MEGA JOURNAL OF CASE REPORTS

OPEN ACCESS

Case Presentation

Compiled Date: December 09, 2024

The Role of Autophagy in CA 19-9 Positive Calculous Cholecystitis Patients

Muhammad Annus Khan[#], Xiaojun Yu[*], Shouwen Wang, Liujin Hou, Wei Wang, Muhammad Ahmad, Fan Huang, Guobin Wang, Farhatul Ain Arshad and Hongchuan Zhao[*]

The First Affiliated Hospital of Anhui Medical University, Hefei, China

[*]Supervisor

[#]**Corresponding author:** Muhammad Annus Khan, The First Affiliated Hospital of Anhui Medical University, University of Science and technology (USTC), Hefei, China

Abstract

Background: Autophagy is an intracellular process responsible for degrading and repairing damaged organelles and proteins. Autophagy may play a role in tissue repair and inflammatory response, whereas elevated levels of CA 19-9, have been associated with severe inflammation. However, the relationship between autophagy and CA 19-9 levels in calculous cholecystitis remains unclear.

Objective: This study aims to assess the expression of autophagy markers Beclin-1 and LC3A in patients with CA 19-9 positive calculous cholecystitis.

Methods: Clinical data of twenty-four patients divided into Group A (high group with CA19-9 >50, U/mL, n = 8), Group B (normal group with CA19-9 <50 U/mL, n = 8), and Group C (negative control group with CA19-9 <50 U/mL, n = 8) who underwent cholecystectomy were analyzed. Immunohistochemical analysis measured the expression of Beclin-1 and LC3A in gallbladder tissue, and clinical parameters, including gallbladder wall thickness and inflammatory markers, were recorded.

Results: Significantly higher Beclin-1 and LC3A expressions were seen in patients with high CA 19-9 levels (mean areas 15.95% and 20.70%, respectively, $p < 0.01$). This suggests

that elevated CA 19-9 levels are linked to an increase in autophagic activity, which may be associated with worse disease outcomes in patients with calculous cholecystitis.

Conclusion: Beclin-1 and LC3A expression suggest the involvement of autophagic activity in elevated CA 19-9 calculous cholecystitis. Particularly for patients with increased CA 19-9 levels, targeting autophagy may provide novel treatment approaches. To investigate these possibilities, more study is required.

Keywords: Calculous cholecystitis, Gallbladder polyps (Cholesterol Polyps), CA 19-9, Autophagy, Beclin-1, LC3A, Gallbladder inflammation

Introduction

Autophagy is a fundamental cellular mechanism, plays a critical role in maintaining cellular homeostasis by degrading and recycling damaged or dysfunctional cellular components [1]. Cholecystitis, an inflammation of the gallbladder, often results from gallstone formation [2]. CA 19-9, a tumor marker predominantly utilised in the detection of pancreatic cancer, has also been linked in the diagnosis and prognosis of other gastrointestinal illnesses, including cholecystitis [3]. It is thought that Beclin-1 and LC3A, two autophagy-related proteins, are crucial in controlling inflammation and cellular reactions in this condition [4]. In cholecystitis, the autophagy pathway might play a protective role by reducing cellular stress and promoting the clearance of damaged cellular components [3]. It is postulated that in calculous cholecystitis, increased CA 19-9 levels could be associated with altered autophagic activity, potentially mediated by Beclin-1 and LC3A [5]. Depending on the cellular environment, changes in Beclin-1 expression can affect the autophagic flow and result in either increased cell death or greater survival [6]. Autophagosome formation is indicated by the change of LC3A by its cytosolic form (LC3-I) to its bound by the membrane phase (LC3-II), which is a frequently used indicator of autophagic activity [7]. In the context of calculous cholecystitis, investigating LC3A expression could reveal how autophagy is modulated in response to gallstone-induced stress and inflammation [8].

The role of autophagy-related proteins in calculous cholecystitis also extends to their potential as biomarkers for disease diagnosis and prognosis. Beclin-1 and LC3A expression levels could serve as indicators of autophagic activity and cellular stress in cholecystitis patients [6]. Beclin-1 and LC3A are a few of the elements that regulate the autophagy process. Beclin-1 is associated with initiation of autophagy and the generation of AVGs; and LC3A participates in the extension and sealing of autophosomal membrane [9]. The dysfunction of such proteins can result in reduced autophagic activity and the diseases linked to them include; cancer, neurodegenerative diseases, and inflammatory diseases [10]. The relationship between autophagy and CA 19-9 levels in calculous cholecystitis has not been clearly described and clarifying this relation might be useful in revealing the pathophysiology of the disease and possible treatment approaches [11]. However, the detailed impact of the alteration of Beclin-1 and LC3A activity in this connection has not been investigated before [12]. Moreover, increased concentrations of the cancerous marker CA 19-9, which is

frequently used to identify pancreatic cancer, are also observed in calculous cholecystitis affected patients according to [13]. Better biomarkers for the aforementioned condition can be created by determining the correlation between CA 19-9 levels and autophytic activity, hence enhancing diagnosis [14]. Current therapeutic strategies mainly include the common surgical treatment of removal of the gallbladder (cholecystectomy) and using antibacterial agents and analgesics for the alleviation of manifestations [15]. These treatments are beneficial to many patients; however, none of them correct the pathophysiological processes of the disease [16]. Further studies on those molecular mechanisms of Beclin-1 and LC3A in calculous cholecystitis would help in formulating targeted therapeutic strategies for patients [17].

Seeing how the rate of gallstone disease has risen in the population and how costly and invasive it is, there is a pressing need for methods and approaches to diagnose and treat calculous cholecystitis to be investigated [17]. Thus, they include autophagy-related proteins, which can be targeted in the development of new therapeutic strategies; for instance, Beclin-1 and LC3A [18]. Thus, in this work, based on analysis of AEs participating in the conditions of increased CA 19-9 levels, it is planned to fill significant gaps in the current knowledge about calculous cholecystitis and to create a basis for designing more efficient diagnostic and therapeutic approaches [19].

Materials and Methods

This study followed a quantitative research design, focusing on numerical data analysis obtained from tissue samples. The research was conducted at the Hepatobiliary Department of the First Affiliated Hospital of Anhui Medical University, Hefei, China, between June 2023 and July 2024. The hospital's Ethical and Scientific Committee granted ethical approval, and all participants provided informed consent before enrollment.

The study included 24 patients (6 males and 18 females) who underwent cholecystectomy. The inclusion criteria were: Patients with elevated serum CA 19-9 levels (>50 U/mL) diagnosed with calculous cholecystitis. Completion of laboratory and imaging tests (routine blood tests, abdominal ultrasonography, and MRI) within one week of surgery. No history of liver disease, bile duct stones, pancreatitis, cancer, or neoplastic conditions. Final diagnosis confirmed by pathological analysis from the Pathology Laboratory of the First Affiliated Hospital of Anhui Medical University. The 24 patients were divided into three groups: Group A (High CA 19-9 group): 2 males, 6 females (mean age: 44.38 ± 13.77 years), Calculous cholecystitis with serum CA 19-9 >50 U/mL. Group B (Normal CA 19-9 group): 3 males, 5 females (mean age: 47.25 ± 17.69 years), Calculous cholecystitis with serum CA 19-9 <37 U/mL. Group C (Negative control group): 1 male, 7 females (mean age: 48.13 ± 11.97 years). Cholesterol polyps with normal serum CA 19-9 levels (<37 U/mL). Gallbladder tissue samples were preserved in 10% formalin solution, which later on embedded in paraffin and processed with hematoxylin and eosin (IHC) staining to analyze histological changes in the mucosa. Optical microscopy was used to assess tissue integrity and pathology, with images captured for further analysis.

Immunohistochemistry

The Envision Flex Kit (Dako Cytomation) was used for immunohistochemical analysis to detect Beclin-1 and LC3A proteins. Tissue sections were formalin-fixed and paraffin-embedded (FFPE), dried at 66°C for 30 minutes before staining. Tissue sections were deparaffinized in xylene and rehydrated through graded ethanol solutions (100%, 95%, and 80%). 3% hydrogen peroxide was used to block endogenous peroxidase activity. Slides were boiled in citrate buffer (pH 6.0) using a pressure cooker for antigen retrieval. After cooling, EBIOGO 3% Peroxidase-Blocking Solution was applied for 5 minutes at 37°C. Slides were rinsed with PBST buffer (Phosphate-Buffered Saline with Tween 20). Primary antibodies (Beclin-1 and LC3A) were applied at 1:200 dilutions (BIOSS Bs-1353R) and incubated for 60 minutes at 37°C. Secondary antibodies (Rabbit/Mouse) were applied for 30 minutes at 37°C. Staining was visualized using EBIOGO DAB color developer, and slides were counterstained with hematoxylin for 2-5 minutes. Slides were washed with PBST, dehydrated through ethanol, cleared in xylene, and mounted with synthetic resin. Beclin-1 and LC3A expression were analyzed under an Olympus CX41 microscope, with two independent observers reviewing the slides for consistency.

Evaluation of Immunohistochemical Staining

Tissue sections were evaluated at 5x magnification using ImageJ software. Areas with moderate to severe inflammation were analyzed, while regions with metaplastic epithelium were excluded. Positive expression (>5%) indicated high Beclin-1 or LC3A levels, while negative expression (<5%) indicated low levels.

Statistical Analysis

Clinical data were managed in Excel. Statistical analyses, including one-way ANOVA, chi-square tests, t-tests, and Tukey's multiple comparisons, were performed using GraphPad Prism 10.1.1 (324). A P-value <0.05 was considered statistically significant. Immunohistochemical results were analyzed using ImageJ software to quantify protein expression.

Results

Table 1 provides a summary of the demographic and clinical characteristics of patients, categorized into three groups based on their serum CA 19-9 levels: High, Normal, and Negative Control groups. In terms of serum CA 19-9 levels, all patients in the High Group had levels exceeding 37 U/ml, with 38% of patients in the 37-111 U/ml range, another 38% in the 111-370 U/ml range, and 24% with levels above 370 U/ml. Both the Normal and Negative groups exhibited serum CA 19-9 levels below 37 U/ml. The difference in CA 19-9 levels between the High Group and both the Normal Group (P1 = 0.0035) and Negative Group (P2 = 0.0032) is statistically significant, but there is no difference between the Normal and Negative groups (P3 = 0.9993).

Table 1: Demographic analysis of calculous cholecystitis patients (high and normal groups) compared to a negative control group.

	High group (n=8)	Normal group (n =8)	Negative group (n =8)	P1	P2	P3
Age (years)	44.38 ±13.77	47.25 ±17.69	48.13 ±11.97	0.9192	0.8668	0.9922
Gender						
Male	2 (25%)	3 (38%)	1 (12%)			
Female	6 (75%)	5 (62%)	7 (88%)			
Serum CA19-9 levels				**0.0035**	**0.0032**	0.9993
<37U/ml	0(0%)	8 (100%)	8 (100%)			
37-111U/ml	3(38%)					
111-370U/ml	3 (38%)					
>370	2 (24%)					

Data are presented as mean ± SD., P1: High group vs. Normal Group, P2: High group vs. Negative Control, P3: Normal Group vs. Negative Control, Analyzed using Tukey's multiple comparisons test.

Table 2 reports the blood indices, liver function tests, inflammatory markers, gallbladder wall thickness, and operation duration across the three patient groups. Notably, the High Group shows a significantly higher White Blood Cell (WBC) count, The difference in WBC count in between the High Group and both the Normal and both the Normal Group (P1 = 0.0024) and Negative Group (P2 = 0.0017) is statistically significant, but there is no difference between the Normal and Negative groups (P3 = 0.9880) suggesting a pronounced inflammatory response. Similarly, the neutrophil (P1 = <0.0001: P2 = <0.0001: P3 = 0.9546) and lymphocyte (P1 = 0.0325: P2 = 0.0425: P3 = 0.9912) have statistically significant difference, which indicate an active immune response. Gallbladder wall thickness, assessed through MRI, is significantly greater in the High Group and have significant statistical difference (P1<0.0001) compared to the Normal (P2<0.0001) and Negative groups (P3=0.7933). This suggests more severe inflammation and structural changes in patients with elevated CA 19-9 levels. Furthermore, operation duration is longer and also have statistical difference in the High Group (approximately 115 minutes, P1 =0.0032) compared to the Normal (75 minutes, P2 = 0.0013) and Negative groups (70 minutes, P3=0.9185). This indicates that higher CA 19-9 levels are associated with more complex surgical procedures.

Table 2: Pre-operative laboratory findings of calculous cholecystitis patients (high and normal groups) compared to a negative control group.

	High group (n =8)	**Normal group (n =8)**	**Negative group (n =8)**	**P1**	**P2**	**P3**
ALB (g/L)	46.88± 1.98	43.39± 3.42	46.94 ± 3.75	0.0916	0.9991	0.0848
TB (µmol/L)	13.87±3.02	11.01±2.89	11.34±4.25	0.2421	0.3248	0.9795
DB (µmol/L)	2.75±1.26	3.85±3.65	3.18±2.20	0.6701	0.9410	0.8584
ALT (U/L)	22.58±13.55	23.69±12.33	25.90±13.75	0.9845	0.8708	0.9403
AST (U/L)	24.21±7.50	22.03±9.164	22.68±6.589	0.8430	0.9187	0.9849
ALP (U/L)	81.39±31.01	86.81±26.87	76.41±35.23	0.9358	0.9457	0.7854
GGT (U/L)	35.80±34.36	83.41±188.7	39.03±23.48	0.6746	0.9982	0.7096
WBC × 10^9/L	9.98±1.58	6.48±2.35	6.35±1.32	**0.0024**	**0.0017**	0.9880
NEUT %	72.28±3.98	53.60±5.25	52.64±9.39	**<0.0001**	**<0.0001**	0.9546
LYM %	41.94±3.61	34.69±5.81	35.03±6.16	**0.0325**	**0.0425**	0.9912
MONO %	5.87±1.06	6.00±2.43	7.18±3.40	0.9945	0.5529	0.6140
NLR	1.73±0.158	1.60±0.41	1.56±0.43	0.7549	0.6087	0.9684
LMR	7.37±1.60	6.52±2.49	5.44±1.85	0.6800	0.1611	0.5464
HGB (g/L)	139.4±5.06	136.9±25.22	142.1±11.47	0.9493	0.9391	0.7968
PLT × 10^9/L	256.6±57.51	226.1±84.89	231.5±41.62	0.6127	0.7152	0.9845
Gallbladder thickness (mm)	7.87±1.97	3.55±0.47	3.13±0.91	**<0.0001**	**<0.0001**	0.7933
Operation duration (min)	113.1±10.67	75.00±26.19	71.00±20.96	**0.0032**	**0.0013**	0.9185

ALB: Albumin, TB: Total bilirubin, DB: Direct bilirubin, ALT: Alanine transaminase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl transpeptidase, WBC: White Blood Cell, NEUT: Neutrophils, LYM: Lymphocytes, MONO.: Monocyte, NLR: Neutrophil-lymphocytes ratio, LMR: lymphocytes-monocyte ratio, HGB: Hemoglobin, PLT: Platelets,, Gall bladder wall thickness: observed by MRI, operation duration: recorded in minutes. Data are presented as mean ± SD., P1: High group vs. Normal Group, P2: High group vs. Negative Control, P3: Normal Group vs. Negative Control, Data analyzed using Tukey's multiple comparisons test.

In **Figure 1A**, patients in the High Group demonstrate significantly elevated CA 19-9 levels compared to both the Normal and Negative groups. The Normal and Negative Control groups show similarly low CA 19-9 levels, suggesting that the elevation is primarily associated with severe calculous cholecystitis. **Figure 1B** shows pre- and postoperative CA 19-9 levels in the High Group, where levels as high as 800 U/ml drop dramatically to near-zero after surgery. This decrease highlights the effectiveness of cholecystectomy in resolving the underlying pathology and normalizing CA 19-9 levels.

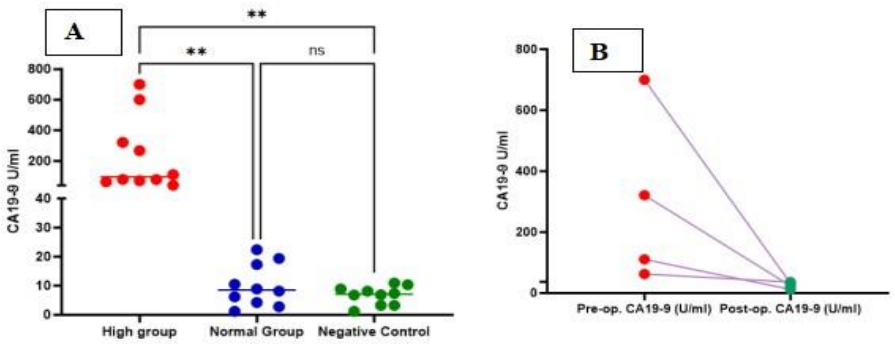

Figure 1: Serum CA 19-9 Comparisons in different groups of patients. Figure 1A: Comparison of serum CA 19-9 levels between the high and normal group of calculous cholecystitis patients with negative control group. Figure 1B: Comparison of serum CA 19-9 levels before and after surgery in the high group of calculous cholecystitis patients. Asterisks (**) denote statistically significant p-values, while "ns" indicates "not significant."

Figure 2A illustrates that patients with elevated CA 19-9 levels (High Group) have thicker gallbladder walls (around 8 mm) compared to the Normal and Negative groups (approximately 3.5 mm). The positive correlation between CA 19-9 levels and gallbladder thickness ($r = 0.8630$, $P < 0.0001$) shown in **Figure 2B** suggests that higher CA 19-9 levels are associated with more severe inflammation.

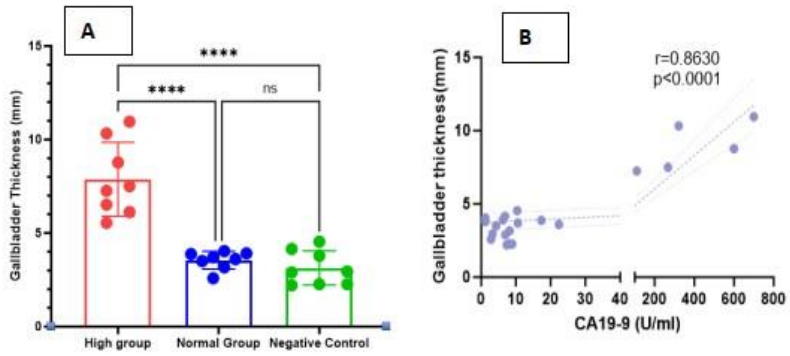

Figure 2: Gallbladder Wall Thickness and Correlation with Serum CA 19-9 in different group of patients. Figure 2A: Comparison of gallbladder wall thickness in calculous cholecystitis patients among the high and normal with negative control group. Figure 2B: Positive correlation between gallbladder wall thickness and serum CA 19-9 levels in different group of patients. "****" indicate for p-values Statistical significance, while Notation "ns" is for “not significant”.

Figure 3A shows significantly elevated WBC counts in the High Group, reflecting a strong systemic inflammatory response. A moderate positive correlation between WBC counts and CA 19-9 levels ($r = 0.5615$, $P = 0.0043$) is depicted in **Figure 3B**, indicating that CA 19-9 may reflect inflammation severity.

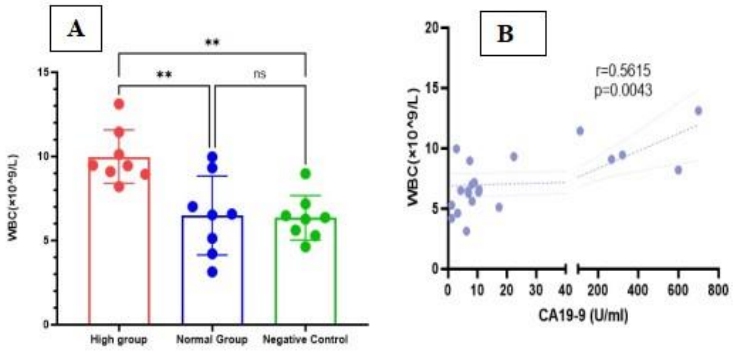

Figure 3: Comparison of WBC counts and correlation with serum CA 19-9 levels in different group of patients. Figure 3A: Comparison of WBC counts in calculous cholecystitis patients among high and normal with negative control group. Figure 3B: Correlation between WBC counts and serum CA 19-9 levels in different group of patients. "**" for P-values indicate statistical significance, while "ns" denoting “not significant.”

Table 3: Area percentage of Beclin-1 expression in gallbladder epithelial cells from high and normal CA19-9 calculus cholecystitis patients compared to negative control group.

Beclin-1	High n=8	Normal n=8	Negative n=8
Positive (Area >5%)	7 (87%)	2 (25%)	2 (25%)
Negative (Area < 5%)	1 (13%)	6 (75%)	6 (75%)

Figure 4A reveals that neutrophil counts are significantly higher in the High Group compared to other groups, suggesting greater immune activation. **Figure 4B** shows a moderate positive correlation between CA 19-9 levels and neutrophil counts (r = 0.6594, P = 0.0005), reinforcing the role of CA 19-9 as an inflammation marker.

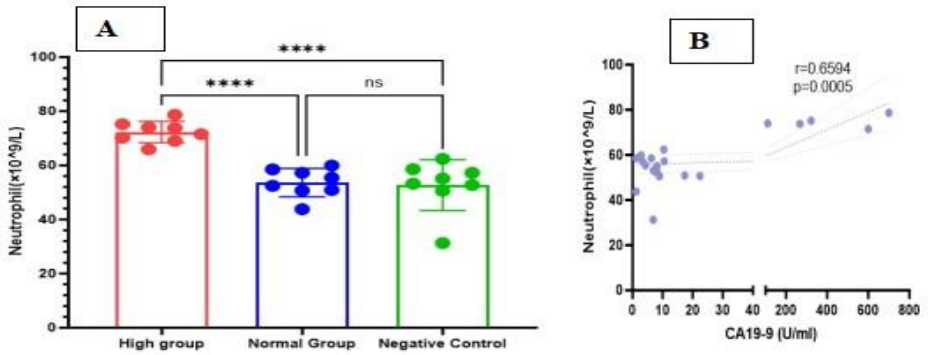

Figure 4: Comparison of neutrophil counts and correlation with serum CA 19-9 levels in different group of patients. Figure 4A: Comparison of neutrophil counts in calculous cholecystitis patients among high and normal with negative control group. Figure 4B: Positive correlation between neutrophil counts and serum CA 19-9 levels in different group of patients. "****" indicate for P-values statistical significance, with "ns" denoting "not significant."

Table 4: Area percentage of LC3A expression in gallbladder epithelial cells from high and normal CA19-9 calculus cholecystitis patients compared to negative control group.

LC3A	High n=8	Normal n=8	Negative n=8
Positive (Area >5%)	7 (87%)	2 (25%)	2 (25%)
Negative (Area < 5%)	1 (13%)	6 (75%)	6 (75%)

Figure 5 shows that lymphocyte counts are elevated in the High Group (approximately 45 x 10^9/L) compared to the other groups. This suggests an enhanced immune response in patients with elevated CA 19-9 levels.

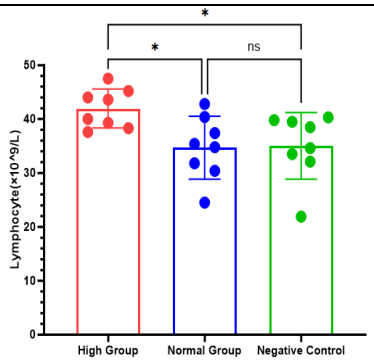

Figure 5: Comparison of Lymphocytes in calculous cholecystitis patients of high and normal with negative control group."*" indicate for p-values Statistical significance, while Notation "ns" is for "not significant".

Figure 6A compares operation duration among the groups, with the High Group showing the longest surgical times. **Figure 6B** presents a moderate positive correlation (r = 0.5620, P = 0.0043) between CA 19-9 levels and operation duration, indicating that elevated CA 19-9 levels are associated with more complex surgical interventions.

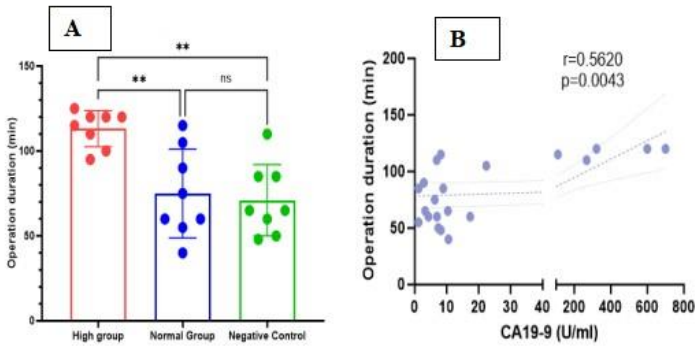

Figure 6: Overview of operation time and correlation with serum CA 19-9 levels in different group of patients. Figure 6A: Comparison of operation time in calculous cholecystitis patients among high and normal with negative control group. Figure 6B: Positive correlation between operation time and serum CA 19-9 levels in different group of patients. "**" indicate P-values statistical significance, with "ns" denoting “not significant.”

Table 3 and Table 4 show that 87% of patients in the High Group exhibit positive Beclin-1 and LC3A expression, indicating elevated autophagy activity. In contrast, only 25% of patients in the Normal and Negative groups show positive expression.

In **Table 5** we assessed that the High group shows significantly higher Beclin-1 expression compared to both the Normal and Negative groups, with P-values of 0.0024 and 0.0022, respectively. There is no significant difference between the Normal and Negative groups (P3 = 0.9994), indicating that Beclin-1 expression is low and similar in these groups. Whereas LC3A shows significantly higher expression in high group compared to the Normal and Negative groups, with very low P-values (<0.0001), indicating a highly significant difference. Similar to Beclin-1, LC3A expression is not significantly different between the Normal and Negative groups (P3 = 0.9446). These findings reinforce the hypothesis that autophagy plays a significant role in the pathophysiology of calculous cholecystitis, particularly in cases with elevated CA 19-9 levels.

Table 5: Statistical difference between Beclin-1 and LC3A expression in gallbladder epithelial cells from high and normal CA19-9 calculus cholecystitis patients compared to negative control group.

	High group (n =8)	**Normal group (n =8)**	**Negative group (n =8)**	**P1**	**P2**	**P3**
Beclin-1	15.95±9.71	3.86±2.34	3.75±4.09	**0.0024**	**0.0022**	0.9994

LC3A	20.70±10.10	4.05±1.25	3.08±2.23	**<0.0001**	**<0.0001**	0.9446

LC3A: isoform of microtubule-associated protein 1 light chain 3. Data are presented as mean ± SD., P1: High group vs. Normal Group, P2: High group vs. Negative Control, P3: Normal Group vs. Negative Control, Data analyzed using Tukey's multiple comparisons test.

In **Figure 7**. We have observed the tissue samples using Immunohistochemistry (IHC) staining, these slides showing Beclin-1 expression in gallbladder epithelial cells across different patient groups: High Group: This section labeled A, B, C: show strong brown staining, indicating higher Beclin-1 expression, particularly in the section labeled A. The intense staining suggests a higher level of autophagy in this group. This could imply that autophagic activity is upregulated, possibly as a cellular response to stress or damage, such as bile obstruction or inflammation. Normal Group: labeled D, E, F shows moderate to lowest staining visible in these panels, with less intensity compared to the High Group. Beclin-1 is expressed, but at lower levels. This indicates normal autophagic activity, which is likely consistent with physiological conditions where there is no excessive cellular stress. Negative Control group: labeled G, H, I show little to no brown staining is observed in these section which serves as a baseline control, confirming that any visible staining in the other groups is specific to Beclin-1. The absence of staining here shows that without specific cellular triggers, autophagy is minimal.

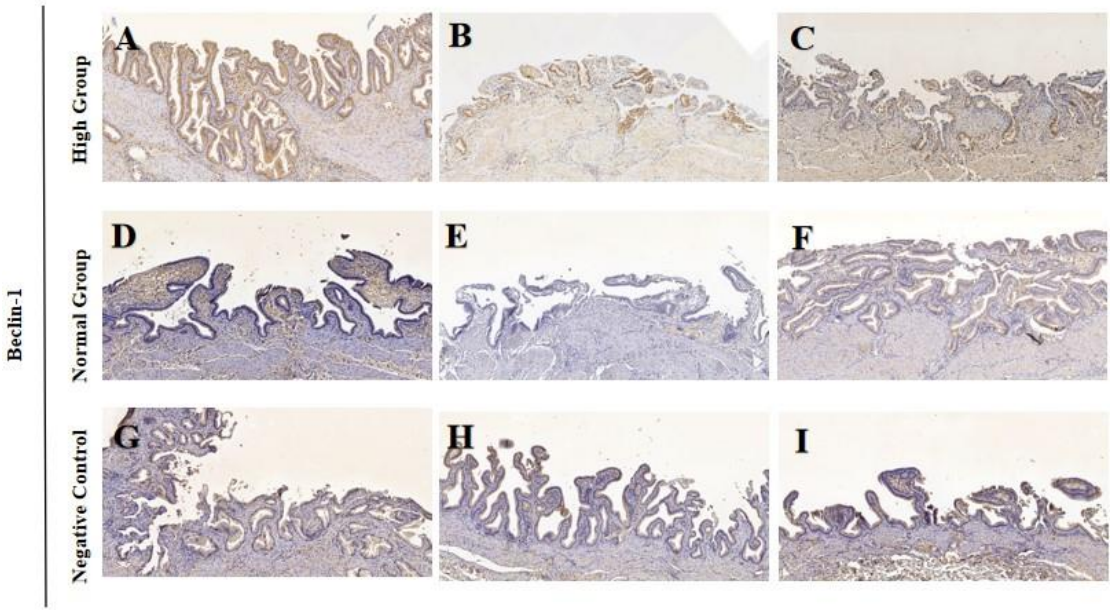

Figure 7: Expression of Beclin-1 in gallbladder epithelial (magnification x05) cells of high and normal CA19-9 calculus cholecystitis patients compared to negative control group.

In **Figure 8**. We have observed the tissue samples using Immunohistochemistry (IHC) staining, these images focuses on the expression of LC3A in gallbladder epithelial cells across different patient groups: High Group: The sections labeled A, B, C shows strong brown staining is observed, similar to the Beclin-1 slide, which indicates high levels of autophagic activity, with LC3A playing a role in the

formation of autophagosomes. It aligns with the Beclin-1 findings, further suggesting elevated autophagy in the High Group, likely as a response to stress (e.g., inflammation or gallstone obstruction). Normal Group: sections labeled D, E, F shows Moderate or light staining is present, showing lower intensity compared to the High Group. The expression of LC3A is relatively normal, representing baseline autophagic activity. This fits with physiological processes in tissues with no significant pathological stress. Negative Control group: sections labeled G, H, I) shows Little to no staining, similar to the Beclin-1 negative control slide, which confirms that the LC3A staining in other groups is specific, and any staining observed in the High or Normal groups reflects true autophagic activity.

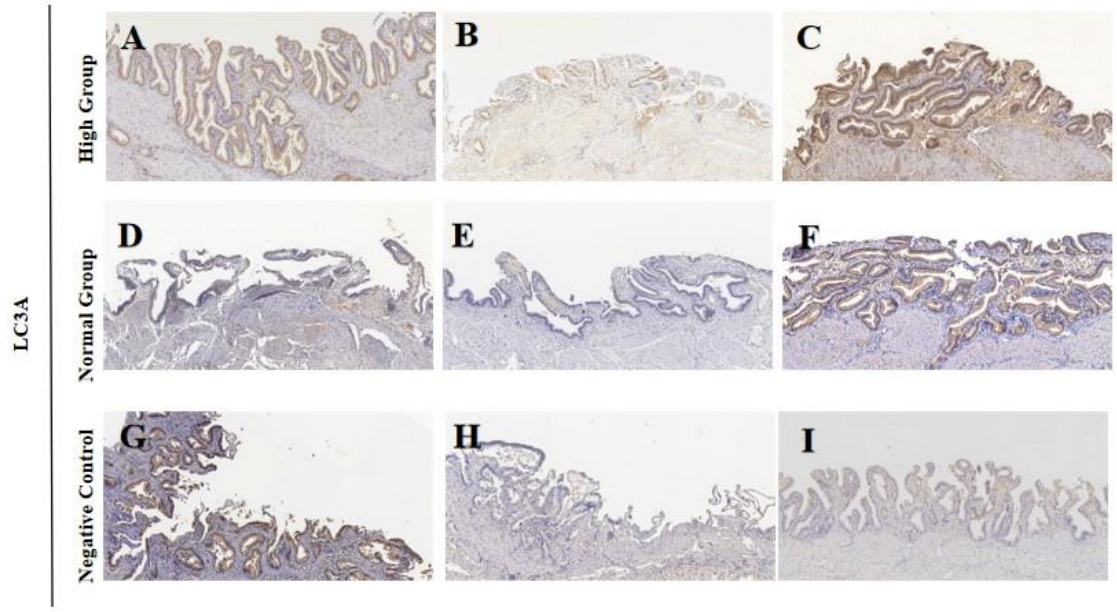

Figure 8: Expression of LC3A in gallbladder epithelial (magnification x05) cells of high and normal CA19-9 calculus cholecystitis patients compared to negative control group.

Correlation with Beclin-1 Findings: Both Beclin-1 and LC3A show elevated expression in the High Group, indicating autophagy is upregulated. This suggests a consistent autophagic response to the pathological state (e.g., inflammation, bile stasis) present in the High Group. These findings reinforce the notion that autophagy could be a key player in modulating the disease's progression.

Figure 9 provides a quantitative comparison of Beclin-1 and LC3A expression, measured using ImageJ software. Both markers show significantly higher expression in the High Group, correlating with increased disease severity. The differences between the High Group and the other groups are statistically significant ($P < 0.01$ for Beclin-1, $P < 0.0001$ for LC3A). These findings confirm that autophagy plays a crucial role in the pathophysiology of calculous cholecystitis, particularly in patients with elevated CA 19-9 levels. The results suggest that CA 19-9 serves as a reliable marker for tracking disease severity and may reflect autophagy-related processes driving gallbladder inflammation.

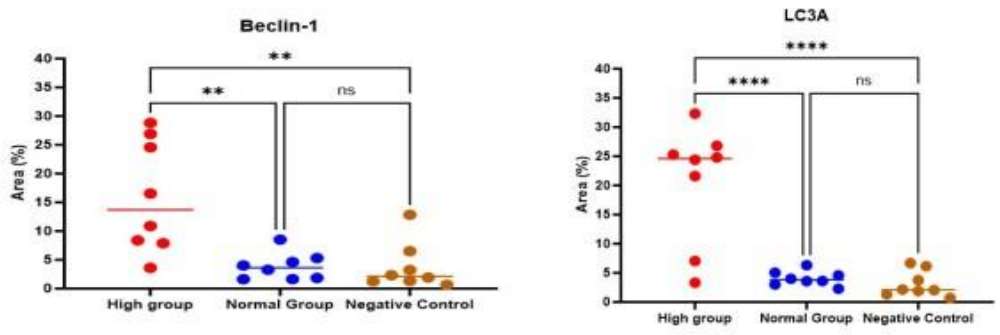

Figure 9: Positive area percentage of Beclin-1 and LC3A expression in gallbladder epithelial cells from high and normal CA19-9 calculus cholecystitis patients compared to negative control group. LC3A: isoform of microtubule-associated protein 1 light chain 3. "*" indicate for p-values Statistical significance, while Notation "ns" is for “not significant”.

Discussion

This present study investigate the expression of two important autophagy markers in gallbladder epithelial cells—Beclin-1 and LC3A—to determine the role of autophagy in patients with calculous cholecystitis, especially those with elevated CA 19-9 levels. The results imply a strong relationship between the severity of calculous cholecystitis, raised CA 19-9 levels, and enhanced autophagy activity. To the best of our knowledge, this work is the first to focus on the autophagy process in the gallbladder epithelium of patients with CA 19-9 positive calculous cholecystitis.

Autophagy Marker Expression Beclin-1 and LC3A

Our results demonstrate that both Beclin-1 and LC3A expressions are significantly elevated in the High Group, characterized by elevated CA 19-9 levels, in contrast to the Normal and Negative Control Groups. The quantitative analysis **(Table 5)** and corresponding IHC images **(Figures 7 and 8)** reveal that patients with high CA 19-9 levels exhibit markedly enhanced staining for Beclin-1 and LC3A, indicating active autophagy. Specifically, the High Group showed an average Beclin-1 expression of 15.95% **(Table 5)**, significantly higher than the 3.86% and 3.75% observed in the Normal and Negative Control Groups, respectively. Similarly, LC3A expression in the High Group averaged 20.70%, compared to 4.05% and 3.08% in the Normal and Negative Control Groups. Given that the High Group exhibited higher expression of these autophagy markers, it is plausible that autophagy plays a key factor in the development and course of calculous cholecystitis in individuals whose CA 19-9 levels are raised. This is consistent with other research showing that autophagy is activated in response to inflammation and cellular stress, which are probably more prominent in individuals with advanced illness. It has long been known that Beclin-1 and LC3A are accurate indicators of autophagic structures **[20]**. Although Ki-67 is frequently used as a prognostic index in a number of cancers, such as chordoma **[21]**, non-muscle invasive bladder cancer **[22]**, superficial non-invasive papillary urothelial neoplasms of the bladder **[23]**, prostate cancer **[24]**, Beclin-1 is similarly linked to carcinogenesis in a variety of neoplastic contexts, including ovarian **[25]**

and gastric cancers [26], as well as breast phyllodes tumours [27], sarcoma, and neuroendocrine tumours of the gastrointestinal tract and pancreas [28]. Additionally, glioblastomas [29], keratoacanthomas [30], and B-cell lymphomas [31] have all been shown to express LC3A. According to [30,32], cutaneous squamous cell carcinomas exhibit LC3A reactivity, which is characterised by unique cytoplasmic, "Stone-Like" Structures (SLS) and cytoplasmic/perinuclear structures, which show up as large, amorphous, densely stained material within cytoplasmic vacuoles [26] found that LC3A exhibits a similar pattern in gastric cancer that is supported by prior research on the protein's behavior under stress [32]. Beclin-1 and LC3A both showed cytoplasmic expression in our investigation.

Clinical Correlation

CA 19-9 Levels, Gallbladder Thickness, and Inflammatory Markers: Further supporting the idea that increased CA 19-9 is a sign of more severe illness is the substantial link seen **(Figure 1A, 2A and 2B)** between CA 19-9 levels, gallbladder wall thickness, and inflammatory markers. In addition to having noticeably thicker gallbladder walls **(Figure 2A)**, patients in the High Group also had higher WBC, neutrophil, and lymphocyte counts **(Figure 3A, 4A and 5)**, which may indicate an elevated inflammatory response. This is in line with the High Group's lengthier operation times **(Figure 6A and B)**, which suggest that these patients probably have more difficult and complicated surgical procedures. Neutrophil count ($r = 0.6594$, $p = 0.0005$), operation duration ($r = 0.5620$, $p = 0.0043$), and WBC ($r = 0.5615$, $p = 0.0043$) all showed positive correlations with CA 19-9 levels, indicating that CA 19-9 may be a valuable biomarker for predicting the degree of inflammation and the complexity of surgical intervention needed. These results highlight the significance of CA 19-9 in calculous cholecystitis not only as a diagnostic marker but also as a possible predictor of disease progression and surgical risk. In animal models, the expression of autophagy has been connected to long-term inflammatory diseases such asthma [33]. On the other hand, the literature on the connection between autophagy and chronic inflammation is scarce. This is the first research to examine the expression of two autophagy markers, Beclin-1 and LC3A, in the gallbladder epithelium of patients with CA 19-9 positive calculous cholecystitis to gallbladder epithelium in normal CA 19-9 calculous cholecystitis patients and gallbladder polyps.

Implications of Autophagy in Disease Progression

The elevated autophagy activity in the High Group, as indicated by increased Beclin-1 and LC3A expression, may have several implications for the pathophysiology of calculous cholecystitis. The role of autophagy in calculous cholecystitis appears to be double-edged, while autophagy may help in managing cellular stress, its excessive activation, as observed in the High Group, could contribute to the persistence of inflammation and tissue damage, thereby exacerbating the disease. This is supported by the observation that patients with the highest autophagy marker expression also had the most severe clinical presentations, including thicker gallbladder walls and longer surgical times. Given that autophagic regulation is crucial in both carcinogenesis and cancer therapy, investigating the autophagy process in chronic inflammation, which could

be cancerous, is imperative [34]. It is well recognised that long-term inflammation leads to genetic and epigenetic changes that may raise the risk of gallbladder calculous epithelial tumour growth [35]. Although autophagy is associated with the inflammatory process, its precise role remains unclear [36,37].

Conclusion

Our results show for the first time a robust association between Beclin-1 expression and LC3A in CA 19-9 positive calculous gallbladder epithelium. Beclin-1 and LC3A overexpression in this situation suggests that gallstones not only increase CA 19-9 levels but also improve autophagy in the gallbladder epithelium. This highlights the need of conducting more research on the function of autophagy in the gallbladder epithelium, especially when bile stones are present, as a possible prelude to cancer.

References

1. Russell RC, KL. Guan. The multifaceted role of autophagy in cancer. The EMBO journal, 2022;41(13):e110031.
2. Helal DS, et al. MicroRNA Let-7a association with glycolysis-induced autophagy in locally advanced gastric cancer: Their role in prognosis and FLOT chemotherapy resistance. Pathology-Research and Practice. 2024;253:154968.
3. Rezazadeh D, et al. Rapamycin reduces cervical cancer cells viability in hypoxic condition: Investigation of the role of autophagy and apoptosis. OncoTargets and therapy. 2020:4239-4247.
4. Devis-Jauregui L, et al. Autophagy in the physiological endometrium and cancer. Autophagy. 2021;17(5):1077-1095.
5. Devis L, et al. Autophagy in the physiological endometrium and cancer. 2020.
6. Qureshi-Baig K, et al. Hypoxia-induced autophagy drives colorectal cancer initiation and progression by activating the PRKC/PKC-EZR (ezrin) pathway. Autophagy. 2020;16(8):1436-1452.
7. Lin F, YT Zhu, ZH Qin. Biomarkers of autophagy. Autophagy: Biology and Diseases: Technology and Methodology. 2021;265-287.
8. Wong MMT, et al. Expression of LC3A, LC3B and p62/SQSTM1 autophagy proteins in hepatocellular carcinoma (HCC) tissues and the predicted microRNAs involved in the autophagy-related pathway. Journal of Molecular Histology. 2024;1-12.
9. Song H, et al. Negative pressure wound therapy promotes wound healing by suppressing macrophage inflammation in diabetic ulcers. Regenerative Medicine. 2020;15(12):2341-2349.
10. Rana T, et al. Exploring the role of autophagy dysfunction in neurodegenerative disorders. Molecular neurobiology. 2021;58(10):4886-4905.
11. Yaribeygi H, et al. Glp-1 Mimetics and Autophagy in Diabetic Milieu: State-of-the-Art. Current Diabetes Reviews. 2024;20(10):93-101.

12. Xue Z, et al. PACAP neuropeptide promotes Hepatocellular Protection via CREB-KLF4 dependent autophagy in mouse liver Ischemia Reperfusion Injury. Theranostics. 2020;10(10):4453-4465.
13. Chen W, et al. High level of tumor marker CA19-9 returned to normal after cholecystectomy in calculous cholecystitis patients. Scandinavian Journal of Gastroenterology. 2023;58(6):643-648.
14. Zhao, X., et al., An overview of autophagy in the differentiation of dental stem cells. Biocell. 2024;48(1):47-64.
15. Van Dijk A, et al. Systematic review of antibiotic treatment for acute calculous cholecystitis. Journal of British Surgery. 2016;103(7):797-811.
16. Pisano M, et al. 2020 World Society of Emergency Surgery updated guidelines for the diagnosis and treatment of acute calculus cholecystitis. World journal of emergency surgery. 2020;15:1-26.
17. Kuhlenschmidt KM, LR Taveras, MW Cripps. Current Management of Acute Calculous Cholecystitis. Current Surgery Reports. 2021;9:1-9.
18. Unalp-Arida A, CE Ruhl. The burden of gallstone disease in the United States population. medRxiv. 2022;2022.07.
19. Bagepally BS, et al. Systematic review and meta-analysis of gallstone disease treatment outcomes in early cholecystectomy versus conservative management/delayed cholecystectomy. BMJ Open Gastroenterology. 2021;8(1):e000675.
20. Klionsky DJ, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2021;17(1):1-382.
21. von Witzleben A, et al. In chordoma, metastasis, recurrences, Ki-67 index, and a matrix-poor phenotype are associated with patients' shorter overall survival. European Spine Journal. 2016;25:4016-4024.
22. Ko K, et al. Significance of Ki-67 in non-muscle invasive bladder cancer patients: a systematic review and meta-analysis. Oncotarget. 2017;8(59):100614.
23. Ogata DC, et al. Superficial papillary urothelial neoplasms of the bladder (PTA E PT1): correlation of expression of P53, KI-67 and CK20 with histologic grade, recurrence and tumor progression. Revista do Colegio Brasileiro de Cirurgioes. 2012;39:394-400.
24. Fisher G, et al. Prognostic value of Ki-67 for prostate cancer death in a conservatively managed cohort. British journal of cancer. 2013;108(2):271-277.
25. Kulshrestha A, et al. Targeting V-ATPase isoform restores cisplatin activity in resistant ovarian cancer: inhibition of autophagy, endosome function, and ERK/MEK pathway. Journal of Oncology. 2019;2019(1):2343876.
26. Giatromanolaki A, et al. LC3A, LC3B and Beclin-1 expression in gastric

cancer. Anticancer research, 2018;38(12):6827-6833.
27. Kleer CG, et al. Pathologic, immunohistochemical, and molecular features of benign and malignant phyllodes tumors of the breast. Modern Pathology. 2001;14(3):185-190.
28. Dhall D, et al. Ki-67 proliferative index predicts progression-free survival of patients with well-differentiated ileal neuroendocrine tumors. Human pathology. 2012;43(4):489-495.
29. Mitrakas AG, et al. Autophagic flux response and glioblastoma sensitivity to radiation. Cancer Biology & Medicine. 2018;15(3):260.
30. Sivridis E, et al. Patterns of LC3A Autophagy Protein Expression in Keratoacanthomas. Head and Neck Pathology. 2020;14:150-155.
31. Giatromanolaki A, et al. Overexpression of LC3A autophagy protein in follicular and diffuse large B-cell lymphomas. Hematology/oncology and stem cell therapy. 2013;6(1):20-25.
32. Zois CE, et al. " Autophagic flux" in normal mouse tissues: focus on endogenous LC3A processing. Autophagy. 2011;7(11):1371-1378.
33. Ran Q, et al. Effects of 3-methyladenine on airway inflammation, airway hyperresponsiveness and mucus secretion in asthmatic mice. Zhonghua jie he he hu xi za zhi= Zhonghua Jiehe he Huxi Zazhi= Chinese Journal of Tuberculosis and Respiratory Diseases. 2019;42(3):185-192.
34. Panda PK, et al. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. in Seminars in cell & developmental biology. 2015. Elsevier.
35. Murata M. Inflammation and cancer. Environmental health and preventive medicine, 2018;23(1):50.
36. Sasaki M, et al. Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Laboratory Investigation. 2010;90(6):835-843.
37. Haq S, et al. Autophagy: roles in intestinal mucosal homeostasis and inflammation. Journal of Biomedical Science. 2019;26:1-14.

Citation of this Article

Khan MA, Yu X, Wang S, Hou L, Wang W, Ahmad M, Huang F, Wang G, Arshad FA and Zhao H. The Role of Autophagy in CA 19-9 Positive Calculous Cholecystitis Patients. Mega J Case Rep. 2024;7(12):2001-2017.