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RESEARCH ARTICLE

Immunohistochemical Evaluation of Precursor Lesions of Gallbladder Carcinoma in Cholecystectomy Specimens with Stones

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Abstract

Objective: Gallbladder carcinoma is an aggressive malignancy with poor prognosis, particularly in high-incidence regions. This study evaluated the immunohistochemical expression of p16, p53, COX-2, and HER2/neu in precursor epithelial lesions of the gallbladder and assessed their potential role in gallbladder carcinogenesis. **Materials and Methods:** A total of 1500 cholecystectomy specimens with gallstones were reviewed over two years. Histopathological examination identified epithelial precursor lesions, including hyperplasia, metaplasia, and dysplasia. Thirty cases each of hyperplasia, metaplasia, and dysplasia were subjected to immunohistochemical analysis for p16, p53, COX-2, and HER2/neu. Immunoreactivity was assessed using staining intensity, percentage positivity, and H-score. HER2/neu expression was evaluated using a modified breast cancer scoring system. Statistical significance was set at $p < 0.05$. **Results:** Precursor epithelial lesions were identified in 287/1500 (19%) cases, comprising hyperplasia (8%), metaplasia (9%), and dysplasia (2%). p16 expression was significantly higher in dysplasia (96%) compared with hyperplasia (10%) and metaplasia (13%) ($p < 0.001$). p53 expression was observed only in dysplasia (16%) ($p < 0.05$). COX-2 expression was detected in all dysplasia cases and in 23% of metaplasia and 16.6% of hyperplasia cases ($p < 0.001$). HER2/neu positivity was seen in 27% of dysplasia, 13% of metaplasia, and 6.6% of hyperplasia cases, suggesting an early molecular alteration in gallbladder carcinogenesis. **Conclusion:** The findings support a stepwise progression from metaplasia to dysplasia in gallbladder carcinogenesis. Careful histopathological and immunohistochemical evaluation of cholecystectomy specimens may aid in identifying high-risk precursor lesions. Our findings suggest a sequential molecular model in which HER2/neu alteration occurs early, p16 dysregulation marks establishment of dysplasia, and p53 accumulation indicates progression. This panel-based approach provides better insight into gallbladder carcinogenesis than individual markers.

Keywords: Hyperplasia- metaplasia- dysplasia- gallbladder- carcinogenesis- immunohistochemistry

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Introduction

Gallbladder carcinoma is one of the most aggressive cancers of GI tract, characterised by late presentation and poor survival rates [1]. Two pathways of its carcinogenesis have been proposed: a) Dysplasia-carcinoma pathway, [2] b) Adenoma-carcinoma pathway [3]. The progression of gallbladder carcinoma is believed to follow a stepwise sequence from hyperplasia to metaplasia, dysplasia, and ultimately carcinoma, commonly referred to as the dysplasia–carcinoma sequence [2]. The exact pathway and relative importance of these pathways in GB carcinogenesis is not clear. Hence the precursor lesions are not yet established. There are few studies in the literature that have investigated the precursor lesions and in most of these studies only one or two immunological markers have been studied in the precursor lesions. There is a paucity of studies assessing their combined expression across all precursor lesions. Such a panel-based approach may provide better insight into the temporal sequence of molecular events and improve risk stratification. The present study was undertaken to

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evaluate the immunohistochemical expression of p16, p53, COX-2, and HER2/neu across hyperplasia, metaplasia, and dysplasia, and to propose an integrated model of gallbladder carcinogenesis

Materials and Methods

Study Setting

Department of Pathology and Department of Surgery, ### and ### Hospital, ###

Study Duration

December 2023 to December 2025

Study Design

Analytical and cross-sectional.

Sample size

Two years retrospective cholecystectomy specimens with gallstones. Sample size was calculated taking 50% as the maximum positivity with same precision. So after taking 50% positivity with 10% precision on either side, the calculated sample size was 93. Since dysplasia is uncommon lesion in cholecystectomy specimen with gallstone and due to limitation of time and resources, 30 cases of dysplasia along with 30 cases of hyperplasia and 30 cases of metaplasia were taken for study randomly for effective comparison and to avoid selection bias.

The patient information, clinical details and all relevant data of the patients was taken from histopathological requisition forms and the confidentiality of the patients was maintained. Formalin fixed paraffin embedded blocks were retrieved from archives. All cholestectomy samples were evaluated for the histological findings (hyperplasia, metaplasia, dysplasia and carcinoma-in-situ).

Study Population

Patients who had undergone cholecystectomy surgery at ### and ## Hospital

Inclusion Criteria

All patients operated for gallstones and specimen received in Department of Pathology at #### and ###Hospital were included in the study.

Exclusion Criteria

Histologically diagnosed cases of invasive gallbladder carcinoma.

Ethical Clearance

Ethical clearance was obtained from institute ethical committee Institutional Ethics Committee-Human Research (IEC-HR) prior to commencement of study.

Informed Consent

Informed consent was taken from all the prospective patients.

Each subject was provided with patient information sheet containing relevant information.

Immunostaining

Formalin fixed and paraffin embedded block of tissue from gallbladder specimen was taken and mounted on Poly-L-lysine coated slides. Commercially available primary antibodies (p16, p53, COX-2 and HER2/neu) respectively were applied using the manufacturer's protocol, using labelled DAKO EnVision kit which is based on polymer chain two step indirect technique, with DAB as chromogen by the following procedure-

1. The sections were de-waxed and brought to water in xylene followed by descending grades of alcohol.

2. The antigen retrieval was done using microwave EZ-Retriever by two cycles of retrieval at 98 degrees Celsius for ten minutes each using Citrate Buffer having a pH 6.5.

3. The slides were then brought to room temperature and given five washes with Tris Buffered Saline or TBS having a pH of 8.6, for three minutes each.

4. The blocking of non-specific tissue epitopes was done using a mixture of 48 ml methanol and 2 ml of 30 percent hydrogen peroxide solution for 45 minutes.

5. Again, five washes with TBS for three minutes each were given to the slides.

6. Following this, the slides were marked using hydrophobic marker and primary antibody was applied to the sections and left overnight in the refrigerator.

7. The next morning, the slides were removed from the refrigerator, allowed to come to room temperature and then washed with TBS five times for three minutes each.

8. The Horse Radish Peroxidase or HRP conjugated secondary antibody was applied to the sections and left at room temperature for 30 minutes.

9. The slides were again washed with TBS five times for three minutes each.

The chromogen was prepared fresh by taking 1 ml of the substrate buffer and a drop of the chromogen, DAB, and mixing properly.

10. The sections were then covered in this freshly prepared chromogen mixture for 5 minutes and then washed in tap water to stop the reaction.

11. The slides were then counterstained with hematoxylin stain for 15 seconds and again washed in running tap water for 5 minutes.

12. The slides were then passed through ascending grades of alcohol and xylene and dried and mounted with DPX.

13. A positive control and negative control, where in the primary antibody was omitted, were put with each batch of the test slides to maintain the validity of the immunostaining procedure.

p16

Immunostaining was performed on at least two representative tissue from all cholecystectomy specimens of patients using p16 monoclonal antibody (clone mouse 16PO4, JC, BSB 5827) according to manufacturer's protocol. Subject confidentiality was maintained in all cases.

Positive control- A positive control of cervical dysplasia was run with each batch of test slides.

Negative control- A negative control wherein the primary antibody was omitted, was also put with each

batch of the test slides to maintain the validity of the immunostaining procedure.

p53

Immunostaining was performed on at least two representative tissues from all cholecystectomy specimens of patients using p53 monoclonal antibody (mouse p53 Ab-5, DO-7, MS-186-PO, thermo) according to manufacturer's protocol.

Positive control- A positive control of adenocarcinoma colon was run with each batch of test slides.

Negative control- A negative control wherein the primary antibody was omitted, was also put with each batch of the test slides to maintain the validity of the immunostaining procedure.

COX-2

Immunostaining was performed on at least two representative tissue from all cholecystectomy specimens of patients using COX-2 monoclonal antibody (clone RBT COX-2, BSB 5358) according to manufacturer's protocol.

Positive control- A positive control of adenocarcinoma colon was run with each batch of test slides.

Negative control- A negative control wherein the primary antibody was omitted, was also put with each batch of the test slides to maintain the validity of the immunostaining procedure.

HER2/neu

HER2/neu immunostaining was performed using monoclonal antibody directed against HER2/neu (clone HER2, BSB 2036) in similar fashion with DAKO EnVision kit on minimum two representative sections from cholecystectomy specimens. A modified scoring criteria was used 0-3 is based on cell membranane staining. Score 0/1+: (negative) no staining or weak, incomplete staining in any proportion of cells. Score 2+: (weakly positive /equivocal) weak to moderate, complete membranous staining in ≥ 10% of cells. Score 3+: (positive) strong complete membranous staining in ≥ 10% of cells

Positive control- Breast cancer with known HER2/neu positivity was taken as positive control with every batch of test slides.

Negative control- A negative control wherein the primary antibody was omitted, was also put with each batch of the test slides to maintain the validity of the immunostaining procedure.

H-score was calculated by multiplying percentage of cells showing immuno positivity with the intensity of staining. The intensity was given as mild, moderate and intense staining. The immuno-expression was calculated using H-score (0-300).

Statistical Analysis

Due to limited number of case availability, time constraint and cost issues, 30 cases of dysplasia were studied. Data obtained were used for descriptive statistics. The expression of p16, p53, COX-2 and HER2/neu was compared. Data was expressed as percentage/proportion and was depicted in the form of bar graphs, pie-charts and histograms to obtain descriptive statistics. The immuno-expression of various histological categories were compared using Mann-Whitney test, chi square test and p value obtained using Kruskal Walli's test.

Results

Out of 1500 cholecystectomy specimens 287 had epithelial changes, of which 122 had hyperplasia, 135 had metaplasia (Figure 1) , and 30 showed features of dysplasia (Figure 2). The remaining 1330 cases had no significant epithelial changes in hyperplasia, metaplasia or dysplasia. Table 1 shows the distribution of theses 287 cases across various histological categories (Table 1).

Among 30 cases of dysplasia adjacent epithelium also showed hyperplasia in 11 cases and intestinal metaplasia in 18 cases.

Patient Demographics

Age distribution

The age of patients showing GB epithelium hyperplasia ranged from 18 to 50 years with mean age being 30 years.

The age of patients with GB epithelium metaplasia ranged from 20 to 50 years with mean age being 35 years.

Thirty patients having GB epithelium dysplasia were in the age group of 21 to 60 years with mean age being 39 years.

Gender

Gender wise distribution of cases across various histological categories was also analysed (Table 2).

Immunohistochemistry

Immunostaining:

Thirty cases from each histological category

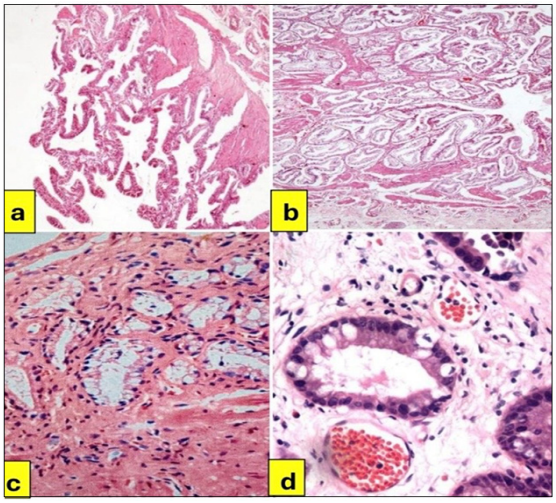

Figure 1. a) H&E (100x) Section Shows Gallbladder Epithelium Showing Papillary Hyperplasia. 1 (b): H&E (200x) section shows gallbladder epithelium showing adenomatous hyperplasia. 1 (c): H&E (40x) Section shows chronic cholecystitis with pyloric metaplasia. 1 (d): H&E (100x) gallbladder mucosa with intestinal metaplasia showing goblet cells

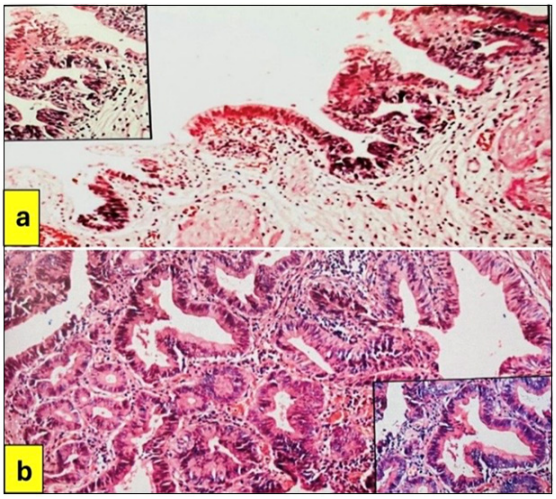

Figure 2. (a) H&E (200X) Low Grade Dysplastic Gallbladder Epithelium. 2(b): H&E(400x) high grade dysplasia

(hyperplasia, metaplasia and dysplasia) were subjected to immunohistochemistry for p16, p53, COX-2 and HER2/neu (Figure 3).

All cases in each histological category were assessed immunohistochemically using 4 different parameters:

1. Subcellular localization: nuclear and cytoplasmic, nuclear, cytoplasmic and membranous.

2. Intensity of staining:

The intensity of staining was graded into three categories: mild, moderate and strong positivity and was given a score of 1, 2 and 3 respectively.

3. Percentage of cells showing immunopositivity

The percentage of immuno-positive cells was calculated by taking into account the number of cells showing immuno-positivity out of the total number of epithelial cells in the tissue section examined.

4. H- score

The result of above parameters across various histological categories were noted (Table 3).

p16

Out of 30 cases of hyperplasia, 3/30 (10%) cases showed focal nuclear and cytoplasmic positivity for p16 and 27/30 (90%) cases were negative for p16 immuno-staining. Amongst the hyperplasia cases moderate to intense staining for p16 was seen in 1/30 (3.3%) while the other 2 cases of hyperplasia showed mild staining. The positive staining in all the three cases was seen in ≤ 20% of cells.

Amongst 30 cases of metaplasia, 4/30 (13%) showed focal nuclear and cytoplasmic positivity for p16 and 26/30 (87%) cases were negative for p16 immuno-staining. Amongst the metaplasia cases showing immunopositivity for p16, 2/30 (6.6%) showed moderate to intense staining while the other 2/30 (6.6 %) showed mild staining. Amongst the 4 cases of metaplasia positive for p16, 3 cases showed positivity in ≤ 20% cells.

Out of 30 cases of dysplasia, 29/30 (96%) cases showed focal or diffuse nuclear and cytoplasmic positivity for p16 and only 1 case of dysplasia was negative. In dysplasia majority 23/30 (77 %) cases showed moderate to intense staining for p16, while 6/30 (20%) cases of dysplasia showed only mild staining. Immunopositivity in > 40% cells was found in 13/30 (43%) cases of dysplasia whereas 16/30 (53%) cases showed immuno-positivity for p16 in ≤ 40% of cells.

H-score: Maximum H-score for hyperplasia, metaplasia and dysplasia was found to be 60, 80 and 260 respectively.

The difference in H-score for p16 between dysplasia-hyperplasia and dysplasia-metaplasia was found to be statistically significant with p-value <0.001. However no statistically significant difference was found between H-score for p16 immuno-expression between hyperplasia and metaplasia with p-value > 0.671.

p53

All cases of hyperplasia and metaplasia were negative for p53 immuno-expression.

Amongst the 30 cases of dysplasia, 5/30 (16%) showed focal nuclear positivity for p53 whereas 25 /30 (84%) were negative for p53 immuno staining.

Amongst dysplasia cases showing immuno-positivity for p53, moderate to intense staining was seen in 2/30 (6.6%) while 3/30 (10%) showed mild staining.

Immuno-positivity for p53 was found in >40% cells in 2 /30 (6.6 %) dysplasia cases while 3/30 (10%) showed immuno-positivity in ≤ 30% cells .

H-score:

Maximum H-score for dysplasia was 210

The difference in H-score for p53 between dysplasia-

Table 1. Distribution of 287 Cases with Epithelial Changes Across Various Histological Categories

No. of Hyperplasia (122)*		No. of Metaplasia (135)*		No. of Dysplasia (30)*	
Papillary	Adenomatous	Pyloric	Intestinal	Low grade	High grade/ Carcinoma-in situ
119	3	107	28	28	2

* Indicates total number of cases in each histological category

Table 2. Gender Wise Distribution of Cases Across Various Histological Categories

Sex	No. of Hyperplasia	No. of Metaplasia	No. of Dysplasia
	(122)*	(135)*	(30)*
Male	40 (33%)	45 (33%)	8 (26%)
Female	82 (67%)	90 (67%)	22 (74%)

* Indicates total number of cases in each histological category.

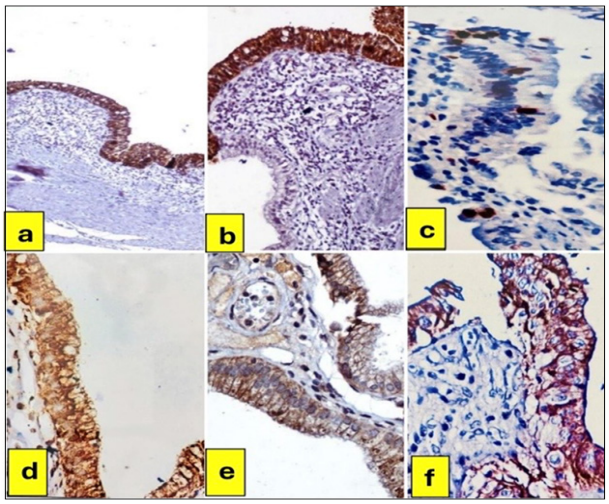

Figure 3. (a) IHC p16 (100x) Shows the Transition Zone of Normal Gallbladder Epithelium Negative for p16 with Dysplastic Epithelium Showing Diffuse Nuclear and Cytoplasmic Positivity. 3 (b): IHC p16 (200x) section shows intense diffuse positivity of p16 in dysplastic gallbladder epithelium. 3 (c): IHC p53 (100x) section shows focal intense nuclear positivity in dysplastic epithelium of gallbladder 3 (d): IHC COX2 (200x) dysplastic gallbladder epithelium showing moderate to intense diffuse cytoplasmic positivity 3 (e): IHC HER2neu (200x) section showing membranous staining for Her/neu in low grade dysplasia of gallbladder 3(f): IHC HER2/neu (200x) high grade dysplasia gallbladder showing membranous staining for HER2/neu.

hyperplasia and dysplasia-metaplasia was found to be statistically significant with p-value <0.001. However no statistically significant difference was found between H-score for p53 immuno-expression between hyperplasia and metaplasia with p-value > 0.60.

COX-2 protein

Out of 30 cases of hyperplasia, 5/30 (16.6%) showed focal cytoplasmic positivity for COX-2 while 25 /30 (83.3%) were negative. Amongst hyperplasia cases showing COX-2 immuno-positivity moderate to intense staining was seen in 1/30 (3.3%) case. Only 1/30 (3.3%) case of hyperplasia showed immuno-positivity for COX-2 in >20 % cells, while other 4/30 (13.3%) showed positivity in ≤ 10 % cells.

In metaplasia, 7/30 (23%) cases showed focal or diffuse cytoplasmic positivity for COX-2, however 23/30 (76%) cases were negative for COX-2 immuno expression. 3/30 (10 %) showed moderate to intense staining. Amongst metaplasia cases immuno-positivity for COX-2 was seen in >20 % cells in 5/30 (16%) cases and ≤20% positive cells in 2/30 (6.7%) cases.

All 30/30 (100%) cases of dysplasia showed focal or diffuse cytoplasmic positivity for COX-2. Out of 30 cases of dysplasia, 24/30 (80 %) showed moderate to intense staining while 6/ 30 (20%) cases showed mild staining for COX-2. More than >40% positive cells were seen in, 20/30 (67 %) cases of dysplasia and rest of cases 10/30 (33%) immuno-positivity in ≤ 40% cells.

H-score:

Maximum H-score for hyperplasia, metaplasia and dysplasia was found 90, 180, 190 respectively.

The difference in H-score for COX-2 between dysplasia-hyperplasia and dysplasia-metaplasia was found to be statistically significant with p-value <0.001. However no statistically significant difference was found between H-score for COX-2 immuno-expression between hyperplasia and metaplasia with p-value > 0.401

HER2/neu

Out of 30 cases of hyperplasia, 2/30 (6.6%) showed focal membranous positivity for HER2/neu and 28/30 (93.3%) were negative for HER2/neu. Amongst hyperplasia cases positive for HER2/neu, moderate staining (score 2) was seen in 1/30 (3.3%) and mild staining (score 2) was seen in 1/30 (3.3%). Both these cases of hyperplasia showed immuno-positivity for HER2/neu in > 20% positive cells.

Out of 30 cases of metaplasia, 4/30 (13%) showed focal membranous positivity for HER2/neu while 26/ 30 (87%) were negative for HER2/neu. Amongst metaplasia cases positive for HER2/neu intense staining (score 3) was seen in 2/30 (6.7%) and mild staining (score 2) was seen in other 2/30 (6.7%). In the metaplasia cases positive for HER2/neu, 2/30 (6.7%) showed positivity in > 20% cells and 2/30 (6.7%) showed positivity in 11-20% cells.

Out of 30 cases of dysplasia 8/30 (27%) showed focal or diffuse membranous positivity for HER2/neu, while 22/ 30 (73%) were negative for HER2/neu. Amongst dysplasia cases positive for HER2/neu, 3/30 (10%) showed intense staining (score 3), whereas 5/30 (16.7%) showed mild staining (score2). In dysplasia cases positive for HER2/ neu, 2/30 (6.6 %) cases showed immuno-positivity in > 40 % cells and rest of the 6/30 (20%) showed immuno-positive cells in the range of 11-40%.

H-score:

Maximum H score of hyperplasia, metaplasia, dysplasia were 60, 80 ,180 respectively.

The difference in H-score of HER2/neu expression between dysplasia and hyperplasia was found to be statistically significant with p-value <0.044. However, the difference in H-score for HER2neu expression between dysplasia-metaplasia and hyperplasia-metaplasia was not statistically significant with p-value 0.186, 0.401 respectively.

Discussion

In our study 1500 cases of cholecystectomy specimens with stones were reviewed and out of these 1213 had no precursor lesions and the epithelial changes were found in 287 cases and showed features of hyperplasia, metaplasia and dysplasia in 122 (8%), 135 (9%) and 30 (2%) cases respectively. In dysplasia cases adjacent epithelium had associated hyperplasia and intestinal metaplasia in 11 and 18 cases respectively. GB Dysplasia is an uncommon lesion as its prevalence is <3% in cholecystectomy specimens [4]. Most of the dysplastic lesions present as a small focus adjacent to invasive GB adenocarcinoma however, our study didn't include invasive carcinoma. We found that majority 18/30 (60%) of dysplasia cases

Table 3. Range and Mean H Score of p16, p53, COX-2, HER2/neu Immuno-expression in Various Histological Categories.

Histological categories	H-score range for p16 (mean H-score)	H-score range for p53 (mean H-score)	H-score range for COX-2 (mean H-score)	H-score range for HER2/ neu (mean H-score)
Hyperplasia	0-60 (2.6)	0	0-90 (5.6)	0-60 (3.33)
Metaplasia	0-80 (4)	0	0-180 (18.3)	0-80 (6)
Dysplasia	0-260 (77.16)	0-210 (12.1)	10-190 (92)	0-180 (16.33)

were associated with intestinal metaplasia, suggesting that metaplasia may be the precursor for dysplasia which may later progress to carcinoma. In the study by Bhawna et al of 900 cholecystectomy specimens they found 34 (3.7%) cases of dysplasia and 11 of those were associated with intestinal metaplasia4 . Kumar et al studied on 400 cholecystectomy specimen and found hyperplasia, metaplasia and dysplasia in 126 (31%), 95 (23%), 7 (1.75%) cases respectively [5]. Digvijay et al found hyperplasia, metaplasia and dysplasia in 62 (4.9%), 124 (9.8%), 19 (1.5%) cases respectively in the study which included 1259 cases of cholecystectomy specimens with stones. In our study the occurrence of precursor lesions is similar to that of Digvijay et al. But the prevalence of precursor lesion in our study was not in concordance with various other studies, the possible explanation for difference in prevalence of hyperplasia, metaplasia, dysplasia could be difference in sample size, selection criteria, criteria used for diagnosis of precursor lesions and population geographical variation.

The ages of patients in our study ages of the patients ranged from 18 years to 60 years, which is similar to that found in other studies (Bhwana et al, Digvijay et al, Khanna et al, Kumar et al). However, the mean ages of hyperplasia, metaplasia and dysplasia in our study was 30, 35 and 39 years respectively. The mean age of dysplasia was 47 years in the study conducted by Bhwana et al. The mean ages of hyperplasia, metaplasia and dysplasia in the study done by Kumar et al were 42, 42 and 56 yrs respectively which is much higher than our study. The mean age of dysplasia in the study conducted by Khanna et al was 40 years [6]. Thus in our study the mean ages of various histological categories were lower compared to the previous studies. The possible explanation for this could be that we noted down the ages from the histological requisition form, and which might not be accurate and correct ages of the patients.

In our study all three types of lesions showed higher incidence in female with male to female ratio being 1:3 which is similar to the finding of various other studies done by Bhwana et al, Kumar et al, Digvijay et al and Khanna et al. The higher prevalence in females can be attributed to female hormones like estrogen, which leads to cholesterol supersaturation resulting in formation of stones and progesterone which retards gallbladder emptying leading to prolong staying of mutagens and resulting in carcinogenesis [7, 8].

p16 protein expression

In our study 29/30 (96%) cases of dysplasia showed p16 positivity (nuclear and cytoplasmic), whereas only 3/30 (10%) hyperplasia, and 4/30 (13%) metaplasia showed p16 positivity. We found p16 expression both in high as well as low grade of dysplasia. High percentage of p16 expression in our study can be attributed to the monoclonal antibodies used for detection of p16. We used 16PO4, JC, BSB5827 clone of mouse monoclonal antibody, which is different from the antibody used by earlier workers.

p16 staining was focal and mild in majority of hyperplasia and metaplasia; intense staining was observed only in 1 case of hyperplasia and 2 cases of metaplasia. Whereas majority of dysplasia cases (27/29) showed intense and diffuse immuno-positivity with p16. The pattern and intensity of staining in dysplasia in our cases is similar to that reported by Choi et a.l [9].

Forty three percent of dysplasia cases showed p16 immuno-positivity in >40% positive cells, in comparison to hyperplasia and metaplasia in which none of the case had >40% positive cells.

Percentage of immuno-positive cells and H-score for p16 was significantly higher ($p < 0.05$) in dysplasia compared to hyperplasia and metaplasia in our study. These results are in concordance with earlier studies done Feng et al, Choi et al and Brigdet et al. However, the p16 expression between hyperplasia and metaplasia was not statistically significant.

A pattern of progressive increase expression was noted from low grade to high grade dysplasia. The high percentage of p16 expression in low as well as high grade dysplasia was similar to that reported in early cases of GB carcinoma [10]. Therefore, our findings support the concept that dysplasia is a precursor lesion that progresses to GB carcinoma and p16 expression plays a significant role in beginning of GB carcinogenesis as its mutation occurs through deletion or epigenetic methylation.

p53 protein expression

In our study 5/30 (16%) cases of dysplasia showed p53 expression (nuclear) whereas none of hyperplasia, metaplasia showed any p53 expression. This is in concordance with the study done by Kamel et al as they found p53 immuno-expression in 2/8 (25%) cases of dysplasia, with no staining in cases of metaplasia [11] Ignaico et al also had similar results in their study with no expression of p53 in normal and metaplastic epithelium.

The intense staining for p53 in our study for dysplasia cases 2/5 (40%) was similar to that found by Wee et al as they reported intense staining in 2/6 (44%) cases of dysplasia. Comparison of H-score of p53 expression in dysplasia to that of hyperplasia and metaplasia turn out to be statistically significant with $p<0.005$ implying

that there is significant difference in p53 expression in dysplasia to that of hyperplasia and metaplasia our results in concordance with the finding of study done by Ignaico et al. [12].

The finding of p53 staining was seen in only in dysplasia with intense staining and in high grade dysplasia, suggests that p53 might play a role in progression of carcinogenesis and not the early phase of initiation.

COX-2 protein expression

In our study COX-2 cytoplasmic expression was seen in 30/30 (100%) cases of dysplasia, 5/30 (16.6%) cases of hyperplasia and 7/30 (23%) cases of metaplasia. In the study done by Legan et al COX-2 expression was seen in 14% normal gallbladder epithelium, 70% of dysplastic epithelium and 60% of carcinoma cases [13].

Similar to the study done by Tsuchida et al we found intense staining in majority of dysplasia 24/30 (80%) and few cases of hyperplasia 1/30 (3.33%) and 3/30 (10%) metaplasia.

Comparison of H-score of COX-2 expression between dysplasia to that of hyperplasia and metaplasia was statistically significant $p<0.05$ which is in concordance with study done by Tsuchida et al and Renato et al.

As COX-2 is an inflammatory mediator and its expression is seen in hyperplasia, metaplasia and dysplasia which can be related to its expression with mild changes in epithelial lining, owing to no role in GB carcinogenesis, it is mostly related to inflammatory changes in gallbladder.

HER2/neu protein expression

HER2/neu (membranous) expression was seen in 8/30 (27%) dysplasia, 2/30 (7%) hyperplasia, 4/30 (13%) metaplasia in our study. It is in contrast to the study done by Toledo et al who reported HER2/neu positivity in 9/10 (90%) of high-grade dysplasia and 40/44 (90%) cases of metaplasia with no expression in normal gallbladder epithelium. In another study done by Puhalla et al only 7/54 (13%) cases of high-grade dysplasia showed expression of HER2/neu and normal epithelium didn't show any reactivity. The difference in percentage positivity in various precursor lesion in our study with that of Toledo et al may be that we considered only membranous staining as positive similar to Puhalla et al who had also found 13% of high-grade dysplasia positive for HER2/neu [14, 15].

Of the positive cases for HER2/neu immuno-expression only 3/30 (10%) dysplasia, 1/30 (3.3%) hyperplasia and 2/30 (6.6%) metaplasia showed moderate to intense membranous staining which is similar to observation by Toledo c et al and Pullah et al.

Majority of hyperplasia 28/30 (93%), metaplasia 28/30 (93%) and even dysplasia 26/30 (86%) showed <20% positive cells and only 2/30 (6.6%) dysplasia showed >40% positive cells.

The difference in HER2/neu H score expression between dysplasia and hyperplasia, was statistically significant $p<0.05$.

Whereas the difference in HER2/neu expression between dysplasia and metaplasia was non-significant with $p>0.05$. This finding was in concordance with Toledoc et al, showing that the cases of metaplasia also express HER2/neu.

The difference in H-score of HER2/neu expression between hyperplasia and metaplasia was non-significant with $p>0.05$.

The expression of HER2/neu varies among the precursor lesions. Even metaplasia cases showed almost similar reactivity for HER2/neu as dysplasia cases showing it links that dysplasia arises from metaplasia and it progresses into carcinoma in-situ. Presence of HER2/neu expression in early cases of metaplasia suggests that its mutation occurs at much early in precursor lesions leading to initiation of carcinogenesis.

This study provides a comprehensive evaluation of four molecular markers across the full spectrum of gallbladder precursor lesions, allowing formulation of a sequential model of carcinogenesis. The high expression of p16 in dysplasia represents a paradox, as p16 is a tumor suppressor. This may be explained by a compensatory overexpression in response to RB pathway dysfunction or oncogenic stress, rather than a direct oncogenic role [13]. The findings suggest that HER2/neu alteration may represent an early event occurring at the stage of metaplasia, while p16 overexpression is associated with the establishment of dysplasia. Accumulation of p53 appears to be a later event, marking progression within dysplastic lesions. COX-2 expression across all lesion categories suggests that it is more closely related to chronic inflammation rather than being a primary driver of neoplastic transformation.

In conclusion, This study highlights the spectrum and frequency of epithelial precursor lesions in cholecystectomy specimens with gallstones and evaluates their immunohistochemical profile. Hyperplasia, metaplasia, and dysplasia were identified in a significant proportion of cases, supporting the concept of a stepwise progression in gallbladder carcinogenesis. This study demonstrates that a panel-based approach provides a more comprehensive understanding of gallbladder carcinogenesis

A progressive increase in p16 expression from hyperplasia and metaplasia to dysplasia suggests its potential role as an early biomarker in the dysplasia–carcinoma sequence. In contrast, p53 expression was restricted to dysplastic lesions, particularly high-grade dysplasia, indicating its probable involvement in the later stages of neoplastic progression. Although COX-2 expression was significantly higher in dysplasia, its presence in hyperplastic and metaplastic epithelium suggests that it may be more closely related to chronic inflammation rather than a specific driver of malignant transformation. HER2/neu expression in both metaplastic and dysplastic lesions indicates a possible early molecular alteration that may contribute to tumor initiation and progression. The study is limited by modest sample size for immunohistochemistry, semi-quantitative scoring (H-score), and lack of follow-up data to assess progression risk.

Overall, the findings support the hypothesis that

metaplasia may precede dysplasia and subsequently carcinoma in gallbladder carcinogenesis. Immunohistochemical evaluation of precursor lesions, particularly p16 and p53, may aid in identifying high-risk epithelial changes in routine cholecystectomy specimens and improve understanding of early molecular events in gallbladder cancer development. The strong and statistically significant expression of p16 and p53 in dysplastic lesions suggests their potential utility as adjunct biomarkers for identifying high-risk epithelial changes. Recognition of such lesions may prompt closer clinical surveillance, especially in younger patients or those from endemic regions.

This study underscores the importance of meticulous histopathological examination and selective immunohistochemical evaluation in routine cholecystectomy specimens, potentially enabling earlier identification of patients at risk for gallbladder carcinoma and contributing to improved preventive and surveillance strategies.

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Statement of Transparency and Principles

- Author declares no conflict of interest
- Study was approved by Research Ethic Committee of author affiliated Institute.
- Study's data is available upon a reasonable request.
- All authors have contributed to implementation of this research.

References

1. Lazcano-Ponce EC, Miquel JF, Muñoz N, Herrero R, Ferrecio C, Wistuba II, Alonso de Ruiz P, et al. Epidemiology and molecular pathology of gallbladder cancer. CA: a cancer journal for clinicians. 2001;51(6):349-364. https://doi.org/10.3322/canjclin.51.6.349
2. Roa I, Aretxabala X, Araya JC, Roa J. Preneoplastic lesions in gallbladder cancer. Journal of Surgical Oncology. 2006 06 15;93(8):615-623. https://doi.org/10.1002/jso.20527
3. Nakajo S, Yamamoto M, Tahara E. Morphometrical analysis of gall-bladder adenoma and adenocarcinoma with reference to histogenesis and adenoma-carcinoma sequence. Virchows Archiv. A, Pathological Anatomy and Histopathology. 1990;417(1):49-56. https://doi.org/10.1007/BF01600109
4. Jain BB, Biswas RR, Sarkar S, Basu AK. Histopathological spectrum of metaplasia, dysplasia and malignancy in gall bladder and association with gall stones. J Indian Med Assoc. 2010;108(2):81-3.
5. Kumar H, Kini H, Tiwari A. Histological evaluation of 400 cholecystectomy specimens. Journal of Pathology of Nepal 5(10):834-840. 2026 05 25;. https://doi.org/10.3126/jpn.v5i10.15640
6. Khanna R, Chansuria R, Kumar M, Shukla HS. Histological changes in gallbladder due to stone disease. Indian J Surg. 2006;68(4):201-4.
7. Everson GT, McKinley C, Kern F. Mechanisms of gallstone formation in women. Effects of exogenous estrogen (Premarin) and dietary cholesterol on hepatic lipid metabolism. The Journal of Clinical Investigation. 1991 01;87(1):237-246. https://doi.org/10.1172/JCI114977
8. Gupta P, Agarwal A, Gupta V, Singh PK, Pantola C, Amit S. Expression and clinicopathological significance of estrogen and progesterone receptors in gallbladder cancer. Gastrointestinal cancer research: GCR. 2012 03;5(2):41-47.
9. Choi H, Yun SS, Kim HJ, Choi JH. Expression of p16 protein in gallbladder carcinoma and its precancerous conditions. Hepato-Gastroenterology. 2010;57(97):18-21.
10. Feng Z, Chen J, Wei H, Gao P, Shi J, Zhang J, Zhao F. The risk factor of gallbladder cancer: hyperplasia of mucous epithelium caused by gallstones associates with p16/CyclinD1/CDK4 pathway. Experimental and Molecular Pathology. 2011 Oct;91(2):569-577. https://doi.org/10.1016/j.yexmp.2011.06.004
11. Kamel D, Pääkkö P, Nuorva K, Vähäkangas K, Soini Y. p53 and c-erbB-2 protein expression in adenocarcinomas and epithelial dysplasias of the gall bladder. The Journal of Pathology. 1993 05;170(1):67-72. https://doi.org/10.1002/path.1711700111
12. Wee A, Teh M, Raju GC. Clinical importance of p53 protein in gall bladder carcinoma and its precursor lesions. Journal of Clinical Pathology. 1994 05;47(5):453-456. https://doi.org/10.1136/jcp.47.5.453
13. Legan M, Luzar B, Marolt VF, Cor A. Expression of cyclooxygenase-2 is associated with p53 accumulation in premalignant and malignant gallbladder lesions. World Journal of Gastroenterology. 2006 06 07;12(21):3425-3429. https://doi.org/10.3748/wjg.v12.i21.3425
14. Toledo C, Matus CE, Barraza X, Arroyo P, Ehrenfeld P, Figueroa CD, Bhoola KD, et al. Expression of HER2 and bradykinin B_1 receptors in precursor lesions of gallbladder carcinoma. World Journal of Gastroenterology. 2012 03 21;18(11):1208-1215. https://doi.org/10.3748/wjg.v18.i11.1208
15. Puhalla H, Wrba F, Kandioler D, Lehnert M, Huynh A, Gruenberger T, Tamandl D, Filipits M. Expression of p21(Wafl/Cip1), p57(Kip2) and HER2/neu in patients with gallbladder cancer. Anticancer Research. 2007;27(3B):1679-1684.