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RESEARCH ARTICLE

Bjelanovic et al: Acid ceramidase in rectal cancer

Acid ceramidase expression and biomarker potential in patients with locally advanced rectal cancer

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ABSTRACT

Acid ceramidase (AC), a pivotal enzyme in sphingolipid metabolism, has been associated with various cancers; however, its specific role in rectal cancer remains poorly understood. This study aimed to explore the clinical significance of AC gene and protein expression in rectal cancer. We analyzed the expression of *ASAH1*, *BAX*, and *BCL2* through quantitative Real-Time PCR in paired tumor and non-tumor tissue samples obtained from patients with locally advanced rectal cancer (LARC) prior to neoadjuvant chemoradiotherapy. Additionally, serum AC levels and standard biochemical parameters were assessed. We further evaluated *ASAH1* expression using RNA-seq data from publicly available TCGA-READ datasets accessed via the UCSC Xena Browser. Two approaches indicated a significant reduction in *ASAH1* expression in tumor tissue (p=0.004 and p<0.001, respectively). Receiver operating characteristic curve analysis revealed a modest capacity for *ASAH1* expression to differentiate between tumor and non-tumor tissue in LARC patients (AUC=0.652, p=0.042). No correlation was observed between *ASAH1* expression and the *BAX/BCL2* ratio in tumor tissue, nor with serum AC levels or the CRP-albumin-lymphocyte (CALLY) index. Conversely, serum AC levels exhibited a negative correlation with the *BAX/BCL2* ratio (rs=−0.536, p=0.002, FDR-adjusted q=0.021). Furthermore, *ASAH1* expression, AC levels, and the CALLY index were not linked to overall survival or treatment response. A key finding of this study is the inverse relationship between serum AC levels and the pro-apoptotic status of tumor tissue, suggesting that circulating AC may provide valuable insights into tumor apoptotic activity. Further large-scale studies are necessary to validate these preliminary findings and elucidate the biomarker potential of AC in rectal cancer.

Keywords: Acid ceramidase, *ASAH1* gene, CALLY index, neoadjuvant chemoradiotherapy, rectal cancer.

INTRODUCTION

Colorectal cancer (CRC) remains a major global health challenge, ranking third in frequency and second in cancer-related mortality according to GLOBOCAN 2022 data [1]. According to U.S. estimates, 46,000 of 153,000 new CRC cases in 2023 were rectal cancers, representing about 30% of all CRCs [2]. Among these, nearly 45% are diagnosed as locally advanced rectal cancer (LARC), defined as stage II and III disease [3]. While early-stage rectal cancer is often treated with surgery alone, LARC typically requires a multimodal approach. Advanced stages are commonly managed with neoadjuvant radiotherapy and/or chemotherapy to reduce tumor size prior to surgical resection [4]. However, response to neoadjuvant therapy is highly variable, with only about 20% of patients achieving a complete pathological response [5]. The clinical course of CRC varies depending on tumor location, with rectal cancer generally associated with poorer relapse-free survival compared to colon cancer [6]. Moreover, systemic inflammation plays a significant role in cancer progression and therapy resistance, further complicating treatment outcomes [7].

Currently used tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), are primarily applied for monitoring disease progression and recurrence in CRC patients. However, their limited specificity and sensitivity reduce their overall clinical utility [8]. Given the limitations of these predictive markers, there is an urgent need for more reliable and specific biomarkers to guide treatment decisions and improve patient outcomes.

In recent years, increasing attention has been given to the role of sphingolipids as potential biomarkers in CRC, given their involvement in tumor growth, therapy resistance, and immune modulation [5, 9, 10]. Notably, recent research has highlighted differences in sphingolipid profiles depending on tumor location, showing significantly lower levels of long-chain ceramides in rectal tissue compared to colon tissue [11]. These findings indicate a potential association between tumor location and sphingolipid metabolism, highlighting the importance of conducting targeted biomarker studies in rectal cancer. Given the interplay between inflammation and sphingolipid metabolism, identifying specific biomarkers that reflect both tumor biology and systemic immune responses could improve patient stratification into those more or less likely to respond to therapy.

Among sphingolipid molecules, ceramides play a crucial role in cancer by regulating key cellular processes such as migration, adhesion, proliferation, differentiation, growth inhibition, and apoptosis [9, 12]. While ceramides represent tumor suppressors, their metabolism is often altered in cancer cells, leading to therapy resistance and disease progression [5, 9]. This imbalance may result not only from altered de novo synthesis but also from increased degradation mediated by acid ceramidase (AC), an enzyme encoded by the *ASAH1* gene. AC breaks down ceramides into sphingosine, which is a precursor for sphingosine-1-phosphate (S1P) [5]. S1P promotes cell survival, proliferation, and resistance to therapy, creating a pro-tumorigenic environment [5, 9]. Tumors often shift sphingolipid metabolism towards S1P and increased AC activity, contributing to systemic inflammation that supports tumor growth [13, 14]. Considering these aspects, AC represents a promising target for novel therapeutic strategies [13, 15, 16]. However, studies specifically focusing on AC in rectal cancer remain scarce [17-19]. A recent study using CRC cell lines and rectal cancer organoids confirmed the role of AC in modulating radiosensitivity, further highlighting its relevance in the response to neoadjuvant chemoradiotherapy (nCRT) [18, 19]. Given the important role of AC in sphingolipid metabolism and its potential impact on cancer progression, this study aimed to assess the expression patterns of AC at both the gene and protein levels in rectal cancer and to explore their clinical relevance as biomarkers.

MATERIALS AND METHODS

Patients

This study included 30 patients with LARC (aged 34–83 years, 63.3% male) recruited at the Clinic for Digestive Surgery - First Surgical Clinic, University Clinical Center of Serbia from April 2019 to February 2022. The inclusion criteria were as follows: histologically confirmed rectal adenocarcinoma, no preoperative treatment, no evidence of distant metastases (M0 status) and signed informed consent. Patients who declined to provide informed consent were excluded from this study. The patients' initial clinical stage was determined using endoscopic ultrasound and pelvic magnetic resonance imaging. The presence or absence of metastases was assessed by computed tomography and/or magnetic resonance imaging.

At the time of diagnosis, biopsy samples of primary tumor tissue and adjacent healthy mucosa were collected from each patient, promptly processed, and stored at −80°C for subsequent RNA extraction and gene expression analysis. Blood samples were also obtained for standard hematological and biochemical analyses, with serum aliquots stored at −80°C for later AC quantification.

On average five weeks after diagnosis, patients underwent clinical re-evaluation before initiating any treatment. Of the 30 patients, 26 were subjected to nCRT followed by surgical resection. Four patients were treated with systemic chemotherapy alone due to poor general condition and/or newly detection of secondary deposits in the lungs and/or liver. Given their deviation from the planned treatment protocol and disease stage exceeding locally advanced disease, they were excluded from the evaluative analysis of therapy response.

The nCRT included a total dose of 50.4 Gy of irradiation in 28 fractions combined with two or three cycles of chemotherapy (5-fluorouracil 425 mg/m2 and Leucovorin 20 mg/m2). After 8-12 weeks, patients were subjected to surgical resection and tumor regression grade (TRG) was determined by histopathological analysis to evaluate the response to nCRT. According to the pathological response to nCRT, patients were stratified into three categories: good responders (n=5) – patients with complete (TRG1) and near-complete (TRG2) tumor regression; moderate responders (n=9) – patients with moderate tumor regression (TRG3); and poor responders (n=12) – patients with minimal tumor regression (TRG4) and no regression (TRG5).

Patient outcomes were evaluated based on overall survival, with follow-up conducted until November 2024.

Gene expression analysis by quantitative real-time PCR

Total RNA was isolated from tissue samples using TRI Reagent Solution (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's guidelines. RNA concentration and purity were assessed by measuring absorbance at 260 nm and 280 nm using a BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and only samples with a 260/280 ratio of 1.8–2.0 were used for further analysis. For the analysis of mRNA expression of target genes, total RNA (2µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's instructions. The reaction was performed under the following conditions: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C.

The mRNA expression levels of the target genes (*ASAH1*, *BCL2* – apoptosis regulator, and *BAX* – BCL2 associated X, apoptosis regulator) were quantified in triplicate using quantitative Real-Time PCR (qRT-PCR) with Power SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with actin beta (*ACTB*) used as the internal housekeeping gene control for normalization. No-template controls were included in all reactions to ensure the absence of contamination. Primer specificity was confirmed prior to the study, and melting curve analysis was conducted for all reactions to ensure product specificity. *ACTB* stability was confirmed across samples and used as the internal housekeeping gene control. Primer sequences and product lengths are provided in Table 1.

qRT-PCR was carried out on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The comparative ΔCt method was employed to calculate relative gene expression, where ΔCt=Ct of target gene − Ct of housekeeping gene. The expression levels of the analyzed target genes were reported as $2^{-\Delta Ct}$ and used for statistical analysis.

Analysis of publicly available sequencing data

RNA-seq data from publicly available datasets were retrieved from the UCSC Xena Browser and analyzed independently to assess *ASAH1* expression in tumor and non-tumor rectal tissues (https://xenabrowser.net; accessed on 12 June 2025). The analysis was performed using the The Cancer Genome Atlas–Rectum Adenocarcinoma (TCGA-READ) dataset. which included transcriptomic data from primary rectal tumor (n=92) and non-tumor tissues (n=10). Only TCGA-READ samples were analyzed, as normal rectal tissues from the Genotype-Tissue Expression (GTEx) Project are not available for this tissue type. Gene expression levels were reported as $\log_2$(FPKM+0.001), where FPKM stands for Fragments Per Kilobase of transcript per Million mapped reads, quantified using the RSEM (RNA-Seq by Expectation-Maximization) method.

Acid ceramidase measurement in serum samples

Quantification of AC levels in serum samples was carried out using a commercial Human ASAH1 ELISA kit (Assay Genie, Dublin, Ireland), following the manufacturer's instructions. Serum samples were incubated with a biotin-conjugated monoclonal antibody specific for AC, followed by the addition of avidin-conjugated horseradish peroxidase. After thorough washing, 3,3',5,5'-tetramethylbenzidine substrate was added, resulting in the development of a blue color. The reaction was stopped by the addition of sulfuric acid, turning the solution yellow. Absorbance was measured at 450 nm using a microplate reader, and the intensity of the colorimetric signal was directly proportional to the concentration of AC in the samples. The calibration range of the assay was 0.156–5 ng/mL, limit of detection was 0.094 ng/mL, limit of quantification was 0.156 ng/mL, intra- assay coefficient of variation was < 8%, inter- assay coefficient of variation was < 10%. A dilution factor of 2 was applied. All samples were measured in duplicate in accordance with good laboratory practice.

Ethical statement

Ethical approval for sample collection was obtained as part of the strategic project MOHERATEKA (grant number F–69) from the Ethics Committee of the Faculty of Medicine, University of Belgrade (Approval No. 1550/V–2; May 31, 2019). Although patient recruitment began in April 2019, sample collection and data acquisition were performed only after this ethical approval had been obtained. The specific analyses performed in this study were additionally approved by the Ethics Committee of the University Clinical Center of Serbia (Approval No. 447/6; October 19, 2021), in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 9.0 (GraphPad Software, LLC, Boston, MA, USA) was used for graphical representation of the results. Continuous variables are presented as medians with interquartile range (IQR) defined as the difference between 75th and 25th percentiles, while categorical variables are presented as numbers (percentages). The Shapiro–Wilk test indicated that the data did not follow a normal distribution, and therefore appropriate non-parametric tests were used for further analysis. Differences between independent samples were evaluated using the Mann-Whitney U test and Kruskal–Wallis test, while matched samples were assessed using the Wilcoxon matched-pairs signed-rank test. The degree of association between variables was calculated using Spearman's rank correlation coefficient (rs). The robustness of the correlations was further evaluated using a bootstrap procedure with 1,000 resamples. Receiver operating characteristic (ROC) curve analysis and area under the curve (AUC) were used to evaluate the discriminatory ability between two variables. AUC values below 0.6 indicate poor, between 0.6 and 0.7 modest, 0.7 to 0.8 good, 0.8 to 0.9 very good, and above 0.9 excellent discriminatory power of the biomarker. Univariate Cox proportional hazards regression analysis was performed to evaluate the association between each variable and overall survival. P values less than 0.05 were considered statistically significant. To control for multiple testing, p-values were adjusted using the False Discovery Rate (FDR) correction according to the Benjamini–Hochberg method.

RESULTS

This study included 30 patients with LARC (all stage III), whose demographic and clinical characteristics are presented in Table 2. Expression levels of the genes *ASAH1*, *BAX*, and *BCL2* were analyzed in biopsied rectal tissue samples before therapy, along with serum levels of AC, standard biochemical parameters, and tumor markers. The ratio of the pro-apoptotic gene *BAX* and the anti-apoptotic gene *BCL2* was calculated to estimate the apoptotic status of tumor tissue samples (*BAX/BCL2*), while the CALLY index was calculated as: albumin level (g/L)×absolute lymphocyte count ($\times 10^9$/L)/CRP level (mg/L)×10, to reflect nutritional, immune, and inflammatory status. *BAX/BCL2* ratio showed a median of 12.1 (IQR 16.0), and the CALLY index had a median of 2.31 (IQR 3.42), reflecting the variability across patients.

In 70% of patients, the expression of *ASAH1* gene was reduced in tumor tissues compared to adjacent non-tumor tissues (median 2.9-fold, IQR 12.6-fold, n=21), whereas in the remaining 30%, *ASAH1* gene expression was increased (median 1.9-fold, IQR 3.8-fold, n=9). The difference in expression between tumor and adjacent non-tumor tissues was statistically significant for the entire cohort (Wilcoxon matched-pairs signed-rank test, p=0.004; Figure 1A). An independent analysis of RNA-seq data from TCGA-READ, performed using the UCSC Xena Browser, showed a significant decrease in *ASAH1* gene expression in primary tumor tissues compared to normal rectal tissues as well (Mann-Whitney U test, $p<0.001$; Figure 1B). ROC analysis demonstrated a modest ability of *ASAH1* gene expression to discriminate between tumor and non-tumor tissue (AUC=0.652, 95% CI 0.508–0.796, n=30, $p=0.042$). The optimal cut-off determined by Youden's index was 0.116, which yielded a sensitivity of 38.5% and a specificity of 92.3% (Figure 2).

Spearman correlation analysis showed no significant association between *ASAH1* gene expression in tumor tissue and serum AC levels ($r_s=-0.013$, 95% BCa CI −0.324 to 0.263, n=30, $p=0.945$, FDR-adjusted $q=0.945$). The association of both *ASAH1* gene expression and serum AC levels with the apoptotic status of tumor tissue (*BAX/BCL2* ratio), as well as with inflammatory (CRP and CALLY index) and tumor markers (CEA and CA 19-9) was further evaluated (Table 3). Among the parameters analyzed, only serum AC levels showed a significant negative correlation with the *BAX/BCL2* ratio ($r_s=-0.536$, 95% BCa CI −0.734 to −0.253, n=30, $p=0.002$, FDR-adjusted $q=0.021$; Figure S1).

To analyze the association of tissue *ASAH1* gene expression, serum AC levels, as well as CALLY index with response to nCRT, patients were categorized according to their pathological response into good (TRG1+TRG2), moderate (TRG3) and poor responders (TRG4+TRG5). However, no significant differences between subgroups were found for any of the analyzed parameters (Kruskal–Wallis test, $p>0.05$; Table 4). The prognostic potential of these markers was evaluated with respect to overall survival in LARC patients. The median follow-up time was 62.5 months (IQR 14 months). At the end of follow-up, 83.3% of patients were alive (25/30). In the univariate Cox regression analysis, none of the examined parameters was significantly associated with overall survival (Hazard ratio ranged from 1.01 to 10.05; all $p > 0.5$), and the corresponding confidence intervals were wide, reflecting the small number of observed events (Table S1).

DISCUSSION

This exploratory study focused on LARC, aiming to assess gene and protein expression profiles of AC and explore their potential clinical relevance, given the limited data compared to colon cancer.

ASAH1 gene expression was analyzed in rectal tissues using two independent approaches: direct quantification by qRT-PCR performed on samples collected in this study, and analysis of publicly available RNA-seq data retrieved from the TCGA-READ dataset. Paired tumor and adjacent non-tumor samples were collected exclusively from treatment-naïve stage III LARC patients. Within this cohort, we observed decreased *ASAH1* gene expression in tumor tissue, together with its modest ability to discriminate between tumor and non-tumor rectal tissue. A similar decrease was also observed when analyzing the TCGA-READ dataset, which included transcriptomic data from primary rectal adenocarcinoma samples of stages II–III. This observation is consistent with a previous study combining colon and rectum TCGA datasets to compare the expression of sphingolipid-related genes between tumor and non-tumor tissues, which reported similar results for *ASAH1* gene [20].It should be noted that tumor–normal pairing was not always available in the Xena Browser. However, despite the differences in platforms, methodologies, and potential variations in sample types, a similar trend was observed in both analyses, suggesting decreased *ASAH1* gene expression in tumor tissue. This reduced expression may reflect functional consequences in tumor biology rather than primarily serving as a biomarker for tissue discrimination. Given the paucity of studies directly quantifying *ASAH1* gene expression in human rectal tissues, particularly in non-tumor samples, our results contribute to a better understanding of *ASAH1* in LARC, especially in the context of limited clinical data.

Based on the *ASAH1* gene expression observed in our study, a corresponding decrease in AC levels might be expected, which contrasts with previous reports of elevated AC expression in CRC [21]. These findings suggest that AC is regulated at multiple levels within the tissue, including transcriptional and post-transcriptional mechanisms, and consequently may reflect the differences in enzyme expression observed between colon and rectum [11]. Since AC levels were not measured in tissue samples, we evaluated the association between serum AC concentrations and *ASAH1* gene expression in tumor tissue, considering gene expression as an indirect indicator of the enzyme. However, no correlation was found, likely to the systemic origin of circulating AC. In addition to tumor cells, AC is secreted by the liver, leukocytes, endothelial cells, and other tissues, reflecting overall rather than tumor-specific sphingolipid metabolism [22-24].

The apoptotic status of tumor tissue is an important determinant of therapy response and may influence patient outcomes. The BAX/BCL2 protein ratio has been previously proposed as a potential indicator of tumor response to adjuvant CRT in rectal cancer patients, highlighting its possible role as a predictive biomarker [25]. In our previous study, we assessed the *BAX/BCL2* ratio at the gene expression level and found no association with nCRT, providing a complementary molecular perspective. In contrast, long-chain ceramides (C20 CER, C22 CER, and C24 CER) were identified as potential indicators of tumor apoptotic status in patients with LARC [26]. Given that ceramides mediate cell cycle arrest and cell death in response to cellular stress, and that AC is responsible for their degradation, in this study we investigated the association of both *ASAH1* gene expression and serum AC levels with the apoptotic status of tumor tissue. Interestingly, while no significant correlation was observed for *ASAH1* gene expression in tumor tissue, we found a negative correlation between serum AC levels and the *BAX/BCL2* ratio. Reduced serum AC levels and the pro-apoptotic status of tumor tissue highlights the possibility that systemic AC measurements could provide insight into tumor apoptotic activity. It is known that lower AC levels may promote apoptosis by allowing ceramide accumulation, since ceramides are not being broken down. Conversely, elevated AC levels not only lead to ceramide degradation but also result in the production of sphingosine, which can be phosphorylated into S1P, a molecule known for its proliferative and pro-survival effects that promote tumor progression [5]. However, further studies are needed to confirm observed relationship. If validated, serum AC could potentially be integrated with tissue-based molecular assessments and other biomarkers for a more comprehensive evaluation.

In our cohort, neither serum AC levels nor *ASAH1* gene expression showed a significant association with treatment response. It should be noted that in addition to gene and protein expression levels, enzymatic activity is a crucial determinant of AC function. For instance, lower enzyme expression with high activity could have similar biological consequences as high expression with moderate activity. Therefore, future studies should evaluate both AC expression and activity in serum and rectal tissue, as their combined assessment may provide more meaningful insights into therapy response and the potential of AC as a therapeutic target. Moreover, no association between these markers and overall survival was observed. Estimates from Cox regression hazard ratios should be interpreted with caution due to the limited number of events, which reduces statistical power. The lack of significant associations with treatment response or survival may reflect the relatively small sample size, few good responders, high overall survival, and the multifactorial nature of tumor biology, which likely involves complex molecular interactions beyond individual biomarkers. Considering the median follow-up of approximately 5 years in our cohort, the proportion of patients alive at the end of follow-up (83.3%) is similar to reported 5-year overall survival rates in LARC (81.6%) [27]. Slight differences between studies may reflect patient selection, treatment protocols, or follow-up duration.

To our knowledge, this is the first study that examined biomarker relevance of the CALLY index in patients with LARC. Although lower preoperative CALLY values have been associated with reduced survival and increased recurrence in CRC, and shown superior prognostic value compared to other inflammatory markers, we found no association with response to nCRT or overall survival [28-31]. However, examining the CALLY index and serum AC levels together in the future studies could provide a more comprehensive insight into the inflammatory, nutritional, and metabolic status of patients with LARC and their treatment outcomes.

This study focused on LARC samples and, given the relatively small sample size, should be considered exploratory in nature. Nevertheless, the preliminary results provide valuable insight into the potential systemic reflection of local tumor biology and may inform future studies with larger and more representative cohorts. Although neither of analyzed parameters showed an association with the treatment response or survival, the role of sphingolipid metabolism in rectal cancer warrants further investigation, particularly given the limited data in the current literature. The interplay between ceramide metabolism, apoptosis, inflammation, and nutritional status should be further explored in larger and more diverse patient cohorts.

CONCLUSION

The results of this study showed a significant decrease in *ASAH1* gene expression in rectal tumor tissue compared to adjacent normal mucosa, highlighting the complex regulation of AC in this cancer type. We observed a negative correlation between reduced serum AC levels and the pro-apoptotic status of tumor tissue, suggesting that systemic AC measurements may provide insight into tumor apoptotic activity.

However, further research with larger patient cohorts is needed to confirm these findings and fully clarify the biomarker relevance of AC in rectal cancer. A deeper understanding of the interplay between ceramide metabolism, inflammation, and tumor progression may improve patient stratification and guide treatment decisions.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Primer sequences and lengths of gene fragments

Gene	**Primer sequence**	**Length of the fragment (bp)**
ASAH1	Forward 5'–TCCTTGATGATCGCAGAACGCC–3' Reverse 5'–ACGGTCAGCTTGTTGAGGAC–3'	121
BCL2	Forward 5'–TCGCCCTGTGGATGACTGA–3' Reverse 5'–CAGAGACAGCCAGGAGAAATC–3'	134
BAX	Forward 5'–TGGCAGCTGACATGTTTTCTGAC–3' Reverse 5'–TCACCCAACCACCCTGGTCTT–3'	195
ACTB	Forward 5'–GGACTTCGAGCAAGAGATGG–3' Reverse 5'–AGGAAGGAAGGCTGGAAGAG–3'	138

Abbreviations: *ASAH1*: N–acylsphingosine amidohydrolase 1; *BCL2:* BCL2 apoptosis regulator; *BAX* : BCL2 associated X, apoptosis regulator; *ACTB:* Actin beta.

Table 2. Demographic and clinical characteristics of patients with locally advanced rectal cancer (*n*=30)

Characteristic	**Value**
Age (years), median (IQR)	66 (13)
Males, *n* (%)	19 (63.3)
Glucose (mmol/L), median (IQR)	5.6 (1.7)
Proteins (g/L), median (IQR)	72 (4.5)
Albumin (g/L), median (IQR)	44 (2)
Cholesterol (mmol/L), median (IQR)	5.4 (1.7)
Triglycerides (mmol/L), median (IQR))	1.5 (0.6)
White blood cells ($\times 10^9$/L), median (IQR)	7 (3)
Lymphocytes ($\times 10^9$/L), median (IQR)	1.9 (1.1)
Neutrophils ($\times 10^9$/L), median (IQR)	4(2)
CRP (mg/L), median (IQR)	3.6 (5.9)
CEA (μg/L), median (IQR)	3.2 (2.9)
CA 19-9 (U/mL), median (IQR)	5.9 (13.2)
T stage at diagnosis, *n* (%)	

T2	1 (3.3)
T3	21 (70.0)
T4	8 (26.4)
N stage at diagnosis, *n* (%)	
N1	7 (23.3)
N2	23 (76.7)
Overall stage, *n* (%)	
IIIA	1 (3.3)
IIIB	7 (23.3)
IIIC	22 (73.4)
Response to nCRT[#], *n* (%)	
TRG1	3 (11.5)
TRG2	2 (7.7)
TRG3	9 (34.6)
TRG4	11 (42.3)
TRG5	1 (3.8)
Outcome, *n* (%)	

Alive	25 (83.3)
Dead	5 (16.7)

#Four patients treated with chemotherapy alone were excluded from the evaluation of nCRT response. Abbreviations: IQR: Interquartile range defined as the difference between the 75th and 25th percentiles; CRP: C-reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; nCRT: Neoadjuvant chemoradiotherapy; TRG: Tumor regression grade.

Table 3. Correlation of acid ceramidase gene and protein expression with serum markers and tumor apoptotic status in patients with locally advanced rectal cancer (*n*=30)

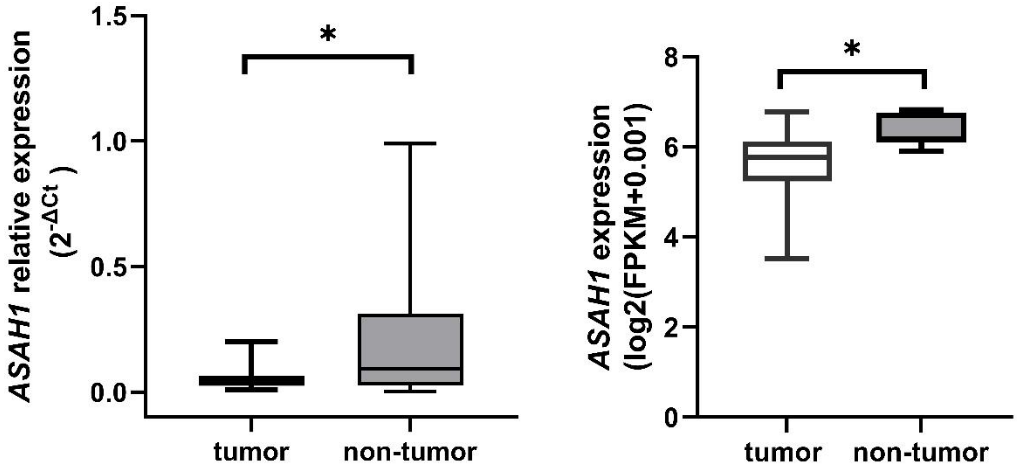
Variable	**CEA (µg/L)**	**CA 19-9 (U/mL)**	**CRP (mg/L)**	**CALLY index**	***BAX/BCL2* ratio**
Tumor tissue *ASAH1* ($2^{-\Delta Ct}$)	rs=0.128	rs=0.215	rs=0.119	rs=−0.164	rs=0.044
	p=0.500 q=0.707	*p*=0.253 q=0.393	*p*=0.530 q=0.707	*p*=0.388 q=0.671	*p*=0.816 q=0.877
	95% BCa CI −0.262–0.488	95% BCa CI −0.167–0.589	95% BCa CI −0.291–0.491	95% BCa CI −0.496–0.207	95% BCa CI −0.367–0.453
Serum AC (ng/mL)	rs=0.013	rs=0.026	rs=0.229	rs=−0.213	rs=−0.536
	p=0.945 q=0.945	*p*=0.890 q=0.890	*p*=0.224 q=0.393	*p*=0.259 q=0.393	*p*=0.002 q=0.021
	95% BCa CI -0.371–0.363	95% BCa CI −0.322–0.352	95% BCa CI −0.118–0.585	95% BCa CI −0.534–0.199	95% BCa CI −0.734–−0.253

Abbreviations: CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CRP: C-reactive protein; CALLY: CRP-albumin-lymphocyte; *BAX*: BCL2 associated X, apoptosis regulator; *BCL2:* BCL2 apoptosis regulator; *ASAH1*: N–acylsphingosine amidohydrolase 1; AC: Acid ceramidase; rs: Spearman correlation coefficient; BCa CI: Bias-corrected and accelerated bootstrap confidence interval; p: Two-tailed p-value from Spearman correlation; q: FDR-adjusted p-value (Benjamini–Hochberg).

Table 4. Association of acid ceramidase at the gene and protein levels, and the CALLY index, with pathological response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer (*n*=26)#

Variable	Good responders (*n*=5)	Moderate responders (*n*=9)	Poor responders (*n*=12)	*p* value
Tumor tissue *ASAH1* ($2^{-\Delta Ct}$), median (IQR)	0.06(0.08)	0.04(0.08)	0.04(0.03)	0.938
Serum AC (ng/mL), median (IQR)	4.2(1.9)	3.9(2.8)	4.1(3.4)	0.451
CALLY index, median (IQR)	3.3(4.9)	2.9 (7.1)	2.4(4.0)	0.845

#Based on the pathological response to therapy, patients were categorized into three groups: good responders—those exhibiting complete (TRG1) and near-complete (TRG2) tumor regression; moderate responders—those with moderate tumor regression (TRG3); and poor responders—those with minimal tumor regression (TRG4) and no regression (TRG5). Statistical significance among these subgroups was evaluated using the Kruskal–Wallis test. Abbreviations: *ASAH1*: N–acylsphingosine amidohydrolase 1; IQR: Interquartile range defined as the difference between the 75th and 25th percentiles; AC: Acid ceramidase; CALLY: CRP-albumin-lymphocyte.



(**A**) (**B**)

Figure 1. Analysis of *ASAH1* gene expression in rectal cancer tissue samples. (A) Relative expression of the *ASAH1* gene in paired tumor (n=30) and adjacent non-tumor (n=30) tissue samples from patients with locally advanced rectal cancer. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test (p=0.004). Data are represented as $2^{-\Delta Ct}$ values in box plots, illustrating the median, interquartile range, and whiskers indicating minimum and maximum values. (B) *ASAH1* gene expression in tumor (n=92) and non-tumor (n=10) rectal tissue samples retrieved from The Cancer Genome Atlas Rectum Adenocarcinoma (TCGA-READ) dataset via the UCSC Xena platform. Statistical significance was assessed using the Mann-Whitney U test (<0.001). Data are presented as $\log_2$-transformed FPKM values with an offset of 0.001, displayed as box plots that show the median, interquartile range, and whiskers indicating minimum and maximum values.

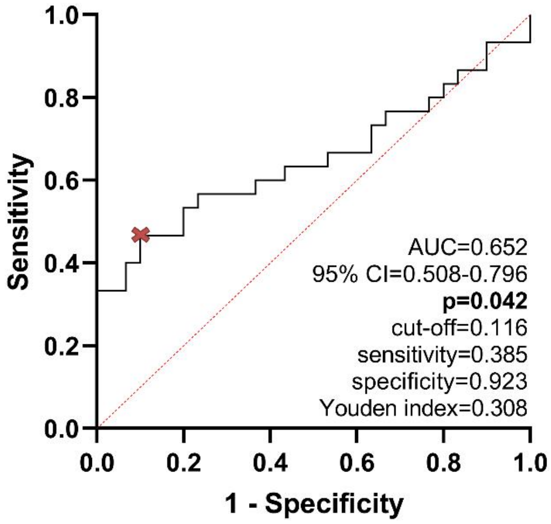


Figure 2. Receiver operating characteristic (ROC) curve for differentiating tumor and non-tumor rectal tissue based on *ASAH1* gene expression. The ROC analysis produced an area under the curve (AUC) of 0.652 (95% CI 0.508–0.796, n=30, p=0.042). The optimal cut-off, indicated by the red symbol, was determined using Youden's index at 0.116, resulting in a sensitivity of 38.5% and a specificity of 92.3%.

SUPPLEMENTAL DATA

Table S1. Univariate Cox regression survival analysis for *ASAH1* gene expression, AC levels, and the CALLY index in patients with locally advanced rectal cancer (*n*=30)

Variable	Hazard ratio (95% CI)	p value
Tumor tissue ASAH1 (2−ΔCt)	10.046 (0.000-65830326.088)	0.773
Serum AC (ng/mL)	1.098 (0.652-1.849)	0.923
CALLY index	1.013 (0.785-1.306)	0.726

Abbreviations: ASAH1: N–acylsphingosine amidohydrolase 1; AC: Acid ceramidase; CALLY: CRP-albumin-lymphocyte.

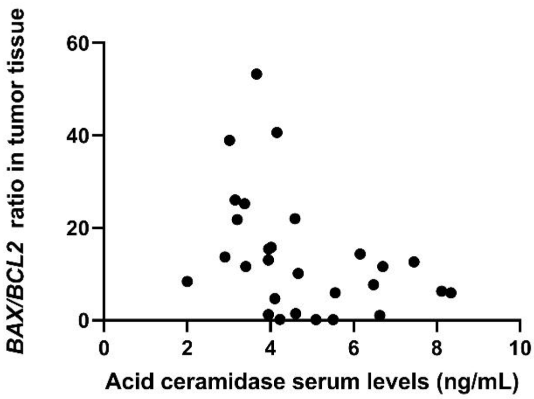


Figure S1. Correlation between serum acid ceramidase (AC) levels and apoptotic status (*BAX/BCL2* ratio) in tumor tissue. The *BAX/BCL2* ratio quantifies the expression ($2^{-\Delta Ct}$) of the pro-apoptotic gene *BAX* relative to the anti-apoptotic gene *BCL2*. The scatter plot illustrates a significant negative correlation between the serum AC levels and the *BAX/BCL2* ratio, assessed using Spearman's rank correlation (rs = −0.536; n = 30; two-tailed p = 0.002; q = 0.021). The p value was adjusted using the False Discovery Rate (FDR) correction according to the Benjamini–Hochberg method. Additionally, the 95% bootstrap confidence interval was calculated, yielding values from −0.734 to −0.253, and the robustness of the correlation was verified through 1,000 bootstrap resamples.