Allele-specific Mutations Involved in the Pathogenesis of Endemic Gallbladder Carcinoma in Chile

Ignacio I. Wistuba, Kenji Sugio, Jaclyn Hung, Yosuke Kishimoto, Arvind K. Virmani, Ivan Roa, Jorge Albores-Saavedra, and Adi F. Gazdar

Abstract

Although gallbladder carcinoma is one of the most frequent neoplasms in Chile, there is limited information about the molecular changes involved in its pathogenesis. We investigated the incidence of ras gene mutations and loss of heterozygosity (LOH) at the p53 locus, DCC, rb, 5q, 3p, 8p, and 9p. We precisely microdissected 194 relevant cases and their accompanying nonneoplastic lesions (which were present in 15 cases) from patients in Chile. The specimens were analyzed by PCR-based assays for LOH, and we designed a RFLP method for ras mutations and immunohistochemistry for p53 protein overexpression. We determined that LOH at p53 (91%), rb (50%), 8p (44%) and DCC (31%) are frequent events and that LOH at p53, rb, and DCC are early events, while ras mutations and LOH at 3p, rb, and 5q occurred occasionally. LOH at p53 occurred more frequently and earlier than protein overexpression. The mean number of mutations present in invasive carcinomas was 2.1, and in six cases, LOH at the p53 gene was the sole mutation detected. The same allele was lost in 61 (93%) of 71 nonneoplastic foci as in the corresponding invasive carcinomas for all four mutations studied. The odds of this occurring by chance are \( \sim 4 \times 10^{-15} \). Although clonality cannot be excluded, allelic loss appears to be highly directed, but the mechanism for allele-specific mutations remains to be determined.

Introduction

GBC is a relatively uncommon neoplasm in the world, but there is considerable geographic variation in its incidence (1). It is one of the most frequent neoplasms in Chile, where it is the leading cause of cancer deaths in females (2). Although GBC has been associated with genetic and environmental risk factors, there is limited information about the molecular changes involved in its pathogenesis. It has been well established that invasive GBC is preceded by preneoplastic lesions, including dysplasia and CIS. In addition, metaplasia of intestinal and pyloric types is frequently present, but its malignant potential is unknown (3). Multiple genetic changes are associated with the development of many human cancers, and molecular studies in several neoplasms have demonstrated their association with the activation of dominant proto-oncogenes and inactivation of recessive tumor suppressor genes. Tumor suppressor genes are frequently inactivated by allelic loss or chromosome deletions. Tumor suppressor genes are frequently inactivated by allelic loss or chromosome deletions. The original primer sequences were modified for nested PCR reactions. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The abbreviations used are: GBC, gallbladder carcinoma; CIS, carcinoma in situ; LOH, loss of heterozygosity; ASM, allele-specific mutations.

Materials and Methods

Details of PCR methodology, the sequences, and the references of all primer pairs used have been deposited with the Editorial Office of the American Association for Cancer Research. Details of PCR methodology, the sequences, and the references of all primer pairs used have been deposited with the Editorial Office of the American Association for Cancer Research and may be obtained from them or from the authors.

Tissue Samples and Histological Diagnosis. Twenty-five GBC (22 adenocarcinomas and 3 small cell carcinomas) obtained by cholecystectomy and containing preneoplastic lesions were selected for study. All cases were retrieved from the files of the Anatomic Pathology Unit, Temuco Hospital, Temuco, Chile. Criteria for histological identification of tumors, preneoplastic lesions (dysplasia and CIS), and metaplasia have been described previously (3). We use the term “nonneoplastic” epithelium for all noninvasive foci examined (including preneoplasia, metaplasia, and normal-appearing epithelium). Serial 5-μm sections were cut from archival, formalin-fixed, paraffin-embedded tissues.

Microdissection and DNA Extraction. Microdissection and DNA extraction were performed as previously described from nonoverslipped hematoxylin and eosin-stained slides (8). Precisely identified areas of stromal lymphocytes, normal epithelium, metaplasia (pyloric and intestinal types), preneoplastic lesions (dysplasia and CIS), and invasive GBC were microdissected under microscopic visualization (Fig. 1). At least 50 cells were used for each PCR reaction.

Polymeric DNA Markers. To evaluate LOH, we used primers flanking microsatellite dinucleotide (CA)n repeat polymorphisms located at the following genes or chromosome locations: 3p14.1-14.3 (D3S1228 locus) and 3p21.2-21.3 (D3S1029 locus) sites of putative recessive oncogenes (9); 5q21-22 (D5S346 locus), 30-70 kb downstream from the APC gene; 8p22 (D8S502 locus), site of a putative recessive oncogene (10); 9p21-22 (JFN A gene and D9S171 loci), regions spanning the CDKN2 gene; rb gene (13q14); and DCC gene (18q21). So as to increase the number of informative cases, LOH at p53 locus and noninformative cases were also analyzed using a pentanucleotide (AAAAAT)n repeat located in intron 1 of the gene. The original primer sequences were modified for nested PCR reactions.

PCR-LOH Analysis. Nested PCR methods were used to amplify dinucleotide repeats from microdissected cells at genes rb, p53, and DCC and at chromosomes 5q and 9p as described previously (11). One round PCR amplification was used to detect polymorphic microsatellites at chromosomes 6p and 3p and to detect the pentanucleotide repeat at the p53 locus (8). To detect (CA)n repeats at 8p and 3p loci and to detect both microsatellites studied at the p53 locus, we used a hot-start PCR method (TaqStart Antibody; Clonotech, Palo Alto, CA). The amplified and labeled fragments thus obtained were subjected to electrophoresis as described previously (8). LOH was scored by visual comparison of the intensity of the allelic bands on autoradiographs.
Detection of K- and N-ras Mutations using a PCR-based Designed RFLP Method. For detection of ras mutations, we used a modification of the designed RFLP method, using nested PCR reactions, as described previously (12). The assays were performed in two steps: (a) a screening test to detect mutations in codons 12, 13, and 61 of K- and N-ras genes; and (b) an identification step, using additional assays to determine the specific base substitution at the codon identified by the screening step.

Immunohistochemical Study of p53 Protein Overexpression. Immunostaining was performed using a streptavidin-biotin immunoperoxidase method. Primary anti-p53 antibody (Ab-6; Oncogene Science) was used at a 1:5 dilution. p53 overexpression was scored by a semiquantitative method evaluating intensity (0–3) and incidence (0–3) of positive cells. A cumulative score greater than or equal to 3 was considered positive for overexpression.

Statistical Analysis. The cumulative binomial test was used to examine the likelihood that the occurrence of a particular event (loss of the same allele in the invasive carcinoma and its corresponding preneoplastic lesions) happens at a particular probability when observed in repeated trials (13). When the results are compared with a chance occurrence or nonoccurrence, the particular probability of comparison is 0.5.

Results

Frequency of p53 Gene Abnormalities. Immunohistochemical evidence of p53 protein overexpression was detected in 13 of 22 (59%) invasive carcinomas, 6 of 13 (46%) CIS, and 4 of 12 (33%) dysplasias (Table 1). Normal (0 of 12) and metaplastic (0 of 6) epithelia did not overexpress p53 protein. The incidence of LOH at the p53 gene (at one or both of the markers studied) in invasive GBC was 91% (20 of 22 informative cases). The concordance for both p53 probes (dinucleotide and pentanucleotide) was 100% (8 of 8 cases; 22 of 22 foci). We found a high frequency of LOH at preneoplastic stages, including CIS (11 of 13; 85%) and dysplasia (7 of 12; 58%). Of interest, we also detected LOH at the earlier stages (normal-
appearing epithelium). We correlated both p53 abnormalities (immunohistochemical p53 protein overexpression and LOH) in all informative cases (n = 22). LOH of the p53 gene (91%) was more frequent than protein overexpression (59%) in invasive carcinomas (Table 1). Of interest, all but one of the immunohistochemically positive tumor cases also demonstrated LOH. Although immunohistochemical detection was limited to dysplasia, CIS, and invasive tumors, LOH was detected at earlier stages, including normal-appearing epithelium and metaplasia. In twelve cases, both LOH and protein overexpression were present in the invasive carcinoma, and in six of these cases, both LOH and protein expression were present in nonneoplastic epithelium. In these six cases, LOH was detected at earlier stages in five cases. In a single case, immunostaining was detected at an earlier stage (dysplasia), while LOH was detected at a later stage (CIS). These findings indicate that LOH precedes protein overexpression in the pathogenesis of gallbladder carcinoma.

Incidence of 9p LOH. We detected a high incidence of 9p LOH (at one or both of the markers studied) in invasive carcinomas (7 of 14 informative cases, 50%; Table 1). The degree of concordance for both 9p probes (IFN A and D9S171) was 71% (5 of 7 cases). We also detected a frequent incidence of 9p LOH in preneoplastic lesions (3 of 4 dysplasias and 5 of 5 CIS), as well as at the earlier stage of metaplasia (2 of 2). No LOH was detected in three cases of normal-appearing epithelium (Fig. 2).

Frequency of DCC Gene LOH. The incidence of LOH at the DCC gene in invasive carcinoma was 31% (4 of 13 informative cases; Table 1). We found LOH in CIS (3 of 3), dysplasia (3 of 3), metaplasia (1 of 2), and normal-appearing epithelium (1 of 2; Fig. 2).

K and N-ras Mutations. We screened foci of 21 invasive GBC for the presence of mutations in K- and N-ras genes at codons 12, 13, and 61 (Table 1; Fig. 2). In two carcinomas (10%), a ras gene point mutation (one K-ras and one N-ras) was present. The K-ras mutation was detected at codon 12 (GGT to GAT), whereas the N-ras mutation was at codon 61 (CAA to AAA). Both of these tumors were heterogeneous for the mutations, which were detected only in poorly differentiated areas of invasive gallbladder adenocarcinomas and not in their associated normal epithelium, metaplasia, preneoplastic lesions, or well-differentiated areas of invasive carcinoma.

Frequency of LOH in Other Markers (Chromosomes 8p, 5q and 3p, and rb Gene). The incidence of LOH at the 8p locus was high in a limited number of invasive GBC cases (4 of 9 informative cases, 44%; Table 1). For the other microsatellite markers studied (5q, rb gene, and 3p loci) the incidences of LOH were less than 25%.

### Table 1: Genetic abnormalities in GBC, normal epithelium, and preneoplastic lesions

<table>
<thead>
<tr>
<th>Locus/genea</th>
<th>No. informative/ tested (%)</th>
<th>Normal epithelium (%)</th>
<th>Metaplasia (%)</th>
<th>Dysplasia (%)</th>
<th>CIS (%)</th>
<th>Invasive carcinoma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 protein expression (IHC)</td>
<td>0/12</td>
<td>0/6</td>
<td>4/12 (33)</td>
<td>6/13 (46)</td>
<td>13/22 (59)</td>
<td></td>
</tr>
<tr>
<td>p53 LOH (either marker)</td>
<td>22/25 (88)</td>
<td>3/12 (25)</td>
<td>1/6 (17)</td>
<td>7/12 (58)</td>
<td>11/13 (85)</td>
<td>20/22 (91)</td>
</tr>
<tr>
<td>(CA)6n</td>
<td>16/25 (64)</td>
<td>3/8</td>
<td>1/5</td>
<td>8/9</td>
<td>9/10</td>
<td>16/16 (100)</td>
</tr>
<tr>
<td>(AAAAT)n</td>
<td>14/21 (67)</td>
<td>0/4</td>
<td>0/2</td>
<td>2/5</td>
<td>4/5</td>
<td>12/14 (86)</td>
</tr>
<tr>
<td>9p (either marker)</td>
<td>14/23 (61)</td>
<td>0/3</td>
<td>2/2</td>
<td>3/4</td>
<td>5/5</td>
<td>7/14 (50)</td>
</tr>
<tr>
<td>9p IFN A</td>
<td>10/23 (44)</td>
<td>0/2</td>
<td>1/1</td>
<td>2/2</td>
<td>3/3</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>9p D9S171</td>
<td>10/20 (50)</td>
<td>0/2</td>
<td>1/1</td>
<td>2/2</td>
<td>3/3</td>
<td>4/10 (40)</td>
</tr>
<tr>
<td>8p</td>
<td>9/14 (64)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4/9 (44)</td>
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<tr>
<td>DCC (18q)</td>
<td>13/20 (65)</td>
<td>1/2</td>
<td>1/2</td>
<td>3/3</td>
<td>3/3</td>
<td>4/13 (31)</td>
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<td>5q (APC)</td>
<td>23/25 (92)</td>
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<td>ND</td>
<td>0/1</td>
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<td>5/23 (22)</td>
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<td>rb (13q)</td>
<td>15/24 (63)</td>
<td>1/2</td>
<td>1/2</td>
<td>2/2</td>
<td>2/2</td>
<td>3/15 (20)</td>
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<tr>
<td>3p</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3/15 (20)</td>
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<td>3p21.3</td>
<td>8/12 (67)</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1/8 (13)</td>
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<tr>
<td>3p14</td>
<td>10/21 (48)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>Either ras gene</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
<td>2/2</td>
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<td>0/1</td>
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<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>1/2</td>
</tr>
</tbody>
</table>

a LOH at the indicated chromosomal locations or genes, except for ras gene point mutations. The percentages for p53 gene LOH and protein expression in noninvasive lesions are presented for ease of comparison. ND, not done; IHC, immunohistochemistry.
Mutations in Gallbladder Carcinoma

Table 2: Allele-specific loss in GBC

<table>
<thead>
<tr>
<th>Lesions</th>
<th>p53a</th>
<th>9pa</th>
<th>DCC</th>
<th>rb</th>
<th>Any locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>3/3</td>
<td>0/0</td>
<td>1/1</td>
<td>1/1</td>
<td>5/5</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>1/1</td>
<td>1/2</td>
<td>1/1</td>
<td>1/1</td>
<td>4/5</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>12/13</td>
<td>4/5</td>
<td>5/5</td>
<td>2/2</td>
<td>23/25</td>
</tr>
<tr>
<td>CIS</td>
<td>17/17</td>
<td>9/11</td>
<td>4/4</td>
<td>4/4</td>
<td>34/36</td>
</tr>
<tr>
<td>Total</td>
<td>33/34 (97%)</td>
<td>14/18 (78%)</td>
<td>11/11 (100%)</td>
<td>8/8 (100%)</td>
<td>66/71 (93%)</td>
</tr>
</tbody>
</table>

a For p53 and 9p, two markers were analyzed, and the data were combined. Thus, a single preneoplastic lesion or p53 demonstrating LOH at both 9p or 9p markers was scored once.

In 25 invasive carcinomas studied, nonneoplastic lesions were present in 15 cases (60%). LOH at one or more of the seven genes/loci examined was present in 14 of these cases. In all of these 14 cases, LOH at the same genes/loci were present in the corresponding invasive carcinomas. p53 protein overexpression was detected in six cases of preneoplasia, and in all six of these cases, p53 protein overexpression was also detected in the corresponding invasive carcinoma. Thus, in all instances in which an abnormality was detected in nonneoplastic epithelium, the same abnormality was present in the corresponding carcinoma.

Patterns of Allelic Loss in Normal Epithelium, Metaplasia, Prenoeoplasias, and Invasive Carcinoma Are Similar. We compared the patterns of allelic loss in normal epithelium, metaplasia, dysplasia, and CIS with those of the corresponding malignant tumors (Table 2 and Fig. 2). For p53 and 9p allelic loss, the pattern was identical in 33 of 34 (97%) and 14 of 18 (78%) of comparisons, respectively, while the pattern of loss for DCC and rb genes was identical in 33 of 34 (97%) and 14 of 18 (78%) of comparisons, respectively.

Discussion

We studied molecular abnormalities (LOH at seven loci/genomes, ras gene point mutations, and p53 protein overexpression) in 25 cases of invasive GBC from Chile. In all but one case, one or more abnormalities were detected (mean, 2.71 per tumor). We determined that certain molecular abnormalities, including LOH at p53 and DCC genes and at chromosome locations 9p and 8p, are frequently present in GBC. In six of 25 invasive carcinomas, LOH at the p53 gene was the sole molecular abnormality detected.

Molecular analyses of small foci of nonneoplastic epithelial lesions are difficult because they require accurate histological identification and localization. The development of a precise microdissection technique and of methodologies for determining polymorphisms and point mutations in small numbers of cells from formalin-fixed, paraffin-embedded tissues have permitted us to analyze the entire spectrum of epithelial lesions in the gallbladder. Of the 25 invasive carcinomas studied, nonneoplastic lesions were present in 15 cases (60%). LOH at one or more of the seven genes/loci examined was present in 14 of these cases. In all of these 14 cases, LOH at the same genes/loci were present in the corresponding invasive carcinomas. p53 protein overexpression was detected in six cases of preneoplasia, and in all six of these cases, p53 protein overexpression was also detected in the corresponding invasive carcinoma. Thus, in all instances in which an abnormality was detected in nonneoplastic epithelium, the same abnormality was present in the corresponding carcinoma.

We have previously reported a high incidence of p53 protein expression (as demonstrated immunohistochemically) in GBC and its preneoplastic lesions (14). In the present study, we found that LOH at the p53 gene (91%) was more frequent than protein overexpression (59%) in invasive carcinomas. Because all but one of the immunohistochemically positive tumor cases also demonstrated LOH, the latter technique is more sensitive than immunohistochemical detection of protein overexpression. While immunohistochemical detection was limited to dysplasia, CIS, and invasive tumors, LOH was detected at earlier stages, including normal-appearing epithelium and metaplasia.

Recessive oncogenes are believed to be inactivated via a two-step process involving both alleles. Knudson (4) has proposed that the first "hit" frequently is a point mutation, while the second allele is subsequently inactivated via a chromosomal deletion, translocation, or other event. While this sequence occurs in inherited cancer states such as the Li-Fraumeni syndrome and familial retinoblastoma, the sequence has not been formally proven in sporadic cancer cases. Our finding of LOH of the p53 and other genes very early in the multistage carcinogenesis process, including histologically normal epithelium, suggest that LOH may precede point mutations, at least in some instances. Of interest, p53 protein overexpression, as demonstrated by immunohistochemistry, occurred at a later stage in the multistep process (dysplasia) but was always (with one exception) present only in lesions that also demonstrated LOH. In six cases, both LOH and p53 overexpression were present in nonneoplastic epithelium. In these six cases, LOH was detected at earlier stages in five cases, while in a single case, immunostaining was detected at an earlier stage (dysplasia), whereas LOH was detected at a later stage (CIS). Because p53 protein "overexpression" usually is due to the longer half-life of the mutant forms of the protein (15), our observations suggest that LOH may precede point mutations in sporadic GBC.

Allelic losses on the short arm of human chromosome 9 have been identified with several cancer types, and a putative oncogene has been identified (16, 17). The gene, known by several names, including Multiple Tumor Suppressor 1 (MTS-1), CDKN2, or p16INK4, encodes the p16 protein, an inhibitor of cyclin-dependent kinase 4. Recently, our laboratory has found that 9p LOH occurs at the earliest stage (hyperplasia) in the pathogenesis of nonsmall cell lung carcinoma (18). A high incidence of 9p LOH was present in our cases of GBC (50%) and could be detected as early as the stage of metaplasia.

Allelic losses of the short arm of chromosome 8 are common in colorectal and other malignancies (10). There is evidence that there are two distinct regions of 8p LOH (8p21 and 8p22) involved in colon carcinoma, suggesting the existence of two independent tumor suppressor genes (10). We detected a high incidence of LOH at the 8p22 locus in GBC (44%), suggesting that a putative suppressor oncogene located in this region may play an important role in its pathogenesis.

Mutations of the DCC (18q21; Ref. 19) and APC (5q21–22; Ref. 20) genes are associated with the development of sporadic colon cancer. In our cases of GBC, LOH of the DCC gene was a relatively frequent (31%) and early event, detected even in normal and metaplastic epithelia, suggesting that it is an important abnormality in the
pathogenesis of this neoplasm. By contrast, 5q LOH near the APC gene was a rare and late event.

Allelic losses in the short arm of chromosome 3 have been associated with the development of several carcinomas, including lung cancer (9). In lung carcinoma, allele losses involve at least three distinct regions located at 3p25, 3p21.3, and 3p14 (9). Presumably, these regions are the sites of yet undiscovered suppressor oncogenes. The low incidence of LOH at 3p21 and 3p14 loci detected in our cases indicates that these abnormalities are not an important event in the pathogenesis of GBC.

Inactivation of the rb gene is associated with hereditary and sporadic cases of retinoblastoma and many other tumors (15). Although the incidence of LOH at the rb gene in GBC was low (3 of 15, 20%), the finding of LOH at the earliest stages, including normal and metaplastic epithelia, suggests that rb mutations, while uncommon, may play an important role in the pathogenesis of a subset of GBC.

There are few reports about the incidence of ras mutations in GBC, and they are limited to the K-ras gene. The reported incidences in the United States and Japan vary from 0–39% (5–7). Our finding of ras point mutations (K- and N-ras genes) only in less differentiated areas of 2 of 21 (10%) gallbladder adenocarcinomas indicates that they are rare and late events, probably related to tumor progression.

GBC, as with several other epithelial tumors of adults, are accompanied by histologically recognizable preneoplastic changes. The most frequent of the associated changes, dysplasia and CIS, are believed to be premalignant (3). Another histological change frequently found in chronic cholecystitis is metaplasia of the pyloric or intestinal type (3). Although the malignant potential of metaplastic lesions has not been determined, the finding of mutations (LOH at p53, 9p, and DCC) (100%), and 13q (rb) (100%). Because in GBC there is a close morphological relationship between invasive carcinoma and its associated preneoplastic lesions, the possibility that the preneoplastic lesions are closely related to the corresponding invasive cancer cannot be excluded. However, the finding of identical patterns of allele loss at all stages of carcinogenesis, including normal epithelium, indicates either: (a) a single clone of mutant cells populated much of the gallbladder epithelium and progressed through the various stages to invasive cancer; or (b) multifocal and multilobular lesions were present, but that allele loss was directed. This phenomenon, which we have termed ASM, has been described previously by us and others in the lung and breast (8, 22, 23). Thus, ASM may represent a highly directed phenomenon, possibly related to differential methylation, heterozygosity of fragile sites, or some form of genomic imprinting (8). In summary, we have demonstrated that allele-specific deletions of the p53 and DCC genes and of chromosome 9p play an important and early role in the pathogenesis of endemic GBC in Chile.

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## References

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