

ABERRANT p53 EXPRESSION AND THE DEVELOPMENT OF GALLBLADDER CARCINOMA AND ADENOMA

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Gallbladder carcinoma (GBC) is a highly lethal but relatively rare neoplasm of the digestive tract. The progression from gallbladder adenoma to carcinoma remains unclear. The *p53* gene is the most frequently mutated tumor suppressor in human cancers. In this study, we analyzed the expression patterns of the *p53* protein in 22 cases of GBC, 17 cases of precursor lesions (16 gallbladder adenomas and 1 cystadenoma), and 15 cases of normal epithelia using immunohistochemical analysis. The results were correlated with clinicopathologic characteristics. We found that *p53* expression was significantly increased in 59.1% (13/22) of GBC cases and in 17.6% (3/17) of gallbladder adenoma cases ($p = 0.009$). There was no *p53* expression in the 15 cases of normal epithelia, and a significant difference was shown between normal epithelium and GBC cases ($p < 0.001$). In addition, the expression pattern of *p53* protein did not show any significant correlation with the histologic type and the differentiation grade of GBC. In conclusion, we suggest that the aberrant *p53* expression may play a role in the occurrence of GBC.

Key Words: *p53*, immunohistochemistry, gallbladder adenoma, gallbladder carcinoma
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Gallbladder carcinoma (GBC) is a highly lethal but relatively rare neoplasm of the digestive tract. Generally speaking, the overall 5-year survival rate is only 5–15% [1,2]. GBC also shows an unusual geographic and demographic distribution. It affects females two to six times more frequently than males, and its incidence increases with age [3]. The highest incidences are observed in North and South America, Poland, and northern India. Low incidences are seen in Singapore and Nigeria [4]. In Taiwan, GBC is also uncommon, and 19.2% of cases are associated with a good operative prognosis [5,6].

These differences in the incidence and mortality rates of GBC suggest that genetic and environmental variables may be involved in its tumorigenesis. Although there have been improvements in diagnostic technique, there is, unfortunately, no promising biomarker that can detect GBC at an early stage. Thus, most cases are diagnosed at an advanced stage with a poor outcome.

Recently, it has been demonstrated that the adenoma-carcinoma sequence is the usual route for the tumorigenesis of GBC [7–9]. However, there is limited evidence concerning the possible mechanisms involved in the transformation. Tumorigenesis is regarded as a multistep process in which genetic alterations accumulate as premalignant lesions progress to invasive carcinomas. Among the many known genetic changes in human carcinogenesis, the most common is an abnormality in the tumor suppressor gene *p53*. Numerous investigators have shown that *p53* mutations are involved in the genesis of many human cancers, including

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GBC [10,11]. However, the role of *p53* in the adenoma-carcinoma sequence of gallbladder tumorigenesis remains unclear.

The accumulated literature suggests the oncogenic properties of *p53* brought about by its mutations [12,13]. Mutations in *p53* have been found in approximately 50% of human tumors. One of the most common mechanisms is point mutation leading to the elevated expression of conformationally altered and functionally defective *p53* proteins. Another is the deletion of one *p53* allele, resulting in the absence of the wild-type *p53* protein. Both mechanisms cause the loss of tumor suppressor function and permit oncogenesis.

The best way to improve the prognosis of GBC is to identify and correct the risk factors that may influence its development. Our objectives were to explore the biologic properties of gallbladder carcinogenesis and to improve the prognosis of GBC. In the present study, we examined the expression pattern of the *p53* protein in 54 tissue specimens, including normal epithelia, precursor lesion, and carcinoma, by using immunohistochemistry.

MATERIALS AND METHODS

Tissue specimens

Tissues from 22 GBC patients (9 men and 13 women, age range 38–87 yrs, mean age 66.3 ± 11.3 yrs) and 17 gallbladder adenoma patients (5 men and 12 women, age range 23–81 yrs, mean age 55.2 ± 16.2 yrs) were obtained from the Department of Surgery of the Kaohsiung Medical University Hospital. Furthermore, 15 normal gallbladder tissues, harvested from patients who underwent right lobectomy to treat hepatocellular carcinoma, were also included in this study. All of these specimens were initially fixed in 10% neutral buffered formalin and then embedded in paraffin after further dehydration processing.

The precursor lesions of the biliary epithelium were defined according to Albores-Saavedra et al [14]. Adenoma is defined as a benign neoplasm, typically polypoid, well-demarcated, single, and arises from glandular epithelium. Cystadenoma is defined as a multiloculated cystic neoplasm containing mucinous or serous fluid and lined by columnar epithelium reminiscent of bile duct or gastric epithelium. GBC is histologically classified according to the World Health Organization Histological Classification of Tumors of the Gallbladder and Extrahepatic Bile Ducts.

Immunohistochemistry

Sections (4 μ m thick) were prepared from the representative tissue blocks on a microtome. Routine immunohistochemistry procedures were followed as described [15]. In brief, the slides were deparaffinized with xylene rinse and rehydrated into distilled water through graded alcohol. Endogenous peroxidase activity was quenched by 10 minutes of incubation in a 3% hydrogen peroxide–methanol buffer. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 15 minutes and allowing the solution to cool slowly at room temperature for 20 minutes. The slides were then incubated with primary mouse monoclonal anti-*p53* antibody (Neomarkers) at a dilution of 1:200 in a humidified chamber for 30 minutes, at room temperature.

The slides were washed three times in phosphate-buffered solution and further incubated with biotinylated secondary antibody for 20 minutes at room temperature. Antigen-antibody complexes were detected by the avidin-biotin-peroxidase method using diaminobenzidine as a chromogenic substrate (DAKO, CA, USA). Finally, the slides were counterstained with hematoxylin and then examined by light microscopy.

Interpretation of p53 immunostaining

Immunohistochemical expression levels of *p53* were scored by a semiquantitative method evaluating the intensity and percentage of positive cells. Only nuclear *p53* staining was regarded as positive staining. Accordingly, the intensity was graded as absent (0), mild (1), moderate (2), and intense (3); while the incidence was categorized as absent (0), less than 10% (1), 10–50% (2), and more than 50% of positive cells (3). Overall staining score was obtained (range 0–6) by adding both variables. A score equal to or greater than 3 was considered as positive for *p53* overexpression. In addition, substituting the primary antibody with the immunoglobulin fraction of nonimmune mouse serum in grade 3 cases served as a negative control. Nuclear staining was determined separately for each specimen, estimated by two independent pathologists. The rare cases with discordant scores were re-evaluated and scored on the basis of the consensual opinion.

Statistical analysis

Statistical differences between categorized groups were analyzed by the χ^2 test and the Fisher's exact test. All statistical analyses were performed with SPSS software package for PC (Version 10.0, SPSS Inc, Chicago, IL, USA). A *p* value less than 0.05 was considered statistically significant.

RESULTS

The expression patterns of the p53 protein in the normal epithelia, precursor lesions, and GBCs are summarized in Tables 1 and 2.

We analyzed 17 precursor lesions, including 16 adenomas and 1 biliary cystadenoma (Table 1). According to the growth pattern, gallbladder adenomas were divided into three types: tubular, papillary and tubulopapillary. In this study, all gallbladder adenomas were of tubular type. Cytologically, of the 16 gallbladder adenomas, 12 cases were pyloric gland type and 4 cases were intestinal type. Positive p53 immunoreactivity was observed in 16.7% (2/12) (Figure 1) of the pyloric gland type and in 25% (1/4) of the intestinal type. In addition, there was no p53 immunostaining in gallbladder cystadenoma. Overall, positive overexpression of p53 protein was observed in 3 (17.6%) of 17 precursor lesions of the gallbladder (Table 1).

The 22 GBC cases in our study were of the following types: 13 adenocarcinoma, 5 papillary adenocarcinoma, 2 adenosquamous carcinoma, 1 mucinous adenocarcinoma, and 1 clear cell carcinoma (Table 2). We noted that p53 overexpression was predominantly seen in two groups, adenocarcinoma (Figure 2A) and papillary adenocarcinoma (Figure 2B), which were 69.2% (9/13) and 60% (3/5), respectively (Table 2). In contrast, p53 overexpression was not observed in adenosquamous carcinoma or clear cell adenocarcinoma specimens. p53 protein was also overexpressed in cases of mucinous adenocarcinoma (Figure 2C) and in 59.1% (13/22) of GBCs (Table 1).

According to the differentiation grade, gallbladder adenocarcinomas were subgrouped into well, moderately, and poorly differentiated adenocarcinomas. We found that p53 immunostaining was overexpressed in 50%, 60%, and 83.3% of well, moderately, and poorly differentiated adenocarcinomas, respectively (Table 2). Although no significant correlation was shown between p53 overexpression and differentiation grade, p53 overexpression was relatively higher in the poorly differentiated adenocarcinoma cases when compared with well-differentiated cases (Table 2).

Normal gallbladder epithelium was examined in 15 specimens resected from patients with hepatocellular carcinoma. We found no positive p53 immunoreactivity in these cases. Meanwhile, normal epithelia in the vicinity of precursor lesion and GBCs also showed no p53 expression.

A statistically significant difference in p53 protein overexpression was present between precursor lesions and

Table 1. The incidence of p53 overexpression in gallbladder pathologic and normal epithelia

Histologic type	Cases tested	Positive cases (%)
Carcinoma	22	13 (59.1) ^{*†}
Precursor lesions	17	3 (17.6)
Adenoma	16	3 (18.8)
Pyloric gland type	12	2 (16.7)
Intestinal type	4	1 (25)
Cystadenoma	1	0 (0)
Normal epithelium	15	0 (0)

* $p < 0.01$ vs normal epithelium; † $p < 0.01$ vs precursor lesions.

Table 2. Correlation of p53 overexpression and histologic features in gallbladder carcinoma

Histologic features	No. tested	No. positive cases (%)
Histologic subtype	22	
Adenocarcinoma	13	9 (69.2)
Papillary adenocarcinoma	5	3 (60)
Adenosquamous carcinoma	2	0 (0)
Mucinous adenocarcinoma	1	1 (100)
Clear cell adenocarcinoma	1	0 (0)
Differentiation grade	13	
Well differentiated	2	1 (50)
Moderately differentiated	5	3 (60)
Poorly differentiated	6	5 (83.3)

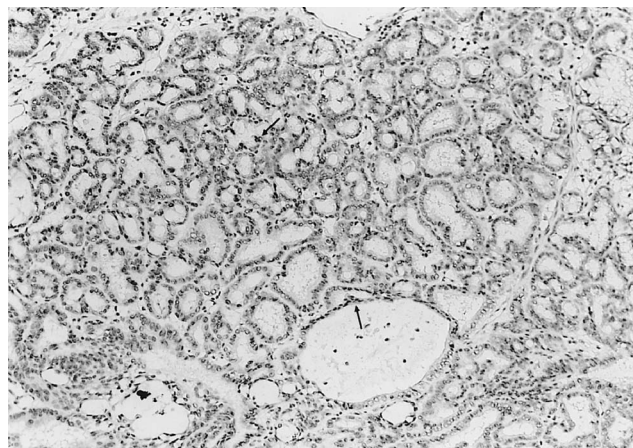


Figure 1. p53 protein overexpression is immunohistochemically detected in the nuclei (arrow) of pyloric gland adenomas (p53; original magnification $\times 20$).

carcinomas of the gallbladder (Table 1, $p = 0.009$). Similarly, a statistically significant difference was present between normal epithelia and carcinomas of the gallbladder (Table 1, $p < 0.001$).

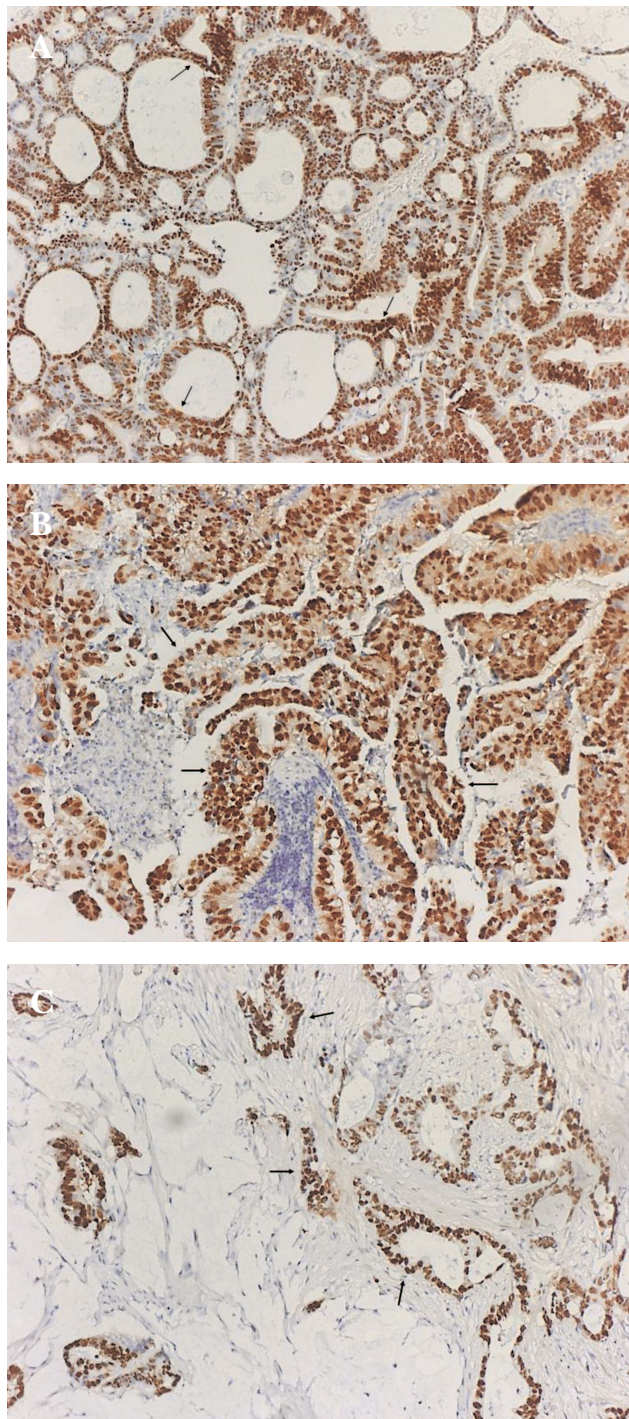


Figure 2. p53 protein overexpression is immunohistochemically detected in the nuclei of (A) adenocarcinoma, (B) papillary adenocarcinoma, and (C) mucinous adenocarcinoma. (arrow: positive nuclear staining) (p53; original magnification $\times 20$).

DISCUSSION

There is abundant evidence proposing that adenomas are the premalignant lesions of the gallbladder. Kozuka et al mentioned that the transition of benign adenoma into carcinoma is histologically traceable [9]. Thus, some authors have claimed that the adenoma-carcinoma sequence is the usual route for tumorigenesis of gallbladder cancers [7–9]. However, there are only limited data about the possible mechanisms involved in this transformation.

The p53 protein has been demonstrated as a potential tumor suppressor [16]. The p53 gene is located on the small arm of chromosome 17 and consists of 11 exons. Mutations in p53 lead to its inactivation and have been found in 50% of human tumors. One of the most common mechanisms is p53 inactivation mediated by point mutation, resulting in expression of a conformationally altered and functionally defective p53 protein, which is immunohistochemically identifiable. On the other hand, a missense mutation or a deletion of one p53 allele often results in the absence of the wild-type p53 protein. This causes the loss of tumor suppressor function and permits the promotion of carcinogenesis.

Carcinogenesis is thought to be a multistep process in which genetic alterations accumulate as premalignant lesions progress to invasive carcinomas. Recent reports have shown that p53 abnormalities are involved in the genesis of GBCs in high-incidence areas of Japan and Chile [10]. Our study provides additional information on how p53 abnormalities may also be involved in the carcinogenesis of GBCs in Taiwan, a low-incidence area. Aberrant p53 expression may play a role in the transition from benign adenoma into GBC.

Accumulated data have shown that the majority of GBCs, which can be of papillary, tubular, mucinous, or signet cell type, are adenocarcinomas (80–95%). Less common types include undifferentiated or anaplastic carcinoma (2–7%), squamous cell carcinoma (1–6%), and adenosquamous carcinoma (1–4%) [17,18]. Adenocarcinomas (13/22) were the most common type of GBCs found in our study (Table 2), followed by papillary adenocarcinomas (5/22) (Table 2). The remaining tumors include two adenosquamous carcinomas, one mucinous adenocarcinoma, and one clear cell adenocarcinoma (Table 2).

Abnormalities of the p53 gene are frequently seen in gallbladder cancers. Although the frequency of p53 immunostaining in GBC varies widely in different studies (range, 35–92%), the majority of studies show a frequency greater than 50% [19]. Well-differentiated tumors have a better prognosis, while poorly differentiated infiltrative tumors are more likely to be associated with lymph node

metastases and liver invasion. In this study, we showed that 59.1% of GBCs overexpressed p53 protein (Table 1). Moreover, although our results showed no significant correlation between p53 overexpression and differentiation grade, p53 protein overexpression seemed to be more prominent in cases of poor differentiation grade (Table 2).

The precursor lesions of GBCs are subgrouped by the classification proposed by Albores-Saavedra et al [14]. Adenoma, a benign glandular tumor, is one of the most common precursor lesions of the gallbladder [20], with a reported incidence of 0.4–1.1% in cholecystectomy specimens. As expected, adenoma constituted the majority of the precursor lesions (16/17) in our study (Table 1). Microscopically, there are three growth pattern types for adenomas: tubular, papillary, and mixed. All of our 16 adenomas were tubular. Cytologically, adenomas can be further classified into three subtypes: pyloric gland, intestinal, and biliary. As reported, tubular adenomas of the pyloric gland type are more common in the gallbladder, while the intestinal type are more common in the extrahepatic bile duct [8,21]. In the present study, the pyloric gland type predominated in 12 of 16 tubular adenomas, and the remaining 4 adenomas were the intestinal type. Some studies also described spindle cell metaplasia, which is occasionally observed in these adenomas. Spindle cell metaplasias, also called squamoid morules, are characterized by nodular aggregates of cytologically bland spindle cells with eosinophilic cytoplasm but without keratinization or intercellular bridges. Its incidence ranges from 5.3–33.3% [22]. In our group, we observed squamoid morules in 3 (25%) of 12 pyloric gland adenomas (data not shown).

It has been suggested that two main histologic pathways exist for the development of gallbladder cancer: (1) the dysplasia-carcinoma in situ sequence and (2) the adenoma-carcinoma sequence. Wistuba and Albores-Saavedra [23] demonstrated the high incidence of p53 overexpression and its presence in dysplasia, even in specimens with invasive carcinomas. Therefore, they suggested that dysplasia-carcinoma in situ is the usual route for gallbladder carcinogenesis and that p53 abnormality is an important and early event [19,23]. On the other hand, Watanabe et al [24] showed a low frequency of p53 overexpression even in the carcinoma portion of carcinoma-in-adenoma cases (6.3%), indicating that the adenoma-carcinoma sequence may also be important. In this study, p53 overexpression was observed in 18.8% (3/16) of the adenomas, and a statistical difference exists between adenomas and carcinomas of the gallbladder (Table 1). It indicated that gallbladder adenomas lack the p53 abnormalities frequently seen in GBCs.

In conclusion, we suggest that p53 overexpression may play a role in GBCs in Taiwan. However, this alteration may not be involved in the putative pathway of gallbladder carcinogenesis: the adenoma-carcinoma sequence. Therefore, other mechanisms or variables may be important in this pathway.

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p53 蛋白質在膽囊癌 及膽囊腺瘤中之異常表現

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膽囊癌是消化道中罕見卻又極具惡性的一種腫瘤，至於它是如何從膽囊腺瘤進展成膽囊癌，其機制目前並不清楚。在人類的惡性腫瘤中，p53 基因是最容易產生突變的一種腫瘤抑制基因。p53 蛋白質如果喪失功能，不僅使癌細胞不易發生凋亡，同時也與某些特定癌症的不良預後有密切關係。在本篇研究中，我們收集了 22 個膽囊癌、17 個膽囊腺瘤以及 15 個正常膽囊黏膜組織，利用免疫組織化學染色方法分析它們個別之 p53 蛋白質表現的情形，並將結果和其臨床及病理組織學的特性作一連結。結果發現，有 59.1% 的膽囊癌及 17.6% 的膽囊腺瘤表現 p53 蛋白質，而且兩者之表現情形呈現統計學上的差異 (P 值等於 0.009)。在正常膽囊黏膜組織方面，則完全沒有呈現 p53 蛋白質免疫染色，同時其表現情形和膽囊癌的結果比較起來，呈現統計學上的差異 (P 值小於 0.001)。另一方面，膽囊癌之 p53 蛋白質表現情形，和其組織學型態及癌細胞之分化情形並沒有呈現統計學上的關聯。本研究的結論支持，異常的 p53 蛋白質表現在膽囊癌的形成上扮演重要的角色。

關鍵詞：p53 蛋白質，免疫組織化學染色，膽囊腺瘤，膽囊癌
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