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p53 and Ki67 immunoexpression and their clinicopathological correlation in primary gall bladder carcinoma: A cross sectional study

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Abstract:
Gallbladder carcinoma is an aggressive malignancy that often presents late, with limited reliable prognostic biomarkers. This cross-sectional observational study of 42 histopathologically confirmed primary gallbladder cancer specimens' evaluated p53 and Ki-67 expression by immunohistochemistry. Most cases were symptomatic, predominantly proliferative and commonly involved the fundus and body; p53 mutant expression was detected in the majority of tumors and was significantly associated with elevated CEA, tumor site and lymph node involvement. Thus, Ki-67 showed a significant correlation with p53 expression, suggesting that both markers reflect tumor proliferation and aggressive biological behavior.

Keywords: Gallbladder carcinoma (GBC), carcinoembryonic antigen (CEA), p53 mutant, Ki-67

Background:
Gall Bladder Carcinoma (GBC) is the sixth most prevalent gastrointestinal cancer and accounts for about two-thirds of biliary tract cancers **[1]**. It originates from the cystic duct and gallbladder's epithelial lining and manifests either a widespread wall thickening or a localized mass **[2]**. Female sex, advanced age, obesity, smoking and particularly cholelithiasis, which cause persistent mucosal irritation and a metaplasia–dysplasia–carcinoma sequence, are major risk factors **[3]**. Majority of GBC cases are unintentionally discovered after surgery or post-operative examination, when the disease is already advanced and survival results are low, due to the disease's subtle beginning and early asymptomatic phases 1. According to GLOBOCAN 2022, India accounts for almost 10% of cases worldwide, with an incidence of 1.2% and a mortality of 0.83% **[4]**. The Indo-Gangetic region, specifically Bihar, Uttar Pradesh, West Bengal, Assam, Odisha and Delhi, bears the brunt of the strain **[5, 6]**. The necessity for trustworthy biomarkers is highlighted by the limited early symptoms and late presentation. Tumor aggressiveness, lymphovascular invasion and early recurrence are related with Ki-67, a nuclear proliferation marker that is lacking from resting cells **[7, 8]**. GBC significantly overexpresses p53, a tumor suppressor gene that is often changed in cancers, when compared to precursor lesions, indicating its role in carcinogenesis **[9, 10]**. Therefore, it is of interest to evaluate the p53 and Ki-67 immunochemistry expression in primary gallbladder cancer and look into their relationship with clinicopathological features in order to increase prognostic information.

Materials and Methods:
The cross–sectional observational single-center, hospital-based study was conducted at AIIMS Patna's Department of Pathology over the period of 2 years. This study included 42 specimens of primary gallbladder cancer that were histopathologically validated and received by the department. Non-epithelial malignancies, autolyzed/necrotic specimens, post-therapy samples and neuroendocrine tumors were excluded. Surgical specimens underwent conventional mechanized tissue processing after being grossed and preserved in 10% neutral buffered formalin. The sections were examined under a light microscope and histomorphological diagnosis was recorded. Tumor grading and histopathological examination were carried out in accordance with the WHO Classification of Digestive System Tumors (5th Edition). After histological analysis, representative tumor blocks were chosen for IHC markers, including p53and Ki-67. Poly-l-lysine-coated slides were used to capture additional 2-3 micron slices from the paraffin-embedded blocks. They underwent immunohistochemical labeling for ki67 antigen (Ventana 30-9 monoclonal mouse antibody) and p53 protein (Ventana clone BP53-11 monoclonal mouse antibody). Immunohistochemical expressions of p53 and ki67 were calculated.

Wild type (normal): Shows scattered nuclear staining with mid epithelial (basal sparing) **[9]**

Aberrant (mutational type): 3 distinct patterns:
a) 80% of cells show strong and diffuse nuclear staining.
b) Complete absence of nuclear staining in all cells.
c) Moderate to strong cytoplasmic staining.

The proportion of tumour cell nuclei with positive staining relative to all tumour cells counted (n = 1000) or 2 mm² (which is equivalent to four 40X fields in Olympus CX23), regardless of the intensity of the staining, was considered positive if >20% of stained cells were present. Radiological results, physical characteristics (tumor size, wall thickness, location, stones), histological features (type, grade, depth of invasion, LVI, PNI, nodal status, TNM stage) and clinical factors (age, gender, clinical features, diet, tumor markers) were all documented. Requisition forms and patient records were used to record all clinical, histological and IHC results, which were then imported into Microsoft Excel (2021) for analysis. The details od interpretation of p53 immunostaining has been illustrated in **Table 1** and interpretation of immunostaining by Ki-67 is illustrated in **Table 2.**

Statistical analysis:
Data was evaluated Jamovi 2.3.18. Demographic, clinical, radiological, gross and histological characteristics were compiled

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using descriptive statistics. Chi-square or Fisher's exact tests were used to assess correlations between p53 and Ki-67 expression and clinicopathological markers; $p < 0.05$ was deemed statistically significant.

Table 1: Interpretation of p53 immunostaining **[11]**

Score	Criteria
0	**Absent**
1	**Mild**
2	**Moderate**
3	**Intense**
Score	**Criteria**
0	**Absent nuclear staining**
1	**<10% staining of tumour cells**
2	**10-50% staining of tumour cells**
3	**>50% staining of tumour cells**

A score of percentage of nuclear staining and intensity will be added and a combined score of ≥ 3 is considered as overexpressed.

Table 2: Interpretation of Ki-67 immunostaining **[12]**

Score	Criteria
0	**0-20%**
1	**20-40%**
2	**40-60%**
3	**>60%**

Scores of 1 and 2 are considered as low expression. Score of 3 is considered as high expression.

Results:

A total of 42 cases of primary GBC were received, processed and reported by the department of Pathology, All India Institute of Medical Sciences, Patna. Out of 42 cases, 41 cases were resected gallbladder specimen and 1 case was core biopsy. The association of p53 and Ki-67 expression with various clinicopathological parameters, including age, gender, clinical presentation, tumor marker, diet pattern, wall thickness, location of tumor, type of growth, tumor size, presence of stone, histological type, grade of carcinoma, depth of invasion of gall bladder wall, lymphovascular invasion, perineural invasion, lymph node involvement and TNM stage were assessed. The median patient age was 55 years (range 18-95), with a female predominance (66.7%) and M: F ratio of 1:2. Majority of patients were symptomatic (98%), with 'pain abdomen' as the most common clinical presentation. Most of tumors appeared as proliferative growths (71.4%) and were found in the body and fundus (83.3%) as the common site. The mean tumor size is 18.3 mm (10mm - 20mm) with a significant predominance (83.3%) of larger tumors (>15 mm) . Majority of these patients (76.2%) did not have associated gallstones. Cases with tumor size ≤15mm show statistically significant association with presence of cholelithiasis and perineural invasion. Most of the patients with cholelithiasis had moderately differentiated adenocarcinoma and a statistically significant association was noted between cholelithiasis and the histological grade of adenocarcinoma **(Table 3)**. Histologically, 73.8% had considerable moderate differentiation with biliary type as most common histological subtype and 23.8% and 33.3%, respectively, had lymph vascular and perineural invasion. Majority of the cases were in TNM stage II (35.7%), followed by TNM stage I (23.8%). p53 mutant expression was seen in 88.1% (37/42), with 52.4% overexpression and 35.7% null pattern. p53 overexpression was significantly associated with increased serum CEA levels and tumor site (neck of gall bladder). Increased serum CEA levels are associated with a higher percentage of p53 overexpression (65%), while p53 overexpression is less frequent (18%) when serum CEA is within the normal limit **(Table 4)**. No statistically significant association was found between the histological grade of primary GBC and p53 expression. Nonetheless, p53 overexpression was observed more frequently in moderately differentiated tumors (52%) and was particularly high in poorly differentiated tumors (**Table 5**). p53 overexpression was observed in a substantial proportion of tumors across various clinicopathological parameters though most associations were not statistically significant, except for increased serum CEA levels and tumor site. All the well differentiated and poorly differentiated adenocarcinoma as well as majority of moderately differentiated adenocarcinoma showed mutant p53 expression, however no statistically significant association was noted **(Table 6).** All 5 GBC cases with liver involvement and 3 cases with distant metastasis exhibit mutant p53 expression. However, no statistically significant association was noted. Mutant type of p53 was statistically significantly associated with symptomatic cases, elevated serum CEA levels and lymph node involvement **(Table 7)**. The most frequent Ki-67 score observed is 3, accounting for 52.4% of the total cases. With no significant correlation with clinicopathological features or TNM stage. Significant correlations between Ki-67 and p53 expressions were common in primary GBC, reflecting their potential role in tumor proliferation and aggressiveness **(Figure 1–6).**

Table 3: Association of cholelithiasis with histological grade **(N=42)**

Cholelithiasis	Histological grade			Total	χ^2 (df)	p value
	Well differentiated n (%)	Moderately differentiated n (%)	Poorly differentiated n (%)			
Present	4 (40%)	5 (50%)	1 (10%)	10	7.12 (2)	**0.034****
Absent	2 (6.3%)	26 (81.2%)	4 (12.5%)	32		
Total	6	31	5	42		

Table 4: Association of p53 expression in primary GBC cases with serum CEA level **(N=42)**

Serum CEA	p53 expression		Total	χ^2 (df)	p value
	Low expression n (%)	Overexpression n (%)			
Increased	11 (35%)	20 (65%)	31	6.99 (1)	**0.008 ****

Within normal range	9 (82%)	2 (18%)	18
Total	20	22	42

Table 5: Association of p53 expression in primary GBC cases with histological grade (N=42)

Histological grade	**p53 expression**		**Total**	**χ^2 (df)**	**p value**
	Low expression n (%)	Overexpression n (%)			
Well differentiated	4 (67%)	2 (33%)	6	2.41 (1)	0.480
Moderately differentiated	15 (48%)	16 (52%)	31		
Poorly differentiated	1 (20%)	4 (80%)	5		
Total	20	22	42		

Table 6: Association of p53 interpretation in primary GBC cases with histological grade (N=42)

Histological grade	**p53 interpretation**		**Total**	**χ^2 (df)**	**p value**
	Mutant n (%)	Wild type n (%)			
Well differentiated	6 (100%)	0 (0%)	6	2.01 (1)	0.778
Moderately differentiated	26 (83.9%)	5 (16.1%)	31		
Poorly differentiated	5 (100%)	0 (0%)	5		
Total	37	5	42		

Table 7: Association of p53 expressions in primary GBC cases with lymph node status (N=34)

Lymph node status	**p53 interpretation**		**Total**	**χ^2 (df)**	**p value**
	Mutant n (%)	Wild type n (%)			
Free of tumor	16 (76.2%)	5 (23.8%)	21	3.63 (1)	**0.05****
Involved	13 (100%)	0 (0%)	13		
Total	29	5	34		

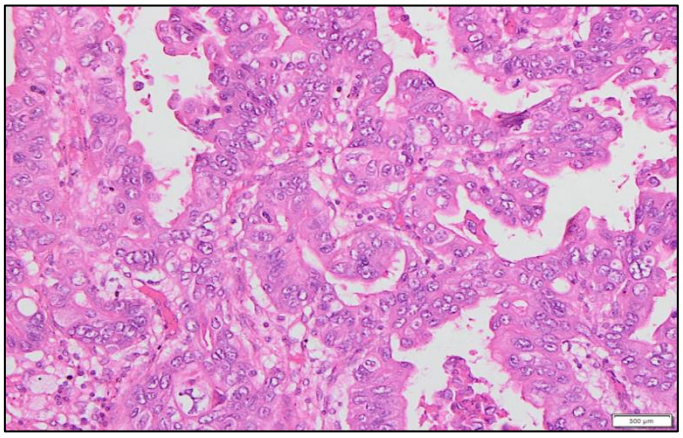

Figure 1: Photomicrograph of biliary type of adenocarcinoma grade 2 (H&E 20X)

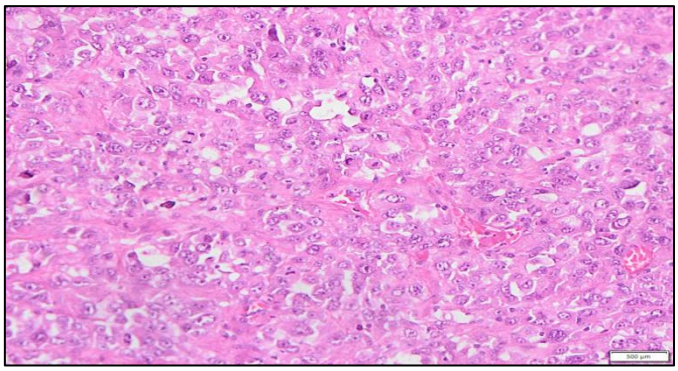

Figure 2: Photomicrograph of poorly differentiated adenocarcinoma (H&E 20X)

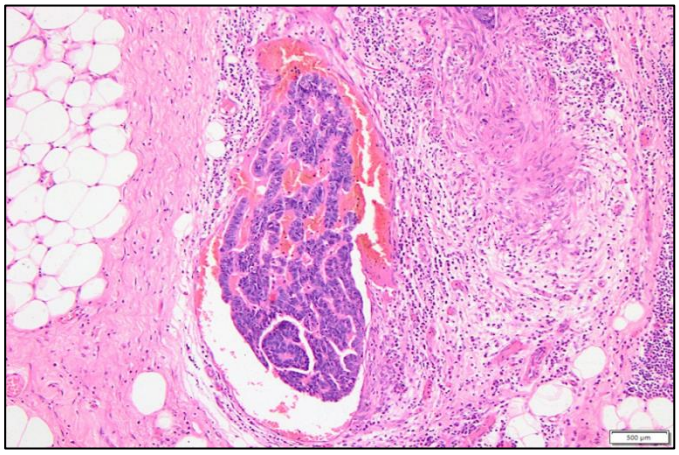

Figure 3: Photomicrograph showing lymph vascular invasion by tumor cells of adenocarcinoma (H&E 4X)

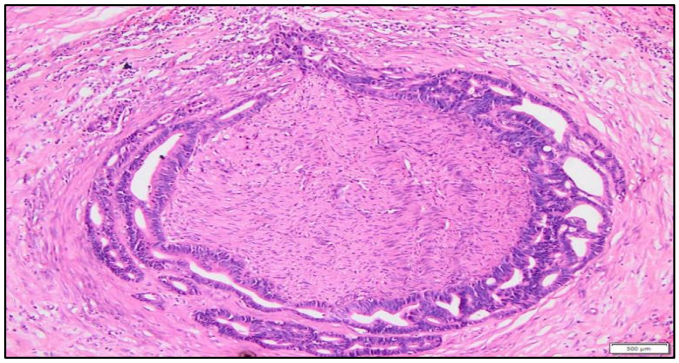

Figure 4: Photomicrograph showing perineural invasion by tumor cells of adenocarcinoma (H&E 4X)

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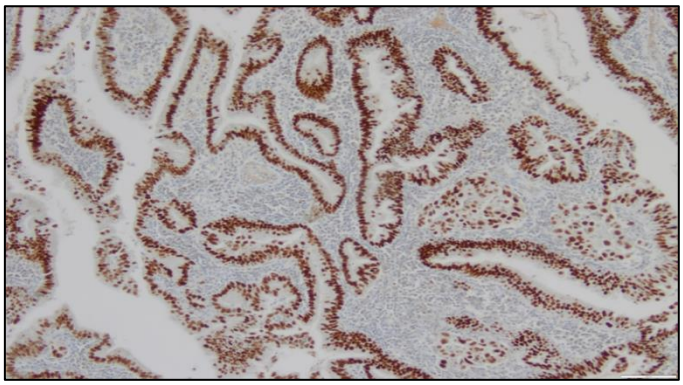

Figure 5: Photomicrograph of adenocarcinoma with intense nuclear staining p53 in 85-90% of tumor cells, p53 overexpression, score 6 (IHC 10X)

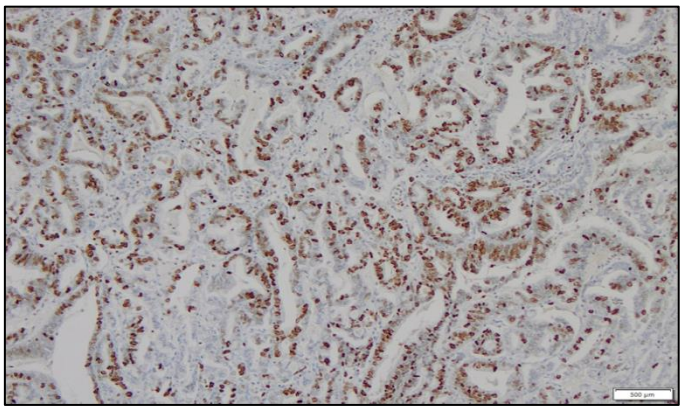

Figure 6: Photomicrograph of adenocarcinoma with nuclear staining of Ki-67 in 55-60% of the tumor cells, score 2 (IHC 20X)

Discussion:

Early-stage GBC is typically asymptomatic, resulting to a late diagnosis and limited therapy possibilities **[13]**. Although personalized treatments are emerging as molecular etiology becomes more known, systemic chemotherapy remains the primary treatment for advanced illness. There is little known about the association between GBC clinicopathological features and p53 and Ki-67, which have been related to a variety of cancers **[14]**. In the present study, the median patient age was 55 years with female preponderance which aligns with the study by Gupta *et al*. **[15]**. This can be attributed to the fact in females' estrogen and progesterone reduce gallbladder motility during the luteal phase and pregnancy, promoting bile stasis and gallstone formation, a key precursor to malignancy. Reproductive factors reflecting prolonged hormonal exposure further heighten this risk, suggesting that the gallbladder functions as a hormone-responsive organ. This concept is supported by the demonstration of estrogen and progesterone receptors in both benign and malignant gallbladder tissues, although their precise clinicopathological significance in gallbladder carcinoma remains debated **[16]**. In GBC, abdominal pain and discomfort predominantly results from direct malignant extension into the adjacent liver tissue and surrounding pericholecystic structures, which stimulates visceral nociceptive pathways. With advancing disease, progressive tumor enlargement leads to stretching of the gallbladder capsule and sustained inflammatory responses, manifesting clinically as a chronic, dull and poorly localized pain in the right upper abdomen causing discomfort **[17]**. In the present cohort, abdominal discomfort constitutes the most frequent presenting complaint which aligns with various other studies where abdominal pain and discomfort was the most prominent feature of gallbladder carcinoma **[18, 19]**. In our study, median serum CA19-9 and CEA levels were 34.2 U/mL and 3.5 ng/mL, respectively, aligning with findings by Sachan *et al*. **[20]**, who reported a progressive increase in CA19-9 from resectable to metastatic gallbladder cancer, while CEA showed no significant variation. Kim *et al*. **[21]** observed lower marker levels, likely reflecting earlier-stage disease, whereas Viner *et al*. **[22]** documented markedly elevated values in metastatic patients, underscoring their association with tumor burden. These differences likely stem from variations in disease stage, cohort composition and assay criteria. Collectively, these markers aid in malignancy detection but have limited sensitivity as standalone diagnostic tools, particularly in early disease. Cholelithiasis is a major risk factor for gallbladder carcinoma and it occurs due to chronic irritation caused by large or long-standing gallstones that promotes malignant transformation through a dysplasia-carcinoma sequence and in our study also 23.8% had cholelithiasis along with gall bladder stones **[23]**. In our cohort, consistent with previous studies, the majority of tumors were located in the fundus and body of the gallbladder. However, only 23.8% of patients had gallstones, a lower prevalence than typically reported, though gallstone presence was significantly linked to higher histological grade. In the present investigation, all tumors were adenocarcinomas, similar with findings by Ramalhosa *et al*. **[24]** and Pujani *et al*. **[25]**. While investigations by Ramesh *et al*. **[26]** revealed that adenocarcinoma NOS was the most prevalent subtype, the biliary subtype predominated. Although some studies reported a majority of well-differentiated tumors, moderately differentiated tumors were the most common , which is consistent with earlier data **[7,26–29]**. In the present study, p53 overexpression was identified in gallbladder carcinoma cases and showed a significant association with elevated serum CEA levels and tumors located at the neck of the gallbladder. Notably, p53 overexpression was more frequent in patients with raised CEA levels compared to those with normal CEA levels, suggesting a link with aggressive tumor behavior. These findings largely align with earlier studies that have reported p53 overexpression in approximately 50–56% of gallbladder cancers, predominantly in malignant rather than benign or inflammatory lesions and more commonly in moderately and poorly differentiated adenocarcinomas. Previous reports by Pavani *et al*. **[9]**, Walvir *et al*. **[29]**, Roa *et al*. **[30],** Ghosh *et al*. **[31]** and Kaur *et al*. **[32]** support the association of p53 alterations with higher tumor grade and advanced disease, indicating its role in tumor progression rather than initiation. While some studies have described p53 changes as an

early event in carcinogenesis, others suggest its persistence during disease progression. Despite its lack of specificity, as stated by Sharma *et al*. **[33]**. CEA's substantial connection with mutant p53 in our research indicates that it may have adjunctive usefulness. Although not statistically significant, mutant p53 predominated across histological subtypes, grades and T stages, which is consistent with Ramesh *et al*. **[26]**. In contrast Kim *et al*. **[27]** exhibited a tendency toward mutant p53 but did not approach significance. This study's generalizability and prognostic interpretation are limited due to the small sample size, single-center methodology and lack of follow-up data. The absence of improved imaging correlation, insufficient evaluation of some pathological characteristics and the possibility of selection bias from mostly symptomatic individuals all limit the usefulness. Future studies using larger multicentric cohorts, molecular profiling and survival analysis is needed to verify these findings and better establish the therapeutic value of p53, Ki-67 and other biomarkers in gallbladder cancer. Similar associations have been reported across the spectrum of gallbladder lesions. Walvir *et al.* **[29]** found p53 overexpression in 42.9% of malignant gallbladder lesions versus 11.98% of inflammatory and 0% of benign/pre-malignant lesions (p=0.003), proposing p53 as a discriminator of malignancy. Kumar *et al.* **[34]** showed a stepwise rise in both p53 score and Ki-67 index from chronic cholecystitis through metaplasia-dysplasia to carcinoma (p=0.0001), with the two markers positively correlated (r^2=0.37, p=0.001), supporting a sequential progression model. Gupta *et al.* **[35]** found Ki-67 correlated with differentiation, jaundice, liver function, gallstones and metastatic site, but not TNM stage which is consistent with the lack of Ki-67-TNM association found in this study. High p53 mutant rate (88.1%) and predominant Ki-67 score of 3 (52.4%) align with the malignant-lesion range reported in these studies, though the carcinoma-only cohort limits direct comparison with their graded spectra.

Conclusion:

p53 mutant expression was highly prevalent (88.1%) in primary GBC and significantly associated with lymph node metastasis, tumor site and elevated CEA, underscoring its prognostic value. Ki-67 showed proliferative activity across most tumors and correlated with p53 expression, reinforcing their shared role in tumor aggressiveness. Combining p53 and Ki-67 immunohistochemistry with routine histology can help in improving prognostic stratification and diagnostic precision in gallbladder carcinoma.

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