Inactivation of Smad4 in Gastric Carcinomas

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Abstract

Allelic loss of chromosome 18q has been noted in intestinal type gastric adenocarcinomas. Smad4 is a gene located at 18q that was recently cloned in humans and found to be significantly altered in pancreatic cancers. We sought to determine whether Smad4 genetic alterations played a significant role in gastric tumorigenesis by studying 35 gastric adenocarcinomas of all histopathological types and pathological stages. Microdissected specimens were used for mutational analysis of Smad4 at the nucleotide level, including the entire coding region and intron/exon boundaries. Allelic imbalance was also analyzed at the Smad4 locus using two nearby microsatellite markers.

One case of apparent biallelic inactivation of Smad4 was found in our study of 35 gastric carcinomas. A nonsense point mutation at codon 334 was demonstrated, which, similar to other Smad4 mutations, is predicted to truncate the conserved COOH-terminal domain of this protein. This Smad4 C to T transition mutation was proven to be somatically acquired. Allelic loss was also noted on chromosome 18q at a marker near Smad4 in this mutated gastric cancer, apparently producing complete inactivation of Smad4 in this tumor. Significant 18q allelic loss (56% of 34 informative cases) was noted in our gastric carcinomas using microsatellite markers near the Smad4 locus, regardless of histological subtype or pathological stage. Additionally, three cases of microsatellite instability were observed.

Thus, Smad4 inactivation was noted in our gastric carcinomas; however, this event was rare. The frequent loss of chromosomal arm 18q observed in gastric cancers suggests the presence of other tumor suppressor genes in this region that are involved in gastric tumorigenesis. Further studies are needed to identify these other targets of inactivation during gastric cancer development.

Introduction

LOH studies have suggested that a gene(s) on chromosome 18q is a target of inactivation in a wide variety of tumors, including intestinal-type gastric carcinomas. Southern blot analysis of 23 intestinal gastric cancers found that 61% had allelic loss using probes on 18q (1). A microsatellite analysis of markers located on 18q in 25 differentiated gastric carcinomas found allelic imbalance in 36% of cases (2).

The Smad4 gene [also known as DPC4 (3)], located at 18q21.1, was recently cloned in humans and was found to be mutated in both alleles in nearly one-half of pancreatic carcinomas studied (4). Analysis of colon cancers found that nearly one-third of cases contained altered Smad4 genes (5). In contrast, Smad4 alterations were noted to be distinctly uncommon in a variety of other tumor types, including those arising from lung, breast, uterine, brain, prostate, renal, and bladder tissues (6). Studies of esophageal cancers failed to identify Smad4 changes (7, 8). A study of 10 gastric cancers and 10 ulcerative colitis-associated neoplasms failed to reveal Smad4 gene mutations (8), whereas a study of six colitis-associated neoplastic lesions found one with biallelic inactivation of Smad4 (9). Because this gene appears to be a candidate tumor suppressor gene, we investigated whether or not alterations of Smad4 occurred in our panel of gastric cancers to determine the role this gene and locus plays in gastric tumorigenesis.

Materials and Methods

Sample Collection. Thirty-eight surgically resected primary gastric adenocarcinoma specimens were collected over the past decade from surgical pathology at Johns Hopkins Hospital and stored at −80°C. Normal tissue or blood samples were obtained from these patients as well. Collection of samples was done according to internal review board-approved protocols. Tumor-node-metastasis staging of resected cancers was assessed according to the consensus criteria adopted by the American Joint Committee on Cancer (10). Histopathology was assessed according to the Lauren classification (11) as either diffuse or intestinal type.

Sample Processing. Cryostat sectioning and microdissection of gastric cancer specimens to enrich for neoplastic cells were performed as described previously (12). Verification of histology and percentage of neoplastic cells were performed in each case by a gastrointestinal pathologist (O. W. C.). Three gastric cancers could not be enriched to at least 50% neoplastic cells and were not further studied. High molecular weight DNA was extracted from the tumor and normal samples according to classic organic methods.

Amplification and Mutational Analysis of Smad4. Exons of the Smad4 gene were amplified with primers located in intron regions using standard PCR methods. PCR products were directly sequenced using ThermoSequenase (Amersham Corp.) according to the manufacturer’s instructions and electrophoresed on 6% denaturing polyacrylamide gels for subsequent autoradiography. Primers for amplification and sequencing are available on the Internet.

LOH Analysis. Two highly polymorphic nucleotide repeat microsatellite markers, D18S4747 and D18S456, located on chromosome 18q near Smad4, were used for LOH analysis according to our published methods (13). The forward primer of each primer pair was fluorescently labeled and used in PCR reactions. PCR products were electrophoresed on a 377 ABI instrument along with a ROX 350 internal standard and data analyzed by Genescan (ABI) software for direct quantitation of alleles in arbitrary units by Genotyper (ABI) software. The relative number of alleles (allele ratio) in each case of paired tumor and normal DNA samples from patients was computed consistently, either the smaller allele to the larger allele or vice versa, to generate comparison ratios greater than 1.0. The comparative ratio was produced by dividing the tumor allele ratio by the normal allele ratio. Comparative ratios greater than 1.5 were considered to represent significant allelic loss and were scored positively.

Results

Gastric adenocarcinoma specimens and paired normal tissue from patients were confirmed histologically for use in subsequent molecular analyses. Gastric tumors were collected and microdissected to enrich the specimens for neoplasia; 35 ultimately consisted of 50% or more neoplastic cells prior to DNA extraction. The pathological stages of these specimens at the time of resection included the full spectrum of all histopathological types and pathological stages.
from early gastric cancers (stage 1a) to advanced lesions (stage IV). Histological classification of the cancers studied according to Lauren's criteria determined that 24 were intestinal type and 12 were diffuse type. Twenty-three of the cancers were located in the stomach body or distal stomach, whereas 12 cancers were located more proximally in the stomach; 6 adenocarcinomas were noted to arise from the gastric cardia or gastroesophageal junction region. Two carcinomas had diffuse neoplastic involvement of the stomach in a "linitis plastica" fashion. None of the 35 patients met consensus criteria for the Hereditary Nonpolyposis Colon Cancer syndrome (14).

We first sought to identify intragenic mutations of Smad4. The entire coding region and intron/exon boundaries were amplified from genomic DNA extracted from the gastric carcinoma specimens. The PCR products were subjected to mutational analysis by direct DNA sequencing, which revealed a nonsense point mutation at nucleotide position 1128 that resulted in a truncating stop codon at position 334 in exon 8 of one gastric carcinoma case (case G7; see Fig. 1). This mutation was confirmed by an independent amplification and sequence analysis and determined to be somatically acquired in this cancer as the corresponding constitutional DNA from this patient did not have this mutation. LOH analysis demonstrated allelic loss of the region near the Smad4 locus in this gastric tumor specimen as well. Thus, biallelic inactivation of Smad4 appears to have occurred during the development of this cancer. No other intragenic or splice site alterations were noted at the nucleotide level in these gastric cancers. The tumor specimen for case G7 could only be enriched to approximately 50% neoplastic cells, which is at the threshold for our studies, yet we were able to detect allelic loss and this intragenic mutation. Because most of our specimens were enriched to greater than 70% neoplastic cells, we are confident that alterations were not overlooked with our analyses.

We extended our analysis to determine the frequency of allelic imbalance at the Smad4 locus in our gastric carcinomas. Two dinucleotide repeat markers, D18S46 and D18S474, which straddle the Smad4 locus, were amplified using genomic DNA extracted from paired normal tissue samples from our patients. There were 24 informative (heterozygous) cases for each marker. Thirty-four of the 35 cases were informative for at least one of the two markers, and 11 were informative for both markers.

For all informative cases, corresponding cancer DNA samples were similarly amplified and analyzed for a given marker. The relative ratio of alleles within each sample was then compared between the tumor and normal templates to give a comparative ratio indicating whether any imbalance occurred in the tumor sample. A clear dimorphic population was seen; most cases did not display imbalance and had ratios near 1.0. On the other hand, 25 comparisons exhibited significant allelic imbalance in their tumor DNA samples; they had comparative ratios above 1.5 and were scored positively for allelic loss (see Fig. 2). Significant allelic imbalance in tumor DNA with reference to at least one of these markers was exhibited in 19 of 34 (56%) informative cases. In those cases that were informative for both markers, allelic balance and imbalance were consistent except for two cases in which loss was noted to occur more centromeric relative to Smad4, at marker D18S474, as opposed to more telomeric, at marker D18S46. Allelic imbalance of this chromosomal region was noted in both intestinal and diffuse-type gastric carcinomas and in both early and more advanced stages of tumor development without significant differences in frequency.

Microsatellite instability was noted in three (8.8%) cases that demonstrated abnormal-sized alleles in the tumor DNA compared to the paired normal DNA (see Fig. 2). Both markers were observed to be unstable in two cases. This finding is consistent with previous reports of a small proportion of gastric cancers demonstrating this phenotype (15–17). The cause of microsatellite instability in this subpopulation of gastric cancers is unknown.

Discussion

Smad4 appears to be a Mad homologue involved in TGF-β ligand superfamily signaling with up to 85% homology in the COOH-terminal region of the gene. This highly conserved COOH-terminal region of Smad4 has been shown to cause transcriptional activation (18). Moreover, tumor-specific mutations of Smad4, including the one found in our gastric cancer case, are noted at sites analogous to an inactivating mutation of the Drosophila Mad gene (19). Thus, altered TGF-β signal transduction appears to have provided a selective growth or survival advantage to our gastric cancer case G7. It is noteworthy that genetically altered TGF-β II and insulin-like growth factor II receptors, both of which are involved in TGF-β signal transduction with repeated nucleotide elements in their coding region, have been observed in gastric cancers that manifest microsatellite instability (20, 21). Taken together, these findings suggest that altered TGF-β signal transduction, known to normally inhibit epithelial growth, is a critical change in a subset of gastric cancers.

We thoroughly examined the Smad4 gene at the nucleotide level and found apparent biallelic inactivation of Smad4 in one case (G7) of 35 gastric carcinomas studied. This carcinoma was a well-differentiated intestinal-type carcinoma of pathological stage IIIA (T3,N1,M0) located proximally in the cardia. The nonsense point mutation in this gastric cancer is tumor specific and is a novel change not previously

![Fig. 1. Mutational analysis of Smad4. The entire coding region and intron/exon boundaries of Smad4 were analyzed by DNA sequencing of gastric carcinomas. Displayed is an autoradiograph of a sequencing gel illustrating sequence of exon 8 of Smad4 from four gastric cancers (Lanes 1–4). Sequence reactions were grouped by nucleotide (A, C, G, and T) to aid in the identification of changes. A transition C to T point mutation resulting in a stop codon was noted at nucleotide position 1128 of the gene product of Smad4 in gastric carcinoma G7 (Lane 3, arrow).](image-url)
reported. It occurs in a region where other inactivating mutations of Smad4 have been found that result in the loss of the conserved COOH-terminal domain of this gene product. As commonly occurs with tumor suppressor genes, the other Smad4 allele appears to have been lost in this gastric cancer.

Our allelic imbalance results suggest the presence of a tumor suppressor gene on chromosomal arm 18q, which is significantly involved in gastric cancer development. Similar significant loss of this chromosome was noted on previous Southern blot analysis in intestinal gastric cancers (1). Over 56% alteration of this region was noted on analysis of two microsatellite markers near the Smad4 locus in our gastric cancers without regard to histological subtype or pathological stage.

Several other genes with potential tumor suppressor functions reside on chromosomal arm 18q. DCC and Smad2 are two genes adjacent to Smad4 located at 18q21, the loci of which have been found homozygously lost in colon cancers (22, 23). Inactivating mutations of Smad2, another Mad-related gene, have been demonstrated in a proportion of colon cancers (23, 24). Moreover, Smad2 is located centromeric to Smad4, and allelic loss was noted to occur in this direction in the few cases showing informativity and discordance with the two markers used that straddle Smad4. DCC, on the other hand, is located telomeric to Smad4. Further studies are needed to determine whether these genes or others on chromosome 18q are commonly altered and inactivated in gastric tumorigenesis.

Overall, we found Smad4 inactivated in our study of gastric carcinomas, but not commonly. Significant allelic loss on chromosome 18q suggests the presence of another tumor suppressor gene(s) in this region, which is the target of inactivation during gastric tumorigenesis. Additional studies are needed to identify the targets of inactivation in gastric cancer development to advance our understanding of this lethal cancer.

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Fig. 2. LOH analysis on chromosome 18q. Two nucleotide repeat markers, D18S474 and D18S46, located near the Smad4 locus on 18q, were analyzed in our panel of gastric carcinomas and paired normal tissues. Data generated by Genotyper through the ABI fluorescent analysis method is illustrated in representative cases. Peaks corresponding to alleles of various size (X axis represents increasing size of bp) and amount (Y axis represents quantity in arbitrary units (AU)). A, an informative case (case G23) for marker D18S474 without allelic loss or instability noted in the tumor sample (T) compared to the normal sample (N). B, an informative case (case G20) for marker D18S46 with loss of the larger allele in the tumor sample compared to the normal sample. C, an informative case (case G6) for marker D18S46 with loss of an allele in the tumor sample; similar to B except that in this case, the loss is of the smaller allele. D, an informative case (case G8) for marker D18S474 with abnormal-sized alleles in the tumor sample.

References

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