

Short Communication

DNA Ploidy Pattern and Tumor Suppressor Gene *p53* Expression in Gallbladder Carcinoma¹

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Abstract

The relationship between *p53* gene expression and DNA content in advanced gallbladder carcinoma was studied. Fifty-three cases of advanced gallbladder carcinoma (45 primary tumors and 8 metastases) were analyzed. *p53* protein expression was determined by immunohistochemistry. DNA content was measured by cytophotometric techniques. Study subjects included 45 (85%) female and 8 male patients, with an overall mean age of 58.6 years. Positive staining for *p53* protein was observed in 27 (51%) cases. In subserosal tumors, the expression was significantly less than that in tumors that reached the serosa ($P = 0.01$). Twenty-nine (55%) cases were diploid and 24 were aneuploid. Sixty-seven % of primary tumors were diploid, whereas 87% of metastases showed an aneuploid DNA content. Both diploid and aneuploid tumors were positive for the *p53* protein in the same proportion, and *p53* was also expressed equally in both primary and secondary tumors. In advanced gallbladder carcinoma, the expression of the *p53* gene was earlier than the accumulation of abnormal quantities of chromosomal DNA in the tumor cells. The determination of these events as markers in preneoplastic lesions is warranted in gallbladder carcinogenesis.

Introduction

In some neoplasias, the importance of tumor suppressor genes, such as the *p53* gene, has been demonstrated (1-4). Although the point mutation of this gene is a late event in carcinogenesis, it has been a useful marker of the dysplasia-carcinoma sequence in gallbladder mucosa (5, 6).

There is limited information on the mechanism of action of the *p53* suppressor gene in gallbladder carcinoma (5-7). Although available monoclonal antibodies do not make it possible to differentiate whether the protein is normal or mutated, its presence implies an overexpression that is not observed in nontumor processes of the gallbladder (5-7). The normal half-

life of *p53* protein is less than 30 min; this rapid degradation makes detection of it by immunohistochemical techniques impossible. However, the mutated protein is structurally abnormal, and its degradation can take many hours. The intranuclear concentrations of mutated *p53* allow for its immunohistochemical detection in tumoral gallbladder mucosa, in which *p53* is expressed from incipient to advanced forms of the disease (5-7). However, in a large number ($n = 191$) of gallbladder carcinomas, we have observed a relationship between differentiation degree, infiltration level, and *p53* protein expression.³

DNA content determination in tumor cells has made it possible to discriminate between different cell populations within the same tumor based on different DNA content (8-14). The presence of abnormal DNA quantities in the tumor cells has been demonstrated as a prognostic factor in some neoplasias (11-21). Although DNA content is unrelated to most other tumor markers, its use as a marker in some malignant neoplasias has been clearly demonstrated (11, 12, 16, 21). In the gallbladder carcinoma, most primary tumors present with a diploid DNA content, whereas the metastases of these tumors are mostly aneuploid (22).

The relationship between the abnormal DNA content in tumor cells and anomalous expression of the *p53* tumor suppressor gene has not been studied in gallbladder carcinoma, and this examination was the aim of the present study.

Materials and Methods

Cases. Fifty-three cases of advanced gallbladder carcinoma that were consecutively diagnosed in the Pathology Department at Temuco Hospital were included. The cases diagnosed in the cholecystectomy histological study were considered primary tumors (45 cases), and the remaining cases were metastases of gallbladder carcinoma and were considered secondary tumors. In all cases, infiltration was determined by serial sections of the surgical sample (mapping), according to preestablished criteria (23, 24). Tumors that had infiltrated the subserosa or serosa layer were considered advanced gallbladder carcinoma.

Clinical parameters (sex, age, and race) and morphological parameters (histological type, degree of differentiation, and infiltration) were determined and included in the analyses.

Protein Expression of the *p53* Tumor Suppressor Gene. Standard immunohistochemical technique of streptavidin-biotin (Biogenex) was performed. Before incubation with the primary antibody (monoclonal antibody anti-*p53*, diluted 1:100; Oncogene Science), histological sections were boiled twice in buffer. Subsequently, samples were incubated with the secondary antibody at 1:320 (multilink biotinylated; Biogenex) for 1 h and finally developed with diaminobenzidine. Negative and positive controls were included with each batch. Normal serum

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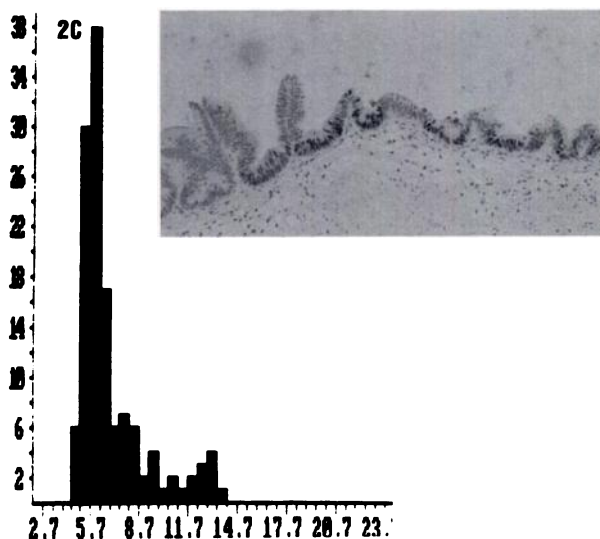


Fig. 1. Early gallbladder carcinoma (well-differentiated type) with only one peak in DNA histogram (diploid DNA content) and p53 protein expression in a high percentage of tumor cells (inset).

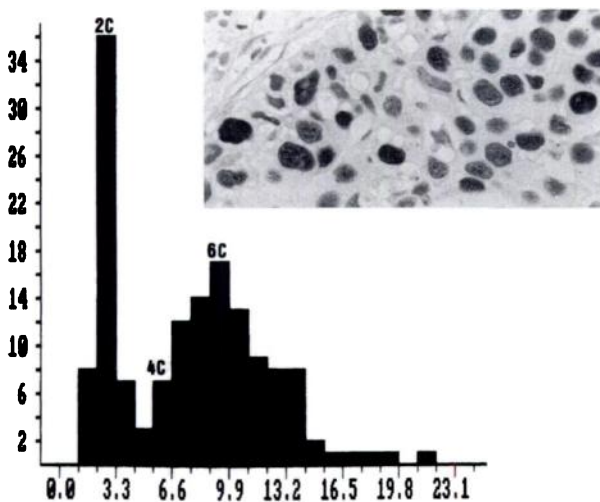


Fig. 2. Advanced gallbladder carcinoma (poorly differentiated type) with second peak corresponding to 6C area (aneuploid DNA content) and a strong positive staining for p53 protein (inset).

served as a negative control, and p53-positive sections of colon carcinoma were used as the positive control.

Measurement of Positivity. Positivity was determined by intranuclear staining of the p53 protein. Those cases in which 5% or more of the cells showed intranuclear staining were considered positive (Figs. 1 and 2). In cases of doubtful positivity, a new test was carried out, and when doubt persisted, these cases were considered negative.

Determination of DNA Content. Two histological sections of 20 μm were obtained from each case, dewaxed in xylene, and hydrated through decreasing alcohol concentrations. Sections were then incubated with 0.5% pepsin, pH 1.5 (Sigma Chemical Co.), at 37°C for 45 min and washed in 9% NaCl. After centrifugation and supernatant elimination, four sections were

Table 1 p53 protein expression in primary gallbladder carcinoma according to infiltration level^a

Infiltration level	p53		Total
	+	-	
Subserosa ^b	6 (30%)	14 (70%)	20
Serosa	17 (68%)	8 (32%)	25

^a Values shown are number of tumors (*n*) and values in parentheses are percentages of total.

^b p53 expression was significantly less in subserosal tumors ($P = 0.01$).

smeared on slides without adherent and postfixed in ethanol at 70°C. These sections were stored at room temperature until staining. Sections were hydrated in physiological serum and were then stained with 0.05% propidium iodide (Calbiochem) and 0.01% (v/v) Triton X-100 in PBS buffer, pH 7.2, for 30 min at 4°C in a darkroom. A Nikon epi-fluorescence microscope with a halogen supply, excitation filters with a wavelength range of 450–490 nm, and a barrier filter of 520 nm were used. The fluorescent emission obtained from the stained nuclei was measured in a Nikon cytophotometer System PI with a sensitivity of 545 mV. A field diaphragm was adjusted according to the maximum size of tumor cells nuclei. Using an automatic scanning stage (Nikon) and Phoscan3 software, 100 tumor nuclei and 30 control nuclei of lymphocytes in the same smear were selected and measured. Obtained histograms were classified as follows. DNA content was considered normal, 2C, or diploid when the case maximum peak coincided with the control peak, and DNA content was considered abnormal or aneuploid (including tetraploid, polyploid, and multiploid types) when there was a second peak farther than 2 SDs from the average of control nuclei measurements (Figs. 1 and 2).

All data were tabulated on a electronic sheet, and the relationships of the data with clinical and morphological parameters were analyzed by means of the χ^2 test and Fisher's exact test.

Results

Of the 53 cases studied, 45 (85%) were females and 8 were males, and cases had an overall mean age of 58.6 years (female mean age, 57.4 years; male mean age, 64 years). Ninety-seven % of the cases were adenocarcinomas, and most (85%) were tumors with moderate or poor differentiation.

In 27 of 53 (51%) cases, positive staining was observed for p53 protein (Fig. 1, inset). No differences were observed in protein expression between primary tumors and metastases. Both control and nontumoral mucosa adjacent to advanced carcinoma did not immunostain positive for p53 protein. Similar proportions of positive cases were observed in both sexes and also in Native American and non-Native American subjects. No differences were detected in the expression of p53 in primary tumors (according to histological differentiation degree). In subserosal tumors, the expression of p53 was significantly less than that in tumors infiltrating the serosa layer ($P = 0.01$; Table 1).

Twenty-nine (55%) of all tumors had a normal or diploid DNA content (Fig. 1), and the remaining 24 tumors (45%) had an abnormal or aneuploid DNA content (Table 2; Fig. 2) Sixty-seven % of the primary tumors showed a diploid DNA content, and 87% of metastases showed an aneuploid DNA content ($P = 0.01$).

Diploid as well as aneuploid tumors showed positivity for

Table 2 DNA content in primary gallbladder cancer and metastases

Tumor type	DNA content		Total
	Diploid	Aneuploid	
Primary tumor ^a	28	17	45
Metastasis	1	7	8
Total	29	24	53

^a Primary tumors showed significantly less aneuploidy than metastases ($P = 0.01$).

Table 3 DNA content and p53 expression

	p53		Total
	+	-	
Diploid	15	14	29
Aneuploid	12	12	24
Total	27	26	53

p53 protein in nearly 50% of the cases (Table 3). No significant differences were observed between primary and secondary tumors.

Discussion

Advanced gallbladder carcinoma and metastases were included in this study. Early gallbladder carcinomas with mucosa or muscular layer infiltration are mostly well-differentiated tumors. Both elements, tumor infiltration level and differentiation degree, present a significant negative association with the immunohistochemical expression of p53 protein.³ Most early gallbladder carcinomas and well-differentiated tumors are diploid, unlike advanced carcinoma and metastases, which present with an abnormal DNA content more frequently (22). Thus, only advanced tumors were selected for this study to compare DNA content and p53 protein expression in those tumors in which these elements are mostly found.

As in previous studies, we have shown that protein expression of p53 gene is detectable by immunohistochemical methods in about 50% of gallbladder carcinomas (5, 7) and 80–90% of gallbladder carcinomas have p53 loss of heterozygosity demonstrated by molecular techniques (6). Another factor that must be considered is the relationship between p53 protein expression and tumor infiltration level in the gallbladder wall. Serosal tumors showed a significantly higher expression than those tumors with subserosa involvement. On the other hand, patients with subserosal infiltration correspond to a heterogeneous group in which it is thus far impossible to determine which of them will have different clinical behavior (25). We have demonstrated that there is a difference in DNA content between primary tumors and metastases (22, 26). It may be that diploid gallbladder tumors are less likely to develop metastases than aneuploid, reflecting a higher malignant potential of aneuploid cells (27). Thus, determination of DNA content may be a useful prognostic indicator in patients with gallbladder carcinoma. In this study, expression of p53 protein and DNA content were not associated and appear to be independent.

Tumor suppressor genes can be inactivated through gene abnormalities (mutations) or chromosomal aberrations (translocations, deletions, nondisjunctions, reduplications, and so on) (28), and consequently, DNA quantity should significantly affect the activity of these genes. Moreover, p53 expression

demonstrated a statistically significant relationship with DNA ploidy in superficial bladder tumor (29) and in colon dysplastic and neoplastic lesions (30). However, nonassociation between p53 mutations and DNA content was demonstrated in head and neck squamous carcinoma (31). There is no consensus about the relationship between DNA content and p53 expression or about which of these is the first to become abnormal in carcinogenesis (29, 30–33). The absence of a relationship between p53 protein expression and DNA content in gallbladder carcinoma suggests that these events are components of different pathways (28). Finally, we must point out the potential use of determination of the p53 protein expression as a malignancy marker of gallbladder epithelium (5, 7) because p53 is not expressed in inflammatory nor in reactive lesions.

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