Definition and Refinement of Chromosome 8p Regions of Loss of Heterozygosity in Gastric Cancer

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ABSTRACT
Loss of heterozygosity at several chromosomal loci is a common feature of the malignant progression of human tumors. These regions are thought to harbor one or more putative tumor suppressor gene(s) playing a role in tumor development. Allelic losses on the short arm of chromosome 8 (8p) have been reported as frequent events in several cancers, and three commonly deleted regions have been defined at 8p11.2–12, 8p21–22, and 8p23.1. To evaluate the possible involvement of these regions in gastric cancer, we used eight microsatellite markers to perform an extensive analysis of allele loss at 8p21–22 in 52 cases of primary gastric adenocarcinoma. We found that 44% of tumors showed allelic loss for at least one marker at 8p21–22. The critical region of loss was found to be between markers LPL and D8S258, which displayed loss of heterozygosity in 39% and 33% of cases, respectively. This region is centromeric to the LPL locus and centered on the D8S258 locus. We conclude that 8p22 deletion is a frequent event in gastric cancer and suggest the presence of a putative tumor suppressor gene near the D8S258 locus. Initial steps were taken toward the identification of this gene, which is likely to play an important role in the pathogenesis of gastric cancer and of other tumors as well.

INTRODUCTION
Stomach cancer is a major health concern for many Americans. Although the death rate in the United States has been declining, nearly 21,900 new cases of gastric adenocarcinoma were expected to be diagnosed in 1999, and 13,700 people will die of the disease (1). Patients with gastric cancer have a relatively poor prognosis, and surgery is currently the only viable option for curative treatment. The resectability rate ranges between 60% and 80% at the time of diagnosis, and curative resection is achieved in 70–80% of cases. Nevertheless, the overall 5- and 10-year survival rates for curative resections are still 40–50% and 36%, respectively (2–4). Early diagnosis has been proven to be effective in improving the outcome of patients in high-risk countries such as Japan (5). In low-risk countries, however, a better understanding of the molecular pathogenesis of this disease is necessary to obtain new diagnostic parameters and to eventually improve prognosis.

The etiology of gastric adenocarcinoma has been extensively investigated, but no single factor has been proven as a direct cause of gastric carcinogenesis. Male gender, age, and lower socioeconomic status are associated with higher incidence of gastric cancer and higher mortality. Diets rich in foods containing salt, nitrates, and nitrites may be correlated to a higher incidence of gastric cancer (6). Helicobacter pylori infection seems to be a risk factor for intestinal-type gastric cancer (7). When untreated, it usually leads to chronic atrophic gastritis and metaplasia, precancerous conditions that can lead to gastric cancer after several years (8). The decline in gastric cancer incidence, particularly in intestinal-type gastric cancer, in developed countries is thought to be correlated with dietary improvements and with treatment of H. pylori infection (9).

In addition to the influence of the environmental factors mentioned above, two characteristics of gastric cancer make it particularly interesting for genetic analysis. First, the two histological types of gastric adenocarcinoma, intestinal and diffuse, seem to develop through two distinct mechanisms (10). Second, the genetic alterations occurring in gastric cancer seem to differ from those occurring in other gastrointestinal neoplasms because the proposed genetic pathway of the adenoma-carcinoma sequence in colorectal cancer has not been found in gastric cancer (11–14). In fact, relatively little is known about the molecular events leading to tumors of the stomach. p53 and p16 have been shown to be inactivated in 30–40% and 12% of gastric tumors, respectively (15–18), and c-myc and/or ras activation occurs in less than 20% of gastric tumors (19, 20). Finally, we and others (21) have recently shown that the FHIT gene is inactivated in a significant number of gastric cancers that do not show Fhit protein expression. To identify additional genetic changes involved in gastric cancer pathogenesis, we focused on the short arm of chromosome 8, a chromosome region frequently showing allelic loss in a variety of human tumors. LOH is one of the most frequent genetic alterations in solid tumors, and the characterization of chromosomal regions displaying a high rate of loss of genetic material will lead to the

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3 The abbreviations used are: LOH, loss of heterozygosity; TSG, tumor suppressor gene; MI, microsatellite instability.
identification of putative TSGs (22, 23). Since the late 1980s, LOH investigations have led to the isolation and identification of some of the most commonly deleted or mutated TSGs in human solid tumors such as the p53 and FHIT genes (24–28).

LOH analyses on chromosome 8p were performed in prostate, colorectal, and esophageal cancers, and allelic imbalance has been found to be frequent at the NEFL locus on 8p11.2–12, at the LPL locus on 8p21–22, and at 8p23.1 (29–37), suggesting the presence of more than one TSG on the short arm of chromosome 8. Studies on different human tumors including breast cancer, small cell lung cancer, and hematological malignancies confirmed the presence of putative TSGs in these chromosomal regions. In particular, LOH at 8p21–22 seems to be associated with cancer progression and advanced stage (38–46). Several candidate TSGs have been identified on 8p. The N33 and PRLT5 genes may be involved in a small fraction of cancers (47, 48). More recently, we identified a novel candidate TSG, FEZ1, at 8p22 whose expression is altered in multiple human tumors (37).

Previous studies suggest that 8p loss also occurs in gastric cancer (49, 50). Using high-density polymorphic markers to screen 52 cases of gastric adenocarcinoma, we defined the minimal region of allele loss harboring a putative TSG playing a role in gastric tumorigenesis. We placed the gene centromeric to the FEZ1 gene, within a 500-kb segment flanked by LPL and D8S258.

**MATERIALS AND METHODS**

Samples. Tumors and normal gastric mucosa were obtained at surgery with informed consent from 52 patients. Seventeen patients were from Padova, Italy (Cittadella Hospital; N cases), and 35 patients were from Tokyo, Japan (National Cancer Center Research Institute; J and NJ cases). The specimens were immediately snap-frozen in liquid nitrogen, and the remaining tissue was routinely processed for histopathological analysis, which confirmed the diagnosis of adenocarcinoma.

DNA Analysis. DNAs from both normal mucosa and tumor were purified by standard techniques (51). The DNA extracted from each sample was amplified by PCR using primers specific for chromosome 8 microsatellite markers. Primer sequences, chromosomal localization, and frequency of heterozygosity were obtained from the Genome Database (William Welch Library, John Hopkins University, Baltimore, MD). Three loci were analyzed at 8p21: (a) NEFL (primers 214/215); (b) D8S136 (primers D8S136CA/GT), and (c) D8S133 (primers D8S133CA/GT). Five loci were analyzed in 8p22: (a) D8S258 (primers AFM107x6bm/a); (b) D8S1715 (AFMa189zg5-F/R); (c) LPL3 (primers LPL3′CA/GT); (d) D8S1145 (CHLC.GATA72C10.P18040F/R); and (e) D8S261 (primers AFM123x5g5m/a). PCR reactions were performed using 100 ng of template in a 50-μl volume using 0.5 unit of Taq polymerase (TaKaRa) with 50 μM each of dATP, dGTP, and dCTP; 5 mM dTTP; and 10 μCi of [α-32P]dCTP. Reaction conditions were optimized for each primer set. The number of cycles was reduced to a number that allowed the differences in allelic band intensity to remain detectable, reflecting real differences in the allelic ratio. PCR products were separated on a 6% polyacrylamide sequencing gel. After electrophoresis, the gel was fixed on 3 MM paper, dried, and exposed to X-ray film for 24–48 h.

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<th>Table 1</th>
<th>Summary of LOH analysis of the five markers tested in all cases</th>
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<td>Marker</td>
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<td>D8S261</td>
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<tr>
<td>LPL</td>
<td>28</td>
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<tr>
<td>D8S258</td>
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<td>D8S133</td>
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<td>NEFL</td>
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Assessment of LOH. Results for all cases were analyzed by visual inspection. In addition, cases presenting equivocal results and cases delimiting critical regions of loss were analyzed as described previously (52) by a comparison of allele intensities in matched normal/tumor DNA using scanning densitometry with a computerized Molecular Dynamics system (Sunnyvale, CA). Quantification was performed using the ImageQuant program. The relative ratio of both tumor and normal alleles was determined, normalized, and then compared. LOH was assigned when the intensity ratio of the two tumor sample alleles differed by at least 30% from that observed on normal DNA.

YAC Clone Analysis. Five polymorphic markers between D8S261 and D8S136 were tested on eight YAC clones that had been mapped between D8S261 and D8S136 by Bookstein et al. (53). PCR amplifications were performed as described previously (53).

RESULTS

Paired normal and tumor samples from 52 gastric adenocarcinomas were screened for LOH at 8p21–22 to determine the frequency of allelic imbalance and to define the minimal region of loss at 8p21–22 in gastric cancer.

Allelic loss for at least one locus was detected in 23 of the 52 (44%) cases examined. Five loci were tested in all of the matched normal/tumor samples (Table 1), and allelic loss ranged from 27% at NEFL to 39% at LPL. LOH was also found in 11 of 33 (33%) informative cases at D8S258, in 11 of 33 (33%) informative cases at D8S133, and in 10 of 34 (29%) informative cases for D8S261. Three more markers, D8S1145, D8S1715, and D8S136, were tested to narrow the minimal region of allele loss in cases showing LOH for at least one of the five markers described above (23 cases). Fig. 1 shows the pattern of allelic loss for each case. In nine cases, we cannot exclude loss of the entire short arm of chromosome 8 (cases N18, J34, J39, J105, J161, J165, NJ2, NJ4, and NJ5). Five cases, on the other hand, retained the most centromeric and telomeric markers and lost the region between LPL and D8S258 (cases N3, N26, J171, J101, and J164). Case J112 in particular shows loss only at D8S258 and shows retention of LPL and D8S133. Cases N8, N11, N33, and J97 retained only the most telomeric marker and lost the proximal region. MI was detected in five tumor samples (data not shown). Two of these showed normal patterns for the most centromeric markers (cases J6 and J9), one showed a normal pattern for the most telomeric marker (case J158), one showed MI only at D8S258 (case J87), and one showed MI for all of the markers tested (case J13). Five cases (N5, J97, NJ1, NJ7, and NJ8) showed loss at the centromeric or
telomeric markers, but not at internal markers. No alterations were found in 44% of the cases examined.

To improve mapping in the region of interest, eight microsatellite loci from chromosome 8p21–22 have been mapped on YAC clones. We could delineate the region more closely, and the final order of the markers included in the lost chromosomal region was tel-D8S1145-D8S1715-LPL-D8S258-D8S298-cen, and markers LPL and D8S258 were found on the same YAC clone, 936c3 (1600 kb), as also reported by Bookstein et al. (53). Although physical distances can be strongly affected by YAC chimerism or internal deletion, we confirm the data reported by Farrington et al. (31) of a short physical distance between LPL and D8S258. YAC clones were isolated and analyzed to construct a contig of the region and to verify the order of the markers.

**DISCUSSION**

This study is the first detailed analysis of allelic loss at 8p21–22 performed on gastric cancer. Allelic imbalance for at least one locus was found in 44% of tumor samples. Our data allowed us to define the region of minimal allelic loss at 8p22 to a segment of less than 1 Mb between loci LPL and D8S258 (Figs. 1 and 2). LOH for these loci was 39% and 33%, respectively, suggesting that the region harbors one or more TSGs involved in the development of gastric cancer (Table 1). In contrast with what has been described in other tumors, the frequency of LOH at the NEFL locus in gastric cancer was relatively low, suggesting that the NEFL region is not involved in this neoplasm. Furthermore, the minimal region of loss in our tumors is centromeric to the FEZ1 gene, suggesting that another TSG distinct from FEZ1 is targeted by LOH in these tumors.

Indeed, previous studies on prostate, colon, breast, and lung cancer indicate that more than one TSG at 8p is involved in the progression of these common human tumors (29–45). Furthermore, a recent wide LOH analysis at 8p21–23 in lung cancer showed an increasing frequency of LOH and a larger size of the deleted region with increasing severity of histopathological preneoplastic changes (45). Moreover, human chromosome 8 can suppress the metastatic potential of highly metastatic rat prostate cancer cell lines (54) as well as the tumorigenicity and invasiveness of a colon carcinoma cell line (55).

Efforts toward positional cloning of TSGs have allowed the isolation of different candidate TSGs at 8p. The PRLTS gene (platelet-derived growth factor receptor-like TSG) is centromeric to the minimal region of allele loss described in this study (48). Alterations of N-acetyltransferase NAT1 and NAT2 genes at 8p22 have also been studied in cancer because of their carcinogen metabolizing action, but no abnormalities were
found in cancer cells (47). The N33 gene was found to be silenced in several cancer cells, although no point mutations have been identified (56). We have recently analyzed 72 esophageal cancers and found LOH at D8S261 locus in 70% of informative cases (37). Positional cloning in the region led to the identification of a putative TSG, FEZ1, at 8p22 around D8S261, centromeric to the MSR region and telomeric to the LPL region. FEZ1 encodes a leucine zipper protein whose expression is altered in multiple human cancers, suggesting that it may play a role in various human tumors. However, in this study, LOH at D8S261 occurred in 29% of informative cases, suggesting a minor role for FEZ1 in gastric cancer.

Our work contributes toward the characterization of 8p, a complex chromosomal region that clearly plays an important role in human cancer, and represents the initial step toward isolation of a putative TSG involved in gastric cancer.

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REFERENCES


Fig. 2 Representative examples of LOH for chromosome 8p in human gastric cancer. Corresponding case number and locus are shown at the top of the radiographs for each normal (right)/tumor (left) matched pair. The arrow indicates an allele lost in tumor DNA. D, difference in the rates between tumor and normal allele.


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