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Methylation and Expression of Human Pepsinogen Genes in Normal Tissues and Their Alteration in Stomach Cancer

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In normal human tissues, pepsinogen A mRNA was expressed only in the fundic mucosa of the stomach, whereas pepsinogen C mRNA was expressed in all regions of the stomach mucosa and also in the proximal duodenal mucosa. The distributions of these mRNAs were consistent with those of pepsinogens A and C in the gastroduodenal mucosa. Methylation analysis of DNAs from normal tissues with methylation-sensitive restriction enzymes, *Hpa*II and *Hha*I, revealed that pepsinogen A and C genes are hypomethylated in tissues producing pepsinogens A and C, suggesting a role of DNA methylation in the regulation of the differential expression of the genes for the two human pepsinogens during normal differentiation. In stomach cancer tissues and cancer cell lines, the expressions of the pepsinogen genes were decreased or lost, in good accordance with their pepsinogen productions. No gross structural changes of the pepsinogen genes were observed in these cancers, but the methylation patterns of the pepsinogen genes were found to be altered in different ways in different cancers. The functional significance of the altered methylation is unknown; however, these results suggest that considerable heterogeneity of the methylation patterns occurs in human stomach cancers.

Key words: Pepsinogen — DNA methylation — Stomach cancer — Human stomach

Human pepsinogens are classified into two biochemically and immunochemically different groups of isozymogens: pepsinogen A (PGA)[9] and pepsinogen C (PGC).[1)] PGA is produced only in the fundic mucosa of the stomach, whereas PGC is produced in all regions of the stomach mucosa and also in the proximal duodenal mucosa.[1)] PGA and PGC are good markers of terminal differentiation of these tissues. Furthermore, as altered pepsinogen isozymogen patterns have consistently been observed in preneoplastic and neoplastic tissues induced experimentally in rat stomach,[2,3)] pepsinogens are also considered to be good markers of preneoplastic and neoplastic changes of the stomach mucosa. Similar changes of the pepsinogen isozymogen and reduction in pepsinogen production have been observed in carcinogen-treated human stomach mucosa *in vitro* and in human stomach cancer.[3–5)] These observations have stimulated interest in the molecular basis of pepsinogen gene regulation.

Recent studies have indicated that DNA methylation may be involved in the regulation of gene expression during normal differentiation.[6–9)] The expressions of many tissue-specific genes are associated with reduced levels of DNA methylation, and the absence of DNA methylation appears to be an essential prerequisite for gene expression, although some findings are not consistent with this idea.[10)] In a previous study, we analyzed the methylation status of PGA genes and found that these genes are hypomethylated in tissues where they are expressed.[11)] To understand the mechanisms involved in the differential expressions of the genes for the two human pepsinogens, we have analyzed the expression and methylation of the gene for the other component of human pepsinogen, PGC, in normal tissues and compared the results with those for PGA genes. As there is recent evidence that the normal process of DNA methylation is deranged in tumor cells[12)] and that this may cause the abnormal gene control characteristic of cancer,[13–15)] we also examined the methylation of the pepsinogen genes in human stomach cancer tissues and cancer cell lines.

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[9] Abbreviations: PGA, pepsinogen A; PGC, pepsinogen C; cDNA, complementary DNA; kb, kilobases; bp, base pairs.

MATERIALS AND METHODS

Human tissues and cell lines Specimens of normal human tissue and stomach cancer were obtained at surgery. Normal parts of resected tissues and cancer tissues were removed, and freed of necrotic tissue. Portions of four of the cancers were transplanted into nude mice and the resulting tumors were transplanted sequentially at intervals of 1 to 3 months. The tumors were removed from the nude mice 1 to 2 months after the last tissue transplantation when they were 5 to 10 mm in diameter. Tissue specimens for biochemical analysis were stored in liquid nitrogen until use. The remaining tissue was examined histologically. The avidin-biotin-peroxidase complex method[16] with rabbit anti-human PGA and PGC antibodies described previously[17] was used to detect PGA and PGC in normal and cancer tissues.

The human stomach cancer cell lines MKN 1, 7, 28, 45, 74, Kato and Okajima[18,19] were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml). Cells were passaged with 0.05% trypsin in isotonic phosphate buffer.

Isolations and analyses of DNA and RNA High-molecular-weight DNA from various human tissues and cancer cell lines was prepared by the method of Gross-Bellard *et al.*[20] and total RNA was isolated by the guanidium/cesium chloride method.[21] A sample of 10 μg of DNA was digested completely with restriction enzymes (5–10 U/μg DNA) under the conditions recommended by the manufacturer. Completeness of digestion was monitored by including λ phage DNA in each reaction mixture. Electrophoresis, transfer to a nitrocellulose filter and detection of DNA were performed by the method of Southern.[22] A sample of 10 μg of RNA was denatured and subjected to electrophoresis on agarose formalin gel by the method of Goldberg.[23] Then the RNA was transferred to a nitrocellulose filter, baked and hybridized as described.[11]

cDNA probes A human stomach cDNA library in PBR-322 was screened at two temperatures, 68°C and 50°C, using a 180 bp *PstI/BglII* fragment of human PGA cDNA, corresponding to amino acid residues 325–372, and containing 33 bp of the 3′-noncoding sequence. A single clone was found to hybridize preferentially at the lower temperature. This 0.8 kb clone was confirmed to be PGC cDNA by partial sequence determination, and was used as a probe for further screening of a full-length cDNA in the human stomach cDNA library in λgt10. Several positive clones were obtained and the longest cDNA insert of about 1.36 kb was subcloned into the PUC13 plasmid and M13 phage for restriction mapping and dideoxy sequencing.[24] The cDNA was 1355 bp in length and included the 5′-noncoding region (50 bp), the PGC coding nucleotide sequence (1164 bp) and the 3′-noncoding region (141 bp). This cDNA insert and a 1.2 kb PGA cDNA insert in the recombinant DNA clone PTM003g-35[11] were both labeled with [α-^{32}P]dCTP by nick-translation[25] and used as hybridization probes.

Assay of pepsinogen level Human tissues and the cell lines were minced and homogenized with 0.1 *M* sodium phosphate buffer (pH 7.4) in a Teflon homogenizer and were centrifuged at 105,000*g* as described previously.[2,3] The PGA and PGC concentrations of the supernatants were determined by radioimmunoassay,[17] and the total activity of gastric acid proteases (pepsinogens and non-pepsin acid proteases[1,26]) was also measured by the method of Anson, with a slight modification.[2,3] The protein contents of the extracts were determined by the method of Lowry *et al.*[27] For identification of non-pepsin acid proteases in the tissue extracts, polyacrylamide gel electrophoresis was performed as described previously.[2,3,5]

RESULTS AND DISCUSSION

Northern blot analysis of total RNA from normal human tissues showed that PGC mRNA of about 1.8 kb was expressed in both the fundic and pyloric mucosa of the stomach and also in the proximal duodenal mucosa (Fig. 1A). RNAs from the large and small intestines, liver, kidney, spleen and lung gave only background hybridization signals, indicating little or no expression of PGC mRNA in these tissues. Immunohistochemical analysis of normal human tissue samples also demonstrated the synthesis of PGC only in the gastroduodenal mucosa (Fig. 2). The PGC mRNA level was high in the fundic mucosa and relatively low in the pyloric and proximal duodenal mucosas, consistent with the mucosal PGC contents of these regions [fundic mucosa, 22.0± 1.8 μg/mg protein; pyloric mucosa, 10.0±1.2 μg/mg

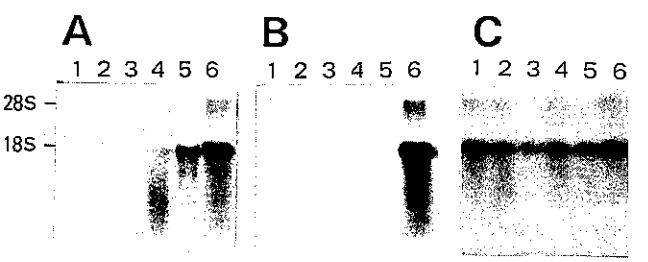

Fig. 1. Northern blot analysis of total RNAs from normal human tissues with PGC (A) or PGA (B) cDNA probe. β-Actin probe (C) was applied as an internal control. The RNA samples (10 μg/lane) analyzed were as follows. Lane: 1, spleen; 2, large intestine; 3, small intestine; 4, duodenum; 5, pyloric stomach; 6, fundic stomach.

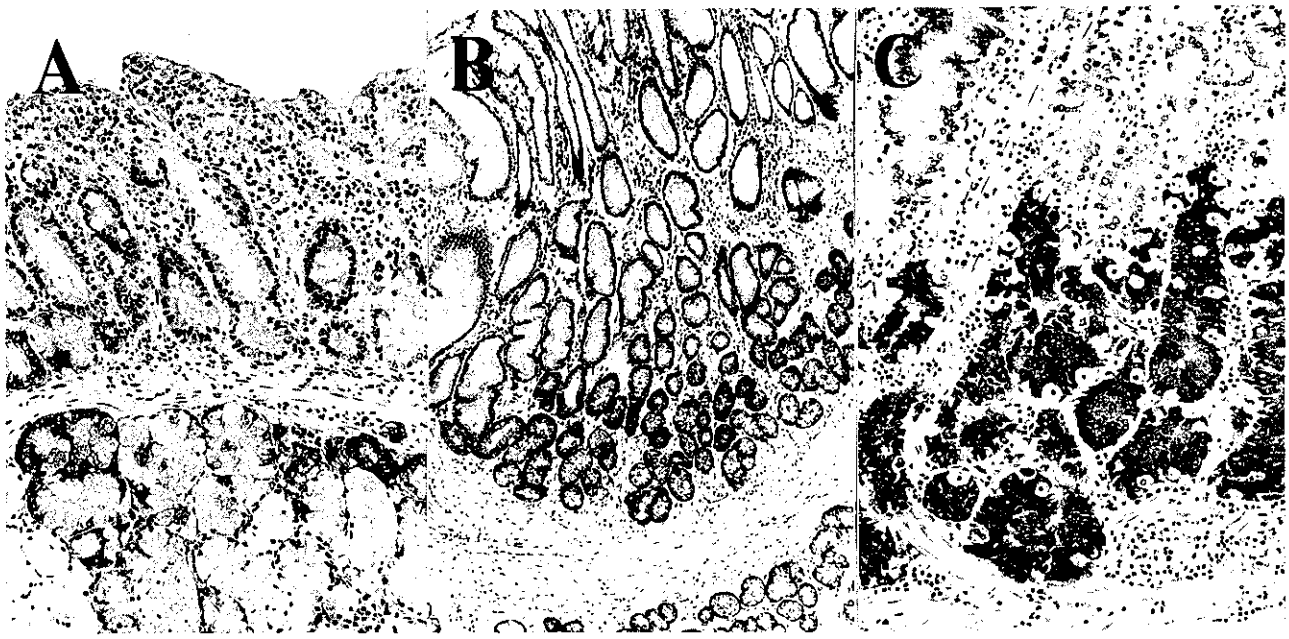

Fig. 2. Immunostaining of gastroduodenal mucosa with anti-PGC antibody by the ABC method. A; duodenal mucosa, ×200, B; pyloric mucosa, ×200, C; fundic mucosa, ×200. Cells staining strongly for PGC are seen in these tissues.

protein; duodenal mucosa 6.7 ± 1.2 μg/mg protein (mean±SE; n=8)]. In contrast, PGA mRNA of almost the same size as PGC mRNA was expressed only in the fundic mucosa of the stomach: no PGA mRNA expression or PGA synthesis was observed in other parts of the gastrointestinal tract (Fig. 1B), as described previously.[11] To control for the quantity and quality of RNAs used in these experiments, we rehybridized Northern blots to a ^{32}P-labeled β-actin probe (Fig. 1C). Approximately equal amounts of expression of β-actin mRNA were observed. These results demonstrate that the productions of the two pepsinogen isozymogens in normal human gastroduodenal mucosa are mainly controlled by the process that regulates expressions of their mRNAs.

In mammals, DNA methylation is predominantly found in the dinucleotide sequence 5′-CG-3′, about 70% of the cytosines being methylated in normal differentiated cells.[6-8] Thus, methylation of specific genes has been studied using methylation-sensitive restriction enzymes that recognize the 5′-CG-3′ doublet. One of the most useful systems is the digestion of DNA with *Hpa*II and *Msp*I. Although both enzymes recognize the DNA sequence 5′-CCGG-3′, *Hpa*II will not cleave the DNA if the internal cytosine is methylated.[28] Thus, by comparing the digests with *Msp*I and *Hpa*II, the existence of CmCGG in genomic DNA can be assessed.[28] When genomic DNAs from several normal tissues were

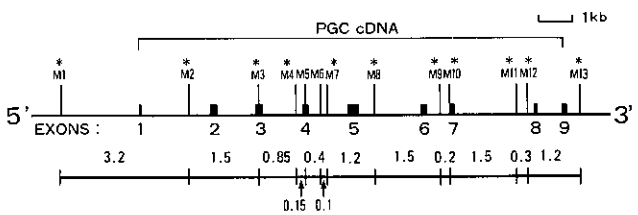

Fig. 3. Methylation map of the human PGC gene. The map shows *Msp*I/*Hpa*II (CCGG) sites and indicates the region of the gene that hybridizes to the cDNA clone used in these studies. *Msp*I/*Hpa*II sites are designated by M1 to M13. The exons are indicated by dark blocks 1 to 9. The sizes of fragments generated by *Msp*I digestion are shown. Asterisks indicate sites that are hypomethylated in the PGC-producing tissues. The methylation condition of the sites M5 and M6 has not been determined. kb, kilobase.

digested with *Msp*I and other methylation-insensitive restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Taq*I and *Bgl*II) and analyzed by Southern blotting with PGC cDNA as a probe, identical patterns were observed. These patterns were in good accordance with the structure of the PGC gene reported previously.[29] Fig. 3 shows the *Msp*I-*Hpa*II sites in the structural map of the human PGC gene. *Msp*I digestion of DNAs invariably generated PGC fragments with sizes of approximately 3.2, 1.5, 1.2

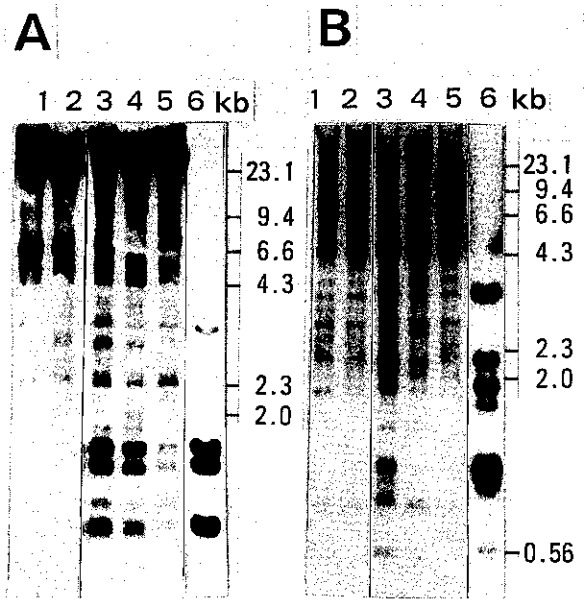

Fig. 4. Methylation status of pepsinogen genes in normal human tissues. DNAs from normal human tissues (lanes: 1, colon; 2, lung; 3, fundic stomach; 4, pyloric stomach; 5 and 6, duodenum) were digested with either *Hpa*II (lanes 1–5) or *Msp*I (lane 6). Southern blots were hybridized with ^{32}P-labeled PGC (A) or PGA (B) cDNA probe.

and 0.85 kb, corresponding to the expected distances between the *Msp*I sites in and around the PGC gene (Fig. 4A, lane 6). Within the PGC gene region, there were additional *Msp*I sites that would have generated hybridizing fragments of 0.4, 0.2 and 0.15 kb. These fragments were too small for efficient transfer to nitrocellulose filters to be detectable by the probe. When the same DNA samples were digested with *Hpa*II, the hybridization patterns were different from those obtained on their digestion with *Msp*I, suggesting methylation of CCGG sites in the PGC gene (Fig. 4A). The patterns were tissue-specific and the extent of methylation differed in different tissues. Digestion of DNAs from PGC-producing tissues, that is, the fundic and pyloric mucosa of the stomach and the proximal duodenal mucosa, with *Hpa*II resulted in release of all the fragments generated by *Msp*I. In contrast, *Hpa*II digests of DNA from non-PGC producing tissues did not contain these *Msp*I fragments. This result was not due to incomplete digestion of DNA samples since λ phage DNA included in the reaction mixture was digested to completion. In addition, when the same filters were hybridized with *Bgl*II-*Bgl*II genomic fragment of insulin gene,[30] an identical hybridization pattern was obtained. Therefore, these results suggest specific hypomethylation of CCGG sites in PGC gene in tissues producing PGC. From the results of the analysis using various-sized cDNA probes, these hypomethylated sites were situated throughout the PGC gene region (Fig. 3). The intensities of the *Msp*I-generated fragments in DNA digests with *Hpa*II of PGC-producing tissues decreased in the order, fundic > pyloric mucosa > duodenal mucosa, indicating that hypomethylation of PGC gene in these tissues was in the same order. These results, together with the results on PGC mRNA expression in these tissues (Fig. 1), appear to substantiate an inverse correlation between the extents of methylation and expression of PGC gene. However, these two events also appear to correlate well with the proportion of the producing cells in each tissue (Fig. 2). Thus, PGC gene is probably hypomethylated in cells producing PGC. The methylation status of PGA genes was also analyzed and compared with that of PGC. As shown in Fig. 4B, the CCGG sites of the PGA genes were more hypomethylated in the fundic mucosa of the stomach, which produces PGA, than in tissues not producing PGA, confirming our previous results.[11] Similar methylation analysis with another methylation-sensitive restriction enzyme, *Hha*I (which cleaves 5′-GCGC-3′, but not 5′-GmCGC-3′), also revealed that the internal cytosine of GCGC sites in PGA and PGC genes was less methylated in each producing tissue than in other tissues (not shown). The results of the present study demonstrate a correlation between hypomethylation and expression of the pepsinogen genes and suggest the possibility that DNA methylation may be involved in the control mechanisms responsible for the differential expressions of the genes for the two human pepsinogen isozymogens in the gastroduodenal mucosa. The role of the observed tissue-specific methylation is unclear. Some recent evidence suggests that DNA methylation may produce transcriptionally inactive chromatin structures.[31, 32] Alternatively, it may inhibit gene expression by affecting the interactions of *trans*-acting factors with specific *cis*-acting DNA sequences.[9, 10] This is best seen in the case of the liver-specific tyrosine aminotransferase gene, in which interactions of liver-specific *cis*-acting elements and specific protein factors are inhibited by DNA methylation.[33] In this context, further studies are required on the *cis*-acting sequence and various *trans*-acting cellular factors required for transcription of the pepsinogen genes. In addition, further analyses using genomic sequencing[34] in and around the regulatory sequence will probably be useful in understanding how much the mechanisms involved in the tissue-specific expression of the pepsinogen genes depend on DNA methylation.

In 21 primary stomach cancers analyzed, the total activity of gastric acid proteases (pepsinogens and non-pepsin acid proteases[1, 26]) was significantly lower than that in normal stomach [mean ± SE: fundic stomach

(n=8), 1526±270 U/g protein; pyloric stomach (n=8), 686±152 U/g protein; stomach cancer (n=21), 78±24 U/g protein]. In these stomach cancers, no PGA biosynthesis was observed, and PGC was greatly reduced in 5 cases (23.8%) and not detectable in the others [PGC concentration in stomach cancer tissue, 931±139 ng/mg protein (mean ± SE)]. Immunohistochemical studies revealed that in the PGC-positive cancers, PGC was present in only a few cells, most cancer cells not producing pepsinogen, in accordance with previous findings.[35] To exclude the possibility of contamination of the cancer tissues with non-neoplastic stomach cells, we transplanted four human gastric cancers into nude mice and examined the pepsinogens in the resulting tumors. In addition, we analyzed 7 stomach cancer cell lines. Only negligible amounts of acid protease activity were detected in the transplanted cancers and 3 cell lines (MKN74, Okajima and Kato). This activity was due to non-pepsin acid proteases, no PGA or PGC production being observed. On Northern blot analysis of total RNAs from these cancer tissues and cell lines, no PGA mRNA expression was observed and only a faint signal of PGC mRNA was detected in PGC-positive primary cancers. Thus, the reduction or disappearance of pepsinogen synthesis in stomach cancer tissues and the cancer cell lines appeared to be due mainly to decreased gene expression.

Altered gene expression could be mediated by various genetic alterations often seen in cancer cells, such as rearrangement, amplification, deletion and mutation.[36] The results of Southern blotting of the stomach cancer DNAs digested with several methylation-insensitive restriction enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Msp*I) did not reveal any evidence of gross change in structure of the PGA or PGC gene in concert with decreased gene expression, although our results did not exclude the possibility of small deletions, insertions or point mutations at sites other than the recognition sites of the restriction enzymes used. In contrast, the methylation patterns of these genes were altered in the stomach cancers. Fig. 5 shows representative results of methylation analysis of the pepsinogen genes in the non-pepsinogen-producing cancer DNAs. The *Hpa*II digestion patterns of the cancer DNAs were different from those of normal tissues that do and do not express the pepsinogen genes and also differed in different cancers, indicating altered and varied methylations of CCGG sites in the PGA and PGC genes in the cancer cells. These altered methylation patterns were also observed in the analysis with *Hha*I and appeared not to be related to the histological type of cancer or its location in the stomach. These results suggest that considerable heterogeneity of DNA methylation occurs in human stomach cancers and are in direct contrast to those of previous studies, which revealed non-random hypomethylation of

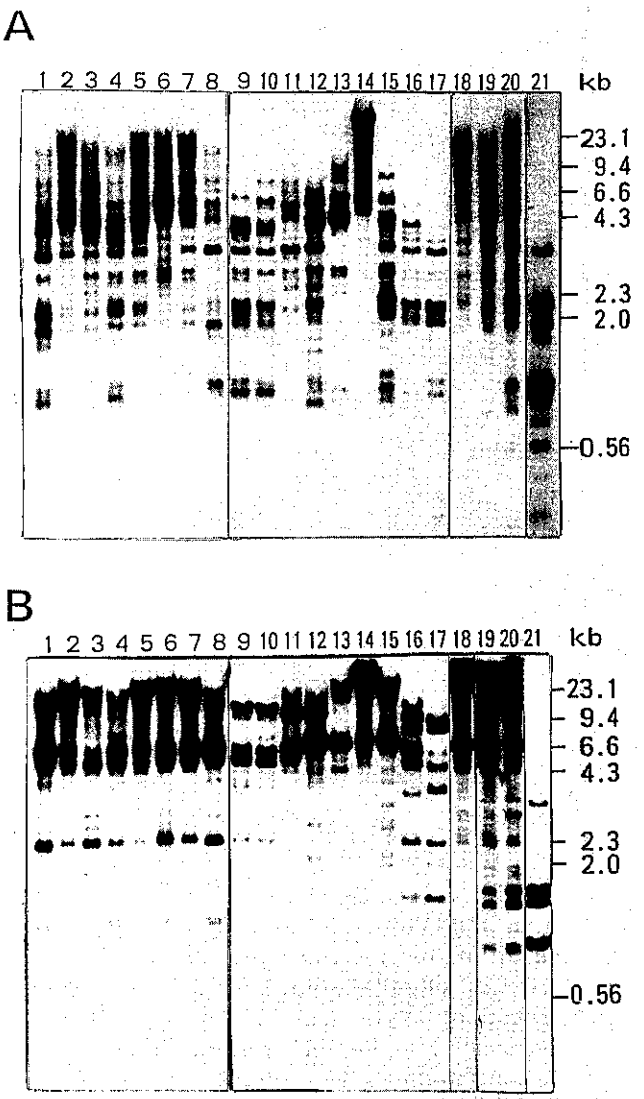

Fig. 5. Methylation status of pepsinogen genes in human stomach cancer. DNAs isolated from stomach cancers (lanes: 1–8, primary stomach cancer; 9 and 10, stomach cancer transplanted into nude mice; 11, MKN 1; 12, MKN 7; 13, MKN 28; 14, MKN 45; 15, MKN 74; 16, Okajima; 17, Kato), human liver (lane 18) and normal human stomach (lanes: 19, pyloric mucosa; 20 and 21, fundic mucosa) were digested with *Hpa*II (A, lanes 1–20) or *Msp*I (A, lane 21). Southern blots were hybridized with ^{32}P-labeled PGA (A) or PGC (B) cDNA probe.

DNA in tumor cells.[37-39] These previously reported hypomethylations might predispose genes that are important for neoplastic growth to be expressed, as suggested by others.[12, 37] Since no simple correlation between methylation and expression of the pepsinogen genes was

observed in the cancers and no information is available about the methylation condition of the sites not analyzed in the present study, the functional significance of the altered methylation of the pepsinogen genes is unknown and requires study. However, if DNA methylation plays a role in the pepsinogen gene regulation, the observed alterations within this mechanism may also be involved in the repression of the differentiated phenotype of stomach mucosa.

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