Large-Scale Alleloype of Pancreaticobiliary Carcinoma Provides Quantitative Estimates of Genome-Wide Allelic Loss

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ABSTRACT

Studies of the alleloype of human cancers have provided valuable insights into those chromosomes targeted for genetic inactivation during tumorigenesis. We present the comprehensive alleloype of 82 xenografted pancreatic or biliary cancers using 386 microsatellite markers and spanning the entire genome at an average coverage of 10 cM. Allelic losses were nonrandomly distributed across the genome and most prevalent for chromosomes arms 9p, 17p, and 18q (>60%), sites of the known tumor suppressor genes CDKN2A, TP53, and MADH4. Moderate rates of loss (at any one locus) were noted for chromosome arms 3p, 6q, 8p, 17q, 18p, 21q, and 22q (40–60%). A mapping of individual loci of allelic loss revealed 11 “hot spots” of loss of heterozygosity (>30%) in addition to loci near known tumor suppressor genes, corresponding to 3p, 4q, 5q, 6q, 8p, 12q, 14q, 21q, 22q, and the X chromosome. The average genomic fractional allelic loss was 15.3% of all tested markers for the 82 xenografted cancers, with allelic loss affecting as little as 1.5% to as much as 32.1% of tested loci, a remarkable 20-fold range. We determined the chromosome location (in cM) of each of the 386 markers used based on mapping data available from the National Center for Biotechnology Information, and we provide the first distance-based estimates of chromosome material lost in a human epithelial cancer. Specifically, we found that the cumulative size of allelic losses ranged from 58 to 1160 cM, with an average loss of 561.32 cM/tumor. Compared to localized allelic loss of each xenografted cancer with known clinicopathological features for each patient and found a significant correlation with smoking status (P < 0.01). These findings offer new loci for investigation of the genetic alterations common to pancreaticobiliary cancers and aid the understanding of mechanisms of allelic loss in human carcinoogenesis.

INTRODUCTION

Structural alterations of chromosomes are a virtually ubiquitous feature of human epithelial neoplasia (1). Gross chromosomal alterations may occur after a variety of events, including chromosomal translocations, rearrangements, amplifications, and deletions, usually leading to general aneuploidy. Telomere dysfunction is thought to be a major mechanism for the development of aneuploidy (2–4), although other factors have also been shown to have a role (5). In some instances, DNA mismatch repair defects without gross chromosomal changes are the underlying feature of epithelial neoplasia (6), as seen in the hereditary nonpolyposis colorectal cancer syndrome (7) or changes are the underlying feature of epithelial neoplasia (6), as seen in the hereditary nonpolyposis colorectal cancer syndrome (7) or

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6 among those reported frequently (9–11). Molecular genetic studies have confirmed these karyotypic findings, with frequent allelic losses of chromosome 1p, 9p, 17p, and 18q and moderate frequencies of loss of additional chromosomal loci (12). Identification of these allelic losses contributed to identification of the mutations of tumor suppressor genes CDKN2A on 9p (14), SMAD4 on 18q (15), LKB1 on 19p (16), and ACVR1B on 12q (17) as playing a role in pancreatic carcinogenesis.

Since the original report of the pancreatic cancer alleloype determined in seven cryostat-dissected neoplasms by Seymour et al. (18) and the subsequent larger manual analysis of 18 pancreatic cancer xenografts by Hahn et al. (19), high-throughput automated techniques have been developed that allow analyses of several-fold more efficient coverage of the genome (19). Using such techniques, the new data presented from a large series of pancreaticobiliary cancers extend and refine the known alleloype maps as well as better define the relationship of global measures of allelic loss to clinicopathological features.

MATERIALS AND METHODS

Xenografted Cancers. The generation of xenografted tumors derived from pancreatic and biliary cancers and their utility for allelotyping studies have been described in detail previously (13). For each xenografted pancreatic or biliary cancer, paired normal duodenal mucosa was also available for analysis. All pancreatic cancer xenografts previously determined to have a medullary phenotype were excluded from analysis (8). Clinical and pathological data were obtained from the Johns Hopkins Hospital Surgical Pathology files. The Johns Hopkins Institutional Review Board approved the study.

Genotyping of Pancreatic Cancers. Genotyping was performed using a modification of the CHLC version 9 marker set (386 microsatellite markers; average spacing, 10 cM). Detailed information can be found online.7 The approximate location of each marker, expressed as cM, was determined using human genome recombination map data derived from the National Center for Biotechnology Information website available as of April 2004.8 Genomic DNA was obtained from pancreatic cancer xenograft tissues, matched normal tissues and pancreatic cancer cell lines using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and diluted to 20 ng/μl in a 96-well deep-well plate. Four blind duplicates as well as 4 positive and 2 negative control samples were included with each batch of 86 samples. PCR was performed using 40 ng of genomic DNA in a final reaction volume of 5 μl. Forward primers were labeled with fluorescent dyes FAM and HEX (obtained from Life Technologies, Inc., Carlsbad, CA) and NED (obtained from Applied Biosystems, Foster City, CA) for detection on an ABI 377 DNA analyzer. Reverse primers were obtained from MWG (High Point, NC). Primer concentrations ranged from 0.035 to 0.170 μM each. Two to three loci were routinely amplified together in multiplex PCR. An average of eight PCR products were pooled together using a Hydra 96 Microdispenser unit (Matrix Technologies, Hudson, NH), and PAGE was performed on the ABI 377 XL DNA analyzers. Gels were prepared using Cambrex Long Ranger XL Single Packs (East Rutherford, NJ). The size standard used for fragment analysis consisted of 21 PCR products labeled with a fluorescent dye (ROX) and generated by PCR amplification of the pUC18 plasmid DNA-amplified fragments. ABI Genescan software was used for

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image analysis of each gel containing fluorescence-labeled PCR products. The inherent error rate for the genome scan, based on paired genotypes from these blind duplicates, was 0.25%. The overall missing data rate was 6.4%.

A subset of 30 microsatellite markers spanning 17 chromosomes with varying frequencies of allelic loss was selected for independent verification of allelic status. PCR products incorporating [α-32P]deoxycytidine were separated on a 6.0% polyacrylamide-8 M urea gel, and autoradiography was performed. Determinations of heterozygosity and the presence or absence of allelic loss were scored by three of the authors (C. A. I-D., M. S. v. d. H., and S. E. K.). Loss of heterozygosity (LOH) was defined as the complete loss of one of the two bands in xenograft DNA as compared with the matched informative normal DNA. A comparison of manual and automated determinations of LOH indicated a concordance rate of >99%.

Statistics. Summary data are expressed as the mean ± SD unless otherwise indicated. When evaluating distributions of fractional allelic loss in relation to age, gender, location, tumor differentiation, and smoking history, a one-tailed Student’s t test was used. When evaluating distributions in relation to age, tumor diameter, and survival, Wilcoxon’s rank-sum test was used. Probability values of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

General Features of the Pancreatic Cancer Allelotype. We analyzed a total of 82 primary cancers representing 73 xenografted pancreatic cancers and 9 xenografted biliary cancers. These 82 pancreatic or biliary cancers were analyzed using 386 microsatellite markers spanning the 22 autosomes and the X chromosome, resulting in a total of 63,304 individual loci analyzed. The average coverage was 16.7 markers/chromosome, and the average spacing of markers was approximately 10 cM. The allelotype determined for 18 of these xenografts at 283 loci has been reported previously (13).

Among the 82 pancreatic or biliary cancer xenografts having matched normal DNA samples, a total of 31,652 determinations of heterozygosity were made, of which 21,203 (67%) were informative. The average informativeness ranged from 0.24 (DXS1068) to 0.90 (D3S2406). LOH was found in 5,398 informative determinations, indicating an average allelic loss of 25.4% of informative markers for the 82 xenografted tumors analyzed (Fig. 1). Rare allelic shifts that extended or shortened the normal allele size by 2–5 bp were also found in 65 xenografted cancers, involving 1–9 individual allele(s)/case. Thus, among this subset of 65 tumors, an average of <0.01% loci/case had minimal shifts, similar to previous reports indicating that microsatellite instability is infrequent among pancreaticobiliary cancers that lack the severe form of the mutator phenotype (18).

Patterns of Allelic Loss Related to Specific Chromosomes. As expected (13), allelic losses were nonrandomly distributed across the genome. The average number of markers showing LOH/chromosome/cancer was 2.57, ranging from 5.41 markers (chromosome 9) to 0.48 markers (chromosome 16). Based on the intermarker distance determined for each marker set for each chromosome, the average cumulative allelic loss was 24.41 cM/chromosome/cancer. The greatest cumulative allelic losses were in chromosomes 9, 18, and 6 (57.46, 53.35, and 52.85 cM, respectively), whereas the smallest were in chromosome 16 (6.01 cM).

The vast majority of markers showing LOH occurred within areas of contiguous loss of whole chromosomes or chromosomal arms. Specifically, 1355 markers with LOH (33.5%) occurred within a region of multimarker LOH (3 or more contiguous markers with LOH), and an additional 1958 markers with LOH (54.6%) were adjacent to a second marker with LOH and flanked on either one or both sides by noninformative markers. The remaining markers with LOH occurred singly, being flanked by two informative markers without LOH (49 markers, 1.2% of all LOH calls), two noninformative markers (129 markers, 3.2% of all calls), or a pattern intermediate to these. Single marker LOH was thus reasonably uncommon relative to contiguous marker LOH.

As a general estimate of allelic loss related to each chromosomal arm, we determined its prevalence as the number of cancers with at least one marker showing LOH for a chromosomal arm divided by all cases that were informative for that same arm (20). The highest prevalence (>60%) was noted for chromosomal arms 9p (84%), 17p (81%), and 18q (80%). Moderate prevalence of allelic loss (40–60%) was noted for chromosomal arms 3p (43%), 6q (54%), 8p (51%), 17q (41%), 18q (41%), 21p (48%), and 21q (46%). In contrast, allelic losses were a rare event for chromosomal arms 1p (0%), 15p (0%), and 16q (8%), similar to previous findings (Ref. 13; Fig. 2). The prevalence rates of multimarker, double marker, and single marker LOH patterns described above correlated with the prevalence of allelic loss determined for each chromosomal arm.

The 386 markers allow mapping of “hot spots” of LOH among the 82 xenografted cancers analyzed, defined as any marker with LOH in ≥30% of informative cases (Figs. 3 and 4). LOH hot spots corresponding to the loci of known tumor suppressor genes included distal chromosome 9p (66–87% LOH), 17p (88–92% LOH), 18q (70–85% LOH), and 19p (33–37% LOH). We also found several loci not previously reported as sites of frequent LOH, located at chromosome 3p (D3S2409, 56% LOH; D3S1766, 51% LOH), distal chromosome 16 (D16S587, 97% LOH; D16S398, 87% LOH), and 17q (D17S740, 85% LOH; D17S466, 71% LOH).
4q (D4S1629, 32% LOH), proximal chromosome 5q (D5S424, 31% LOH; D5S641, 32% LOH), chromosome 12q (D12S1052-D12S392, 31–42% LOH), chromosomes 21 and 22 (range, 40–42% LOH for all markers), and the X chromosome (DXS1001, 46% LOH). Two different hot spots at chromosome 6q (D6S1040, 61% LOH; D6S2436, 55% LOH) were also found, as well as a hot spot at distal chromosome 8p (D8S1130, 53% LOH). Chromosomal arms 6q and 8p have been reported previously to have high-frequency allelic loss, although no gene-specific genetic inactivations in pancreatic cancers have yet been found (21).

The homozygous deletion of tumor suppressor genes is a known mechanism of genetic inactivation in pancreatic cancer (14, 16, 22, 23). Therefore, we screened the allelotypes of the 82 cancers for potential homozygous deletions, represented by apparent PCR failures for xenografted tumor DNA (but not within the matched normal DNA) that were flanked by at least two or more contiguous sites of LOH among adjacent markers. Forty-two xenografted cancers contained one or more potential homozygous deletions. All chromosomes contained at least one potential homozygous deletion (range, 1–3), with the exception of chromosomes 2, 11, and 20. In 39 of 42 cases, repeat amplification using the identical markers generated a PCR product of the appropriate size, indicating a simple initial amplification failure for these individual markers. For the remaining three potential deletions, repeat amplification did not generate a product, despite multiple controls and redesigning of primers flanking the region, indicating the likely presence of a homozygous deletion for these loci. An analysis of the location of these loci revealed that one corresponded to chromosome 9p21 in a pancreatic cancer xenograft having a known homozygous deletion for CDKN2A (PX28; Ref. 14), and the remaining two corresponded to chromosome 18q in two different pancreatic cancers with known homozygous deletion of MADH4 (PX182 and PX191; Ref. 15). Thus, our data indicate that homozygous deletions are rare at the sites of the microsatellite markers used (<0.0001%). Because our sampling of the genome occurred at an average spacing of 10 cM, it is likely that 10 cM represents only a crude size limit for a homozygous deletion (such a large size was already known to be unlikely) and that potential smaller deletions would not be effectively screened by our markers.

Patterns of Allelic Loss Related to Individual Cancers. The comprehensiveness of this data set also allowed a determination of the relationship of the genomic fractional allelic loss [G-FAL (defined as the number of markers with LOH divided by all informative markers/carcinoma)] to the overall allelotype for each cancer analyzed. Our data indicate a wide spectrum of allelic loss among pancreatic and biliary cancers. For example, in individual tumors, the G-FAL ranged from 124 of 386 markers (32%; sample PX171) to 6 of 386 markers (1.5%; sample PX147) with an average of 59 markers (15%; median of 56 markers) lost per cancer. The G-FAL was also determined in units of cumulative cM of chromosomal material lost and ranged in size from 58 cM (PX147) to 1160 cM (PX171) [average for all cancers, 561 cM; median, 520 cM].

Two specific features of the pancreaticobiliary cancer allelotype were noted. First, although G-FAL was distributed across a spectrum, a special importance of certain chromosomes could be seen. As shown in Fig. 3, the G-FAL is distributed across a spectrum from 0 to 1.00, with a median of 0.56, indicating that the majority of cancers have a low fractional allelic loss. The G-FAL is also distributed across a spectrum from 0 to 1.00, with a median of 0.56, indicating that the majority of cancers have a low fractional allelic loss. The G-FAL is also distributed across a spectrum from 0 to 1.00, with a median of 0.56, indicating that the majority of cancers have a low fractional allelic loss.
in Fig. 3, allelic losses (of any length) involving chromosomes 9, 17, 18, or 6 occurred in the majority of cases analyzed. Even in those cancers with G-FAL < 5%, at least one of these chromosomes had allelic loss. For one of these cases (PX147), allelic losses of chromosomes 6 and 18 were the sole loci of LOH noted. Second, we noted a less deterministic pattern. With increasing G-FAL, there was a tendency toward allelic losses of increasing length and involvement of an increasing number of chromosomes. For example, among those cases with a G-FAL < 10%, the average number of chromosomes showing allelic loss of any length was 7.2, and the average cumulative amount of material lost was 243 cM. In contrast, among those cases with G-FAL > 20%, the average number of chromosomes affected reached 14.5, with an average loss of 942 cM. No specific differences between pancreatic and biliary cancers were found.

In recent years, chromosome instability has been recognized as a predominant mechanism underlying aneuploidy in solid human tumors (1). Our data support this claim. However, we also show that within the spectrum of chromosome instability-positive pancreaticobiliary cancers, a 20-fold range of G-FAL values exists. At one end of this spectrum, pancreatic cancers were remarkable for minimal allelic losses (PX147; G-FAL = 1.55%), whereas at the opposite end of the spectrum, widespread allelic losses affecting the majority of chromosomes were found (PX179; G-FAL = 32%). Similar findings have been reported for other human tumor types, including lung carcinomas (24), colorectal carcinomas (20), and endocrine tumors of the pancreas (25). One possible explanation offered is that structural alterations of chromosomes may not act as the major mechanism of genetic instability in all microsatellite instability-negative cases (26). Alternatively, the fundamental property of chromosome instability may be the same for all cases, but some tumors may “fortuitously” acquire key changes early in carcinogenesis. This study also represents one of the first estimates of the actual cumulative lengths of allelic loss in a human cancer as determined from the human genome data. Several other large-scale allelotypes of human tumors have also been reported, but the data presented have not included LOH in relation to physical distances (24, 25, 27–31).

Relationship of Allelic Loss to Clinicopathological Characteristics. The ages and genders were similar among patients with primary pancreatic ductal adenocarcinomas and primary distal common bile duct carcinoma. When compared with the G-FAL determined for each case, no significant differences were found among the G-FAL values for each tumor type with respect to patient age, gender, lymph node status, tumor diameter, tumor location, or overall survival. A trend was noted for increasing G-FAL to be associated with worse tumor differentiation, but this trend did not reach statistical significance. However, we identified a significant difference in G-FAL in relation to a history of smoking ($P = 0.01$), with greater G-FAL noted in smokers ($16.7 \pm 5.6\%$) than in nonsmokers ($13.2 \pm 4.8\%$). High frequencies of LOH were also found in lung cancers obtained from smokers as compared with nonsmokers (32). Cigarette smoking is a known major risk factor for the development of several tumor types, including pancreatic cancer (33, 34). Cigarette smoke contains hundreds of toxic chemicals, including the carcinogens benzo(a)pyrene, nitrosamines, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (34). Metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane have been shown to bind DNA and induce activating point mutations in the TP53 or KRAS2 genes (35, 36) as well as stimulate the formation of mitogenic metabolites that stimulate DNA synthesis and proliferation of pancreatic cancer cells (37). Thus, our finding of increased G-FAL in the pancreatic cancers from smoking patients suggests that one or more carcinogens may potentiate genetic instability in this tumor type.

REFERENCES


Fig. 4. Loss of heterozygosity (LOH) maps determined in 82 pancreatic and biliary cancers. Schematic maps of each chromosome depict the proportion of tumors having LOH at each of 386 markers. Data are plotted from pter to qter, based on chromosome location as determined from recombination map data (see “Materials and Methods” for details). The approximate location of the centromere is depicted by a black circle within each schematic chromosome. The prevalence of LOH as a fraction of all informative cases for each marker is represented by the width of each chromosome, normalized to a baseline representing the set of informative markers (shown below each chromosome). For illustrative purposes, a standardized chromosome at bottom right (gridded) has 0% LOH (corresponding to 100% width) at the q arm terminus and 100% LOH (0% width) at the p arm terminus. Hot spots were defined as those loci having \( \geq 50\% \) LOH among all informative tumors for that marker. Arrowheads indicate the sites of both known (9p, 17p, and 18q) and novel (3p, 4q, 5q, 6q, 8p, 12q, 14q, 21q, 22q, and Xq) hot spots of LOH.


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