Genome-wide Allelotyping Analysis Reveals Multiple Sites of Allelic Loss in Gallbladder Carcinoma


Departments of Anatomic Pathology (I. W., M. T., H. A., P. T.) and Surgery (F. P.), P. Universidad Catolica de Chile, Santiago, Chile, and Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas, Southwestern Medical Center, Dallas, Texas 75390 [A. M., A. F. G.]

ABSTRACT
Although gallbladder carcinoma (GBC) is a highly malignant neoplasm, there is very limited information about the molecular changes involved in its pathogenesis. To identify the chromosomal locations of putative tumor suppressor genes involved in the pathogenesis of GBC, we conducted a genome-wide allelotyping or loss of heterozygosity (LOH) analysis of GBCs. Microdissected tissue from 24 archival GBCs and their matched control DNAs were analyzed for PCR-based LOH using 169 microsatellite markers spanning all nonacrocentric autosomal arms and the X chromosome. The chromosomal arms with the greatest frequencies of LOH (≥60%) were 3p, 6q, 7q, 8p, 9p, 9q, 11q, 12q, 17p, 18q, 19p, 22q, and Xq. The average fractional allele loss index in GBC cases was high (0.43) and frequent breakpoints were detected in gallbladder tumors. Of interest, 21 different regions of frequent LOH (hot spots) defined as ≥50% for individual GBC samples were detected in this neoplasm, nearly half of them confined to one microsatellite marker. We conclude that in GBC at least 21 chromosomal regions with frequent allelic losses are involved, suggesting that several putative tumor suppressor genes are inactivated in its pathogenesis. Overall, these data provide global estimates of the extent of genetic changes leading to GBC and will be useful for the identification of new tumor suppressor genes and for multiple new markers for translational research.

INTRODUCTION

GBC is a relatively uncommon neoplasm which demonstrates considerable geographic and gender variation in incidence (1). Mortality rates are highest among American Indian women from the southwestern United States and Chilean and Japanese women (2). It is one of the most frequent neoplasms in Chile, where it is the leading cause of cancer deaths in females (3, 4). GBC is a highly malignant neoplasm with poor prognosis, and most cases are diagnosed at an advanced stage (5). Although GBC has been associated with genetic and environmental risk factors, there is very limited information about the molecular changes involved in its pathogenesis (6). Most studies have focused on mutations of the dominant oncogene K-ras and the tumor suppressor genes TP53 and p16INK4/CDKN2. Recently, cytogenetic analysis of short-term cultured GBCs demonstrated several nonrandom chromosomal aberrations and imbalances in this neoplasm (7). The delineation of genetic alterations that occur in GBC may be important for the development of molecular markers for early detection and the prediction of response to chemotherapy, as well as for the development of gene therapy strategies.

It is now well recognized that tumorigenesis is a multistep process resulting from the accumulation of sequential genetic alterations (8).

MATERIALS AND METHODS

Archival Tumor Specimens. Paraffin-embedded material from 24 surgically resected primary GBCs were analyzed. They were obtained from cases resected by cholecystectomy between 1990 and 1998 at the Catholic University Medical School Hospital, Santiago, Chile. The patients consisted of 19 women and 5 men ranging in age from 51 to 85 years (mean age, 68 years). The cases for study were randomly selected from those in which sufficient tumor tissue was available for microdissection and analysis of multiple markers. All tumors were adenocarcinomas, and they consisted of 13 (54%) tubular adenocarcinomas, 5 (21%) papillary adenocarcinomas, 5 (21%) tubulopapillary adenocarcinomas, and 1 (4%) adenosquamous carcinoma. They consisted of 6 (25%) well-differentiated, 11 (46%) moderately differentiated, and 7 (29%) poorly differentiated adenocarcinomas. Most of the tumors were advanced GBCs (20 of 24 cases, 83%) with invasion of the gallbladder serosa; the rest of the cases (4 of 24 cases, 17%) were early GBCs, with invasion of the submucosa (1 case, 4%) and muscularis propria (3 cases, 12%) of the gallbladder. The demographic and pathological features of the 24 GBC cases studied are similar to the cases diagnosed in our institution over the 1990–1998 period. Serial 5-μm sections were cut from archival, Formalin-fixed, paraffin-embedded tissues. All slides were stained with H&E, and one of the slides was coverslipped. The coverslipped slide was used as guide to localize regions of interest for microdissection of the other slides.

Microdissection and DNA Extraction. Microdissection from archival paraffin-embedded tissues was precisely performed under microscopic visualization using a micromanipulator, as described previously (12). From multiple sections of each case, 4000–5000 sectioned tumor cells were microdissected. A representative example of microdissection of a GBC specimen is shown in Fig. 1. DNA extraction was performed as described previously (12). Dissected lymphocytes or normal stromal cells from the same slide were used as a source of constitutional DNA from each case. After DNA extraction, 5 μl of the proteinase K-digested samples containing DNA from at least 200 cells were used for each multiplex PCR.

Microsatellite DNA Markers and PCR-LOH Analysis. To perform a genome-wide allelotyping study, we used 182 microsatellite markers spanning all 39 nonacrocentric autosomal arms and both arms of chromosome X. One hundred sixty-nine (93%) of the microsatellite markers amplified satisfactorily in all GBC specimens studied. The microsatellite markers tested are shown in

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1 Supported by Grant Fondo Nacional de Desarrollo Cientifico y Tecnologico 1990489 to I. W.
2 To whom requests for reprints should be addressed, at Department of Anatomic Pathology, P. Universidad Catolica de Chile, Maroquete 367, P.O. Box 114-D, Santiago, Chile. Phone: (56-2) 686-3209; Fax: (56-2) 639-5871; E-mail: iwistuba@med.puc.cl.

The abbreviations used are: GBC, gallbladder carcinoma; TSG, tumor suppressor gene; LOH, loss of heterozygosity; FAL, fractional allelic loss.
Fig. 2. We examined a mean of 4.7 microsatellite markers per chromosomal arm (range, 1–10). The markers were selected from the Genome Database (http://www.gdb.org/). Because we used DNA extracted from archival paraffin-embedded tissues, the primers selected amplify fragments no larger than 256 bp. A two-round PCR strategy (multiplex PCR followed by uniplex PCR) was used to amplify each marker, as described previously (12). We optimized the PCR conditions so as to perform 23 multiplex PCRs containing all 169 markers used in this study. Each multiplex PCR contained six to eight microsatellite markers. The list of multiplex sets and specific optimization conditions we used are available upon request. A 10°C “touch-down” PCR strategy (13) was used spanning the primers’ annealing temperature followed by 25 cycles at the optimal annealing temperature. The final product was separated on a 6% denaturing polyacrylamide gel and subjected to autoradiography. LOH was scored by visual detection of complete absence of one allele of informative cases (Fig. 3).

Data Analysis. The data were analyzed using a series of Microsoft Visual Basic programs specifically written for various computations or repetitive tasks in Microsoft Excel, including LOH frequencies, FAL index, breakpoint frequencies, color-coded formatting of LOH patterns, and clustering analysis, as described previously (14).
RESULTS

LOH Analysis of GBC Reveals Frequent Deletions throughout the Genome. Overall, the average marker informative (i.e., heterozygosity rate) as determined from normal tissues was 63%. This heterozygosity rate is very similar to the mean heterozygosity rate expected for all markers examined. The average percentage of heterozygosity rate for normal tissues was 63%. This heterozygosity rate is very similar to the mean heterozygosity rate expected for all markers examined, indicating that our archival tissues were suitable for this type of molecular analysis. To increase the efficiency of the allelotyping study performed from microdissected material, a multiplex PCR strategy for LOH analysis was used (12). Thus, all 169 microsatellite markers were combined in 23 multiplex PCRs containing 6–8 markers. Multiplex PCR is a useful strategy for allelotyping analysis of archival invasive tumors or precursor lesions in which there are not tumor cell lines or fresh tumor specimens readily available (17–21).

Our allelotyping analysis revealed that GBC demonstrated a very high incidence of deletions affecting multiple chromosomal loci. We identified the smallest regions of frequent allelic loss, so-called “hot spots.” Twenty-one such regions were found and are listed in Table 1 along with other human tumors in which those regions have been frequently associated, and we list the most important potential candidate genes located within these regions. Of those, 11 regions of loss were confined to 1 microsatellite marker and the others consisted of 2–5 markers (Fig. 2).

Breakpoint Frequencies and Clustering Analysis in GBC. To determine the chromosomal regions with very frequent breakpoints in the tumors, we calculated the frequencies of breakpoints on the chromosomal arms demonstrating sites of frequent loss. Breakpoints were defined as the junction between a marker showing LOH and an adjacent marker retaining heterozygosity in a given tumor sample DNA. The highest percentages of breakpoints (≥30%) were found at 1q12-31, 3p22-25, 2p22-25, 2q33-37, 4p16, 5q11-13, 5q23-32, 6q13, 7p15-21, 7q32-34, 8p24, 8q21-22, 9q31-33, 10q24, 11q12-13, 11q14, 11q24-25, 17p13, 17q21, and 19p13. Several of these regions were located near a hot spot of allelic loss. For individual tumors, the number of breakpoints was 33 ± 6 (range, 23–41). Cluster analysis to examine if allelic loss at one chromosomal region was linked to changes at another region (14) did not reveal any concordance between markers from different chromosomes.

DISCUSSION

In this report, we performed an intermediate-density genome-wide allelotyping analysis of GBC using 169 microsatellite markers (average of 4.7 markers at each chromosomal arm) spanning all nonacrocentric autosomal arms and X chromosome. Because archival Formalin-fixed and paraffin-embedded GBC specimens were studied, a precise microdissection technique of histological-sectioned tumor samples was used. Dissected lymphocytes or stromal cells from the same histological slide were used as a source of constitutional DNA from each case. Overall, the average marker heterozygosity rate for normal tissues was 63%. This heterozygosity rate is very similar to the mean heterozygosity rate expected for all markers examined, indicating that our archival tissues were suitable for this type of molecular analysis. To increase the efficiency of the allelotyping study performed from microdissected material, a multiplex PCR strategy for LOH analysis was used (12). Thus, all 169 microsatellite markers were combined in 23 multiplex PCRs containing 6–8 markers. Multiplex PCR is a useful strategy for allelotyping analysis of archival invasive tumors or precursor lesions in which there are not tumor cell lines or fresh tumor specimens readily available (17–21).

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genes at 8p22, have been proposed as candidate TSGs in this region (37–39). The frequent losses at chromosome regions 9p21-23 and 17p12-13 correlate with previously reported findings of TP53 (17p13) (reviewed in Ref. 6) and CDKN2; p16 Ink4 /CDKN2; p15 INK4b /CDKN2 (10, 40) gene abnormalities in this neoplasm. On the other hand, we detected relatively low incidences of LOH at the 5q11-23 (APC), 13q14 (RB gene), and 18q22 (DCC gene) regions compared to other reports (10, 11), although we had previously reported a low incidence of loss at 5q (10).

The most prominent novel hot spots in GBC that we detected were 1p34-36 (70%), 3q13 (67%), 4p16 (58%), 6q21 (73%), 7q32 (71%), 7q36 (62%), 9q22-31 (60%), 9q34 (67%), 11q13-14 (50%), 12q21-23 (60%), 18q12 (64%), 19p12-13 (86%), 21q11-20 (55%), Xp11 (54%), and Xq4 (82%). As shown in Table 1, all but one (3q13) of those regions has been previously involved in the development of one or more human tumors and most of them harbor putative and known TSGs. Recently, Gorunoeva et al. (7) reported a cytogenetic analysis of short-term cultured GBCs which demonstrated several nonrandom chromosomal aberrations and imbalances in this neoplasm. They described 15 chromosomal arms having frequent partial or whole- arm losses (3p, 4q, 5q, 9p, 10p, 10q, 11p, 14p, 14q, 15p, 17p 19p, 21q, and Xq). However, few of those arms showed a very high frequency of deletions (≥50%) and contain regions of frequent LOH (≥50%; hot spots) in the present study. Future investigations, using higher resolution of microsatellite markers will be crucial to narrow down these regions of frequent deletions in GBC, combined with mutation analysis of putative or known TSGs in these regions will be necessary to further understand the multistage pathogenesis of GBC. On average, nine regions of high LOH frequency (hot spots) were found in each GBC specimen, giving an indication of the possible number of TSGs inactivated in each GBC. Some of the allelic losses may be caused by genomic instability or the presence of fragile sites (41). In fact, many (n = 19) breakpoints were identified that were recurrent in a significant fraction (≥30%) of tumors. Several of those sites (4p16, 7q32-34, 8p24, 9q31-33, 11q12-13, 11q14, 17p13, and 19p13) are located near a hot spot of frequent allelic loss and some of these losses may therefore be the consequence of recurrent alterations

cated on several chromosomal arms. On average, 39% of markers showed loss in individual tumors and 13 chromosomal arms demonstrated a very high (≥60%) incidence of LOH. In addition, GBC cases demonstrated a relatively high LOH frequency expressed by the FAL index by cases (average, 0.43; range, 0.25–0.55). Similar high FAL indices have been demonstrated in genome-wide allelotyping analyses in other malignant tumors (14–16, 22–26).

Several sites of frequent allele loss were detected in this study. The location of these sites was based on the determination of the minimal regions of loss which are defined by the occurrence of “breakpoints” surrounding regions of frequent LOH. These sites are likely to represent the major TSG regions which are lost in GBC and warrant further investigation. Overall, we found at least 21 different sites of frequent (≥50%) allelic loss (hot spots) in GBC, and the majority (n = 16) of them have not previously been described in this neoplasm.

Only two reports using a small number of microsatellite markers have demonstrated LOH at several chromosomal regions in GBC, including 3p14-21 (20–52%), 5q11-23 (APC-MCC regions, 22–66%), 8p22-23 (44%), 9p21-22 (p16ink4a/CDKN2 region, 50–52%), 13q13-14 (20–33%), 17p11-13 (TP53 region, 58–91%), and 18q22-23 (DCC region, 30–40%) (10, 11). Our present data confirm and extend these findings. In the present study, high incidences of LOH in GBC have been confirmed on chromosomes 3p12-21 (83%), 8p21-23 (60–71%), 9p21-22 (50%), 9p22 (71%), 10p12 (47%), 11q13-14 (50%), 12q21-23 (60%), 13q13-14 (50%), 14p16 (58%), 14q12 (64%), 19p12-13 (86%), 21q11-20 (55%), Xp11 (54%), and Xq4 (82%). As shown in Table 1, all but one (3q13) of those regions has been previously involved in the development of one or more human tumors and most of them harbor putative and known TSGs. Recently, Gorunoeva et al. (7) reported a cytogenetic analysis of short-term cultured GBCs which demonstrated several nonrandom chromosomal aberrations and imbalances in this neoplasm. They described 15 chromosomal arms having frequent partial or whole- arm losses (3p, 4q, 5q, 9p, 10p, 10q, 11p, 14p, 14q, 15p, 17p 19p, 21q, and Xq). However, few of those arms showed a very high frequency of deletions (≥60%) and contain regions of frequent LOH (≥50%; hot spots) in the present study. Future investigations, using higher resolution of microsatellite markers will be crucial to narrow down these regions of frequent deletions in GBC, combined with mutation analysis of putative or known TSGs in these regions will be necessary to further understand the multistage pathogenesis of GBC.

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### Table 1 Chromosomal regions with incidence (≥50%) of LOH in GBC obtained from genome-wide allelotyping analysis

<table>
<thead>
<tr>
<th>Chromosome arm</th>
<th>Flanking markers</th>
<th>GBC region</th>
<th>N</th>
<th>%</th>
<th>Other malignant tumors*</th>
<th>Known or putative TSGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p</td>
<td>1p34-36</td>
<td>1420</td>
<td>70</td>
<td>Melanoma; gliomas; colon, breast, head-neck, and ovarian carcinomas</td>
<td>p73</td>
<td></td>
</tr>
<tr>
<td>3p</td>
<td>3p12-21</td>
<td>1923</td>
<td>83</td>
<td>Mesothelioma; lung, renal, head-neck, breast, and cervical carcinomas</td>
<td>DUTT1; FHIT</td>
<td></td>
</tr>
<tr>
<td>3p</td>
<td>3p22-24</td>
<td>510</td>
<td>50</td>
<td>Lung, breast, and cervical carcinomas</td>
<td>RARB</td>
<td></td>
</tr>
<tr>
<td>3q</td>
<td>3q13</td>
<td>69</td>
<td>67</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>4p</td>
<td>4p16-3</td>
<td>712</td>
<td>58</td>
<td>Urinary bladder, breast and cervical carcinomas</td>
<td>RNF4; SH3BP2</td>
<td></td>
</tr>
<tr>
<td>6q</td>
<td>6q21</td>
<td>811</td>
<td>73</td>
<td>Lymphoma; mesothelioma; melanoma; breast, prostate, and pancreatic carcinomas</td>
<td>APE2, CD24, BLM1P, CCNC</td>
<td></td>
</tr>
<tr>
<td>7q</td>
<td>7q32</td>
<td>1014</td>
<td>71</td>
<td>—</td>
<td>SMOH</td>
<td></td>
</tr>
<tr>
<td>7q</td>
<td>7q36</td>
<td>813</td>
<td>62</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>8p</td>
<td>8q21-23</td>
<td>2424</td>
<td>100</td>
<td>Urinary bladder, lung, head-neck, breast, colon, prostate, and hepatocellular carcinomas</td>
<td>PGDFR β-like, N33; FEZI</td>
<td></td>
</tr>
<tr>
<td>9p</td>
<td>9p21-23</td>
<td>1422</td>
<td>68</td>
<td>Mesothelioma; melanoma; gliomas; thyroid, urinary bladder, lung, breast, head-neck, colon, and pancreatic carcinomas</td>
<td>p16ink4a/CDKN2, p15ink4b/CDKN2</td>
<td></td>
</tr>
<tr>
<td>9q</td>
<td>9q22-23</td>
<td>610</td>
<td>60</td>
<td>Basal cell carcinoma of skin; urinary bladder, breast, lung, and esophageal carcinomas</td>
<td>NBBCS, DECI</td>
<td></td>
</tr>
<tr>
<td>9q</td>
<td>9q34</td>
<td>1421</td>
<td>67</td>
<td>Lymphoma; lung and esophageal carcinomas</td>
<td>TSCI</td>
<td></td>
</tr>
<tr>
<td>10p</td>
<td>10p12</td>
<td>47</td>
<td>57</td>
<td>Glomias; neuroblastoma; sarcomas</td>
<td>CACNB2</td>
<td></td>
</tr>
<tr>
<td>11q</td>
<td>11q13-14</td>
<td>1020</td>
<td>50</td>
<td>Neuroendocrine tumors; head-neck, thyroid, and pancreatic carcinomas</td>
<td>MEN-1</td>
<td></td>
</tr>
<tr>
<td>12q</td>
<td>12q21-23</td>
<td>915</td>
<td>60</td>
<td>Seminoma; salivary gland, head-neck, ovarian, and pancreatic carcinomas</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>17p</td>
<td>17p12-13</td>
<td>1823</td>
<td>78</td>
<td>Most human tumors</td>
<td>TP53</td>
<td></td>
</tr>
<tr>
<td>18q</td>
<td>18q12</td>
<td>711</td>
<td>64</td>
<td>Esophageal, pancreatic, and cervical carcinomas</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>19p</td>
<td>19p12-13</td>
<td>1922</td>
<td>86</td>
<td>Leukemia; lung, cervical, pancreatic, and hepatocellular carcinomas</td>
<td>STK11/LKB1</td>
<td></td>
</tr>
<tr>
<td>22q</td>
<td>22q11-12</td>
<td>1120</td>
<td>55</td>
<td>Glomias; breast, colon, head-neck, and ovarian carcinomas</td>
<td>NF2</td>
<td></td>
</tr>
<tr>
<td>Xp</td>
<td>Xp11-3</td>
<td>713</td>
<td>54</td>
<td>Renal and ovarian carcinomas</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Xq</td>
<td>Xq34</td>
<td>911</td>
<td>82</td>
<td>Breast carcinoma</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Other human tumors in which high frequency of allelic loss at chromosome regions has been reported. —, Unknown.
at common, potentially fragile sites. Of interest, three of the most frequently observed common fragile sites of the human genome (41, 42) showed either frequent allelic losses (3p14.2/2FRA3B and 18q12/2FRA18A) or frequent breaks (breakpoint, 7q32/2FRA7H) in our GBC cases. Although we used the term breakpoint to describe LOH-heterozygous junctions, these abnormalities could occur by physical chromosomal deletion or by other mechanisms such as mitotic recombination (43).

Cluster analysis to search for correlation between the loss or retention of different markers in our GBCs was performed using a method previously published (14). The algorithm that was used is similar to one that has been used for cluster analysis of microarray data (44) and is well adapted to allelotyping studies (14). The cluster analysis performed with the allelotyping data in our study did not reveal any concordance between markers from different chromosomes. It is possible that the analysis of more tumor specimens could provide some concordances between microsatellite markers because it would increase the number of informative data (heterozygosity).

In summary, allelic losses at multiple sites of the genome are frequent in GBC. Our data identified at least 21 chromosomal regions with frequent allele loss in this neoplasm suggesting that multiple putative TSGs are inactivated, most of which remain to be identified. Although our allelotyping analysis was limited in some chromosomal arms, and these data may represent a partial picture of what losses may be occurring on GBC, they provide global estimates of the extent of genetic changes leading to GBC. Our findings may be useful for the positional cloning of new TSGs and for the identification of multiple new markers for translational research.

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