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Original Article

High plasma histidine concentration predicts a favorable prognosis in patients with ampullary cancer

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Abstract: Circulating amino acid levels differ between patients with cancer and healthy individuals, and plasma histidine levels are lower in patients with periampullary cancer. This study examines histidine-related metabolic signaling in ampullary cancer. In total, 106 cancer specimens and 49 plasma samples from ampullary cancer patients were analyzed using immunohistochemistry, high-performance liquid chromatography for histidine levels, and enzyme-linked immunosorbent assays for histamine levels. Additionally, three ampullary cancer cell lines (TGBC-18 TKB, SNU-478, and SNU-869) were treated with histidine or histamine to assess growth. Plasma histidine levels were lower in patients with ampullary cancer than in healthy controls, whereas plasma histamine levels were similar between these groups. Elevated plasma histidine level was correlated with improved recurrence-free and overall survival in patients with ampullary cancer, as demonstrated by Kaplan-Meier survival analysis and multivariate Cox proportional hazards modeling. Expression of histidine-catabolic enzymes, namely histidine decarboxylase and histidine ammonia-lyase, was synergistic and positively correlated with early-stage cancer. Histidine treatment suppressed cancer cell proliferation, whereas histamine promoted cell proliferation of TGBC-18 TKB ampullary cancer cells. These findings suggest that plasma histidine is a prognostic survival factor, and combined treatment with histidine may offer therapeutic potential in patients with ampullary cancer.

Keywords: Ampulla of Vater carcinoma, biomarkers, histamine, histidine

Introduction

Cancer cell metabolism differs from that of normal cells, with amino acid consumption playing a critical role [1]. Dysregulated amino acid metabolism is frequently observed in cancer cells [2]. Circulating amino acid levels vary across patients with cancer due to tumor demand, especially for essential amino acids [3]. Histidine, an essential amino acid, has conflicting roles in cancer. A histidine-free diet has been shown to promote tumor growth without affecting body weight in a rat model of subcutaneous Walker tumor transplantation [4]. Elevated circulating histidine levels were associated with a reduced risk of colorectal cancer (odds ratio: 0.80 per standard deviation) in the European Prospective Investigation into Cancer and Nutrition, and UK Biobank cohorts [5]. Conversely, higher plasma histidine concentrations were correlated with an increased risk of breast cancer onset in premenopausal women (odds ratio: 1.7 per standard deviation) [6]. These conflicting findings underscore the complexity of histidine metabolism in cancer and suggest that histidine-related mechanisms may vary across cancer types. To date, no studies have reported plasma histidine levels or dietary histidine supplementation in patients with ampullary cancer.

Histidine is converted into urocanate by histidine ammonia-lyase (HAL) and into histamine by histidine decarboxylase (HDC) [7]. It is essential for nucleotide synthesis and influences the cellular pool of tetrahydrofolate. Histidine supplementation enhances cancer cell sensitivity to methotrexate by upregulating degradation pathways [8]. Its safety has been studied in a

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mouse model of pediatric leukemia, where it improved cancer therapy outcomes [9]. Histidine cotreatment with gemcitabine induces cytotoxicity of pancreatic cancer cells (SW1990 and Colo357) and extends mouse survival [10]. It also improves tyrosine kinase inhibitor sensitivity in hepatocellular carcinoma HepG2 cells [11]. Thus, further investigation of histidine supplementation's clinical applications is warranted.

Although ampullary cancer is the most common malignancy of the small intestine, it remains a rare disease, with an incidence of 0.5 to 0.9 cases per 100,000 individuals [12] and a five-year cancer-specific survival rate of 47.3% [13]. Its progression involves Wnt signaling, cancer stemness, the tumor immune microenvironment, and bile acid and lipid metabolism [13-20]. Preoperative plasma levels of histidine, lysine, threonine, isoleucine, leucine, and valine are lower in patients with periampullary cancer than in those with benign diseases [21]. Plasma metabolite profiles may help distinguish cancer from normal conditions, but further research is required to clarify histidine's role in ampullary cancer.

This study aimed to evaluate histidine-related metabolism signaling in ampullary cancer. Specifically, circulating histidine and histamine levels were assessed in patients with cancer, and cell culture models were employed to examine clinical applications.

Materials and methods

Patients and blood samples

In total, 134 patients with ampullary cancer were enrolled from April 1989 to January 2010 at National Cheng Kung University Hospital (NCKUH). All underwent radical surgery, standard adjuvant therapy, and received regular follow-ups until April 2024. Body weight, laboratory data, pathological reports, recurrence status, disease-specific survival, and overall survival were available in the retrospective chart review. During the study period, adjuvant therapy was not routinely administered. Adjuvant chemotherapy was provided to three patients, and adjuvant radiotherapy was performed in another three, based on the physician's choice.

Some patients underwent surgery in NCKUH, but refused to donate the residual specimens were excluded from the study. Plasma samples were collected before the operation from the patients who were willing to undergo additional blood draws. Formalin-fixed paraffin-embedded (FFPE) tumor sections, or stored plasma, were obtained from the Human Biobank, Research Center of Clinical Medicine, NCKUH, with appropriate written informed consent. The present study was approved by the Institutional Review Board of the NCKUH (IRB no. ER-100-343). Samples with insufficient volume for experimental procedures were excluded from the analysis. Finally, we collected 106 FFPE samples and 49 plasma samples. Another 15 healthy anonymous volunteers were enrolled during the same time period. All plasma samples were stored in -80°C freezer. The plasma level of histamine and histidine was measured retrospectively from the stored samples.

Immunohistochemistry staining

Expression of the histidine-catabolic enzymes was studied, including HDC and HAL. FFPE sections (5 μm thick) were mounted on slides, deparaffinized, and rehydrated for immunohistochemistry analysis. Epitope retrieval was performed via heat autoclave, and nonspecific peroxidase activity was blocked. Sections were incubated overnight at 4°C with mouse monoclonal anti-HDC and anti-HAL antibodies (Santa Cruz sc-68940 and sc-133646, Dallas, TX). The primary antibody was diluted at a ratio of 1:100 (HDC) or 1:200 (HAL) using a commercial diluent (DAKO, Cat. No. S3022). A horseradish peroxidase-labeled polymer-conjugated secondary antibody (Dako REAL EnVision Detection System, Cat. No. K5007, DAKO, Santa Clara, CA) was applied for 1 hour at room temperature, followed by 3-amino-9-ethylcarbazole (ScyTec, Logan, UT) for target protein detection. Hematoxylin was used for counterstaining. IHC sections were examined using an inverted microscope. All slides were evaluated by a single experienced researcher (H.P.H.) to minimize inter-observer variability and ensure consistency in the assessment process. The immunoreactivity of HDC and HAL proteins was assessed using a semi-quantitative approach based on the Remmele and Stegner immunoreactive scoring (IRS) system [22]. IRS scores ranged from 0 to 12 and were categorized as low (IRS less than 3) or high (IRS $\geq$ 3).

Ampullary cancer cell lines

The TGBC-18 TKB cell line, derived from a papillotubular adenocarcinoma of the Vater's papilla, was obtained from the RIKEN Bioresource Center, Japan (Tsukuba, Ibaraki, Japan) (https://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=RCB1169&lang=) [23]. SNU-478 and SNU-869 cell lines were gifted from Prof. Li-Tzong Chen (Center of Cancer Research, Kaohsiung Medical University), obtained from the Korean Cell Line Bank in Seoul National University College of Medicine. The tumor of SNU-478 was a poorly-differentiated adenocarcinoma, with deletion of p15/p16, mutation of TP53/MLH1, and loss of E-cadherin. The tumor of SNU-869 was a well-differentiated adenocarcinoma with focal papillary features, mutation of TP53, and preservation of E-cadherin [24, 25]. The TGBC-18 TKB cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, Thermo-Fisher, Carlsbad, CA) with 10% fetal bovine serum (FBS; Hyclone™, Cytiva Wilmington DE) and 1% antibiotics mixture (10,000 units of penicillin, 10,000 units of streptomycin, and 25 μg amphotericin B; Caisson Laboratories, North Logan, UT). SNU-478 and SNU-869 cells were maintained in RPMI-1640 medium (GIBCO) with 10% FBS and 1% antibiotics mixture. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Cell proliferation assay

Cancer cells (5 × 10^3 cells/well) were seeded into 96-well plates one day before treatment. Each well was filled with 200 μL of serum-free medium. Histidine (Sigma-Aldrich, Burlington, MA) was directly dissolved in serum-free DMEM medium to the target concentration (0, 1, 5, 10, and 25 mM). Histamine (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mM stock solution. Serial dilutions were performed and added to the respective wells to achieve the desired final concentrations (0, 0.01, 0.1, 1, and 10 μM). After exposure to histidine (0-25 mM) or histamine (0-10 μM) for 24 or 48 hours, 180 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT, Sigma-Aldrich) solution was added per well and incubated for 3 h. Absorbance at 570 nm was then measured using a spectrophotometer. The dosage of histidine and histamine was based on protocols established in previous research [10, 26].

Enzyme-linked immunosorbent assay (ELISA)

Histamine ELISA was performed using a commercial kit (LDN Cat. No. BA E-5800; LDN, Nordhorn, Germany) according to the manufacturer's instructions. Standard histamine was diluted into appropriate concentrations (0, 0.25, 0.5, 1.5, 5, 15, and 50 ng/mL) to establish the standard curve. Pure plasma samples from ampullary cancer patients or anonymous healthy donors without any dilution were utilized. A total of 25 μL of standards or samples was added into the appropriate wells of commercialized microtiter strips and incubated with goat anti-histamine antibody (BA E-1010, LDN) at room temperature for 3 hours. After washing, the enzyme conjugate (Donkey anti-goat immunoglobulins conjugated with peroxidase, BA E-1040, LDN) was added and incubated at room temperature for 30 minutes, followed by a chromogenic substrate containing tetramethylbenzidine, substrate buffer, and hydrogen peroxide. The reaction was stopped after 25-30 min with the addition of 50 μL of stop solution, and absorbance was measured at 450 nm.

High-performance liquid chromatography (HPLC)

Plasma was deproteinized by mixing 1 part plasma with 3 parts of 10% trichloroacetic acid, then centrifuged at 16,200 g for 20 minutes at 4°C, and filtered through a 0.22 μm filter to collect supernatant. Phenylisothiocyanate was added and UV-detection using a reverse phase C18 HPLC column at 200 nm (Merck-Hitachi D-7000 system; Labexchange, Burladingen, Germany). The gradient elution was established from 100% phosphate buffer, and then a linear increase in the concentration of methanol. Standard L-histidine (Sigma-Aldrich) was used for quantification.

Statistical analysis

Statistical analyses were conducted using STATA version 16.0 (College Station, TX, USA). MTT assays were performed in 12 replicates to assess cell proliferation. Categorical variables were analyzed using the chi-square for comparisons involving three or more categories, and Fisher's exact test was applied for comparisons between two groups. Continuous variables were analyzed using the nonparametric statistical methods. The Kruskal-Wallis H

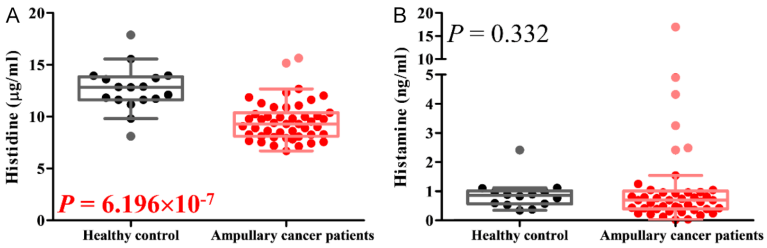

Figure 1. Plasma histidine and histamine levels. A. Histidine levels in HPLC analysis. B. Histamine level in ELISA. The Wilcoxon rank-sum test was employed to compare healthy controls and patients with ampullary cancer.

test was applied for comparison involving three or more groups, whereas the Wilcoxon rank-sum test was used for comparisons between two groups, also the post hoc test. Outliers in plasma histidine levels measured by HPLC and histamine concentrations assessed by ELISA were excluded from the analysis and treated as missing data. Associations between specific markers and recurrence-free survival (RFS) or overall survival (OS) were assessed using the Kaplan-Meier method, and significance was tested using the log-rank test. The Cox proportional hazards model was utilized for multivariate analysis of predictors of recurrence-free survival, with age and gender included as covariates. Histidine and histamine expression levels were categorized based on the median value, with levels below the median defined as low expression and levels equal to or above the median defined as high expression. The median value was calculated based on histidine/histamine levels measured in both ampullary cancer patients and healthy individuals. $P < 0.05$ was considered statistically significant.

Results

Plasma levels of histidine and histamine in patients with ampullary cancer

In total, 49 patients with ampullary cancer and 15 healthy people were enrolled to examine plasma histidine and histamine levels. The clinical and demographic characteristics of the 49 patients were summarized in Table S1. HPLC was performed by Prof. Shu-Chu Shiesh (Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University). Preoperative plasma was collected from patients with cancer. Median plasma histidine levels were 12.9 μg/mL (range: 11.2-17.9 μg/mL, mean ± standard deviation: 13.2 ± 1.8 μg/mL) in healthy controls and 9.3 μg/mL (range: 6.7-15.7 μg/mL, mean ± SD: 9.6 ± 1.9 μg/mL) in patients with ampullary cancer (**Figure 1A**, $P = 6.196 \times 10^{-7}$). Median plasma histamine levels were 0.9 μg/mL (range: 0.3-2.4 μg/mL, mean ± SD: 0.9 ± 0.5 μg/mL) in healthy controls and 0.7 μg/mL (range: 0-17.0 μg/mL, mean ± SD: 1.4 ± 2.8 μg/mL) in patients with ampullary cancer (**Figure 1B**, $P = 0.332$). Plasma histidine and histamine levels did not correlate with patient demographics or pathological status (**Table 1**). Patients with ampullary cancer had lower preoperative plasma histidine levels compared with healthy individuals, but this was unrelated to cancer severity. Preoperative plasma histamine levels were similar between the two groups.

Expression of metabolism-related enzymes in ampullary cancer

Expression of the histidine-catabolic enzymes, HDC and HAL, was analyzed in 106 patients with ampullary cancer. Immunohistochemistry staining of HDC and HAL was performed in FFPE sections, with cytoplasmic staining of HDC and HAL detected (**Figure 2**). Expression levels of HDC and HAL were positively correlated (**Table 2**). High HDC expression was correlated with early-stage tumors (pT1) and localized cancer [American Joint Committee on Cancer tumor node metastasis (AJCC TNM) stage I] (**Table 3**). High HAL expression was also linked to localized cancer (AJCC TNM stage I) (**Table 4**). These findings suggest that histidine-catabolic enzymes are correlated with favorable tumor characteristics. However, HDC and HAL expression did not correlate with preoperative plasma histidine or histamine levels (**Figure 3**).

Correlation between histidine/histamine levels and survival in patients with ampullary cancer

HDC and HAL expression levels were not correlated with ampullary cancer recurrence (**Tables 5**, **6**). However, patients with cancer recurrence exhibited lower preoperative histidine levels, especially those with peritoneal carcinomatosis (**Table 7**). Preoperative plasma histamine

Table 1. Correlation between plasma histidine or histamine levels and demographic or histopathological characteristics in patients with ampullary cancer (N = 49)

	Histidine		Histamine	
	Plasma level (μg/mL)	*P*	Plasma level (μg/mL)	*P*
Gender		0.313		0.200
Female	8.8 (6.7-15.7)		0.5 (0-17.0)	
Male	9.5 (7.4-15.2)		0.8 (0-8.1)	
Operative method		0.548		0.840
Whipple's operation	9.3 (7.1-15.7)		0.9 (0.2-17.0)	
Pylorus-preserving pancreaticoduodenectomy	9.2 (6.7-15.2)		0.7 (0-8.1)	
Excision	9.8 (7.8-12.7)		0.6 (0.2-2.5)	
Histological differentiation		0.245		0.686
Well-differentiated	9.8 (7.6-15.7)		0.6 (0-17.0)	
Moderately differentiated	8.5 (6.7-15.2)		0.7 (0.1-8.1)	
Poorly differentiated	9.0 (7.4-11.1)		0.8 (0.7-1.0)	
Pancreatic invasion		0.334		0.191
Negative	9.7 (6.7-15.7)		0.5 (0-17.0)	
Positive	9.0 (7.1-15.2)		0.8 (0.1-8.1)	
Tumor stage		0.480		0.731
T0	9.4 (8.1-9.8)		0.5 (0.3-2.5)	
T1	10.1 (8.1-15.7)		0.5 (0.2-17.0)	
T2	8.9 (6.7-11.9)		0.7 (0-4.9)	
T3	8.9 (7.1-15.2)		0.8 (0.1-8.1)	
T4	9.1 (7.8-10.0)		0.4 (0-3.2)	
Lymph node metastasis		0.800		0.633
Negative	9.3 (6.7-15.7)		0.7 (0-17.0)	
Positive	8.9 (7.4-15.2)		0.8 (0-8.1)	
AJCC TNM stage		0.580		0.428
Stage 0	9.4 (8.1-9.8)		0.5 (0.3-2.5)	
Stage I	9.8 (6.7-15.7)		0.5 (0-17.0)	
Stage II	8.8 (7.1-15.2)		0.8 (0.1-8.1)	
Stage III	9.2 (7.9-10.1)		0.5 (0-3.2)	

The non-parametric Kruskal-Wallis H test was employed for statistical analysis involving three or more groups, while the Wilcoxon rank-sum test was used for comparisons between two groups. Abbreviation: AJCC TNM stage, American Joint Committee on Cancer tumor, node, metastasis staging system.

levels were not associated with any type of cancer recurrence (**Table 7**). Plasma histidine and histamine levels were stratified into high and low expression groups and subsequently analyzed in relation to patient demographics and survival outcomes. Plasma histidine or histamine levels showed no significant correlation with the clinical demographics of ampullary cancer patients (**Tables 8**, **9**). Higher plasma histidine levels were correlated with better RFS and OS (**Figure 4A**, **4B**), whereas plasma histamine levels were not correlated with RFS or OS (**Figure 4C**, **4D**). In multivariate analysis, plasma histidine level and AJCC TNM stage were identified as poor predictors of recurrence-free survival (**Table 10**). These findings suggest that preoperative histidine level is a prognostic marker for ampullary cancer.

Plasma histidine levels were significantly higher in the three patients who received adjuvant chemotherapy (adjusted *P* = 0.0246, **Figure 5A**), and modestly elevated in the three patients who underwent adjuvant radiotherapy (adjusted *P* = 0.0879, **Figure 5A**) compared to those who did not receive adjuvant therapy. Patients treated with adjuvant chemotherapy or radiotherapy demonstrated improved RFS (**Figure**

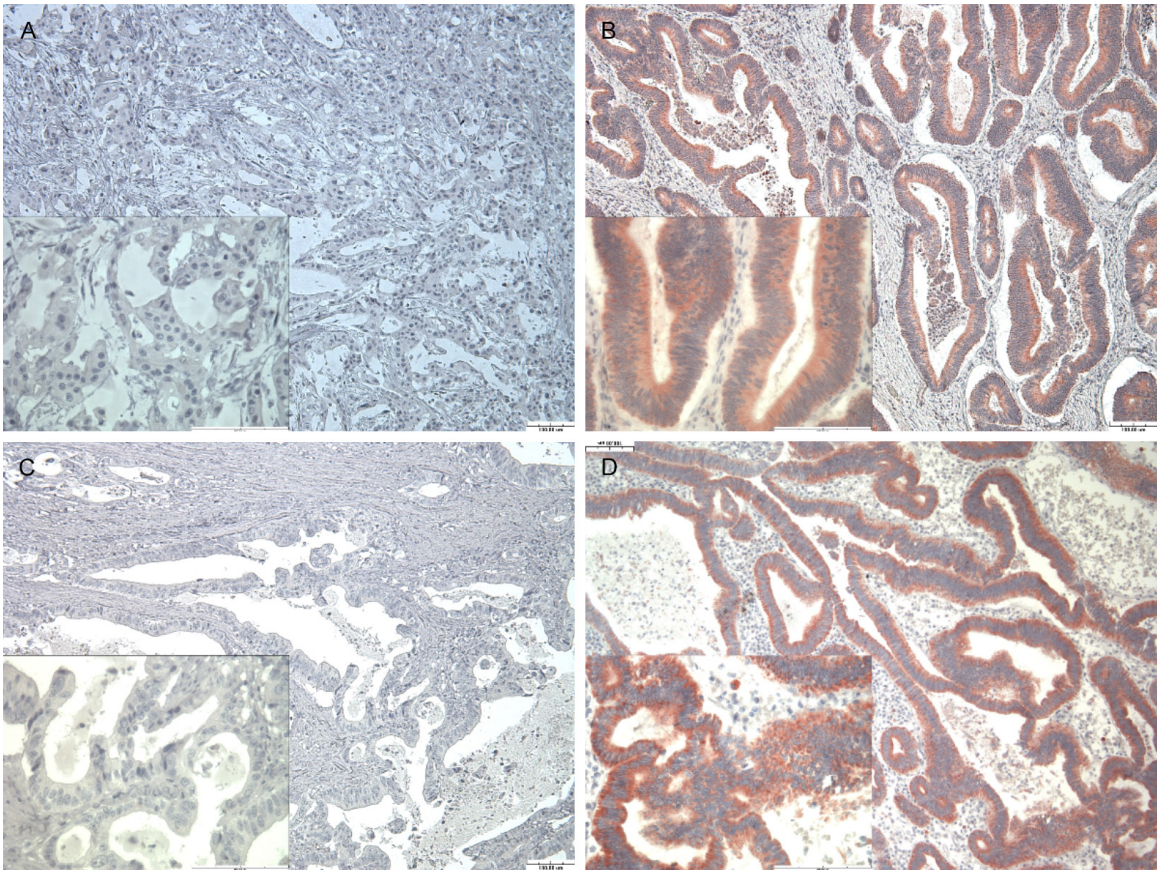

Figure 2. Immunohistochemistry staining of histidine decarboxylase (HDC) and histidine ammonia-lyase (HAL) in ampullary cancer tissues. (A) Negative expression and (B) strong immunoreactivity of HDC. (C) Negative and (D) high HAL expression. The primary images were captured at a magnification of ×100, while the inset image in the lower right quadrant was acquired at a magnification of ×400.

Table 2. Correlation between histidine decarboxylase (HDC) and histidine ammonia-lyase (HAL) expression levels in patients with ampullary cancer ($P < 0.001$)

		HDC expression	
		Low	High
HAL expression	Low	29 (81%)	22 (32%)
	High	7 (19%)	47 (68%)

Fisher's exact test was employed for statistical analysis.

5B). Notably, none of the patients with low plasma histidine levels received adjuvant therapy (**Figure 5C**). Among patients with high plasma histidine levels, those who received adjuvant radiotherapy exhibited superior RFS outcomes (**Figure 5D**). However, the limited sample size constrained the statistical power to confirm the significance of a potential synergistic therapeutic effect between elevated plasma histidine and adjuvant therapy.

Effects of histidine and histamine on ampullary cancer cell lines

The effect of histidine on ampullary cancer was examined in cell cultures, with histamine employed as a control. Three ampullary cancer cell lines were tested. Histidine treatment suppressed ampullary cancer cell proliferation (**Figure 6A-C**). Drug sensitivity was assessed using dose-response curves and the half-maximal inhibitory concentration (IC_{50}) after 48 h of histidine treatment, which was found to inhibit cancer cell survival (**Figure 6D-F**). Conversely, histamine treatment promoted the proliferation of TGBC-18 TKB ampullary cancer cells (**Figure 7A**, **7D**). In contrast, SNU-478 cell proliferation remained unaffected following histamine exposure (**Figure 7B**, **7E**), while SNU-869 cells exhibited an inhibition at higher histamine concentrations (**Figure 7C**, **7F**). These findings suggest that histidine supplementation may exert anti-

Table 3. Correlation between histidine decarboxylase (HDC) expression and demographic or histopathological characteristics of patients with ampullary cancer

	HDC expression		*P*
	Low	High	
Patient, count	36	69	
Age (year), median (range)	54 (32-90)	66 (35-84)	0.589
Gender			0.838
Female	16 (44%)	29 (42%)	
Male	20 (56%)	40 (58%)	
Body weight (kg), median (range)	57.8 (36-76.5)	64 (38-92.5)	0.096
Total bilirubin (mg/dL), median (range)	3.5 (0.3-19.6)	2.1 (0.2-16.8)	0.312
Direct form of bilirubin (mg/dL), median (range)	2.7 (0-18)	1 (0-15.7)	0.174
AST (U/dL), median (range)	74 (9-330)	74 (19-621)	0.448
ALT (U/dL), median (range)	97 (10-850)	100 (10-464)	0.557
Albumin (g/dL), median (range)	3.5 (2.8-4.6)	3.6 (2.5-4.8)	0.758
Globulin (g/dL), median (range)	3.3 (2.4-4.6)	3.6 (2.4-4.8)	0.609
Alkaline Phosphatase (U/L), median (range)	311 (61-1129)	323 (70-144)	0.369
BUN (mg/dL), median (range)	14 (4-36)	14 (3-42)	0.686
Creatinine (mg/dL), median (range)	0.9 (0.6-2.1)	0.8 (0.4-2.7)	0.301
CEA (ng/mL), median (range)	2.2 (0.1-8.1)	1.9 (0.01-296.3)	0.583
CA125 (U/dL), median (range)	16.3 (7.2-47.8)	13.3 (0.5-164.1)	0.364
CA19-9 (U/dL), median (range)	62.7 (0.6-1802.6)	44.2 (0.3-7512.9)	0.408
Tumor size (cm), median (range)	2 (0.7-8)	2.5 (0.8-6.5)	0.266
Histological differentiation*			0.098
Well-differentiated	14 (40%)	22 (48%)	
Moderately-differentiated	16 (46%)	33 (49%)	
Poorly-differentiated	5 (14%)	2 (3%)	
Perineural invasion	6 (17%)	10 (14%)	0.780
Pancreatic invasion	22 (61%)	28 (41%)	0.064
Lymphovascular invasion	17 (47%)	22 (32%)	0.140
Tumor stage			0.028
T1	0	11 (16%)	
T2	14 (39%)	28 (41%)	
T3	17 (47%)	18 (26%)	
T4	5 (14%)	12 (17%)	
Axillary lymph node metastasis*			0.090
Negative	17 (47%)	38 (66%)	
Positive	19 (53%)	20 (34%)	
Positive lymph node count	0 (0-8)	0 (0-9)	0.078
Total resected lymph node count	7 (0-36)	7 (0-57)	0.466
AJCC TNM stage			0.049
Stage I	9 (25%)	34 (49%)	
Stage II	21 (58%)	22 (32%)	
Stage III	5 (14%)	12 (17%)	
Stage IV	1 (3%)	1 (1%)	

Fisher's exact test was used to assess the correlation between two categorical variables, and the chi-square test was applied for analyses involving larger sample sizes or other categorical comparisons. The Wilcoxon rank-sum test was used to compare continuous variables across different levels of HDC expression. *Excluding missing data. Abbreviations: AJCC TNM, American Joint Committee on Cancer tumor node metastasis; ALK-P, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CA125, cancer antigen 125; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen.

Table 4. Correlation between histidine ammonia-lyase (HAL) expression and demographic or histopathological characteristics in patients with ampullary cancer

	HAL expression		*P*
	Low	High	
Patient, count	52	54	
Age (year), median (range)	65 (32-90)	64 (35-89)	0.977
Gender			0.698
Female	21 (40%)	24 (44%)	
Male	31 (60%)	30 (56%)	
Body weight (kg), median (range)	57.6 (36-79.8)	62 (38-92.5)	0.285
Total bilirubin (mg/dL), median (range)	2.6 (0.3-19.6)	2.2 (0.2-16.8)	0.452
Direct bilirubin (mg/dL), median (range)	2.1 (0-18)	1.2 (0-15.7)	0.367
AST (U/dL), median (range)	84.5 (9-330)	64 (23-621)	0.451
ALT (U/dL), median (range)	106 (13-850)	91 (10-561)	0.284
Albumin (g/dL), median (range)	3.5 (2.5-4.8)	3.7 (2.6-4.8)	0.150
Globulin (g/dL), median (range)	3.3 (2.4-4.8)	3.6 (2.4-4.4)	0.557
Alkaline Phosphatase (U/L), median (range)	288 (61-1129)	381 (70-1404)	0.786
BUN (mg/dL), median (range)	13 (4-36)	15 (3-42)	0.321
Creatinine (mg/dL), median (range)	0.9 (0.5-2.1)	0.8 (0.4-2.7)	0.271
CEA (ng/mL), median (range)	2.0 (0.1-296.3)	2 (0.01-41.4)	0.766
CA125 (U/dL), median (range)	15.6 (2.7-164.1)	13.3 (0.5-66.7)	0.379
CA19-9 (U/dL), median (range)	55 (0.6-7512.9)	50.5 (0.3-2328.8)	0.575
Tumor size (cm), median (range)	2.2 (0.7-8)	2.5 (1-6)	0.749
Histological differentiation*			0.842
Well-differentiated	22 (43%)	25 (48%)	
Moderately-differentiated	25 (49%)	24 (46%)	
Poorly-differentiated	4 (8%)	3 (6%)	
Perineural invasion	8 (53%)	8 (44%)	> 0.999
Lymphovascular invasion	20 (65%)	19 (54%)	0.841
Tumor stage			0.151
T1	3 (6%)	8 (15%)	
T2	18 (35%)	25 (46%)	
T3	21 (40%)	14 (26%)	
T4	10 (19%)	7 (13%)	
Axillary lymph node metastasis*			0.217
Negative	27 (53%)	29 (66%)	
Positive	24 (47%)	15 (34%)	
Positive lymph node count	0 (0-9)	0 (0-6)	0.131
Total resected lymph node count	7 (0-36)	7 (0-57)	0.547
AJCC TNM stage			0.028
Stage I	14 (27%)	30 (55%)	
Stage II	27 (52%)	16 (30%)	
Stage III	10 (19%)	7 (13%)	
Stage IV	1 (2%)	1 (2%)	

Fisher's exact test was used to assess the correlation between two categorical variables, and the chi-square test was applied for analyses involving larger sample sizes or other categorical comparisons. The Wilcoxon rank-sum test was used to compare continuous variables across different levels of HAL expression. *Excluding missing data. Abbreviations: AJCC TNM, American Joint Committee on Cancer tumor node metastasis; ALK-P, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CA125, cancer antigen 125; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen.

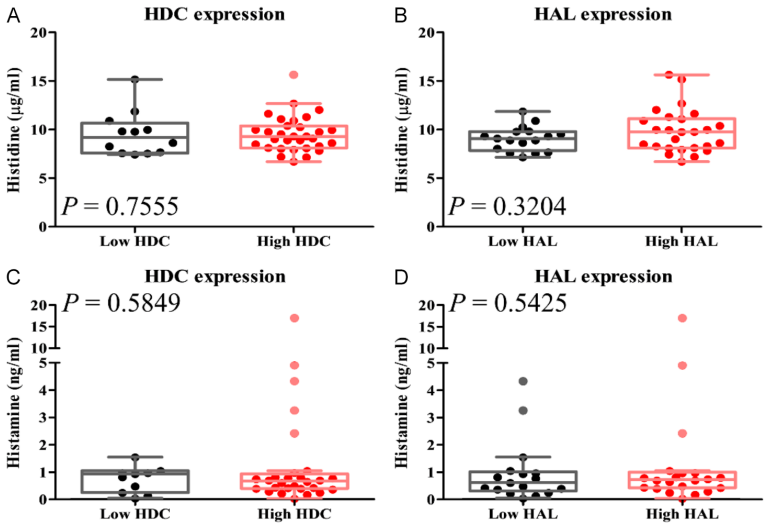

Figure 3. Correlation between plasma histidine or histamine levels and immunohistochemistry staining of histidine decarboxylase (HDC) or histidine ammonia-lyase (HAL) in patients with ampullary cancer. Correlations between (A) histidine and HDC, (B) histidine and HAL, (C) histamine and HDC, and (D) histamine and HAL. The Wilcoxon rank-sum test was used to compare histidine/histamine levels between groups stratified by HDC/HAL expression levels.

proliferative effects, whereas histamine may promote proliferation in selected cancer types.

Discussion

Histidine is an essential amino acid in humans. In this study, patients with ampullary cancer exhibited lower plasma histidine levels compared with healthy controls, and higher preoperative plasma histidine levels were correlated with a better prognosis. The histidine-catabolic enzymes, HDC and HAL, were correlated with favorable tumor characteristics. *In vitro* assessments confirmed that histidine suppressed cell proliferation of three ampullary cancer cell lines (TGBC-18 TKB, SNU-478, and SNU-869), whereas histamine enhanced cell proliferation of TGBC-18 TKB cells. These findings suggest that preoperative plasma histidine is a prognostic biomarker for ampullary cancer, and histidine supplementation may help suppress tumor proliferation.

In our previous study, genes regulated by peroxisome proliferator-activated receptor alpha and retinoid X receptor involved in lipid metabolism were found to be overexpressed in complementary DNA (cDNA) microarray data from five patients with ampullary cancer and the NCBI GSE39409 dataset [20]. In contrast, the expression levels of genes involved in aerobic and anaerobic respiration, glycolysis, mitochondrial metabolism, and hypoxia were comparable between ampullary cancer tissues and matched normal duodenal samples [20]. The present study investigated histidine catabolism in ampullary cancer.

Higher histidine concentrations have been linked to reduced all-cause mortality risk [27], with a stronger association observed in males compared to females [28]. However, findings related to its role in cancer are inconsistent. Plasma histidine levels are lower in patients with non-small cell lung, pancreatic, breast, and gastric cancers relative to those in healthy controls [29-32] but higher in patients with colorectal cancer and lymph node metastasis compared to those without such metastasis [33]. Higher plasma histidine levels are correlated with an increased risk of breast cancer [5] but reduced risk of colorectal cancer [6]. Additionally, histidine levels decrease with advancing colorectal cancer stage, being highest in those with benign polyps and lowest in those with stage IV cancer [34]. In ampullary cancer, we found that plasma histidine levels were lower in patients with ampullary cancer compared to healthy controls. Notably, higher plasma histidine concentrations were significantly associated with improved RFS and OS. These findings were supported by our *in vitro* experiments, where histidine treatment inhibited cancer cell proliferation, suggesting a potential therapeutic benefit. Cancer cells primarily utilize anaerobic glycolysis for energy production, rather than oxidative phosphorylation [35]. Consequently, their metabolic response to extrinsic amino acids differs from that of normal cells [36]. Supplementation with histidine in cancer cells enhances histidine catabolism and leads to depletion of tetrahydrofolic acid under methotrexate treatment [8]. In a previous study, a diet enriched with essential amino acids (including histidine) activates ATF4 (activating transcription factor 4), resulting in the suppres-

Table 5. Correlation between histidine decarboxylase (HDC) expression and disease recurrence in patients with ampullary cancer

	HDC expression		*P* value
	Low	High	
Patient, count	36	69	
Recurrence and ampullary cancer-related events	21 (58%)	43 (62%)	0.748
Liver metastasis	7 (19%)	20 (29%)	0.288
Local recurrence	11 (31%)	24 (35%)	0.663
Peritoneal carcinomatosis	4 (11%)	11 (16%)	0.502
Bone metastasis	3 (8%)	7 (10%)	0.764
Other metastasis	8 (22%)	11 (16%)	0.428
Other cause-related events	10 (28%)	18 (26%)	0.910

Fisher's exact test was used to assess the correlation between two categorical variables.

Table 6. Correlation between histidine ammonia-lyase (HAL) expression and disease recurrence in patients with ampullary cancer

	HAL expression		*P* value
	Low	High	
Patient, count	52	54	
Recurrence and ampullary cancer-related events	35 (67%)	30 (56%)	0.214
Liver metastasis	13 (25%)	15 (28%)	0.746
Local recurrence	15 (29%)	21 (39%)	0.275
Peritoneal carcinomatosis	6 (12%)	9 (17%)	0.449
Bone metastasis	6 (12%)	4 (7%)	0.467
Other metastasis	7 (13%)	12 (22%)	0.240
Other cause-related events	12 (23%)	16 (30%)	0.447

Fisher's exact test was used to assess the correlation between two categorical variables.

Table 7. Correlation between plasma histidine or histamine levels and disease recurrence in patients with ampullary cancer

	Histidine		Histamine	
	Plasma level	*P*	Plasma level	*P*
No recurrence	9.8 (6.7-15.2)		0.5 (0-8.1)	
Recurrence and ampullary cancer-related events	8.6 (7.1-15.7)	0.039	0.7 (0-17.0)	0.659
Liver metastasis	9.2 (7.5-15.7)	0.896	0.7 (0.1-17.0)	0.533
Local recurrence	8.8 (7.2-15.7)	0.803	0.8 (0-17.0)	0.797
Peritoneal carcinomatosis	7.6 (7.1-8.1)	0.063	0.3 (0.2-0.5)	0.215
Bone metastasis	8.2 (7.5-15.7)	0.674	0.9 (0.1-17.0)	0.313
Other metastasis	8.4 (7.4-11.1)	0.151	1.0 (0-4.3)	0.315

The Wilcoxon rank-sum test was used to compare continuous variables with recurrence or metastasis.

sion of glycolysis and mammalian target of rapamycin complex 1 (mTORC1) signaling, ultimately inhibiting tumor growth [3]. These findings provide insight into the potential antiproliferative mechanism associated with histidine supplementation. Normal cells predominantly generate energy via oxidative phosphorylation, and appropriate histidine supplementation is unlikely to elicit adverse effects [37]. Dietary histidine intake at doses of 4.0-4.5 g per day has been associated with reductions in body mass index, adiposity, markers of glucose homeostasis, proinflammatory cytokines, and oxidative stress. However, excessive intake exceeding 24 g per day may lead to decreased serum zinc levels and cognitive impairment

Table 8. Correlation between plasma histidine levels and demographic or histopathological characteristics in patients with ampullary cancer (N = 49)

	Histidine		*P*
	Low	High	
Patient, count	23	26	
Age (year), median (range)	67 (40-82)	66 (36-80)	0.125
Gender			0.117
Female	14 (61%)	10 (39%)	
Male	9 (39%)	16 (62%)	
Operative method			0.863
Whipple's operation	6 (26%)	7 (27%)	
Pylorus-preserving pancreaticoduodenectomy	13 (57%)	13 (50%)	
Wide local excision	4 (17%)	6 (23%)	
Histological differentiation*			0.305
Well-differentiated	7 (32%)	12 (54%)	
Moderately-differentiated	13 (59%)	9 (41%)	
Poorly-differentiated	2 (9%)	1 (5%)	
Pancreatic invasion	12 (55%)	9 (35%)	0.165
Tumor stage			0.514
Adenoma	1 (4%)	2 (8%)	
T1	2 (9%)	7 (27%)	
T2	8 (35%)	7 (27%)	
T3	9 (39%)	8 (30%)	
T4	3 (13%)	2 (8%)	
Lymph node metastasis*			0.257
Negative	11 (58%)	15 (75%)	
Positive	8 (42%)	5 (25%)	
AJCC TNM stage			0.764
Adenoma	1 (4%)	2 (8%)	
Stage I	8 (35%)	12 (45%)	
Stage II	11 (48%)	9 (35%)	
Stage III	3 (13%)	3 (12%)	

Fisher's exact test was used to assess the correlation between two categorical variables, and the chi-square test was applied for analyses involving larger sample sizes or other categorical comparisons. The Wilcoxon rank-sum test was used to compare continuous variables between low and high histidine levels. *Excluding missing data. Abbreviation: AJCC TNM stage, American Joint Committee on Cancer tumor, node, metastasis staging system.

[37]. In the present study, plasma histidine levels did not show a significant correlation with histological differentiation, tumor stage, lymph node involvement, or overall cancer stage. However, higher plasma histidine concentrations were associated with a lower incidence of peritoneal carcinomatosis. These findings suggest that plasma histidine is not derived from ampullary cancer itself, but rather reflects systemic histidine metabolism, which may influence the host response to tumor progression. Adjuvant therapy was not routinely administered, and we failed to confirm the significance of a potential synergistic therapeutic effect between elevated plasma histidine and adjuvant therapy due to the small sample size. We boldly hypothesize that dietary histidine supplementation may represent a potential therapeutic strategy for patients with ampullary cancer.

Histidine is degraded by HDC into histamine and by HAL into urocanate. HAL is highly expressed in pancreatic cancer [10], and HDC expression is found in melanoma, pancreatic neuroendocrine tumors, and small cell lung carcinoma, as well as colon, breast, and other can-

Table 9. Correlation between plasma histamine levels and demographic or histopathological characteristics in patients with ampullary cancer (N = 49)

	Histamine		*P*
	Low	High	
Patient, count	21	28	
Age (year), median (range)	68 (52-82)	66 (36-80)	0.108
Gender			0.117
Female	13 (62%)	11 (39%)	
Male	8 (38%)	17 (61%)	
Operative method			0.855
Whipple's operation	5 (24%)	8 (29%)	
Pylorus-preserving pancreaticoduodenectomy	11 (52%)	15 (53%)	
Wide local excision	5 (24%)	5 (18%)	
Histological differentiation*			0.292
Well-differentiated	9 (47%)	10 (40%)	
Moderately-differentiated	10 (53%)	12 (48%)	
Poorly-differentiated	0	3 (12%)	
Pancreatic invasion	6 (29%)	15 (56%)	0.062
Tumor stage			0.526
Adenoma	2 (10%)	1 (4%)	
T1	5 (24%)	4 (14%)	
T2	6 (28%)	9 (32%)	
T3	5 (24%)	12 (43%)	
T4	3 (14%)	2 (7%)	
Lymph node metastasis*			0.818
Negative	11 (69%)	15 (65%)	
Positive	5 (31%)	8 (35%)	
AJCC TNM stage			0.460
Adenoma	2 (10%)	1 (4%)	
Stage I	10 (47%)	10 (36%)	
Stage II	6 (29%)	14 (49%)	
Stage III	3 (14%)	3 (11%)	

Fisher's exact test was used to assess the correlation between two categorical variables, and the chi-square test was applied for analyses involving larger sample sizes or other categorical comparisons. The Wilcoxon rank-sum test was used to compare continuous variables between low and high histidine levels. *Excluding missing data. Abbreviation: AJCC TNM stage, American Joint Committee on Cancer tumor, node, metastasis staging system.

cers [38-40]. Increased HDC expression correlates with a better prognosis in breast cancer cases [41]. However, histidine metabolism in ampullary cancer has not been reported previously. We observed synergistic expression of HDC and HAL in ampullary cancer, with HAL being more prevalent in stage I cancer and HDC in T1 tumors. HDC and HAL are key enzymes involved in histidine catabolism. In our study, the expression levels of HDC and HAL were not associated with clinical outcomes in patients with ampullary cancer. This lack of correlation further emphasizes the potential significance of histidine itself in the context of ampullary cancer.

Under normal physiological conditions, histamine is highly concentrated in the stomach, lymph nodes, and thymus and is released by mast cells in response to allergies and inflammation. Exogenous histamine treatment has been shown to induce cell apoptosis and suppress breast cancer in mouse models [41], and it also reduces chronic inflammation-related colorectal tumorigenesis [42]. In the present study, histamine treatment enhanced the cell

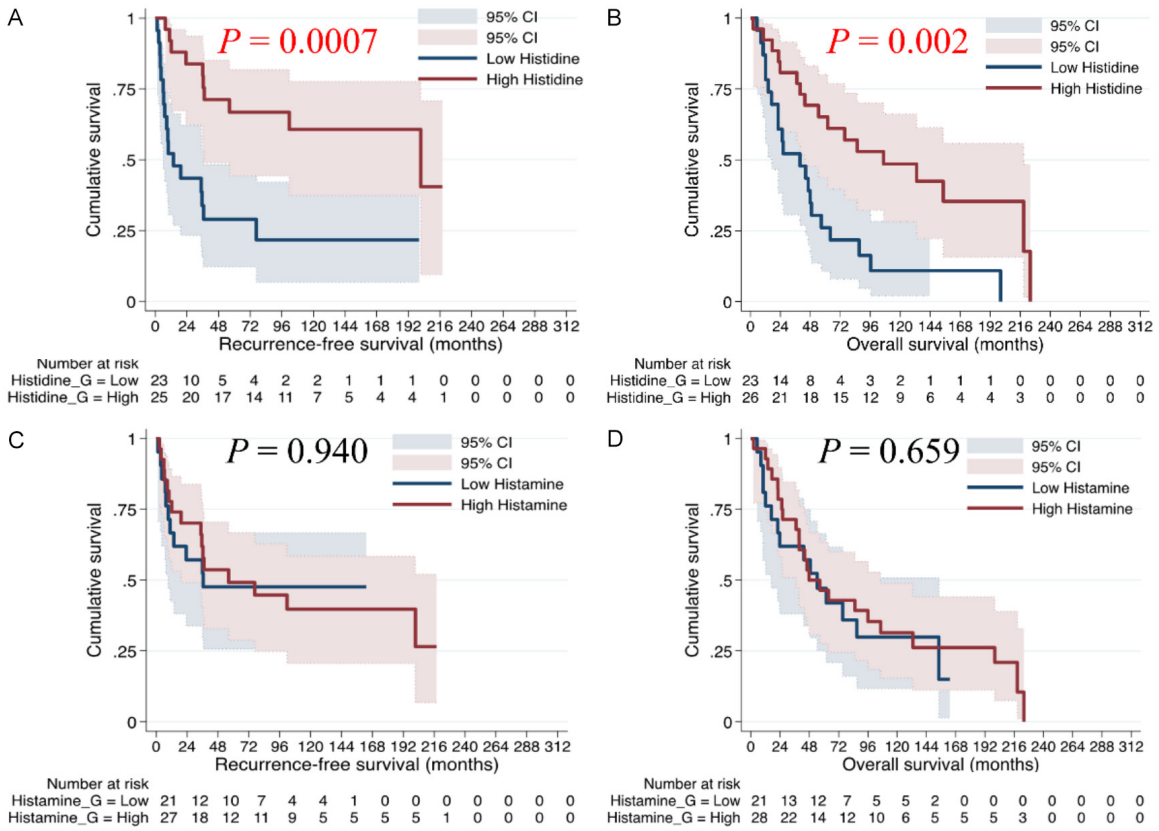

Figure 4. Correlation between histidine or histamine levels and survival outcomes in patients with ampullary cancer. (A) Recurrence-free survival and (B) overall survival curves based on low or high plasma histidine levels. (C) Recurrence-free survival and (D) overall survival curves based on low or high plasma histamine levels. Kaplan-Meier survival curves were compared using the log-rank test.

Table 10. Cox proportional hazards model to identify predictors of recurrence-free survival in patients with ampullary cancer

	HR	95% CI	*P*
Age	1.029	0.989-1.070	0.159
Gender			0.089
Female	1		
Male	0.481	0.206-1.119	
Histidine	0.745	0.565-0.983	0.037
AJCC TNM stage			
Adenoma	1		
Stage I	4.58×10^8	1.24×10^8-1.69×10^9	< 0.001
Stage II	2.16×10^9	7.33×10^8-6.35×10^9	< 0.001
Stage III	3.38×10^8	N.C.	< 0.001

The initial model included the following variables: age, sex, plasma histidine level, tumor stage, pancreatic invasion, histological differentiation, lymph node metastasis, and cancer stage. N.C., not calculated, because 5 out of 6 stage III patients experienced cancer recurrence. Abbreviation: AJCC TNM stage, American Joint Committee on Cancer tumor, node, metastasis staging system.

growth of TGBC-18 TKB cancer cells. However, treatment with histamine didn't have any effect on the growth of SNU-478 cells. Treatment with medium-dose histamine (1 μM) didn't have any effect on the growth of SNU-869 cells. The growth of SNU-869 cells was suppressed by high-dose histamine (10 μM). TGBC-18 TKB cells were derived from a primary papillotubular adenocarcinoma of the Vater's papilla [23], SNU-478 cells from a primary adenocarcinoma of the ampulla of Vater, and SNU-869 from an adenocarcinoma with focal papillary features [24, 25]. The differences in the origin of these cancers may contribute to the varying responses of the three cell lines to extrinsic histamine. Human cancers are inherently heterogeneous, consisting of multi-

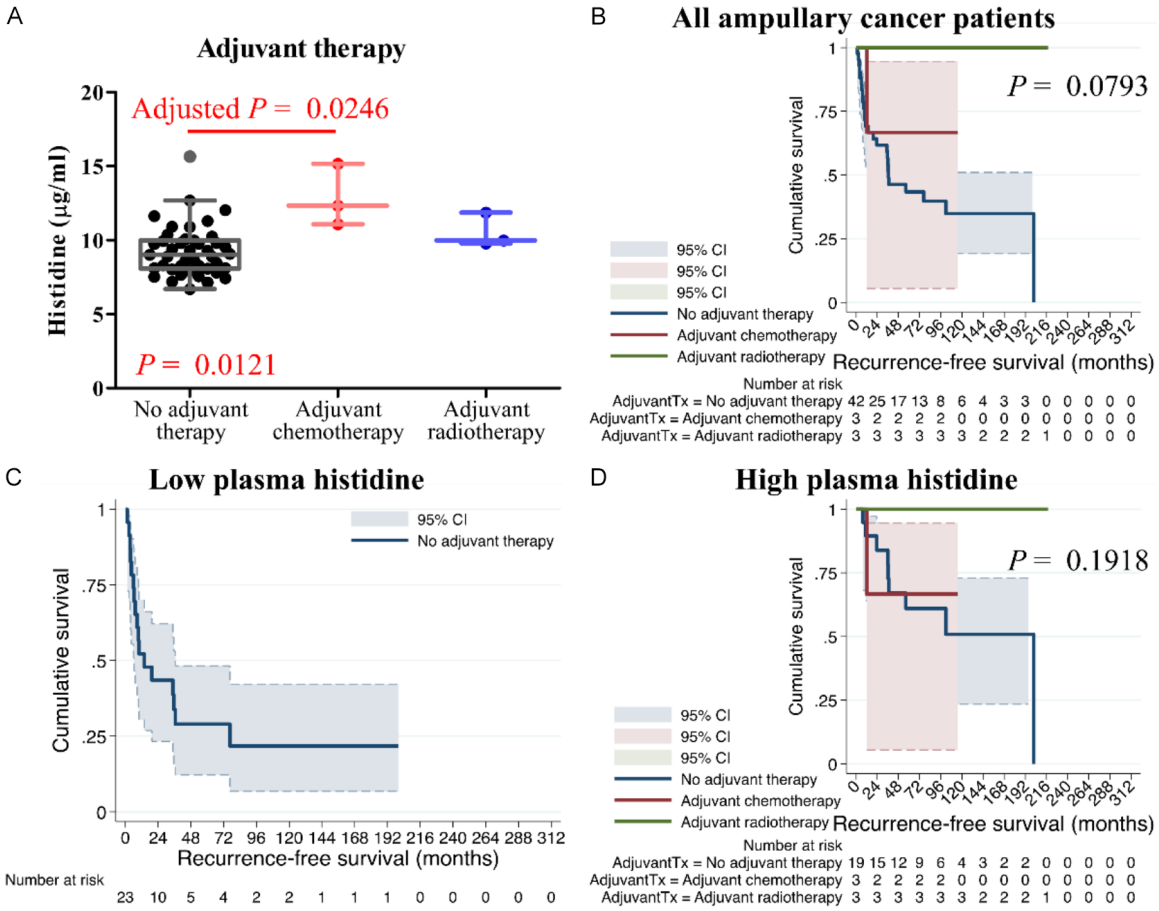

Figure 5. Impact of adjuvant therapy and plasma histidine levels on survival outcomes in patients with ampullary cancer. (A) Plasma histidine levels in patients stratified by adjuvant therapy status: no therapy, chemotherapy, or radiotherapy. The Wilcoxon rank-sum test was employed to compare adjuvant therapy status. (B) Recurrence-free survival was analyzed based on adjuvant therapy status. Recurrence-free survival was evaluated in patients with low (C) or high (D) plasma levels of histidine. Statistical comparisons between survival curves were performed using the log-rank test.

ple clones of cancer cells. Although a single-cell-derived cell line may initially be homogeneous, genomic instability rapidly leads to the development of heterogeneity within the cell population [43]. It is not surprising that the three cell lines derived from the ampulla of Vater exhibit distinct characteristics. Therefore, antihistamine receptor agents may be potential therapeutics for a certain type of ampullary cancer.

A limitation of this study is the small sample size, owing to the rarity of ampullary cancer (incidence: 0.063%-0.210% in autopsy studies). Patient enrollment and specimen collection were also time-consuming. In the present study, plasma levels of histidine and histamine were analyzed only in 49 ampullary cancer patients. Because of the limited sample size, only a minimal number of variables could be included in the multivariate analysis. Plasma histidine level and AJCC TNM stage emerged as predictors of worse recurrence-free survival. A small sample size may reduce statistical power and increase the risk of false negatives. Apart from this, the ampulla of Vater is a unique organ located over the confluence of the bile duct and pancreatic duct. Only large mammals have the ampulla of Vater, e.g., cat, pig, or primates. There is no such organ in mice or rats. Therefore, to support our clinical findings, we used cell culture models with human ampullary cancer cell lines to validate the effects of histidine and histamine. No *in vivo* animal model could be used.

Furthermore, the physiological plasma concentration of histidine ranges from approximately

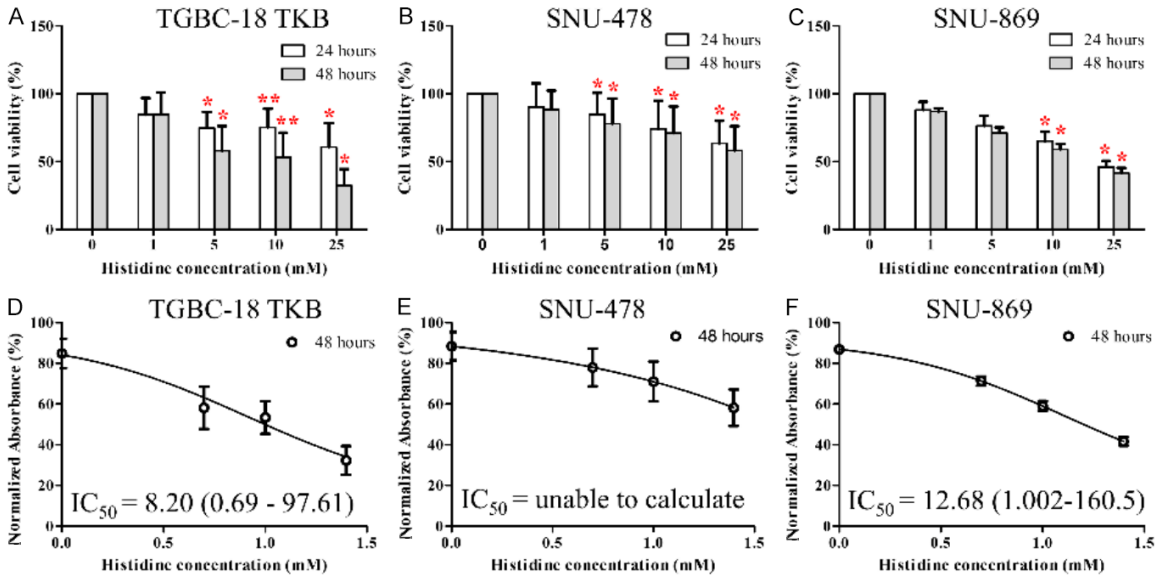

Figure 6. Cell growth ability and drug sensitivity of ampullary cancer cells under histidine treatment. A. MTT assay of TGBC-18 TKB cells. B. MTT assay of SNU-478 cells. C. MTT assay of SNU-869 cells. D. Half maximal inhibitory concentration (IC_{50}) of TGBC-18 TKB cells. E. IC_{50} of SNU-478 cells. F. IC_{50} of SNU-869 cells. Post hoc analyses of cell growth rates at the target concentration were compared between treated and non-treated groups using the Wilcoxon rank-sum test. *$P < 0.05$.

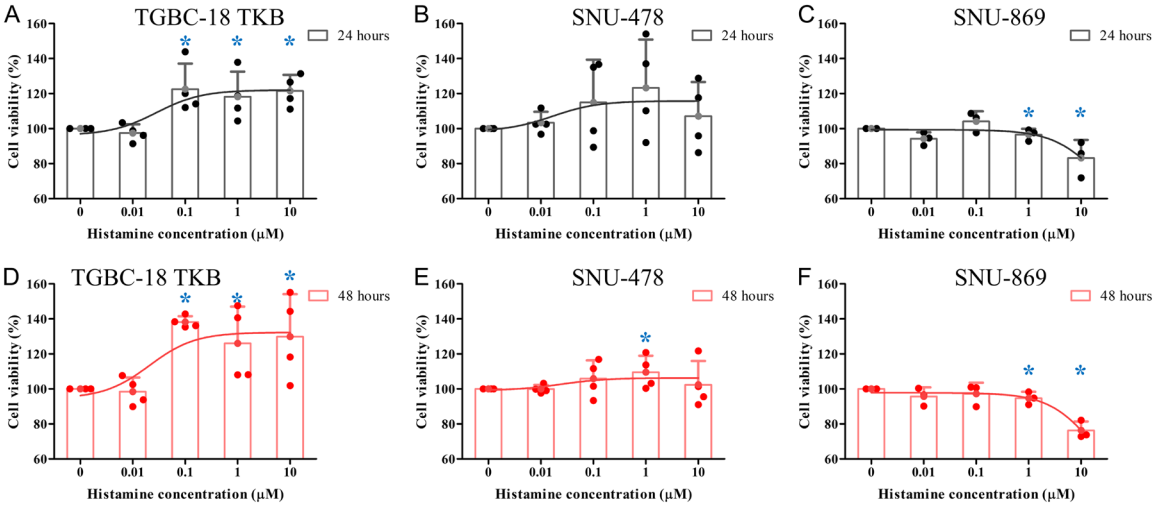

Figure 7. Dose-response curves showing the effect of histamine on ampullary cancer cell growth. (A) TGBC-18 TKB cells, (B) SNU-478 cells, and (C) SNU-869 cells treated with histamine for 24 h. (D) TGBC-18 TKB cells, (E) SNU-478 cells, and (F) SNU-869 cells treated with histamine for 48 h. Post hoc analyses of cell growth rates at the target concentration were compared between treated and non-treated groups using the Wilcoxon rank-sum test. *$P < 0.05$.

70 to 110 μM, whereas the concentrations used *in vitro* MTT assay were relatively high (0-25 mM). Plasma histidine level reflects systemic circulation rather than the intratumoral or intraintestinal environment. To our knowledge, no studies have investigated intratissue or intraintestinal histidine concentrations. We hypothesize that oral histidine supplementation maintains a high local concentration in direct contact with the ampulla of Vater, which is anatomically located in the second portion of the duodenum. To mimic tumor microenvironmental conditions, we employed an *in vitro* MTT assay. As there are no prior studies investigating the local effects of histidine in ampullary cancer, the dosage used in this project was

based on protocols established in previous research involving pancreatic cancer cell lines [10]. Although this dosage was based on prior studies [10], higher levels of histidine may induce osmotic stress or alter pH balance, potentially impacting cell viability and experimental outcomes. Translating *in vitro* findings into clinical applications presents inherent challenges, and cell proliferation assays alone are insufficient to conclusively determine the therapeutic effects of histidine supplementation. Further meta-analysis or larger multicenter studies may help to confirm the significance of plasma histidine level and the treatment potential of oral histidine supplement in patients with ampullary cancer.

Another limitation of the present study is the lack of access to AI-based or other automated tools for quantifying IHC staining results at our research center. Consequently, the IHC-stained sections were evaluated manually by a senior faculty member. The reliability of these assessments is supported by consistency with findings reported in several previously published studies [14-19]. Moreover, plasma histidine levels were retrospectively analyzed using stored samples rather than measured at the time of blood collection, which may have led to underestimation.

Conclusion

In conclusion, the expression levels of the histidine-catabolic enzymes, HDC and HAL, were elevated in early-stage ampullary cancer. Plasma histidine levels were lower in patients with ampullary cancer than in healthy controls, and higher histidine levels were correlated with improved prognosis. Furthermore, histidine treatment inhibited cancer cell proliferation in TGBC-18 TKB, SNU-478, and SNU-869 cell lines. These findings suggest that plasma histidine may serve as a prognostic biomarker for survival, and histidine supplementation might have therapeutic potential in the management of ampullary cancer.

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Written informed consent was obtained from all patients before study participation.

Disclosure of conflict of interest

None.

Abbreviations

95% CI, 95% confidence intervals; AJCC TNM, American Joint Committee on Cancer tumor node metastasis; ALK-P, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CA125, cancer antigen 125; CA19-9, cancer antigen 19-9; CEA, carcinoembryonic antigen; HAL, histidine ammonia-lyase; HDC, histidine decarboxylase; FFPE, formalin-fixed paraffin-embedded; IC_{50}, half-maximal inhibitory concentration; RFS, recurrence-free survival; OS, overall survival.

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Table S1. Clinical and demographic characteristics of the 49 patients with ampullary cancer evaluated for plasma histidine/histamine levels

Variable	N (%)
Age (year), median (range)	67 (36-82)
Gender	
Male	24 (49%)
Female	25 (51%)
Body weight (kg), median (range)	62.4 (41-87)
Total bilirubin (mg/dL), median (range)	1.5 (0.2-16.8)
Direct bilirubin (mg/dL), median (range)	0.7 (0-15.7)
AST (U/dL), median (range)	56 (16-336)
ALT (U/dL), median (range)	79 (10-561)
Albumin (g/dL), median (range)	3.7 (2.8-4.8)
Globulin (g/dL), median (range)	3.4 (2.9-3.4)
Alkaline Phosphatase (U/L), median (range)	286 (70-1073)
BUN (mg/dL), median (range)	14 (1-42)
Creatinine (mg/dL), median (range)	0.8 (0.3-2.7)
CEA (ng/mL), median (range)	2.0 (0.2-13.0)
CA125 (U/dL), median (range)	15.0 (2.7-66.7)
CA19-9 (U/dL), median (range)	44.2 (0.6-1747)
Operation methods	
Whipple	13 (27%)
Pylorus-preserving pancreaticoduodenectomy	26 (53%)
Wide local excision	10 (20%)
Tumor size (cm), median (range)	2.4 (0.7-5.5)
Pancreatic invasion	21 (43%)
Tumor stage	
Adenoma	3 (6%)
T1	9 (18%)
T2	15 (31%)
T3	17 (35%)
T4	5 (10%)
Lymph node metastasis*	
Negative	26 (67%)
Positive	13 (33%)
Histological differentiation*	
Well-differentiated	19 (43%)
Moderately-differentiated	22 (50%)
Poorly-differentiated	3 (7%)
AJCC TNM stage	
Adenoma	3 (6%)
Stage I	20 (41%)
Stage II	20 (41%)
Stage III	6 (12%)

*Excluding missing data. Abbreviation: AJCC TNM stage, American Joint Committee on Cancer tumor, node, metastasis staging system.