynthesis and redox balance. Co-inhibition of glutaminolysis and one-carbon metabolism resulted in a marked accumulation of reactive oxygen species (ROS), which sensitized ATC cells to chemotherapeutic agents, including tyrosine kinase inhibitors (TKIs) like lenvatinib and sorafenib. This combination therapy significantly enhanced the efficacy of these TKIs, leading to increased tumor cell death. Our data suggest that this synergistic effect is due to the dual depletion of glutathione (GSH) and disruption of the pentose phosphate pathway (PPP), resulting in elevated ROS levels and impaired ATC cell viability. Given the metabolic plasticity of ATC cells and their ability to adapt to nutrient deprivation, targeting both glutamine and one-carbon metabolism represents a promising therapeutic approach. By exploiting these metabolic vulnerabilities, this strategy could improve the efficacy of existing therapies for ATC and offer new treatment avenues for patients with this aggressive cancer. Further clinical studies are warranted to validate these findings and explore the potential of metabolic inhibitors in combination with standard treatments for ATC.

Key words : anaplastic thyroid cancer, glutamine metabolism, one-carbon metabolism

I. INTRODUCTION

Anaplastic thyroid carcer (ATC) is one of the most aggressive solid tumors in humans. ATC originate from follicular thyroid cells and are associated with the highest risk of death of all tumors arising in the thyroid gland, but account for only a minority of all thyroid cancer cases[1-3]. Most patients present with extensive locoregional invasion, and distant metastases are found at diagnosis in 15%-50% of cases[4,5]. The median survival for patients with anaplastic thyroid carcinoma (ATC) has historically been approximately 5 months, with a 1-year overall survival rate of 20%[6]. In contrast to differentiated thyroid cancer (DTC) cells, ATC cells lack the biological characteristics and functions of normal follicular cells, including iodine uptake, thyroglobulin synthesis, and TSH dependence, the latter of which regulates the growth and biosynthetic activities of thyrocytes. [7,8]. Unlike DTC, thyroidectomy and radioactive iodine therapy are often ineffective for ATC. As a result, ATC is typically treated with systemic therapies, such as chemotherapy and multikinase inhibitors, which have limited efficacy, with a response rate of approximately 15% [9-16].

Glutamine is an abundant amino acid in our body, used as a nitrogen source and a precursor for synthesizing glutathione, which maintains redox balance [17,18]. Particularly, due to its potential to induce inflammation, glutamine can aid tissue regeneration by suppressing inflammatory responses. Several studies have demonstrated the critical role of glutamine metabolism in shaping the tumor microenvironment of ATC. Moreover, patients with ATC exhibit the highest expression levels of glutaminase 1 (GLS1) and glutamate dehydrogenase (GDH) compared to other types of thyroid cancer. [19,20].

For glutamine to be used as an energy source, it needs to be broken down by an enzyme called Glutaminase (GLS). Only after glutamine is converted to glutamate by GLS, can glutamate enter mitochondria. Once inside mitochondria, glutamate is converted to alpha-ketoglutarate, which can then enter the mitochondrial TCA cycle. Consequently, glutamine serves as an energy source that can generate ATP through the TCA cycle. Especially, many cancer cells utilize glutamine as a prominent energy source, so there is currently active development of drugs that inhibit the enzyme GLS responsible for breaking down glutamine. The biochemical reaction that converts glutamine to glutamate by GLS, allowing it to be used as an energy source, is called glutaminolysis, and it operates very strongly in various cancer cells [21,22].

Phosphoglycerate dehydrogenase (PHGDH) is known as the rate-limiting enzyme that can control serine synthesis and one-carbon metabolism. PHGDH is an enzyme that synthesizes serine

from 3-phosphoglycerate generated in glucose metabolism. This pathway is linked to one-carbon metabolism, facilitating mechanisms that promote nucleotide synthesis, NADPH synthesis, and methyl donor synthesis [23]. In particular, nucleotide synthesis is essential for synthesizing the substances needed to produce DNA and RNA when cells divide rapidly. Therefore, rapidly dividing cells utilize one-carbon metabolism to synthesize nucleotides. Furthermore, PHGDH-mediated one-carbon metabolism synthesizes both NADH and NADPH, which help maintain redox balance [24]. Consequently, by removing reactive oxygen species (ROS) generated in the mitochondria, it reduces cellular stress and inhibits cell death. In cancer cells, upregulation of PHGDH expression promotes the proliferation of cancer cells and confers resistance to anticancer agents by maintaining a redox balance that can lower ROS levels [23,25].

In this paper, we confirmed that ATC utilizes glutamine as an energy source. We investigated whether inhibition of PHGDH-mediated one-carbon metabolism, which disrupts the anticancer effect, along with inhibition glutaminolysis, could result in superior anticancer effects in ATC. The results revealed that simultaneous inhibition of glutaminolysis and PHGDH could maximize the anticancer effects of tyrosine kinase inhibitors such as lenvatinib and sorafenib.

Finally, there is an urgent need for the development of novel drugs that inhibit glutaminolysis and PHGDH. Such drugs would maximize the anticancer effects of existing tyrosine kinase inhibitors (TKIs), thereby overcoming refractory cancers.

II. MATERIALS AND METHODS

1. Human studies with ethical considerations

This study was a retroactive, single center examination of patients diagnosed with ATC (September 2021–January 2022). All courses entailing patients were achieved in proportion to the institutional ethical standards, whole applicable national/local regulations, and guidelines of the 1964 Helsinki Declaration and its later amendments. The study procedures were authorized by the Institutional Review Board (IRB) of Gangnam Severance Hospital, Yonsei University College of Medicine (IRB protocol: 3-2021-0043).

2. Inhibitors for glutaminolysis, one-carbon metabolism and tyrosine kinase

BPTES (HY-12683, MedChemExpress, Monmouth, Oregon, USA), CBR-5884 (HY-100012, MedChemExpress, Monmouth, Oregon, USA), SHIN1 (HY-112066, MedChemExpress, Monmouth, Oregon, USA), and sorafenib (HY-10201, MedChemExpress, Monmouth, Oregon, USA), Trolox (S3665, Selleckchem, Houston, Texas, USA), lenvatinib (S1164, Selleckchem, Houston, Texas, USA).

3. Reagents for in vitro studies

The following reagents were commercially acquired. Crystal violet (V5265, Thermo Fisher Scientific, Waltham, MA, USA), H2DCFDA (D399, Thermo Fisher Scientific, Waltham, MA, USA), MitoSOXTM Red (M36008, Thermo Fisher Scientific, Waltham, MA, USA), monobromobimane (mBBr) (M1378, Thermo Fisher Scientific, Waltham, MA, USA), Propidium Iodide (P3566, Thermo Fisher Scientific, Waltham, MA, USA), and H2O2 (216763, Sigma-Aldrich, St. Louis, MO, USA).

4. Antibodies for western blot

The following antibodies were commercially acquired. PHGDH (sc-100317, Santa Cruz Biotechnology, Dallas, TX, USA), SHMT2 (sc-390641, Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (sc-47778, Santa Cruz Biotechnology, Dallas, TX, USA), MTHFD2 (98116S, Cell Signaling Technology, Danvers, MA, USA).

5. Cell culture & drug treatment

SNU-80 cells were purchased from Korean Cell Line Bank (KCLB) and were grown RPMI- 1640 medium (10-041-CV, Corning, NY, USA)-1% penicillin-streptomycin (15140122, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (35-015-CV, Corning, NY, USA). Glutamine-full medium consists of medium (LM-001-05, Welgene, Daegu, Korea, TPC-1; 10-041-CV, Corning, NY, USA)-1% penicillin-streptomycin (15140122, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (35-015-CV, Corning, NY, USA). Glutamine-free medium consists of DMEM medium (LM-001-08, Welgene, Daegu, Korea)-1% penicillin-streptomycin (15140122, Gibco, Waltham, MA, USA) supplemented with 10% dialyzed fetal bovine serum (26400044, Gibco, Waltham, MA, USA); MTT, 0.2 mg/ml; Crystal violet solution, 0.1%; Hoechst 33342, 2 ug/ml; mBBr, 10 uM; H2DCFDA, 10 uM; MitoSOXTM Red, 5 uM; BPTES, 10 uM; CBR-5884, 60 uM; H2O2, 10uM; Trolox, 25 uM; lenvatinib, 50 uM; sorafenib, 10 uM.

6. Cell Proliferation assay

For cell proliferation assay, SNU-80 cells (3 × 103 cells/well) were seeded in 96-well plates and exposed to glutamine-free medium or reagents for the indicated time. For cell viability assay, SNU-80 cells (5 × 103 cells/well) were seeded in 96-well plates and were treated with glutamine-free medium or reagents for the indicated times. Cell proliferation was measured at 570 nm using a Multiskan GO spectrophotometer (51119300, Thermo Fisher Scientific, Waltham, MA, USA). Synergy effect of multiple-drugs treatment was calculated with the following equation provided at Synergy Finder (https://synergyfinder.fimm.fi/synergy/synfin_docs/).

1) SHSA = EA, B, C – max(EA, EB, EC)
2) SBliss = EA, B, C – 100(1-(1-EA/100)(1- EA/100))(1- EC/100))

EX means the percentage of viability inhibition by chemical X in indicated concentration.

7. Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (15596018, Invitrogen, Waltham, MA, USA). cDNA was synthesized using 1 ug total RNA with Reverse Transcriptase (A3803, Promega, Madison, WI, USA). Real-time RT-PCR was performed using TOP Real ™ qPCR 2X Pre-MIX (RT501S, Enzynomics, Daejeon, Korea). Gene expression was normalized to the expression level

of 36B4.

8. Immunoblot assays

Protein lysates were lysed in mammalian lysis buffer (25 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 10% glycerol) with Protease Inhibitor Cocktail (P3100-001, GenDEPOT, Katy, TX, USA). Lysate samples were loaded and separated by SDS-PAGE (8-10%) and transferred to nitrocellulose membranes (10600001, Amersham, Buckinghamshire, UK). Blocking solution was prepared with 5% skim milk-Tris-0.1% Tween 20. Primary antibodies were incubated at 4 °C for overnight. After incubation with HRP-conjugated secondary antibodies for 1 h at room temperature, immunoblot signals were measured by Clarity Western ECL Substrate (BR1705061 and 1705062, Bio-Rad, Hercules, CA, USA).

9. Transcriptomics analysis

The raw FASTQ data files were retrieved from SRA accession number PRJNA954580, comparing ATC cells under full media conditions and glutamine deprivation. The raw data underwent quality control using FastQC (version 0.11.7), followed by trimming with Trimmomatic (version 0.38). Mapping was performed using HISAT2 (version 2.1.0). The genes were assembled, and gene and transcript expression levels were quantified as read counts or fragments per kilobase of transcript per million mapped reads (FPKM) using StringTie (version 2.1.3b). To address systematic bias, read count data were normalized using the Trimmed Mean of M-values (TMM) method implemented in the edgeR package. Gene Set Enrichment Analysis (GSEA) was conducted to compare pathway terms between the two groups. Furthermore, motif analysis was performed utilizing the Integrated System for Motif Activity Response Analysis (ISMARA), selecting the top five motifs with the most significant differences in z-values.

10. Chromatin Immunoprecipitation assay

Chromatin and proteins were cross-linked with 1% (w/v) methanol-free formaldehyde (28908, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 5 minutes. The cross-linking reaction was quenched by adding glycine to a final concentration of 0.125 M. Cells were subsequently washed with cold PBS and lysed using cell lysis buffer (150 mM NaCl, pH 7.8, 50

mM Tris-HCl, pH 7.6, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, and 1X protease inhibitor). The resulting cell suspension was passed through a 1 mL insulin syringe to disrupt cell membranes, followed by centrifugation at 12,000 × g for 1 minute at 4°C. The pellet was resuspended in shearing buffer (1% SDS, pH 7.6, 10 mM EDTA, pH 8.0, 50 mM Tris-HCl, and 1X protease inhibitor), and chromatin was sheared using a Covaris ultrasonicator (M220).

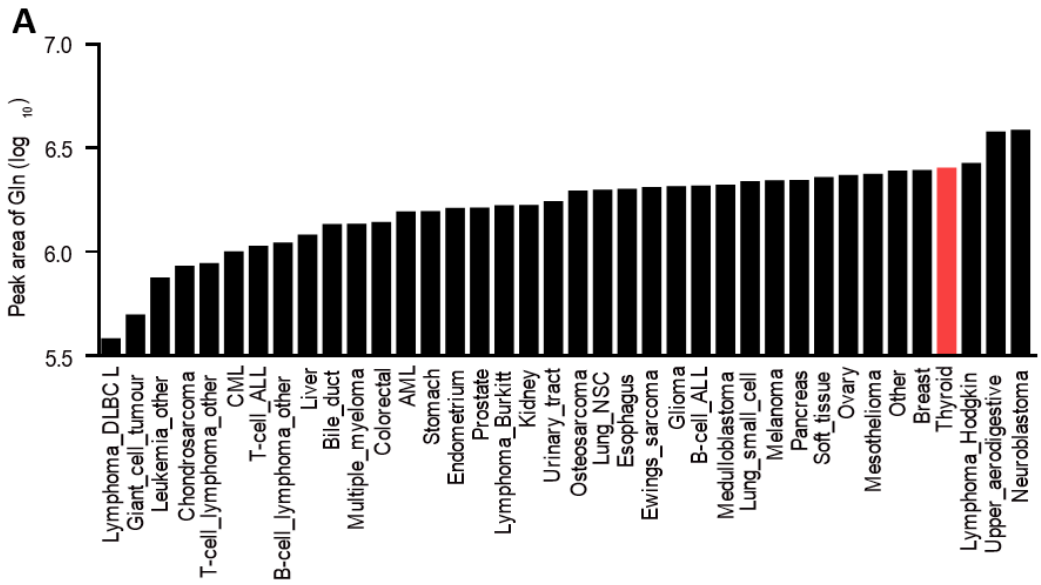
The sheared samples were diluted with dilution buffer (150 mM NaCl, pH 8.0, 20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1% Triton X-100, and 1X protease inhibitor) and subjected to immunoprecipitation with specific antibodies, including control rabbit IgG (2729, Cell Signaling Technology, Danvers, MA, USA), ATF4 (11815, Cell Signaling Technology, Danvers, MA, USA), and CEBPB (sc-7962, Santa Cruz Biotechnology, Dallas, TX, USA). Samples were incubated with a protein A and protein G bead slurry for 1 hour at 4°C. The antibody-chromatin complexes were sequentially washed three times with the following buffers: buffer 1 (150 mM NaCl, pH 8.0, 20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), buffer 2 (500 mM NaCl, pH 8.0, 20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), buffer 3 (0.25 M LiCl, pH 8.0, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% NP-40, 1% deoxycholate), and TE buffer (pH 8.0, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA).

Cross-linking was reversed by incubating the complexes in elution buffer (1% SDS, pH 8.0, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA) overnight at 65°C, followed by proteinase K treatment. DNA was purified using the QiAquick PCR Purification Kit (QIAGEN 28106, Sigma-Aldrich, St. Louis, MO, USA).

III. RESULTS

1. Glutamine level is dramatically high in ATC cells

To examine whether ATC obtains energy through glutaminolysis, we analyzed metabolomics data from various cancer types using the CCLE public database [26]. The analysis of CCLE metabolomics data showed markedly elevated glutamine levels in thyroid cancer cells, implying that glutamine serves as an essential energy source for their biological processes (Figure 1A) [27]. Notably, we found that glutamine levels were higher in ATC cell lines compared to non-ATC groups, suggesting that glutamine could play a pivotal role in fueling ATC cell metabolism (Figure 1B).



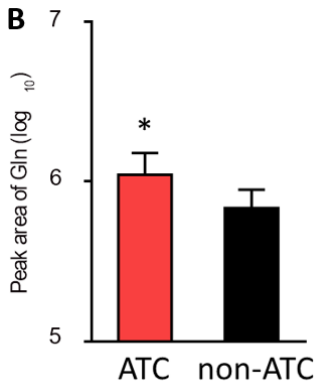


Figure 1. ATC might be highly dependent on glutamine metabolism. The bar graph illustrates the peak area of glutamine levels across various cancer tissues. All data were obtained from the CCLE metabolomics database. Statistical analyses were conducted using a two-tailed unpaired Student's t-test, with significance defined as $P < 0.05$.

2. Glutaminolysis Inhibition prohibits ATC proliferation

To investigate the impact of glutamine as a key energy source for the proliferation of ATC cells, we attempted to remove glutamine from ATC cell culture media. When ATC cells were cultured in glutamine-deficient media, we observed a significant decrease in the rate of cell growth (Figure 2A). Glutaminase (GLS) is the key enzyme involved in glutaminolysis. When the function of GLS is inhibited, glutaminolysis is suppressed as it cannot break down glutamine [28]. Therefore, to inhibit glutaminolysis, we administered BPTES, known as a GLS inhibitor, and measured the growth of ATC cells. The results demonstrated a notable decrease in cell proliferation upon inhibition of glutaminolysis through treatment with BPTES. Therefore, based on these findings, we have shown that the proliferation and growth of ATC cells depend on glutamine and glutaminolysis.

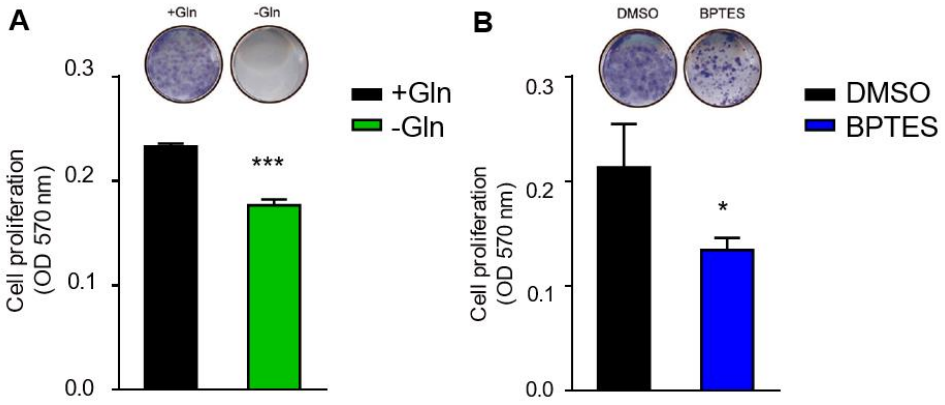


Figure 2. Inhibition of glutamoinolysis compromises cell proliferation in SNU-80 ATC cells. Bar graph represents proliferation of SNU-80 ATC cells in response to glutamine deficiency (A) and glutaminolysis inhibitor (B). Colony formation assays were stained with crystal violet solution, followed by dissolution in methanol for quantification. Statistical analyses were performed using a two-tailed unpaired Student's t-test. (*$P < 0.05$; ***$P < 0.001$)

3. Glutaminolysis Inhibition has no impacts on ATC cell death

Since the inhibition of glutaminolysis suppressed the proliferation of ATC cells, we conducted experiments to determine whether ATC cells die when glutaminolysis is inhibited. The results showed that even when ATC cells were cultured in glutamine-deficient media, there was no significant difference in cell viability compared to ATC cells cultured in normal media (Figure 3A). Likewise, inhibiting glutaminolysis with BPTES did not result in any significant change in cell viability compared to the control group (Figure 3B). Therefore, based on these results, it seems that while glutamine is crucial for the growth and proliferation of ATC cells, there are specific molecular mechanisms that allow ATC cells to maintain viability even when glutaminolysis is inhibited.

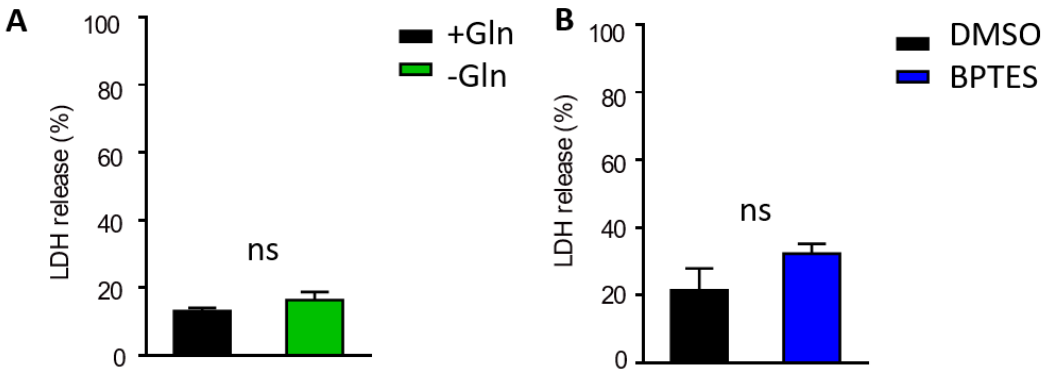


Figure 3. Cell death was not detected under glutaminolysis inhibition. The bar graph depicts apoptotic cell death in SNU-80 ATC cells under conditions of glutamine deficiency (A) and treatment with a glutaminolysis inhibitor (B). Statistical analyses were conducted using a two-tailed unpaired Student's t-test. (ns, not significant)

4. Glutamine deficiency induces one-carbon metabolism in ATC cells to enhance cell viability

Since inhibiting glutamine metabolism does not induce cell death, we hypothesized that ATC cells might activate alternative pathways to circumvent this inhibition. To explore this, we analyzed differentially expressed genes (DEGs) in cells cultured under glutamine-rich and glutamine-deficient conditions. The analysis revealed that glutamine deficiency significantly influences pathways related to one-carbon metabolism (Figure 4A) [29-31]. Additionally, gene set enrichment analysis (GSEA) revealed an enrichment of pathways related to serine metabolism in cells deprived of glutamine (Figure 4B).

One-carbon metabolism is a molecular mechanism activated by environmental stress to protect cells from apoptosis. To investigate its activation in ATC cell lines under glutamine deprivation, we cultured the cells in glutamine-rich and glutamine-free media and conducted mRNA sequencing. Analysis of the sequencing data identified significant alterations in signaling pathways linked to gene expression changes. Under glutamine-starved conditions, the expression of one-carbon metabolism-related genes, including PSAT1, PHGDH, MTHFD2, and SHMT2, was significantly upregulated, whereas genes associated with cell division, such as SHMT1, were downregulated (Figure 5A). Protein-level analysis further confirmed increased expression of key one-carbon metabolism enzymes, including PHGDH, SHMT2, MTHFD2, and PSPH, in glutamine-starved ATC cells (Figure 5B). These findings suggest that one-carbon metabolism is induced by glutamine starvation as a survival mechanism to prevent cell death.

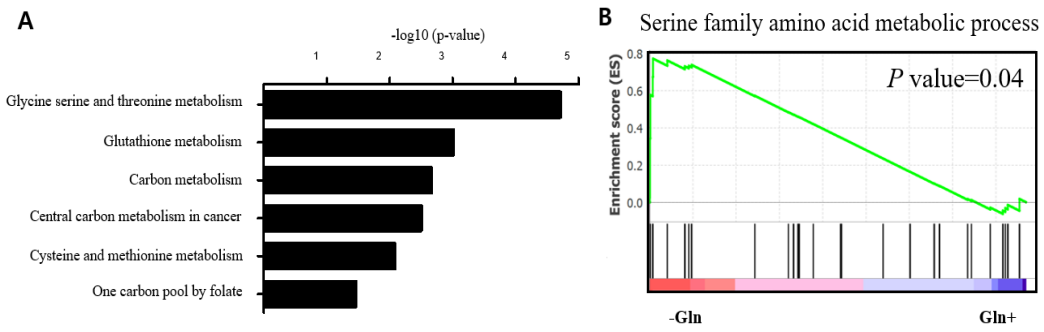


Figure 4. Glutamine deprivation enhances one-carbon metabolism in ATC. A KEGG analysis of differentially expressed genes (DEGs) revealed an upregulation of one-carbon metabolism-related pathways after 24 hours of glutamine deprivation. **B** The serine metabolic process was also significantly upregulated under glutamine-deprived conditions in ATC cells. Gene Set Enrichment Analysis (GSEA) highlighted the critical role of the serine metabolic process in supporting the survival of ATC cells during glutamine deprivation.

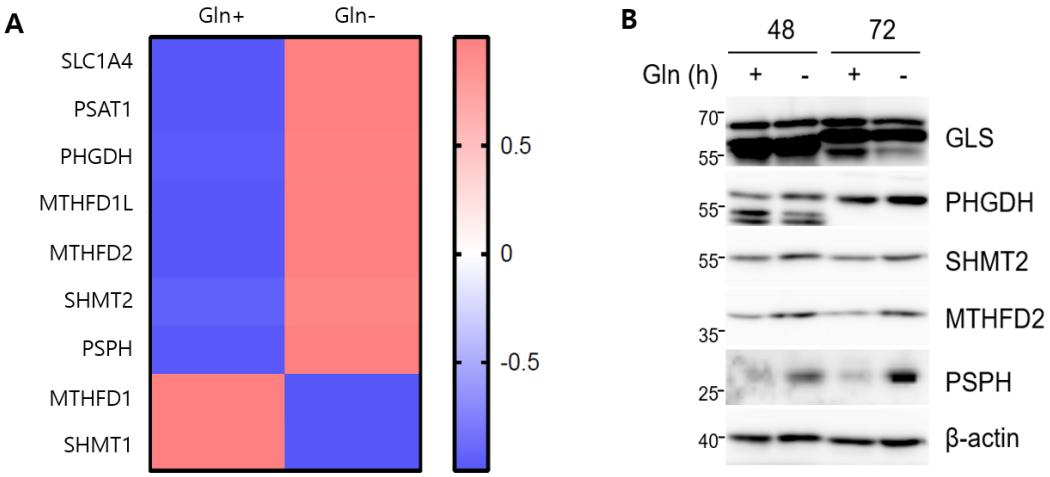


Figure 5. Gene expression profiles of key genes and the expression of key enzymes in one-carbon metabolism of ATC cells in the presence or absence of glutamine. A Glutamine deprivation induced key genes of mitochondrial 1C metabolism, including SHMT2, MTHFD2 as well as rate-limiting enzyme PHGDH. **B** Immunoblotting of key enzymes involved in one-carbon metabolism during glutamine starvation in ATC cells.

5. ATF4 is a dominant regulator of one-carbon metabolism during glutamine deprivation

Transcription factor motif analysis was performed to uncover the molecular mechanisms underlying alterations in the serine metabolic process and one-carbon metabolic pathways, as inferred from mRNA sequencing data. This analysis aimed to identify the transcription factors responsible for regulating specific biological processes. The results revealed that regulatory mechanisms involving ATF4 and CEBPB were significantly activated under glutamine-deprived conditions (Figure 6). Notably, ATF4 is known to upregulate one-carbon metabolism-related genes through transcriptional dysregulation[32,33]. ATF4 and CEBPB are well-recognized as pivotal transcription factors that facilitate cell survival under environmental stress. Notably, their activity is suppressed in glutamine-rich conditions but significantly upregulated during glutamine deprivation, activating the cell's stress response mechanisms. Our in vitro experiments demonstrated that the upregulation of one-carbon metabolism-related proteins induced by glutamine deprivation was completely abolished by genetic inhibition of ATF4 (Figure 6). These findings indicate that ATF4 serves as a critical transcriptional regulator of one-carbon metabolism in ATC cells under glutamine-deprived conditions.

Chromatin immunoprecipitation (ChIP) assays were performed to determine whether ATF4 is recruited to the promoter regions of PHGDH and SHMT2, key genes involved in the one-carbon metabolism pathway identified in this study. This technique is used to confirm the binding of specific proteins to designated DNA regions. The results revealed that the recruitment of ATF4 to the promoter regions of PHGDH and SHMT2 was significantly enhanced in ATC cells under glutamine-deprived conditions (Figure 7). These findings establish that ATF4 binds to the promoters of PHGDH and SHMT2 during glutamine starvation, thereby upregulating their expression.

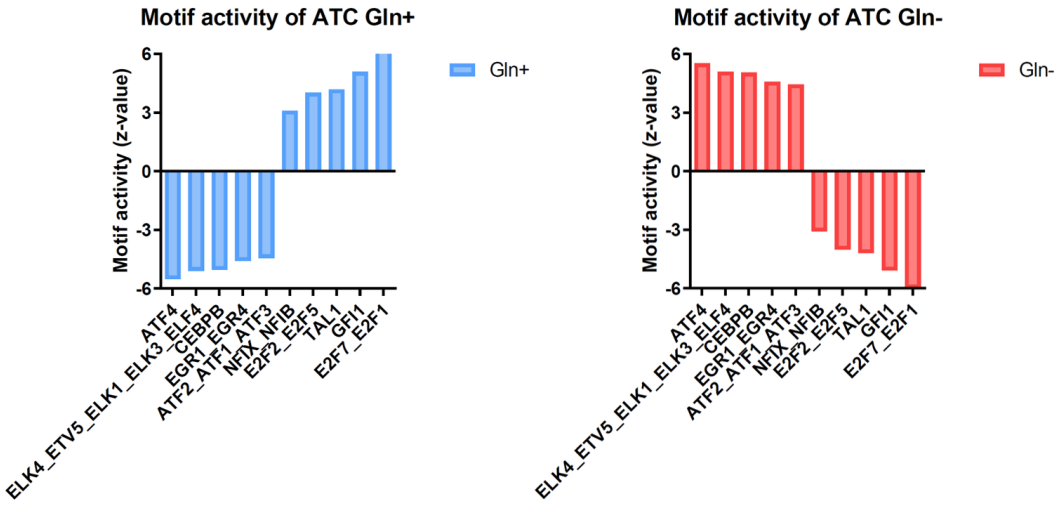


Figure 6. Transcription factor motif analysis in ATC cells in response to glutamine deprivation. ISMARA analysis has revealed that ATF4 and CEBPB transcriptional activity were dramatically activated in ATC cells with glutamine deprivation.

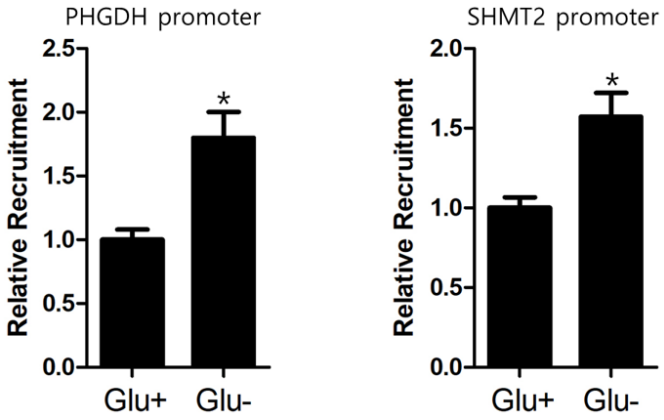


Figure 7. Recruitment of ATF4 at the promoter of PHGDH and SHMT2 in response to glutamine deprivation in ATC cells. Chromatin Immunoprecipitation was performed to examine the recruitment of ATF4 at the promoter of PHGDH and SHMT2 in ATC cells. *$P < 0.05$.

6. PHGDH is highly expressed in ATC tissue compared to PTC tissue

PHGDH is a key rate-limiting enzyme in one-carbon metabolism and plays a pivotal role in serine synthesis. Previous studies have reported significant upregulation of one-carbon metabolism enzymes under glutamine-deprived conditions. Notably, tissue analyses from patients revealed substantially higher PHGDH expression in ATC tissues compared to PTC tissues (Figure 8). Since PHGDH has been associated with preventing cancer cell death, these findings suggest that ATC cells depend on glutamine as an energy source while promoting survival through the upregulation of PHGDH and the activation of one-carbon metabolism.

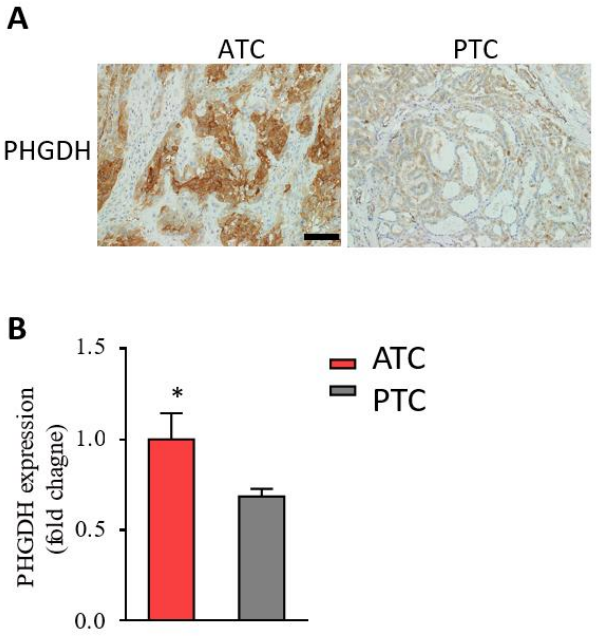


Figure 8. PHGDH is highly upregulated in ATC patients compared to PTC. PHGDH expression in human tissues from ATC patients and PTC patients. (A) Immunohistochemistry analysis of PHGDH protein expression (B) mRNA expression. Statistical analyses were conducted using two-tailed unpaired Student's *t* test (*$P < 0.05$)

7. Co-inhibition of glutaminolysis and PHGDH exhibits synergistic effects to inhibit ATC cell proliferation

The results suggest that ATC cells rely on glutamine as an energy source and utilize PHGDH-mediated one-carbon metabolism to promote cell growth and inhibit apoptosis [34,35]. To investigate whether inhibiting glutamine utilization and PHGDH activity could suppress ATC cell growth, we utilized CBR-5884, a PHGDH inhibitor. Both glutamine starvation and CBR-5884 treatment significantly reduced ATC cell proliferation compared to the control group (Figure 9A). Furthermore, the combination of glutamine starvation and CBR-5884 produced a synergistic effect, resulting in a more pronounced reduction in cell proliferation than either treatment alone (Figure 9A).

In a subsequent experiment, glutaminolysis was inhibited using BPTES instead of glutamine starvation. Consistent with previous findings, treatment with either BPTES or CBR-5884 alone significantly inhibited ATC cell proliferation (Figure 9B). Notably, the combination of BPTES and CBR-5884 further enhanced the suppression of cell proliferation compared to single treatments (Figure 9B). These results suggest that dual inhibition of glutaminolysis and PHGDH is a promising therapeutic strategy for ATC.

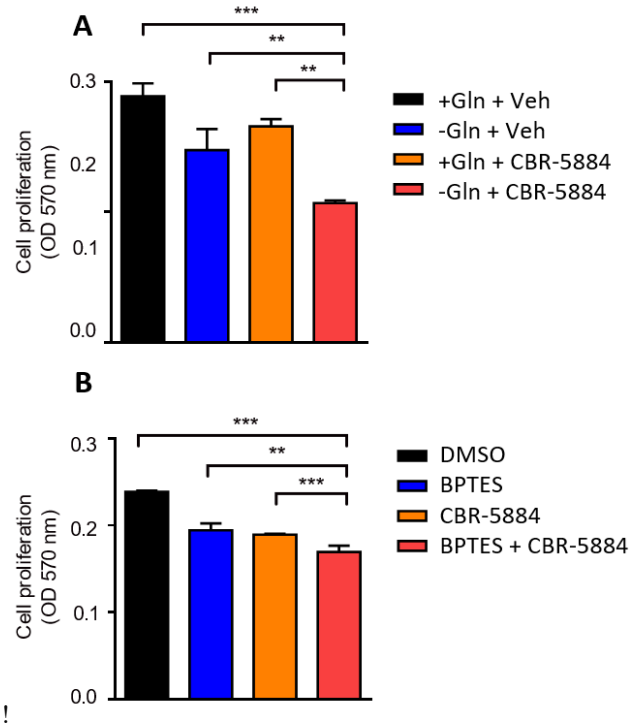


Figure 9. Inhibition of glutaminolysis and PHGDH reduces cell proliferation in SNU-80 cells. Cell proliferation assay in SNU-80 cells. (A) Bar graph represents proliferation of SNU-80 ATC cells in response to glutamine deficiency and PHGDH inhibitor, CBR-5884 (A) Bar graph represents cell proliferation of SNU-80 ATC cells with GLS inhibitor, BPTES and PHGDH inhibitor, CBR-5884 (B). Statistical comparisons were performed using two-tailed unpaired Student's *t* test (***P* < 0.01; ****P* < 0.001)

8. Co-inhibition of glutaminolysis and PHGDH increases intracellular ROS level in ATC

Reactive oxygen species (ROS) are well-known for their role in reducing cell viability. To evaluate the impact of co-inhibiting glutaminolysis and PHGDH on ROS levels, we measured ROS levels in ATC cells treated with BPTES, CBR-5884, and lenvatinib. Interestingly, co-treatment with lenvatinib and BPTES did not elevate intracellular ROS levels. In contrast, co-treatment with lenvatinib and CBR-5884 significantly increased intracellular ROS levels. Furthermore, the triple combination of lenvatinib, BPTES, and CBR-5884 resulted in a markedly high intracellular ROS level in ATC cells (Figure 7A).

Notably, the combination of BPTES, CBR-5884, and sorafenib did not produce a synergistic increase in ROS levels compared to the combination of sorafenib and CBR-5884 alone (Figure 7B). These findings suggest that specific tyrosine kinase inhibitors (TKIs) may exhibit synergistic effects when combined with the co-inhibition of glutaminolysis and PHGDH. Identifying TKIs with such synergistic properties could be crucial for developing more effective therapeutic strategies for ATC.

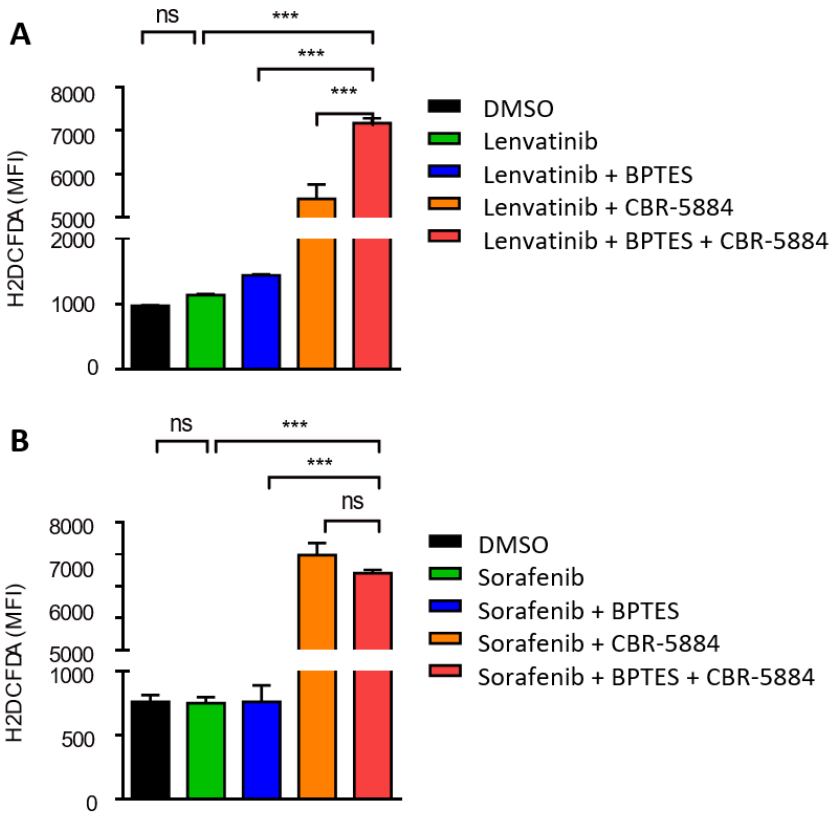


Figure 10. Co-inhibition of glutaminolysis and PHGDH synergistically enhances chemotherapy efficacy by accumulating ROS level. Intracellular ROS level in SNU-80 cells with treatment of BPTES, CBR-5884 in the presence of TKIs, Lenvatinib or Sorafenib. (A) Bar graph represents ROS level in SNU-80 ATC cells in response to combination of indicated drugs with lenvatinib (B) Bar graph represents ROS level in SNU-80 ATC cells in response to combination of indicated drugs with sorafenib. Statistical comparisons were performed using two-tailed unpaired Student's *t* test (***$P < 0.001$; ns, not significant)

9. Co-inhibition of glutaminolysis and PHGDH enhances chemotherapy efficacy to reduce cell viability in ATC

Previous findings demonstrated that the co-inhibition of glutaminolysis and PHGDH enhances the efficacy of lenvatinib and increases intracellular ROS levels. To further investigate, we examined

whether this co-inhibition, in combination with lenvatinib, reduces ATC cell viability. Consistent with earlier results, we confirmed that the co-inhibition of glutaminolysis and PHGDH significantly enhances the anticancer effects of lenvatinib, leading to a marked reduction in ATC cell viability (Figure 8A). Similarly, co-inhibition of glutaminolysis and PHGDH also potentiated the anticancer effects of sorafenib, resulting in decreased cell viability (Figure 8B).

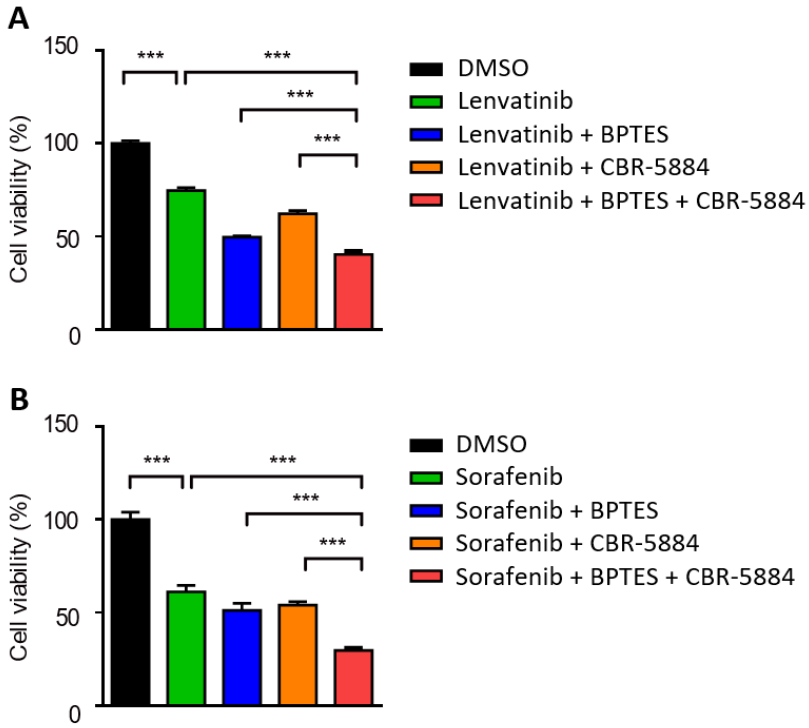


Figure 11. Co-inhibition of glutaminolysis and PHGDH synergistically enhances chemotherapy efficacy to reduce cell viability in ATC cells. Cell viability of SNU-80 cells with treatment of BPTES, CBR-5884 in the presence of TKIs, Lenvatinib or Sorafenib. (A) Bar graph represents cell viability of SNU-80 ATC cells in response to combination of indicated drugs with lenvatinib (B) Bar graph represents cell viability of SNU-80 ATC cells in response to combination of indicated drugs with sorafenib. Statistical comparisons were performed using two-tailed unpaired Student's *t* test (***$P < 0.001$)

10. Co-inhibition of glutaminolysis and PHGDH with TKIs exhibits synergistic effects targeting for ATC

We investigated the synergistic effects of co-inhibiting glutaminolysis and PHGDH in combination with tyrosine kinase inhibitors (TKIs) for ATC treatment. Using Bliss and HSA scores to evaluate drug combination efficacy, with scores above 10 indicating synergy, we found that co-inhibition of glutaminolysis and PHGDH demonstrated synergistic effects with both lenvatinib and sorafenib (Figure 9). These findings suggest that combining glutaminolysis and PHGDH co-inhibition with existing TKIs may offer a promising therapeutic strategy for ATC, which currently lacks effective treatment options.

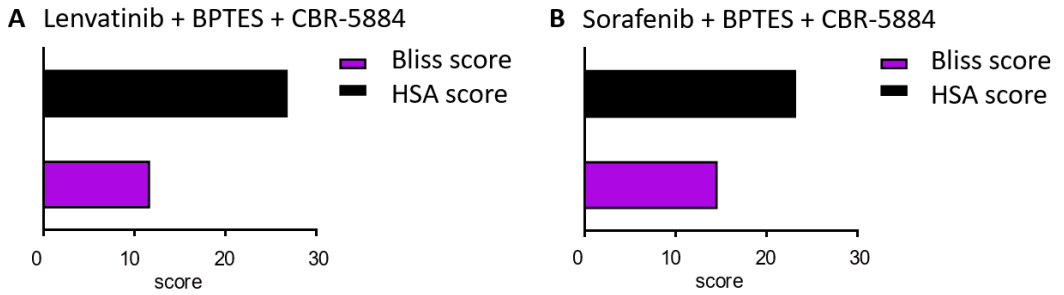


Figure 12. Triple treatment of Co-inhibition of glutaminolysis and PHGDH with TKIs exhibits synergistic effect targeting for ATC. Both score values are above 10, co-inhibition of glutaminolysis and PHGDH exhibits 'synergistic effect' with TKIs.

IV. DISCUSSION

The treatment of ATC remains a formidable challenge despite extensive research efforts. ATC is defined by its aggressive progression, high metastatic potential, and poor response to conventional therapies. The lack of clarity regarding the molecular drivers of tumor progression further hampers the development of effective targeted therapies. Currently, cytotoxic chemotherapy, including regimens such as paclitaxel/carboplatin and docetaxel/doxorubicin, or monotherapy with agents like doxorubicin, constitutes the mainstay of ATC treatment. However, overall survival (OS) rates have shown minimal improvement over the past several decades, underscoring the limited efficacy of traditional chemotherapy.[37-39]. The highly aggressive nature of ATC, coupled with its resistance to conventional therapies, highlights the critical need for innovative therapeutic strategies that target the molecular pathways underlying its progression and therapy resistance.

Recent advancements in tumor-agnostic therapies have introduced new possibilities for patients with anaplastic thyroid cancer (ATC) harboring specific genetic mutations. Notably, the combination of dabrafenib and trametinib has demonstrated significant efficacy in patients with BRAF V600E-mutant ATC. In a cohort of 36 patients, this combination therapy achieved an overall response rate of 56%, with a median duration of response of 14.4 months[37]. These findings underscore the potential of targeted therapies in improving outcomes for this aggressive cancer subtype. Furthermore, the addition of pembrolizumab to dabrafenib and trametinib has significantly extended the median survival of patients compared to dabrafenib and trametinib alone (17 months vs. 9 months), underscoring the potential of combination therapies to improve clinical outcomes. However, despite these advancements, the efficacy of such therapies is often restricted to specific patient subgroups, as many ATC cases lack targetable mutations such as BRAF V600E. Consequently, there is a pressing need for innovative approaches that target ATC's metabolic vulnerabilities to provide broader therapeutic options.

ATC has been shown to exhibit higher metabolic activity than other thyroid cancer subtypes, including elevated levels of enzymes such as GLS1 and GDH[20,40]. These enzymes play critical roles in glutamine metabolism, which is frequently exploited by cancer cells to sustain rapid growth and proliferation. Analysis of publicly available datasets, including CCLE and GEO, has further confirmed that glutamine levels are markedly higher in ATC cells compared to non-ATC thyroid cancer cells [26]. Based on these observations, we hypothesized that targeting glutamine metabolism could significantly impair ATC cell viability, offering a potential therapeutic strategy. Consistent

with this hypothesis, our study demonstrated that ATC cells are highly dependent on glutamine metabolism for growth. Pharmacological inhibition of glutaminolysis using agents such as BPTES resulted in a marked reduction in cell proliferation, confirming the reliance of ATC cells on this metabolic pathway for survival. However, glutamine metabolism inhibition alone did not induce cell death, indicating the presence of compensatory mechanisms that enable ATC cells to evade apoptosis and sustain viability.

One of the key compensatory pathways activated in ATC cells under glutamine deprivation is one-carbon metabolism, which is essential for maintaining cellular homeostasis, particularly redox balance[24]. Our findings demonstrate that one-carbon metabolism plays a critical role in safeguarding ATC cells against oxidative stress induced by nutrient deprivation. The transcription factor ATF4 was identified as a key regulator of one-carbon metabolism under glutamine-deprived conditions, driving the expression of essential enzymes such as PHGDH and SHMT2. These enzymes facilitate the serine biosynthesis pathway, supplying vital intermediates for nucleotide synthesis, antioxidant defense, and methylation reactions—processes essential for ATC cell survival during metabolic stress. Chromatin immunoprecipitation assays further confirmed that ATF4 is recruited to the promoter regions of PHGDH and SHMT2 under glutamine deprivation, underscoring its pivotal role in enabling the metabolic adaptation of ATC cells.

Lenvatinib and sorafenib are currently used in ATC treatment. However, clinical studies have reported that these agents show limited efficacy when used as monotherapy in ATC patients[12,41]. Recent research has suggested that combining TKIs with other therapeutic approaches may significantly enhance their effectiveness[42-45]. Our findings support this notion, demonstrating that the co-inhibition of glutamine metabolism and one-carbon metabolism significantly enhances the therapeutic efficacy of lenvatinib and sorafenib. This enhanced efficacy is primarily attributed to the increased accumulation of reactive oxygen species (ROS), triggered by the simultaneous disruption of these metabolic pathways. ROS accumulation is well-documented for its role in inducing oxidative damage and promoting cancer cell death, and our results indicate that ATC cells are particularly vulnerable to ROS-mediated tumor suppression under these conditions.

Although clinical evidence for targeting glutamine and one-carbon metabolism in ATC patients is currently limited, studies in other cancer types suggest the potential effectiveness of such strategies. For instance, in solid tumors, glutamine levels decrease with increasing distance from blood vessels,

potentially activating one-carbon metabolism in the hypoxic tumor core[15,46]. These observations highlight the potential of dual targeting of glutamine and one-carbon metabolism as a promising therapeutic approach for ATC.

The activation of one-carbon metabolism under metabolic stress conditions, such as glutamine deprivation, enables ATC cells to sustain their proliferation and survival. Cancer cells can produce serine, a critical component of one-carbon metabolism, through multiple pathways, including autophagy, glycolysis, and serine uptake via specific transporters[31,35,47-49]. Our study demonstrated that inhibition of glutamine metabolism leads to an upregulation of enzymes involved in the serine biosynthesis pathway, such as PHGDH, enabling ATC cells to sustain one-carbon metabolism. Conversely, a decrease in the mRNA levels of enzymes such as SHMT1 and MTHFD1, both critical components of cytosolic one-carbon metabolism, was observed. [49-51]. These changes suggest that one-carbon metabolism is intricately linked to ATC progression and therapy resistance.

The adaptability of cancer cells to metabolic stress through epigenetic modifications is well-established. Metabolic reprogramming frequently coincides with alterations in gene expression, regulated by epigenetic changes such as histone methylation, histone acetylation, and DNA methylation[52-54]. Our bioinformatics analysis revealed that glutamine deprivation led to increased transcription factor activity, particularly involving ATF4, which in turn activated the one-carbon metabolism pathway. These findings highlight the dynamic interplay between metabolic stress and epigenetic regulation in ATC, where metabolic reprogramming supports cancer cell survival during therapy.

Another critical finding of our study was the impact of glutamine metabolism inhibition on intracellular GSH levels. GSH is a vital antioxidant that protects cancer cells from oxidative stress and is synthesized through pathways including NADPH production via the pentose phosphate pathway (PPP) and one-carbon metabolism[24,47]. Inhibiting glutamine metabolism reduced total GSH levels in ATC cells, which in turn led to increased ROS levels. Previous studies have shown that glutamine deprivation deactivates PPP in cancer cells, contributing to ROS accumulation[17,41,55]. Our data suggest that one-carbon metabolism also plays a role in GSH depletion, further enhancing the oxidative stress experienced by ATC cells. Considering the critical role of GSH as a major antioxidant, its depletion likely explains the substantial ROS accumulation observed during the co-inhibition of glutamine and one-carbon metabolism. Additionally, our experiments confirmed that ROS directly regulates ATC cell proliferation, as demonstrated by the impact of Trolox, an

antioxidant that effectively reduces ROS levels. These results align with findings in other cancer types, such as glioblastoma and leukemia, where ROS-induced oxidative stress has been shown to suppress tumor growth[56,57].

In summary, our study emphasizes the critical roles of glutamine and one-carbon metabolism in the survival and proliferation of ATC cells. Targeting these metabolic pathways significantly enhanced the efficacy of existing chemotherapeutic agents, such as lenvatinib and sorafenib, by promoting ROS accumulation and inducing oxidative stress. These findings suggest that dual inhibition of glutamine and one-carbon metabolism represents a promising therapeutic strategy for ATC. However, further clinical studies are needed to validate these results and explore the integration of metabolic inhibitors with standard-of-care treatments for ATC patients. Given the aggressive and treatment-resistant nature of ATC, novel therapeutic approaches that exploit its metabolic vulnerabilities may offer new opportunities to improve patient outcomes.

V. CONCLUSION

In conclusion, this study has highlighted the critical role of glutamine and one-carbon metabolism in the survival and proliferation of anaplastic thyroid cancer cells. Inhibiting these metabolic pathways significantly impairs ATC cell growth and enhances the efficacy of chemotherapy by promoting ROS accumulation. The co-inhibition of glutaminolysis and PHGDH, in combination with TKIs, presents a novel therapeutic strategy that could improve clinical outcomes for patients with ATC, an aggressive cancer with limited treatment options. Further research is warranted to confirm the clinical relevance of these findings and to develop targeted therapies that exploit the metabolic vulnerabilities of ATC cells.

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Abstract in Korean

역형성 갑상선암에서 글루타민 대사와 단일 탄소 대사의 차단으로 ROS 축적을 통한 항암효과 향상

역형성 갑상선 암은 가장 위험한 암 중 하나로 진단 시 사망률이 매우 높고 치료하기 어려운 희귀 암으로 분류된다. 역형성 갑상선암을 치료하기 위해 다양한 항암제가 개발되었지만 지금까지는 뚜렷한 효능이 관찰되지 않았으며 유의미한 효과를 보이는 약물은 거의 없다. 역형성 갑상선암은 빠른 성장을 하고 국소 전이와 원격전이가 잘 일어나며 이러한 특성을 가진 암세포는 일반적으로 다양한 에너지원을 활용하여 성장한다고 알려져 있다. 글루타민은 우리 몸에 풍부하게 존재하는 아미노산으로 항산화 특성과 질소 공급 역할을 하며 다양한 암세포의 성장 및 전이 과정에 활용되는 것으로 알려져 있다. 특히 악성도가 높은 암세포는 에너지원으로 글루타민을 선호한다는 연구 결과가 다양한 암 유형에서 보고되고 있다. 그러나 역형성 갑상선암에서 에너지원으로 글루타민을 사용하는 것에 대한 연구는 보고되지 않았다. 따라서 본 논문에서는 역형성 갑상선암이 성장을 위해 글루타민에 의존하는지 조사하고자 한다. 또한 글루타민 공급이 중단되었을 때 역형성 갑상선암이 어떻게 반응하는지 살펴보고자 한다. 연구 결과 역형성 갑상선암 세포가 글루타민에 높은 의존성을 보인다는 것을 확인할 수 있었다. 또한 글루타민 공급이 차단되면 암세포의 성장이 억제된다는 것을 확인했다. 그러나 흥미롭게도 암세포가 성장이 억제되었지만 죽지 않고 살아 있다는 것도 관찰했다. 즉, 암세포가 분열을 멈추었지만 생존 가능한 상태를 유지했다는 것을 확인했다. 전사체 분석을 통해 역형성 갑상선암 세포가 글루타민 공급이 차단되었을 때 죽지 않고 생존하기 위해 ATF4 매개 단일 탄소대사를 증가시켜 ROS 항상성을 조절하고 세포의 증식 억제를 회피함을 확인했다. 다음으로 글루타민 공급을

차단하는 동시에 단일 탄소 대사를 억제하는 억제제를 투여하면 역형성 갑상선암 세포의 사멸을 촉진할 수 있다는 것을 확인했다. 또한 기존 티로신 키나제 억제제의 효능을 향상시킬 수 있다는 것을 확인할 수 있었다. 결론적으로 단일 탄소 대사의 억제는 역형성 갑상선암에서 항암 효과를 높여 환자의 생존율을 높일 수 있는 치료적 목표가 될 수 있으며, 글루타민 공급을 차단하고 단일 탄소 대사를 억제하는 것이 역형성 갑상선암을 치료하는 새로운 치료 전략이라고 제안한다.

핵심되는 말: 역형성 갑상선암, 글루타민 대사, 단일 탄소 대사

PUBLICATION LIST

Hwang YS, Yun HJ, Jeong JW, Kim MK, Joo SY, Lee HK et al. Co-inhibition of glutaminolysis and one carbon metabolism promotes ROS accumulation leading to enhancement of chemotherapeutic efficacy in anaplastic thyroid cancer. Cell Death and Disease 2023;14(8):515.