Age-associated loss of heterozygosity of tumor suppressor genes in the gastric mucosa of humans

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Moragoda, Lathika, Richard Jasiewski, Prasad Kulkarni, and Adhip P. N. Majumdar. Age-associated loss of heterozygosity of tumor suppressor genes in the gastric mucosa of humans. Am J Physiol Gastrointest Liver Physiol 282: G932–G936, 2002. First published January 30, 2002; 10.1152/ajpgi.00312.2001.—The current study is based on the hypothesis that aging predisposes gastric mucosa to carcinogenesis through altered expression and/or mutations of genes involved in cell growth. To test this hypothesis, we investigated the age-associated changes in mutation of adenomatous polyposis coli (APC), deleted in colorectal cancer (DCC), p53, and K-ras genes in the gastric mucosa of 19 healthy subjects of varying ages (25–91 yr). Specifically, we studied the loss of heterozygosity (LOH) of these genes in cardia, body, and antrum of the stomach. We observed that 3 of 19 subjects (16%) over 60 yr of age show LOH of at least one of the tumor suppressor genes. In the subjects over 60 yr of age, the incidence of LOH is 38% (3/8). Two of three subjects had mutations in more than one tumor suppressor gene. In all three affected subjects, mutation in APC, DCC, or p53 was located mainly in the body of the stomach, suggesting increased susceptibility of this region to neoplastic changes. However, no LOH of K-ras was observed in these subjects. Our observation that subjects over 60 yr of age show mutation in one or more of the tumor suppressor genes suggests an age-related increase in predisposition of the stomach to neoplasia.

aging; mutations; p53; adenomatous polyposis coli; deleted in colorectal cancer; K-ras; neoplasia

Although earlier observations in the mouse suggest that proliferative activity of the small intestine either decreases (16, 24) or remains unchanged (17) with aging, recent morphological and biochemical studies from our own and other laboratories have demonstrated that in barrier-reared Fischer 344 rats, aging is associated with increased mucosal proliferative activity in the stomach and small and large intestines (3, 14, 21, 22, 27–29). In both gastric and colonic mucosa, the age-related rise in proliferation could partly be attributed to enhanced transition from G1 to S phase as well as progression through the S phase of the cell cycle (42, 44). Moreover, in the gastric mucosa, aging is also associated with increased activation of extracellular regulatory kinases and c-Jun NH2-terminal kinase and transcriptional activity of AP1 and nuclear factor κB (43). These changes are also accompanied by increased activation and expression of epidermal growth factor (EGF) receptor (37, 38). Overexpression of EGF receptor has been associated with many malignancies, including the colon and stomach (5, 6). These and other relevant observations have led to the postulation that aging may predispose the gastrointestinal tract to neoplasia (3, 23, 29).

Many probable reasons have been suggested for the age-dependent rise in malignancies, including altered carcinogen metabolism and cumulative effects of long-term exposure of cancer-causing agents (8, 10). The possibility that aging may render target cells more susceptible to carcinogenesis through mutation of tumor suppressor genes has not been investigated. Inactivation of tumor suppressor genes has been linked to the development and progression of carcinogenesis (25, 41).

The tumor suppressor gene p53 plays a crucial role in cellular proliferation and apoptosis and as the guardian of genomic integrity (4, 34). Similarly, the loss or inactivation of the tumor suppressor gene adenomatous polyposis coli (APC), which initiates genomic instability that may produce adenomas in the colon predisposing it to carcinogenesis, is well documented (11, 30). Mutation and loss of heterozygosity (LOH) of APC is also known for other cancers, including gastric cancer (33–36, 40). Similarly, deleted in colorectal cancer (DCC) is a tumor suppressor gene that has been shown to have allelic deletions and/or loss of expression in gastrointestinal and other carcinomas (13, 19). We have also studied K-ras, which is one of the protooncogenes most frequently mutated in many malignancies, including colorectal cancer, and is associated with increased uncontrolled cell proliferation (9).
Table 1. LOH analysis of p53, DCC, and APC genes in 19 healthy subjects of various ages

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+, Mutation of both alleles; +, mutation of one allele; −, normal allele; LOH, loss of heterozygosity; DCC, deleted in colorectal cancer gene; APC, adenomatous polyposis coli gene.

One of the possibilities for the age-related rise in gastrointestinal cancers, particularly gastric cancer, could be that the rate of mutation(s) in tumor suppressor genes in the stomach increases with aging. To test this possibility, we have examined the incidence of mutation in p53, DCC, APC, and K-ras genes in different regions (cardia, body, and antrum) of the stomach during advancing age in humans. The reason for analyzing mutations in the cardia, body, and antrum is to determine whether and to what extent different regions of the stomach are affected by aging.

METHODS

Biopsies. Patients aged more than 18 yr of age undergoing clinically indicated esophageagastroduodenoscopy (e.g., Hemoccult-positive stool, gastroesophageal reflux, iron deficiency anemia etc.) who were found to have macroscopically normal-appearing gastric mucosa were eligible to participate. Pairs of mucosal forcep biopsies were obtained from cardia, body, and antrum regions of the stomach and snap frozen in liquid nitrogen and stored at −80°C.

DNA extraction. Genomic DNA was extracted from each biopsy specimen using DNA Stat-60 reagent (Tel-Test, TX) according to the manufacturer's protocol. Briefly, samples were homogenized in DNA Stat reagent and DNA precipitated from the aqueous phase using isopropanol.

Detection of LOH of p53. LOH in p53 was studied essentially according to the procedure described by Ara et al. (2). This method used restriction fragment length polymorphism (RFLP) exhibited by codon 72, which can be detected by restriction digestion with BstU1, following PCR amplification. Standard PCR reactions containing template DNA were denatured at 94°C for 1.5 min and amplified at 94°C for 40 s, 60°C for 40 s, and 72°C for 45 s for 40 cycles followed by extension at 72°C for 15 min. The primers used were sense 5′-TTGCGGTCTCCAAAGCAATGGATGA-3′ and antisense 5′-TCTGGGAAGGGACAGAAGATGAC-3′ to amplify a 200-bp fragment. PCR products were digested with BstU1 (New England Biolabs) and separated on 2.5% agarose gels (FMC Bioproducts).

Detection of LOH of DCC. To study LOH of DCC, variable number of tandem repeats within the DCC gene (19, 26) was amplified. PCR reactions containing sample DNA were denatured at 94°C for 1.5 min and run at 94°C for 30 s, 58°C for 40 s, and 72°C for 40 s for 40 cycles followed by 15 min of extension at 72°C. The primers used were sense 5′-GATGACATTITCCTCTTAG-3′ and antisense 5′-GTGTTATTTGCTTTGAAATGAC-3′ (26). The PCR products were separated on 2.5% agarose gels (FMC).

Detection of LOH of APC. LOH of APC was studied according to the procedure described by Tandle et al. (35). This method involves studying RFLP of APC in exon 11 by digesting with Rsal following PCR amplification. PCR reactions containing sample DNA were denatured first at 94°C for 1.5 min and amplified at 94°C for 40 s, 59°C for 40 s, and 72°C for 40 s for 35 cycles followed by 15 min of extension at 72°C. The primers used were sense 5′-GGACTACAGGCGCATTT-3′ and antisense 5′-GCCATCATCTCTCAAGATTCAAG-3′. The amplified 133-bp fragment was digested with Rsal (NEB), and products were separated on 2.5% agarose gels.

Detection of LOH in K-ras. Mutations in K-ras were studied using the technique described by De Jong et al. (9). This technique detects a G-to-A transition, which creates a restriction site for HpH1 in codon 12 of this gene. Briefly, PCR is performed using a mismatched primer pair (sense 5′-ACTTTGTGGTAGTTGAGGTG-3′ and antisense 5′-TCCACAAAGTGTCTTGAAT-3′) to generate a 75-bp product. The products were then digested with HpH1, which generates 46- and 26-bp bands if the gene is mutated. Wild-type products remain uncut by HpH1. PCR conditions used were identical to those described for APC amplification.

RESULTS

To determine whether aging is associated with genetic changes in the gastrointestinal tract, incidence of mutations in p53, DCC, and APC genes, as assessed by LOH, was examined in biopsy specimens from different regions of the stomach (cardia, body, and antrum) in 19 subjects aged 25–91 yr (mean, 55.1) whose routine endoscopy revealed macroscopically normal appearing gastric mucosa (Table 1). Indications for upper endoscopy included gastroesophageal reflux disease (n = 10), nonulcer dyspepsia (n = 6), and Hemoccult-positive stools with negative colonoscopy and evidence of anemia (n = 3). LOH of p53 was studied using the occurrence of RFLP in codon 72 of this gene (Fig. 1). Nine of nineteen

Fig. 1. Representative figure showing loss of heterozygosity (LOH) analysis of p53. Heterozygous condition is indicated by the presence of all 3 bands. Homozygous individuals have either a 199-bp band or 113- and 86-bp bands. LOH is shown by a 64-yr-old subject in the body. A, antrum; B, body; C, cardia; M, DNA marker.
subjects were homozygous, five of nine carried the allele for arginine (113- and 86-bp bands) and four of nine carried the allele for proline (199-bp band). Of the 10 subjects who were heterozygous, one subject, age 64 yr, showed LOH of p53 (Fig. 1). This LOH was observed only in the body of the stomach but not in the other two regions.

With respect to DCC, we observed eight homozygous individuals having either a 200-bp allele or a 160-bp allele and 11 heterozygous individuals with both alleles (Fig. 2). Two of the heterozygous individuals showed alleles that were larger than the normal 200-bp allele and smaller than the 160-bp allele (Fig. 2, lane 8). These size differences may be due to insertions or deletions. However, these deviations were observed in all three regions of the stomach and hence, were not considered as age-associated mutations. LOH in DCC was observed in 10% (2/19) of the subjects whose ages were 64 and 91. Interestingly, the 64-yr-old individual was the same subject who exhibited LOH in p53 (Table 1 and Fig. 2, lane 6). In the 91-yr-old subject, both DCC alleles were observed to be lost in the body, although both alleles were present in cardia and antrum (Fig. 2, lane 9). Similar to what we observed for p53, LOH in DCC was also detected in the body of the stomach.

In the case of APC, 11 individuals were observed to be heterozygous, possessing the 133-bp band corresponding to an allele uncut with Rsa1 and 85- and 68-bp bands representing alleles cut with Rsa1. The rest were homozygous, having either cut or uncut allele (Fig. 3). Interestingly, the 91-yr-old subject, who was homozygous with respect to antrum and cardia, showed a gain of heterozygosity in the body region (Fig. 3). This may be explained by the occurrence of a mutation at the restriction site of one allele, thus rendering this site unavailable for cutting. A similar phenomenon was observed with a 72-yr-old subject who showed homozygosity with respect to body and cardia but heterozygosity with respect to antrum (Fig. 3). Both of these patients had histologically normal gastric mucosa without evidence of Helicobacter pylori infection.

Investigation of K-ras gene, on the other hand, revealed no mutations in codon 12 in the cardia, antrum, or main body of the stomach of any subject (data not shown). Codon 12 was selected to analyze mutational status of K-ras, because this region is considered to be most relevant to the progression of colorectal cancer.

**DISCUSSION**

Aging is associated with many gastrointestinal dysfunctions (20, 23). One of the most consistent pathological observations in senescent animals is increased incidence of many types of malignancies, including gastric and colorectal cancers. Gastric cancer rarely occurs before the age of 40 yr, but its incidence increases subsequently, with peak incidence occurring in the seventh decade. Many probable reasons, including altered carcinogen metabolism and long-term exposure of cancer-causing agents, have been offered for the age-dependent rise in malignancies (8, 10).

Carcinogenesis, which is a multistep process, results from the accumulation of mutations during progression from normal epithelium to carcinoma (12). Genetic changes that occur at different stages of epithelial cell carcinoma have been extensively studied by Vogelstein and colleagues (11) in human colon cancer. At least for colon cancer, it has been suggested that the loss or inactivation of the tumor suppressor gene APC initiates genomic instability that may produce phenotypic appearance of an adenoma. The advanced tumors, however, possess mutations and/or deletion of a number of oncogenes and tumor-suppressor genes not seen in early adenoma (7, 11, 12). Although such detailed analysis of genetic alterations has not been performed for gastric cancer, inactivation of several tumor suppressor genes, including APC, p53, and DCC, has been observed in gastric cancer (1, 39, 45) and inactivation of K-ras in the case of colorectal cancer (9). However, to the best of our knowledge, no information is available whether aging, which is thought to predispose the
The gastrointestinal tract to carcinogenesis, is associated with increased inactivation of tumor suppressor genes. Our current data, for the first time, show that in humans, the incidence of mutations of several tumor suppressor genes, specifically APC, DCC, and p53 in the gastric mucosa, is higher in older subjects. We have observed that 3 of 19 subjects (16%) who are over 60 yr of age show LOH of APC, DCC, or p53 gene in the body of the stomach. However, among the subjects over 60 yr of age, the rate of this incidence is 38% (3/8). This rise in mutations of tumor suppressor genes among the older subjects could not be accounted for by the presence of cancer in the stomach or any other organ. None of the subjects were diagnosed with any type of cancer or precancerous lesions at the time of endoscopy or were treated for these symptoms earlier. Foci of atrophic gastritis typically coalesce, resulting in a state of reduced gastric acid that may progress to chronic atrophic gastritis and associated intestinal metaplasia. These lesions are thought to be most closely associated with the subsequent development of dysplasia and carcinoma (18). Although some of the subjects had mild gastritis, this condition could not be related to LOH of the tumor suppressor genes, because the three subjects who demonstrated LOH of one of the tumor suppressor genes were devoid of this symptom. More importantly, none of our study subjects with genetic aberrations had gastric mucosal evidence of intestinal metaplasia, atrophic gastritis, or H. pylori infection, each of which is thought to be associated with the development of gastric cancer (31, 32). Finally, none of our study patients had clinical evidence of pernicious anemia or a history of gastric surgery, conditions known to predispose to gastric carcinogenesis (18). Although mutational activation of K-ras protooncogene is widely implicated in the development and progression of colorectal cancer (15), we observed no mutations of this protooncogene in the gastric mucosa of any of the subjects. Thus, unlike the tumor suppressor genes, aging does not appear to induce mutations of the protooncogene K-ras.

Although gastric carcinoma occurs in all regions of the stomach, we have observed that the majority (4/5) of mutations are located in the body of the stomach. The reason for this is not fully understood. One plausible explanation could be that with aging, the body of the stomach, which is primarily composed of parietal cells, becomes more susceptible to genetic alterations. Whether this is the result of prolonged exposure to endogenous acid or exogenous noxious agents remains to be determined.

Interestingly, two of three subjects who show mutations also show mutation in more than one gene. Although the reasons for this are not fully understood, one plausible explanation could be that they may possess defective DNA repair mechanisms. Another possibility could be due to inherent genetic susceptibility of tumor suppressor genes to mutations and to endogenous and/or exogenous noxious agents. Undoubtedly, further detailed analysis with a larger number of subjects is needed to gain an understanding in this and other related matters.

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REFERENCES


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