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Histopathological and Immunohistochemical Expression of Ki-67 and Alpha-Fetoprotein as Indicators in Various Gallbladder Diseases

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ABSTRACT

Background: Gallbladder disease encompasses a spectrum of conditions affecting the gallbladder, including cholelithiasis (gallstones), cholecystitis (inflammation), gallbladder polyps, and gallbladder cancer. These disorders are influenced by multiple factors such as genetic predisposition, metabolic dysfunction, inflammatory pathways, and biliary stasis. **Aim:** This study aimed to evaluate the histopathological and immunohistochemical expression of Ki-67 and alpha-fetoprotein (AFP) in gallbladder tissue.

Methods: This study was conducted at the College of Science, Wasit University, Iraq. Samples were collected from the Al Karama Teaching Hospital and Al Zahraa Hospital between October 2023 and July 2024. The study included 40 patients with cholecystitis (9 males and 31 females, aged 20–80 years) and 10 normal samples. The pathological classification categorized samples into acute cholecystitis, chronic cholecystitis, and hyperplasia. Immunohistochemical analysis was performed to assess *in situ* the Ki-67 and AFP expression.

Results: Ki-67 expression increased progressively across the type disease recorded 1.2% in controls, 5.7% in acute cholecystitis, 8.93% in chronic cholecystitis, and 13.9% in hyperplasia, with a statistically significant association ($p < 0.001$). Overall, 72.5% of the samples tested positive for Ki-67 expression. In contrast, AFP expression remained low (1.0% in controls and acute cholecystitis, 2.05% in chronic cholecystitis, and 3.73% in hyperplasia) and showed no significant association with the disease type ($p > 0.05$). Only 15.0% of the samples tested positive for AFP.

Conclusion: These findings indicate Ki-67 expression is significantly associated with gallbladder disease and serves as a strong marker of histopathologic lesion progression, while AFP expression remains minimal and statistically insignificant.

Keywords: *Ki-67, alpha-fetoprotein, gallbladder, cholecystitis, IHC*

1. INTRODUCTION

The gallbladder plays a crucial role in bile storage and secretion, primarily by aiding digestion and facilitating elimination of waste products from the body (Rajab et al., 2020). Histopathological examination of the gallbladder is essential to understand the structural and cellular changes associated with various pathological conditions. Inflammatory diseases of the gallbladder are characterized by thickening and increased mucosal contrast enhancement of the affected bile ducts and gallbladder wall, as observed using magnetic resonance imaging (MRI) (Bilgin et al., 2009). Chronic inflammation can lead to cholecystitis, impairing gallbladder motility and cytoprotective function (Behar, 2013).

Gallbladder inflammation is most commonly caused by gallstones, but can also occur due to obstruction from tumors or scarring of the bile duct (Sethi and Johnson, 2011). The primary risk factor for calculous cholecystitis is gallstone formation, which is influenced by various factors including female sex, advanced age (> 60 years), pregnancy, oral contraceptive use, obesity, diabetes mellitus, rapid weight loss, and medications such as hormone replacement therapy in menopausal women (Casper and Lammert, 2011). Chronic cholecystitis and cholelithiasis are associated with an increased risk of gallbladder cancer (Lazcano-Ponce et al., 2001).

Ki-67 is a well-established proliferative marker expressed in all phases of the cell cycle, except G0, and is detected in the nuclei of proliferating tumor cells (Doval et al., 2014). Recently, Ki-67 has gained significance as an important immunohistochemical marker owing to its universal expression in proliferating malignant cells, but is absent in normal cells. Thus, Ki-67 is a valuable marker for assessing tumor proliferation, particularly in breast carcinoma (Kumar et al., 2021). The immunohistochemical detection of Ki-67 is widely accepted as an indicator of cell proliferation**.** Papillary Hyperplasia Characterized by diffuse papillary projections of the mucosa without structural abnormalities, can mimic neoplastic conditions(Baba et al.,2014).Metaplastic alterations such as pyloric and intestinal metaplasia are frequently associated with gallbladder cancer (GBC). Although metaplasia is commonly observed within tumor areas, it is more frequently present in the surrounding tissue. Dysplasia, which may represent a transitional stage from metaplasia to cancer, has also been found in these regions (Roa et al., 2006).

Alpha-fetoprotein (AFP) is a serum glycoprotein secreted by embryonic liver cells and yolk sac tissues, and is widely used as a diagnostic marker for hepatocellular carcinoma (HCC) and yolk sac tumors (Ng and Ng, 1995). Given the shared embryological origin of the liver and gallbladder, AFP secretion by gallbladder cancer (GBC) cells may occur. AFP has multiple functions in medicine, primarily serving as a clinical tumor marker for HCC and hepatoblastoma (Liu et al., 2022). In addition to its role as a biomarker, AFP has been implicated in immune modulation and tumor progression, making it a potential target for cancer therapies (Yeo et al., 2024). This study aimed to analyze gallbladder diseases using histopathological and immunohistochemical assessments. It examines the morphological and structural changes in gallbladder tissues and evaluates Ki-67 and alpha-fetoprotein (AFP) expression as potential diagnostic and prognostic markers. The findings aim to enhance the understanding and improve diagnostic strategies for gallbladder pathology.

2. MATERIAL AND METHODS

2.1 Samples collection

The study included 40 patients (9 males and 31 females) diagnosed with different cholecystitis, aged between 20 and 80 years, and 10 normal samples were collected from Al-Karama Teaching Hospital and Al-Zahraa Hospital between October 2023 and July 2024 following surgical gallbladder removal. The samples were categorized by a pathologist as acute cholecystitis, chronic cholecystitis, and hyperplasia. Each sample was preserved in 10% formalin for 72 h, followed by washing with water for 2–3 h. The samples then underwent standard histological processing, including dehydration using graded ethanol concentrations, clearing in xylene, infiltration with paraffin, and embedding in paraffin blocks. Thin sections were cut and stained with hematoxylin and eosin (H&E) to examine the general tissue structure. Additionally, immunohistochemical staining was performed to detect Ki-67 and alpha-fetoprotein (AFP) following the specified protocol.

2.2 Immunohistochemistry

Immunohistochemical detection of Ki-67 and alpha-fetoprotein (AFP) was performed. Gallbladder tissue samples were fixed in 10% formalin, dehydrated in ethanol, Clearing by xylene, embedded in paraffin wax, and sectioned at 5 µm thickness. The staining process began with deparaffinization in xylene, followed by rehydration using a graded ethanol series. Antigen Retrieval (30 minutes 110 ˚C) in the Chamber Boiling method. Endogenous enzyme activity was blocked by incubating the slides with 3% hydrogen peroxide (H_2O_2) at room temperature for 30 minutes. After washing in distilled water, Novocastra Protein Block was applied to prevent non-specific binding, and the samples were incubated at room temperature for 20 min before discarding the excess liquid. Primary antibodies for Ki-67 and AFP were then applied and incubated for 40 min at 37°C in a humid chamber. After washing, the slides were incubated with Novocastra Post Primary for 10 min at 37°C, followed by another washing step. Streptavidin-HRP was added and incubated for 10 minutes at 37°C. After a final wash, the tissues were stained with diaminobenzidine (DAB) substrate for 20 min, leading to the formation of a brown precipitate at the antigen sites. The stained tissues were counterstained with Harris hematoxylin for 1 min and differentiated using acid alcohol. The slides were dehydrated using graded ethanol, cleared in xylene, and mounted for microscopic examination. All reagents, including Novocastra Peroxidase Block, Novocastra Protein Block, Novocastra Post Primary, Novolink Polymer, and DAB substrate buffer, were purchased from Leica Biosystems, UK.

3. STATISTICAL ANALYSIS

The immunohistochemical (IHC) expression of Ki-67 and AFP in this study was assessed by identifying distinct brown cytoplasmic or nuclear staining, utilizing ImageJ software for analysis. Staining was categorized as positive or negative based on intensity and distribution. To ensure the accuracy of the scoring, slides were ranked from lowest to highest according to staining intensity and extent, with specific localization noted for each marker. Statistical analysis was performed using SPSS version 26 and Microsoft Excel 2010. Data were collected, summarized, and analyzed accordingly. Numerical data are presented as mean ± standard deviation. The Kolmogorov-Smirnov test was employed to assess the normality of data distribution, which informed the selection between parametric and non-parametric statistical methods.

4. RESULT

4.1 Distribution according to the type of diagnosis.

The frequency distribution of patients according to their clinical features is shown in table (4.1). The frequency distribution of cholecystitis patients according to severity of disease was as follows: 10 (25.0%) of cholecystitis patients with acute severity, 19 (47.5 %) of cholecystitis patients with chronic severity, and 11 (27.5 %) of cholecystitis patients with hyperplasia.The frequency distribution of patients according to cholelithiasis was as follows: 34 (85.0 %) of cholecystitis patients with calculas and 6 (15.0%) of cholecystitis patients without calculas, and the differences were highly significant (p= 0.001).

This result agrees with Jones et al. (2024), reported the gallstone-associated cholecystitis, also known as calculous cholecystitis, is the most common form of gallbladder inflammation. It occurred when gallstones obstruct the cystic duct, leading to bile accumulation, increased pressure, and subsequent inflammation of the gallbladder wall. This result is consistent with other studies showing that 88% of cases had calculus cholecystitis and 8% had calculus cholecystitis. (Al-Saltany,2017).

Table (4-1): The frequency distribution patients according to some clinical features

Characteristic	Patients *n* (%)	P value
Severity of disease		
Acute, *n* (%)	10 (25.0%)	0.161 ¥ NS
Chronic, *n* (%)	19 (47.5 %)	
Hyperplasia, *n* (%)	11 (27.5 %)	
Cholelithiasis		
Yes, *n* (%)	34 (85.0 %)	0.001 ¥ S
No, *n* (%)	6 (15.0 %)	

n: number of cases; ¥: Chi-square test; S: significant at P< 0.05.

4.2 Histopathological Evaluation of Acute and Chronic Cholecystitis

Histopathological examination of gallbladder specimens from patients with acute and chronic cholecystitis revealed a spectrum of morphological changes reflecting varying stages and severity of inflammatory responses. In acute cholecystitis (AC**)**, the gallbladder mucosa displays widespread epithelial atrophy, interspersed with areas of focal hyperplasia and gastric metaplasia. Acute inflammation was characterized by a significant infiltration of neutrophils throughout all layers of the gallbladder wall, ranging from the mucosa to the serosa. This was accompanied by vascular congestion, bleeding, swelling, localized areas of fibrosis, and the formation of granulation tissue as shown in figures (4.1, 4.2, 4.3). These findings are in agreement with those described by Mencarini et al. (2024), who outlined the pathophysiological basis of AC as an acute response to cystic duct obstruction by gallstones, leading to ischemic injury and secondary bacterial infection. Gately (1983) highlighted that acute acalculous cholecystitis, a less common form, is prevalent among critically ill or trauma patients and has a higher mortality rate, which can be mitigated with timely surgical intervention such as cholecystectomy.

In contrast, chronic cholecystitis (CC**)** was characterized by long-standing inflammation resulting in significant structural remodeling of the gallbladder wall. Histological examination revealed prominent muscle hypertrophy glandular proliferation, and extensive subepithelial fibrosis. The wall was markedly thickened with erosion of the mucosa and prominent mononuclear inflammatory cell infiltrates composed primarily of lymphocytes, plasma cells, and histiocytes. Notably, several specimens exhibited features consistent with chronic xanthogranulomatous cholecystitis, a rare but clinically important variant marked by granuloma formation, multinucleated giant cells, and the presence of lipid-laden (foamy) macrophages (Figures 4.4) (Figures 4.5) (Figures 4.6). These findings support prior literature, including the studies by Kaur et al. (2012) and Sood et al. (2016), Singh et al. (2018), and Bano et al. (2020) described chronic cholecystitis as a condition of persistent inflammation often secondary to repeated episodes of acute attacks. Over time, this leads to epithelial atrophy, muscular hypertrophy, and fibrosis, frequently resulting in contracted and dysfunctional gallbladder. Additional metaplastic changes such as intestinal and gastric metaplasia were also noted, potentially reflecting an adaptive response to chronic injury

and irritation.

Chronic inflammation is believed to be perpetuated by sustained obstruction of the cystic duct, most commonly due to gallstones, along with repeated bouts of chemical irritation from retained bile acids, ischemia, and recurrent bacterial infections. Collectively, these factors contribute to progressive mucosal damage, submucosal fibrosis, and disruption of the functional architecture of the gallbladder. Importantly, increased mucosal thickness and lymphoplasmacytic infiltration observed in chronic cases may serve as early histological indicators of preneoplastic transformation. This is particularly relevant given the emerging evidence linking chronic inflammation with an increased risk of gallbladder dysplasia and carcinoma

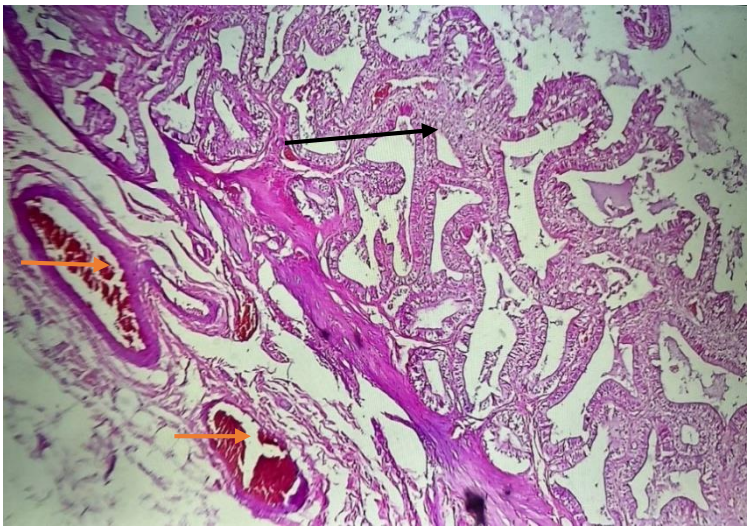


Figure (4.1) histological section of gall bladder showing acut cholecystitis include hyperplastic (black arrow) and congestion of blood vessels (oranage arrow) (H&E stain, 4X)

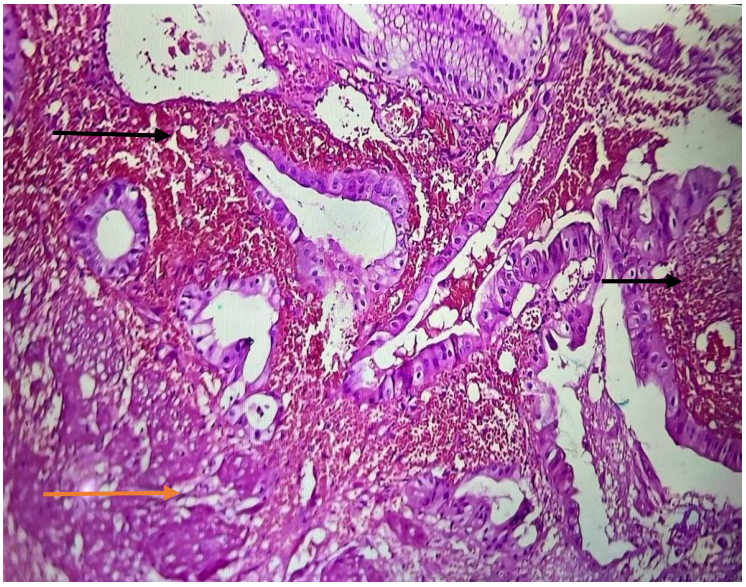


Figure (4.2) histological section of gall bladder showing acut cholecystitis include fibrosis (oranage arrow), hemorrhagic zones(black arrow) (H&E stain, 20x).

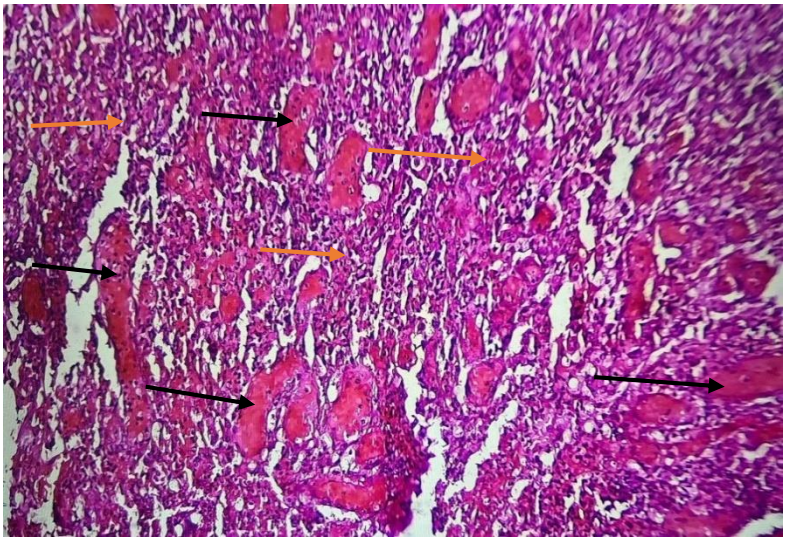


Figure (4.3) histological section of gall bladder showing acut cholecystitis include edema (black arrow) with infiltration of inflammatory cells (mostly neutrophils &eosinophils) (orange arrow) (H&E stain, 20x).

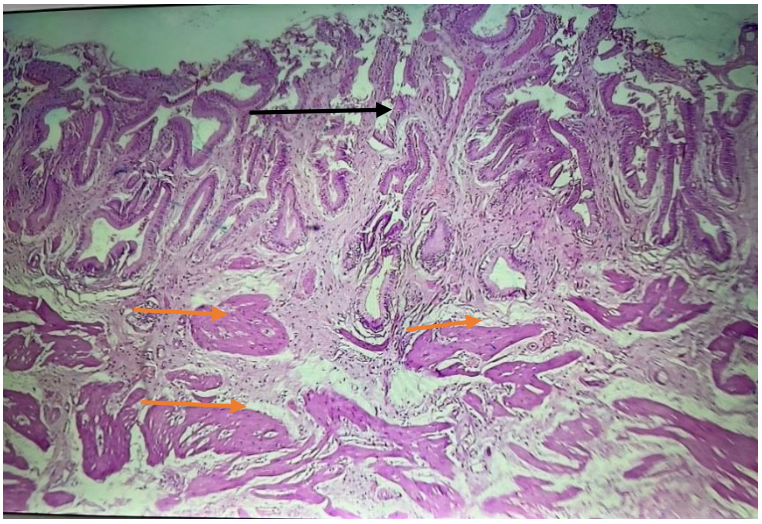


Figure (4.4) Histological section of gall bladder showing chronic cholecystitis include glandular hyperplasia mucosal epithelium (black arrow) & muscle hypertrophy (oranage arrow) (H&E stain, 4x).

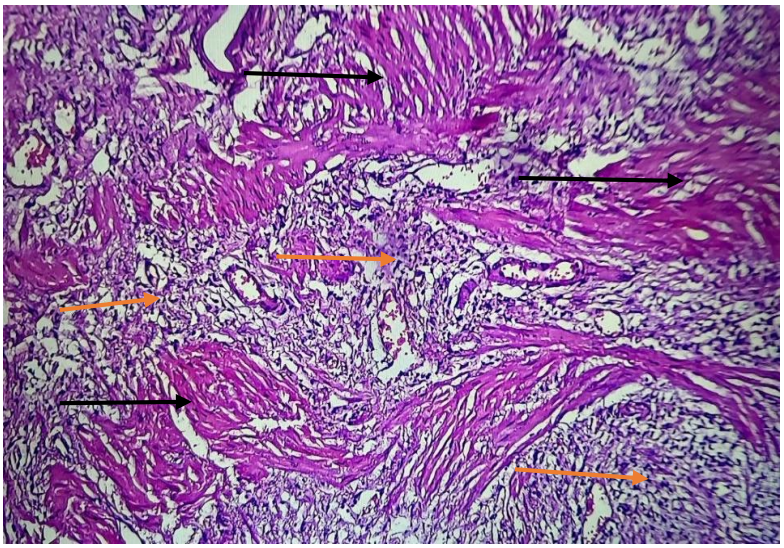


Figure (4.5) Histological section of gall bladder showing chronic cholecystitis included fibrosis (black arrow) & infiltrated inflammatory cells, intermixed with bland spindle cells extending to the serosal layer (orange arrow) (H&E stain,20x).

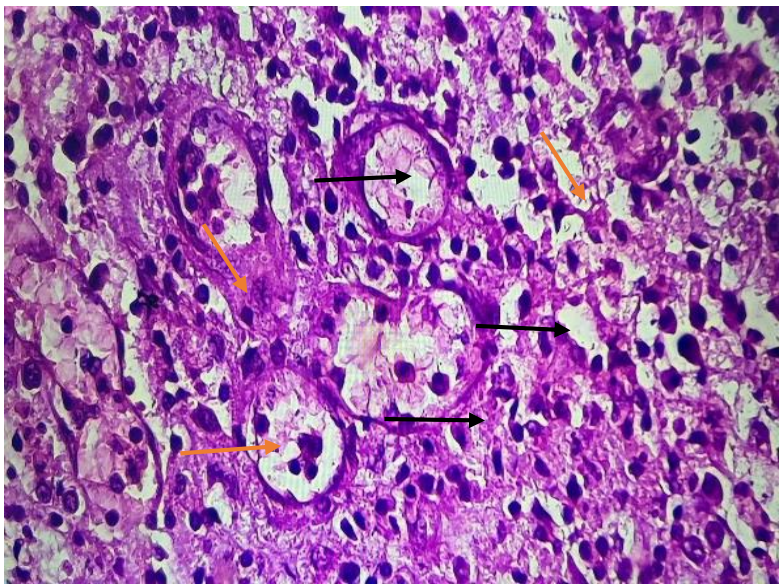


Figure (4.6): Histological section of gall bladder showing chronic cholecystitis include infiltrated foamy histiocytes(orange arrow) and (Xanthogranulomatous cholecystitis) (black arrow) (H&E stain,40x).

4.3. Immunohistochemical results of KI67

The results of the Ki-67 expression, measured by immunohistochemistry, exhibited significant differences among patient groups with various forms of cholecystitis. The mean Ki-67 expression levels were notably higher in patients with acute cholecystitis, chronic cholecystitis, and hyperplasia than those in the healthy control group. The highest Ki-67 expression was observed in the hyperplasia group, indicating a markedly increased cellular proliferation rate, which is likely associated with tissue changes characteristic of hyperplasia. Numerically, the mean ± SE values for Ki-67 expression were as follows: 28.10 ± 6.49, 48.52 ± 5.99, 69.54 ± 6.17, and 0.58 ± 0.10, in patients with acute cholecystitis, chronic cholecystitis, hyperplasia, and healthy control groups, respectively. Statistical analysis revealed a highly significant difference ($p < 0.001$**)** between all patient groups and the healthy control group (Table 4.2), further emphasizing the role of Ki-67 as a cellular proliferation marker in gallbladder pathology.

Relationship between the presence of gallstones (cholelithiasis) and ki67 expression in gallbladder tissues of the total sample, 29 (72.5%) were positive for Ki-67, whereas none of the patients without stones (no stone) showed positive Ki-67 expression, with 6 (15.0%) remaining negative for the marker. This indicated that Ki-67 positivity is strongly linked to the presence of cholelithiasis ($p<0.001$). Table (4.3) presents the association between Ki-67 expression and the presence of stones (cholelithiasis)

The results of expression analysis scoring percentage of ki67 using image j software shown in the table (4.4) revealed marked variation according to gallbladder disease tissue, the percentage expression of normal gallbladder tissue illustrated very little expression recorded (1.2%) of total area recorded as negative in figure (4.7) Additionally, in some of samples exhibited negative results, as demonstrated in (Figure 4.8 A, B). In acute cholecystitis, the Ki67 expression percentage was illustrated with increased expression was (5.7%) of the total area, which was weak with an intensity score of 1+. (figure 4.9 A, B). Furthermore, the results of percentage expression of chronic cholecystitis tissue also illustrated high present expression than acute cholecystitis recorded (8.93%) of total area these records were weak with an intensity score of 1+. (figure 4.10 A, B). The ki67 expression percentage gradually increased in disease types, which elevated in hyperplasia were (13.9%) with an intensity score of 2+. (figure 4.11 A, B) (figure 4.12 A,B)

we observed varying levels of Ki-67 expression in different gallbladder conditions associated with inflammation and gallstones. Our findings indicate the increasing Ki-67 expression is not solely indicative of malignancy; rather, it may also reflect benign processes in which cellular proliferation is part of a regenerative or inflammatory response this variation of increasing expression of ki-67 were equally to increase the severity of gall bladder disease.

In addition, the study demonstrated a significant correlation between increased Ki-67 expression and the presence of gallstones in the gallbladder tissue, supporting the hypothesis that gallstones may induce cellular proliferation in response to

chronic irritation and inflammation. This observation is consistent with previous reports showing that Ki-67 expression can be elevated in areas exhibiting regenerative changes, atypia, dysplastic lesions, and chronic inflammation within the gallbladder (Ramesh et al., 2024).

Specifically, we found that Ki-67 in progressive expression were lowest in acute cholecystitis, moderate in chronic cholecystitis, and highest in hyperplastic lesions. In acute cholecystitis, the inflammatory process is rapid and predominantly mediated by an immediate immune response, resulting in minimal regenerative activity and low Ki-67 expression (Vakkala et al., 2007). In contrast, chronic cholecystitis, which involves repeated cycles of tissue injury and repair, exhibits a moderate increase in Ki-67 expression, reflecting a controlled and sustained regenerative response to ongoing irritation from gallstones (Raziq & Ahmed, 2016).

Although elevated Ki-67 levels are often associated with malignancy, in this context, they likely represent benign reactive hyperplasia, a compensatory mechanism in response to chronic irritation from gallstones. The absence of cytological atypia and architectural disorganization in these lesions further supports a benign diagnosis (Scholzen & Gerdes, 2000; Raziq & Ahmed, 2016). Moreover, studies have shown that the Ki-67 labeling index (LI) in gallbladder hyperplasia is significantly higher than that in normal mucosa, with high-grade hyperplasia exhibiting a markedly elevated LI compared with low-grade hyperplasia (Tanno et al., 1998).

The findings of this study demonstrated Ki-67 expression increased with disease severity, from acute to chronic cholecystitis and hyperplasia, indicating enhanced cellular proliferation linked to inflammation and tissue remodeling. Its significant correlation with gallstones suggests chronic irritation drives these changes. While often associated with malignancy, elevated Ki-67 in benign conditions reflects regenerative processes. The highest expression in hyperplasia supports its role in sustained inflammation. These findings highlight Ki-67 as a useful marker of disease progression in gallbladder pathologies.

Table (4.2): **Comparison of Immunological Evaluations (ki67) in patients and healthy controls**

Groups		**ki67**
Acute cholecystitis	**Mean ± SE**	**28.10 ± 6.49**^{A}
	Range	5.00-63.00
Chronic cholecystitis	**Mean ± SE**	**48.52 ± 5.99**^{B}
	Range	5.00-74.00
Hyperplasia	**Mean ± SE**	**69.54** ± 6.17^{C}
	Range	10.00-96.00
Control	**Mean ± SE**	**0.58** ± 0.10^{D}
	Range	0.30-1.50
p-value		**0.001****

p < 0.01 → *(**)* Strong statistical significance (99% confidence level).

Table (4.3): The association between the of ki67 and stone in the study sample .

ki67	**Cholelithiasis**		**Total**	**P value**
	stone	**No stone**		
Negative	**5 (12.5%)**	6 **(15.0%)**	**11 (27.5%)**	**0.001**
Positive	**29 (72.5%)**	0%	**29 (72.5%)**	

Table (4.4): The expression intensity for ki67 by image J.

Disease type	Expression percentage (%)	Expression intensity scores	Categories
Normal	**1.2%**	**0**	**Negative**
Acute cholecystitis	**5.7%**	**1**	**Weak**
Chronic cholecystitis	**8.93%**	1	**Weak**
Hyperplasia	**13.9%**	2	**Moderate**

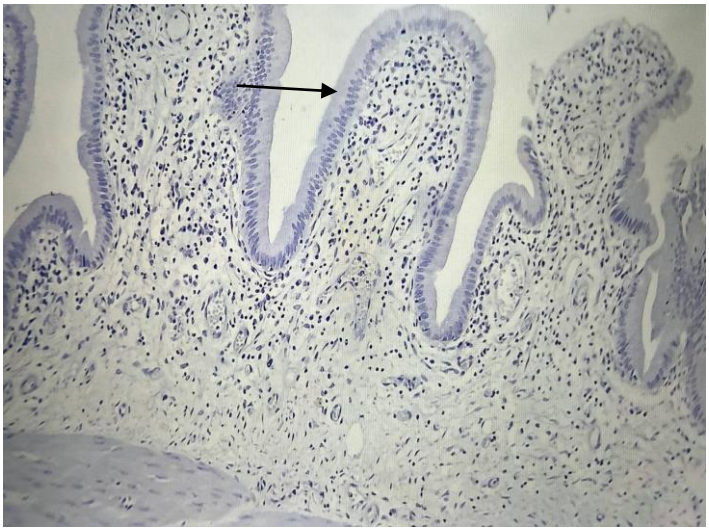
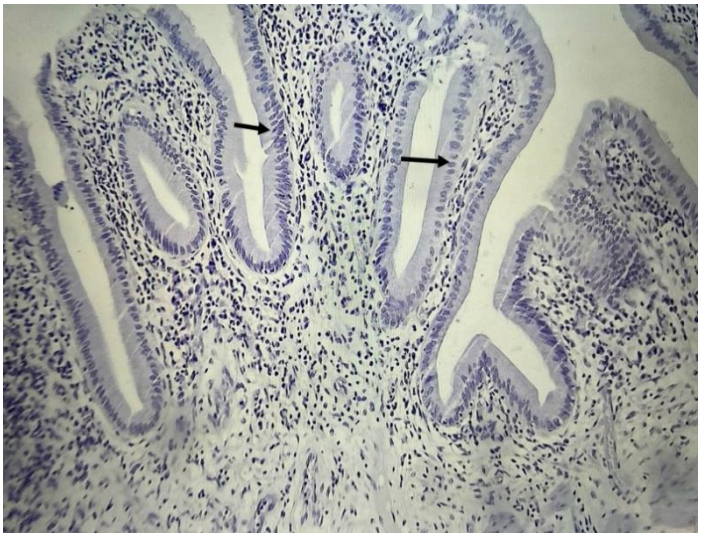


Figure (4.7) Immunohistochemical cross-section of normal gall bladder tissue showing the expression of Ki-67 appeared negative in simple columnar epithelial (arrow) used as Control (10X).



Figure(4.8A): Immunohistochemical cross-section of chronic cholecystitis with mucosal hyperplasia, showing the expression of Ki-67 appear negative (arrows) (20X).

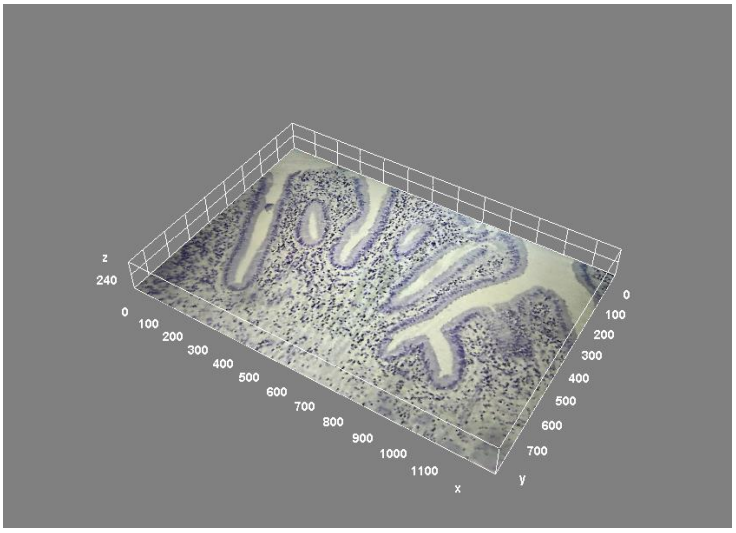


Figure (4.8B): The surface plot of gallbladder with hyperplasia lesion shows the percentage of expression negative of Ki-67

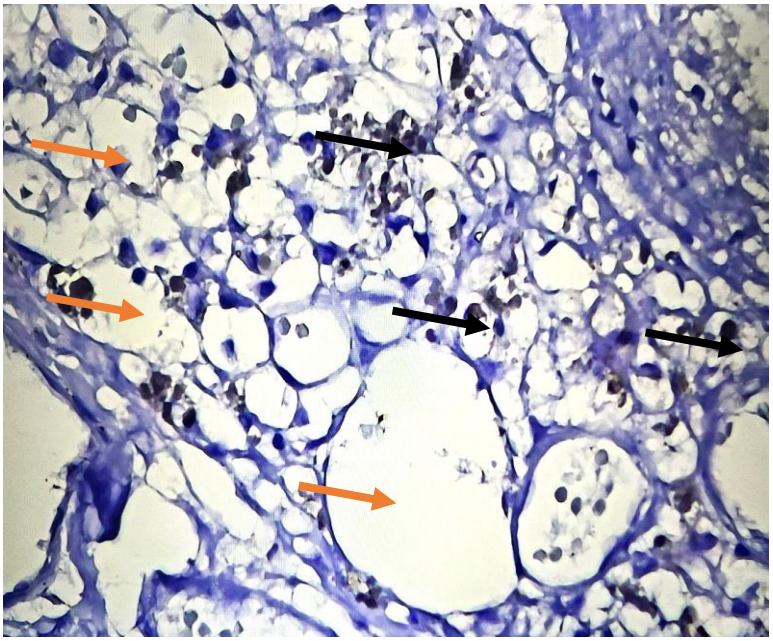


Figure (4.9 A): Immunohistochemical cross-section of Xanthogranulomatous Cholecystitis (XGC) with foamy macrophages (orange arrow), showing the expression of KI67 appears weak to low index proliferation (black arrow) (20X).

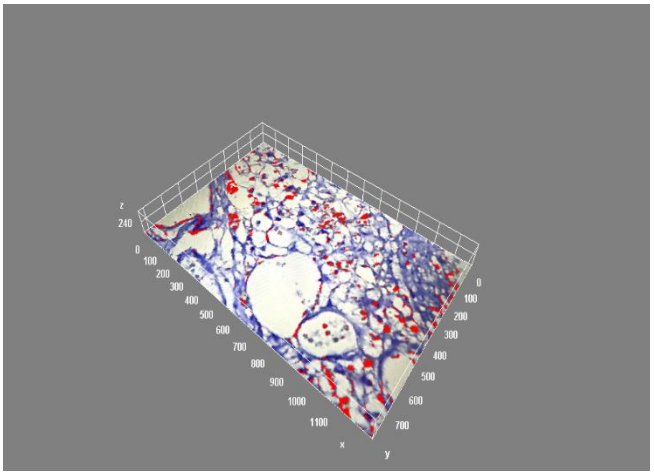


Figure (4.9 B): Surface plot of gall bladder showing the percentage of expression positive of Ki-67.

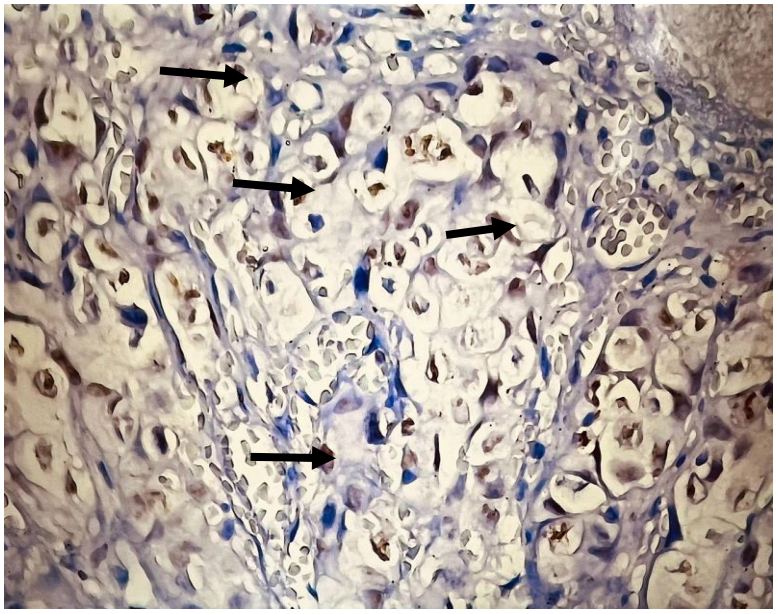


Figure (4.10A): Immunohistochemical cross-section Chronic cholycystitis may lead to increased cell turnover showing higher KI67 expression (arrow) (20X).

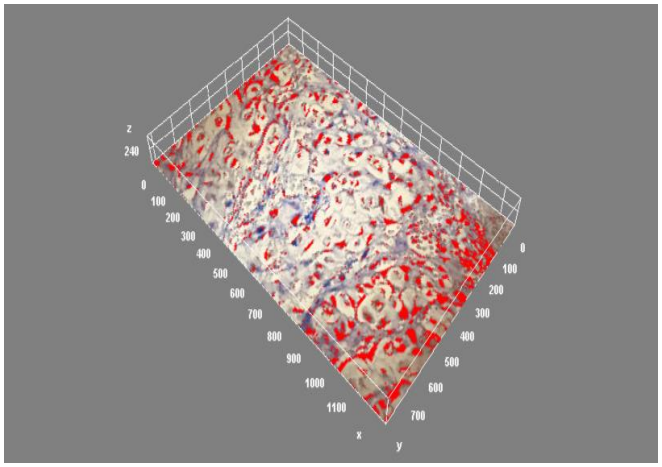


Figure (4.10B): Surface plot of gall bladder showing the percentage of expression positive of Ki-67.

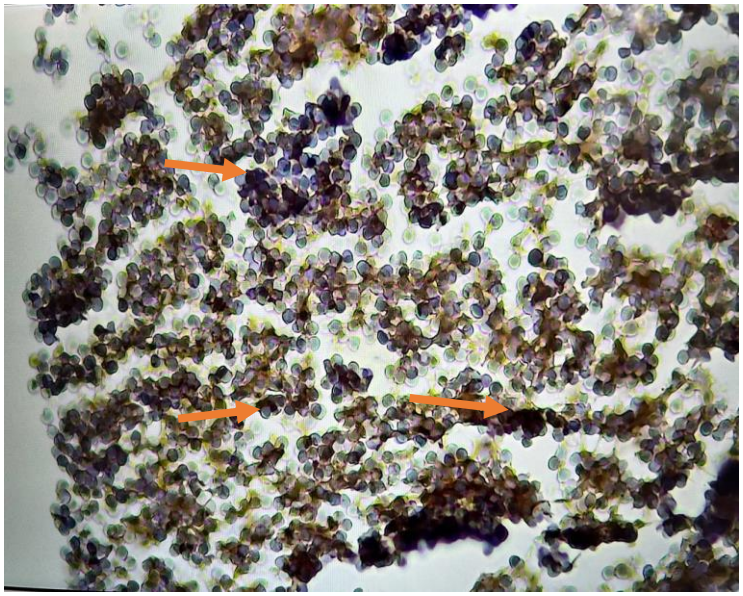


Figure (4.11A): Immunohistochemical cross-section of Dysplasia Pre-cancerous changes in the gallbladder epithelium showing high proliferation index of KI67-positive cells expression (arrow) (20X).

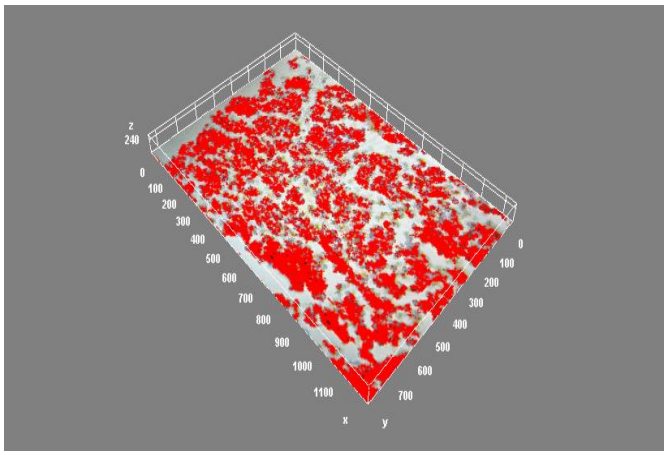


Figure (4.11B): Surface plot of gall bladder showing the percentage of expression positive of Ki-67.

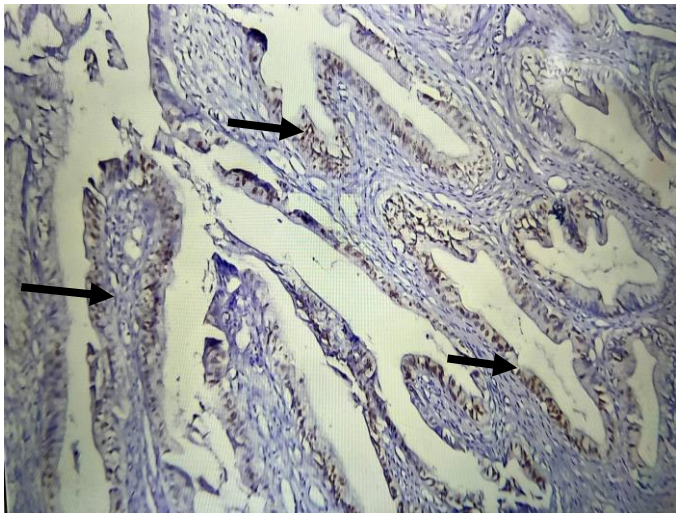


Figure (4.12A): Immunohistochemical cross-section of hyperplasia in the gallbladder epithelium shows moderate to

strong KI67-positive cells, suggesting active cell proliferation within the gallbladder mucosa (arrow) (20X).

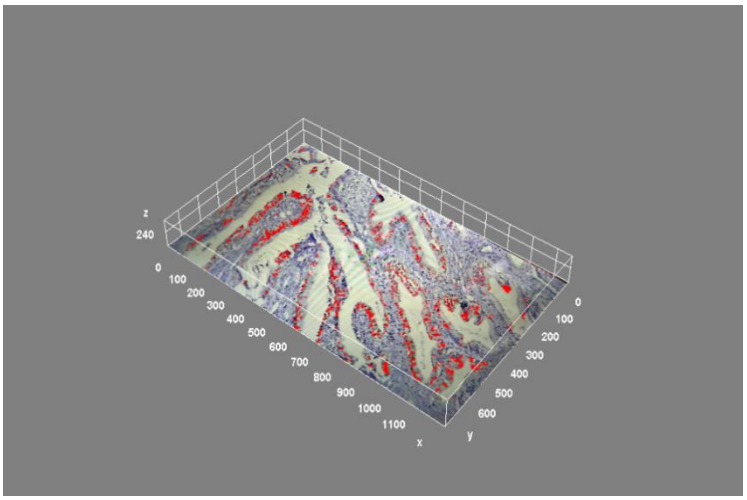


Figure (4.12B): Surface plot of gall bladder showing the percentage of positive expression of Ki-67.

4.5. Immunohistochemical result of Alphafetoprotein

The immunohistochemical analysis of alpha-fetoprotein (AFP) expression revealed distinct variations among different cholecystitis conditions. In acute cholecystitis, AFP levels remained stable, indicating minimal involvement of this marker in the disease process. In chronic cholecystitis, AFP levels exhibited a moderate increase, suggesting a possible link between AFP expression and disease progression or severity. The most significant elevation was observed in hyperplasia, where AFP levels were markedly higher, potentially correlating with increased cellular proliferation or pre-neoplastic changes. In contrast, the control group displayed minimal AFP expression, reinforcing the distinction between normal and diseased gallbladder tissues.

Numerically, the mean ± SE values for AFP levels were 3.00 ± 0.0 in acute cholecystitis, 7.26 ± 1.35 in chronic cholecystitis, and 14.45 ± 3.95 in hyperplasia, while the control group had the lowest expression with a mean of 0.46 ± 0.07. Despite these observed differences, statistical analysis revealed no significant association ($p > 0.05$), suggesting that while AFP levels varied across conditions, they may not serve as a definitive marker for distinguishing between disease states as shown in table (4.5).

Relationship between gallstones (cholelithiasis) and alpha-fetoprotein (AFP) expression in gallbladder tissue. Of the total sample, 29 cases (72.5%) with gallstones and five cases (12.5%) without gallstones exhibited negative AFP expression, accounting for 85% of the total sample. In contrast, positive AFP expression was observed in five cases (12.5%) with gallstones and in one case (2.5%) without gallstones, totaling 15% of the sample. The P> 0.05 indicates no statistically significant association between AFP expression and the presence of gallstones as shown in table (4.6).

The expression analysis scoring percentage of alpha using ImageJ software shown in the table revealed marked variation of disease tissue. The percentage expression of normal gallbladder tissue illustrated very little expression recorded (1.0 %)of the total area these records were negative figure (4.13). Additionally, the majority of the samples exhibited negative results, as demonstrated in (figure 4.14 A, B).

In acute and chronic cholecystitis, the expression remains low (1.0% and 2.05%, respectively) of total area these records were weak with an intensity score of 1+ (Figure4.15 A, B). The alpha expression percentage gradually increased in disease types, which elevated in hyperplasia were (3.73%) with an intensity score of 1+ (Figure 4.16 A, B). Also the present results showed that counts of alpha among patients with Hyperplasia were significantly higher tissue of other groups (P value ≤ 0.05) (table 4.7)

Alpha-fetoprotein (AFP) is not typically expressed in normal gallbladder tissue and is primarily associated with several disorder related to hepatocellular carcinoma and gastrointestinal cancers. However, elevated AFP levels have been observed in rare cases of gallbladder carcinoma, specifically in patients with hepatoid differentiation (Lee et al., 2011). This atypical AFP expression underscores the necessity for further research to elucidate its role in non-malignant and pre-neoplastic gallbladder conditions.

Littman et al. (2023) reported a statistically significant association between AFP levels and hyperplasia, suggesting that AFP may serve as a biomarker for distinguishing between benign and potentially precancerous gallbladder conditions. The progressive increase in AFP levels from acute to chronic cholecystitis and hyperplasia further supports the hypothesis that

AFP may contribute to the pathophysiology of gallbladder diseases, particularly in conditions involving enhanced cellular activity.

Conversely, Brown and Roberts (1992) found no significant correlation between AFP level and cholelithiasis. This finding suggests that while gallstones induce inflammatory and structural changes in the gallbladder, they do not consistently correspond to AFP elevation. Instead, AFP expression appears to be more closely associated with advanced pathological processes such as hyperplasia or preneoplastic transformations.

The presence of AFP in gallbladder inflammation remains an intriguing finding as its expression is conventionally linked to malignant conditions. Chronic inflammation can stimulate AFP expression through cellular regeneration and proliferation, leading to activation of markers typically associated with malignancy (Potapovich et al., 2009). Furthermore, chronic tissue injury and prolonged inflammatory stimuli have been implicated in tumorigenic pathways, even under non-cancerous conditions (Hibino et al., 2021). This raises the possibility that AFP plays a role in the early tissue alterations that may precede neoplastic transformation.

Additionally, gallbladder inflammation has been linked to an increased risk of dysplastic transformation, which may lead to altered protein expression patterns, including AFP. Dixit et al. (2021) documented a case in *Gastroenterology* of a 60-year-old woman with incidental gallbladder carcinoma and elevated serum AFP levels. Similarly, Mita et al. (2022) described a case of *gastrointestinal tumor* involving a 69-year-old woman with AFP-producing poorly differentiated adenocarcinoma with signet ring cells. These findings suggest a potential association between AFP expression and pre-neoplastic or early malignant changes in the gallbladder.

AFP expression was absent in normal and acute cholecystitis cases but slightly increased in chronic cholecystitis and hyperplasia. This suggests a link between AFP expression, chronic inflammation, and early preneoplastic changes. Its lack of association with gallstones indicates AFP may reflect tissue remodeling rather than direct inflammatory damage. Further studies are needed to assess its potential as an early biomarker for gallbladder disease

Table (4.5): Comparison of Immunological Evaluations (Alpha) in patients and healthy controls

Groups		**Alpha**
Acute cholecystitis	**Mean ± SE**	**3.00** ± 0.0^{A}
	Range	3.00-3.00
Chronic cholecystitis	**Mean ± SE**	**7.26** ± 1.35^{A}
	Range	3.00-35.00
Hyperplasia	**Mean ± SE**	**14.45** ± 3.95^{B}
	Range	3.00.50.00
Control	**Mean ± SE**	**0.46** ± 0.07^{A}
	Range	0.10-1.00
p-value		**0.033***

Table (4.6): The association between the presence of alpha and stone in the study sample.

alpha	**Cholelithiasis**		**Total**	**P value**
	stone	**No stone**		
Negative	**29 (72.5%)**	5 **(12.5%)**	**34 (85.0%)**	**0.901**
Positive	**5 (12.5%)**	1 **(2.5%)**	**6 (15.0%)**	

Table (4.7): The expression intensity for alpha by image J.

Disease type	Expression percentage (%)	Expression intensity scores	Categories
Normal	**1.0 %**	**0**	**Negative**
Acute cholecystitis	**1.0 %**	**1**	**Weak**
Chronic cholecystitis	**2.05%**	1	**Weak**
Hyperplasia	**3.73%**	1	**Weak**

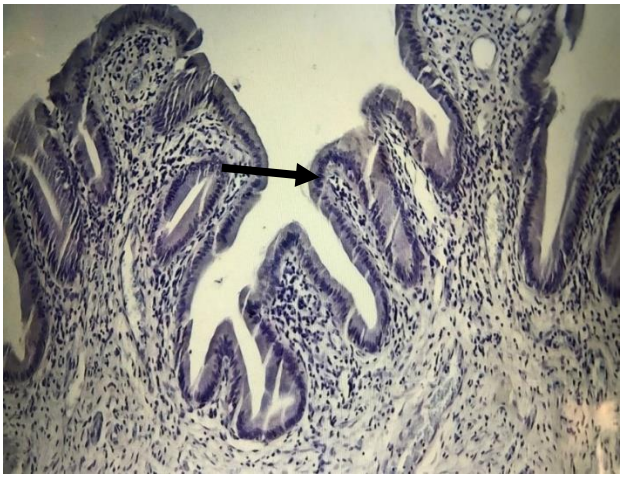
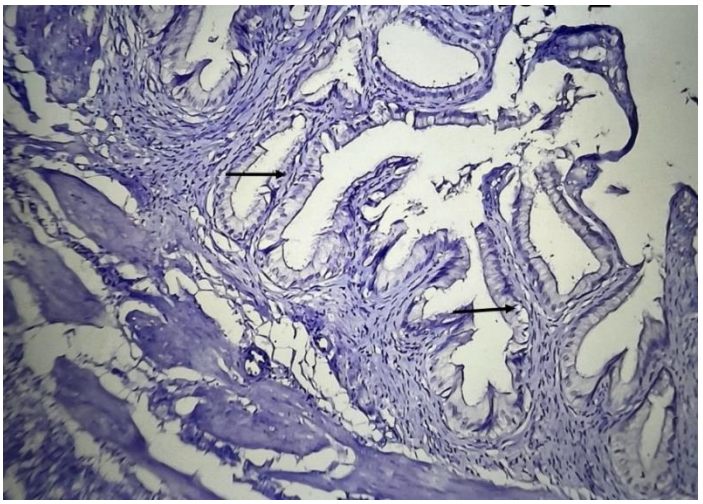


Figure (4.13) Immunohistochemical cross-section of normal gall bladder tissue showing the expression of AFP appeared negative in simple columnar epithelial used as Control(arrow) (20X).



Figure(4.14A): Immunohistochemical cross-section of gall bladder Hyperplasia showing the expression of AFP appear negative (arrows) (20X).

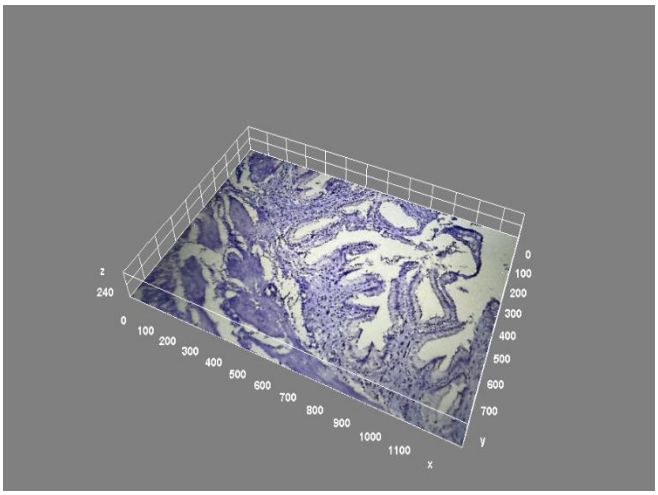


Figure (4.14B): The surface plot of gallbladder with hyperplasia lesion shows the percentage of expression negative of AFP

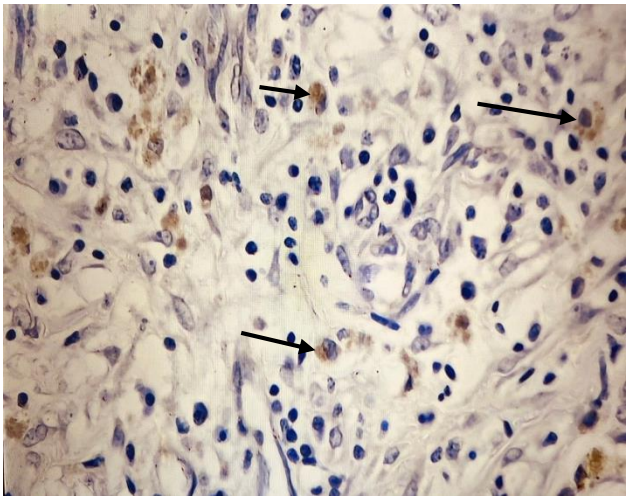


Figure (4.15A): Immunohistochemical cross-section of mesenchymal gall bladder (chronic cholecystitis) showing the expression of AFP appears focal and weak to moderate(arrow) (no stone)(20X).

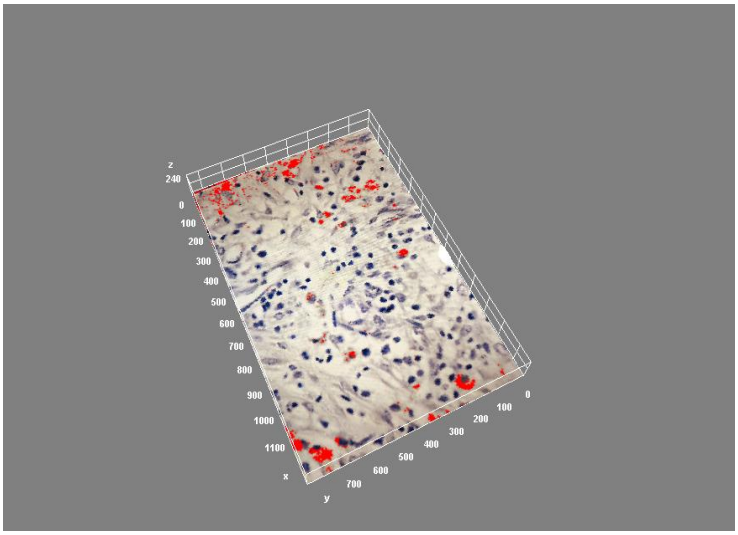


Figure (4.15B): Surface plot of gall bladder showing the percentage of expression positive of AFP

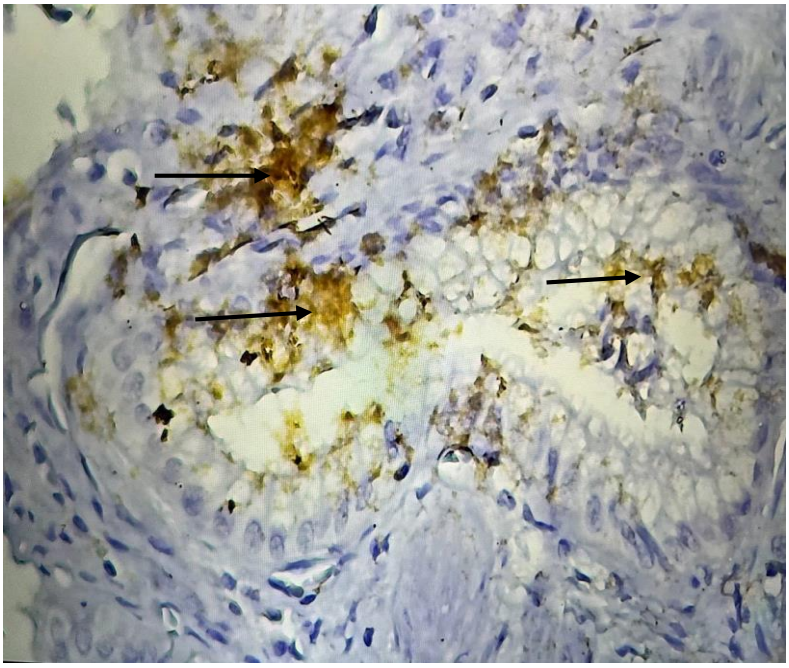


Figure (4.16A): Immunohistochemical cross-section of gall bladder showing the expression of AFP appears glandular structures with an irregular arrangement, possibly indicating dysplastic showing strong positivity (arrow) while others remain unstained or weakly positive. (20X)

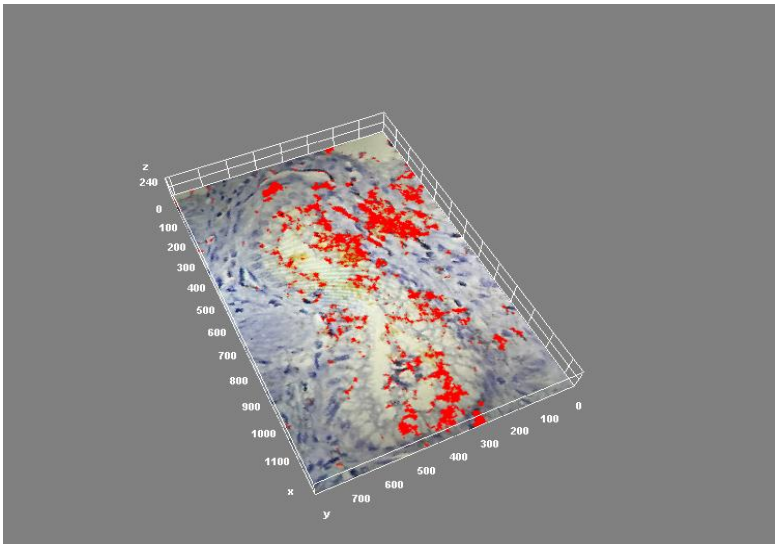


Figure (4.16B): The surface plot of gallbladder shows the positive expression percentage of AFP.

5. CONCLUSION

Ki-67 expression shows a significant association with gallbladder disease, increasing with disease severity and serving as a reliable marker for histopathological lesion progression. In contrast, AFP expression remains low and statistically insignificant across disease types, through its gradual increase may suggest a potential link to cellular proliferation and early preneoplastic changes.

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