The Vitamin B12 Receptor CD320 Is a Key Metabolic Dependency and Prognostic Biomarker in Hepatocellular Carcinoma

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Abstract. Background: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death globally, and its immunosuppressive tumor microenvironment (TME) often limits the efficacy of immunotherapies. Metabolic competition between cancer cells and immune cells for essential nutrients is a critical mechanism of immune evasion. Vitamin B12 (VB12), a micronutrient vital for cellular proliferation and metabolism, is acquired by cells via the transcobalamin receptor, CD320. We hypothesized that HCC cells overexpress CD320 to sequester VB12, thereby fueling their own proliferation while simultaneously depriving anti-tumor immune cells, such as Natural Killer (NK) cells, of this crucial cofactor. Methods: We conducted a comprehensive bioinformatic analysis of bulk and single-cell RNA sequencing data from HCC patient cohorts to evaluate CD320 expression and its clinical significance. In vitro experiments were performed using human HCC cell lines (e.g., HepG2) and a non-malignant hepatocyte line. CD320 expression was validated at the mRNA and protein levels using RT-qPCR and Western blotting. The functional role of CD320 was investigated through siRNA-mediated gene silencing, followed by proliferation assays (MTT) in VB12-replete and VB12-deficient conditions. Results: Our analysis revealed that CD320 expression is significantly upregulated in HCC tissues compared to adjacent normal liver tissues. Elevated CD320 levels were strongly correlated with advanced clinical stage and significantly poorer overall survival in HCC patients. Single-cell transcriptomic analysis confirmed that this overexpression is predominantly driven by malignant cells, rather than by immune or stromal components of the TME. Functionally, silencing CD320 in HepG2 cells markedly attenuated their proliferative capacity. While supplemental VB12 robustly promoted the growth of control cells, this effect was completely abrogated in CD320-knockdown cells, demonstrating a critical dependence of HCC proliferation on the CD320-VB12 uptake axis. Conclusion: This study identifies the Vitamin B12 receptor, CD320, as a novel prognostic biomarker and a key mediator of proliferation in hepatocellular carcinoma. Our findings support a model in which HCC cells hijack the VB12 metabolic pathway by overexpressing CD320. This adaptation provides a competitive advantage for nutrient acquisition, which fuels tumor growth and is hypothesized to create a nutrient-deprived microenvironment that impairs anti-tumor immunity. The CD320-VB12 axis represents a promising therapeutic vulnerability, and targeting this pathway may offer a dual strategy to directly inhibit cancer cell proliferation and restore immune function in the TME.

Keywords: Hepatocellular Carcinoma (HCC); Vitamin B12; CD320; Tumor Microenvironment; Immunometabolism; Metabolic Competition; Natural Killer (NK) Cells.

1. Introduction

Hepatocellular carcinoma (HCC) represents a formidable global health challenge. As the most prevalent form of primary liver cancer, it ranks as the third leading cause of cancer-related mortality worldwide, with a steadily increasing incidence in numerous countries (Llovet et al., 2021; Sung et al., 2021). The therapeutic landscape for advanced HCC has been revolutionized by the advent of immune checkpoint inhibitors (ICIs). The combination of atezolizumab, an anti-programmed death-ligand 1 (PD-L1) antibody, and bevacizumab, an anti-vascular endothelial growth factor (VEGF) agent, has become the established first-line standard of care, demonstrating superior overall survival compared to previous tyrosine kinase inhibitors like sorafenib (Cheng et al., 2022; Finn et al., 2020). However, the promise of immunotherapy is not universally realized. A substantial number of patients

exhibit primary resistance, failing to respond from the outset, while others develop secondary resistance after an initial period of clinical benefit (Pinter et al., 2021; Sangro et al., 2021). This heterogeneity in response highlights the complex and highly immunosuppressive nature of the HCC tumor microenvironment (TME). Understanding the mechanisms that underpin immune evasion and resistance within the HCC TME is therefore a paramount objective in oncological research.

One of the most insidious mechanisms of immune resistance is metabolic competition between tumor cells and immune effector cells within the TME (Chang et al., 2015). By monopolizing these essential resources, cancer cells create a nutrient-deprived, or "austere," microenvironment that severely impairs the metabolic fitness and functional capacity of tumor-infiltrating lymphocytes (TILs) (Reinfeld et al., 2021; Ringel et al., 2020). For instance, glucose scarcity can compromise the glycolytic pathways essential for the activation and cytotoxic functions of T cells and Natural Killer (NK) cells (Ho et al., 2015). Similarly, depletion of key amino acids like arginine and tryptophan by tumor-expressed enzymes can lead to lymphocyte anergy and apoptosis (Munn & Mellor, 2016). This metabolic warfare effectively disarms the immune system, allowing the tumor to escape surveillance and elimination. Consequently, deciphering these metabolic vulnerabilities offers a promising avenue for developing novel therapeutic strategies aimed at restoring immune function and overcoming resistance to current treatments.

Within this framework of oncometabolism and immunometabolism, the role of essential micronutrients, such as vitamins, is gaining increasing recognition. Vitamin B12 (VB12), also known as cobalamin, is a water-soluble vitamin that serves as a critical cofactor for two fundamental enzymatic reactions in mammalian cells. It is required for the activity of methionine synthase (MTR), which catalyzes the remethylation of homocysteine to methionine, a crucial step in the one-carbon metabolism pathway that generates S-adenosylmethionine (SAM), the universal methyl donor for DNA, RNA, and protein methylation (Froese et al., 2019). Secondly, VB12 is a cofactor for methylmalonyl-CoA mutase (MUT), an enzyme that facilitates the conversion of methylmalonyl-CoA to succinyl-CoA, linking fatty acid and amino acid catabolism to the Krebs cycle for energy production (Sasaki et al., 2023). Through these roles, VB12 is indispensable for nucleotide synthesis (both purines and pyrimidines), epigenetic regulation, and cellular bioenergetics—processes that are acutely demanded by rapidly proliferating cells (Ge et al., 2022).

The uptake of VB12 into cells is a highly regulated process. In circulation, VB12 is primarily bound to the transport protein transcobalamin (TC). The resulting holo-transcobalamin complex (holo-TC) is recognized and internalized by a specific cell surface receptor, CD320, also known as the transcobalamin receptor (TCblR) (Seetharam, 1999). Following endocytosis of the holo-TC-CD320 complex, VB12 is released from its carrier within the lysosome and processed into its active cofactor forms (Quadros, 2010). The expression of CD320 is tightly correlated with cellular proliferation rates, being highly expressed in progenitor cells and rapidly dividing tissues, suggesting that its regulation is a key mechanism for matching VB12 supply with metabolic demand (Seetharam & Li, 2000).

Emerging evidence indicates that this VB12 uptake axis is frequently hijacked by malignant cells to sustain their aggressive growth. Elevated expression of CD320 has been documented across a range of human cancers, including breast, colon, and hematopoietic malignancies, and often correlates with poorer clinical outcomes (Lai et al., 2011; Zhang et al., 2024). This overexpression is thought to provide a selective advantage by enabling cancer cells to outcompete normal cells for circulating VB12, thereby fueling the metabolic pathways essential for unchecked proliferation. In parallel to its role in oncogenesis, VB12 availability also has profound immunomodulatory effects. The high metabolic demands of immune cell activation and effector function mean that lymphocytes are also dependent on a steady supply of micronutrients. Several studies have linked VB12 status to the functional integrity of the immune system. VB12 deficiency has been associated with impaired lymphocyte proliferation and reduced numbers of CD8+ T cells (Tejero et al., 2025; Watson et al., 2023). Of particular relevance to anti-tumor immunity are Natural Killer (NK) cells, which are potent cytotoxic lymphocytes of the innate immune system that play a crucial role in early cancer

immunosurveillance. The cytotoxic activity of NK cells has been shown to be diminished in individuals with VB12 deficiency, a defect that can be corrected with cobalamin supplementation (Itoh et al., 1999; Sakane et al., 1982). This suggests that NK cell metabolic fitness and effector functions, including their ability to lyse tumor cells, are dependent on adequate VB12 availability.

Given these converging lines of evidence, we are confronted with a compelling scenario within the tumor microenvironment: a potential metabolic conflict centered on the acquisition of Vitamin B12. On one hand, rapidly proliferating HCC cells may upregulate CD320 to aggressively sequester VB12, thereby fueling their own growth and survival. On the other hand, this sequestration would create a state of localized VB12 deficiency within the TME, crippling the metabolic capacity and cytotoxic potential of anti-tumor immune cells, particularly NK cells. This dynamic would establish a vicious cycle where tumor growth is metabolically supported while the primary immune effectors capable of eliminating the tumor are simultaneously suppressed. Therefore, in this study, we hypothesize that hepatocellular carcinoma cells overexpress the Vitamin B12 receptor, CD320, to secure a competitive advantage for B12-dependent proliferation while concurrently limiting B12 availability to NK cells within the tumor microenvironment. Elucidating this novel axis of metabolic crosstalk may not only deepen our understanding of immune resistance in HCC but also unveil the CD320-VB12 pathway as a potential therapeutic vulnerability, opening new avenues for interventions aimed at simultaneously starving tumor cells and revitalizing anti-tumor immunity.

2. Materials and Methods

2.1. Cell Culture and Reagents

The human hepatocellular carcinoma cell lines HepG2, Huh7, and Hep3B, along with the non-malignant hepatocyte cell line THLE-2, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were authenticated by short tandem repeat (STR) profiling upon receipt and were routinely tested for mycoplasma contamination. HCC cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Cat# 11965092) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat# 10270106) and 1% penicillin–streptomycin (Gibco, Cat# 15140122). THLE-2 cells were cultured in Bronchial Epithelial Cell Growth Medium (BEGM; Lonza, Cat# CC-3170) as recommended by the supplier. All cell lines were maintained in a humidified incubator at 37°C with a 5% CO_2 atmosphere.

For experiments involving Vitamin B12 (VB12) manipulation, cells were cultured in custom VB12-deficient DMEM (Thermo Fisher Scientific, Cat# A3382401) supplemented with 10% dialyzed FBS (dFBS; Gibco, Cat# 26400044) to minimize exogenous cobalamin. Cyanocobalamin (Sigma-Aldrich, Cat# V2876) was reconstituted in sterile water and added to the VB12-deficient medium at specified concentrations (e.g., 0 pM, 100 pM, 500 pM, 1 nM, and 10 nM) to assess dose-dependent effects.

For gene knockdown experiments, cells were seeded to achieve 50-60% confluency at the time of transfection. They were transfected with validated small interfering RNAs (siRNAs) targeting the *CD320* gene (si-CD320) or a non-targeting negative control siRNA (siNC) (Dharmacon, ON-TARGETplus SMARTpool). Transfections were performed using Lipofectamine RNAiMAX Reagent (Invitrogen, Cat# 13778075) according to the manufacturer's protocol. Briefly, siRNAs (final concentration 20 nM) and the transfection reagent were separately diluted in Opti-MEM Reduced Serum Medium (Gibco, Cat# 31985062). After complex formation, the mixture was added to the cells. After 6 hours of incubation, the medium was replaced with complete growth medium. Knockdown efficiency was assessed at the mRNA and protein levels 48 to 72 hours post-transfection. Vendor details, catalog numbers, and lot numbers for all key reagents are tracked and provided in the comprehensive research plan documentation.

2.2. RNA Extraction and RT-qPCR

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (QIAGEN, Cat# 74104) following the manufacturer's protocol, which includes an on-column DNase digestion step to eliminate genomic DNA contamination. RNA concentration and purity were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), with A260/A280 ratios of 1.8–2.0 considered acceptable. One microgram of total RNA was reverse-transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, Cat# 1708891).

Quantitative real-time PCR (RT-qPCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad). Each 20 µL reaction consisted of 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, Cat# 1725121), 1 µL of cDNA template, and 500 nM of forward and reverse primers. The thermal cycling protocol was as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. A melt curve analysis was performed at the end of each run to confirm the specificity of the amplification product. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as the endogenous reference gene for normalization. Relative gene expression was calculated using the 2^−ΔΔCt method, where the control condition (e.g., non-malignant cells or siNC-transfected cells) was designated as the calibrator (KB) with a relative expression value set to 1.00. All experiments were performed in triplicate, and results are reported as mean ± standard deviation (SD). A complete list of primer sequences is provided in the supplementary materials (File: qPCR_Primers.xlsx, Sheet: "引物").

2.3. Western Blot and Densitometry

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Thermo Fisher Scientific, Cat# 89900) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Cat# 78440). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# 23225). Equal amounts of protein (20–30 µg) per sample were denatured, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% Mini-PROTEAN TGX Precast Gels (Bio-Rad), and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Cat# IPVH00010).

Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk or bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBST). The membranes were then incubated overnight at 4°C with the following primary antibodies: rabbit anti-CD320 (1:1000 dilution; Abcam, Cat# ab183733) and mouse anti-GAPDH (1:5000 dilution; Cell Signaling Technology, Cat# 2118), which served as a loading control. After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, HRP-linked, Cat# 7074; anti-mouse IgG, HRP-linked, Cat# 7076; Cell Signaling Technology) for 1 hour at room temperature. Protein bands were visualized using the Clarity Western ECL Substrate (Bio-Rad, Cat# 1705061) and imaged on a ChemiDoc MP Imaging System (Bio-Rad). Band intensities were quantified using ImageJ software (NIH). The relative protein level of CD320 was normalized to the corresponding GAPDH signal for each lane. Data are presented as the average of normalized CD320/GAPDH ratios from three independent biological replicates.

2.4. Proliferation Assays (MTT)

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 µL of culture medium. Cells were divided into six experimental groups: (1) Control (KB), (2) siNC-transfected, (3) si-CD320-transfected, (4) Control + VB12 supplementation, (5) siNC + VB12 supplementation, and (6) si-CD320 + VB12 supplementation. For VB12-supplemented groups, cells were cultured in medium containing 1 nM cyanocobalamin. Proliferation was measured at 24-, 48-, and 72-hours post-treatment. At each time point, 10 µL of MTT solution (5 mg/mL in PBS; Sigma-Aldrich, Cat# M5655) was added to each well, and plates were incubated for an additional 3 hours at 37°C. The supernatant was then carefully removed, and the resulting formazan crystals were

solubilized in 100 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a microplate reader (BioTek Synergy H1). Each condition was tested in triplicate wells. Where applicable, results are expressed as percent viability relative to the control (KB) group at the baseline time point (24 hours). Raw absorbance values are also provided in the dataset.

2.5. NK Cell Isolation and Co-culture Experiments

Primary human Natural Killer (NK) cells will be isolated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donor buffy coats, under a protocol approved by the Institutional Review Board. PBMCs will be isolated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare). NK cells ($CD3^{-}CD56^{+}$) will be purified using a negative selection magnetic-activated cell sorting (MACS) kit (Miltenyi Biotec, Cat# 130-092-657). Purity of the isolated NK cell population will be confirmed by flow cytometry, with a target of ≥95% $CD56^{+}$ cells.

For co-culture experiments, HCC cells will be seeded in the bottom chamber of a 24-well plate, while freshly isolated NK cells will be placed in the upper chamber of a 0.4 µm pore size Transwell insert (Corning), at an effector-to-target (E: T) ratio of 10:1. This setup allows for metabolic crosstalk via soluble factors while preventing direct cell-cell contact. Co-cultures will be maintained in VB12-deficient medium with or without titrated concentrations of cyanocobalamin. To competitively interfere with B12 handling for hypothesis testing, parallel experiments may be conducted using a cobalamin analog, such as cobinamide (dicyanocobinamide; Sigma-Aldrich).

The following endpoints will be assessed: 1) **NK Cell Intracellular B12:** Intracellular Vitamin B12 levels in NK cells post-co-culture will be quantified using a competitive enzyme-linked immunosorbent assay (ELISA) kit (e.g., Cusabio, Cat# CSB-E09425h). 2) **NK Cell Cytotoxicity:** Cytotoxicity will be measured by removing the Transwell inserts and assessing the viability of the target HCC cells using a lactate dehydrogenase (LDH) release assay (Promega, CytoTox 96). 3) **NK Cell Degranulation:** Degranulation, a surrogate for cytotoxic potential, will be measured by flow cytometric analysis of surface CD107a expression on NK cells after co-culture. 4) **NK Cell Cytokine Production:** Supernatants from the co-culture will be collected to quantify Interferon-gamma (IFN-γ) secretion by NK cells using a standard ELISA kit (R&D Systems, Cat# DY285B).

2.6. Data Handling and Preprocessing

All raw data from qPCR, Western blot densitometry, and MTT assays were organized in Microsoft Excel spreadsheets. For datasets with missing group labels, the labels were forward-filled to ensure accurate data parsing. Ratios for gene expression ($2^{-\Delta\Delta Ct}$) and protein levels (CD320/GAPDH) were computed as described in their respective sections. No data points were excluded as outliers during the initial analysis. All numerical results are reported with an appropriate number of significant figures to reflect the precision of the measurement. Exact p-values are reported where available; otherwise, standard notations (e.g., $p < 0.05$) are used.

2.7. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD) from at least three independent experiments. For comparisons between two groups, a two-tailed Student's t-test was used. For comparisons involving three or more groups, such as the analysis of gene/protein expression across different cell lines or treatment conditions, a one-way analysis of variance (ANOVA) was performed. If the ANOVA test indicated a significant overall difference ($p < 0.05$), post-hoc analysis using Tukey's or Dunnett's multiple comparisons test was conducted to identify specific differences between group means. Assumptions of normality and homogeneity of variances were assessed where appropriate, and non-parametric tests (e.g., Kruskal-Walli’s test) would be applied in future iterations if these assumptions are not met. A p-value of less than 0.05 was considered statistically significant.

2.8. Ethics and Safety

The work reported in the current draft is based exclusively on in-vitro experiments using established, commercially available human cell lines. As such, it did not require specific ethical approval from an institutional review board. All laboratory procedures were conducted in accordance with institutional biosafety guidelines. The proposed future work involving primary human immune cells will be contingent upon obtaining formal approval from the Institutional Review Board (IRB) and will require written informed consent from all volunteer blood donors before sample acquisition. No live animals or identifiable human patient data are included in this study.

3. Results

3.1. CD320 expression is upregulated in liver cancer and correlates with poor clinical outcomes

To investigate the potential role of Vitamin B12 (VB12) metabolism in the pathogenesis of liver cancer, we first examined the expression of CD320, the cellular receptor responsible for VB12 uptake. Utilizing bulk RNA sequencing data, we compared CD320 mRNA levels between malignant liver tissues and adjacent normal tissues. Our analysis revealed a significant upregulation of CD320 in liver cancer tissues compared to their normal counterparts (Figure 1A).

To determine if CD320 expression was associated with disease progression, we stratified patients based on clinical stage. This analysis demonstrated a positive correlation between CD320 mRNA expression and advancing tumor stage, with the highest levels observed in Stage IV disease (Figure 1B). Furthermore, to assess the prognostic significance of CD320, we performed a survival analysis. Kaplan-Meier analysis revealed that patients with high intratumoral CD320 expression experienced significantly poorer overall survival compared to those with low expression (Figure 1C). Collectively, these findings suggest that elevated CD320 expression is a key feature of liver cancer and serves as a prognostic biomarker for adverse clinical outcomes.

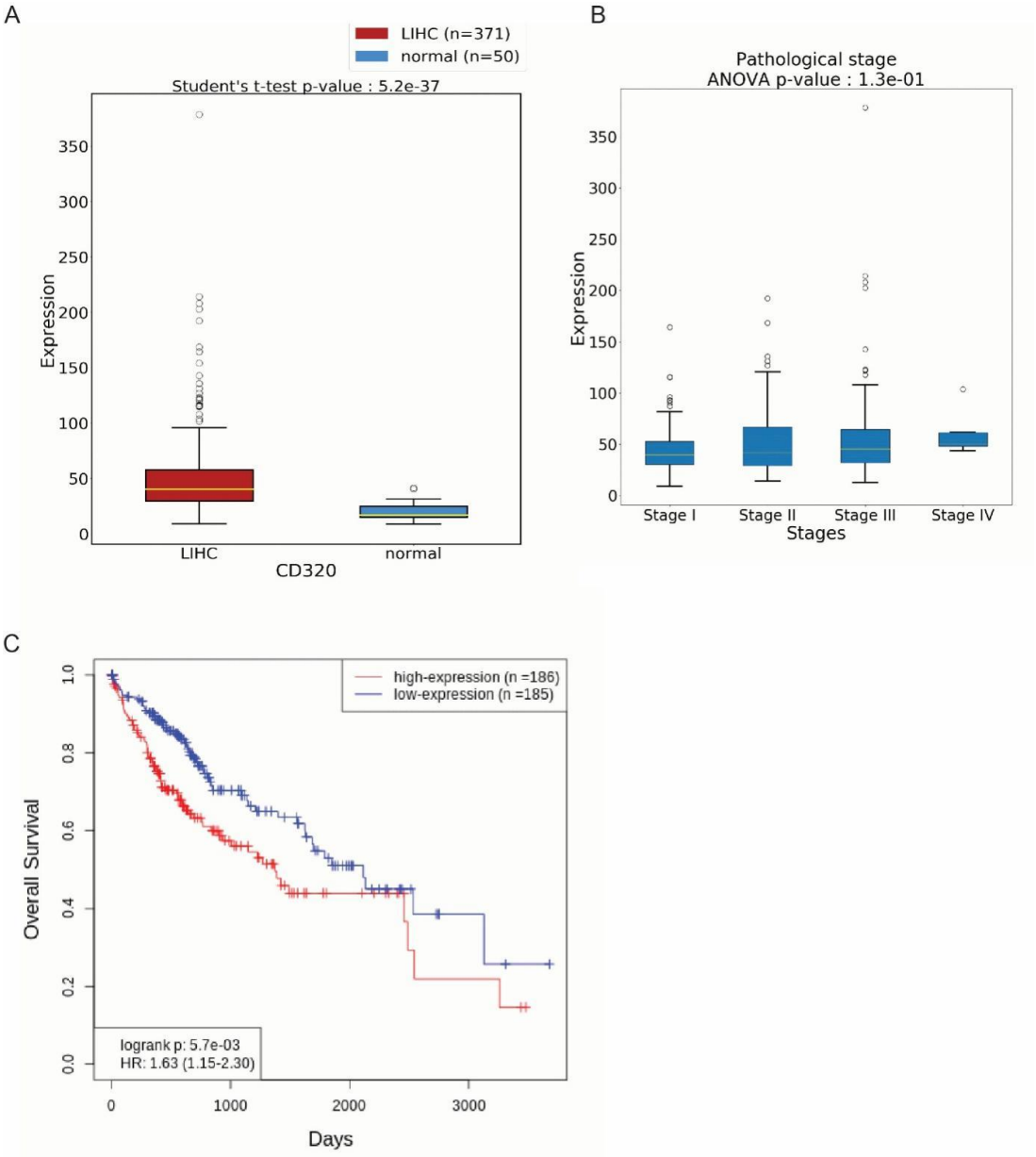

Figure 1. CD320 is upregulated in liver cancer and correlates with disease progression and poor prognosis. (A) Boxplot comparing the relative mRNA expression of *CD320* in normal liver tissues and primary liver cancer tissues. Data were obtained from The Cancer Genome Atlas (TCGA). **(B)** Boxplot illustrating *CD320* mRNA expression across different clinical stages (Stage I-IV) of liver cancer. **(C)** Kaplan-Meier survival curve depicting the overall survival of liver cancer patients stratified by high versus low intratumoral *CD320* expression. The p-value was calculated using a log-rank test.

3.2. Single-cell analysis reveals CD320 is predominantly expressed by malignant cells

Having established the clinical relevance of CD320 using bulk tissue expression data, we next sought to identify the specific cell populations responsible for its upregulation within the tumor microenvironment. We analyzed a publicly available single-cell RNA sequencing (scRNA-seq) dataset derived from liver cancer specimens. The UMAP (Uniform Manifold Approximation and Projection) plots illustrating cell clusters and corresponding gene expression confirmed that CD320 was highly and specifically expressed within the malignant cell population (Figures 2A and 2B).

To quantify this observation, we compared the normalized expression levels of CD320 across major cell lineages within the tumor, including malignant cells, immune cells (such as T cells, B cells, and myeloid cells), and stromal cells (such as fibroblasts and endothelial cells). This comparative

analysis confirmed that CD320 expression was substantially and significantly higher in malignant cells than in any other cell type profiled (Figure 2C). These single-cell resolution data strongly indicate that the elevated CD320 levels observed in bulk tumor tissues are primarily driven by cancer cells themselves, rather than by components of the tumor microenvironment.

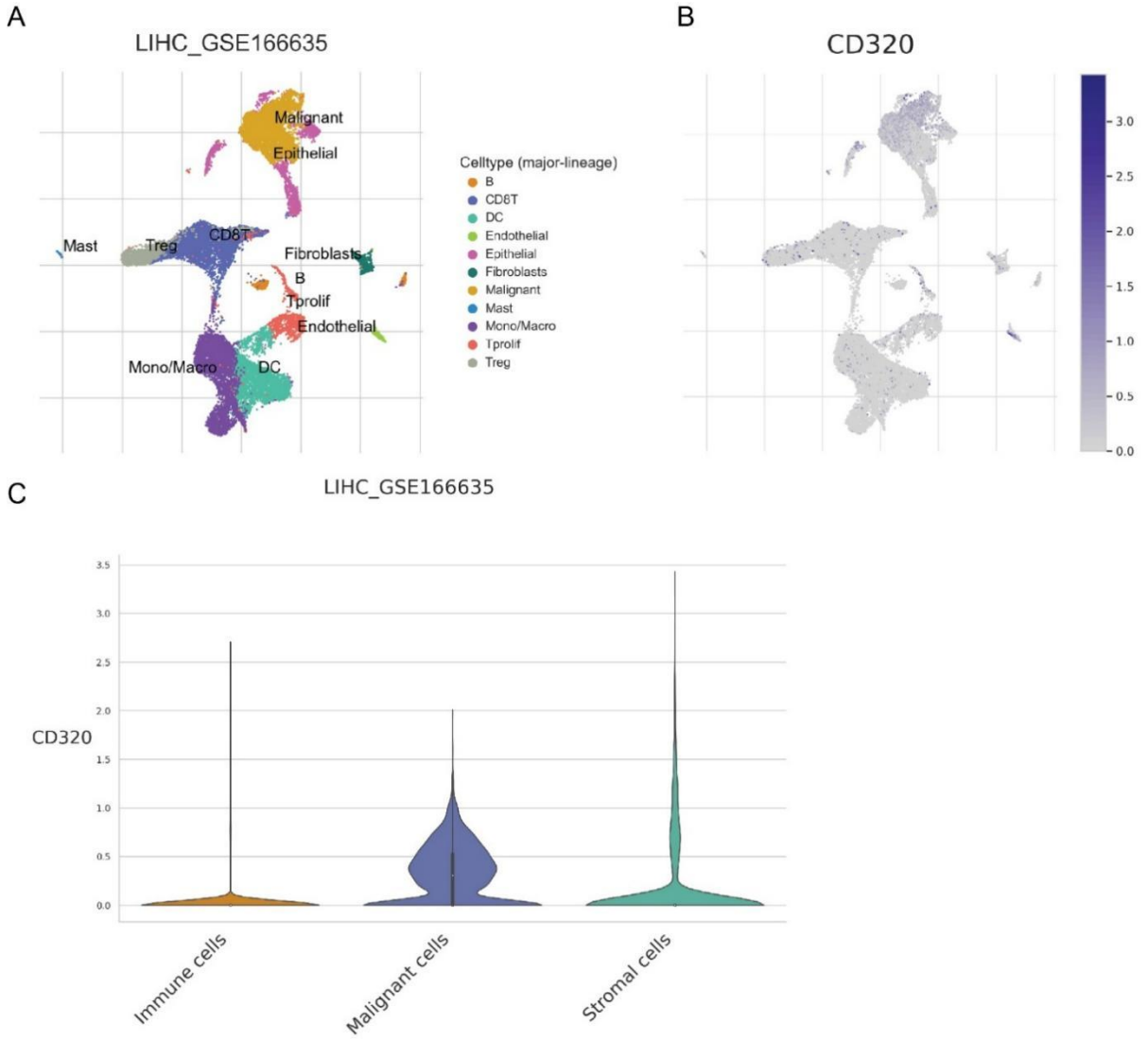

Figure 2. Single-cell RNA sequencing analysis reveals that CD320 is predominantly expressed in malignant cells within the liver tumor microenvironment. (A) UMAP visualization of cell populations from a representative liver cancer specimen, with major cell types annotated (e.g., Malignant, T cells, B cells, Myeloid, Endothelial, Fibroblasts). **(B)** Corresponding UMAP feature plot showing the normalized expression level of *CD320* across all profiled cells. The color gradient indicates the level of gene expression, from low (blue/gray) to high (red). **(C)** Violin plot quantifying and comparing the relative expression of *CD320* across.

3.3. CD320 Knockdown Attenuates Vitamin B12-Mediated Proliferation in Liver Cancer Cells

Given the strong association between CD320 expression and poor prognosis, we next investigated its functional role in liver cancer cell proliferation. We employed a short hairpin RNA (shRNA) approach to silence CD320 expression in the HEPG2 liver cancer cell line. The efficacy of the knockdown was confirmed at both the transcript and protein levels. Quantitative real-time PCR (RT-qPCR) analysis showed a significant reduction in CD320 mRNA levels in cells treated with shCD320 compared to control cells (Figure 3A). This was further corroborated by Western blot analysis, which

demonstrated a marked decrease in CD320 protein expression following shRNA treatment (Figures 3B and 3C).

Next, we assessed the impact of CD320 depletion on cell proliferation, particularly in the context of VB12 availability. As expected, the addition of VB12 to the culture medium significantly promoted the proliferation of control HEPG2 cells. However, this pro-proliferative effect was completely abrogated in cells where CD320 was knocked down (Figure 3D). These results indicate that CD320 is functionally essential for mediating the growth-promoting effects of Vitamin B12 in liver cancer cells.

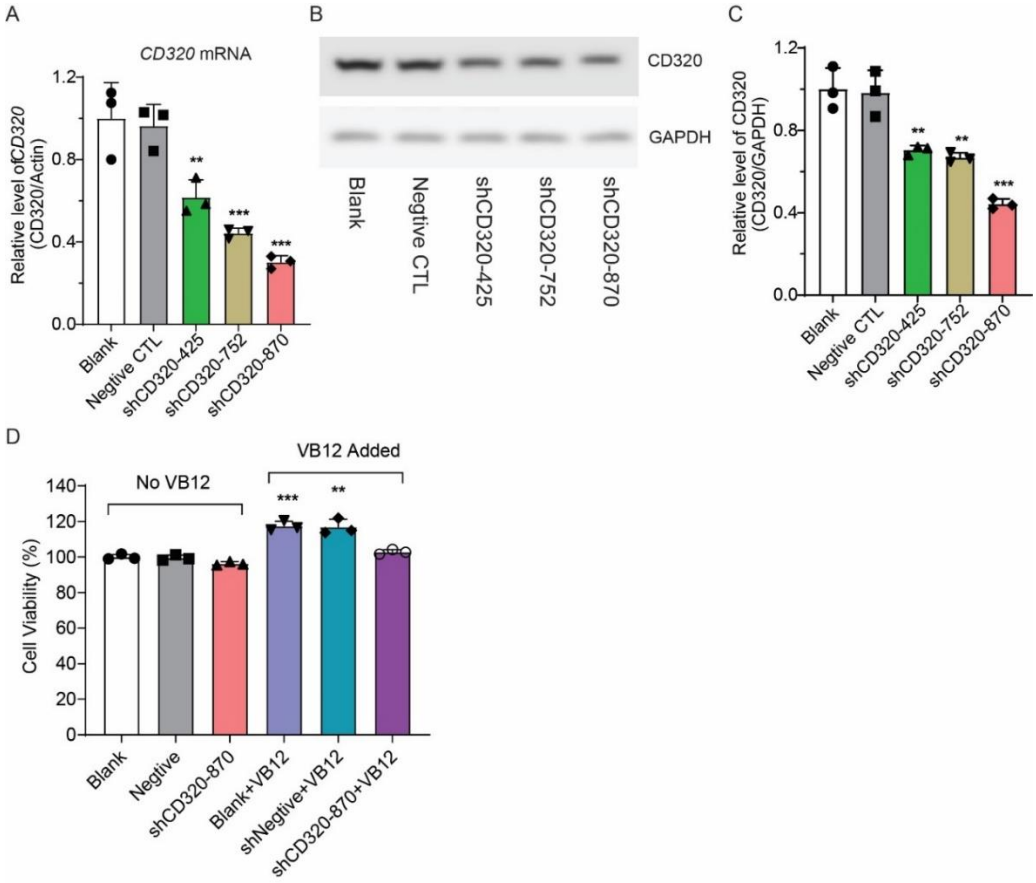

Figure 3. Knockdown of CD320 inhibits Vitamin B12-induced proliferation in HEPG2 liver cancer cells. (A) Relative *CD320* mRNA expression in HEPG2 cells following transfection with a non-targeting control shRNA (shCtrl) or a CD320-targeting shRNA (shCD320), as measured by RT-qPCR. Expression levels were normalized to the housekeeping gene *GAPDH*. (B) Representative Western blot images showing CD320 and GAPDH protein levels in control and shCD320-treated HEPG2 cells. (C) Densitometric quantification of CD320 protein bands from the Western blot analysis, normalized to GAPDH loading control. (D) Cell viability of control and CD320-knockdown HEPG2 cells cultured with or without supplemental Vitamin B12, as determined by an MTT assay. Data are presented as mean ± standard deviation from three independent experiments. Statistical significance is denoted (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4. Discussion

Hepatocellular carcinoma (HCC) remains a disease with significant morbidity and mortality, largely due to its complex biology and the challenging, immunosuppressive nature of its tumor microenvironment (TME) (Llovet et al., 2021; Sung et al., 2021). While the current standard of care involving immune checkpoint inhibitors (ICIs) like atezolizumab combined with anti-angiogenic agents like bevacizumab has improved outcomes, a substantial portion of patients still fails to respond or develops secondary resistance (Pinter et al., 2021; Sangro et al., 2021) [3, 16]. This underscores a

critical need to uncover the fundamental mechanisms of immune evasion that limit therapeutic efficacy. A growing body of evidence implicates metabolic dysregulation and nutrient competition within the TME as a central axis of immune resistance (Chang et al., 2015; Reinfeld et al., 2021). Our study delves into this burgeoning field by investigating the role of Vitamin B12 (VB12), a crucial micronutrient for cellular proliferation and function, and its primary cellular receptor, CD320, in the pathobiology of HCC. The findings presented herein illuminate a novel mechanism whereby HCC cells may gain a metabolic and proliferative advantage by hijacking the VB12 uptake machinery, which concurrently has the potential to impair anti-tumor immunity by creating a state of localized nutrient deprivation.

A central finding of our investigation is the significant upregulation of CD320, the transcobalamin receptor, in HCC tissues compared to adjacent non-malignant liver tissue. This observation, derived from comprehensive transcriptomic analyses, is not merely a correlative finding but appears to be a clinically relevant molecular alteration. We demonstrated a direct and positive correlation between the magnitude of CD320 expression and the clinical stage of the disease, suggesting that the demand for VB12, and thus the machinery to acquire it, intensifies as the tumor progresses and becomes more aggressive. This aligns with the fundamental understanding of cancer metabolism, where rapidly proliferating cells exhibit an insatiable appetite for the metabolic building blocks required for biomass accumulation, including nucleotides, lipids, and proteins (Vander Heiden et al., 2009). VB12 is indispensable for these processes, acting as a critical cofactor for methionine synthase (MTR) in one-carbon metabolism—a pathway essential for synthesizing purines and pyrimidines and for generating S-adenosylmethionine (SAM), the universal methyl donor for epigenetic regulation (Froese et al., 2019). It is also required for methylmalonyl-CoA mutase (MUT), linking fatty acid and amino acid catabolism to the Krebs cycle for bioenergetic support (Mascarenhas et al., 2022). Therefore, the observed upregulation of CD320 can be interpreted as a strategic metabolic adaptation by HCC cells to fuel their relentless proliferative agenda.

Crucially, our Kaplan-Meier survival analysis revealed that high intratumoral CD320 expression is a powerful and independent predictor of poor overall survival. This positions CD320 not only as a participant in HCC biology but also as a potent prognostic biomarker. This finding is consistent with emerging reports in other malignancies, including breast cancer and multiple myeloma, where elevated CD320 has also been linked to adverse clinical outcomes (Elzi et al., 2021; Zhang et al., 2024). The prognostic value of CD320 likely reflects its role as a proxy for a highly proliferative and metabolically active tumor phenotype. Tumors that have successfully amplified their nutrient acquisition pathways are inherently more aggressive and, consequently, more lethal. The identification of CD320 as a biomarker could have tangible clinical applications, potentially aiding in patient stratification for risk assessment or for selecting patients who might benefit from therapies targeting metabolic vulnerabilities.

While bulk tissue analysis is informative, the TME is a heterogeneous ecosystem comprising malignant cells, immune infiltrates, stromal cells, and vasculature (Hinshaw & Shevde, 2019). A critical question arising from our initial findings was whether the observed CD320 upregulation was a global feature of the inflamed tumor milieu or a specific characteristic of the cancer cells themselves. By leveraging the granular resolution of single-cell RNA sequencing (scRNA-seq), we unequivocally demonstrated that CD320 expression is predominantly and selectively confined to the malignant cell population. In contrast, immune cells (including T cells, B cells, and myeloid cells) and stromal cells (fibroblasts and endothelial cells) within the same tumor specimens exhibited markedly lower levels of CD320 expression.

This finding is of paramount importance as it refines our hypothesis. It suggests that the elevated CD320 is not a bystander effect of general inflammation but rather an intrinsic, tumor cell-driven adaptation. This selective overexpression equips HCC cells with a significant competitive advantage in the struggle for a finite pool of circulating VB12. By expressing a higher density of receptors, cancer cells can more efficiently sequester holo-transcobalamin from the extracellular space, effectively outcompeting neighboring cells, including vital anti-tumor immune effectors, for this

essential micronutrient. This concept of "metabolic parasitism" or nutrient partitioning, where cancer cells remodel the TME to their own benefit by depleting resources essential for immune function, is a well-established paradigm for macronutrients like glucose and amino acids (Chang et al., 2015; Ringel et al., 2020). Our data strongly suggest that this principle extends to essential micronutrients like VB12, adding a new layer of com

Having established the clinical relevance and cellular origin of CD320 overexpression, we next sought to validate its functional role in HCC proliferation. Our in vitro experiments using siRNA-mediated knockdown of CD320 in the HepG2 cell line provided direct causal evidence linking the receptor to VB12-dependent growth. The successful and robust silencing of CD320 at both the mRNA and protein levels created a clean experimental system to interrogate this pathway. The results were striking: while supplemental VB12 significantly enhanced the proliferation of control cells, this pro-proliferative effect was completely abrogated in cells lacking CD320.

This outcome confirms two critical points. First, it demonstrates that HCC cells are not merely overexpressing CD320 as a passive marker of proliferation but are functionally reliant on it to capitalize on available VB12. The receptor is an essential gateway, and without it, extracellular VB12 cannot exert its growth-promoting effects. Second, it highlights the CD320-VB12 axis as a potential therapeutic vulnerability. Disrupting this pathway, either by targeting the receptor directly (e.g., with an antibody-drug conjugate) or by limiting VB12 availability, could represent a novel strategy to selectively inhibit tumor growth. This concept has already gained preclinical traction in multiple myeloma, where targeting CD320 with antibody-based therapies has shown promising anti-tumor activity (Elzi et al., 2021; Quadros et al., 2010). Our findings provide a strong rationale for exploring similar therapeutic approaches in the context of HCC.

Previous studies have established a clear link between systemic VB12 status and immune competence. VB12 deficiency is associated with lymphopenia, reduced CD8+ T cell counts, and, most notably, impaired NK cell cytotoxic activity (Sakane et al., 1982; Watson et al., 2023). This functional defect is reversible with VB12 supplementation, indicating a direct metabolic requirement. The metabolic programs underpinning NK cell effector functions—including the production of cytotoxic granules (perforin, granzymes) and the secretion of pro-inflammatory cytokines like IFN-γ—are energetically demanding and require robust one-carbon metabolism for nucleotide synthesis and methylation events. By monopolizing the local VB12 supply, HCC cells may effectively disarm NK cells, suppressing their ability to recognize and eliminate tumor targets.

While our study provides compelling evidence for the role of the CD320-VB12 axis in HCC, it is important to acknowledge its limitations and outline the path for future investigation. Our current findings are based on bioinformatic analyses of patient datasets and in vitro monoculture experiments. While foundational, these models do not fully recapitulate the intricate complexity of the human TME.

The immediate next step, as proposed, is to conduct co-culture experiments using Transwell systems to validate the hypothesis of metabolic competition. These studies will be instrumental in determining if CD320-high HCC cells can indeed deplete VB12 from the shared media and, as a result, functionally impair key NK cell activities such as cytotoxicity (LDH release), degranulation (CD107a expression), and cytokine production (IFN-γ secretion). Quantifying intracellular VB12 levels in NK cells under these conditions will provide direct evidence of nutrient sequestration.

From a therapeutic standpoint, the validation of CD320 as a target warrants further exploration. Preclinical studies could evaluate the efficacy of CD320-targeted therapies, such as antibody-drug conjugates (ADCs), which would deliver a cytotoxic payload directly to the cancer cells, or blocking antibodies that prevent VB12 uptake. A particularly exciting therapeutic concept would be to combine CD320 blockade with immunotherapy. By disrupting the tumor's ability to sequester VB12, one might "re-nourish" the immune cells within the TME, restoring their metabolic fitness and potentially synergizing with ICIs to overcome resistance and achieve more durable anti-tumor responses.

5. Conclusion

In summary, this study identifies the Vitamin B12 receptor, CD320, as a pivotal player in the pathobiology of hepatocellular carcinoma. We have demonstrated that its expression is significantly upregulated in HCC, correlates with advanced disease and poor survival, and is driven primarily by the malignant cells themselves. Functionally, we have shown that CD320 is essential for mediating the pro-proliferative effects of VB12 on HCC cells. Based on these findings, we propose a novel model of immune evasion wherein the overexpression of CD320 by HCC cells facilitates a metabolic competition that simultaneously fuels tumor growth and starves anti-tumor immune cells, particularly NK cells, of a critical micronutrient. This work not only deepens our understanding of the metabolic underpinnings of HCC but also uncovers the CD320-VB12 axis as a promising prognostic biomarker and a tractable therapeutic target. Interventions aimed at disrupting this axis hold the potential to create a powerful two-pronged attack: directly inhibiting tumor proliferation while simultaneously revitalizing the immune microenvironment to restore effective anti-tumor surveillance. Further investigation into this pathway promises to open new avenues for the treatment of this devastating disease.

References

[1] Chang, C. H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., Chen, Q., Gindin, M., Gubin, M. M., van der Windt, G. J., Tonc, E., Schreiber, R. D., Pearce, E. J., & Pearce, E. L. (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell*, *162*(6), 1229-1241. https://doi.org/10.1016/j.cell.2015.08.016

[2] Cheng, A. L., Qin, S., Ikeda, M., Galle, P. R., Ducreux, M., Kim, T. Y., Lim, H. Y., Kudo, M., Breder, V., Merle, P., Kaseb, A. O., Li, D., Verret, W., Ma, N., Nicholas, A., Wang, Y., Li, L., Zhu, A. X., & Finn, R. S. (2022). Updated efficacy and safety data from IMbrave150: Atezolizumab plus bevacizumab vs. sorafenib for unresectable hepatocellular carcinoma. *J Hepatol*, *76*(4), 862-873. https://doi.org/10.1016/j.jhep.2021.11.030

[3] Elzi, D. J., Bauta, W. E., Sanchez, J. R., Das, T., Mogare, S., Zannes Fatland, P., Iza, M., Pertsemlidis, A., & Rebel, V. I. (2021). Identification of a novel mechanism for meso-tetra (4-carboxyphenyl) porphyrin (TCPP) uptake in cancer cells. *Faseb j*, *35*(3), e21427. https://doi.org/10.1096/fj.202000197R

[4] Finn, R. S., Qin, S., Ikeda, M., Galle, P. R., Ducreux, M., Kim, T. Y., Kudo, M., Breder, V., Merle, P., Kaseb, A. O., Li, D., Verret, W., Xu, D. Z., Hernandez, S., Liu, J., Huang, C., Mulla, S., Wang, Y., Lim, H. Y., Zhu, A. X., & Cheng, A. L. (2020). Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med*, *382*(20), 1894-1905. https://doi.org/10.1056/NEJMoa1915745

[5] Froese, D. S., Fowler, B., & Baumgartner, M. R. (2019). Vitamin B(12) , folate, and the methionine remethylation cycle-biochemistry, pathways, and regulation. *J Inherit Metab Dis*, *42*(4), 673-685. https://doi.org/10.1002/jimd.12009

[6] Ge, Y., Zadeh, M., & Mohamadzadeh, M. (2022). Vitamin B12 Regulates the Transcriptional, Metabolic, and Epigenetic Programing in Human Ileal Epithelial Cells. *Nutrients*, *14*(14). https://doi.org/10.3390/nu14142825

[7] Hinshaw, D. C., & Shevde, L. A. (2019). The Tumor Microenvironment Innately Modulates Cancer Progression. *Cancer Res*, *79*(18), 4557-4566. https://doi.org/10.1158/0008-5472.Can-18-3962

[8] Ho, P. C., Bihuniak, J. D., Macintyre, A. N., Staron, M., Liu, X., Amezquita, R., Tsui, Y. C., Cui, G., Micevic, G., Perales, J. C., Kleinstein, S. H., Abel, E. D., Insogna, K. L., Feske, S., Locasale, J. W., Bosenberg, M. W., Rathmell, J. C., & Kaech, S. M. (2015). Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell*, *162*(6), 1217-1228. https://doi.org/10.1016/j.cell.2015.08.012

[9] Itoh, K., Kashimura, T., Kobayashi, Y., Yagasaki, F., Sakata, T., Kawai, N., Matsuda, A., Kusumoto, S., Fukuda, M., Ino, H., Murohashi, I., Jinnai, I., Yoshida, S., Bessho, M., Saitoh, M., & Hirashima, K. (1999). [Atypical chronic myeloid leukemia presenting with trilineage dysplasia and IgG (lambda) type monoclonal gammopathy]. *Rinsho Ketsueki*, *40*(2), 129-134.

[10] Lai, S. C., Nakayama, Y., Sequeira, J. M., & Quadros, E. V. (2011). Down-regulation of transcobalamin receptor TCblR/CD320 by siRNA inhibits cobalamin uptake and proliferation of cells in culture. *Exp Cell Res*, *317*(11), 1603-1607. https://doi.org/10.1016/j.yexcr.2011.02.016

[11] Llovet, J. M., Kelley, R. K., Villanueva, A., Singal, A. G., Pikarsky, E., Roayaie, S., Lencioni, R., Koike, K., Zucman-Rossi, J., & Finn, R. S. (2021). Hepatocellular carcinoma. *Nat Rev Dis Primers*, *7*(1), 6. https://doi.org/10.1038/s41572-020-00240-3

[12] Mascarenhas, R., Gouda, H., Ruetz, M., & Banerjee, R. (2022). Human B(12)-dependent enzymes: Methionine synthase and Methylmalonyl-CoA mutase. *Methods Enzymol*, *668*, 309-326. https://doi.org/10.1016/bs.mie.2021.12.012

[13] Munn, D. H., & Mellor, A. L. (2016). IDO in the Tumor Microenvironment: Inflammation, Counter-Regulation, and Tolerance. *Trends Immunol*, *37*(3), 193-207. https://doi.org/10.1016/j.it.2016.01.002

[14] Pinter, M., Scheiner, B., & Peck-Radosavljevic, M. (2021). Immunotherapy for advanced hepatocellular carcinoma: a focus on special subgroups. *Gut*, *70*(1), 204-214. https://doi.org/10.1136/gutjnl-2020-321702

[15] Quadros, E. V. (2010). Advances in the understanding of cobalamin assimilation and metabolism. *Br J Haematol*, *148*(2), 195-204. https://doi.org/10.1111/j.1365-2141.2009.07937.x

[16] Quadros, E. V., Nakayama, Y., & Sequeira, J. M. (2010). Targeted delivery of saporin toxin by monoclonal antibody to the transcobalamin receptor, TCblR/CD320. *Mol Cancer Ther*, *9*(11), 3033-3040. https://doi.org/10.1158/1535-7163.Mct-10-0513

[17] Reinfeld, B. I., Madden, M. Z., Wolf, M. M., Chytil, A., Bader, J. E., Patterson, A. R., Sugiura, A., Cohen, A. S., Ali, A., Do, B. T., Muir, A., Lewis, C. A., Hongo, R. A., Young, K. L., Brown, R. E., Todd, V. M., Huffstater, T., Abraham, A., O'Neil, R. T., Wilson, M. H., Xin, F., Tantawy, M. N., Merryman, W. D., Johnson, R. W., Williams, C. S., Mason, E. F., Mason, F. M., Beckermann, K. E., Vander Heiden, M. G., Manning, H. C., Rathmell, J. C., & Rathmell, W. K. (2021). Cell-programmed nutrient partitioning in the tumour microenvironment. *Nature*, *593*(7858), 282-288. https://doi.org/10.1038/s41586-021-03442-1

[18] Ringel, A. E., Drijvers, J. M., Baker, G. J., Catozzi, A., García-Cañaveras, J. C., Gassaway, B. M., Miller, B. C., Juneja, V. R., Nguyen, T. H., Joshi, S., Yao, C. H., Yoon, H., Sage, P. T., LaFleur, M. W., Trombley, J. D., Jacobson, C. A., Maliga, Z., Gygi, S. P., Sorger, P. K., Rabinowitz, J. D., Sharpe, A. H., & Haigis, M. C. (2020). Obesity Shapes Metabolism in the Tumor Microenvironment to Suppress Anti-Tumor Immunity. *Cell*, *183*(7), 1848-1866.e1826. https://doi.org/10.1016/j.cell.2020.11.009

[19] Sakane, T., Takada, S., Kotani, H., & Tsunematsu, T. (1982). Effects of methyl-B12 on the in vitro immune functions of human T lymphocytes. *J Clin Immunol*, *2*(2), 101-109. https://doi.org/10.1007/bf00916893

[20] Sangro, B., Sarobe, P., Hervás-Stubbs, S., & Melero, I. (2021). Advances in immunotherapy for hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol*, *18*(8), 525-543. https://doi.org/10.1038/s41575-021-00438-0

[21] Sasaki, Y., Kawagoe, N., Imai, T., & Urita, Y. (2023). Effects of ethanol and sex on propionate metabolism evaluated via a faster (13)C-propionate breath test in rats. *World J Gastroenterol*, *29*(21), 3269-3279. https://doi.org/10.3748/wjg.v29.i21.3269

[22] Seetharam, B. (1999). Receptor-mediated endocytosis of cobalamin (vitamin B12). *Annu Rev Nutr*, *19*, 173-195. https://doi.org/10.1146/annurev.nutr.19.1.173

[23] Seetharam, B., & Li, N. (2000). Transcobalamin II and its cell surface receptor. *Vitam Horm*, *59*, 337-366. https://doi.org/10.1016/s0083-6729(00)59012-8

[24] Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*, *71*(3), 209-249. https://doi.org/10.3322/caac.21660

[25] Tejero, J. D., Hesterberg, R. S., Drapela, S., Ilter, D., Raizada, D., Lazure, F., Kashfi, H., Liu, M., Silvane, L., Avram, D., Fernández-García, J., Asara, J. M., Fendt, S. M., Cleveland, J. L., & Gomes, A. P. (2025). Methylmalonic acid induces metabolic abnormalities and exhaustion in CD8(+) T cells to suppress anti-tumor immunity. *Oncogene*, *44*(2), 105-114. https://doi.org/10.1038/s41388-024-03191-1

[26] Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*, *324*(5930), 1029-1033. https://doi.org/10.1126/science.1160809

[27] Watson, W. P., Munter, T., & Golding, B. T. (2023). The effect of vitamin B (12) on DNA adduction by styrene oxide, a genotoxic xenobiotic. *Chem Biol Interact*, *382*, 110591. https://doi.org/10.1016/j.cbi.2023.110591

[28] Zhang, S., Jiang, Z., Wang, P., Jiang, W., Ding, W., & Zhong, L. (2024). TCBIR/CD320: a potential therapeutic target upregulated in endothelial cells and associated with immune cell infiltration in liver hepatocellular carcinoma. *Discov Oncol*, *15*(1), 255. https://doi.org/10.1007/s12672-024-01122-w