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Adenocarcinomas of the gallbladder from United States patients demonstrate less frequent molecular change for several genetic markers than other intra-abdominal cancers*

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ABSTRACT

Context: The incidence of gallbladder cancer is quite low in the US, with an estimate (2013) for new cases of less than 10,000. The rarity suggests a possible shared molecular pathology that might facilitate a greater understanding of this tumor. Objective: We wished to assess the molecular genetic profile of this tumor, particularly *KRAS* gene mutations, which are frequent in tumors associated with chronic inflamemation elsewhere within the abdomen. Design: We ascertained 25 cases of gallbladder adenocarcinoma from our pathology department records for 2000-2012. PCR based techniques were used to evaluate the DNA for loss of heterozygosity of the *APC* and *DCC* genes; for point mutations in the *KRAS* gene, codons 12 and 13; for point mutation in the *BRAF* gene, codon 600; for point mutation in the *GNAS* gene, codon 201; and for microsatellite instability. Results: Patients included 5 males and 20 females. Approximately three-quarters of cases were associated with gallstones, inflammation and dysplasia. Microsatellite instability and *GNAS* mutation, both present in just 4% of cases, and *BRAF* mutation present in no cases, do not appear to be significant parts of carcinogenesis of gallbladder carcinoma. We detected a *KRAS* gene mutation in only 8% of the cases. Loss of heterozygosity for the *APC* was detected in 16.7% of informative cases; and for the *DCC* gene, in 34.8% of informative cases. Conclusions: Many molecular genetic changes frequently seen with tumors arising from other intra-abdominal organs are *infrequent* in this tumor type. In particular, *KRAS* mutations were uncommon, in contra-distinction to other malignant tumors developing in the setting of chronic inflammation/infection.

Keywords: Gallbladder Carcinoma; Molecular Genetic Changes; *KRAS* Mutation; *GNAS* Mutation; *BRAF* Mutation; Microsatellite Instability; Loss of Heterozygosity

1. INTRODUCTION

The gross appearance and invasive characteristic of carcinoma of the gallbladder were first described in 1777 [1]. Almost two and one-half centuries later, typical features continue to be with late diagnosis, limited treatment options and poor prognosis. The incidence of gallbladder cancer is quite low in the United States, with an estimated number of new cases of gallbladder plus other biliary cancers of just 10,300 for 2013 [2]. Yet, this very uniqueness suggests the possibility of a common pathophysiology and shared molecular pathology that might facilitate a greater understanding of this tumor, and thereby perhaps enhance our knowledge regarding other gastrointestinal and abdominal tumors.

There is a worldwide association between chronic inflammation, or cholelithiasis, and gallbladder cancer, as well as accumulating data to support the relationship of chronic infection with gallbladder cancer [3]. Information about the molecular genetic changes involved in

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gallbladder carcinogenesis is quite limited and variable, and the relationships between chronic infection, inflamemation, pre-cancerous changes, and carcinoma have not been adequately explored.

We have evaluated the surgical, histological and molecular findings in a cohort of 25 cases of gallbladder carcinoma seen at our hospital. We evaluated each cancer for genetic changes that have been reported previously with regard to gallbladder cancer [4-8], as well as for mutations in the *GNAS* gene, seen frequently in appendiceal mucinous neoplasms, an organ, like the gallbladder, may demonstrate inflammation [9]. Specifically, we assayed for loss of heterozygosity for *APC* and *DCC* genes, microsatellite instability, *KRAS*, *BRAF* and *GNAS* common point mutations.

2. METHODS

Clinical material primarily reflects a suburban community of middle economic level, with representations from various minority groups (African-American, Asian) of both middle and low economic status. The computer files of the Department of Pathology were reviewed for the years 2000 to 2011. One clinical pathologist reviewed all histological slides and indicated the areas for molecular study. Inflammation was graded on the basis of infiltrate, fibrosis and activity, according to published guidelines [10]. Dysplasia was characterized according to the criteria of Sasatomi [11]. We also randomly selected cases of acute and chronic cholecystitis from the computer files for the years 2010 through 2011. The study was approved by the hospital Institutional Review Board.

2.1. DNA Extraction and Purification

All tissue specimens were formalin-fixed and paraffin-embedded. Histological slides stained with H&E were examined and the area of relevant tissue was identified and marked, as was an area of normal tissue (**Figure 1**). Paraffin blocks were available for all cases. Consecutive unstained slides from the blocks were prepared and the corresponding areas were isolated under a dissecting microscope by manual dissection. The paraffin wax was removed by xylene and ethanol washes. Cellular material was lysed in a proteinase K buffer solution. DNA was isolated and purified using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). DNA concentration was determined using a DanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2. Microsatellite Analysis for Loss of Heterozygosity and for Microsatellite Instability

In all primer sets the forward primer contained a 5'-fluorescent label and the reverse primer contained a 5'-GTGTCTT tail (Applied Biosystems Custom Oligo Synthesis Service, OligosUS@appliedbiosystems.com). All PCR reactions used Applied Biosystems reagents (Roche Molecular Systems, Inc., Branchburg, NJ) with a final 1.5 mM $MgCl_2$ concentration. Reactions were run on an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 5 minutes denaturation at 94˚C, followed by 35 cycles of a 30 second denaturetion at 94˚C, 30 second annealing at 55˚C, and a 60 second elongation at 72˚C, with a final 30 minute extension at 72˚C. PCR products were separated by capillary electrophoresis with an ABI 3130 Genetic Analyzer, and the data were processed with GeneMapper software (Applied Biosystems, Foster City, CA).

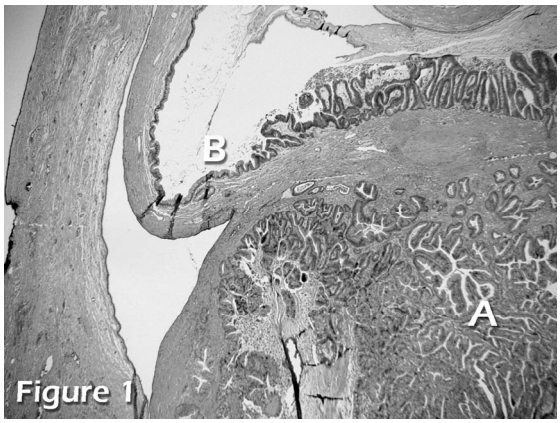

Figure 1. Histological section of gallbladder. A) an area of adenocarcinoma showing packed glandular formation; B) adjacent normal mucosa with uniform thickness and consistent invaginations. 40× magnification, H&E stain.

Loss of heterozygosity of the *APC* gene was determined by amplification of the CA repeat region within the D5S346 locus. Samples homozygous for this locus were analyzed using repeats within the D5S1965 and/or D5S492 loci. Loss of heterozygosity of the *DCC* gene was determined by amplification of the CA repeat markers within the D18S58, D18S61 or D18S1407 loci. Primer sets used as previously reported [12]. For all loss of heterozygosity studies, neoplastic tissue was evaluated simultaneously with normal colonic mucosal tissue from the same patient. Loss of heterozygosity was defined as previously [13].

MSI was detected using the Bethesda panel of markers that includes two mononucleotide markers BAT25 and BAT26, and three dinucleotide markers D2S123, D5S346, and D17S250. A tumor was defined as "microsatellite unstable-high" if two or more of the five markers had a changed allele pattern, and this is referred to as "MSI".

2.3. Sequence Analysis for *KRAS*, *BRAF*, and *GNAS*

The codon 12/13 region in exon 2 of the *KRAS* gene was

amplified using the primer set
5'-AAGGCCTGCTGAAAATGACTG-3' and
5'-GGTCCTGCACCAGTAATATGCA-3'. The codon 600 region in exon 15 of the *BRAF* gene was amplified using the primer set
5'-CATAATGCTTGCTCTGATAGGAAA-3' sense and 5'-GATCCAGACAACTGTTCAAACTG-3'. The codon 201 region in exon 8 of the *GNAS* gene was amplified using the primer set
5'-ACTGTTTCGGTTGGCTTTGGTGA-3' and
5'-AGGGACTGGGGTGAATGTCAAGA-3.

Hot-start PCR was performed in 50 µl volumes with AmpliTaq Gold polymerase and ABI reagents (Applied Biosystems, Foster City, CA) using 100 ng of template DNA, 50 pmols of each primer, and 2.0 mM $MgCl_2$ on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). PCR consisted of an initial 8 minute denaturation at 94˚C, followed by 40 total cycles of a 30 second denaturation at 94˚C, 30 second annealing, and one minute elongation at 72˚C, with a final 30 minute extension at 72˚C. For *KRAS* and *BRAF*, the annealing temperature was stepped down at 62˚C, 60˚C, and 58˚C for 5, 15, and 20 cycles, respectively. For *GNAS* the annealing temperature was stepped down at 65˚C, 63˚C, and 61˚C for 5, 15, and 20 cycles, respectively.

The post-PCR products were quality checked by agarose gel and then purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) prior to sequencing. The sequencing reactions were performed in 20 µl volumes using 0.5X BigDye Terminator Cycle Sequencing Reagents (Applied Biosystems, Foster City, CA), 5.0 pmol of either the reverse *KRAS* primer, the forward *BRAF* primer, or the reverse *GNAS* primer and 1.0 µl of the purified PCR reaction. Reactions were run on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) for 25 cycles using a 2 minute extension time. The sequencing reaction fragments were cleaned using isopropanol precipitation. Sequencing products were separated by capillary electrophoresis with an ABI 3130 Genetic Analyzer and the data was processed with Sequencing Analysis (Applied Biosystems, Foster City, CA) software.

2.4. Statistical Methods

Descriptive statistics were used to characterize the gallbladder cancer cases. We used mean and range to describe continuous variables such as age, and frequency distributions to describe categorical variables.

3. RESULTS

We identified a total of 25 cases of primary cancer of the gallbladder through the computer search. Slides and paraffin blocks were available for all 25 cases. The patients included 5 males and 20 females. The average age for the group was 73.7 years, with a span from 49 to 89 years. The average for males was 74.6 years and 73.5 years for females. Twenty-three of the patients were Caucasians, one female was of Korean ancestry and one female was of Japanese ancestry.

All cancers were adenocarcinomas. Histological findings are summarized in **Table 1**. We attempted to evaluate the case material for the presence of bacteria by staining newly cut slides with the Brown and Brenn Gram stain. No definite clusters of bacteria could be identified in any of the cases, despite extensive histologic areas of acute and chronic inflammation. Cultures at the time of surgery were not possible for these retrospective cases.

Loss of heterozygosity for the *APC* gene was de-

Table 1. Histologic findings in 25 cases of adenocarcinoma of the gallbladder.

Histologic feature	No. (%)
Gallstone	25
present	19 (76)
absent	6 (24)
Differentiation	25
Well differentiated	5 (20)
Moderately differentiated	13 (52)
Poorly differentiated	7 (28)
Inflammatory activity*	25
None	5 (20)
Grade 1	0
Grade 2	8 (32)
Grade 3	12 (48)
Dysplasia†	25
None	8 (32)
Mild	2 (8)
Moderate	4 (16)
Severe	11 (44)
Tumor invasion	25
Nerve	10 (40)
Blood vessel	10 (40)
Lymphatics	10 (40)
Peri-gallbladder lymph node‡	5 (22)

*Inflammation grading based on Barcia. (ref); †Dysplasia grading based on Sasatomi. (ref); ‡Lymph node status unknown for two cancers.

tected in 4 of 24 (16.7%) informative cases, and for the *DCC* gene in 8 of 23 (34.8%) informative cases. Microsatellite instability was found in just one of 25 (4%) tumors. A *KRAS* mutation was present in 2 of 25 (8%) tumors, both were c.35G > A. All tumors were *BRAF* wild type and one tumor (4%) contained a *GNAS* mutation in codon 201 (**Table 2**). We also studied 7 cases of acute cholecystitis and 26 cases of chronic cholecystitis. Gallstones were present in 28 of these 33 (84.8%) cases, and 28 (84.8%) had moderate or severe inflammatory changes. However, none of these 33 cases contained any molecular genetic change of loss of heterozygosity for *APC* or *DCC*, *KRAS*, *BRAF* or *GNAS* mutation, or microsatellite instability. Additionally, no molecular genetic changes were detected in one case each of a gallbladder adenoma, bile duct carcinoma, gallbladder dysplasia, and gallbladder mucinous metaplasia.

4. DISCUSSION

The estimate for new cases of gallbladder cancer for 2013 in the United States is approximately one seventh that of pancreatic carcinoma and one-twentieth that of colorectal cancer [2]. More than 90% of gallbladder cancers are adenocarcinomas. During embryological development, the liver, biliary apparatus and pancreas arise as diverticula from the foregut, suggesting possibly some commonality to the tissues of the gallbladder and pancreas. Two pathways for the development of gallbladder carcinoma have been identified. The first is associated with a specific congenital abnormality of the pancreaticbile duct junction, in which the pancreatic and common bile ducts join together before reaching the duodenal wall, allowing reflux of pancreatic secretions into the gallbladder. This anomaly is particularly common in Japan [14].

Table 2. Molecular findings in 25 cases of adenocarcinoma of the gallbladder.

Molecular change	No. (%)
KRAS mutation	25
Mutated	2 (8)
Wild type	23 (92)
BRAF	25
Mutated	0
Wild type	25 (100)
GNAS	25
Mutated	1 (4)
Wild type	24 (96)
APC	25
Loss of heterozygosity	4 (16)
Not studied	1 (4)
Normal	20 (80)
DCC	25
Loss of heterozygosity	8 (32)
Homozygous	2 (8)
Normal	15 (60)
Microsatellite instability	25
Present	1 (4)
Absent	24 (96)

The more common pathway for the development of gallbladder carcinoma is associated with gallbladder disease, usually in the form of cholelithiasis combined with cholecystitis [3]. Similar to other epithelial cancers, such as colon cancer, a series of pre-malignant changes defined as metaplasia, dysplasia and carcinoma *in situ* has been described for gallbladder carcinoma [15,16]. These precursor changes may be detected in the mucosa adjacent to carcinomas, and they may incidentally be detected in gallbladders removed for cholelithiasis. Gallstones are found with cancer in about 80% of cases, and diffuse calcification of the gallbladder ("porcelain gallbladder") is found in about 15% of cases.

A link has been proposed between chronic infection of the gallbladder with *Salmonella typhi* and gallbladder cancer [17], but whether the link is specific to this organism, and whether the link is specifically modulated by chronic inflammation and specific molecular genetic changes, has not been clarified. Additionally, an association has been reported between *Helicobacter bilis* and gallbladder cancers in 3 of 11 cases, in which bacterial DNA was detected in the cancer tissues [18]. Gallbladder cancer is more common in areas of the world reporting mixed gallstone disease, rather than pure cholesterol stones. The mixed type of gallstone is more likely to originate from a nidus of infection. Our inability to demonstrate the presence of bacteria by histology is consistent with the findings of others [19]. Gallbladder carcinoma is more frequent in females than males in all populations evaluated. The female/male ratio of 4:1 for our cases is slightly higher than most reported ratios. [20].

Information about the molecular genetic changes involved in gallbladder carcinogenesis is quite limited and inconsistent. Most of the reported molecular studies are of cases from Chile and Japan. Gallbladder carcinoma is approximately five times more prevalent in Japan than in the US [20], and findings in Japanese cases may not be representative of cases in the United States. Nagahashi *et*

al studied Japanese and Hungarian cases of gallbladder cancer and reported a *KRAS* mutation in just 1 of 42 (2.4%) tumors [4]. A second Japanese study involving different patients found *KRAS* mutations in 30 of 51 (59%) cases of gallbladder cancer. [5] A study from Chile found only 1 of 21 (4.8%) gallbladder cancers to have a *KRAS* mutation [6]. A more recent study from China detected a *KRAS* gene mutation in just 2 of 75 (2.7%) gallbladder cancers [21]. One study of 29 US cases found 2 (6.9%) with a *KRAS* mutation and none with a *BRAF* mutation [22].

Other molecular changes have been evaluated in gallbladder carcinoma. A point mutation in the *p*53 gene has been demonstrated in 50% or more of the Japanese and Hungarian cases mentioned above [4,23]. Increased expression of *p*53 in 13 of 20 (65%) gallbladder adenocarcinomas was recently reported from Australia [24]. A *BRAF* mutation was found in 7 of 21 (33.3%) gallbladder carcinomas from Greece [8]. A recent study of 30 cases of biliary intraepithelial neoplasia and intrahepatic cholangiocarcinomas found none with a *GNAS* mutation [25].

Nagahashi *et al.* determined microsatellite instability to be present in 8 of 19 (42.1%) Japanese cases and just 1 of 15 (6.7%) Hungarian cases [4]. A 1995 study of 25 gallbladder cancers from Chile found loss of heterozygosity for 5q21 (*APC* and *MCC* genes) in 5 of 23 (21.7%) cases tested; loss of heterozygosity for 9p21 (*CDKN*2 gene) in 4 of 10 (40%) cases, and loss of heterozygosity for 18q21 (*DCC* gene) in 4 of 13 (30.8%) cases [6]. In a study of 32 gallbladder cancers from Korea, 20 (62.5%) demonstrated LOH for chromosome 5q, and 10 (31.2%) demonstrated LOH for 18q [7]. In a more recent study of cases from Chile, using high resolution analysis of 81 microsatellite markers, Wistuba *et. al.* found a frequency of loss of heterozygosity of 88% to 100% for chromosomal regions 3p, 8p, 9q and 22q [26]. They also demonstrated allelic losses in normal and dysplastic gallbladder epithelium. A recent study of cases from northern India, another highly endemic area, suggested that genetic variants in certain germ line DNA repair pathways might be involved in gallbladder carcinogenesis. Odds ratios of about 2.0 were found for variants of the genes *EERCC*2, *MSH*2, and *OSGGI* [27].

There are two associations that might suggest a high frequency of *KRAS* gene mutations in cases of gallbladder carcinoma. First, both the gallbladder and the pancreas arise from a foregut cell lineage, and *KRAS* mutations are quite frequent in pancreatic cancer, reported as occurring in 73 of 79 (92.4%) cases [28]. The second association involves the relationship of gallbladder carcinoma with inflammation. *KRAS* mutations have been found in a high percentage of other intra-abdominal processes associated with inflammation, such as mucinous cystadenomas of the appendix [29] and chronic gastritis associated with *H. pylori* [30]. However, only 3.8% of the intestinal-type gastric cancers related to *H. pylori* contained a *KRAS* mutation [29]. The low incidence of *KRAS* mutation in *H. pylori*-associated gastric cancer is similar to our finding of 8% in gallbladder cancers. This pattern of a high incidence of *KRAS* mutation in the precursor benign tissue, but not in the malignant tissue, was also reported for gallbladder tumors [22], and is worthy of further systematic study of paired tissue samples from the same patients.

Our study has several limitations. The number of cases is just 25, but this represented thirteen years of material. Evaluation of this tumor would benefit from pooling of pathological tissue. We did not study every possible gene, but we focused on several genes important in intra-abdominal tumorigenesis. Evaluation with newer techniques should facilitate an expanded molecular study.

Our results suggest that microsatellite instability, present in just 4% of cases, does not appear to be a significant part of the carcinogenesis for gallbladder carcinoma. None of our cases demonstrated a *BRAF* mutation and we found only one *GNAS* mutation. As *BRAF* is located "downstream" from *KRAS*, it appears that at least two specific members of this pathway are not contributing to gallbladder carcinogenesis. In conclusion, our results show that in a series of United States cases of gallbladder carcinoma, approximately three-quarters are associated with gallstones, inflammation and dysplasia. Almost half show local nerve, blood vessel and lymphatic invasion. However, many molecular genetic changes commonly seen in tumors arising from other intra-abdominal organs are found infrequently in this tumor type. Additionally, *KRAS* gene mutations, frequent in some other tumors associated with chronic inflammation, are infrequent in gallbladder adenocarcinomas. Further study will be necessary to understand these differences and to clarify which molecular genetic changes are critical for the development of gallbladder cancer.

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