Mitochondrial DNA Mutation at the D310 (Displacement Loop) Mononucleotide Sequence in the Pathogenesis of Gallbladder Carcinoma

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ABSTRACT

Purpose: Mutations in the mitochondrial DNA (mtDNA) have been observed frequently in human neoplasia, in both coding and noncoding regions. A mononucleotide repeat (poly-C) between 303 and 315 nucleotides (D310) within the regulatory displacement loop has been identified recently as a frequent hot spot of deletion/insertion mutations in tumors. We investigated the frequency and pattern of D310 abnormalities in the pathogenesis of gallbladder carcinoma (GBC).

Experimental Design: DNA extracted from neoplastic and nonneoplastic archival gallbladder tissue including 123 tumors, 53 dysplastic areas, and 90 histologically normal epithelia adjacent to GBC, chronic cholecystitis, and 15 normal gallbladders were examined by PCR-based assay for D310 mutations, followed by sequencing in a subset of cases.

Results: D310 mutation was a relatively frequent (47 of 123; 38%) abnormality in GBC. A very high frequency of mutations were detected in dysplastic (8 of 14; 57%) and normal-appearing gallbladder epithelia (10 of 22; 46%) accompanying GBC, showing a clonal relationship compared with the corresponding tumors. D310 mutations were also detected in dysplastic (8 of 39; 21%) and normal (17 of 68; 25%) epithelia obtained from chronic cholecystitis. A single case of 15 normal gallbladders showed a D310 abnormality. Overall, deletions (67 of 91; 74%) at D310 were more frequent than insertions.

Conclusions: D310 mutation at the mtDNA displacement loop is a relatively frequent and early event in the sequential pathogenesis of GBC, being detected in normal-appearing epithelium from chronic cholecystitis. Our findings suggest that mtDNA mutations should be additionally investigated in GBC pathogenesis, and D310 mononucleotide abnormalities could be included in a panel of molecular biomarkers for GBC early detection strategy.

INTRODUCTION

Gallbladder carcinoma (GBC) is a relatively uncommon neoplasm that shows considerable geographic variation in incidence (1). In Chile, this neoplasm is the leading cause of cancer deaths in females, and there has been a striking and consistent increase in incidence during the last few decades (2). GBC is a highly malignant neoplasm, usually diagnosed at advanced stages of the disease (1). As with other epithelial neoplasms, GBC is preceded by preneoplastic lesions, including dysplastic gallbladder epithelia (3). Although GBC has been associated with genetic and environmental risk factors, there is limited information about the molecular abnormalities involved in its pathogenesis (4).

Human mitochondrial DNA (mtDNA) is composed of a 16.6-kb, double-stranded, closed-circular DNA molecule (5). Many common polymorphisms have been described in the mtDNA, and most of them accumulate in the regulatory region or displacement loop (D-loop; Ref. 6). Human mtDNA has a mutational rate that is at least 10 times higher than nuclear DNA (7), and it has been reported that mutations accumulate with age in some systems, such as brain and skeletal muscle (8). In recent years, somatic mutations in the mtDNA have also been observed in human neoplasms (9–21). In studies that extensively analyzed the mitochondrial genome using direct sequencing, more than half of the tumors showed mtDNA abnormalities, mostly base substitutions and deletion/insertion mutations in both coding and noncoding regions (9–20). A mononucleotide repeat between 303 and 315 nucleotides (D310) has been identified recently as a frequent hot-spot of deletion/insertion mutations in tumors (18). This homopolymeric C-stretch (CCCCCCC..TC-CCCCC) is part of the conserved sequence block II located within the regulatory D-loop region and involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mtDNA heavy-strand replication (22).

In our series of studies investigating the molecular pathogenesis of GBC, we have found recently that mutations affecting the tumor suppressor gene TP53 occur as a frequent (67%) and early event in the pathogenesis of GBC. The consistent mutational pattern (mostly C to T transitions) of TP53 detected in our GBCs suggests that the lithiasis-related inflammatory process of the gallbladder may play an important role in the TP53 muta-
tions in this neoplasm. Because it is generally accepted that mtDNA mutations are generated during oxidative phosphorylation through pathways involving reactive oxygen species, we hypothesized that mtDNA mutations should be detected frequently in GBC pathogenesis. Thus, we investigated the frequency and pattern of D310 mononucleotide sequence abnormalities, as an expression of mtDNA somatic damage, in the sequential pathogenesis of GBC by examining neoplastic and non-neoplastic archival gallbladder tissue from 123 tumors, 53 dysplasias, 90 histologically normal epithelia, and 15 normal gallbladders.

MATERIALS AND METHODS

Archival Tumor Specimens. Formalin-fixed, paraffin-embedded material from 123 surgically resected primary invasive GBCs was obtained from cholecystectomy specimens resected between 1990 and 1998 at the Catholic University Medical School Hospital and Hospital Dr. Sotero del Rio (Santiago, Chile), as part of an Institutional Review Board approved study. The patients consisted of 83 women and 40 men ranging in age from 45–83 years (mean age, 58 years). Twenty-eight (23%) were well differentiated, 42 (34%) were moderately differentiated, and 53 (43%) were poorly differentiated tubulo-papillary adenocarcinomas. The majority of the tumors were advanced GBCs (101 cases; 82%) with invasion of the gallbladder serosa; the remaining were early GBCs, with invasion of the submucosa (13 cases; 11%) or muscularis propria (9 cases; 7%) of the gallbladder.

Gallbladder Normal Epithelium and Preneoplastic Lesions. Thirty-six discrete normal-appearing and dysplastic gallbladder epithelia were identified adjacent to GBCs, each consisting of at least 1000 cells. These included 22 normal-appearing epithelia and 14 dysplasias. Normal and dysplastic epithelia from 107 gallbladder specimens with chronic cholecystitis and without carcinoma were also selected. These included 39 gallbladder specimens with dysplasia, and 68 with only normal epithelia. In the dysplastic and nondysplastic cases, the whole gallbladder specimen was histologically examined to rule out the presence of invasive carcinoma and dysplasia, respectively. The dysplastic lesions were scored using published criteria for their histopathological identification in the gallbladder epithelium (3). In addition, 15 normal gallbladder specimens were obtained, and their normal epithelium was examined. Representative examples of histology for each category are
show in Fig. 1A. Microdissected normal stromal cells and inflammatory cells from the same slide were used as a source of constitutional mtDNA for each case.

Microdissection and DNA Extraction. Five-μm sections were cut from archival, formalin-fixed, paraffin-embedded tissue. Microdissection and DNA extraction were performed as described previously from non-cover-slipped H&E-stained slides (23). Precisely identified areas of stromal cells, invasive carcinoma, and epithelia from gallbladder specimens were microdissected under microscopic visualization.

Genotyping Assay of the D310 Repeat. Five μl of DNA extraction buffer containing at least 200 microdissected nuclei were used to amplify the D310 repeat from paired normal stromal cells and tumor/epithelial samples. The sequence for the forward primer was 5′-ACAATTTGACGTCTGACAGCCACTT-3′ and for the reverse primer 5′-GGCAAGATTTGTTAAGTGCTG-3′ (18). A two-round PCR strategy was used to amplify D310 repeat. The PCR conditions were as described previously (18), and in both reaction exactly the same conditions were used. The product from the first PCR was diluted 1:10 to be used as a template for the second reaction. The second PCR product was labeled using [32P]dCTP electrophoresed on a 6% denaturing polyacrylamide gel and subjected to autoradiography. D310 mutations were scored by visual detection of shifted band in radiography (Fig. 1). To circumvent the possibility of artificial D310 shifted bands occurring in DNA extracted from paraffin-embedded tissue, the assay was repeated for all of the paired normal stromal cells and tumor/epithelial samples that showed D310 alterations. In addition, DNA quality of the samples examined was tested amplifying at least three microsatellite markers in other studies performed in our laboratory. To confirm D310 mutations, in a subset (30 of 91 D310 alterations; 33%) of tumor (n = 15) and epithelial samples (n = 15) exhibiting D310 mutations in radiographs both strands were manually sequenced (Fig. 1C). Briefly, the PCR product was reamplified, visualized on a 2% agarose gel, and purified. Sequence analysis was performed manually using the USB Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB, Cleveland, OH) using the same primers as for the PCR amplification. Sequencing reactions were analyzed on 8% denaturing polyacrylamide gel electrophoresis.

Data Analysis. Statistical analysis was performed using nonparametric Wilcoxon and χ2 tests. The cumulative binomial test was used to examine the likelihood that the occurrence of a particular event (the same D310 sequence mutation pattern in carcinoma and an associated epithelial sample) occurs at a particular probability when observed in repeated trials. When the results are compared with a chance occurrence or nonoccurrence, the particular probability of comparison is 0.5. Probability values of P < 0.05 were regarded as statistically significant.

RESULTS

D310 Mutation Frequency. We detected D310 repeat mutations in the tumor compared with normal stromal cells in 38% (47 of 123) of GBC (Table 1). A progression in the frequency of D310 mutations according to increasing histopathological grade was observed. One of 15 (7%) normal gallbladders and approximately one-fourth of normal (17 of 68; 25%) and dysplastic (8 of 39; 21%) epithelia from chronic cholecystitis demonstrated D310 mutations. Statistically significant differences were detected comparing normal gallbladders versus chronic cholecystitis (P = 0.009) and chronic cholecystitis versus samples obtained from cancer specimens (normal epithelium, dysplasia, and invasive tumors; P = 0.002; Table 1). Normal-appearing epithelia (10 of 22; 46%) and dysplasias (8 of 14; 57%) accompanying GBC demonstrated higher frequency of mutations than the same histology obtained from chronic cholecystitis; however, they were obtained only from tumors having D310 abnormalities. No correlation between D310 deletion/insertion mutation changes and clinicopathological data in GBC, dysplastic, and chronic cholecystitis specimens was detected.

D310 Mutation Pattern. Radiographic film examination indicated that in gallbladder specimens, base deletions (73%) of the D310 repeat were much more frequently detected than insertions (27%; Table 1). To confirm D310 deletion/insertion mutations detected by visual examination of radiographic films, a subset of tumors (n = 15) and epithelial samples (n = 15) showing repeat changes were sequenced, including their corresponding stromal cells (normal control; Fig. 1). This analysis confirmed all of the deletion/insertion mutations detected by examination of radiographic films. Also, sequencing analysis

Table 1. Somatic deletions/insertions mutations in the D310 repeat in the sequential pathogenesis of gallbladder carcinoma (GBC).

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of cases</th>
<th>No. of alterations</th>
<th>Deletions</th>
<th>Insertions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−1 bp</td>
<td>&gt;−1 bp</td>
</tr>
<tr>
<td>Normal gallbladder</td>
<td>15</td>
<td>1 (7%)</td>
<td>1 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Chronic cholecystitis</td>
<td>107</td>
<td>25 (23%)</td>
<td>14 (74%)</td>
<td>5 (26%)</td>
</tr>
<tr>
<td>Normal epithelium</td>
<td>68</td>
<td>17 (25%)</td>
<td>10 (71%)</td>
<td>4 (29%)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>39</td>
<td>8 (21%)</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Cancer specimens a</td>
<td>159</td>
<td>65 (41%)</td>
<td>31 (67%)</td>
<td>15 (33%)</td>
</tr>
<tr>
<td>Normal-appearing epithelium c</td>
<td>22</td>
<td>10 (46%)</td>
<td>4 (57%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td>Dysplasia c</td>
<td>14</td>
<td>8 (57%)</td>
<td>5 (71%)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>123</td>
<td>47 (38%)</td>
<td>22 (66%)</td>
<td>10 (31%)</td>
</tr>
<tr>
<td>Total</td>
<td>281</td>
<td>91 (32%)</td>
<td>46 (70%)</td>
<td>20 (30%)</td>
</tr>
</tbody>
</table>

a Statistical significant differences: normal gallbladder vs. chronic cholecystitis, P = 0.009 and vs. invasive carcinoma, P = 0.01; chronic cholecystitis vs. cancer specimens, P = 0.002 and vs. invasive carcinoma, P = 0.01.

b Sum of samples obtained from GBC specimens, including normal epithelium, dysplasia and invasive carcinoma.

c Normal and dysplastic epithelium accompanying GBC were obtained only from tumors with the D310 mutation.
confirmed in all of the cases the designation of single (−1 or +1) or multiple (≥ −1 or +1) bp alterations based on radiographs visualization. Overall, single bp deletions were far more frequently found than multiple bp changes (Table 1). In addition, in two GBCs sequenced, a T to C point mutation was detected in the D310 sequence leading to a polycytidylic acid tract not interrupted by T.

**D310 Mutation and Microsatellite Instability Data in GBC.** Because 24 GBCs tested for D310 insertion/deletion mutations have been examined previously for allele loss and microsatellite instability at chromosomes 3p, 8p, 9q, and 22q using 81 polymorphic markers (24), we correlate on those tumors D310 mutation and microsatellite instability data. Ten of those 24 (42%) GBCs demonstrated the D310 mutation. Although GBCs with D310 mutation demonstrated higher microsatellite instability index (number of microsatellite instability divided by number of marker tested) compared with tumors without mutation (mean index 0.11 versus 0.07, respectively), no significant differences were detected (Wilcoxon test, P = 0.14).

**D310 Mutation as Clonal Marker in GBC Pathogenesis.** Twenty-two normal-appearing epithelia and 14 dysplasias accompanying 26 GBCs with D310 repeat abnormalities were examined (Fig. 2). In 10 GBCs both types of samples (normal-appearing epithelium and dysplasia) were obtained. Analysis of the D310 abnormality pattern in GBCs and their corresponding normal and dysplastic epithelia showed two main findings (Fig. 2): (a) in all cases in which epithelial samples demonstrated D310 alterations, those were also present in the corresponding invasive tumor; and (b) in 19 of 22 (86%) comparisons identical D310 change was detected in epithelial samples and their corresponding GBCs. The possibility that this occurred by chance alone is remote as tested by the cumulative binomial test (cumulative binomial test, P = 2.4 × 10⁻⁴). Only 3 normal-appearing epithelia demonstrated a different pattern of D310 repeat alteration compared with their corresponding GBCs.

**DISCUSSION**

Our data indicate that D310 mononucleotide repeats in mtDNA are a hot spot (38%) for somatic deletion/insertion mutations in GBC. Whereas those abnormalities have been described as frequent events in a number of human primary tumors, including head and neck (37%), breast (29%), and colorectal carcinomas (28%; Ref. 18), to date they have not been reported previously in gallbladder cancer. In addition, our findings indicate that D310 repeat alteration is an early event in the sequential pathogenesis of GBC, being detected in preneoplastic lesions (dysplasia) and normal-appearing epithelium accompanying invasive GBC, and in gallbladder specimens with chronic inflammation. The increase of the deletion/insertion frequency of the D310 repeat from 7% in normal gallbladders, to 21% in normal and dysplastic epithelia from gallbladders with chronic inflammation, and 38% in invasive GBCs indicated that this abnormality is associated with neoplastic transformation of gallbladder epithelium. At least three possibilities could be proposed to explain such an increase: (a) selective growth advantage; (b) defective cellular maintenance mechanism for DNA integrity; and (c) an increased chance due to clonal selection of a particular cell (25). The significance of a mutation in the D310 repeat located at D-loop of the mtDNA has not yet been elucidated. The D-loop functions as a promoter for both the heavy and light strands of the mtDNA, but does not encode any functional proteins (5). As with other tumors (18), most of the alterations in D310 we detected in GBC were 1-bp deletion/insertions, which meant that they were in the polymorphic length range (between 7-C and 9-C; Ref. 6). These observations suggest that most D310 variants in tumors are unlikely to lead to a functional impairment of the mitochondria.

Similar frequencies of deletions and insertions at D310 region have been reported in other tumors (18). However, the gallbladder malignant and nonmalignant specimens demonstrated a significantly higher frequency of D310 deletions (73%) than insertions. Our gallbladder specimens showed similar higher rates (73%) of 1-bp changes compared with other tumor types (18) and head and neck preneoplastic lesions (26). Our finding of 2 GBC cases harboring a T to C transition at the D310 sequence leading to a polycytidylic acid tract not interrupted by T have been reported previously in colon cancer cells (27). On the other hand, although a study on gastric carcinoma linked mtDNA somatic mutations to nuclear genome instability (28), this association has not been detected in breast cancer (13) and in our GBCs, giving additional support to the hypothesis that different systems are responsible for mitochondrial and nuclear genomic instability in tumor cells.

Similar to the deletions/insertions measured by microsatellite analysis, the D310 deletions/insertions appear to be good markers to determine clonal origin of epithelial lesions (25). Our analysis of precisely microdissected normal and dysplastic epithelia accompanying GBC indicated that in all of the cases in which epithelial samples demonstrated D310 alterations, those were also present in the corresponding tumor, and in 86% of comparisons identical D310 change was detected in epithelial...
samples and corresponding GBC. These data support the concept of D310 changes as a reliable marker for clonal assessment in malignant transformation of gallbladder epithelium. We established recently (24) that based on microsatellite allele loss and instability patterns most (84%) normal and dysplastic gallbladder epithelia accompanying GBC arise as independent clones. Thus, we suggest that D310 mutations represent a clonal selection occurring at very early stages of gallbladder epithelium proliferation. The similar frequency of D310 deletions/insertions detected in the present study in normal and dysplastic epithelia from both malignant (GBC) and nonmalignant (chronic cholecystitis) gallbladder specimens also support this hypothesis. The higher frequency of D310 mutations detected in GBCs may indicate later waves of clonal expansion associated with tumor development and progression.

Mitochondrial DNA deletions/insertions and base changes mainly involve purine transitions, which have been assumed to occur after the action of reactive oxygen species (29). Thus far, renal (30), breast (31), and colon cancer cells (10) have been reported to show a variable spectrum of reactive oxygen species-induced mutations. The present findings of frequent mutations in the mtDNA and our unpublished data of very frequent T to C transitions in *TP53* during the sequential pathogenesis of GBC suggest that both types of mutations are generated during oxidative phosphorylation through pathways involving reactive oxygen species. Mambo et al. (32) demonstrated recently that the D310 region is highly susceptible to mutations induced by exposure to the oxidant agent tert-butyl hydroperoxide. These findings may explain the high frequency of homoplasmic D310 somatic mutations in many tumor types.

Our findings indicate that D310 insertions/deletions at the mtDNA D-loop are a relatively frequent and early event in the sequential pathogenesis of GBC, appearing in chronic cholecystitis stage. Worldwide, gallstones and chronic cholecystitis are established risk factors for gallbladder cancer (1). Because only a small fraction of patients with cholelithiasis and chronic cholecystitis stage. Worldwide, gallstones and chronic cholecystitis are established risk factors for gallbladder cancer (1). Because only a small fraction of patients with cholelithiasis and chronic cholecystitis develop gallbladder cancer (1) it is important to identify the factors that induce malignant progression. The development of epithelial cancer, including GBC (4, 24, 33–35), requires multiple mutations. It is possible that those preneoplastic lesions that have accumulated multiple mutations are at higher risk for progression to invasive cancer. Our finding of relatively frequent (24%) mutation at the D310 sequence in chronic cholecystitis without cancer supports the hypothesis that a subset of gallbladders with chronic inflammation may be at greater risk of progression to cancer. Of interest, in our previous studies similar frequencies of allele loss and genetic instability (24), gene aberrant methylation (35), and *TP53* gene abnormalities have been detected in chronic cholecystitis specimens.

All of the epidemiological studies have shown that gallstones are the primary risk factor for GBC (reviewed in Ref. 1). Because primary prevention of GBC is not expected in the near future, secondary prevention oriented to surgical treatment of gallstones by laparoscopy cholecystectomy needs to be emphasized in endemic areas. However, the high prevalence of cholelithiasis in those areas and the elevated cost of the surgical procedures suggest that novel approaches for GBC early detection are also needed. D310 sequence mutations and other mtDNA changes have been successfully detected recently in bodily fluid samples obtained from cancer patients (11, 36), including plasma in hepatocellular cancers (37) and serum in colon carcinomas (38). Our finding of 38% of GBC demonstrating D310 sequence mutations suggests that this may be a potentially useful marker for GBC early detection, especially if is included in a panel of serum biomarkers containing, among others, *TP53* mutations, genetic instability, and gene abnormal methylation.

**ACKNOWLEDGMENTS**

We thank Dr. Anirban Maitra, Department of Pathology, Johns Hopkins University School of Medicine (Baltimore, MD) for critical review of the manuscript.

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