



# Significance of HER2 and Ki-67 in Preneoplastic Lesions and Carcinoma of Gallbladder

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## Abstract

**Background** HER2 is an oncoprotein which is overexpressed in several cancers including breast and stomach. Several studies have shown that HER2 is overexpressed in gallbladder cancer and in precancerous lesions. The present study was undertaken to assess pattern and level of expression of HER2 in metaplasia, dysplasia, and different stages of gallbladder carcinoma, which would determine its suitability as a prognostic biomarker in neoplastic transformation of gallbladder epithelium. The study was also aimed at to find the significance of Ki-67 index in these lesions.

**Methods and Materials** One hundred and twenty-eight patients who underwent cholecystectomy comprised the study group. Among them, 108 (84.4%) specimens showing metaplasia, dysplasia, and carcinoma on routine histopathology were considered as cases and 20 (15.6%) specimens of chronic cholecystitis having non-metaplastic mucosa were considered as control. Immunohistochemistry (IHC) was performed for HER2 and Ki-67. For HER2 interpretation ASCO/CAP guideline for breast cancer was followed. Chi-square test was used to find out the significance of HER2 expression in dysplasia/metaplasia/carcinoma. The ANOVA and Tukey-Kramer Multiple Comparisons Test were used for determining the association of Ki-67 with malignant transformation.

**Results and Conclusions** Overexpression of HER2 was observed in 48% ( $n = 12$ ) of adenocarcinomas, 58% ( $n = 7$ ) of high-grade dysplasia, 47% ( $n = 8$ ) of low-grade dysplasia, and 74% ( $n = 25$ ) of intestinal metaplasia. Ki-67 index increases in a non-linear fashion as the precursor lesions progress toward malignancy. In the future, these markers might be used as a prognostic biomarker for gallbladder carcinoma and its precursor lesions and it might become a valid indication for targeted therapies for gallbladder cancer.

**Keywords** Preneoplastic conditions of gallbladder · Adenocarcinoma of gallbladder · HER2 · Ki-67

## Introduction

Gallbladder carcinoma is an uncommon malignancy with a high mortality rate. It stands out from among the other gastrointestinal tract malignancies for its unique epidemiological profile, proclivity for females, definitional ambiguities, ability to escape early diagnosis, and relative absence of effective treatment [1]. However, in Chile and India, gallbladder cancer remains a major problem [2] (age-standardized rates from 3.9 to 8.6/100,000) with the vast majority of patients presenting with advanced disease.

The incidence of gallbladder cancer in north and central India is very high. It is the commonest gastrointestinal cancer in women [3]. Even Indian migrants to different countries have a higher risk of having gallbladder cancer as compared to the natives. The incidence of gallbladder cancer parallels the prevalence of gallstone disease; gallstone disease is common in north India and occurs at a younger age than in western populations. Moreover, patients with gall stone disease present for treatment a long time after the onset of symptoms. Both these factors result in prolonged exposure of the gallbladder to stones [4].

Two distinct independent biological pathways leading to gallbladder cancer are hypothesized, based on morphological, genetic, and molecular evidence: (1) a metaplasia-dysplasia-carcinoma sequence and (2) an adenoma-carcinoma sequence, contributing only 3% of cases [5].

The HER2 gene is located on the 17q12-q21 chromosomal region and acts as an oncogene in several human cancers [6].

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HER2 protein overexpression, either as the product of gene amplification or transcriptional deregulation, is observed in approximately 20% of breast and ovarian cancers [7] and 12% of gastric cancer [8].

Ki-67 is a nuclear protein encoded by the MKI67 gene on the long arm of chromosome 10 (10q26). It is closely and strictly associated with cell proliferation. Some studies have found that Ki-67 expression is higher in moderately differentiated than in well-differentiated gallbladder carcinoma [9].

An estimated 10–30% of gallbladder cancers show HER2 protein overexpression, depending upon the criteria used for interpretation of HER2 [10]. However, data regarding the efficacy of HER2 therapy for gallbladder cancer is scarce [11].

So far, very few studies have been undertaken to determine HER2 overexpression in gallbladder metaplasia and dysplasia [12].

The present study was undertaken to assess pattern and level of expression of HER2 in metaplasia, dysplasia, and different stages of gallbladder carcinoma, which would determine its suitability as prognostic biomarker in early neoplastic transformation of gallbladder epithelium. The study also aimed to find the significance of Ki-67 index in these lesions.

### Materials and Methods

The present study was an observational, prospective, as well as retrospective study, conducted in the Department of Pathology, ESIPGIMS Maniktala, Kolkata. This study was commenced after obtaining ethical clearance from the ethical committee of the institution. The sample consisted of 128 cholecystectomy specimens. Inclusion criteria for cases were metaplastic, dysplastic, or carcinomatous changes in the epithelium on routine histopathologic examination done by hematoxylin and eosin (H&E) staining. Exclusion criteria were extensively denuded mucosa or fixation artifacts. Based on these inclusion and exclusion criteria, 108 (84.4%) specimens were selected as cases. Twenty (15.6%) specimens of gallbladder with no such changes in the mucosa were selected as controls. Included cases did not include any cases which were known to be metastatic. For retrospective study, slides were reviewed and corresponding

paraffin blocks were retrieved. Cases and controls were selected. On H&E-stained sections, intestinal metaplasia cases showed mucosa composed of tall columnar cells with brush borders, variable proportion of goblet cells, and Paneth cell metaplasia. Cases of pyloric metaplasia showed small branching glands consisting of columnar and cuboidal cells with abundant bubbly cytoplasm and basally located flattened nuclei. Combined Periodic Acid-Alcian Blue staining (ABPAS) at low pH (pH 1) were done to distinguish between these two. Dysplasia was found in 29 cases, they were categorized into low-grade and high-grade dysplasia. Low-grade dysplasia had pseudostratification, mild nuclear atypia limited to basal 2/3rds of epithelium. High-grade dysplasia had stratification, with marked nuclear atypia involving whole thickness of the epithelium. Carcinoma was diagnosed in 25 cases. All of them were incidental findings. They were again classified into well-differentiated and poorly differentiated; staging was done according to TNM staging.

Immunohistochemistry (IHC) was done on the formalin-fixed paraffin-embedded sections. HER2 was done using rabbit monoclonal HER2 antibody, clone SP3, Cell Marque. Immunohistochemistry for Ki was performed using rabbit monoclonal Ki-67 antibody of clone SP6. Assessment for HER2 and Ki-67 was done by two pathologists individually. ASCO/CAP guideline for interpretation of HER2 in breast [13] was followed for scoring of HER2 in this study. FISH-positive HER2 3+ breast carcinoma tissue was considered as positive control for HER2. Whole section was scanned at low magnification, and “hotspots” (areas where Ki-67 staining is particularly prevalent) were identified. Ki-67 score was calculated in each case as the percentage of positively nuclear stained cells among the total number of malignant/dysplastic/metaplastic or normal non-metaplastic epithelial cells, whichever is implicated.

Chi-square test was used to find out the significance of HER2 expression in dysplasia/metaplasia/carcinoma. The ANOVA and Tukey-Kramer Multiple Comparisons Test were used for determining the association of Ki-67 with malignant transformation. Chi-square test was used to find out the significance of HER2 expression in different stages and grades of

**Table 1** Demographic characteristics of patients, HER2 score and Ki-67 expression in various preneoplastic lesions and carcinoma of gallbladder

Type of lesion	Age (± SD)	M:F ratio	HER2 0/+1 (%)	HER2 2+/3 (%)	Ki-67 in percent
Carcinoma (n = 25)	53.58 (±9.5)	1:24	n = 13 (52)	n = 12 (48)	43.75
High-grade dysplasia (n = 12)	49.16 (± 15.83)	0:12	n = 5 (41.7)	n = 7 (58)	40.87
Low-grade dysplasia (n = 17)	47.88 (± 15.49)	2:15	n = 9 (53)	n = 8 (47)	36.06
Intestinal metaplasia (n = 34)	45.05 (± 11.62)	3:31	n = 9 (26.4)	n = 25 (74)	14.16
Pyloric metaplasia (n = 20)	41.15 (± 9.75)	1:19	n = 19 (95)	n = 1 (5)	1
Normal (n = 20)	38.75 (± 11.06)	1:19	n = 20 (100)	n = 0 (0)	1

**Table 2** HER2 score in carcinoma, dysplasia, metaplasia, and non-metaplastic epithelium of Gallbladder

HER2 score	Carcinoma <i>n</i> = 25 (%)	HER2 score in carcinoma according to grade (%)			High-grade dysplasia <i>n</i> = 12 (%)	Low-grade dysplasia <i>n</i> = 17 (%)	Intestinal metaplasia <i>n</i> = 34 (%)	Pyloric metaplasia <i>n</i> = 20 (percent)	Normal mucosa <i>n</i> = 20 (%)
		Well differentiated <i>n</i> = 18	Moderately differentiated <i>n</i> = 4	Poorly differentiated <i>n</i> = 3					
0	6 (24)	2 (11.1)	1 (25)	3 (100)	1 (8)	2 (11.8)	1 (2.9)	9 (45)	8 (40)
1	7 (28)	5 (27.8)	2 (50)	0 (0)	4 (33)	7 (41.2)	8 (23.5)	10 (50)	12 (60)
2	8 (32)	7 (38.9)	1 (25)	0 (0)	6 (51)	8 (47)	23 (67.6)	1 (5)	
3	4 (16)	4 (22.2)	0 (0)	0 (0)	1 (8)	0 (0)	2 (6)	0 (0)	

carcinoma. Statistical analysis was done using Statistical Package for Social Services (SPSS), version 17.

## Results

The study group comprised of 108 cases of which 25 (23.2%) cases were adenocarcinoma, 12 (11.1%) cases were of high-grade dysplasia, 17 (15.7%) cases were of low-grade dysplasia, 34 (31.5%) cases were of intestinal metaplasia, and 20 (18.5%) cases were of pyloric metaplasia. Twenty cases of non-metaplastic mucosa were taken as control. Patients' characteristics are summarized in Table 1. It was observed in the present study that the whole spectrum of gallbladder epithelial changes is more common in females than in males (female:male = 15:1) and cholecystitis with non-metaplastic mucosa was observed at lower age group (mean age 38.75 years), whereas carcinoma occurred in higher age groups (mean age 53.58 years). Mean age increases as the precursor lesions progress toward malignancy. Considering both 2+ and 3+ positivity, the present study shows overexpression of HER2 in the intestinal type of metaplasia (2+ in 67.6% and 3+ in 6%, with a total of 74% cases of high positivity), in low-grade dysplasia (2+ in 47%), and in high-grade dysplasia (2+ in 50% and 3+ in 8%, with a total of

58% cases showing high positivity). HER2 overexpression was seen in 48% (3+ in 16%, 2+ in 32%) of overall carcinoma.

Well-differentiated carcinoma shows increased HER2 expression (3+ in 22% and 2+ in 39%, with a total of 61% cases of high positivity), whereas decreased HER2 expression is seen in moderately and poorly differentiated carcinoma (HER2 2+ in 14%), pyloric metaplasia (2+ in 5%, 0 or 1+ in 95%), and non-metaplastic mucosa (0 or 1+ in 100%) (Tables 2 and 3).

Association of increased HER2 expression in intestinal metaplasia, low-grade dysplasia, and well-differentiated carcinoma of gallbladder (category 1), in comparison with low HER2 expression in pyloric metaplasia, advanced carcinoma, and normal epithelium of the gallbladder (category 2), has been found to be statistically significant using chi-square test (*p* value is less than 0.00001 and chi-square statistic is 42.2518) (Table 3).

Increased HER2 overexpression (2+ or 3+) in well-differentiated carcinomas (61%) compared with decreased incidence of HER2 overexpression (14%) in higher grade (moderate and poorly differentiated carcinoma) (Tables 2 and 4). This association is statistically significant at *p* < 0.05.

Increased HER2 overexpression was also observed in carcinoma of lower stage (stages T1 and T2) in comparison to carcinoma of higher stage (T3 and T2N1) in this study. This

**Table 3** Categorization of lesions according to level of HER2 expression, considering 0 and 1+ as low expression and 2+ and 3+ as high expression. High HER2 expression in category 1 lesions compared with low HER2 expressions of category 2 lesions using chi-square test

Category	Type of lesion (total number)	HER2 score 0 OR 1 (%)	HER2 score 2 or 3 (%)
Category 1 (lesions showing predominantly increased HER2 expressions)	Intestinal metaplasia (34)	9 (26)	25 (74)
	Low-grade dysplasia (17)	9 (53)	8 (47)
	High-grade dysplasia (12)	5 (42)	7 (58)
	Well-differentiated carcinoma (18)	7 (39)	11 (61)
Category 2 (lesions showing decreased HER2 expressions)	Pyloric metaplasia (20)	19 (95)	1 (5)
	Moderately and poorly differentiated carcinoma (7)	6 (85.7)	1 (14.3)
	Normal epithelium (20)	20 (100)	0 (0)

The chi-square statistic is 42.2518. The *p* value is < .00001. The result is significant at *p* < .05

**Table 4** HER2 score in carcinoma according to tumor grade

Grade	HER2 Score 0 and 1+ (%)	HER2 score 2+ and 3+ (%)
Well-differentiated carcinoma (N = 18)	7 (39%)	11 (61%)
Moderately and poorly differentiated carcinoma (N = 7)	6 (86%)	1 (14%)

association is also significant at  $p < 0.05$  ( $p$  value 0.035366) (Tables 5 and 6).

It was observed in the study that Ki-67 increased in a non-linear fashion in the following sequence: cholecystitis and pyloric metaplasia → intestinal metaplasia → low-grade dysplasia → high-grade dysplasia → carcinoma (Figs. 1, 2, 3, and 4). To test the significance of this association, the one-way analysis of variance (ANOVA) test was applied for the result. Using the data obtained with ANOVA, Tukey-Kramer Multiple Comparisons Test was done. If the value of  $q$  is greater than 4.108, then the  $p$  value is less than 0.05 (Table 7).

### Discussion

Epidemiologic data from published studies have shown that age-adjusted incidence rates in gallbladder malignancy (per 100,000) range from 0.16 for those 20–49 years to 1.47 for those 50–64 years, to 4.91 for 65–74 years, and to 8.69 for individuals over the age of 75 years [14]. Thus, there is a trend for cholecystectomy specimens to be more likely to have malignant changes with increasing age.

In the present study, mean age of the patients increases in the following order: normal non-metaplastic mucosa, metaplasia, dysplasia and carcinoma. Like other studies, it has been found that gallbladder disease is more common in females and the incidence of premalignant lesions and carcinoma of the gallbladder increases with increasing age [15].

All the malignancies in this study were incidental finding, predominantly of lower grade and stage, associated with cholelithiasis, and mostly occurred in higher age groups than all

**Table 5** HER2 score in carcinoma according to TNM staging

TNM staging	Number of carcinoma cases	HER2 score			
		0	1+	2+	3+
T1	11	2	2	3	4
T2	7	0	3	4	
T3	4	2	2		
T2 N1	3	2		1	

**Table 6** Expression HER2 according to stage of carcinoma considering HER2 0 and 1+ as negative and HER2 2+ and 3+ as positive and contingency table for association between HER2 score and tumor staging. The chi-square statistic is 4.4274. The  $p$  value is 0.035366. The result is significant at  $p < 0.05$ . The values in each cell represent the observed cell totals, (the expected cell totals), and the [chi-square statistic] for each cell

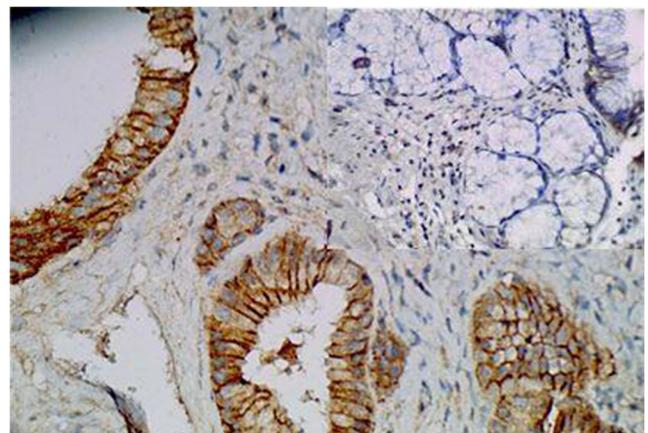
Staging	HER2 0 and 1+	HER2 2+ and 3+
T1 and T2 (n = 18)	7 (39%) (9.36) [0.60]	11 (61%) (8.64) [0.64]
T3 and T2 N1 (n = 7)	6 (86%) (3.64) [1.53]	1 (14%) (3.36) [1.66]

other lesions. Like all other gallbladder lesions, carcinoma is also more prevalent in females.

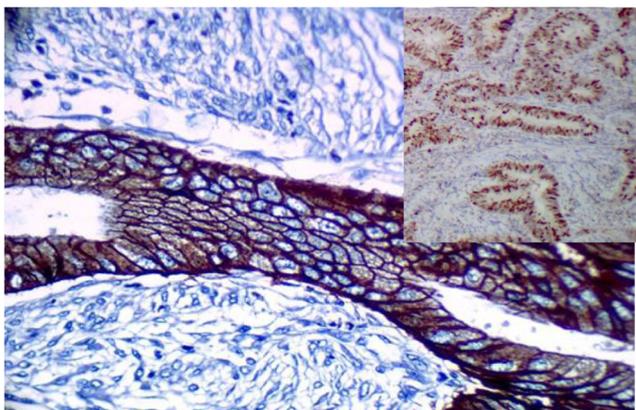
The percentage of overexpression of HER2 was in concordance with the studies, which adopted the ASCO/CAP guidelines for interpretation of HER2 score [16]. In contrast to the present study, Toledo et al. [10] observed 33% HER2 expression in malignancy (considering membrane staining only, disregarding intensity and extent), whereas the finding of HER2 in high-grade dysplasia was similar to our study. Chaube et al. [17] observed 25% of HER2 positivity (considering both cytoplasmic and membrane staining) in carcinoma.

In the study by Ashai et al. [18], dysplastic epithelium adjacent to carcinoma showed similar IHC pattern to that of carcinoma, similar to the present study. In the other studies, detailed above, there is no reported HER2 positivity in dysplastic epithelium. In the present study, dysplasia was classified into high-grade and low-grade dysplasias with 47% of low-grade dysplasia showed overexpression for HER2. In the available literature, there is no separate description for HER2 expression in low-grade dysplasia.

In cases of intestinal metaplasia, expression of HER2 was in accordance with the study of Toledo et al. [10] who found 91.7% positivity in metaplasia (without considering intensity and extent of membrane staining), whereas in this study, 74% positivity has been found in intestinal metaplasia (considering



**Fig. 1** HER2 score of 3+ in intestinal metaplasia. Inset shows HER2 score 0 in pyloric metaplasia (HER 2 immunostain × 400)

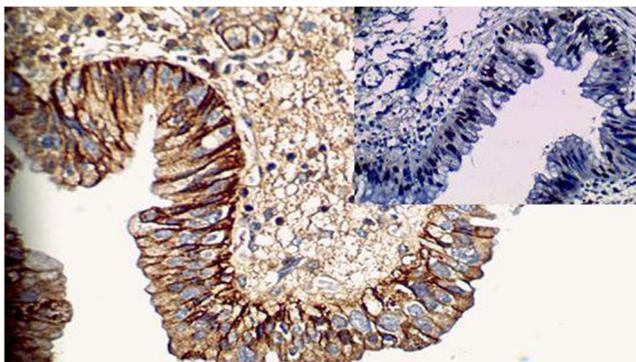


**Fig. 2** HER2 score 3+ in well-differentiated carcinoma (HER2 immunostain × 400). Inset showing high Ki-67 index (80%) in well-differentiated carcinoma (Ki-67 immunostain × 100)

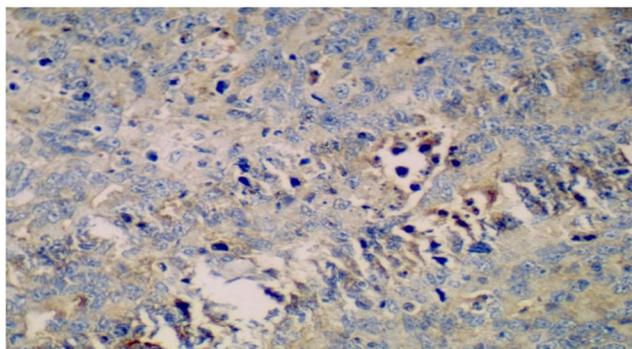
both 2+ and 3+ positivity). None of the other studies referred above have found any HER2 overexpression in metaplasia. Similar to the findings of the present study, Toledo et al. [10] found no expression for HER2 in pyloric metaplasia.

In normal epithelium, Toledo et al. [10] and Ashai et al. [18] found no expression for HER2 similar to the findings of this study. However, Kawamoto et al. [19] found overexpression of HER2 in 12.5% of non-malignant gallbladder diseases (cholecystitis), considering both 2+ and 3+ positivity, but they did not specify whether any associated metaplastic or dysplastic changes were present in these cases of cholecystitis.

HER2 overexpression has been observed to be more frequent in better differentiated carcinomas in some of the studies [20]. However, Roa et al. [6] reported a lower incidence of HER2 overexpression in the early carcinomas (mucosal and muscular), compared with the advanced stage carcinomas (subserosal and serosal) (7.1% vs 13.8%); however, this difference was not statistically significant. The significance of our finding of increased HER2 expression in lower grade and stage carcinomas (compared to higher grade and stage lesions) cannot be fully explained with the available data. However, we hypothesize that this might be a consequence



**Fig. 3** HER2 score of 3+ in high-grade dysplasia (HER2 immunostain × 400). The inset showing raised Ki-67 index in high-grade dysplasia (Ki-67 immunostain × 100)



**Fig. 4** HER2 score of 0 in a case of poorly differentiated carcinoma (HER2 immunostain × 400)

of HER2 receptor downregulation in higher stage and grade lesions. Further studies are required to confirm these findings as well as this hypothesis.

Pujani et al. [21] found increased HER2 expression in 24% of gallbladder carcinoma, considering 3+ positivity according to ASCO-CAP guidelines. Similar to this study, HER2 expression decreased with increased grade of tumor. However, the association was not statistically significant.

No correlation was seen between IHC staining pattern and tumor grade by Ashai et al. [18], but frequency of HER2 positive cases was highest in advanced stage in their study.

Our study did not include cases of metastatic gallbladder carcinoma. Other studies that have included cases of metastatic carcinoma have showed no significant difference in HER2 expression between localized and metastatic disease ( $P = 0.289$ ). [12] However, this differentiation is increasingly relevant as monoclonal therapies targeting HER2 in other cancers, like breast and gastric, are largely employed in the metastatic setting. Other studies with larger cohorts will perhaps help clarify this.

Xuan et al. [22] found in their study that there is higher expression of Ki-67 in gallbladder carcinoma than in precursor lesions. Ki-67 expression was also more prevalent in older patients and in advanced stages of carcinoma.

**Table 7** Significance of the (non-linear) increase of Ki-67 index tested using the Tukey-Kramer multiple association test

Comparison	Mean difference	<i>q</i>	<i>P</i> value
carcinoma vs dysplasia	7.530	2.396	>0.05
carcinoma vs intestinal metaplasia	29.590	11.446	<0.001
Carcinoma vs pyloric metaplasia	42.750	15.206	<0.001
Carcinoma vs normal	42.750	15.206	<0.001
Dysplasia vs intestinal metaplasia	22.060	8.533	<0.001
Dysplasia vs pyloric metaplasia	35.220	12.528	<0.001
Dysplasia vs normal mucosa	35.220	12.528	<0.001
Intestinal vs pyloric metaplasia	13.160	6.066	<0.001
Intestinal vs normal	13.160	6.066	<0.001
Pyloric metaplasia vs normal	0.000	0.000	

In a study by Stancu et al. [23], Ki-67 index was found to be increased in the carcinogenesis process of gallbladder. Ki-67 index rose from simple dysplasia to severe dysplasia and to carcinoma. Pujani et al. [23] also found increased Ki-67 index in gallbladder carcinoma which was statistically significant.

Similarly, the present study also revealed that a higher or increasing Ki-67 index indicates a higher likelihood of malignant transformation to carcinoma. The difference in Ki-67 index was found to be significant in between the following groups.

- Carcinoma versus intestinal metaplasia/pyloric metaplasia/normal mucosa
- Dysplasia versus intestinal metaplasia/pyloric metaplasia/normal mucosa
- Intestinal metaplasia versus pyloric metaplasia/normal mucosa

## Conclusion

The expression of HER2 and Ki-67 in gallbladder carcinoma and its precursor lesions is of importance in identifying subgroups of patients who would likely benefit from targeted therapies using monoclonal antibodies like trastuzumab.

The study provides evidence that increasing HER2 expression is indicative of ongoing transformation from normal to carcinomatous epithelium in the gallbladder and that there is significant loss of expression of HER2 in advanced stage and grade of carcinoma.

Increasing Ki-67 percentage is indicative of greater proliferation resulting in impending transition from metaplasia to dysplasia.

It can be concluded from the result of this study that HER2 and Ki-67 studies can prognosticate cases of neoplastic proliferation of gallbladder. These markers can also be used to identify those patients most likely to benefit from treatment with targeted monoclonal antibody therapies. Further characterization might also enable HER2 and Ki-67 expression levels to be used in prognostication.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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