Microsatellite instability in preneoplastic and neoplastic lesions of the gallbladder

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Background. Gallbladder cancer is very common in Chile and is the leading cause of cancer deaths in women aged over 40 years. However, there is limited information about the molecular changes involved in its pathogenesis. Microsatellite analysis was performed using polymerase chain reaction (PCR)-based assays to identify genetic loci that were altered in neoplastic and preneoplastic conditions of early and advanced gallbladder cancer. Our findings were then correlated with clinicopathological variables and survival time.

Methods. We selected 59 surgical specimens of gallbladder adenocarcinomas (29 early cancers and 30 advanced cancers) and 22 surgical specimens from patients with chronic cholecystitis from a high-risk area for gallbladder cancer (Temuco, Chile). Laser capture microdissection (LCM) was used to harvest tumor cells and preneoplastic lesions. Microsatellite analysis was performed using 13 different markers. The tumors and preneoplastic lesions were also examined with immunohistochemistry for hMLH1, hMSH2, and hMSH6.

Results. We found that 10% (6/59) of gallbladder cancers showed high-frequency microsatellite instability (MSI-H), with identical proportions in both early and advanced cancers. In premalignant lesions adjacent to the six MSI-H tumors, we detected instability in two of six examples of intestinal metaplasia (33%) and five of six examples of dysplasia (83%). All MSI-H cases showed an altered pattern with the antibodies studied. MSI status was not associated with survival or other clinicopathological features. No MSI or immunohistochemical alterations were found in the chronic cholecystitis group.

Conclusions. Microsatellite instability was observed in equal proportions in early and late cancers, and it was also found in premalignant lesions, indicating that inactivation of mismatch repair genes occurs early in gallbladder carcinogenesis.

Key words: gallbladder cancer, preneoplastic lesions, microsatellite instability, loss of heterozygosity, laser capture microdissection

Introduction

Gallbladder cancer is the leading cause of cancer deaths in Chile in women aged over 40 years. This disease has a mortality rate of 35.2/100 000 in this group and 11.8/100 000 in the general population.¹² In contrast, incidence rates are low in the United States (less than 2 per 100 000 for both sexes, with the exception of American Indian populations in New Mexico (12.5 per 100 000 in women and 4.9 per 100 000 in men).³⁴ In spite of its high incidence in Chile, there is limited information about the molecular changes involved in its pathogenesis, especially regarding preneoplastic lesions.

Gallbladder carcinoma is preceded by a lengthy pre-malignant process taking several decades.⁵⁶ This process is characterized by chronic inflammation, lithiasis, intestinal metaplasia, and dysplasia in the surrounding mucosa. The process is also characterized by chronic cholecystitis, a very common disease in Chile. It has been reported in 40%–50% of the adult female population and in 20%–30% of males.⁷ Our previous work, utilizing mapping and histopathologic description of cholecystectomy specimens, identified an association of neoplastic lesions with lithiasis in 90% of cases, with chronic cholecystitis in 100%, with intestinal metaplasia in 65.6%, and with dysplasia in 81.3% of cases.⁸ We proposed a progression model based on these observations, where the period required to progress from dysplasia to advanced gallbladder carcinoma would be approximately 15 years.⁶

The identification of dysplasia as a premalignant lesion in gall-bladder cancer is also supported by studies examining tumor-related genes and gene products.
Reduced p21 (WAF/CIP1) expression was detected by immunohistochemistry in dysplasias and carcinomas. The accumulation of p53 protein, as detected by immunohistochemistry, has been reported in dysplasias, as well as in their adjacent carcinomas. Loss-of-heterozygosity studies, using markers near the TP53 gene and others, report losses in dysplasias resembling those in the adjacent tumor. For a review, see Sasatomi et al. The status of metaplasia as a premalignant condition is less clear, though it is implicated by its frequent association with carcinoma. Microsatellite instability (MSI) is a defect of tumor DNA characterized by an alteration in the number of simple sequence repeats of microsatellite markers, caused by the insertion or deletion of repeat units. Such alterations are normally repaired by the DNA mismatch repair (MMR) system. These proteins, which correspond to several MutS and MutL homologues from the prokaryotic MutHL DNA repair system, hMLH1 (human MutL Homologue 1), and hMSH2, hMSH3, and hMSH6 (human MutS homologues 2, 3, and 6), bind the mismatched DNA, allowing excision and repair of the sequence. In the absence of a normal mismatch repair function, due to mutation or promoter methylation of critical MMR genes, alterations in repeat sequences remain unrepaired, and alleles of different sizes will be formed at the next replication. This condition, called MSI, was first described in hereditary nonpolyposis colorectal cancer (HNPPC), but it also affects a subset of sporadic pancreatic, endometrial, prostate, and gastric carcinomas. Although the phenotype was first observed in analyses of microsatellite markers from tumor DNA, the defect most likely alters tumor biology by causing the inactivation of genes containing repeat sequences. Examples of these vulnerable genes are transforming growth factor beta receptor type II, BAX, and E2F4. A National Cancer Institute conference in the United States made recommendations regarding nomenclature and criteria for the identification of colorectal tumors as having MSI. This conference recommended the use of a panel of five mononucleotide and dinucleotide markers (BAT25, BAT26, D2S123, D5S346, and D17S250), and it defined three categories of tumors: MSI of high frequency (MSI-H) and of low frequency (MSI-L), and tumors which are unaffected, called microsatellite stable (MSS). Although the NIH guidelines were recommended to apply only to colorectal cancer, they are useful standards to apply to other tumor types, in order to facilitate comparisons between studies. In prior studies of gallbladder cancer, there are only a few reports, with small numbers of cases, using varied numbers and types of microsatellite markers, so that comparisons with other tumor types are difficult. Using the NCI criteria, we analyzed a large series of early and advanced gallbladder cancers for MSI. In addition, we examined pre-malignant glands for the same alterations as those seen in the adjacent tumors, and correlated our results with the immunohistochemical (IHC) expression of the MMR system and the clinicopathological findings.

Methods

Formalin-fixed, paraffin-embedded cholecystectomy specimens from 59 patients with gallbladder cancer were selected from patients having adequate material to analyze (29 early cancers; defined by infiltration as far as the muscle layer) and 30 advanced gallbladder cancers; showing infiltration beyond the muscle layer). Twenty-one patients were younger than 40 years old. Specimens from 22 patients with chronic cholecystitis were obtained from a high-risk area for gallbladder cancer (Temuco, Chile). Authorization for using the material was obtained from the Ethics Committee of the Faculty of Medicine, Universidad de la Frontera. Serial 6-µm sections were cut from paraffin blocks, using a technique designed to reduce the risk of cross-contamination at the microtome. Five different areas in early and advanced cancers were harvested, including stromal lymphocytes, normal epithelium, intestinal metaplasia, dysplasia, and carcinoma. In specimens from patients under 40 years old, normal control (stromal lymphocytes) and areas of carcinoma were examined. In addition, normal mucosa and intestinal metaplasia from 22 gallbladders with no tumor were analyzed. A subset of patients were identified as belonging to an Amerindian ethnic group (Mapuche) by their distinctive surnames.

Microdissection and DNA extraction

Slides were prepared for laser capture microdissection (LCM) as described previously. Microdissection was performed using a PixCell I microscope (Arcturus Engineering, Mountainview, CA, USA), using a 30-µm-diameter laser beam and 50- to 100-ms pulse width. Areas were harvested by LCM from non-cover-slipped methyl green-stained sections onto optically clear plastic caps, as shown in Fig. 3. Harvested tissue was digested from the caps with 50–100 µl of protease K (1 mg/ml, in 50 mM Tris-HCl buffer, pH 8, with 1 mM ethylene diamine tetraacetic acid (EDTA), and 0.45% Tween-20) at 52°C overnight. The protease K was denatured at 95°C for 15 min. DNA solutions were stored at −20°C.