Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2′-deoxycytidine treatment and oligonucleotide microarray

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To identify novel methylation-silenced genes in gastric cancers, we carried out a chemical genomic screening, a genome-wide search for genes upregulated by treatment with a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC). After 5-aza-dC treatment of a gastric cancer cell line (AGS) 579 genes were upregulated 16-fold or more, using an oligonucleotide microarray with 39 000 genes. From these genes, we selected 44 known genes on autosomes whose silencing in gastric cancer has not been reported. Thirty-two of these had CpG islands (CGI) in their putative promoter regions, and all of the CGI were methylated in AGS, giving an estimated number of 421 ± 75 (95% confidence interval) methylation-silenced genes. Additionally, we analyzed the methylation status of 16 potential tumor-related genes with promoter CGI that were upregulated four-fold or more, and 14 of these were methylated in AGS. Methylation status of the 32 randomly selected and 16 potential tumor-related genes was analyzed in 10 primary gastric cancers, and 42 genes (*ABHD9*, *ADFP*, *ALDH1A3*, *ANXA5*, *AREG*, *BDNF*, *BMP7*, *CAV1*, *CDH2*, *CLDN3*, *CTSL*, *EEF1A2*, *F2R*, *FADS1*, *FSD1*, *FST*, *FYN*, *GPR54*, *GREM1*, *IGFBP3*, *IGFBP7*, *IRS2*, *KISS1*, *MARK1*, *MLF1*, *MSX1*, *MTSS1*, *NT5E*, *PAX6*, *PLAGL1*, *PLAU*, *PPIC*, *RBP4*, *RORA*, *SCRN1*, *TBX3*, *TFAP2C*, *TNFSF9*, *ULBP2*, *WIF1*, *ZNF177* and *ZNF559*) were methylated in at least one primary gastric cancer. A metastasis suppressor gene, *MTSS1*, was located in a genomic region with frequent loss of heterozygosity (8q22), and was expressed abundantly in the normal gastric mucosa, suggesting its role in gastric carcinogenesis. (*Cancer Sci* 2006; 97: 64–71)

Epigenetic alterations are involved in cancer development and progression, and methylation of promoter CGI leads to transcriptional silencing of their downstream genes.[1] In various human cancers, silencing of tumor-suppressor genes, such as *CDKN2A* (*p16*), *CDH1* (E-cadherin) and *MLH1*, is known to be one of the major mechanisms for their inactivation, along with mutations and LOH. To identify genes silenced by promoter methylation by genome-wide screenings, various techniques have been developed.[2] Most techniques are based on the methylation status of genomic DNA, including MS-RDA and restriction landmark genomic scanning. In contrast, Suzuki *et al*. developed a technique that screens genes re-expressed after treatment with a demethylating agent, 5-aza-dC, using a microarray.[3] The chemical genomic screening technique is simple and is effective in identifying genes silenced in cell lines. It has been applied to colon, bladder, esophageal, pancreatic and prostate cancers.[3–7]

Gastric cancer is the second most common cause of cancer death in the world.[8] As its molecular basis, deep involvement of aberrant DNA methylation has been indicated by the higher incidences of aberrant DNA methylation of known tumor-suppressor genes than of mutations.[9] We previously searched for genes silenced in MKN28 and MKN74 cell lines using MS-RDA,[10] and identified lysyl oxidase as a novel tumor-suppressor gene.[11] However, the entire picture of methylation-silenced genes in gastric cancers is still unclear, and further searches for methylation-silenced genes are necessary.

In the present study, we carried out a chemical genomic screening of methylation-silenced genes in the human gastric cancer cell line AGS.

Materials and Methods

Tissue samples, cell lines and 5-aza-dC treatment

Ten primary gastric cancer samples (male/female = 7/3, aged 38–81 years) and two normal gastric mucosae were obtained from 10 patients undergoing gastrectomy at Aichi Cancer Center (Nagoya, Japan) with informed consent. These samples were frozen and stored at −80°C until extraction of DNA or RNA. Gastric cancer cell lines AGS, MKN28, MKN45 and KATOIII were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and American Type Culture Collection (Manassas, VA, USA). Two gastric cancer cell lines, HSC44 and HSC57, were gifted by Dr Kazuyoshi Yanagihara at the National Cancer Center Research Institute (Tokyo, Japan). AGS cells were seeded at a density of 3×10^5 cells/10 cm dish on day 0 and treated with freshly prepared 1 μM 5-aza-dC (Sigma) for 24 h on days 1, 3 and 5. After each treatment, the cells were placed in fresh medium and harvested on day 6. Genomic DNA was extracted by standard phenol/chloroform procedures. Total RNA was extracted using ISOGEN

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Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; CGI, CpG island; LOH, loss of heterozygosity; MS-RDA, methylation sensitive-representational analysis; MSP, methylation-specific PCR; PCR, polymerase chain reaction.

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(Nippon Gene, Tokyo, Japan) and purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA).

Oligonucleotide microarray analysis

Oligonucleotide microarray analysis was carried out using GeneChip Human Genome 133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) with 54 000 probe sets and 47 400 transcripts from 39 000 genes. From 8 μg of total RNA, the first-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, Groningen, the Netherlands) and a T7-(dT)24 primer (Amersham Bioscience, Buckinghamshire, UK), and the double-stranded cDNA was then synthesized. From the double-stranded cDNA, biotin-labeled cRNA was prepared using a BioArray HighYield RNA transcript labeling kit (Enzo, Farmingdale, NY, USA). Labeled cRNA (20 μg) was fragmented, and the GeneChips were hybridized. The arrays were stained and scanned according to the protocol from Affymetrix. The data were processed using GeneChip Operating Software. The signal intensities were normalized so that the average of all of the genes on a GeneChip would be 500. The *P*-values for different expression (change *P*-value) were calculated in each probe by statistical algorithms based on the Wilcoxon's signed rank test. The change *P*-values of 0.003 and 0.997 were used as thresholds to define genes with increased and decreased expression, respectively. Expression data for the normal tissues using GeneChip were obtained from the database RefEXA (http://www.lsbm.org/site_e/database/index.html),[12] with kind permission from Dr H. Aburatani.

Methylation-specific polymerase chain reaction

DNA (1 μg) digested with *Bam*HI was denatured in 0.3 M NaOH at 37°C for 15 min. Then, 3.6 M sodium bisulfite (pH 5.0) and 0.6 mM hydroquinone were added, and the sample underwent 15 cycles of 30-s denaturation at 95°C and a 15-min incubation at 50°C. The sample was desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI, USA) and desulfonated in 0.3 M NaOH. DNA was ethanol-precipitated and dissolved in 40 μL of Tris-EDTA buffer. MSP was carried out with a primer set specific to the methylated or unmethylated sequence (M or U set), using 0.5 μL of the sodium-bisulfite-treated DNA. A region 200 bp or less upstream of a putative transcriptional start site was analyzed, except for *BDNF* (−401 to −214). Primer sequences and PCR conditions are shown in Table 1. DNA methylated with *Sss*I methylase was used to determine specific conditions of PCR for M sets.

Results

Oligonucleotide microarray analysis

AGS cells were treated with 1 μM of 5-aza-dC, which caused growth suppression at 49%, and upregulated genes were searched for using an oligonucleotide microarray. Among the 39 000 genes (54 000 probe sets) analyzed, 1430 genes (1747 probes) were upregulated four-fold or more (signal log ratio > 2) and 579 genes (678 probes) were upregulated 16-fold or more (signal log ratio > 4). To identify silenced genes with known functions from the 579 genes, we excluded genes on chromosome X (95 probes, 70 genes) and genes without known functions (i.e. *FLJ* genes, *KIAA* genes, *LOC* genes, *MG* genes and *Orf* genes [149 probes, 141 genes]).

Among the remaining 368 genes (434 probes), we found eight genes (14 probes) whose methylation-silencing had been reported in gastric cancers (*BNIP3*,[13] *CDKN2A* (*p16*),[14] *CHFR*,[15] *ID4*,[16] *RBP1*,[17] *RUNX3*,[18] *THBD*,[10] *TIMP*[19]). The remaining 360 genes (420 probes) were considered as candidates for novel methylation-silenced genes in gastric cancers (Table 1).

Methylation analysis of genes upregulated by 5-aza-dC treatment

From the 360 genes upregulated 16-fold or more, we selected 44 genes randomly (Table 2). Among these 44 genes, 32 genes (73%) had CGI in their 5′ regions, which were considered as promoter regions (Table 2). To examine whether the induction of these genes by 5-aza-dC treatment was really due to promoter demethylation, the methylation status of these 5′-CGIs were analyzed by MSP. For all the 32 genes, only methylated molecules were detected before 5-aza-dC treatment, and unmethylated DNA molecules were detected after the treatment in AGS, suggesting silencing of the 32 genes by methylation of their 5′-CpG islands (representative results in Fig. 1).

Analysis of five additional gastric cancer cell lines (MKN28, MKN45, HSC44, HSC57, KATOIII) showed that five genes (*ANXA5*, *AREG*, *CAV1*, *IL6R*, *TBX3*) were methylated only in AGS, and 27 genes were methylated in multiple gastric cancer cell lines (Table 3). The microarray analysis of KATOIII and HSC57 showed that none of the 32 genes were expressed when unmethylated DNA molecules were not present.

We next selected 16 potential tumor-related genes with promoter CGI and four-fold or greater upregulation as the above analysis suggested that a considerable number of silenced genes were still present among the genes with upregulation of 16-fold or less (Table 2). The potential tumor-related genes were selected based on their tumor-related function and location in genomic regions with frequent LOH (5q21-23,[20,21] 8p22,[20,21] 9p12-24[20–22]) or with DNA loss by comparative genomic hybridization (19p13.12-p13.3[23]) in gastric cancers. MSP showed that 14 of these 16 genes were methylated in AGS before 5-aza-dC treatment (Table 3). *CDKN2D* and *SNAI1* were not methylated even before 5-aza-dC treatment, suggesting that they were induced as a stress response by 5-aza-dC treatment.

Presence of methylation in primary gastric cancers

The methylation status of the above 48 genes (32 selected randomly and 16 tumor-related genes) were examined in 10 primary gastric cancers. It was shown that 42 genes (*ABHD9*, *ADFP*, *ALDH1A3*, *ANXA5*, *AREG*, *BDNF*, *BMP7*, *CAV1*, *CDH2*, *CLDN3*, *CTSL*, *EEF1A2*, *F2R*, *FADS1*, *FSD1*, *FST*, *FYN*, *GPR54*, *GREM1*, *IGFBP3*, *IGFBP7*, *IRS2*, *KISS1*, *MARK1*, *MLF1*, *MSX1*, *MTSS1*, *NT5E*, *PAX6*, *PLAGL1*, *PLAU*, *PPIC*, *RBP4*, *RORA*, *SCRN1*, *TBX3*, *TFAP2C*, *TNFSF9*, *ULBP2*, *WIF1*, *ZNF177* and *ZNF559*) were methylated in at least one gastric cancer (Table 3). The numbers of methylated genes in each case ranged from one to 10. Case 6 had a large number of methylated genes, which was similar to AGS (Table 3). The expression levels of the 48 genes in the normal gastric mucosae were obtained from the RefEXA database (Table 3).

Table 1. Primers for methylation-specific polymerase chain reaction

Genes	M/U	Forward primer		Reverse primer		Annealing (°C)	No. cycles
		Position†	Sequence	Position	Sequence		
ADFP	M	−169	GGTCGGGTTTTCGTTCGGTTTTC	−36	ACCCGAATATCACCCTCGAACACG	55	34
	U	−170	AGGTTGGGTTTTTGTTTGGTTTTT	−36	ACCCAAATATCACCCTCAAACACA	57	34
ALDH1A3	M	−108	TCGGTTTCGTAGTTAATTAGGC	−18	GACTCGACCCGAACACTACGCA	55	35
	U	−108	TTGGTTTTGTAGTTAATTAGGT	−18	CAACTCAACCCAAACACTACACA	49	34
ANXA5	M	−164	TATTTAGGTTCGCGAGATTAGC	−48	CCAAAACCCCAACCGCAAACCG	57	34
	U	−154	TGTGAGATTAGTGGGATAGTTT	−48	ACCAAAACCCCAACCACAAACCA	55	34
AREG	M	−173	TTTTTAGCGAATTTTTACGTAC	−22	ATAAAACGACGCGCACCTACCG	55	35
	U	−165	GAATTTTTATGTATGAGGGAGGT	−22	ATAAAACAACACACACCTACCA	55	34
BDNF	M	−401	TACGTAAATAGCGAGGTTAGTC	−214	AACTCCGACGAAACTAAATTCG	55	34
	U	−411	GTGAGTTGGTTATGTAAATAGT	−214	AACTCCAACAAAACTAAATTCA	52	34
CAV1	M	−80	TTTCGGGACGTTTTTCGGTGGT	−6	TAAAAACGTTTCTCCCGCGCTA	59	34
	U	−95	GAAAATATTTGTTTTTTTTGGGAT	−3	ACAAATAAAAACATTTCTCCCACA	55	35
EEF1a2	M	−248	GTTTCGTTTTTCGGGTTCGTC	−28	GCCCTACAACACGCCAATACG	57	34
	U	−250	TTGTTTTGTTTTTTGGGTTTGTT	−28	ACACCCTACAACACACCAATACA	58	34
F2R	M	−187	TTAGGAGGGTCGAGACGGTCGC	−96	TCCTCTAAACACCGTTAATTCG	55	34
	U	−189	TTTTAGGAGGGTTGAGATGGTTGT	−98	TCCTCTAAACACCATTAATTCACA	55	35
FADS1	M	−234	GTTCGTTTGACGTTAGGAAGTC	−34	GCCCAAAACCAACCGCCTACG	55	35
	U	−234	GTTTGTTTGATGTTAGGAAGTT	−34	CACCCAAAACCAACCACCTACA	55	34
FSD1	M	−159	AGGGTTTTGGGCGAGGTTAGC	−25	AAACTACCTTTACCGCGACCG	56	34
	U	−158	GGGTTTTGGGTGAGGTTAGTGT	−25	CAAACTACCTTTACCACAACCA	58	34
FST	M	−172	TTTAGATTTAAAGCGCGGTTGC	−47	ACGAATAACTCGAACGAACG	55	34
	U	−173	GTTTAGATTTAAAGTGTGGTTGT	−47	ACAAATAACTCAAACAAACA	55	34
GREM1	M	−134	CGTCGGTATTTAAACGGGAGAC	−35	GAAACTCGACGCGAAATCAACG	55	35
	U	−134	TGTTGGTATTTAAATGGGAGAT	−35	CAAAACTCAACACAAAATCAACA	55	34
IGFBP7	M	−195	GGGTCGGTTACGTCGGGTGTTC	−18	GACAAAAACGCGAATAAACCG	55	35
	U	−197	ATGGGTTGGTTATGTTGGGTGTTT	−18	CAACAACAAAAACACAAATAAACCA	60	35
IL6R	M	−117	TTTTTATAGCGTAATTTCGTTTAC	78	AACCGAAACGAATAACGCAACA	48	35
	U	−124	GGTGTGTTTTTTATAGTGTAATTTT	65	TAACACAACAACCCCACACACCA	60	34
IRS2	M	−127	GCGGCGTTAATGCGAGGTAGC	−29	TAAATAACACATCGCGCACCG	55	35
	U	−128	TGTGGTGTTAATGTGAGGTAGT	−24	CACACAATAAATAACACATCACA	60	35
KISS1	M	−198	AAAGTTTCGTTTCGGAGGGTTC	−49	CTTTTATAAAACCCGAAATAACG	58	34
	U	−198	AAAGTTTTGTTTTGGAGGGTTT	−49	CCTTTTATAAAACCCAAAATAACA	55	33
MARK1	M	−268	TTTAGACGATCGTAAATTTTGC	−26	TCAAAAAAAACGACCCGAACCG	52	34
	U	−216	GGATAGGTGGGTAAGAGAGTGT	−32	AAAACAACCCAAACCAACTACA	55	34
MLF1	M	−118	GGGTAGCGGCGTATTGTTTTTC	−16	CTCACTCGCCGCGACGCAAACG	55	35
	U	−120	TAGGGTAGTGGTGTATTGTTTTTT	−7	ACAAACAACACCTCACTCACCACA	60	35
MSX1	M	−178	CGTCGTTTGGGTTTTGTTTTGC	−18	CCGACTCCGAACCCTACCG	55	34
	U	−160	TTGTGTGTTTTTAGGTTTAGTGT	−18	CCAACTCCAAACCCTACCA	58	34
MX1	M	−178	GGGTTCGGGTTCGAGAATTTGC	−21	TTCGCCTCTTTCACCCCG	55	34
	U	−179	TGGGTTTGGGTTTGAGAATTTGT	−21	ACTTCACCTCTTTCACCCCA	55	36
NT5E	M	−183	AGTCGATAGTCGCGTTAGGGTC	−36	GAACAACTAAAACCGAAACTCG	55	35
	U	−184	TAGTTGATAGTTGTGTTAGGGTT	−41	AACTAAAACCAAAACTCAATACC	53	35
PLAGL1	M	−195	GTTCGGGTTTATTTGCGTTAGC	−47	AACCCCTAACGAAAACGTCACG	60	33
	U	−196	GGTTTGGGTTTATTTGTGTTAGT	−47	CCCCTAACAAAAACATCACA	60	34
PPIC	M	−162	GTTTTTCGTATTCGTTTAAGGC	−33	AAAATAAAAATCGAACAATCCG	55	35
	U	−165	GGTGTTTTTTGTATTTGTTTAAGGT	−57	AAAAACAAAAACCCAAAACACA	55	34
PYCARD	M	−186	CGGGGAATCGCGGAGGTTTC	−36	AATAAAACCCGAAAAAAAACCG	55	35
	U	−190	GGTTTGGGGAATTGTGGAGGTTTT	−13	ATCACACCCTCCAACTAACCTACA	55	35
RBP4	M	−32	TTCGGGTTTCGGTGAGTTAGGGC	69	CCGCTACTTTATAACGCCG	58	34
	U	−33	GTTTGGGTTTTGGTGAGTTAGGGT	69	ACCCCACTACTTTATAACACCA	60	33
RGS2	M	−184	ACGTTAGTAGCGTTTCGGTTTC	−37	GTCGCAACATTTATAAAACCTCG	55	35
	U	−185	GATGTTAGTAGTGTTTTGGTTTT	−37	CATCACAACATTTATAAAACCTCA	60	34
SCRN1	M	−106	GAGGGTGGGTTCGCGGTTAC	−14	CTACAATAACGAAAACGACCG	55	35
	U	−106	GAGGGTGGGTTTGTGGTTATGT	−21	CAATAACAAAAACAACCACCAAACA	60	35
TBX3	M	−98	TTGGTTCGAAAGCGTTAAAGAG	−22	ACCGAACGTCTACTCGACGACT	53	35
	U	−110	GTAGTAATATAATTGGTTTGAAAGT	−33	CTACTCAACAACTCTAAAAAATCA	55	35
TFAP2C	M	−146	GCGTTGCGTTAGGTTCGGGTGC	40	CGCGAATATCAAAACCGCTCCG	55	35
	U	−148	TGGTGTTGTGTTAGGTTTGGGTGT	40	ACCACAAATATCAAAACCACTCCA	60	35
ULBP2	M	−213	TGAGTTTGTCGTGGAAGGAATC	−89	GTCAAACGAATCATAACGTCACG	55	35
	U	−193	TTGTGTTTTGGTAGGAGTTGGGT	−71	ATCAAACAAATCATAACATCACA	52	34

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Table 1. Continued

Genes	M/U	Forward primer		Reverse primer		Annealing (°C)	No. cycles
		Position†	Sequence	Position	Sequence		
WIF1	M	−131	CGTTCGCGTTTTATTTTTTTGC	−27	AACGCGTCGCCTCCCGACCTAA	53	35
	U	−126	GTGTTTTATTTTTTTGTGTGATTT	−21	AACAACTAAACACATCACCTCCCA	55	34
ZNF559	M	−147	GGTTCGGGAATTCGAGGTTTC	−43	TACCTCAAACGCCAACGAAAACG	58	34
	U	−149	TGGGTTTGGGAATTTGAGGTTTT	−70	CTATTAAAATAACAACCATTATACA	52	34
ABHD9	M	−195	CGTGAGTTATCGTATTCGGTTC	−115	TCCTATACGAAACTTAAAACCG	59	33
	U	−197	GGTGTGAGTTATTGTATTTGGTTT	−102	ACAAAACCTAACAAATCCTATACA	55	34
BMP7	M	−227	GTTTTTTCGTTGTTTTTTCGGC	−82	ATACTAACCCCGAACCCCTCG	55	34
	U	−231	GTTTGTTTTTTTGTTGTTTTTTTGGT	−82	AATACTAACCCCAAACCCCTCA	59	34
CDH2	M	−217	GCGGTGTCGTTATATAGTAGC	−128	ACTCTAAACCTACGCCGCCG	50	34
	U	−294	GTAAAATTATGAGTTTGAAATTTTGT	−114	AAAAAAAAACATATAAACATCTACA	55	34
CDKN2D	M	−122	GCGGTGTCGTTATATAGTAGC	−15	ACTCTAAACCTACGCCGCCG	55	34
	U	−164	GGTTTTGTGGGTGGAATGTT	−15	CTACTCTAAACCTACACCACCA	55	34
CLDN3	M	−111	AGGTTTTGGAGAGCGCGGTTTC	−47	ACCCTAAACTAAAACCGATACG	50	34
	U	−105	TGGAGAGTGTGGTTTTGTTTTTATT	−47	CTAACCCTAAACTAAAACCAATACA	55	34
CTSL	M	−182	GATTTTATTTTGCGTCGTTTC	−40	ACGCTACGATTAACTATACCG	55	34
	U	−186	GTTTGATTTTATTTTGTGTTGTTTT	−40	ACTACACTACAATTAACTATACCA	48	34
FYN	M	−228	TCGTACGTATTTTGGGATGTTC	−141	CTACGAACCGCAACCATTAACG	55	34
	U	−229	ATTGTATGTATTTTGGGATGTTT	−129	ACCCCTTAAAAACTACAAACCA	55	34
GPR54	M	−200	TTATAAACGTTCGGTCGTAGC	−54	CAAAATTACGCCCTAACACCG	52	34
	U	−206	TATGGGTTATAAATGTTTGGTT	−54	CAAAATTACACCCTAACACCA	58	34
IGFBP3	M	−99	TTTCGGTTTTTATATAGCGGTC	−37	AAAAAACGACTAATCCTCAACG	55	34
	U	−102	TTATTTTGGTTTTTATATAGTGGTT	−37	AACAAAAAACAACTAATCCTCAACA	48	34
MTSS1	M	−130	GAGAGCGCGTTTTCGTTTGGC	−32	CGCCTCCTTTTCACTCCTACG	59	34
	U	−130	GAGAGTGTGTTTTTGTTTGGT	−32	CCACCTCCTTTTCACTCCTACA	55	34
PAX6	M	−188	AGGGAGTATTTAATCGGTTGGC	−47	CTCCTACGCCTAAACCAAAACG	59	34
	U	−138	GTAATATTTTGTGTGAGAGTGAGT	−47	TCCTCCTACACCTAAACCAAAACA	55	34
PLAU	M	−177	TTTGTGAGCGTTGCGGAAGTAC	−51	ACGATCTCCGCACTATACTACG	55	34
	U	−153	GGGGTTTGGGTTGTTGAGT	−51	CTACAATCTCCACACTATACTACA	50	34
RORA	M	−213	GGTTGGAGAAGTTTTCGTTAGC	−111	GACGAACGAACAAACAAAAACG	55	34
	U	−215	TTGGTTGGAGAAGTTTTTGTTAGT	−123	CAAACAAAAACACAAAAAAACACA	55	34
SNAI1	M	−155	ATTTGTTCGGGGAGTGGTTTTC	−91	AAAACGAAACCTTATCTACCACG	55	34
	U	−213	GGAGTTTTTGTTTGGGTTTTTATT	−91	AAAAACAAAACCTTATCTACCACA	55	34
TNFSF9	M	−198	GTCGAGTTTGGAAGGTCGGAAAC	−65	AAAAAACCACGCCCCTCCG	56	34
	U	−199	GGTTGGAAATGGAAAGGAGAGT	−65	AAAAAAAACCACACCCCTCCA	55	34
ZNF177	M	−119	GTAGGAGTATTTGCGATGTTTC	−12	AAAATAACGAAACGACGAACG	58	34
	U	−97	GTTTTTAAGTTTTTAGGGTGAATTT	−22	AAACAACAAACACCCACTTCCA	55	34

†Transcription start site = 0. All primers were designed on the top strand sequences. M, specific to methylated DNA; U, specific to unmethylated DNA.

Table 2. Genes upregulated after 5-aza-dC treatment in the AGS cell line

Probe set	Gene title	Symbol	Fold change	CGI
The 44 genes picked randomly from the genes showing greater than 16-fold upregulation after 5-aza-dC treatment				
209122_at	*Adipose differentiation-related protein*	ADFP	36.0	Yes
203180_at	*Aldehyde dehydrogenase 1 family, member A3*	ALDH1A3	46.2	Yes
200782_at	*Annexin A5*	ANXA5	32.5	Yes
205239_at	*Amphiregulin (schwannoma-derived growth factor)*	AREG	59.3	Yes
206382_s_at	*Brain-derived neurotrophic factor*	BDNF	29.2	Yes
203065_s_at	*Caveolin 1, caveolae protein, 22 kDa*	CAV1	37.2	Yes
210140_at	*Cystatin F (leukocystatin)*	CST7	22.1	No
219424_at	*Epstein–Barr virus induced gene 3*	EBI3	18.5	No
204540_at	*Eukaryotic translation elongation factor 1 alpha 2*	EEF1A2	25.0	Yes
203989_x_at	*Coagulation factor II (thrombin) receptor*	F2R	59.3	Yes
208962_s_at	*Fatty acid desaturase 1*	FADS1	23.0	Yes
203240_at	*Fc fragment of IgG binding protein*	FCGBP	16.8	No
1570515_a_at	*Filamin A interacting protein 1*	FILIP1	34.8	No
219170_at	*Fibronectin type 3 and SPRY domain containing 1*	FSD1	16.0	Yes
226847_at	*Follistatin*	FST	29.2	Yes

Table 2. Continued

Probe set	Gene title	Symbol	Fold change	CGI
218469_at	*Gremlin 1 homolog, cysteine knot superfamily*	GREM1	59.3	Yes
213620_s_at	*Intercellular adhesion molecule 2*	ICAM2	18.5	No
201162_at	*Insulin-like growth factor binding protein 7*	IGFBP7	38.4	Yes
205945_at	*Interleukin 6 receptor*	IL6R	26.0	Yes
209185_s_at	*Insulin receptor substrate 2*	IRS2	24.0	Yes
205563_at	*KiSS-1 metastasis-suppressor*	KISS1	54.8	Yes
221047_s_at	*MAP/microtubule affinity-regulating kinase 1*	MARK1	17.6	Yes
1552456_a_at	*Methyl-CpG binding domain protein 3-like 2*	MBD3L2	38.4	No
206560_s_at	*Melanoma inhibitory activity*	MIA	23.0	No
204784_s_at	*Myeloid leukemia factor 1*	MLF1	27.0	Yes
205932_s_at	*Msh homeo box homolog 1 (Drosophila)*	MSX1	16.0	Yes
202086_at	*Myxovirus (influenza virus) resistance 1*	MX1	18.5	Yes
205581_s_at	*Nitric oxide synthase 3 (endothelial cell)*	NOS3	18.5	No
203939_at	*5′-nucleotidase, ecto (CD73)*	NT5E	53.3	Yes
207002_s_at	*Pleiomorphic adenoma gene-like 1*	PLAGL1	46.2	Yes
204517_at	*Peptidylprolyl isomerase C (cyclophilin C)*	PPIC	23.0	Yes
221666_s_at	*PYD and CARD domain containing*	PYCARD	25.0	Yes
219140_s_at	*Retinol binding protein 4, plasma*	RBP4	43.6	Yes
202388_at	*Regulator of G-protein signaling 2, 24 kDa*	RGS2	33.6	Yes
201462_at	*Secernin 1*	SCRN1	31.4	Yes
204614_at	*Serine (or cysteine) proteinase inhibitor, clade B, member 2*	SERPINB2	36.0	No
202627_s_at	*Serine (or cysteine) proteinase inhibitor, clade E, member 1*	SERPINE1	26.0	No
208539_x_at	*Small proline-rich protein 2 A*	SPRR2A	22.1	No
224167_at	Likely ortholog of mouse spermatogenic Zip 1	SPZ1	18.5	No
219682_s_at	*T-box 3*	TBX3	36.0	Yes
205286_at	*Transcription factor AP-2 gamma*	TFAP2C	32.5	Yes
221291_at	*UL16 binding protein 2*	ULBP2	29.2	Yes
204712_at	*WNT inhibitory factor 1*	WIF1	31.4	Yes
224518_s_at	*Zinc finger protein 559*	ZNF559	30.3	Yes
Genes showing greater than four-fold upregulation after 5-aza-dC treatment, having CpG islands, and having cancer related function or having chromosomal location in the region of frequent loss in gastric cancer.				
220013_at	*Abhydrolase domain containing 9*	ABHD9	8.0	Yes
209591_s_at	*Bone morphogenetic protein 7 (osteogenic protein 1)*	BMP7	13.9	Yes
203440_at	*Cadherin 2, type 1, N-cadherin (neuronal)*	CDH2	26.0	Yes
210240_s_at	*Cyclin-dependent kinase inhibitor 2D (p19)*	CDKN2D	4.4	Yes
203953_s_at	*Claudin 3*	CLDN3	45.3	Yes
202087_s_at	*Cathepsin L*	CTSL	13.0	Yes
216033_s_at	*FYN oncogene related to SRC, FGR, YES*	FYN	78.8	Yes
242517_at	*G protein-coupled receptor 54*	GPR54	6.3	Yes
210095_s_at	*Insulin-like growth factor binding protein 3*	IGFBP3	9.8	Yes
203037_s_at	*Metastasis suppressor 1*	MTSS1	6.5	Yes
205646_s_at	*Paired box gene 6 (aniridia, keratitis)*	PAX6	18.4	Yes
205479_s_at	*Plasminogen activator, urokinase*	PLAU	137.2	Yes
210479_s_at	*RAR-related orphan receptor A*	RORA	55.7	Yes
219480_at	*Snail homolog 1 (Drosophila)*	SNAI1	4.6	Yes
206907_at	*Tumor necrosis factor superfamily, member 9*	TNFSF9	4.8	Yes
207417_s_at	*Zinc finger protein 177*	ZNF177	4.4	Yes
Genes reported as silenced genes in gastric cancer and showing greater than 16-fold upregulation after 5-aza-dC treatment				
201848_s_at	*BCL2/adenovirus E1B 19 kDa interacting protein 3*	BNIP3	20.3	Yes
207039_at	*Cyclin-dependent kinase inhibitor 2 A (p16)*	CDKN2A	33.6	Yes
223931_s_at	Checkpoint with forkhead and ring finger domains	CHFR	16.8	Yes
209291_at	*Inhibitor of DNA binding 4*	ID4	57.8	Yes
203423_at	*Retinol binding protein 1, cellular*	RBP1	54.8	Yes
204198_s_at	*Runt-related transcription factor 3*	RUNX3	27.0	Yes
203888_at	*Thrombomodulin*	THBD	29.2	Yes
201147_s_at	*Tissue inhibitor of metalloproteinase 3*	TIMP3	65.6	Yes

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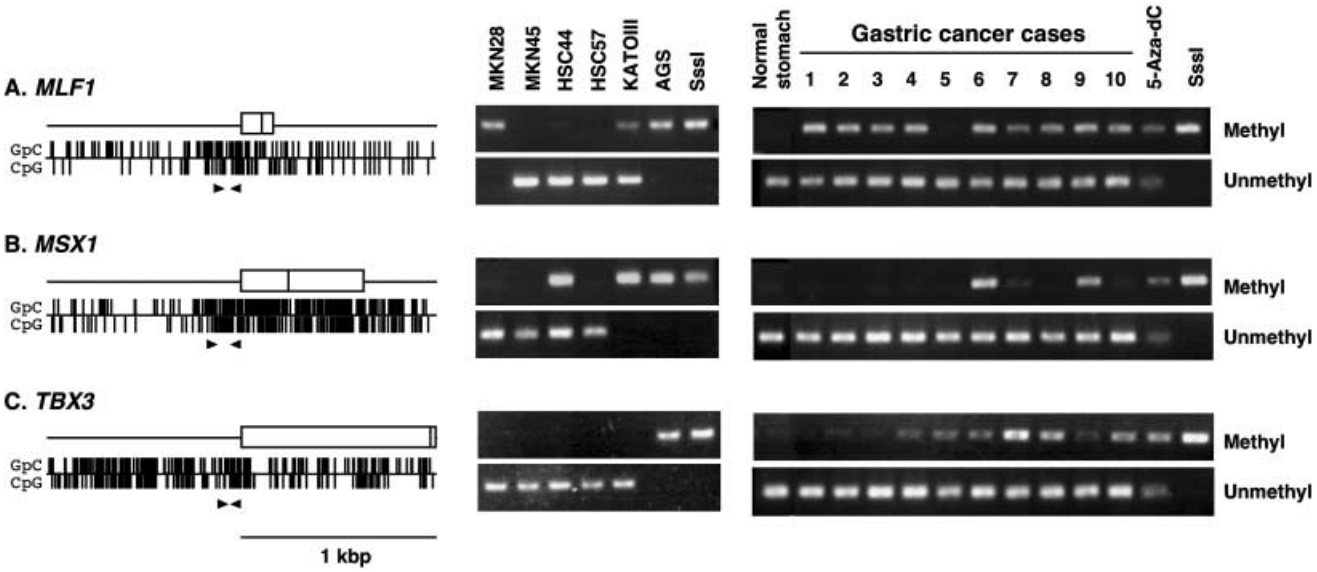

Fig. 1. A representative result of methylation analysis. (A) *MLF1*; (B) *MSX1*; and (C) *TBX3*. The left sides of each panel represent the 5′ CpG islands and regions analyzed by methylation-specific polymerase chain reaction (MSP). Vertical marks, individual GpC and CpG sites; Open boxes, non-coding and coding exons; and arrowheads, positions of MSP primers (M sets). The right sides show the results of MSP in gastric cancer cell lines, normal gastric mucosa and primary gastric cancers. 5-aza-dC, AGS cells after treatment with 5-aza-2′-deoxycytidine; *Sss*I, genomic DNA methylated with *Sss*I methylase.

There remained a possibility that these silenced genes were normally methylated or were methylated tissue-specifically. Therefore, we selected 11 genes with relatively high chances of having methylated CGI, based on their low expression in the normal gastric mucosae (*CLDN3*, *FADS1*, *KISS1*, *PAX6*, *PLAGL1*, *RBP4*, *RORA*, *ULBP2*, *WIF1*, *ZNF177* and *ZNF559*). Along with three additional genes (*MLF1*, *MSX1* and *TBX3*), their methylation status was examined in the normal gastric mucosae. However, none of the 14 genes were methylated.

Discussion

Chemical genomic screening revealed that a considerable number of genes were methylation-silenced in the AGS gastric cancer cell line. After 5-aza-dC treatment of AGS, 579 genes were upregulated 16-fold or more. When we analyzed 44 selected genes, 32 of them had CGI in their promoter regions, and all of the 32 genes turned out to be methylation-silenced. Because 32 of the 44 genes selected from 579 genes were silenced, it was estimated that 421 ± 75 (95% confidence interval) genes were silenced in AGS. To avoid overestimation, we randomly selected 44 genes from the 360 genes after excluding: (i) genes on chromosome X, which harbors many normally methylated genes like *MAGE*; (ii) genes that have not been characterized yet; and (iii) genes whose methylation-associated silencing was already known in gastric cancers. Among the 16 potential tumor-related genes, 10 were upregulated 16-fold or less, and eight of the 10 genes were found to be methylation-silenced. If genes with relatively small upregulation were analyzed, the number of silenced genes in AGS was expected to be larger.

As for the number of methylation-silenced genes in a cancer, Costello *et al*. estimated that an average of 600 CGI in the whole genome were methylated aberrantly in the tumors.[24] However, the number was calculated by analyzing CGI in any location against a gene, and the number of genes silenced, for which methylation of promoter CGI is necessary, was not determined. Using chemical genomic screening, Sato *et al*. estimated that an average of 140 genes would be methylated aberrantly in pancreatic cancers.[6] Compared with this number, the number of genes silenced in the AGS cell line was considered to be much larger. We recently found that AGS had an increased rate of *de novo* methylation,[25] and this could be one of the mechanisms.

By methylation analysis of 48 genes (Table 3), 46 genes were found to be methylated in AGS, and 42 genes were methylated in at least one primary gastric cancer. Among the 42 genes, eight genes (*CAV1*,[26,27] *IGFBP3*,[28] *IGFBP7* [*MAC25/IGFBP-rP1*],[29] *PAX6*,[30] *PLAGL1* [*ZAC/LOT1*],[31,32] *PLAU* [*uPA*],[33,34] *RBP4*[35] and *WIF1*[36]) were reported to be silenced with functional relevance in cancers other than gastric cancers. In addition, two genes (*CDH2*[37] and *FYN*[38]) were reported to be methylated in some cancers, but their functional significance needs clarification.

Also among the 32 genes whose silencing was novel, we were able to find potential tumor-related genes. To achieve this, some genes were selected based on (i) antioncogenic cellular functions or (ii) location in genomic regions with frequent LOH in gastric cancers. Candidate tumor-related genes were further selected based on (iii) the presence of methylation of promoter CGI in primary gastric cancers, and (iv) expression in normal gastric mucosae when various tissues were compared. *MTSS1/MIM/BEG4* met all of these criteria, and was a good candidate for a novel tumor-related gene. It mediates Sonic hedgehog signaling by potentiating Gli-dependent transcription,[39] and is known as a metastasis suppressor gene in bladder cancers.[40] Although LOH was not frequent in their locations, *ANXA5*, *AREG*, *GREM*, *IGFBP7*, *IRS2*, *BMP7*, *CTSL* and *IGFBP3* were expressed in the normal

Table 3. Methylation profiles in gastric cancer

Gene Symbol	Function	Chromosomal location	Methylation status in gastric cancer cell line†						Expression (signal intensity of GeneChip)					Methylation status in gastric cancer‡										
													+5-aza-dC	Stomach§	Case1	Case2	Case3	Case4	Case5	Case6	Case7	Case8	Case9	Case10	
			MKN28	MKN45	HSC44	HSC57	KATOIII	AGS	HSC57	KatoIII	AGS	AGS	(RefEXA)		tub1	tub2	por2	por	tub2	por1	por2	por	por2	por2	Normal
32 genes selected randomly and with GGI																									
ADFP	Fatty acid transport	9p22.1	M	U	M/U	U	M	M	2191	74	33	1186	154		U	U	U	U	U	M	U	U	U	U	–
ALDH1A3	Vitamin A metabolism	15q26.3	U	U	U	U	M	M	322	69	16	1435	65		U	U	U	U	U	M	M	U	M–	U	–
ANXA5	Apoptosis	4q28-q32	U	U	U	U	U	M	1740	4933	144	6960	433		U	U	U	U	U	M	U	U	U	U	–
AREG	Cell proliferation	4q13-q21	U	U	U	U	U	M	9568	9103	7	2677	137		U	U	U	U	U	M	U	U	U	U	–
BDNF	Growth factor activity	11p13	M	M	M	M/U	U	M	50	20	5	447	32		M	U	M	M	U	M	M	M	M	M	–
CAV1	Cell aging	7q31.1	U	U	U	U	U	M	24	167	20	1411	102		U	U	U	U	U	M	U	U	U	U	–
EEF1A2	Translational elongation	20q13.3	U	U	M	U	M	M	240	60	112	7234	58		U	U	U	U	U	M	U	U	U	U	–
F2R	G-protein signaling	5q13	U	U	M/U	U	M	M	140	7	4	820	26		U	U	U	U	U	M	M	U	U	U	–
FADS1	Fatty acid desaturation	11q12.2-q13.1	U	U	M/U	U	M	M	375	19	22	542	40		U	U	U	U	U	M	M–	M	M	U	–
FSD1	Microtubule depolymerization	19p13.3	U	M/U	M/U	U	M	M	49	25	31	362	5		U	U	U	U	U	M	U	U	M	U	U
FST	Transcription factor	5q11.2	U	U	M	U	M	M	6	4	7	278	–		U	U	U	U	U	M	M	U	M	U	–
GREM1	Signal transduction	15q13–15q15	M	M	M	U	M	M	77	33	5	2128	184		M	M	M	M	M	M	M	M	M	M	–
IGFBP7	Angiogenesis	4q12	M	U	M	U	M	M	13	11	9	934	483		U	U	M–	U	U	M	M	U	M	M–	–
IL6R	Immune response	1q21	U	U	U	U	U	M	124	227	12	574	72		U	U	U	U	U	U	U	U	U	U	–
IRS2	Signal transduction	13q34	U	U	U	U	M	M	6602	57	30	1245	274		U	U	U	U	U	U	U	U	M–	U	–
KISS1	Metastasis suppressor	1q32	M/U	M	M	U	U	M	19	26	21	3697	7		U	M–	M–	M–	U	M–	M–	M–	M–	M–	U
MARK1	Protein amino acid phosphorylation	1q41	U	U	U	U	M	M	79	85	45	845	43		U	U	U	U	U	M	U	M	U	U	–
MLF1	Cell differentiation	3q25.1	M	U	U	U	M/U	M	936	1078	22	709	42		M	M	M	M	U	M	M	M	M	M	U
MSX1	Transcription factor	4p16.3-p16.1	U	U	M/U	U	M	M	524	13	43	2675	32		U	U	U	U	U	M	M–	U	M	U	U
MX1	Response to virus	21q22.3	M	U	M	U	U	M	856	1533	48	996	223		U	U	U	U	U	U	U	U	U	U	–
NT5E	DNA metabolism	6q14–6q21	U	U	U	U	M/U	M	115	394	4	821	31		U	U	U	U	U	U	M–	U	U	U	–
PLAGL1	Induction of apoptosis	6q24–6q25	M	M	M	M/U	M	M	456	16	4	668	81		M–	M	M	M	M	M	M	M	M	M	U
PPIC	Signal transduction	5q23.2	U	U	U	U	M	M	2724	59	49	1621	186		U	U	U	U	U	M	M–	U	U	U	–
PYCARD	Signal transduction	16p12–16p11.2	M	M	M	U	U	M	2739	557	9	420	39		U	U	U	U	U	U	U	U	U	U	–
RBP4	Vitamin A metabolism	10q23–10q24	M	U	M	U	U	M	86	643	10	880	11		U	U	U	M–	M–	M	M	M–	U	M–	U
RGS2	G-protein signaling	1q31	U	U	U	U	M/U	M/U	27	90	27	1727	575		U	U	U	U	U	U	U	U	U	U	–
SCRN1	Exocytosis	7p14.3-p14.1	M/U	U	U	U	M	M	1595	57	19	1306	127		U	U	U	U	U	M	U	U	M	U	–
TBX3	Morphogenesis	12q24.1	U	U	U	U	U	M	1280	881	15	1714	38		U	U	U	M	M	M	M	M	M–	M	U
TFAP2C	Transcription factor	20q13.2	U	U	M/U	U	M	M	1486	26	27	2446	51		U	M–	M	U	U	M	M	U	M	U	–
ULBP2	T cell proliferation	6q25	U	U	U	U	M/U	M	365	510	29	1286	8		U	U	U	U	U	M	U	U	U	U	U
WIF1	Wnt signaling	12q14.3	M	M	M	M/U	M	M	9	41	7	241	2		U	U	M	M	M–	M	M	M	M	M–	U
ZNF559	Transcription factor	19p13.2	M	U	M/U	U	M	M	609	7	7	532	–		M–	M	M–	M–	U	U	U	M	M–	M–	U
16 genes with potential tumor-related functions																									
ABHD9	Response to chemical substance	19p13.12	M	M	M	U	M	M	19	18	13	140	3		U	M	U	M–	M	M	M	M	M	M–	–
BMP7	Cell proliferation	20q13	U	U	M	U	M/U	M	4166	19	61	1185	68		U	U	U	M–	U	M	M	M–	M	M–	–
CDH2	Cell adhesion	18q11.2	M	M	M	U	M	M	13	13	9	487	57		M	U	M	M	M–	M	M	M	M	M	–
CDKN2D	Cdk inhibitor	*19p13*	U	U	U	U	U	U	47	339	119	489	28		U	U	U	U	U	U	U	U	U	U	–
CLDN3	Cell adhesion	7q11.23	U	U	M/U	U	U	M	7584	69	7	359	1		U	U	U	U	U	M	M	M–	M	U	U
CTSL	Protein processing	9q21–9q22	U	U	M	U	M/U	M	1017	102	50	1753	118		U	U	U	U	M–	M	M	M	U	M	–
FYN	Proto-oncogene	6q21	U	U	U	U	U	M	341	1046	5	797	64		U	U	U	U	U	M	U	U	U	U	–
GPR54	G-protein signaling	*19p13.3*	U	U	M	U	M	M	89	85	35	210	–		U	U	U	U	U	M	U	U	U	U	–
IGFBP3	Induction of apoptosis	7p13–7p12	M/U	M/U	M/U	U	M	M	106	15	31	277	422		M	U	M	M	M–	M	M	M	M	M	–
MTSS1	Cytoskeletal organization	8p22	M/U	U	M	U	U	M	17	694	73	625	400		M	U	U	U	U	M	U	M	U	U	–
PAX6	Transcription factor	11p13	M	U	M/U	U	M	M	17	21	29	417	6		U	U	M	M	U	M	M	M	M	M–	U
PLAU	Angiogenesis	10q24	M	U	U	U	M	M	1060	168	11	2950	80		U	U	U	U	U	M–	U	U	U	U	–
RORA	Signal transduction	15q21-q22	U	U	M	U	M	M	195	49	2	340	20		M	M	U	U	M	M–	M	M	M	M	U
SNAI1	Transcriptional repressor	20q13.1-q13.2	U	U	U	U	U	U	235	40	52	319	8		U	U	U	U	U	U	U	U	U	U	–
TNFSF9	Apoptosis	19p13.3	U	U	U	U	U	M	615	1133	84	916	23		U	U	U	U	U	M	U	U	U	U	–
ZNF177	Transcription factor	19p13.2	M	M	M	M	U	M	76	59	47	222	14		M	M	M	M	M	M	M	M	M	M	U

From genes upregulated 16-fold or more, 44 genes were selected randomly, and the methylation status of 32 genes with CGI were analyzed. In addition, 16 genes with potential tumor-related functions and CGI were selected for methylation analysis. †In cancer cell lines, M: only methylated molecules detected, U: only unmethylated molecules detected, M/U: both methylated and unmethylated molecules detected. ‡In primary cancer samples, 'M' and 'U' indicate detection and absence, respectively, of methylated molecules. 'M–' indicates slight detection of methylated molecules. §Obtained from the RefEXA database. Underlined chromosomal locations, regions of frequent loss in gastric cancer.

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gastric mucosae and had potential antioncogenic functions, such as mediation of SMAD signaling (*BMP7*)[41] and induction of apoptosis (*IGFBP3*).[42] There is a possibility that silencing of these genes is causally related to development and progression of gastric cancers. However, considering the large number of methylation-silenced genes, it was likely that the majority of the genes silenced in AGS did not have causal roles in gastric carcinogenesis.

In summary, we found a considerable number of methylation-silenced genes in a gastric cancer cell line AGS. Potential tumor-related genes were selected based on their known functions, chromosomal locations, methylation in primary samples and expression in normal gastric mucosae. The usefulness of chemical genomic screening was confirmed.

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