Figure 3 Cumulative incidence of all the metachronous gastric cancers for patients in quartile (Q1–Q4) of methylation levels of *miR-124a-3*, *EMX1*, and *NKX6-1*. The Q4 methylation had higher incidences of a metachronous gastric cancer than the Q1 methylation with p values of 0.17, 0.08 and 0.54 for *miR-124a-3*, *EMX1* and *NKX6-1*, respectively, by the log-rank test.

Nevertheless, several limitations of the study should be mentioned. First, the patient population was heterogeneous at enrolment for the past history of ER or the years since the first ER. These factors were associated with the occurrence of metachronous gastric cancer (table 4). Therefore, in addition to the multivariate analysis involving these factors, we conducted analyses stratified by these factors and found consistent results in each stratum, although the results were not statistically significant owing to the limited number of events (see online supplementary table S4). Thus, it is unlikely that these factors biased the association between the quartiles of *miR-124a-3* and metachronous gastric cancer. Second, owing to a relatively small number of events, inevitable for a prospective study of cancer risk, we could not conclude which pattern, dose–response or threshold pattern, was obeyed by the relationship between methylation level of *miR-124a-3* and cancer risk. However, we found an association of *miR-124a-3* with authentic gastric cancer risk for the Q4 (highest) using Q1 (lowest) or the combination of all the other quartiles (Q1–Q3) as a reference (table 5 and see online supplementary table S1). This finding may support the threshold curve. Since the incidence of a metachronous gastric cancer is stable long after *H. pylori* eradication,[25] [37] further long-term follow-up is expected to strengthen the correlation and clarify an appropriate model of relationship.

From a molecular viewpoint, we tried to measure the accumulation of aberrant methylation in stem cells by analysing gastric

mucosae without *H. pylori* infection, at least 6 months after *H. pylori* eradication. It is known that the methylation level in gastric mucosa decreases after *H. pylori* eradication,[21–23] and that the persistent methylation level in gastric mucosa without *H. pylori* infection is correlated with gastric cancer risk.[14] [19] [24] Since methylation induced in stem cells can only persist for a long time without its inducer, the methylation level in gastric mucosa without *H. pylori* infection could be considered to reflect epigenomic damage accumulated in stem cells. However, we do not have direct evidence to support the hypothesis, because it is still impossible to analyse DNA methylation of specific genes in histological sections. The higher methylation level and smaller HR in patients with *H. pylori* infection before the enrolment (see online supplementary table S3) might have indicated that the methylation levels had not reached baseline after eradication and were superimposed with methylation in progenitor cells.

We analysed three preselected marker genes. Ideal marker genes should be methylated in parallel with overall methylation levels of driver genes in stem cells, but at much higher levels for accurate measurement. *miR-124a-3* is a tumour-suppressor gene (driver gene) with relatively high methylation levels,[31] and was considered to have met the criteria. Two other genes (*EMX1* and *NKX6-1*) were homeobox genes and considered to be passenger genes. In addition to the analysis using the methylation level of a single gene, we performed an exploratory analysis by combining methylation of three marker genes. Simple addition of the methylation levels of the three genes did not improve the prediction power (data not shown). However, when we scored the number of genes within the Q4 (highest) using the three marker genes, the patients with the highest scores displayed a high HR (95% CI) (2.23 (1.12 to 4.44); p=0.022) with a statistically significant trend p of 0.038 using the authentic metachronous gastric cancers. The smaller p value in this combined analysis also supports a threshold model.

In our previous study, we performed biopsies from three positions (antrum, middle body and upper body).[19] It was shown that methylation levels of the three positions were different and that their mean had the highest association with gastric cancer risk. However, patients with a high mean methylation level had higher methylation levels in any of the three positions. In the current study, we performed biopsy from one position, considering the merits (smaller risk of bleeding) and demerits (less precise reflection of the cancer risk) of limiting the positions of biopsy. Since the utility of epigenetic analysis of gastric tissue was demonstrated here, an increase of biopsy positions in future studies may be considered.

In summary, the methylation level of *miR-124a-3* was associated with an increased risk of developing metachronous gastric cancers. Assessment of an epigenetic field defect using methylation levels in normal tissue is a promising biomarker for cancer risk that takes account of life history.

Stomach

Competing interests None.

Patient consent Written informed consent was obtained from all the patients.

Ethics approval Study approved by the institutional review board at each hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

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Frequent involvement of chromatin remodeler alterations in gastric field cancerization

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ABSTRACT

A field for cancerization, or a field defect, is formed by the accumulation of genetic and epigenetic alterations in normal-appearing tissues, and is involved in various cancers, especially multiple cancers. Epigenetic alterations are frequently present in chronic inflammation-exposed tissues, but information on individual genes involved in the formation of a field defect is still fragmental. Here, using noncancerous gastric tissues of cancer patients, we isolated 16 aberrantly methylated genes, and identified chromatin remodelers ACTL6B and SMARCA1 as novel genes frequently methylated in non-cancerous tissues. SMARCA1 was expressed at high levels in normal gastric tissues, but was frequently silenced by aberrant methylation in gastric cancer cells. Moreover, somatic mutations of additional chromatin remodelers, such as ARID1A, SMARCA2, and SMARCA4, were found in 30% of gastric cancers. Mutant allele frequency suggested that the majority of cancer cells harbored a mutation when present. Depletion of a chromatin remodeler, SMARCA1 or SMARCA2, in cancer cell lines promoted their growth. These results showed that epigenetic and genetic alterations of chromatin remodelers are induced at an early stage of carcinogenesis and are frequently involved in the formation of a field defect.

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Introduction

A field for cancerization (field defect) refers to a non-cancerous tissue predisposed to carcinogenesis [1,2], and is involved in the development of various types of cancers, especially in those associated with chronic inflammation and multiple cancers [2,3]. The formation of a field defect has been initially explained by the accumulation of genetic alterations in non-cancerous tissues [1]. Over the past decade, the involvement of epigenetic alterations in the formation of a field defect (epigenetic field defect) was shown by the higher levels of aberrant DNA methylation in non-cancerous tissues of cancer patients than in normal tissues of non-predisposed individuals [4]. The presence of an epigenetic field defect has been suggested for various types of cancers, such as gastric [5,6], colorectal [7], esophageal [8], liver [9], and renal cancers [10].

Especially in gastric tissues, it is known that aberrant DNA methylation of various but specific genes is induced by exposure to chronic inflammation triggered by Helicobacter pylori (H. pylori) infection [5,11,12], the almost exclusive cause of gastric cancers [13]. The degree of accumulation of aberrant methylation is highly correlated with risk of gastric carcinogenesis [6]. A multicenter prospective cohort study demonstrated that the degree of accumulation of aberrant methylation can be used as a risk marker for the occurrence of metachronous multiple gastric cancers after endoscopic submucosal dissection (ESD) [14]. Nevertheless, information on the tumor-suppressor genes involved in the formation of an epigenetic field defect is still limited, except for CDH1 (E-cadherin) and CDKN2A (p16) [5].

Driver genes in gastric cancers include those in at least eight cancer-related pathways [15–20]. Three growth-promoting pathways, the AKT/mTOR, mitogen-activated protein kinase (MAPK), and WNT pathways, are activated not only by activating mutations (or amplification) of oncogenes but also by methylation-silencing of their negative regulators. Four tumor-suppressive pathways, cell adhesion, cell cycle regulation, mismatch repair, and p53 pathways, are inactivated by both genetic alterations and methylation-silencing.

In addition to genes involved in these tumor-suppressive pathways, inactivation of chromatin remodelers by genetic alterations was recently identified [21–23]. Chromatin remodelers consist of multiple subunits, such as *ARID1A* (BAF250A), *SMARCA2* (BRM), and *SMARCA4* (BRG1), and are involved in the regulation of transcription by modulating chromatin accessibility [24]. However, it is mostly unclear what genes among these pathways are involved in the formation of an epigenetic field defect.

In this study, we aimed to clarify genes involved in the formation of a field defect as drivers by comprehensively analyzing aberrant DNA methylation of genes with promoter CpG islands (CGIs) and involved in the major eight gastric cancer-related pathways in non-cancerous tissues of gastric cancer patients. Identification of such genes will provide a basis for establishing a novel cancer risk diagnosis and early intervention in individuals highly at risk.

Materials and Methods

Clinical samples

One hundred and thirty-two gastric cancer samples and 89 non-cancerous samples were collected from patients undergoing gastrectomy (123 cancer and 80 non-cancerous samples) or endoscopic biopsy (nine cancer and nine non-cancerous samples). Forty-three normal gastric tissue samples were endoscopically collected from healthy volunteers. In addition, 19 non-cancerous samples were collected by endoscopic biopsy from gastric cancer patients for an association analysis between methylation and expression. The samples were stored in RNAlater (Life Technologies, Carlsbad, CA, USA) at –80 °C until the preparation of genomic DNA or RNA. All samples were collected with informed consents, and the study was approved by the Institutional Review Boards.

Cell lines

Seven human gastric cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD) (AGS and N87) and Japanese Collection of Research Bioresources (Tokyo, Japan) (KATO III, MKN7, MKN45, MKN74, and NUGC-3). HGEC6B was established from non-cancerous fundic gland cells of a gastric cancer patient by introducing *CCND1*, mutant *CDK4*, and *TERT* genes using lentiviral or retroviral vectors [25,26]. Details of cultivation and immortalization of gastric epithelial cell will be published elsewhere. Six human gastric cancer cell lines were kindly provided by Dr. K. Yanagihara (58As9, HSC39, HSC44, and HSC57), Dr. M. Tatematsu (GC2), and Dr. W. Yasui (TMK1). All gastric cancer cell lines were maintained in RPMI1640 containing 10% (v/v) FBS. HGEC6B was maintained in Keratinocyte-SFM containing 5 ng/ml EGF, 50 μg/ml bovine pituitary extract, 10% (v/v) FBS, 2 mM N-acetylcysteine (NAC), and 0.2 mM ascorbic acid 2-phosphate (Asc-2P). To analyze cell growth, cells were seeded on day 0, and the cell number was counted on days 1–5. The differences of cell numbers were evaluated by the Welch t-test.

Treatment with 5-aza-dC

AGS and GC2 were seeded on day 0, and were treated with freshly-prepared 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St. Louis, MO) for 24 hours on days 1, 3, and 5. Cells were cultured in fresh medium without 5-aza-dC for 24 hours on days 2 and 4. Cell numbers were counted on day 6, and the cells were harvested. This treatment protocol efficiently inhibited growth of AGS and GC2 cells (Appendix S1: Supplementary Fig. S1).

Preparation of genomic DNA and total RNA

Genomic DNA was extracted by the standard phenol/chloroform method, and was quantified by a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Total RNA was extracted by ISOGEN (Nippon Gene, Tokyo, Japan).

Analysis of DNA methylation

A genome-wide DNA methylation analysis was conducted using an Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA), as described previously [20,27]. The methylation level of an individual probe was obtained as a β value, which ranged from 0 (unmethylated) to 1 (fully methylated). A total of 482,421 probes for CpG sites were assembled into 296,494 genomic blocks, collections of probes classified by their locations against transcription start sites (TSSs) and CGIs [27], and the methylation level of a block was defined as a mean β value of the probes

in the block. The methylation level of a block covering a CGI within 200 bp from a TSS (TSS200 CGI), whose methylation is known to be critical for gene silencing, was analyzed for 65 genes involved in the Akt/mTOR pathway (*FBXW7*, *PHLPP1*, *PTEN*, and *THEM4*), cell adhesion (*CDH1*), cell cycle regulation (*CHFR*), the MAPK pathway (*RASSF1*), mismatch repair (*MLH1*, *MSH2*, *MSH3*, and *MSH6*), the p53 pathway (*BAX*, *CASP3*, *CD82*, *CDKN1A*, *CHEK2*, *CYCS*, *DDB2*, *EI24*, *GADD45B*, *GADD45G*, *GTSE1*, *IGFBP7*, *MIR-34b*, *PERP*, *PMAIP1*, *RPRM*, *RRM2*, *SESN1*, *SESN2*, *SIAH1*, *STEAP3*, *THBS1*, *TNFRSF10B*, *ZMAT3*), the WNT pathway (*APC*, *AXIN2*, *CSNK1A1*, *CTBP1*, *CTBP2*, *DKK3*, *GSK3B*, *NKD1*, *NKD2*, *SENP2*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *SOX17*, *WIF1*) [20], and chromatin remodeling [*ACTL6A* (BAF53A), *ACTL6B* (BAF53B), *ARID2* (BAF200), *ATRX*, *SMARCA1* (SNF2L), *SMARCA4*, *SMARCAL1*, *SMARCB1* (SNF5), *SMARCC1* (BAF155), *SMARCC2* (BAF170), *SMARCD1* (BAF60A), *SMARCD3* (BAF60C), *SMARCE1* (BAF57), and *PBRM1* (BAF180)]. The methylation level of TSS200 CGIs was substituted by that of a block in the 1st exon/5′-UTR CGI for three genes involved in cell cycle regulation (*CDKN2A*) and chromatin remodeling (*ARID1A* and *SMARCA2*) because these genes did not have probes located in TSS200 CGIs although they had promoter CGIs. A gene was defined as unmethylated (β value = 0–0.2), partially methylated (β value = 0.2–0.3 for non-cancerous tissues and 0.2–0.4 for cancers), and methylated (β value = 0.3–1.0 for non-cancerous tissues and 0.4–1.0 for cancers).

Gene-specific methylation analysis was conducted by qMSP, as described previously [28], using primers specific to methylated or unmethylated DNA (Appendix S2: Supplementary Table S1). We were able to design primers for *SMARCA1* only within the 1st exon, although its CGI spanned from its promoter region to exon 1. DNA methylation levels were calculated as the fraction of methylated molecules among the total DNA molecules (methylated molecules + unmethylated molecules). Methylation status in a cell line was defined as unmethylated (U), partially methylated (P), and completely methylated (C) when its methylation level was 0–20%, 20–80%, and 80–100%, respectively. A difference in methylation levels was evaluated by the Mann–Whitney U-test. Bisulfite sequencing was conducted, as described previously [28] using primers listed in Appendix S2: Supplementary Table S1.

Analysis of gene expression

Genome-wide analysis of gene expression was conducted by a SurePrint G3 Human Gene Expression 8x60K v2 Microarray (Agilent Technologies, Santa Clara, CA). cRNA labeled with Cy3 was synthesized from 200 ng of total RNA by using a Low Input Quick Amp Labeling Kit (Agilent Technologies), and 600 ng of Cy3-labeled cRNA was fragmented and hybridized to SurePrint G3 Gene Expression Microarray at 65 °C for 17 hours. Then, the microarray was scanned by using an Agilent G2565BA microarray scanner (Agilent Technologies). The obtained signals were processed by Feature Extraction Ver.9.1 (Agilent Technologies), and analyzed by GeneSpring Ver.12.5 (Agilent Technologies). The signal intensity of each probe was normalized so that the 75 percentile of signal intensity of all probes would be 1.0, and the mean signal intensity of all the probes within a specific gene was used as an expression level of the gene. Quantitative RT-PCR (qRT-PCR) was conducted by measuring actual copy numbers, as described previously [29], using primers listed in Appendix S2: Supplementary Table S2. The copy number of an individual gene was normalized to that of *GAPDH*.

Analysis of somatic mutations

A library DNA containing 672 PCR amplicons of 18 chromatin remodelers [*ACTL6A*, *ACTL6B*, *ARID1A*, *ARID1B* (BAF250B), *ARID2*, *ATRX*, *PBRM1*, *PHF10* (BAF45A), *SMARCA1*, *SMARCA2*, *SMARCA4*, *SMARCAL1*, *SMARCB1*, *SMARCC1*, *SMARCC2*, *SMARCD1*, *SMARCD3*, and *SMARCE1*] was prepared from 80 ng of genomic DNA of each sample by multiplex PCR using an Ion AmpliSeq™ Library Kit 2.0 (Life Technologies) and customized primers (Appendix S2: Supplementary Table S3). The 672 PCR amplicons covered 86.5–100% of the coding regions of 18 chromatin remodelers (Appendix S2: Supplementary Table S4). The library DNA prepared was uniquely barcoded with an Ion Xpress Barcode Adaptors 1–96 Kit (Life Technologies), and libraries from 58 samples were pooled. The pooled library was mixed with Ion Spheres, and emulsion PCR was conducted using the Ion OneTouch 2 (Life Technologies) with an Ion PI Template OT2 200 Kit v2 (Life Technologies). Then, the complexes of Ion Spheres with amplified DNA were washed, concentrated using Ion OneTouch ES (Life Technologies), and loaded onto an Ion PI Chip (Life Technologies). Sequencing was conducted using an Ion Proton Sequencer (Life Technologies) with an Ion PI Sequencing 200 Kit v2 (Life Technologies), and the obtained sequences were mapped, as described previously [27].

Somatic mutations in an individual gastric cancer sample were identified by excluding sequence variations also present in a corresponding non-cancerous sample. Reading depths of individual regions are shown in Appendix S2: Supplementary Table S5. Identified somatic mutations were confirmed by dideoxy sequencing, as described previously [27] using primers listed in Appendix S2: Supplementary Table S6.

Estimation of cancer cell content in a primary cancer sample

The cancer cell content of a primary gastric cancer sample was estimated by the profile of DNA methylation levels of 40,333 genomic blocks with CGIs unmethylated in normal
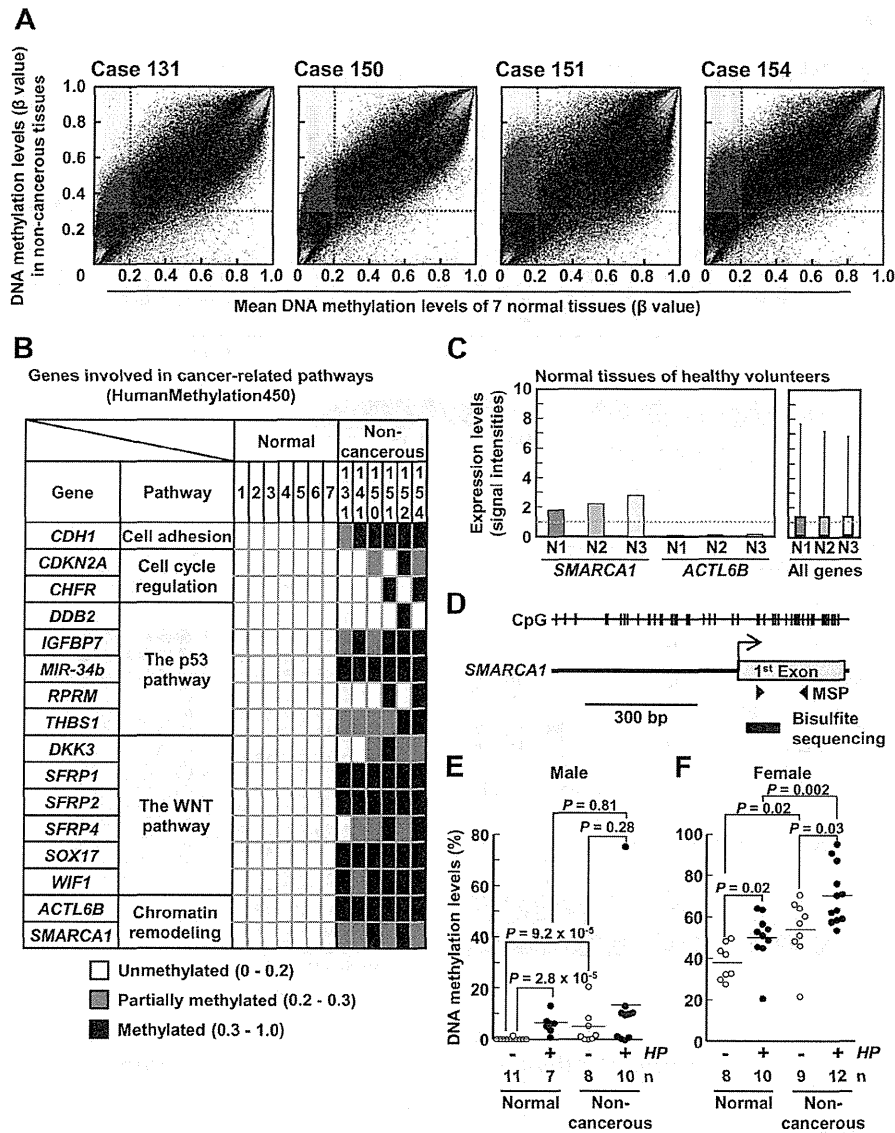
Fig. 1. Aberrant DNA methylation of genes involved in gastric cancer-related pathways in non-cancerous gastric tissues. (A) Comprehensive analysis of DNA methylation in non-cancerous tissues of male gastric cancer patients. Methylation levels of 296,494 genomic blocks analyzed by a BeadChip array were compared with those in normal gastric tissues of healthy volunteers without *H. pylori* infection. 3.2–5.7% of genomic blocks were aberrantly methylated in four non-cancerous gastric tissues. (B) Aberrant DNA methylation of genes involved in gastric cancer-related pathways in non-cancerous gastric tissues. Among the 68 genes in eight pathways, 16 genes in five pathways, including chromatin remodelers, *ACTL6B* and *SMARCA1*, were frequently methylated. (C) Gene expression levels of *SMARCA1* and *ACTL6B* in normal gastric tissues without *H. pylori* infection analyzed by a microarray. *SMARCA1* was expressed at high levels while *ACTL6B* was not. Box plots represent expression levels of all the genes analyzed by a microarray. (D) Genomic structure around the *SMARCA1* promoter region. Positions of CpG sites are shown by vertical lines. Open box, 1st exon; arrow, TSS; arrowheads, primers for qMSP; and closed box, a region analyzed by bisulfite sequencing. (E) The *SMARCA1* methylation level in gastric tissues (mucosae) of male healthy volunteers (n = 18) and gastric cancer patients (n = 18) analyzed by qMSP. It was higher in individuals with *H. pylori* infection, and also in cancer patients. (F) The *SMARCA1* methylation level in gastric tissues (mucosae) of female healthy volunteers (n = 18) and gastric cancer patients (n = 21). It was higher in individuals with *H. pylori* infection although there was a fluctuation in the methylation level even in healthy volunteers without *H. pylori* infection.

gastric tissues without *H. pylori* infection. A fraction (0.1–25.8%) of the blocks are specifically methylated in cancer cells, and their methylation levels are known to reflect cancer cell content [30]. Based on a histogram of distribution of methylation levels of the 40,333 blocks in a cancer tissue, a peak of methylation level was determined, and the level was used as the cancer cell content (Appendix S1: Supplementary Fig. S2).

Depletion of chromatin remodelers by shRNA

SMARCA1 or SMARCA2 was depleted in gastric cancer cell lines using three independent shRNAs (Appendix S2: Supplementary Table S7) designed by using siDirect version 2.0 [31], as described previously [32]. shRNA for firefly luciferase (shLuc)

was used as the control. The depletion of SMARCA1 or SMARCA2 proteins was confirmed by western blotting, as described previously [33] using anti-SMARCA1 antibody (1:1000; MABE366; Millipore, Billerica, MA) and anti-SMARCA2 antibody (1:1000; ab15597; Abcam, Cambridge, UK), respectively. Protein bands were quantified by using ImageJ 1.47v software.

Gene expression changes by *SMARCA1* or *SMARCA2* depletion were analyzed using a SurePrint G3 Human Gene Expression 8x60K v2 Microarray. Genes whose signal intensities were decreased by two fold or more and had signal intensities of 1.0 or more in cells with control shRNA and normal gastric mucosae without *H. pylori* infection (n = 3) were defined as down-regulated genes. Genes whose signal intensities were increased by two fold or more and had signal intensities of 1.0 or more in SMARCA1-depleted or SMARCA2-depleted cells were defined as up-regulated genes.

Fig. 2. Methylation-silencing of SMARCA1 in gastric cancers. (A) SMARCA1 methylation levels in 14 gastric cancer cell lines and one normal epithelial cell line analyzed by qMSP. SMARCA1 was completely methylated in five of 14 gastric cancer cell lines. M, a male-derived gastric cancer cell line; and F, a female-derived gastric cancer cell line. M-DNA, completely methylated DNA; and U-DNA, completely unmethylated DNA. (B) The association between DNA methylation and loss of SMARCA1mRNA expression. The expression levels were analyzed by qRT-PCR in the 14 gastric cancer cell lines and one normal gastric epithelial cell line. SMARCA1 mRNA was not detected in any of the five cell lines with complete methylation of SMARCA1, but was detected in five of 10 cell lines without complete methylation. C, completely methylated; P, partially methylated; and U, unmethylated. (C) SMARCA1 protein expression in gastric cancer and normal cell lines analyzed by western blotting. SMARCA1 protein was not expressed in gastric cancer cell lines with complete SMARCA1 methylation. (D) Re-expression of SMARCA1 by 5-aza-dC treatment. After 5-aza-dC treatment, unmethylated DNA molecules were detected, and SMARCA1 mRNA was re-expressed. (E) Confirmation of SMARCA1 demethylation by bisulfite sequencing. Some DNA molecules showed demethylation of all the CpG sites analyzed. Due to unavailability of primers for the promoter CGI, an exonic CGI whose methylation status is likely to be the same as that of the promoter CGI was analyzed. (F) SMARCA1 methylation levels in the initial set of 50 gastric cancers (42 male and 8 female cancers) analyzed by a BeadChip array. SMARCA1 was methylated in 11.9% of male gastric cancers. A cut-off value of 0.4 was adopted for methylation in primary cancer samples, as used previously [20,27]. (G) Validation of SMARCA1 methylation in another set of 82 gastric cancers (50 male and 32 female cancers) by qMSP. SMARCA1 was methylated in 8% of male gastric cancers with a cut-off value of 6%, as used previously [37].

Gene ontology analysis

Enrichment of specific biological processes in gene ontology criteria among genes whose expression levels were changed by *SMARCA1* or *SMARCA2* depletion was analyzed by using DAVID bioinformatics resources [34,35].

Results

Aberrant DNA methylation of genes involved in various cancer-related pathways in non-cancerous gastric tissues

From the genes involved in the eight major gastric cancer-related pathways (the Akt/mTOR, cell adhesion, cell cycle regulation, MAPK, mismatch repair, p53, WNT, and chromatin remodeling pathways), 68 genes with promoter CGIs were identified. DNA methylation statuses of these genes were analyzed by a BeadChip array in six non-cancerous tissues of male gastric cancer patients (Fig. 1A and Appendix S1: Supplementary Fig. S3). Among the 68 genes, a total of 16 genes involved in cell adhesion (*CDH1*), cell cycle regulation (*CDKN2A* and *CHFR*), the p53 pathway (*DDB2, IGFBP7, MIR-34b, RPRM*, and *THBS1*), the WNT pathway (*DKK3, SFRP1, SFRP2, SFRP4, SOX17*, and *WIF1*), and chromatin remodeling (*ACTL6B* and *SMARCA1*) were frequently methylated in non-cancerous gastric tissues (Fig. 1B).

Methylation-silencing of most of the 16 genes in non-cancerous tissues was novel, and, especially, that of two genes encoding chromatin remodelers, *ACTL6B* and *SMARCA1*, has not been reported, even in cancers. Therefore, we then analyzed the expression levels of the

two genes in normal gastric tissues. By a microarray analysis of three normal gastric tissues of healthy volunteers without *H. pylori* infection, *SMARCA1* showed a higher expression level than most of the genes on the microarray, but *ACTL6B* did not (Fig. 1C). Since a gene with high expression in normal cells but with aberrant DNA methylation in cancer cells is known to have a high chance of being a driver gene [32], it was suggested that *SMARCA1* was a potential driver gene involved in the formation of an epigenetic field defect.

High SMARCA1 methylation levels in normal gastric tissues with H. pylori infection

SMARCA1 was located on chromosome X, and *H. pylori* infection has been established as an inducer of an epigenetic field defect in the stomach [12,36]. Therefore, the *SMARCA1* methylation level was analyzed in gastric tissues (mucosae) of male healthy volunteers (non-predisposed mucosae) and gastric cancer patients (predisposed mucosae) classified by *H. pylori* infection status using quantitative methylation-specific PCR (qMSP) (Fig. 1D). Both among the healthy volunteers ($P = 2.8 \times 10^{-5}$) and cancer patients ($P = 0.28$), the *SMARCA1* methylation level was higher in individuals with *H. pylori* infection (Fig. 1E). Also, both among individuals with *H. pylori* infection ($P = 0.81$) and among those without ($P = 9.2 \times 10^{-5}$), the DNA methylation levels were higher in cancer patients. In female healthy volunteers and gastric cancer patients, the methylation level was also higher in individuals with *H. pylori* infection although there was a variation in the methylation level even in volunteers without

Fig. 3. Frequent mutations of genes encoding chromatin remodelers in gastric cancers. (A) Mutations were analyzed in the initial set of 50 gastric cancers by an Ion Proton Sequencer. Among the 18 genes analyzed, nine genes, *ARID1A, ARID1B, ARID2, SMARCA1, SMARCA2, SMARCA4, SMARCB1, PBRM1*, and *PHF10*, were mutated in one or more gastric cancers. As reported previously [21,23], *ARID1A* (10%) was most frequently mutated in the 50 gastric cancers. In addition, *SMARCA4* (10%) was also frequently mutated. The presence of somatic mutations is shown by a filled box. (B) Localization of somatic mutations of the three genes that encode ATPase subunits of chromatin remodelers. Mutations were detected at various positions of the coding regions. QLQ, glutamine-leucine-glutamine domain; and HSA, small helicase/SANT-associated domain.

H. pylori infection (Fig. 1F). These results suggested that aberrant methylation of chromatin remodeler was induced by H. pylori infection and that its accumulation was involved in the formation of a field defect.

Methylation-silencing of SMARCA1

To examine methylation-silencing of SMARCA1 in gastric cancers, its DNA methylation was first analyzed in 14 gastric cancer cell lines by qMSP. SMARCA1 was completely methylated in one of eight male-derived gastric cancer cell lines and in four of six female-derived cell lines (Fig. 2A). Then, an association between the complete methylation of SMARCA1 and its loss of mRNA expression was analyzed in the 14 gastric cancer cell lines and one normal epithelial cell line. SMARCA1 mRNA was not detected in any of the five cell lines (44As3, AGS, GC2, HSC44, and KATO III) with complete methylation while its high expression was detected in five (58As9, MKN7, MKN45, N87, and HGEC6B) of the 10 cell lines without complete methylation (Fig. 2B). SMARCA1 protein was also not detected in the cell lines with complete methylation of SMARCA1 (Fig. 2C). In contrast, such association was unclear by analysis of 19 non-cancerous samples (Appendix S1: Supplementary Fig. S4).

Thirdly, re-expression of SMARCA1 after treatment with a DNA demethylating agent, 5-aza-dC, was analyzed in two gastric cancer cell lines with complete methylation, AGS and GC2. After treatment with 0.3 µM of 5-aza-dC, unmethylated (demethylated) DNA was detected by MSP (Fig. 2D), and complete demethylation of multiple CpG sites (+56 to +111 from the TSS) was confirmed by bisulfite sequencing (Fig. 2E and Appendix S1: Supplementary Fig. S5). Accordingly, SMARCA1 was re-expressed in both cell lines, and more re-expression was induced by higher doses of 5-aza-dC (Fig. 2D). These results showed that SMARCA1 was silenced by aberrant DNA methylation in gastric cancers.

The presence of SMARCA1 methylation in primary gastric cancers

To examine the presence of SMARCA1 methylation in primary gastric cancers, SMARCA1 methylation was analyzed in an initial set of 50 primary gastric cancers by a BeadChip array (CpG site at -13 from the TSS). It was methylated in five of 42 male gastric cancers, but an increase was unclear in eight female gastric cancers (Fig. 2F). The presence of SMARCA1 methylation was further validated in another set of 82 primary gastric cancers by qMSP. In male gastric cancers, SMARCA1 was methylated (DNA methylation level ≥ 6%) in four of 50 cancers (Fig. 2G). In female gastric cancers, detecting an increase was difficult due to the fluctuation of basal methylation levels, but at least one of 32 cancers had a clearly increased level.

Frequent mutations of chromatin remodelers in gastric cancers

To further support the importance of disruption of chromatin remodelers in gastric cancers, somatic mutations of 18 genes encoding chromatin remodelers were analyzed in the initial set of the 50 gastric cancers by target sequencing using a next-generation sequencer. Among the 18 genes, nine (ARID1A, ARID1B, ARID2, SMARCA1, SMARCA2, SMARCA4, SMARCB1, PBRM1, and PHF10) were mutated in one or more of the 50 gastric cancers (Table 1 and Fig. 3A). In total, 15 (30%) of the 50 gastric cancers had at least one somatic mutation of a chromatin remodeler (s). Among the 14 gastric cancer cell lines, potential somatic mutations were detected in nine of them (Appendix S1: Supplementary Fig. S6 and Appendix S2: Supplementary Table S8).

Mutations of ATPase subunits of SWI/SNF complex, SMARCA2 and SMARCA4, were detected in 4 and 5, respectively, of the 50 gastric cancers, and two mutations of an ATPase subunit of ISWI complex, SMARCA1, were detected in one of the 50 gastric cancers. The

mutations of SMARCA2, SMARCA4, and SMARCA1 were distributed at various positions of their coding regions (Fig. 3B). Among the nine mutated genes, SMARCA1 and SMARCA2 were also aberrantly methylated in the initial set of the 50 gastric cancers (Fig. 2F and Appendix S1: Supplementary Fig. S7).

The presence of the somatic mutations in the majority of cancer cells of individual cases

To assess the timing of occurrence of the somatic mutations, a fraction of cancer cells with a specific mutation of a chromatin remodeler was analyzed in individual gastric cancer samples. For each of the 15 gastric cancer samples with a somatic mutation (s), its cancer cell content was estimated by the profile of cancer-specific DNA methylation [30] (Appendix S1: Supplementary Fig. S2 and Appendix S2: Supplementary Table S9), and the mutant allele frequency was obtained using the read numbers of the mutant and wild-type sequences (Table 1). For most cancers, the mutant allele frequency was close to the cancer cell content, and the correlation between these two values was 0.64 ($P = 7 \times 10^{-4}$) (Fig. 4). This result showed that mutations of the chromatin remodelers were present in the majority of cancer cells of individual cases, when present, and it was suggested that they were induced at an early stage of gastric carcinogenesis.

Tumor-suppressive function of chromatin remodelers in gastric cancers

The tumor-suppressive function of chromatin remodelers in gastric cancers was analyzed. SMARCA1 or SMARCA2 was depleted by two independent short hairpin RNAs (shRNAs) in gastric cancer
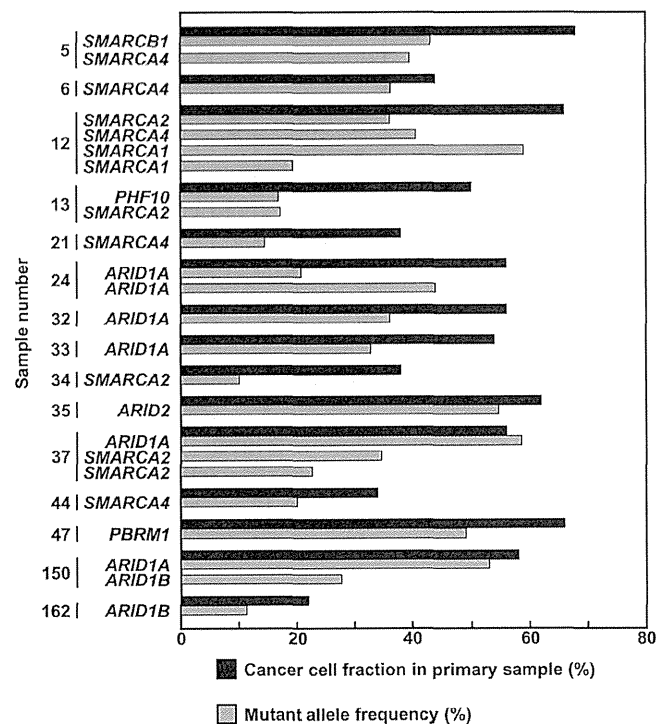

Fig. 4. The cancer cell content and the mutant allele frequency in individual cancers. The cancer cell content was estimated by a profile of cancer-specific DNA methylation, and the mutant allele frequency was calculated using read numbers of the mutated and wild-type sequences. For most cancers, the mutant allele frequency was close to the cancer cell content.

Table 1
List of somatic mutations of genes encoding chromatin remodelers in the 50 GCs.

Sample number	Gene	Coverage	Mutant allele frequencies (%)	Nucleotide change	Amino acid change
1	No mutation				
2	No mutation				
3	No mutation				
4	No mutation				
5	SMARCB1	88	43.2	c.1130G > A	p.Arg377His
	SMARCA4	477	39.6	c.3460C > T	p.Leu1154Phe
6	SMARCA4	897	36.3	c.2573C > T	p.Thr858Met
9	No mutation				
10	No mutation				
11	No mutation				
12	SMARCA2	512	36.1	c.548G > A	p.Arg183Gln
	SMARCA4	1334	40.6	c.1631_1633delAGA	p.Gln544_Lys545delinsGln
	SMARCA1	1067	59	c.2127delA	p.Lys709fs
	SMARCA1	1107	19.3	c.538_539insA	p.Gly180fs
13	PHF10	2144	16.9	c.937C > T	p.Arg313Trp
	SMARCA2	778	17.2	c.2455C > T	p.Arg819Trp
14	No mutation				
15	No mutation				
16	No mutation				
17	No mutation				
18	No mutation				
19	No mutation				
20	No mutation				
21	SMARCA4	324	14.5	c.3727C > T	p.Arg1243Trp
22	No mutation				
23	No mutation				
24	ARID1A	1901	20.7	c.2757_2758insC	p.Gln920fs
	ARID1A	373	44	c.3458delC	p.Ser1153fs
25	No mutation				
26	No mutation				
31	No mutation				
32	ARID1A	61	36.1	c.1619C > T	p.Thr540Met
33	ARID1A	497	32.8	c.4000C > T	p.Gln1334*
34	SMARCA2	159	10.1	c.1199T > A	p.Met400Lys
35	ARID2	940	54.7	c.1311_1312delAA	p.Ala437fs
36	No mutation				
37	ARID1A	2048	58.5	c.3196C > T	p.Gln1066*
	SMARCA2	185	34.6	c.579_588delCCGAGGCCAG	p.Ala193fs
	SMARCA2	877	22.7	c.590C > T	p.Pro197Leu
39	No mutation				
40	No mutation				
42	No mutation				
43	No mutation				
44	SMARCA4	304	20.1	c.2654G > T	p.Arg885Leu
45	No mutation				
47	PBRM1	522	49	c.436C > T	p.Arg146*
51	No mutation				
53	No mutation				
54	No mutation				
124	No mutation				
131	No mutation				
137	No mutation				
141	No mutation				
150	ARID1A	905	53	c.3688delA	p.Lys1230fs
	ARID1B	1094	27.7	c.5695G > T	p.Glu1899*
151	No mutation				
152	No mutation				
154	No mutation				
162	ARID1B	388	11.3	c.4726C > T	p.Pro1576Ser

* Termination codon.

cell lines with their expression (Fig. 2C and Appendix S1: Supplementary Fig. S8), and changes of cell growth were analyzed. For SMARCA1, its protein levels were reduced to 6–63% (44–63% by shRNA#1 and 6–7% by shRNA#2) of those in cell lines with a control shRNA. All the three SMARCA1-depleted cell lines showed significant increases of cell growth rates (1.2- to 1.7-fold), compared with those with a control shRNA (Fig. 5A).

For SMARCA2, protein levels were reduced to 8–49% (8–20% by shRNA#1 and 12–49% by shRNA#3) of those in cell lines with a control shRNA. Two of three SMARCA2-depleted cell lines, GC2 and

HSC57, showed significant increases of cell growth rates (1.1- to 1.4-fold) compared with those with control shRNA (Fig. 5B). These results confirmed that chromatin remodelers, SMARCA1 and SMARCA2, were tumor-suppressor genes in gastric cancers.

Downstream genes affected by depletion of chromatin remodelers

Finally, gene expression profiles were compared between cell lines with depletion of chromatin remodelers and control cells. In SMARCA1-depleted cells (58As9), 63 genes were
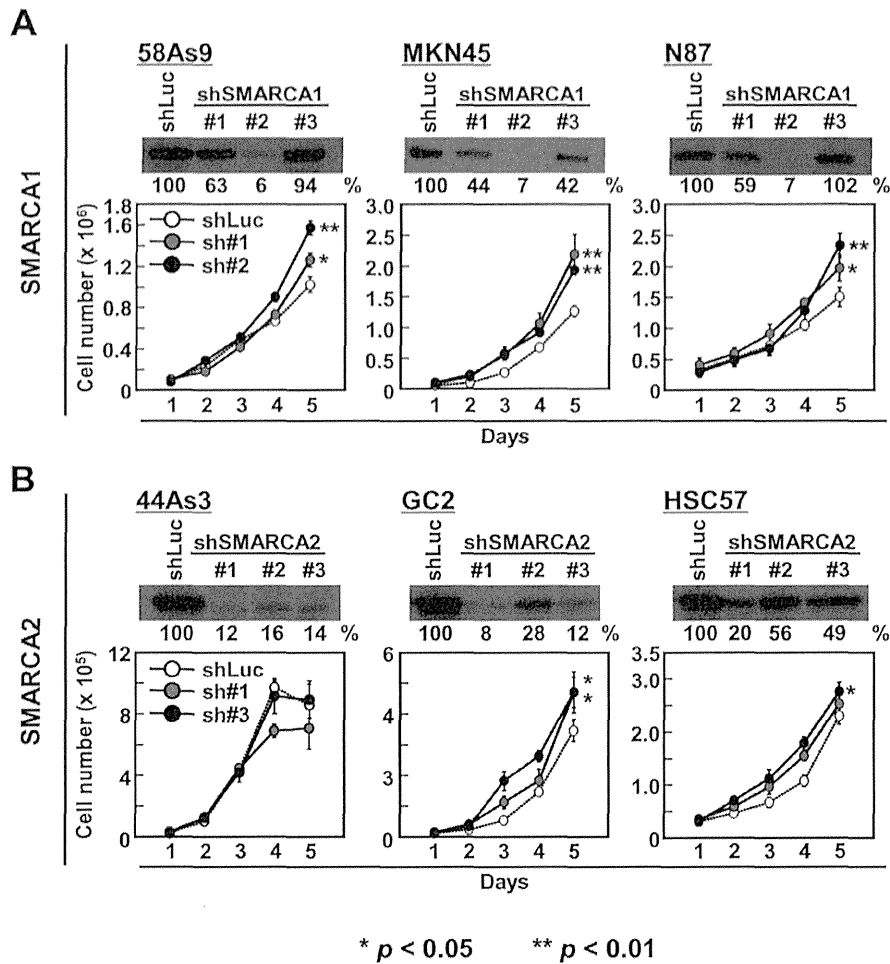
Fig. 5. Growth inhibitory function of chromatin remodelers in gastric cancers. SMARCA1 (A) or SMARCA2 (B) was depleted by two independent shRNAs in three gastric cancer cell lines, and their cell growth was analyzed. All the three SMARCA1-depleted cell lines and two of three SMARCA2-depleted cell lines showed significant increases of cell growth rates.

down-regulated and 41 genes were up-regulated (Fig. 6A and C). Among the down-regulated genes, genes related to cellular homeostasis and response to stimulus were enriched (Fig. 6C). No specific enrichment was observed among the up-regulated genes. In SMARCA2-depleted cells (GC2), 26 genes were down-regulated and 165 genes were up-regulated (Fig. 6B and D). Among the up-regulated genes, genes related to development of epithelial cells were enriched (Fig. 6D). No specific enrichment was observed among the down-regulated genes. These results suggested that alterations of genes encoding chromatin remodelers are involved in gastric carcinogenesis by affecting various cellular functions.

Discussion

A chromatin remodeler, SMARCA1, was frequently methylated in non-cancerous tissues of gastric cancer patients, and was methylation-silenced in gastric cancers. In addition, chromatin remodelers, such as ARID1A, SMARCA1, SMARCA2, and SMARCA4, were frequently mutated in gastric cancers. Since cancer cell content ranged from 20% to 70% depending upon samples, target sequencing using a next-generation sequencer was effective to detect mutations in a relatively small fraction of DNA samples. When detected, mutations of chromatin remodelers were present in the majority of cancer cells of individual cases. Depletion of SMARCA1

or SMARCA2 in gastric cancer cell lines promoted their growth. This suggested for the first time that disruption of chromatin remodelers occurs at an early stage of gastric carcinogenesis, and is involved in the formation of a field defect as a driver.

An early onset of mutations of chromatin remodelers, including ARID1A and SMARCA4, in esophageal cancers was observed in never-dysplastic Barrett's esophagus [38]. In addition, early onset of mutations of a DNA demethylating enzyme TET2 is known to be involved in the development of hematological malignancies [39]. TET2 mutations can be induced in hematopoietic stem/progenitor cells, and are involved in the generation of pre-malignant clones, and acquirement of additional alterations leads to development of myeloid malignancies. As epigenetic modifiers, mutations of chromatin remodelers may be induced at an early stage of carcinogenesis, and involved in the generation of pre-malignant clones, some of which will eventually evolve into a cancer cell.

Depletion of SMARCA1 in cancer cell lines promoted their growth, indicating that SMARCA1 functions as a tumor-suppressor gene. SMARCA1 is located on chromosome X and can be potentially inactivated by a single hit. Therefore, SMARCA1 is considered to be a "risky" gene compared to other tumor-suppressor genes located on autosomes. So far, only a small number of such genes have been reported. FHL1 is inactivated by aberrant DNA methylation in gastric cancers and colon cancers [28]. WTX, FOXP3, and PHF6 are inactivated by
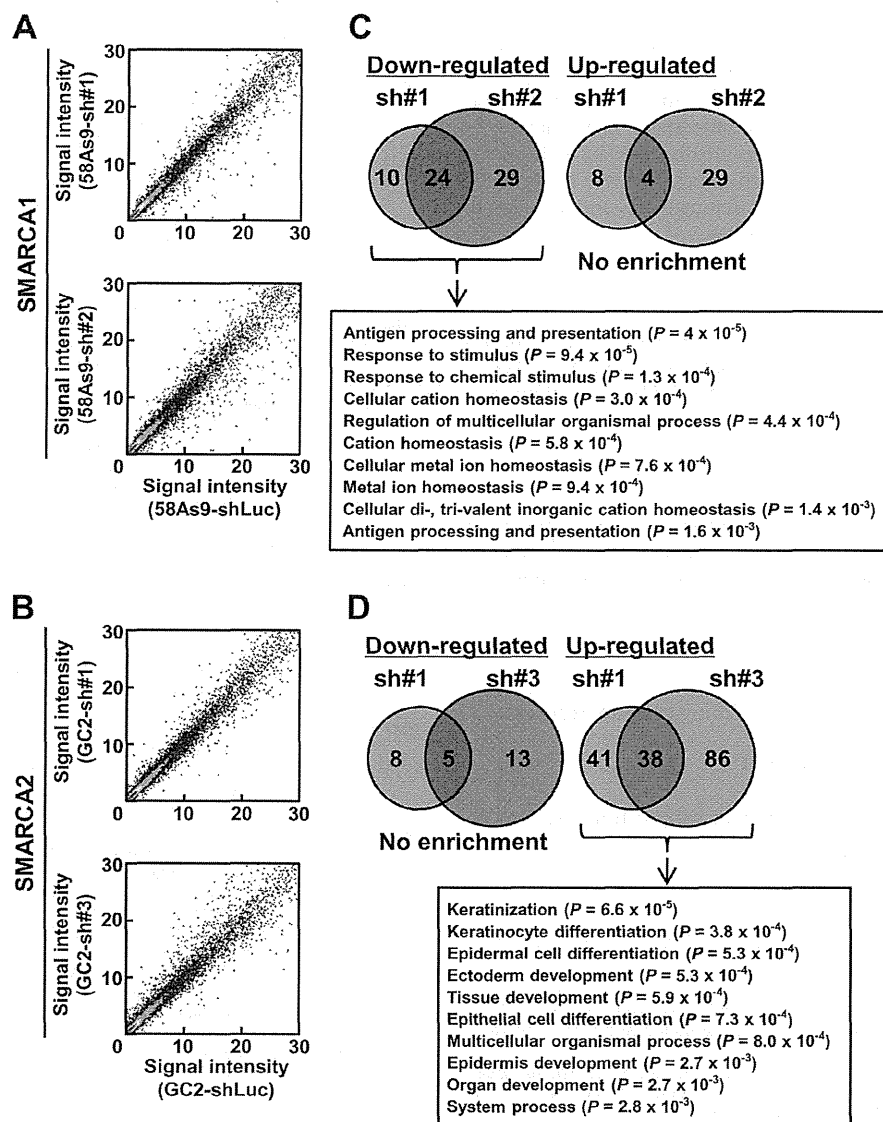
Fig. 6. Downstream genes affected by depletion of chromatin remodelers. (A) and (B) Analysis of gene expression changes by depletion of chromatin remodelers. Expression changes of 32,078 genes by the depletion of SMARCA1 or SMARCA2 were analyzed. In SMARCA1-depleted 58As9 cells, 63 genes were down-regulated and 41 genes were up-regulated (A). In SMARCA2-depleted GC2 cells, 26 genes were down-regulated and 165 genes were up-regulated (B). (C) and (D) Ontology of genes whose expression levels were changed by depletion of chromatin remodelers. Among the genes down-regulated by SMARCA1 depletion, genes related to cellular homeostasis and response to stimulus were enriched. Among the genes up-regulated by SMARCA2 depletion, genes related to development of epithelial cells were enriched. The top 10 significantly enriched biological processes are shown.

genetic alterations in Wilms tumors, breast and prostate cancers, and T-cell acute lymphoblastic leukemia, respectively [40–43].

SMARCA1 protein was undetected not only in gastric cancer cell lines with complete methylation of *SMARCA1*, but also in those without, namely HSC39, HSC57, and NUGC-3. Among these cell lines, NUGC-3 had a *SMARCA1* mutation. Since, in general, a misfolded protein generated by a gene mutation can be rapidly degraded by proteasome [44], it was possible that SMARCA1 was misfolded by a mutation and degraded by proteasome. As for HSC39 and HSC57, the absence of transcription factors that regulate *SMARCA1* expression was considered as a possible mechanism.

Despite its abundant expression in normal gastric tissues, *SMARCA1* was aberrantly methylated in non-cancerous gastric tissues and gastric cancers. Generally, genes with abundant expression in normal cells are resistant to aberrant DNA methylation induction [29,45–52]. As a possible mechanism of how aberrant methylation is induced in a

gene with abundant expression, its expression may be temporarily suppressed by exposure to carcinogenic factors, such as *H. pylori* infection, and such decreased expression can lead to increased susceptibility to aberrant methylation induction.

Chromatin remodelers, *SMARCA1* and *SMARCA2*, showed a growth-suppressive function in gastric cancers, but the effect of their knockdown on enhancement of cell growth was moderate. Chromatin remodelers are known to regulate transcription of genes involved in a variety of biological functions, such as cell cycle, DNA repair, and apoptosis [53]. Indeed, expression of genes involved in various biological processes, such as cellular homeostasis, response to stimulus, and development of epithelial cells, was changed by depletion of chromatin remodelers. Therefore, it is considered that disruption of chromatin remodelers by genetic and epigenetic alterations can be involved in cancer development by affecting not only cell growth but also multiple biological functions.

The association in non-cancerous tissues between *SMARCA1* methylation and its expression levels was unclear. Since non-cancerous tissues contain not only cells with *SMARCA1* methylation but also a large number of cells without, it might have been difficult to observe a clear association in non-cancerous tissues.

In conclusion, chromatin remodelers are disrupted by both genetic and epigenetic alterations, and such alterations are likely to be induced at an early stage of gastric carcinogenesis and involved in the formation of a field defect as drivers.

Conflict of interest

The authors have declared that no competing interests exist.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.11.038.

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