Original Article

p53 and Beta-Catenin Expression in Gallbladder Tissues and Correlation with Tumor Progression in Gallbladder Cancer

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ABSTRACT

Background/Aim: The inactivation of the tumor suppressor gene and activation of the proto-oncogene are key steps in the development of human cancer. p53 and beta-catenin are examples of such genes, respectively. In the present study, our aim was to determine the role of these genes in the carcinogenesis of the gallbladder by immunohistochemistry. **Patients and Methods:** Sections from paraffin-embedded blocks of surgically resected specimens of gallbladder cancer (GBC) (80 cases), chronic cholecystitis (60 cases), and control gallbladders (10 cases) were stained with the monoclonal antibody p53, and polyclonal antibody beta-catenin. Results were scored semiquantitatively and statistical analysis performed. p53 expression was scored as percentage of the nuclei stained. Beta-catenin expression was scored as type of expression—membranous, cytoplasmic, and nuclear staining. Beta-catenin expression was correlated with tumor invasiveness, differentiation, and stage. **Results:** Over-expression of p53 was seen in 56.25% of GBC cases and was not seen in chronic cholecystitis or in control gallbladders. p53 expression in gallbladder cancer was significantly higher than in inflammatory or control gallbladders ($P < 0.0001$). p53 expression increased with increasing tumor grade ($P = 0.039$). Beta-catenin nuclear expression was seen in 75% cases of gallbladder cancer and in no case of chronic cholecystitis and control gallbladder. Beta-catenin nuclear expression increased with tumor depth invasiveness, and grade ($P = 0.028$ and $P = 0.0152$, respectively). **Conclusion:** p53 and beta-catenin nuclear expression is significantly higher in GBC. p53 expression correlates with increasing tumor grade while beta-catenin nuclear expression correlates with tumor grade and depth of invasion, thus suggesting a role for these genes in tumor progression of GBC.

Key Words: Beta-catenin, gallbladder cancer, p53, tumor invasiveness

Received: 14.01.2012, Accepted: 07.04.2012
How to cite this article: Ghosh M, Sakhuja P, Singh S, Agarwal AK. p53 and beta-catenin expression in gallbladder tissues and correlation with tumor progression in gallbladder cancer. Saudi J Gastroenterol 2013;19:34-9.

Carcinogenesis of gallbladder carcinoma is not well understood. Only a limited amount of information is available regarding the molecular carcinogenesis. Oncogenes (beta-catenin) and tumor suppressor genes (p53) have been shown to be involved in the development and progression of gallbladder carcinoma. Inactivation of the p53 tumor suppressor gene is the most common genetic alteration in human cancers, and the prognostic significance of p53 over-expression has been reported in several malignancies, including those of the stomach,[1] colon,[2] and endometrium.[3] Beta-Catenin is a key regulator of the cadherin-mediated cell adhesion system, and altered expression and mutation of beta-catenin have been identified in many human malignancies.[4,5] In the present study, we investigated expression patterns of beta-catenin and p53 in different gallbladder tissues to elucidate the role of p53 and beta-catenin in gallbladder carcinogenesis. In addition, we evaluated the relationship with tumor invasion, differentiation, metastasis, nodal involvement, and stage of the lesions.

Access this article online

Quick Response Code:

Website: www.saudijgastro.com

DOI: 10.4103/1319-3767.105922

PATIENTS AND METHODS

Tissue specimen

The study was conducted on sections from 80 surgically resected specimens of gallbladder adenocarcinoma operated at the GB Pant Hospital between 2005 and 2008 and randomly selected 60 specimens of chronic cholecystitis with gallstone disease, 10 specimens of gallbladder controls from resections of gallbladder as part of other procedures with no pathology in

the gall bladder. Cases were selected on the basis of their final histopathologic diagnosis and their clinicopathologic data were analyzed. This study was conducted in our institution which is a tertiary referral teaching center.

Standard TNM system of AJCC was used to classify the primary tumors (T category)[6] as follows: T1: Tumor limited to lamina propria or muscle layer; T1a, Tumor limited in the mucosa, T1b, Tumor extends to muscle layer. T2: Tumor invades perimuscular connective tissue, but no extension beyond the serosa or into the liver. T3: Tumor perforates the serosa (visceral peritoneum) or directly invades one adjacent organ (stomach, duodenum, colon, pancreas, omentum, extrahepatic bile ducts). T4: Tumor invades main portal vein or hepatic artery or invades multiple extrahepatic organs or structures. Tumors were then staged according to the UICC/AJCC TNM system.[6]

The tumor grades were also noted in each case as Well differentiated (WD), Moderately differentiated (MD), and Poorly differentiated (PD) using criteria by Albores-Saavedra.[7]

Antibodies

Immunohistochemical staining was performed with monoclonal antibody p53 (DakoCytomation ready to use), monoclonal antibody, and beta-catenin (Santa cruz biotechnology) in all cases.

Immunohistochemistry

Tissues of gallbladder cancer and normal gallbladder, chronic cholecystitis and gallbladder were preserved in 10% formalin, embedded in paraffin, serially sectioned at 4-μm thickness, onto poly-L lysine-coated slides and deparaffinized. Antigen retrieval was done in citrate buffer in a microwave for 2 minutes at half power followed by 2 minutes at full power for p53. Antigen retrieval for beta-catenin was for 15 minutes in citrate buffer in a microwave. After washing two times with Tris buffer, primary antibody was applied (p53 from ready to use solution, beta-catenin was used at 1: 200 dilution). Negative control was made by replacing primary antibody by tris buffer, while tissue from breast and colonic cancer was used as positive control. After the application of primary antibodies, slides were incubated overnight in a humid chamber. Tissue sections were washed thrice with tris buffer. Next, biotinylated secondary antibodies were applied for 1 hour in same chamber at room temperature. After washing thrice with tris buffer, streptavidin peroxidase reagent was applied and incubated in the same chamber for 1 hour at room temperature. Finally, reaction product was visualized by developing color by incubating the slides with solution of diaminobenzidine tetrahydrochloride. Counter staining was done with hematoxylin. The section was mounted in crystal mount and then coverslipped in xylene.

Evaluation of p53 and beta-catenin expression

The expression of p53 in the epithelium was examined under a light microscope. p53 expression was judged positive when more than 5% cells were stained. The staining cell fraction was evaluated as Grade I when 5 to 30% cells in one section were stained, Grade II: 30-60% cells were stained, and Grade III: more than 60% cells were stained. The results were scored semiquantitatively and statistical analysis performed. p53 expression was scored as percentage of the nuclei stained.

Beta-catenin expression was judged positive when more than 5% cells were stained. Staining was evaluated as membranous, cytoplasmic, or nuclear based on the location of staining.

Statistical analysis was done using chi square test.

RESULTS

Among the 80 cases of Gallbladder cancer, 28 were well differentiated, 37 moderately and, 15 were poorly differentiated. When categorized by TNM staging, 13, 29, 37, and 1 case was of T1, T2, T3, and T4 category, respectively. Nodal involvement was seen in 17 cases, while in 63 cases no nodal involvement was present. Metastasis was present in four while 76 cases had no metastasis. Thus, 36 cases had Stage1 disease, 40 cases had Stage 2 disease, and 4 cases were in stage 4.

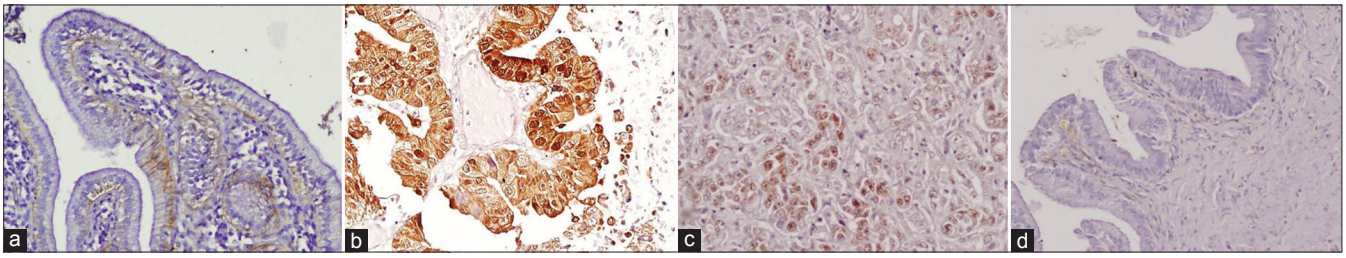

Figure 1: Microscopic photograph of the diseased and control gallbladder stained by immunoreactive beta-catenin and p53 antibody (a) Beta-catenin expression in chronic cholecystitis with membranous staining. Original magnification ×200; (b) Beta-catenin expression in gallbladder cancer with membranous, cytoplasmic, and nuclear staining, Original magnification ×200; (c) Gallbladder cancer with high p53 expression with nuclear staining. Original magnification ×200; (d) Chronic cholecystitis with no p53 expression. Original magnification ×200

p53 expression in the gallbladder tissue specimens [Table 1]

p53 expression was positive when nucleus stained positive. In each high positive area, 1000 cells were counted and for each case five such areas were selected.

p53 staining was nuclear with high level of p53 staining in gallbladder cancer [Figure 1]. In gallbladder cancer, expression was positive in 45 of 80 cases (56.25%). Expression was not observed in chronic cholecystitis (0 of 60) and in control gallbladders (0 of 10). P53 expression in gallbladder cancer was significantly higher than in chronic cholecystitis and normal gallbladders ($P < 0.0001$).

Correlation of p53 expression with different parameters in gallbladder cancer is shown in Table 2. Difference in the level of expression of p53 was studied between tumor grades (WD vs. PD and MD, taken together) and was found to be significant ($P = 0.039$), with higher p53 scores in MD and PD groups. P53 scores showed no significant difference with tumor category (T category) ($P = 0.425$) and tumor staging ($P = 0.456$).

Beta-catenin expression in the gallbladder tissue specimens [Table 1]

Beta-catenin expression was membranous, cytoplasmic, or nuclear with the latter pattern of staining only in tumors [Figure 1].

In gallbladder cancer, Beta-catenin expression was seen in all 80 cases (100%). Expression was heterogeneous, strong in the tumor area and the expression pattern was predominantly membranous and altered to cytoplasmic and focal nuclear in 75% (60 of 80 cases). In 60 chronic cholecystitis and 10 control gallbladder specimens, Beta-catenin expression was seen in all cases but expression pattern was focal and membranous. Thus, Beta-catenin expression pattern shifted to nuclear and cytoplasmic with higher staining intensity in GBC than chronic cholecystitis and control gallbladder tissues, which showed a membranous pattern.

Correlation of nuclear pattern expression of Beta-Catenin with different parameters in gallbladder cancer is shown in Table 3. The nuclear expression of beta-catenin shows significant difference between T category ($P = 0.028$) as tumors restricted to mucosa and muscle (T1) showed lower positivity as compared with tumors extending beyond muscle (T2-T4). Similar difference was found with tumor differentiation or grade when comparing WD to MD and PD ($P = 0.0152$). Beta-catenin positivity score was lower in the WD group. There is an increased nuclear staining of beta-catenin from tumor stage 1 to stage 4; however, results are not found significant ($P = 0.06$). In addition, no significance of nuclear pattern expression was found with nodal status or metastasis.

DISCUSSION

Molecular markers like p53 and beta-catenin have been identified in GBC in very few studies, and in most cases comprising a small number of GBC specimens.[8-13] Thus, their prognostic significance remains to be extensively validated. In this study, we have included a reasonable number of cases of gallbladder cancer and compared them with inflammatory and control gallbladder tissues.

Our results suggest that (1) there is significant p53 over-expression in gallbladder carcinoma, (2) beta-catenin

Table 1: p53 and beta-catenin expression in gallbladder tissues

Gallbladder tissues	Gallbladder cancer (*n*=80) (%)	Cholecystitis (*n*=60) (%)	Control gallbladder (*n*=10) (%)	*P* value
p53 expression	45/80 (56.25)	0/60 (0)	0/10 (0)	(*P*=.0001)
Beta-catenin (Nuclear expression)	60/80 (75)	0/60 (0)	0/10 (0)	(*P*=.0001)

Table 2: p53 expression in gallbladder cancer (*n*=80)

	p53 staining positive (%)	p53 staining negative (%)	Comparison	*P* value
T1 (*n*=13)	7/13 (54)	6/13 (46)	T1 vs. T2-4	*P*=0.425
T2 (*n*=29)	14/29 (48.2)	15/29 (51.7)		
T3 (*n*=37)	24/37 (65)	13/37 (35)		
T4 (*n*=1)		1/1 (100)		
WD (*n*=28)	12/28 (42.8)	16/28 (57.14)	WD vs. MD and PD	*P*=0.039
MD (*n*=37)	23/37 (62)	14/37 (38)		
PD (*n*=15)	10/15 (66.7)	5/15 (33.3)		
Stage 1 (*n*=36)	20/36 (56)	16/36 (44)	Stage 1 vs. Stage 2-4	*P*=0.456
Stage 2 (*n*=40)	24/40 (61)	16/40 (39)		
Stage 4 (*n*=4)	1/4 (25)	3/4 (75)		

WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

Table 3: Beta-catenin nuclear expression in gallbladder cancer (*n*=80)

	Nuclear expression (%)	Absent nuclear expression (%)	Comparison	*P* value
T1 (*n*=13)	7 (54)	6 (46)	T1 vs. T2-4	*P*=0.028
T2 (*n*=29)	22 (76)	7 (24)		
T3 (*n*=37)	30 (81)	7 (19)		
T4 (*n*=1)	1 (100)			
WD (*n*=28)	17 (60.1)	11 (39.2)	WD vs. MD and PD	*P*=0.0152
MD (*n*=37)	29 (78.4)	8 (21.6)		
PD (*n*=15)	14 (93.3)	1 (6.7)		
Stage 1 (*n*=36)	24/36 (67)	12/36 (33)	Stage 1 vs. Stage 2-4	*P*=0.06
Stage 2 (*n*=40)	33/40 (82.5)	7/40 (17.5)		
Stage 4 (*n*=4)	3 (75)	1 (25)		

WD: Well differentiated, MD: Moderately differentiated, PD: Poorly differentiated

expression shifts from cytoplasmic pattern in inflammatory and control gallbladder to nuclear pattern in gallbladder carcinoma, (3) p53 over-expression increases with worsening of tumor grade, (4) Nuclear pattern of beta-catenin increases with tumor invasiveness (T category), differentiation, and worsening of tumor grade.

In our study, 54% of gall bladder carcinoma cases showed p53 protein nuclear expression. In other studies of gallbladder carcinoma, the p53 protein nuclear expression ranges from 39.6% to 92% of cases,[8-10] similar to our results. The variability of results could depend on the different methods used to analyze the p53 nuclear expression, although all these authors evaluated their results by a semiquantitative method including both the number of cells stained and the intensity of staining. Legan *et al*. found p53 over-expression in 84.6% of gallbladder carcinomas and in 18.7% of patients without carcinoma. They suggested that accumulation of the wild-type p53 protein owing to a failure of degradation might push the cell into cell cycle arrest and apoptosis. Such cells would occur rather sparsely in tumors because they could not proliferate as malignant clones. It therefore seems very likely that the accumulation of p53 in dysplastic and malignant gallbladder epithelium resulted from p53 mutation. This suggests that p53 gene mutations are involved in the carcinogenesis of the gallbladder epithelium.[11]

The level of expression of p53 increased significantly with the grade of tumor ($P = 0.039$). Low-grade tumor (well differentiated) shows low expression of p53 (42.8%) in comparison to that in the moderately differentiated (62%) and poorly differentiated tumors (66.7%). However, p53 expression did not correlate with depth of tumor invasion (T category) ($P = 0.425$) or tumor stage (0.456) in our cases. The results of our study corroborate those of others in which p53 over-expression was not related to the level of gallbladder wall invasion by the tumor.[14] These results may indicate that p53 has a role in gallbladder carcinogenesis and in progression of the cancer from low-grade tumor to higher grade but not in tumor invasiveness. The higher p53 expression with the increasing grade of GBC suggests its role in tumor progression rather than initiation.

Wee *et al*.[15] found no correlation of p53 expression with tumor invasion and found p53 over-expression even in dysplasia and carcinoma-*in-situ* unassociated with invasive malignancy. In this initial study, we have not included dysplasia and noninvasive malignancy, but a larger study with few number of such cases included is planned and will throw light on the role of p53 in carcinogenesis. These findings indicate a role of p53 gene mutation in carcinogenesis. Oohashi *et al*. reported p53 over-expression as an early event unrelated to tumor grade, stage, or size etc. p53 expression could thus differentiate benign from malignant lesions of gallbladder.[14]

There are studies indicating that high-grade (poorly differentiated) gallbladder carcinomas tend to over-express p53 protein more than low-grade tumors (well differentiated) but most of these series are too small for statistical analysis.[15-17] Kalekou and Miliaras found that the expression of p53 protein was significantly higher in deeply invasive tumors ($P = 0.028$) and in moderately and poorly differentiated carcinomas ($P < 0.05$).[18] Kamel *et al*. raised the hypothesis that p53 is an early event in the carcinogenesis of gallbladder carcinoma. p53-positive dysplasia evolves to a more aggressive type of tumor, which is associated with high tumor grade and p53 positivity.[17] Chaube *et al*.[19] demonstrated that role of p53 seems to be limited to the initiation of gallbladder carcinogenesis as none of the premalignant lesions nor grade I GBC showed p53 over-expression. Roa *et al*. studied 191 cases of gallbladder carcinoma and similar to our study, they observed increased p53 expression with increasing tumor grade and absence of expression in controls and in normal mucosa adjacent to tumors.[20] Chang *et al*. showed poor survival of GBC with abnormal p53 expression.[21]

There are very limited studies evaluating the role of beta-catenin in gallbladder cancer. Beta-catenin mutations are

associated with cytoplasmic accumulation of free beta-catenin with subsequent nuclear translocation, thus resulting in nuclear expression pattern. In our study, we have observed nuclear expression of beta-catenin in 70% of cases with GBC, while this was not seen in controls or chronic cholecystitis.

Puhalla *et al*. showed significant differences in expression pattern of beta-catenin between normal, inflamed, and cancerous tissues. Our results are similar to their results. The beta-catenin membranous expression decreased from cholecystitis and normal to malignant tissue.[13] These results show that beta-catenin may be related with gallbladder carcinogenesis.

Hirata *et al*. demonstrated that the expression of E-cadherin or beta-catenin frequently diminishes as the tumor progresses, and abnormalities of beta-catenin expression were associated with decreased apoptosis in gallbladder cancers.[22] Kimura *et al*.[23] reported similar results, demonstrating that beta-catenin protein expression was seen in both the nucleus and cytoplasm of cancer tissues, while the non-cancerous tissues displayed intense membranous staining. They also found significant correlation between cytoplasmic or nuclear beta-catenin immunoreactivity and clinicopathological status of gallbladder carcinoma, especially in the poorer histological differentiation grade ($P < 0.05$). They suggested that beta-catenin alteration might be a minor contributor to the development of gallbladder carcinomas through abnormal Wnt-wingless expression; however, decreased membranous expression of beta-catenin might be correlated to carcinoma progression through loss of cell adhesive function in E-cadherin-catenin fashion.

In our study, the nuclear expression of beta-catenin showed a significant increase with the grade of tumor, thus suggesting a role in tumor progression ($P = 0.0152$). The expression level of beta-catenin increased from 60.1% in well-differentiated tumor (low grade) to 93.33% in poorly differentiated (high grade) tumor. With increasing depth of invasion of tumor, there is also an increase in the nuclear expression of beta-catenin ($P = 0.028$).

Chang *et al*. shows that the cytoplasmic and nuclear expression of beta-catenin in carcinomas was correlated with less-aggressive tumor behavior; in particular, cytoplasmic expression was associated with improved patient outcome ($P = 0.028$).[5]

Moon *et al*. also showed that beta-catenin was expressed in 80% of non-malignant specimens, exclusively in the cell membrane, while the cancer specimens showed cytoplasmic and/or nuclear staining. Significantly higher beta-catenin expressions were present in cancer cells of the invasive front than in the tumor central areas ($P < 0.001$), and these expressions were significantly ($P = 0.01$) associated with the invasion depth.[24]

From our study, it is observed that p53 and beta-catenin expression are altered in gallbladder cancer, thus suggesting a role in carcinogenesis and beta-catenin may have a role in tumor progression. In our study, we have not included preinvasive lesions such as dysplasia and CIS. However, we did have four cases which were metastatic. These cases were positive with p53 80-90% staining. But due to the small numbers, no deductions could be drawn.

For beta-catenin, expression was heterogeneous, strong in the tumor area, and expression pattern was predominantly nuclear. Thus, the pattern is similar to high-grade or invasive GBC. But due to the small numbers, no deductions could be drawn. Further studies with these will give a better insight into the role of these genes in carcinogenesis and progression. More detailed and extensive studies are required to further clarify the role of p53 and beta-catenin in gallbladder carcinogenesis.

Metaplasia and hyperplasia was observed along with chronic cholecystitis and dysplasia with carcinoma. But these were not assessed separately in this study, which is a limitation of this study. Even an analysis of precancerous lesions and carcinoma *in situ* would be worthwhile

ACKNOWLEDGEMENT

This study was financially supported by the Department of Science and Technology, India, Fast Track Scheme. NO.SR/FTP/L-125/2004. The authors also wish to thank Ms Arpita Singh, Research fellow, Department of pathology, for her help in processing the tissues and conducting the laboratory work.

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Source of Support: This study was financially supported by the Department of Science and Technology, India, Fast Track Scheme,
Conflict of Interest: None declared.