Identification of Silencing of Nine Genes in Human Gastric Cancers

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ABSTRACT

Aberrant methylations in human gastric cancers were searched for by a genome scanning technique, methylation-sensitive representational difference analysis. Nine CpG islands (CGIs) in the 5’ regions of nine genes, LOX, HRASLS, BA305P22.2, FLNC (+/−flipABPL), HAND1, a homologue of RIKEN 2210016F16, FLJ32130, PGAR (HFRAP/ANGPTL4/ARP4), and thrombomodulin, were found to be methylated in two gastric cancer cell lines, MKN28 and MKN74, but unmethylated in the normal sample. Their expressions were lost in the two cell lines, and the causal roles of the methylations in gene silencing were shown by treatment with 5-aza-2′-deoxycytidine. In 41 primary gastric cancers, methylations of these CGIs in association with reduced expressions were observed at high incidences (29–41%) for five genes, including LOX and HRASLS, which have been reported to have tumor-suppressive activities. The other four genes were methylated at low incidences (0–7%). By analysis of the numbers of aberrant methylations in individual cancers, a subset of cancers with high prevalence of aberrant methylations was identified, and all of the 11 cancers in the subset were diffuse type. To analyze the possible involvement of decreased fidelity of maintenance methylation in this subset of cancers, aberrant hypomethylations of three normally methylated CGIs were examined. Cancers with high prevalence of hypomethylations of normally methylated CGIs, however, constituted a different subset. It is expected that these nine genes may include important genes in gastric cancer development and would be useful to identify a distinct subset of gastric cancers.

INTRODUCTION

Epigenetic alterations, as well as genetic alterations, are involved in cancer development and progression (1, 2). Methylation of CGIs4 in the 5’ regions of tumor suppressor genes is known to inhibit transcriptional initiation and cause permanent silencing of the genes. Gene silencing is now regarded as one of the major mechanisms to inactivate tumor suppressor genes, along with gene mutations and deletions. On the other hand, methylation of CGIs in downstream exons and introns does not block elongation of mRNA in mammalian cells (1, 3). To identify genes involved in human cancers, such as novel tumor suppressor genes, it seems reasonable to search for aberrant methylations in the 5’ regions of genes as their markers. For this purpose, we previously established a genome-scanning method, MS-RDA (4). By this method, DNA fragments that are differentially methylated in two genomes can be isolated after amplification of DNA fragments obtained by digestion with a methylation-sensitive restriction enzyme and a series of subtractive hybridizations and selective amplifications.

Gastric cancer is one of the major current causes of cancer death in Asian countries (5). As for its molecular basis, silencing of the p16 gene has been reported in 42.0–42.2% of cancers (6, 7). Microsatellite instability is observed at incidences ranging between 31 and 67% (8, 9), and the majority of the microsatellite instability-positive gastric cancers show methylation of the hMLH1 gene (10, 11). RUNX3 was shown recently to be involved in human gastric cancers, and the major mechanism for its inactivation is also promoter methylation (12). The most prevalent genetic alteration in human gastric cancers is the p53 mutation, which is observed in 37–43% of intestinal-type gastric cancers (13). E-cadherin is inactivated by mutations in 17–56% of diffuse gastric cancers (14, 15) and by promoter methylation in a similar fraction (15).

In the present study, we applied the MS-RDA method to human gastric cancer cell lines using three restriction enzymes, HpaII, SacII, and NarI, and identified silencing of nine genes.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Forty-one primary gastric cancer samples were obtained from 40 patients undergoing gastrectomy, with informed consent. Two independent gastric cancer samples (4C-1 and 4C-2) were obtained from case 4. For 28 of the 41 cancers, noncancerous gastric epithelial tissues (normal samples) were also obtained by scraping off the mucosae. These samples were frozen and stored at −80°C until extraction of DNA or RNA. Six gastric cancer cell lines, KATOIII, MKN28, MKN45, MKN74, NUGC3, and AGS, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC93, HSC44, and HSC57, were established by K. Y. DNA was extracted by standard phenol/chloroform procedures. RNA was isolated with ISOGEN (Nippon Gene, Tokyo, Japan).

MS-RDA. Three series of MS-RDA were performed using HpaII, with some modifications on the original method (4, 16), and two other methylation-sensitive restriction enzymes, SacII and NarI, respectively.

Genomic DNAs of a normal sample (sample 21N), MKN28 (established from an intestinal-type gastric cancer, 70-year-old female), and MKN74 (established from an intestinal-type gastric cancer, 37-year-old male) were digested with a methylation-sensitive restriction enzyme. An R adaptor specific to the restriction enzyme was ligated to the digestion product of the normal sample and to the mixture of digestion products of MKN28 and MKN74. An amplicon was prepared by PCR using an R primer in the presence of 1 μM betaine (Sigma Chemical Co., St. Louis, MO), which facilitated amplification of G + C-rich sequences (17, 18). The R adaptor was then removed by digestion and gel filtration. For only the normal sample, a new J adaptor was ligated. The ligation product was mixed with an excess amount of the amplicon of the cancer cell lines and underwent denaturation and reannealing. Using the product, PCR was performed with a J primer, and DNA fragments present in only the amplicon of the normal sample were selectively amplified. After switching the J adaptor to an N adaptor, the second cycle of competitive hybridization and selective amplification was performed in the same manner. After the third competitive hybridization and selective amplification using the J adaptor again, the PCR product was cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI). Ninety-six clones were sequenced and tested for independence. Adaptors for SacII and NarI were as follows: 5′Sac26/9, AGC ACT TCT CAG CCT TCT ACC CCC CGG GTG CGT GAG; 5′Sac26/9, ACC GAC GTC GAC TAT CCA TGA ACC GCG GTG TCA TGG; 5′Sac26/9,
AGG CAA CTG TGC AGG ACC GC/GCT CGG; RNar24/11, AGG CAA CTG TGC AGG ACC/GCG CTG AG; JNar24/11, GCC GAT GTC TAT CTA TAA GCA/GCG CTC CTT CG; andNNar24/11, AGG CAA CTG TGC AGG ACC/GCG CTG CTC GG.

DNA Sequencing and Database Search. Cycle-sequencing was performed with a BigDye Terminator kit (PE Biosystems, Foster City, CA) and an ABI automated DNA sequencer (PE Biosystems). Repetitive sequences were screened by the Repeat Masker software (University of Washington Genome Center), and sequence homology was searched using the BLASTN algorithm.

Sodium Bisulfite Modification, Bisulfite Sequencing, and MSP. One μg of DNA, digested with BamHII, underwent sodium bisulfite modification as described previously (19) and was suspended in 20 μl of H2O. For bisulfite sequencing, 1 μl of the solution containing bisulfite-modified DNA was used for PCR with the primers common for methylated and unmethylated DNA sequences. The primers and PCR conditions are available as supplementary data. PCR products were cloned into pGEM-T Easy vector, and 101 clones from each sample were cycle-sequenced using T7 and Sp6 primers.

For MSP, 1 μl of the solution was used for PCR with primers specific for methylated (M) or unmethylated (U) sequences. Using the DNA from the normal sample and DNA that was methylated with SsSI methylase, annealing temperatures specific for M and U primers were determined. Minimal PCR cycles to yield bands for these positive controls were determined, and four cycles were added for test samples. These primers and PCR conditions are available as supplementary data. MSP for p16 and hMLH1 were performed as described previously (11, 20). For normally unmethylated CGIs, a sample was regarded as methylation (+) when PCR product was obtained only with the U set and was regarded as methylation (+) when PCR product was obtained with the M set or with both the U set and M set. For NM-CGIs, a sample was regarded as demethylation (−) when PCR product was obtained only with the M set and was regarded as demethylation (+) when PCR product was obtained with the U set or with both the M set and U set.

Quantitative RT-PCR. Total RNA was treated with DNase I (Ambion, Austin, TX), and cDNA was synthesized from 3 μg of total RNA using a Superscript II kit (Life Technologies, Inc., Rockville, MD). Real-time PCR was performed using SYBR Green PCR Core Reagents (PE Biosystems, Warrington, United Kingdom) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of molecules of a specific gene in a sample was measured by comparing its amplification with the amplification of standard samples that contained 102 to 106 copies of the gene. The quantity of mRNA of each gene was normalized with that of PCNA.

RESULTS

MS-RDA. DNA fragments aberrantly methylated in the gastric cancer cell lines MKN28 and MKN74 were searched for by three series of MS-RDA using three methylation-sensitive restriction enzymes, HpaII, SacII, and NarI, respectively. Of the 96 clones sequenced in each series, 36, 17, and 18 clones were found to be independent, respectively. A database search revealed that 17, 17, and 17, respectively, of them were derived from CGIs, and that 3, 7, and 6, respectively, were derived from CGIs in the 5′ regions of genes (Table 1).

Methylation Status of the 5′ Regions of 16 CGIs. As for the 16 CGIs in the 5′ regions, their methylation status in the normal sample (21N) and two gastric cancer cell lines (MKN28 and MKN74) were analyzed by bisulfite sequencing. Nine CGIs, which were in the 5′ regions of LOX, HRA5LS, hA305P22.2.3, FLNL (γ-filamin/ABPL), HAND1, a homologue of RIKEN 221016F16, Membrane transporter gene XPCT, Liver dipeptidyl peptidase (peHDP7), Retinoid homeobox protein (RX), and Fibroblast growth factor homologue (FGF-4), were methylated in the two cancer cell lines but not in the normal sample (Fig. 1). For all of these nine genes, all of the DNA molecules analyzed were methylated in the cancer cell lines, and it was indicated that no unmethylated allele was retained. Especially for the HRA5LS gene, a cytosine at −292 had a C/G polymorphism (Fig. 1B), and it was confirmed that both the C allele and G allele were methylated. MSP primers were developed for these nine CGIs to detect their methylation (Fig. 2).

As for the remaining seven CGIs, five CGIs at the 5′ regions of XPCT, peHDP7, a homologue of Chimerin 1, DFKZp566K023, and KIAA0247 were unmethylated both in the normal sample and in the cancer cell lines, and two CGIs of RX and FHF-4 were methylated in both (data not shown).

Confirmation of Silencing for the Nine Genes. Expressions of the nine genes were analyzed in the normal sample (21N) and nine gastric cancer cell lines, including MKN28 and MKN74, by quantitative RT-PCR (Fig. 3). Their expressions in MKN28 and MKN74 were reduced to less than 1/10, generally less than 1/100, of 21N, whereas their expressions were retained in other gastric cancer cell lines without methylation of the CGIs in the 5′ regions.

To examine the role of methylation of the CGIs in gene silencing, MKN28 and MKN74 were treated with 5-aza-dC, a demethylating
agent. Demethylation of the 5' regions of the nine genes was observed in both cell lines (Fig. 2), and reexpression was induced (Fig. 3). These data showed that the methylation of the 5' regions of the nine genes caused their silencing.

Presence of Methylation and Reduced Expression in Primary Gastric Cancers. Using 41 primary gastric cancers and nine gastric cancer cell lines, MSP was performed to examine the methylation status of the nine CGIs identified here and two CGIs in the promoter regions of p16 and hMLH1 (Figs. 2 and 4). The CGIs in the 5' regions of FLNc, TM, HRASLS, HAND1, and LOX were methylated at high incidences (29–41% in primary cancers), whereas those of the other four genes were methylated at low incidences (0–7%). As for the normal samples, methylation was detected in 0–5 of the 28 samples (Fig. 4).

Expression levels of the nine genes, as well as p16 and hMLH1, were examined in the 41 primary gastric cancers and eight normal samples by quantitative RT-PCR (Fig. 5). Expression levels in cancer samples with methylation were generally reduced, but each gene had a unique expression profile. As for LOX, HRASLS, HAND1, PGAR, and TM, both overexpression and reduced expression were observed in the cancers without methylation, and reduced expression was observed in the majority of cancers with methylation. This pattern was similar to p16. As for hMLH1 and FLNc, reduced expression was observed in the cancers without methylation, and the reduction was clearer in the cancers with methylation. This pattern was similar to hMLH1. A homologue of RIKEN 220016F16 showed a similar tendency, although primary cancers with methylation were not available. As for FLNc, remarkable overexpression was observed in some cancers, both without methylation and with methylation.

Methylation and Hypomethylation Profile in the Primary Gastric Cancers. When the number of aberrant methylations in individual cancers was analyzed using the nine CGIs identified in this study, 11 cancers had aberrant methylations in four or more CGIs, whereas 16 cancers had no aberrant methylation at all (Fig. 4). Aberrant methylations of p16 and/or hMLH1 were detected in 10 of the 11 cancers with high prevalence of aberrant methylations but in 2 of the 16 cancers with no aberrant methylation (P < 0.0001, χ² test). All of the 11 cancers with high prevalence of aberrant methylations were diagnosed as diffuse-type gastric cancers, whereas 6 of the 16 cancers with no aberrant methylation were diffuse type (P < 0.001, χ² test). To examine the possible presence of aberrant hypomethylation of the NM-CGIs in the 5' regions, MAGE-A1, MAGE-A3, and MAGE-B2 (21–23) were selected. A high prevalence of hypomethylation of the NM-CGIs was observed in a different subset of gastric cancers (Figs. 2 and 4).

DISCUSSION

Nine CGIs in the 5' regions of nine genes were identified by MS-RDA as being completely methylated in two gastric cancer cell lines, MKN28 and MKN74 (Fig. 1). The nine genes were silenced in both cancer cell lines by the CGI methylation, but the normal sample and other unmethylated cancer cell lines retained expression (Figs. 2 and 3). The gene expressions in methylated cancer cell lines were not detected at all, or at very low levels less than 1/100 of cell lines without methylation. The residual expression could be derived from a tiny fraction of cells whose
methylation was removed spontaneously, or leaky expression could take place even if the promoter region was methylated.

Five of the nine genes were methylated at high incidences (29–41%) in 41 primary cancers, and two of the five, LOX and HRA SLS, were previously reported to have tumor-suppressive activities. Although the roles of LOX and HRA SLS in human gastric carcinogenesis still need to be studied, their methylations in cancers were first demonstrated here.

FLNc is a member of the filamin family, which is known to organize actin polymerization in response to various signals (28). In our previous study (29), a subunit of Arp2/3 complex, p41-Arc, was found to be reduced in human gastric cancers partly because of promoter methylation, and this complex is known to join the filamin family to regulate actin polymerization (28). These findings suggest that abrogation of normal actin polymerization may be important in gastric carcinogenesis. HAND1 encodes a basic helix-loop-helix transcriptional factor, which is essential for placent al development and cardiac morphogenesis (30). Its expression in normal gastric mucosa and silencing in gastric cancers suggested that HAND1 might have a role in the maintenance of differentiated status of gastric epithelium.

Analysis of primary cancers is difficult because contamination of normal cells in cancer tissues is inevitable, and “cancers with methylation” still have a chance to retain a normal allele. Therefore, the expressions of LOX, HRA SLS, ba305P22.2.3, HAND1, FLJ32130, PGAR, and TM can be regarded as reduced in association with their methylation, as in the case of p16 and hMLH1 (Fig. 5). This showed that the silencing found in the cell lines were also present in the primary cancers. LOX, HRA SLS, HAND1, PGAR, and TM showed both overexpression and reduced expression in cancers without methylation, similar to p16. An increase in the number of cells in cell cycle causes overexpression of p16, whereas loss of p16 contributes to tumorigenesis (32, 33). It was speculated that some genes with this type of expression profile, such as p16, might have roles in the negative regulation of cell growth. In contrast, ba305P22.2.3, a homologue of RIKEN 2210106F16 and FLJ32130, similar to hMLH1, showed only reduced expression in cancers without methylation. It was speculated that genes in this group may not be directly involved in the regulation of cell growth but that some of them might be involved in carcinogenesis, e.g., by maintaining genome stability.

Eleven of the 41 primary cancers showed aberrant methylations frequently (4 CGIs or more), and a significantly high incidence of p16 and hMLH1 methylations was observed in this subset of cancers (Fig. 4). This was in good agreement with a previous report that the “CGI methylator phenotype (CIMP)” was present in 41% of human gastric cancers (34). CIMP is known to be associated with p16 and hMLH1

Fig. 2. MSP analysis of the 5’ CGIs of the nine genes identified (A-I) and three MAGE genes (J-L). 1N, 5N, 6N, 17N, 18N, 21N, and 28N, normal samples. 4C, 5C, 6C, 17C, 18C, and 28C, cancer samples. A-aza, treated with 5-aza-dC. Ssxl genomic DNA of 21N treated with Ssxl methylase as a positive control for methylated DNA. A-I, specific amplification of unmethylated DNA by U primers and that of methylated DNA by M primers were confirmed by observing a band with U primers in 21N and a band with M primers in Ssxl-treated DNA. The 5’ CGIs of the nine genes were methylated in both MKN28 and MKN74, and their demethylation was induced by treatment with 5-aza-dC. Representative analysis of a cell line without methylation and primary samples with or without methylation are also shown. J-L, the 5’ CGIs of the three MAGE genes were methylated in normal samples (17N and 18N). Demethylation (−) in 17C and demethylation (+) in 18C were observed for all of the three genes.

Fig. 3. Expression of the nine genes in the normal sample, an unmethylated gastric cancer cell line, and MKN28 and MKN74 before (−) and after (+) 5-aza-dC treatment. Expression levels (shown in the logarithmic scale) were analyzed by quantitative RT-PCR and normalized with that of PCNA. The methylation status of each sample is shown in Fig. 2. For each of the nine genes, the expression in MKN28 and MKN74 was reduced to less than 1/10, generally less than 1/100, of 21N, whereas the expression was retained in a gastric cancer cell line without methylation. Treatment of MKN28 and MKN74 with 5-aza-dC resulted in reexpression of a gene to a level comparable with the expression level of a cell line without methylation.
methylations and with loss of imprinting of IGF2 (35). It is noteworthy, however, that the 11 cancers with high prevalence of aberrant methylations here were significantly associated with diffuse-type gastric cancers, although the original MS-RDA was performed using cell lines from intestinal-type cancers. It could be interpreted as: (a) genetic or epigenetic instability underlying CIMP led to the poorly differentiated histology; and/or (b) some of the methylations that had been considered as "aberrant" reflected the methylations that were present in the precursor cells of diffuse-type gastric cancers.

The molecular basis underlying CIMP is unknown. It was hypothesized that if decreased fidelity of maintenance methylation was involved in CIMP, aberrant hypomethylation would also be observed in CIMP+ cancers. We examined aberrant hypomethylation of three NM-CGIs at the 5′ regions of three MAGE genes (21–23). Five gastric

Fig. 5. Expression analysis of the nine genes, p16 and hMLH1. Quantitative RT-PCR was performed using 41 primary gastric cancers and eight normal samples. The expression levels were normalized with that of PCNA and shown by dots with their means; bars, SE. N, normal samples. C(U), cancer samples without methylation. C(M), cancer samples with methylation. Numbers of samples were shown at the bottom of each graph. In C(M), expression levels were generally reduced for the nine of the 11 genes. In C(U), expression levels of LOX, HRASLS, HAND1, PGAR, and TM, similar to p16, ranged from overexpression to reduced expression. Expression levels of baA305P22.2.3, a homologue of RIKEN 2210016F16, and FLJ32130 were consistently reduced, similar to hMLH1. Overexpression of FLNc was observed in some cancer samples.
cancers showed hypomethylation of all of the three but constituted a subset different from CIMP(+) cancers. Unusual clustering of hypomethylation was observed, and it was significant (P < 0.05) by a method reported previously (34). This indicated that a new phenotype, which could be designated as “CGI hypomethylator phenotype (CHOP),” is present and that CIMP and CHOP are caused by mechanisms other than decreased fidelity of maintenance methylation. Interestingly, four of five CHOP(+) gastric cancers were intestinal type, whereas 5 of 17 cancers with no aberrant hypomethylation were intestinal type (P < 0.05, χ² test). MAGE products are known as carcinotesticular antigens recognized by autologous cytotoxic T cells, which are used for cancer immunotherapy (36, 37). Therefore, there is a possibility that CHOP(+) cancers constitute a group of cancers where immunotherapy is effective.

MS-RDA was performed using a mixture of DNAs of two cancer cell lines as the driver. This strategy might have neglected infrequent methylations that occurred in one cell line only, because MS-RDA recognizes differentially methylated regions by selectively amplifying unmethylated fragments, and thus MS-RDA preferentially isolates DNA fragments methylated almost completely in the driver.6 But this strategy was effective to isolate aberrant methylations that commonly occurred in the two cell lines, and thus possibly in other cell lines and primary cancers as well. However, confirmation of aberrant methylation in primary cancers is still necessary to exclude methylation restricted to cell lines.

In the present study, we found that silencing of nine genes is present in human gastric cancers, that gastric cancers with high prevalence of aberrant methylations are associated with diffuse-type histology, and that a new phenotype, CHOP, exists.

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