Original Article

Role of Morphology and Immunohistochemistry in the Diagnosis of Incidental Cancers in Gallstone Disease

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Abstract

Introduction: The majority of gallbladder adenocarcinomas have been known to develop from dysplasia or carcinoma in situ, without pre-existing adenomas, hence observation of these non-neoplastic or preneoplastic lesions is important as it may underlie an occult malignancy. To study the role of morphology and immunohistochemistry in the diagnosis of incidental cancers in cases of gall stone disease by adopting a universal histopathological screening policy for all the gallstone disease cases operated at our tertiary care center. **Material and Methods:** The present study was designed as an observational cross sectional study completed over a time period of 14 months. A total of 176 cases were included in the study including all the patients of gall stone disease in the adult age group. All the indeterminate and frankly malignant cases were subjected to immunohistochemistry with a panel of antibody markers including p53, CEA and Ki-67 using Streptavidin Biotin immunoperoxidase method Fisher-exact test was used to compare the data taking '*p*' value less than 0.05 as statistically significant. **Results:** Mean age of patients was 40.55±13.25 years. Majority of patients (n=141; 80.1%) were females. The final diagnosis revealed a dominance of non-neoplastic inflammatory (n=168; 95.5%) pathology. There were 8 (4.5%) of malignancy, of these 7 were adenocarcinoma and 1 was adenosquamous carcinoma. Atypia was seen in 5 (2.8%) cases and 3 (1.7%) cases were of dysplasia. Histopathology detected carcinoma in 7 (4%) cases whereas addition of immunohistochemistry helped to find out one more unidentified case, thus the diagnostic yield increased to 8 (4.5%) cases. Out of three dysplasia cases, one (33.3%) was found positive on immunohistochemistry, which was later confirmed to be malignant. None of the cases of atypia were positive on p53, Ki-67 and CEA immunohistochemistry. On comparison between the three groups, the results were statistically significant (*p*=0.00062). **Conclusion:** Immunohistochemistry has a useful role in detection of incidental gallbladder carcinoma when morphology is limited by indeterminate results. A panel of immunohistochemical markers including p53, Ki-67 and CEA may prove very useful to pick up cases of malignancy in such cases of diagnostic dilemma.

Keywords: Atypia, carcinoembryonic antigen, carcinoma gallbladder, dysplasia, Ki-67, p53

Introduction

Gallbladder carcinoma (GBC) ranks sixth among gastrointestinal cancers and is associated with high mortality rate and poor survival.[1] The percentage of patients diagnosed to have gallbladder cancer after cholecystectomy for presumed gallbladder stone disease ranges from 0.2% to 3.3%.[2] It has been shown that more than 80% of gallbladder adenocarcinomas develop from dysplasia or carcinoma *in situ*, which were *de novo* cancers without preexisting adenomas, thus indicating the role of nonneoplastic or preneoplastic lesions to be equally important.[3]

The pathogenesis of GBC holds a relevance to identify each of the gallstone disease specimens for histopathological changes such as metaplasia and dysplasia that are commonly found in mucosa close to invasive carcinoma. In addition, atypical hyperplasia may progress to carcinoma *in situ* and a proportion of such occult cancer of gallbladder cases to invasive carcinoma, hence extensive sampling of the gallbladder and ancillary immunohistochemical panels may be helpful.[4]

The role of immunohistochemistry (IHC) becomes important in doubtful cases presenting with atypia, in which

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immunohistochemical markers such as p53, carcinoembryonic antigen (CEA), and Ki-67 can be used successfully to establish the diagnosis completely.[5,6] The present study was carried out to determine the role of morphology and IHC in the diagnosis of incidental cancers in cases of gallstone disease operated at our center.

Materials and Methods

Study design

The study was designed as an observational cross-sectional study.

Sample size

A total of 176 cases were included in the study including all the patients of gallstone disease in the adult age group.

Study setting and duration

The study was carried out in the Department of Pathology of our tertiary care institute over 1 year and 2 months from September 2021 to November 2022.

Procedure

The study was carried out following the Helsinki Declaration for research on human subjects after institutional ethical approval (RD-01/09-21). Informed consent for the study was obtained from all the participants.

Patient details regarding age and gender were noted. The gross morphology of the gallbladder specimens received along with wall thickness, presence of any growth or lesion, and type of stones were taken into account. Microscopic details were noted after hematoxylin and eosin staining. On microscopic examination, the lesions were grouped as frankly benign (inflammatory, with metaplasia and benign proliferative lesions), frankly malignant (carcinoma), and indeterminate categories (including those reported as atypia or dysplasia) where definite characterization into benign or malignant could not be done on histopathological examination alone.

All the indeterminate and frankly malignant cases were subjected to IHC with a panel of antibody markers including p53, CEA, and Ki-67 using the Streptavidin–Biotin immunoperoxidase method. Deparaffinization and rehydration were done with consecutive solution changes of xylene, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, and distilled water. Heat-induced epitope retrieval was done by microwave irradiation. The slides were then allowed to cool at room temperature. Incubation with the primary antibody at appropriate dilutions overnight at room temperature was done. After washing in buffer, the sections were incubated with horse radish peroxidase-conjugated secondary antibody at appropriate dilution. After washing in buffer, the slides were developed with diaminobenzidine for 10 min. The slides were then rinsed with tap water, counterstained with hematoxylin, and mounted after consecutive changes in ethanol and xylene.

The p53 antibody (DO-7, Dako) was used in 1:200 dilution. The degree of p53 overexpression was scored using a semiquantitative method evaluating the intensity and incidence of positively stained cells. Nuclear staining for the marker was considered and expressed in percentage.

Positive: Two types of positive (mutated) staining patterns were taken.[7]

a. If >60% of cells positive, diffuse positive due to missense mutation of p53
b. If <5% of the cells positive, null positive staining due to nonsense mutation of p53.

A known case of p53-positive (mutated) gastric carcinoma was taken as a positive control.

For evaluation of CEA, Clone II-7, Dako, was taken at 1:100 dilution). An IHC-positive case of carcinoma colon was taken as a positive control. CEA is normally limited to the apical membrane of benign cells, and cytoplasmic positivity is seen in tumor cells. In this study, cytoplasmic as well as membranous positivity was considered positive.

For Ki-67 immunoexpression, clone IA6 mouse monoclonal antibody was used and an IHC-positive section from carcinoma breast was taken as control. Ki-67 index was calculated as the percentage of positively stained tumor cell nuclei out of the total tumor cells counted. A percentage of >20% stained cells was considered to be positive, regardless of the intensity of staining.

For all the IHC assessments, the negative control was a separate section of patient tissue processed using the same reagents and epitope retrieval protocol as the patient test slide, except that the primary antibody was omitted and replaced by diluent/buffer solution in which the primary antibody was diluted.

The data obtained were tabulated for frequency distribution. Fisher's exact test was used to compare the data. Fisher's exact test was applied using IBM SPSS Statistics for windows, Version 26 (IBM Corp., Armonk, N.Y., USA) and advanced Excel. P <0.05 was considered statistically significant.

Observations and Results

The age of patients ranged from 18 to 73 years. The mean age of patients was 40.55 ± 13.25 years. There was no significant association between the final diagnosis and the age of patients (P = 0.184).

Majority of patients (n = 141; 80.1%) were females. There were 35 (19.9%) males. The sex ratio (M: F) was 1:4. Majority of specimens had normal wall thickness (n = 144; 73.5%). There were 54 (26.5%) specimens with thickened wall. Gross mucosal abnormalities were seen in 12 (6.8%) cases, with 4 cases having ulceration with thickened area, 2 cases having thickened mucosa, and 1 each with atrophied, polypoidal, fatty streaks with polypoidal projections, growth, thickened area, partial ulceration with thickened area, and ulceration with diffused thickening, respectively. Majority of stones were identified as mixed stones (53.4%), followed by cholesterol stones (23.8%) and pigment stones (18.8%), respectively.

The final diagnosis revealed a dominance of nonneoplastic (n = 168; 95.5%) pathology (all diagnosed as chronic cholecystitis) of inflammatory nature. There were 8 cases(4.5%) which were diagnosed as carcinoma, of these 7 were adenocarcinoma and 1 was adenosquamous carcinoma. In 95 (54%) cases, only inflammation was seen. There were 57 (32.4%) cases in whom inflammation with pyloric metaplasia was seen, whereas in 19 (10.8%) cases, inflammation with intestinal metaplasia was seen. A total of 63 (35.8%) cases showed hyperplastic changes transforming into RA sinus. Inflammation with hyperplasia was seen in 5 (2.8%) cases. A total of 7 (4.0%) cases were identified as carcinoma gallbladder, with 6 being adenocarcinoma and 1 being adenosquamous carcinoma [Table 1 and Figure 1]. Atypia was seen in 5 cases(2.8%), all of which also showed chronic inflammation. These included 2 cases which were associated with pyloric and intestinal metaplasia, 2 cases with associated hyperplastic epithelium and a single case with moderate chronic mononuclear inflammatory infiltrate. A total of 3 (1.7%) cases had dysplasia [Table 1].

These five cases with atypia and three cases with dysplasia were subsequently subjected to IHC evaluation. There was 1 (12.5%) case that was positive for all the three IHC markers.

Table 1: Histopathological diagnosis

Finding	Number of cases (%)
Chronic cholecystitis	161 (91.48)
Atypia	5 (2.8)
Dysplasia	3 (1.7)
Carcinoma (6 – adenocarcinoma, 1 – adenosquamous carcinoma)	7 (3.98)

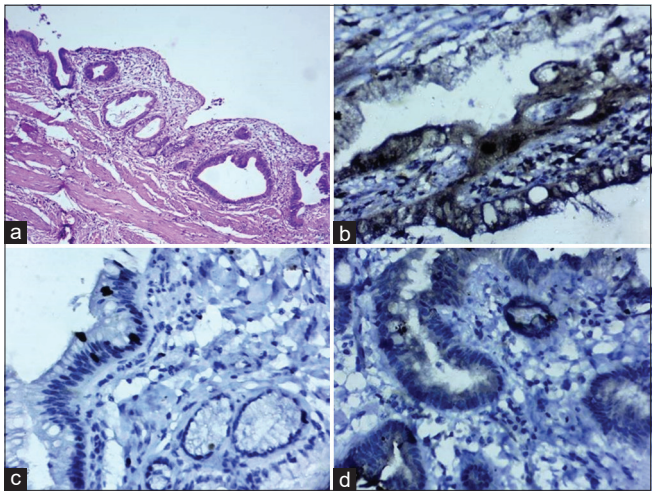

Figure 1: Microphotograph of focal atypia. (a) Section showing focal atypia of the epithelium (H and E, ×10 view), (b) P53 negative (positive nuclear staining in 25% of epithelial cells) in a case of focal atypia (×40 view), (c) Ki-67 negative (positive nuclear staining in 6% of epithelial cells) in a case of focal atypia (×40 view), (d) Carcinoembryonic antigen negative, showing only membranous positivity in a case of focal atypia (×40 view)

Thus, the diagnostic yield of IHC was 12.5%. Histopathology detected GBC in 7 (4%) cases, whereas the addition of IHC helped to find one more unidentified GBC case, thus the diagnostic yield increased to 8 (4.5%) cases. On comparing the data statistically, the change in diagnostic yield was highly significant (P = 0.00062). None of the atypia cases were found to be positive on IHC [Figure 1]. Out of 3 dysplasia cases, 1 (33.3%) was found positive on IHC, which was later confirmed on additional sectioning when invasive glands were identified in one of the deeper sections [Figure 2]. IHC was positive in all 7 cases of GBC (100%) [Figure 3]. On comparison of IHC results between the three categories, the difference was highly significant (P = 0.00062).

Discussion

Incidental malignancies comprise up to two-fifth of total gallbladder cancer cases,[8] and hence, it is essential that all the cholecystectomy specimens obtained from gallstone disease patients must be evaluated for malignancy. Generally, this evaluation is based on histomorphological assessment; however, in the recent years, newer and more sensitive markers have been identified that are helpful in settling the doubtful cases. Ever since the early evaluations in the 1990s exploring the association of p53 gene in gallbladder lesions,[9] a number of candidate genes have been found to be associated with various neoplastic and nonneoplastic lesions of gallbladder. With the emergence of immunohistochemical techniques,

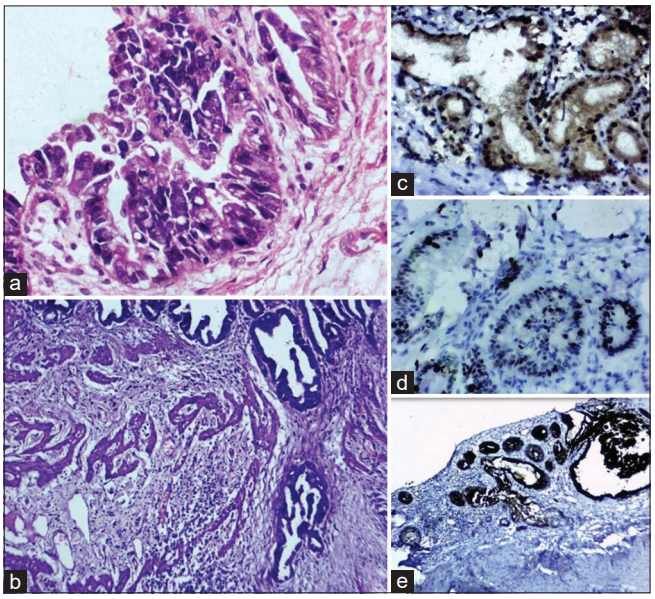

Figure 2: Microphotograph of a single case of dysplasia which showed malignancy on immunohistochemistry (IHC) and further sectioning. (a) section showing high-grade dysplasia (H and E, ×40), (b) IHC was suggestive of malignancy, and on additional sectioning, malignant glands were noted to be going in stroma. The case was proved to be adenocarcinoma, (c) P53 high nuclear positivity in > 75% of the epithelium (×40 view), (d) High Ki-67 index (45%) (×40 view), (e) Carcinoembryonic antigen – positive, showing both cytoplasmic and membranous positivity (×10 view)

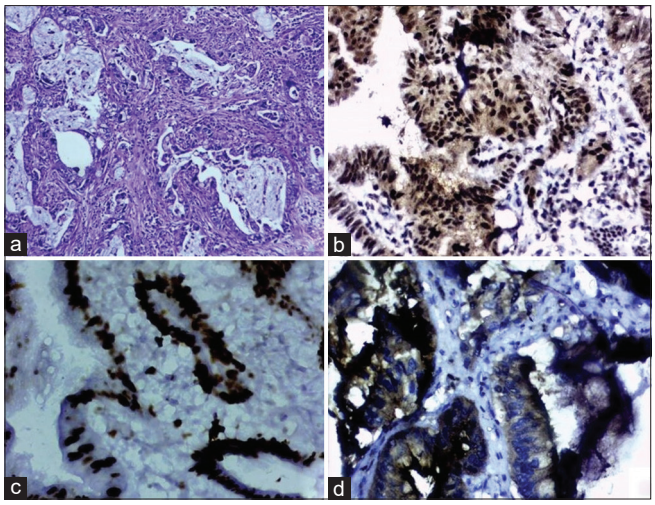

Figure 3: Microphotograph showing adenocarcinoma, gallbladder. (a) Section showing malignant glands invading the stroma in a case of gallbladder adenocarcinoma (H and E, ×10 view), (b) P53 high nuclear positivity in > 90% tumor epithelium seen in a case of adenocarcinoma gallbladder (×40 view), (c) Ki-67 index = 92%, in a case of adenocarcinoma gallbladder (high-power view), (d) Carcinoembryonic antigen showing nuclear positivity in a case of adenocarcinoma gallbladder (high-power view)

the assessment of genetic link to gallbladder diseases has become much easier to study and has helped to understand the pathogenesis, differentiation, prognosis, and treatment of GBC effectively.

For this purpose, a total of 176 gallbladder specimens obtained from cases of gallstone disease (aged 18–73 years; mean age: 40.55 ± 13.25 years; 80.1% of women) were enrolled in the study. The majority of patients(104 cases) in our study were <40 years of age (59%). The gall bladder in all the cases, were examined for gross and histomorphological changes to look whether they are doubtful or suspicious for malignancy. Immunohistochemistry for p53, CEA, Ki-67 was done on only those cases which were doubtful/suspicious for malignancy.

A study similar to the present study reported a dominance of females (84.1%); however, in their study, majority of the patients were aged >40 years (58%).[10] A dominance of females with their proportion ranging from 70.1% to 85.9% has been widely reported.[11-13] The mean age of patients has also been reported between 35 and 45 years in different studies from India.[12,13]

In the present study, gross and histomorphological evaluation confirmed incidental GBC in 7/176 (4%) cases. An additional eight cases (five cases diagnosed as atypia and three cases diagnosed as dysplasia) were also considered to have potential/suspicion of malignancy and were subsequently subjected to IHC evaluation using p53, CEA, and Ki-67 IHC stains that were found to be positive for all the three stains in one specimen (in dysplasia group) and was additionally diagnosed as carcinoma. Thus, in total, the prevalence of incidental GBC in gallstone disease cases was 8/176 (4.5%).

Except for one study by Jain *et al*.,[14] all the studies have used only histomorphology for evaluation of incidental carcinoma and most of them[12,13,15-20] have reported incidental GBC in <1% of cases. There are few studies that have reported it to be 1%–3%.[11,21-23] Only two studies have reported it above 5%; of these, one study[14] has used a combination of histomorphology and IHC, whereas the other study[24] has used histomorphology alone. From this overview, it can be expected that IHC helps to identify the additional burden of GBC.

It has been documented in an earlier study that the IHC helped in the additional detection of six cases from the originally detected only one case of GBC, thus increasing the overall burden by seven folds.[14] Compared to their study, in the present study, we could find this increment to increase by only 1.14 folds. Comparing the GBC prevalence of histomorphologically diagnosed cases in the present study (4.0%) with previous studies, the findings of the present study are close to that reported by Giridharan and Madhivadhanam[13] and Sarangi and Mohanty, and 2.5% respectively[18] who reported it to be 2.9 and 2.5%, respectively. However, there is one study that has reported it to be as high as 10.12%.[24]

It may be noted that there is high fluctuation in incidental GBC detection in studies with smaller size, and most of the studies having a sample size above 1000 have found this detection rate to be within 1% range. In such circumstances, more reliable diagnostic markers and additional diagnostic tools need to be utilized to rule out underdetection.

In the present study, out of eight cases subjected to IHC evaluation, five were atypia and three were dysplasia as diagnosed histomorphologically. Of these, only one case with dysplasia was confirmed as carcinoma on IHC, and none of the atypia cases were confirmed as GBC on IHC. Compared to the present study, Jain *et al*.[14] in their study had all the IHC-evaluated cases with atypia and found 50% of them to be carcinoma through IHC-guided additional sectioning as the IHC positivity rate in their study was much higher (10/12 = 83.3%) compared to only 12.5% in the present study.

In their study, 6 out of 10 IHC-positive cases (60%) were confirmed as GBC on additional sectioning. In the present study, the single IHC-positive case was confirmed to be GBC, thus showing a diagnostic yield of 100%.

The high utility of IHC markers such as p53 has been reported in cases of atypia.[25] Moreover, markers like CEA have been found to be 100% specific in nature.[26] Ki-67 is also known to be a good diagnostic as well as prognostic marker of GBC apart from showing a strong correlation histopathologically.[27-29] In the present study, all the three markers were positive in the single case showing positive expression of IHC markers, thus showing it to be 100% sensitive as well as 100% specific in the diagnosis of GBC. The present study followed a protocol to minimize the need of IHC in a sequential assessment pattern where only cases with high histomorphological suspicion need IHC evaluation.

In the present study, a comparison of IHC positivity rate between atypia, dysplasia, and malignant cases yielded statistically significant results. Despite its limitations, the findings of the present study reflected that the development of protocols for the evaluation of cholecystectomy specimen obtained from patients of gallstone disease must include IHC evaluation for suspicious specimen and should undergo a thorough diagnostic workup to tap the additional burden of GBC. Further studies on the issue on a larger sample size with inclusion of other IHC markers are recommended.

Limitations

The major limitation of this study was the low number of cases of atypia and dysplasia observed over the duration of the study.

Conclusion

The findings of the study showed that IHC has a useful role in the detection of incidental GBC when histomorphological evaluation is indeterminate and/or is suspicious for malignancy. Further studies on a larger sample size including only cases with atypical histopathological findings with inclusion of other IHC markers are recommended to further strengthen the evidence in this direction.

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Conflicts of interest

There are no conflicts of interest.

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