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Clinicopathological Study of Ki67 Expression In Dysplastic And Malignant Gallbladders

Dr. Mridul Singh[1], Dr. Pranita Medhi[1], Dr. Dhrubajyoti Borgohain[2], Dr. Lucky Moni Duara[1], Dr. UttamKonwar[3]

[1](Pathology, Assam Medical College and Hospital, Dibrugarh,India)
[2](Anaesthesia, Lakhimpur Medical College and Hospital, India)
[3](General Surgery, Assam Medical College and Hospital, Dibrugarh, India)

Abstract
BACKGROUND*Gallbladder carcinomas are common in North-East, India. Ki67 expression has shown good correlation with dyplastic and malignant gallbladder. However, with conflicting results. We aimed to assess the expression and clinicopathological association of Ki67 in biliary intraepithelial neoplasia (BillN) and carcinomas.*
MATERIAL AND METHODS*It was a hospital based cross-sectional study and was carried out on all gallbladder BillN and carcinoma specimens received in one year from July 2020 to June 2021 in a tertiary care institute.Statistical analysis was performed using SPSS 16.0.*
RESULTS*Ki67 expression progressively increased with significant statistical association between low and high grade BillN (p=0.0026) and between BillN and carcinoma (p=0.0001). Significant association was observedbetween presence of gallstones and Ki67 score (p=0.0479). Ki67 score also increased with histological tumour differentiation(p=0.0392).*
CONCLUSION*Gallbladder carcinomas are aggressive with poor survival and remainsa therapeutic challenge.The full potential of Ki67 as a marker of aggression, therapy and prognosis needs to be tappedfor better management of patients.*
Keyword:*Biliary intraepithelial neoplasia (BillN), Gallbladder carcinoma, Ki67 expression*

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I. Introduction

In India, gallbladder carcinoma ranks 16th among the cancer related deaths.[1] There is a rising trend in gallbladder carcinomas in Dibrugarh district with 7.81% increase in the year 2016.[2]In countries where gallbladder carcinomas are endemic, low-grade dysplasia is seen in as many as 15% of gallbladders with lithiasis, and high grade dysplasia in 1-3.5%.[3]

Understanding the molecular events in gallbladder carcinogenesis may provide a novel targeted therapeutic approach. There is a good correlation of Ki67 labelling index and morphologic aggression indicators of hyperplastic, dysplastic and malignant diseases of gallbladder in addition to its prognostic significance.[4] However, several disparities exist among various studies in regard to Ki67 expression in gallbladder.[4-8]Taking into consideration the lacunae regarding Ki67 expression we aim to assess the expression of Ki67 in biliary intraepithelial neoplasia (BillN) and gallbladder carcinomas (GBC) and to find the association of Ki67 positivity with different clinicopathological parameters.

II. Material And Methods

A hospital based cross-sectional study was carried out on all gallbladder BillN and carcinoma specimens received in the Department of Pathology, Assam Medical College and Hospital, Dibrugarh, Assam from July 2020 to June 2021.During the study, 450 cholecystectomy specimens were received out of which 30 Biliary intraepithelial neoplasias and 18 gallbladder carcinomas were detected.

Study Design: Hospital based cross-sectional study

Study Location: This was a tertiary care teaching hospital-based study done in Department of Pathology, Histopathology section at Assam Medical College, Dibrugarh, Assam

Study Duration: July 2020 to June 2021.

Sample size calculation: This was an open study with all cases of gallbladder carcinomas and Biliary intraepithelial neoplasia (dysplasia) referred to Histopathological section during the study period fulfilling the inclusion criteria.

Subjects & selection method: The study population was drawn from the cholecystectomy specimens (premalignant and malignant lesions) received in the Department of Pathology at Assam Medical College and Hospital, Dibrugarh.

INCLUSION CRITERIA:1. Patients from Department of Surgery and Medicine, Assam Medical College and Hospital with complete clinical examination indicating gallbladder cancer.

2.Incidentally detected gallbladder dysplasias (Biliary intraepithelial neoplasias) and carcinomas.

EXCLUSION CRITERIA:1.Patients with metastasis to gallbladder.2.Autolysed specimens.

Procedure methodology

After written informed consent was obtained, a well-designed questionnaire was used to collect the data of the recruited patients retrospectively. The questionnaire included socio-demographic characteristics such as age, gender, height, weight, and clinical history along with radiological and pathological investigations.

Histopathological examination of the gallbladder was performed using standard protocols. Immunohistochemistry for Ki67 was also done following standard protocol and scoring was done following the stated guidelines.[6]

A total of 25 microscopic fields were examined for Ki67 score and for each microscopic field, scoring was done. Quantitative assessment was done according to the number of stained cells nuclei.

- 0 : score 0
- 1-10% : score 1
- 10-50% : score 2
- 50-100% : score 3

For final score, the sum of 25 microscopic fields were taken in order to express the final score out of 100. The obtained score was expressed as % expression.[6]

External positive and negative controls were put while performing IHC staining in each batch.

Statistical analysis Statistical analysis was done using SPSS 16.0.Approval taken by Institutional Ethical committee reference number: No. AMC/EC/PG/8751

III.RESULTS

During the study, 450 cholecystectomy specimens were received out of which 30 Biliary intraepithelial neoplasias and 18 gallbladder carcinomas were detected.

		BillN		Carcinoma	
		Number	(%)	Number	(%)
Age group (in years)	<30	9	30.00	1	5.56
	31-40	8	26.67	0	0.00
	41-50	9	30.00	8	44.44
	51-60	3	10.00	6	33.33
	61-70	1	3.33	3	16.67
	>71	0	0.00	0	0.00
Total		30	100.00	18	100.00
MEAN		38.27±12.7		52.2±10.0	

Sex	**Male**	**9**	**30.00**	**6**	**33.33**
	Female	**21**	**70.00**	**12**	**66.67**
	Total	**30**	**100.00**	**18**	**100.00**
MALE: FEMALE		**1:2.3**		**1:2**	
Clinical features	**Pain abdomen**	**30**	100.00	**18**	100.00
	Jaundice	**0**	0.00	**2**	11.11
	Weight loss	**2**	6.67	**8**	44.44
	Nausea	**4**	13.33	**2**	11.11
	Vomiting	**2**	6.67	**3**	16.67
	Anorexia	**2**	6.67	**4**	22.22
Number of stones	**None**	**2**	6.67	**6**	33.33
	Single	**6**	20.00	**5**	27.78
	Multiple	**22**	73.33	**7**	38.89
	Total	**30**	**100.00**	**18**	**100.00**

Table 1: Demographic profile and clinical features and gallstones

HISTOLOGY			**Frequency**	**Percentage**
BillN	**Low grade**		**26**	**86.67**
	High grade		**4**	**13.33**
Histological type	**Adenocarcinoma**	**Biliary**	**9**	**50.00**
		Intestinal	**1**	**5.56**
		Mucinous	**1**	**5.56**
		Poorly cohesive	**1**	**5.56**
	ICPN with associated invasive carcinoma		**6**	**33.33**
	TOTAL		**18**	**100.00**

Differentiation	**Well differentiated**	**4**	**22.22**	
	Moderately differentiated	**13**	**72.22**	
	Poorly differentiated	**1**	**5.56**	
	Undifferentiated	**0**	**0.00**	
	Total	**18**	**100.00**	
STAGE		**T stage**	**No. of primary gall bladder carcinoma cases**	**Percentage**
Invasion upto	**Carcinoma in situ**	**Tis**	**4**	**18.18**
	Lamina propria	**T1a**	**0**	**0.00**
	Muscular layer	**T1b**	**7**	**31.82**
	Perimuscular connective tissue, on peritoneal side with no extension to serosa	**T2a**	**6**	**27.27**
	Perimuscular connective tissue, on hepatic side with no extension into the liver	**T2b**	**0**	**0.00**
	Perforates serosa, and/or directly invades liver and/or other adjacent organ or structure	**T3**	**5**	**22.73**
	Invades portal vein or hepatic artery or two or more extrahepatic organs or structures	**T4**	**0**	**0.00**
	TOTAL		**22**	**100.00**

Table 2: Histological profile of BillN and Gallbladder carcinoma

Ki67 Score	**BillN Low-grade**		**BillN High-grade**		**Carcinoma**	
	Number	**(%)**	**Number**	**(%)**	**Number**	**(%)**
0	**3**	10.00	**0**	0.00	**1**	5.56
1	**19**	63.33	**0**	0.00	**1**	5.56
2	**4**	13.33	**4**	13.33	**3**	16.67
3	**0**	0.00	**0**	0.00	**13**	72.22

		Ki67 (0+1)	**Ki67 (2+3)**	**p-value**
BillN	**Low grade**	**22**	**4**	**0.0026***
	High grade	**0**	**4**	
BillN		**22**	**8**	**0.0001***

CARCINOMA		**2**	**16**	
Gallstones	**Present**	**23**	**17**	**0.0479***
	Absent	**1**	**7**	
Differentiation	**Well**	**2**	**2**	**0.0392***
	Moderate and Poorly	**0**	**14**	
Histological type	**Adenocarcinoma**	**1**	**10**	**0.5294***
	ICPN with associated invasive carcinoma	**0**	**6**	
Invasion till	**Tis, T1**	**1**	**10**	**1***
	T2, T3 and T4	**1**	**10**	

*p value calculated using Fisher's exact test

Table 3: Ki67 expression and cinicopathological association

Ki67 SCORE IN BillN AND CARCINOMA

Low grade BillN, 10% (3 patients), 63.33% (19 patients) and 13.33% (4 patients) had Ki67 expression as score 0, 1, 2 respectively. Among high grade BillN, all the 4 patients (100%) had Ki67 expression score as 2.

Gallbladder carcinomas showed variation in Ki67 expression, 5.56% (1 patient), 5.56% (1 patient), 16.67% (3 patients) and 72.22% (13 patients) showed Ki67 expression as score 0, 1, 2 and 3 respectively.

Low and high grade BillN and Ki67 expression showed significant statistical association (p=0.0026).

BillN and carcinoma Ki67 expression showed significant statistical association (p=0.0001).

We found statistically significant association ($p<0.05$) between presence and absence of gallstones and Ki67 expression (p=0.0479).

Mean Ki67 expression for well differentiated carcinomas was 36.25±38.02 and for moderate and poorly differentiated carcinomas was 63.00±20.08.

There was equal distribution of Ki67 expression among well differentiated tumours. However, all cases of moderately and poorly differentiated tumours showed Ki67 expression of more than 10%. There was statistical significance (p=0.0392) between histological grade and Ki67 expression.

The mean Ki67 expression for adenocarcinoma and ICPN with invasive carcinoma were 46.08±25.18 and 79.00±10.00 respectively.

Among the different types of carcinomas, majority of the gallbladders had Ki67 expression of more than 10%. Only one gallbladder adenocarcinoma had Ki67 expression less than 10%. However, there was no statistical significance between types of carcinomas and Ki67 expression (p=0.5294).

In our study, majority of the patients had Ki67 expression of more than 10% inspite of differences in staging of tumours. p=1 was obtained because of similar expression of Ki67 in different groups. There was no significant association between Ki67 expression and depth of tumour infiltration ($p>0.05$).

IV.DISCUSSION

1. HISTOLOGICAL TYPE

BILIARY INTRAEPITHELIAL NEOPLASIA

Solsini L et al. (2014) found low grade dysplasia (10/18) to be more common than high grade dysplasia (2/18). Low grade dysplasia was seen in 55.5% and high grade in 27.8% patients.[9]

Jain K et al. (2014) also found low grade dysplasia (47/55) to be more common than high grade dysplasia (8/55). Low grade dysplasia accounted for 13.4% and high grade 2.3% of all the 350 gallbladder specimens studied.[10]

Our findings are consistent with the studies cited above. Low grade BillN is more common than high grade BillN. This may be attributed to the fact that for low grade BillN will be seen with less mucosal insult and hence more commonly found in routine cholecystectomy cases.

CARCINOMA

Dutta U et al. (2019) in their study of 29 primary gallbladder carcinoma found most common histological type to be adenocarcinoma (16/29), 11 (37.93%), 4 (13.79%) and 1 (3.45%) were biliary, intestinal and mucinous

adenocarcinoma respectively. 10 gallbladders (34.48%) were ICPN with associated invasive carcinoma and only 1 case (3.45%) was reported as undifferentiated, adenosquamous and squamous cell carcinoma respectively.[11]
Gupta A et al. (2021) in their study of 326 patients noted adenocarcinoma as the most common type. A total of 149 patients (45%) had adenocarcinoma, 6 (2%) had poorly differentiated carcinoma and only 1 case (1%) had adenosquamous carcinoma.[12]
Among the carcinoma cases, adenocarcinoma is the most common. However, in our study the second most common is ICPN with associated invasive carcinoma which only matched the study conducted in Dibrugarh showing the common occurrence of this tumour in upper Assam.[11]

2. DIFFERENTIATION

A study conducted in Dibrugarh by Dutta U. et al. (2019) found that out of 29 cases of GBC, the most common were well differentiated (20 gallbladders, 68.97%), followed by moderately (7 gallbladders, 24.13%) and poorly differentiated (1 gallbladder, 3.45%).[11]
Dubey AP et al. (2018) states that out of 68 cases of GBC, 16 cases (23.53%), 24 (35.29%) and 28 (41.18%) were well, moderate and poorly differentiated respectively.[13]
Our study presents a unique picture where most cases are moderately differentiated followed by well and poorly differentiated. This may be because of unique ethnicity of the patients.

3. HISTOPATHOLOGICAL INVASION AND T STAGING

A study conducted by Dutta U et al. (2019) in Dibrugarh with 29 primary GBC cases showed that in terms of invasion, T staging showed T1b, T1a, T2 and T3 stages in 13 (44.83%), 5 (17.24%), 6(20.69%) and 5 (17.24%) respectively.[11]
Samuel S et al. (2018) in their study comprising of 4738 GBC specimens, observed T1, T2, T3, and T4 in 27.2% (1291), 29.5% (1396), 38.2% (1812) and 5.0% (239) cases respectively.[14]
Our study shows similar finding with the previous study conducted in Dibrugarh.[11] Most of the cases present in T1b followed by T2a and T3. We are of the opinion that surgery as a treatment modality is offered only in earlier T stages of gallbladder carcinoma. Hence maximum cases fall into the T1a and T2a category. Carcinomas presenting late are given chemotherapy.

4. Ki67 SCORING IN BillN AND CARCINOMA

In a study by Singh AK et al. (2017) found that in 47 cases of premalignant lesions Ki67 expression in all of them (100%) were <10%. However, among the carcinoma cases 75.3% (64 cases) had Ki67 expression below 10%, 16.5% (14 cases) had expression between 10-25% and 8.2% (7 cases) had Ki67 expression >25%.[4]
Pujani M et.al. (2016) compared 25 carcinoma and 25 benign lesions of gallbladder with their Ki67 expression. For 60% of carcinoma cases (15 cases) and 1% (4 cases) of benign cases had Ki67 was more than 20%. This expression was statistically significant (p=0.0001).[7]
Our study has shown comparable results. Low grade BillN has shown maximum cases below 10% Ki67 expression. Compared to that High grade BillN has shown more expression and lastly carcinoma maximum cases have shown expression between 50-100%. Progressive increase in Ki67 has been noted with statistical significance between low and high grade BillN (p=0.0026) and again between BillN and carcinoma cases (p=0.0001).

5. AGE AND Ki67 SCORE

In a recent study by Gupta A et al. (2021) showed that among the 50 carcinoma cases of gallbladder, patients below the age of 40 had mean Ki-67 expression of 65 compared to 52 for above 40 years of age patients. There was weak negative correlation and not statistically significant (p=0.308).[8]
Singh AK et al. (2017) noted that even though Ki67 expression was higher in patients above 40 years of age but statistically (p=0.176) the results were not significant.[4]
Our study results are consistent with results of Gupta A et al.[8] and Singh AK et al.[6] where Ki67 was higher in age groups >40 years but the results were not statistically significant for both the studies.

6. SEX AND Ki67 SCORE

Gupta A et al. (2021) found that females and males had mean Ki-67 expression of 57.7 and 48.2 respectively but the p was 0.095.[8]
Even though Ki67 expression was higher among females in the study by Singh AK et al. (2017) the p was 0.957 (statistically non-significant).[4]
Our study is consistent with the above studies. Ki67 expression is not associated statistically with sex.

7. CLINICAL FEATURES AND Ki67 SCORE

Singh AK et al. (2017) found no statistical significance association between abdominal pain other clinical features (p=0.313).[4]
Our study is consistent with Singh AK et al.[4] where they did not find any statistical association.

8. GALLSTONES AND Ki67 SCORE

In the study by Singh AK et al. (2017) they had found statistically significant association between presence and absence of gallstones and Ki67 score ($p<0.001$).[4]

Gupta A et al. (2021), in their study found mean expression of Ki67 to be 57.86±23.54 and 46.79±17.28 for presence and absence of gallstones respectively. However, there was no significant statistical association ($p=0.186$).[8]

Our study results are like Singh AK et al.[4] Gallstones are considered a risk factor for development of carcinoma. Hence, their presence indicates higher proliferation of the gallbladder epithelium as indicated by the Ki67 score. However, more number of samples may be needed to comment confidently on the statistical significance of association of gallstones with Ki67 expression as different studies show discordant results.

9. HISTOLOGICAL DIFFERENTIATION AND Ki67 SCORE

Study by Ojha A et al. (2018) found that among 44 patients of gallbladder carcinoma, mean Ki-67 labelling index was 27.0±4.2, 32.5±6.1 and 33.14±2.5 for well, moderately and poorly differentiated carcinomas and the variation of Ki67 for the differentiation was statistically significant ($p=0.010$).[5]

Garg C et al. (2019) reported mean Ki67 expression among well, moderately and poorly differentiated carcinomas as 21, 35 and 62 respectively. The p value was 0 and statistically significant.[15]

Gupta A et al. (2021) noticed progressive increase in mean Ki-67 expression with increasing differentiation. Well, moderately and poorly differentiated tumors had mean Ki67 expression as 45.83±19.60, 46.11±23.82 and 64.44± 22.84. However, the results were statistically non-significant ($p=0.158$).[8]

Gupta P. et al. (2016) found that in 25 GBC, mean expression was 28.20±7.83% among different histological group, 37.50±3.77% (poorly differentiated) to 25.50±6.73% (well differentiated). This difference was statistically significant ($p=0.015$).[6]

Our study is consistent with Ojha A et al.[5], Garg C. et al.[15] and Gupta P et al.[6] indicating that Ki67 expression varies and progressively increases with differentiation. Thus, Ki67 can be taken as a marker for aggression.

10. HISTOLOGICAL TYPES AND Ki67 SCORE

Singh AK et al. (2017) compared the various histological types of adenocarcinomas and Ki67 expression ($p=0.853$) and the results were not statistically significant.[4]

Gupta P et al. (2016) observed that mean Ki67 in adenocarcinoma, mucinous, papillary and signet cell carcinoma were 29.80±7.93, 26.33±5.69, 26.83±8.52 and 18 respectively. The results were not statistically significant ($p=0.468$).[6]

Our studies and the cited studies show Ki67 is not associated with histological types as it's a marker of proliferation.

11. TUMOUR INFILTERATION AND Ki67 SCORE

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A study from Spain (2004) showed mean MIB-1 index for Tis, T1, T2, T3 and T4 as 12.0%, 34.8%, 33.8%, 27.5% and 13.9% respectively. The p was <0.05 and statistically significant.[16]

Gupta A et al. (2021) noticed the mean Ki67 expression in T1, T2, T3 and T4 as 67.50±17.68, 70.00±35.36, 55.36±19.46 and 70.00±0. The p was calculated as 0.616 (statistical non-significant).[8]

Garg C et al. (2019) observed mean MIB 1 LI to be 0, 26.5, 37.4 in invasion upto mucosa, muscularis propria and full thickness. The expression is however statistically non-significant ($p=0.561$).[15]

Our results are the same as Gupta AK et.al and Garg C et.al. No association of Ki67 index with T staging. This comes as a surprise to us. The more the T staging the more infilterative the carcinoma is. We expected Ki67 to show statistical significance as Ki67 is a marker of aggression. This may be because of the variable expression of Ki67 in different types of carcinomas.

V.CONCLUSION.

In the present study, Ki67 expression showed progressive increase from low grade to high grade Biliary intraepithelial neoplasia and to carcinoma. This helps us to understand the pathological proliferative gallbladder epithelium and in turn the pathogenesis. There was significant statistical association between presence of gallstones and Ki67 score indicating higher proliferative mucosa in the presence of gallstones. Ki67 also showed positive correlation with tumour differentiation indicating that Ki67 can be taken as a marker of aggression. Hence, cases with high Ki67 index should be followed up. Ki67 is an established prognostic marker for various cancers such as breast, bladder and prostrate and same can be used in gallbladder. Researches are under way to use Ki67 as a therapeutic marker.

The present study was carried out for a short duration of one year with very limited number of patients. The novel Coronavirus 19 made it difficult for patients to seek medical treatment and allow for follow-up.
We believe in the recent future, the full potential of Ki67 as a marker of aggression, therapy and prognosis will be tapped leading to better management of patients with gallbladder cancers.

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