BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Identification of Long-Range Epigenetic Silencing on Chromosome 15q25 and Its Clinical Implication in Gastric Cancer

Jee-Youn Kang,^{*†} Sang-Hyun Song,^{*} Jiyeon Yun,^{*†} Mi-Seong Jeon,^{*†} Yongjun Cha,^{*} Si-Hyun Lee,^{*†} Hwang-Phill Kim,^{*} Eun-Goo Jeong,^{*} Sae-Won Han,^{*‡} Nam-Yun Cho,^{*§} Myeong Chel Kook,[¶] Gyeong Hoon Kang,^{*§} and Tae-You Kim^{*†‡}

From the Cancer Research Institute^{*} and the Department of Pathology,[§] Seoul National University College of Medicine, Seoul; the Department of Molecular Medicine and Biopharmaceutical Sciences,[†] Graduate School of Convergence Science and Technology, Seoul National University, Seoul; the Department of Internal Medicine,[‡] Seoul National University Hospital, Seoul; and the Department of Pathology,[¶] National Cancer Center, Goyang, Republic of Korea

Accepted for publication
November 4, 2014.

Address correspondence to
Tae-You Kim, M.D., Ph.D.,
Department of Internal Medi-
cine, Seoul National University
Hospital, Seoul 110-744,
Republic of Korea; or Gyeong
Hoon Kang, M.D., Ph.D.,
Department of Pathology,
Seoul National University
College of Medicine, Seoul
110-799, Republic of
Korea. E-mail: kimty@snu.ac.kr
or ghkang@snu.ac.kr.

Recent genome-wide epigenomic and transcription profiling studies have demonstrated that epigenetic silencing can encompass multiple neighboring genes, termed as long-range epigenetic silencing (LRES). Herein, we identified a novel LRES region by comparing gene expression of human colon cancer HCT116 cells with their DNA methyltransferase 1 and DNA methyltransferase 3B double-knockout derivative double-knockout cells. Ten consecutive genes spanning 3 Mb of chromosome 15q25 were coordinately silenced, with eight genes showing promoter CpG island hypermethylation and enrichment of repressive histone marks, which were evaluated by bisulfite sequencing analysis and chromatin immunoprecipitation assay. Comparison of primary gastric tumor specimens with normal tissue confirmed that the long-range silencing of this region was tumor specific. Methylation of genes within the LRES region was evaluated in 190 gastric tumor tissues using the MethyLight assay, and their association with clinicopathological features, such as older age, high-grade differentiation, and diffuse or mixed-type histology, was determined. LRES-positive gastric cancer patients (six or more methylated genes) showed lower recurrence and better survival. Our findings emphasize the differential dynamics of DNA methylation and histone modification, indicating the importance of studying the relationship of each epigenetic modification in the context of chromatin domains. Patients with LRES showed lower recurrence and better prognosis, indicating that stratifying patients according to underlying molecular features, such as LRES regions, may better predict recurrence and survival. (*Am J Pathol* 2015, 185: 666–678; <http://dx.doi.org/10.1016/j.ajpath.2014.11.022>)

Epigenetic events, defined as heritable changes in gene expression that are not associated with DNA sequence alterations, are gaining evidence as a fundamental hallmark of the cancer.^{1–4} The cancer epigenome is featured by global hypomethylation of normally methylated silent regions and *de novo* methylation of specific CpG islands within gene promoters, resulting in transcriptional silencing.^{5,6} In concert with DNA methylation aberrations, post-translational modification of histones is also deregulated, affecting genome-wide chromatin configuration and gene transcription.^{7,8} Transcriptional repression mediated by abnormal gains of CpG island methylation was generally thought to occur at local chromosomal region of discrete gene promoters during tumor initiation and progression, and the identification of hypermethylated genes by candidate approaches has contributed to a better knowledge of cancer epigenetics.^{9–11}

Gastrointestinal cancer, which includes gastric and colorectal cancer, is one of the main causes of global cancer mortality.¹² Clinicopathological features, such as tumor size, lymph node, and metastasis (TNM) stage, have

Supported by the National R&D Program for Cancer Control grant 0720540 funded by the Ministry of Health & Welfare (T-Y.K.), the Basic Science Research Program through the National Research Foundation of Korea grant NRF-2011-0021123 funded by Ministry of Education (S-H.S.), the Mid-career Researcher Program through NRF grant NRF-2013R1A2A2A01009297 funded by Ministry of Science, ICT and Future Planning (T-Y.K.), the Science Research Center/Engineering Research Center Program through NRF grant NRF-2011-0030049 funded by the MSIP (G-H.K.), and the Korea Health Technology R&D Project grant HI14C1277 through the Korea Health Industry Development Institute funded by MOHW, Republic of Korea (T-Y.K.).

J.-Y.K. and S.-H.S. contributed equally to this work.

Disclosures: None declared.

been used to predict patients' outcomes.^{13,14} However, these features do not completely account for the patients' recurrence or survival, indicating that other factors complicate the prognosis.^{13,14} Therefore, identifying molecular aberrations may help categorize patients into clinically relevant subgroups to better predict prognosis and therapeutic response.¹⁵ In this regard, several studies have reported that a subset of colon cancers show widespread concurrent methylation of numerous promoter CpG island loci, known as CpG island methylator phenotype,¹⁶ which is associated with prognosis in colon cancer. However, its prognostic value is still controversial in other types of cancer,^{17–21} due to the absence of a standardized CpG island methylator phenotype marker panel for specific cancer type. Thus, identification of standardized epigenetic markers that can precisely stratify patients according to subtypes is needed.

Recent genome-wide epigenomic and transcription profiling studies have reported large chromosomal regions that are frequently hypermethylated in cancer cells. This epigenetic silencing affecting multiple neighboring genes is termed as long-range epigenetic silencing (LRES).²² LRES was first described in colorectal cancer, affecting a 4-Mb gene-rich region in chromosome 2q14.2.²³ Interestingly, neighboring genes spanning the region showed suppression of most of the genes, hypermethylation of specific CpG islands, and enrichment of inactive histone mark H3K9me2 along the region.^{23,24} Concurrent silencing of multiple neighboring genes associated with CpG island promoters was also observed in other chromosomal regions, including the protocadherin cluster at 5q31²⁵ and Ikaros gene family in colorectal cancer.²⁶ In addition to colorectal cancer, LRES has been also demonstrated in prostate cancer,^{27,28} breast cancer,²⁹ and Wilms' tumor,³⁰ indicating that coordinated epigenetic regulation over a large chromosomal region may be a common event in tumor progression. Compared with previous studies investigating individual genes with limited resolution, genome-wide studies addressing LRES might reveal much more about the extent and exact landscape of epigenetic aberrations in cancer; however, their underlying mechanism and potential as a prognostic marker are still unclear.^{9–11}

Herein, we report the coordinated silencing of a new set of multiple neighboring genes in gastrointestinal tract cancer, which feature tumor-specific CpG island promoter methylation and aberrant chromatin structures. In particular, we found that gastric cancers with this LRES region are associated with older age of onset, high-grade differentiation, diffuse or mixed-type histology, lower recurrence rate, and better survival, indicating LRES as a potential biomarker.

Materials and Methods

Patients and Tissues

A consecutive series of 109 advanced gastric cancer cases (T2–T4) were operated on at the National Cancer Center

(Goyang, Republic of Korea). The study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (Seoul, Republic of Korea). Written informed consent was received from the patient. The cases included patients who underwent curative surgery and extended lymph node dissection (D2); cases of gastric carcinomas that were excised with palliative intent were not included. Patients who received neoadjuvant therapy, had other primary malignancies, or died from other causes were excluded. Median patient age was 59 years (range, 22 to 79 years), and the male/female ratio was 2.11:1. The following pathological parameters were assessed through gross and microscopic examination: gross type (Borrmann classification), tumor differentiation, histological type (Lauren classification), and TNM stage (American Joint Committee on Cancer, 6th edition). Tumor differentiation was categorized as high ($\geq 50\%$ glandular area) or low ($< 50\%$ glandular area). Tumors were classified as mixed type when they showed both intestinal- and diffuse-type components in excess of 40%. For multiple synchronous tumors, data were derived from the higher-stage tumor or the larger tumor if the two synchronous tumors were of the same stage. After identifying tumor areas by light microscopy, areas of approximately 1 cm², where the tumor cells were densest and represented the most prevalent histological type, were marked and scraped from the tissue glass slide with a knife.

Cell Culture and Treatment

Cells were cultured in McCoy 5A [HCT116 and double knockout (DKO)] or RPMI 1640 (SNU1, SNU5, SNU16, SNU216, SNU484, SNU601, SNU620, SNU638, SNU668, SNU719, AGS, SNUC1, SNUC4, SNUC5, SNU61, SNU283, SNU1033, HT15, HT29, LOVO, and LS174T) medium supplemented with 10% fetal bovine serum and gentamicin (10 μ g/mL).^{31–33} 5-Aza-2'-deoxycytidine (5-aza-CdR; Sigma, St. Louis, MO) was administered as indicated.

RT-qPCR Assay

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Total RNA (2 μ g) was used to synthesize cDNA with random hexamers and ImProm-II Reverse Transcriptase (Promega, Madison, WI).³⁴ Amplification was performed using the StepOnePlus real-time quantitative PCR (qPCR) system (Applied Biosystems, Foster City, CA) and Premix Ex Taq (TaKaRa, Shiga, Japan) with SYBR Green I (Molecular Probes, Eugene, OR).

Methylation-Specific PCR and Bisulfite Genomic Sequencing Analysis

Genomic DNA was isolated with the QIAamp DNA mini kit (Qiagen, Hilden, Germany), and 1 μ g genomic DNA was

modified with the EpiTect Bisulfite kit (Qiagen), according to the manufacturer's instructions.³⁵ Sodium bisulfite–modified DNA was amplified using primer sets specific for methylated or unmethylated sequences. PCR products were gel purified and cloned into a TA cloning vector (RBC Bioscience, Taipei, Taiwan). The inserted PCR fragments of individual clones were then sequenced as per the primers listed in Table 1.

ChIP Assay

The chromatin immunoprecipitation (ChIP) assay was performed as previously described.³⁶ Briefly, cells were

cross-linked with 1% formaldehyde for 10 minutes and then incubated with 0.125 mol/L glycine for 5 minutes at room temperature to terminate the reaction. Nuclei were prepared and digested with 50 U micrococcal nuclease at 37°C for 15 minutes and sonicated to yield chromatin fragments (200 to 400 bp). The precleared chromatin was incubated overnight with antibodies at 4°C, and then incubated with protein A agarose (Millipore, Billerica, MA), which was pre-equilibrated with sonicated salmon sperm DNA and bovine serum albumin. Immunoprecipitated material was washed extensively, and the cross-links were reversed. DNA from the eluted chromatin was purified by phenol extraction and ethanol

Table 1 List of Primers Used

Primer name	Forward primer	Reverse primer
Methylation-specific PCR		
RPS17 (U)	5'-TTATTGTTTATGGTTTGGATTGT-3'	5'-TCCCCTCAATTAACCTAACTCACT-3'
RPS17 (M)	5'-TTTTATTGTTTACGGTTTGGATC-3'	5'-CCCCTCGATTAACTTAACTCG-3'
CPEB1 (U)	5'-TGTTTTTAAAAGTGTAGAGGTAGTG-3'	5'-AAAAACCACAAAAAATCTTACAC-3'
CPEB1 (M)	5'-GCGTTTTTAAAAGTGTAGAGGTAGC-3'	5'-AAAAACCACGAAAAAATCTTACG-3'
HOMER2 (U)	5'-GTTTTTTGTTTTTGGTTTGTGTT-3'	5'-CAACTACCACTACTACCACTACCAC-3'
HOMER2 (M)	5'-TCGTTTTTTGGTTCGTTGTC-3'	5'-CGACTACCACTACTACCACTACCG-3'
BTBD1 (U)	5'-GAGGTTTTGGAGGTGTTTTTTT-3'	5'-CTCCAACACACAATACCCACAT-3'
BTBD1 (M)	5'-TTTTCGGAAGCGTTTTTTTC-3'	5'-AACTACGCGATTACCCACGT-3'
TM6SF1 (U)	5'-AGTTAGGTTTAAAAATTGGATGTTG-3'	5'-AAATCATCTATAAAAAACCCACAA-3'
TM6SF1 (M)	5'-GAGTTAGGTTTAAAAATCGGATGTC-3'	5'-AAATCGTCTATAAAAAACCCACG-3'
HDGFRP3 (U)	5'-AGAATTTTATAGAGTGGTATGGATGG-3'	5'-ACCAATTAATTCTTAAATACACAAC-3'
HDGFRP3 (M)	5'-GAGAATTTTATAGAGTGGTACGGAC-3'	5'-ACCGATTAAATCTTAAATACACGAC-3'
BNC1 (U)	5'-GATGTTTTGGAGTTGGTAAATG-3'	5'-AAAAATAACATATAAAATACCCACCAA-3'
BNC1 (M)	5'-GACGTTTCGGAGTTGGTAAAC-3'	5'-ATAACGTATAAAATACCCGCCG-3'
SH3GL3 (U)	5'-GTTTTGGAATGTAGTGTTGTTATTGG-3'	5'-ACAAAATAATCTCAAACTCAAC-3'
SH3GL3 (M)	5'-GTTTTGGAACGTAGTGTTGTTATC-3'	5'-AAACGAAATAATCTCAAACTCGAC-3'
ADAMTSL3 (U)	5'-AGTTTTTGTTTAGTTTTATTTAATTTTGT-3'	5'-AACTCAATACCCACCTTACAC-3'
ADAMTSL3 (M)	5'-TCGTTTTAGTTTATTTAATTTTCGC-3'	5'-ACTCGATACCCACCTTACG-3'
ZSCAN2 (U)	5'-TTAAATATAGGAATATTATGTGTGA-3'	5'-CTTAAAAAACCTTTTCAACTACAAA-3'
ZSCAN2 (M)	5'-TTAAATATAGGAATATTACGTGCGA-3'	5'-CTTAAAAAACCTTTTTCGACTACGAA-3'
Bisulfite genomic sequencing		
RPS17	5'-GGTTTTTATGTTTATAGAGAGGTTTGGG-3'	5'-TTCTATAATAACCCRAACCRCTTCTTC-3'
CPEB1	5'-TAGGAGGGTAGAGTTATAYGAGTAGGTAGT-3'	5'-ACAAAACAATTACCATAACAAACCCCTCC-3'
HOMER2	5'-GTGTATATAGTGTGTTTTTGYGTTTTTGG-3'	5'-TCACTACCCCTACTCCRACRGTAC-3'
BTBD1	5'-TGAGTTAGGATTAGTTTTTAYGTTTTTGT-3'	5'-ATTCRCRCTACAAAAAACAAAAACCC-3'
TM6SF1	5'-GTTAGGGGTATTTGTTTTTGYGGGGGTGT-3'	5'-ACRAAAACCCCRATAACCRCAAAACACTC-3'
HDGFRP3	5'-GAGAATAATAAAGATYGTGTTTGYGGTTG-3'	5'-ATAAACRAAAACRCAACAAACCCRACAAATC-3'
BNC1	5'-TGAGAAGAGYGTAGAGAATTTTAGAG-3'	5'-AAACARCTCCCCAAACRCCAAACTAC-3'
SH3GL3	5'-TAGGATATTTTAYGTTTAGTTTTTAGGT-3'	5'-ACTAATTTTATAAACTACTTCTTCAAC-3'
ADAMTSL3	5'-GGAGTGGTTTTTAGGAAGYGGYGGAGG-3'	5'-TCRTACRAAATCCRACACAAACCTTCCC-3'
ZSCAN2	5'-GGTGTGTTTTTATTAATAGAAAGGTAG-3'	5'-CTAACTCAAACCCACTATTCCCTCACC-3'
ChIP assay		
RPS17	5'-AGCAGGTATCCCCTGGCTAT-3'	5'-GTACTTGGGCCCTTCACAAA-3'
CPEB1	5'-CACAGAGCCTTCTGGTCTCC-3'	5'-GCCTGGAGTCACCTGAGTGT-3'
HOMER2	5'-GTCTTGATGCCACCTCCACT-3'	5'-AATATTGCAGACCCCACTGC-3'
BTBD1	5'-GGAGGTGTAAACAGGACTGGACTC-3'	5'-CCCCAGACCTACTGAGTCAGAATA-3'
TM6SF1	5'-TTTCCATACCCGTAGGCATC-3'	5'-CTACACCACCTCCTGCGATT-3'
HDGFRP3	5'-ACGTACCCAGGACAATGATAG-3'	5'-TAAATCCTGGAAAGTGGTAGTGGT-3'
BNC1	5'-TCTCCGCAGTTCTCCTCAGT-3'	5'-AGCCCAGGACGTTAGGACTT-3'
SH3GL3	5'-CCGGTACCCATGACAATCTC-3'	5'-GTGGAGACCTGGGCTACTTG-3'
ADAMTSL3	5'-GTCCAGCCCCATTTAACTT-3'	5'-CTTGCGCCGAGAAGTAT-3'
ZSCAN2	5'-AAGGACCAGCTTCCAGAAT-3'	5'-GGGATGCACAGATGGAACCTT-3'

ChIP, chromatin immunoprecipitation; M, methylated; U, unmethylated.

Figure 1 Long-range epigenetic silencing (LRES) of chromosome 15q25 is mediated by epigenetic modification. **A:** Schematic representation of chromosome 15q25 with the locations of 12 Reference Sequence genes and their associated CpG islands are indicated as **black boxes** and vertical bars, respectively, according to the University of California, Santa Cruz, genome browser (chromosome 15: 80,124,510-83,152,382). **B:** Relative gene expression of HCT116 and double-knockout (DKO) cells was derived from expression arrays. Among the 12 genes shown, *RPS17* and *ZSCAN2* were classified as boundary of the LRES region. **C:** Differential gene expression between HCT116 cells (gray bars) and DKO cells (black bars) was determined by real-time quantitative RT-PCR, and normalized to 18S ribosomal RNA. Data are presented as the averages of three independent RNA preparations, with error bars representing $\pm$ SD. **D:** Bisulfite genomic sequencing results for CpG islands of gene promoters in the LRES region from HCT116 and DKO cells. **Squares** denote CpG sites across PCR products amplified from bisulfite-treated DNA (gray squares, methylated; white squares, unmethylated). **E and F:** Characterization of chromatin modification patterns around chromosome 15q25. A chromatin immunoprecipitation assay was performed with antibodies specific for active histone mark H3K4me3 (**E**) and inactive histone mark H3K27me2 (**F**) in HCT116 and DKO cells. Error bars represent SEM for triplicate chromatin preparations. The 1775 site (amplicon 8) of the P21 locus and NECDIN (amplicon 9) served as positive and negative controls, respectively.³⁶

precipitation. Differences in DNA enrichment for ChIP samples were determined by qPCR using 2.5% of the precipitated sample DNA and 0.02% of the input DNA. The primers used for ChIP assay are listed in Table 1.

MethylLight Assay

Eight genes located within the LRES were analyzed by the MethylLight assay using primers and probes targeting promoter sequences or the first exon. Briefly, two sets of primers and probes designed to bind to bisulfite-converted DNA were used in the reaction; one set of primers and a probe were designed for every methylated target (methylated reaction), and one set of primers and a probe were directed to the reference locus, *Alu* (control reaction), used to normalize for input DNA and control for DNA amplification. We used M.SssI-treated placental genomic DNA as a

constant reference sample to determine the percentage of methylated reference at a particular locus. Percentage of methylated reference was defined as follows:

$$100 \times \frac{(\text{methylated reaction} / \text{normalization control reaction})_{\text{sample}}}{(\text{methylated reaction} / \text{normalization control reaction})_{\text{M.SssI-Reference (placental gDNA)}} \quad (1)$$

A CpG island locus was considered to be methylated if the percentage of methylated reference value was greater than four.

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences version 12.0 (SPSS, Inc., Chicago, IL). Relationships between clinicopathological parameters and LRES status were evaluated by Pearson χ^2 test. Multivariate regression analysis was performed using Cox proportional hazards model to

Figure 2 Long-range epigenetic silencing (LRES) of chromosome 15q25 in gastric cancer cells. **A:** Gene expression within the LRES region in 11 gastric cancer cells was analyzed by real-time quantitative RT-PCR (RT-qPCR), using 18S ribosomal RNA as an internal control. **B:** Differential gene expression of SNU601 and SNU484 cells was determined by RT-qPCR, and normalized to 18S ribosomal RNA. **C:** Methylation-specific PCR for genes within the LRES region. Bisulfite-modified genomic DNA derived from SNU601 and SNU484 cells was amplified with unmethylated (U) or methylated (M) primers. **D** and **E:** Characterization of chromatin modification profiles for the chromosome 15q25. A chromatin immunoprecipitation assay was performed with antibodies specific for active histone mark H3K4me3 (**D**) and inactive histone mark H3K27me2 (**E**) in SNU601 and SNU484 cells. Error bars represent the $\pm$ SEM of triplicate chromatin preparations. The 1775 site (amplicon 8) of the P21 locus and NECDIN (amplicon 9) served as positive and negative controls, respectively.³⁶ Data are presented as the averages of three independent RNA preparations, with error bars representing $\pm$ SD (**B**). DKO, double knockout; DW, distilled water (negative control).

define specific risk factors for overall survival and disease-free survival. All *P* values were two sided, and *P* < 0.05 was considered significant.

Cell Growth Inhibition Assay

A tetrazolium dye (MTT; Sigma) assay was used to evaluate the inhibitory effect of chemotherapeutic agents (doxorubicin, cisplatin, oxaliplatin, and paclitaxel) on cell growth. HCT116 and DKO cells were seeded in 96-well plates, incubated for 24 hours, and then treated with chemotherapeutic agents for 72 hours at 37°C with indicated doses. After treatment with the drug, MTT solution was added to each well and the cells were incubated for 4 hours at 37°C before the medium was removed.

Next, the samples were treated with dimethyl sulfoxide (Sigma), after which the absorbance of the converted dye in the living cells was measured using a microplate reader (Versa-Max; Molecular Devices, Sunnyvale, CA) at a wavelength of 540 nm. Six replicate wells were used for each analysis, and at least three independent experiments were performed.

Results

Epigenetic Silencing of Multiple Neighboring Genes Across Chromosome 15q25 in HCT116 Cells

Recent genome-wide studies have revealed that large chromosomal regions with multiple CpG island promoters

Figure 3 Drug-induced gene reactivation and epigenetic modification. SNU601 cells were treated with dimethyl sulfoxide (DMSO) or 5-aza-2'-deoxycytidine (5-aza-CdR or 5AZA; 5 μ mol/L) for 5 days. **A:** Restoration of previously silenced genes within the long-range epigenetic silencing (LRES) region after 5-aza-CdR treatment was determined by real-time quantitative RT-PCR. Each value was normalized to 18S ribosomal RNA. Error bars represent the SD for three independent RNA preparations. **B:** Bisulfite genomic sequencing analysis for CpG islands of LRES gene promoters after 5-aza-CdR treatment. Each square denotes a CpG site across the PCR product amplified from bisulfite-treated DNA (gray squares, methylated; white squares, unmethylated). **C** and **D:** Chromatin remodeling across the LRES region after 5-aza-CdR treatment. Enrichment of active histone mark H3K4me3 (**C**) and inactive histone mark H3K27me2 (**D**) was measured by chromatin immunoprecipitation assay and reported relative to the total input. The 1775 site (amplicon 8) of the P21 locus and NECDIN (amplicon 9) served as positive and negative controls, respectively.³⁶ Data are presented as the averages of three independent RNA preparations with error bars representing means $\pm$ SD (**A**); means $\pm$ SEM ($n = 3$) (**C** and **D**).

are often transcriptionally repressed by DNA methylation, termed as LRES. To search for genomic regions with multiple neighboring genes silenced by concordant epigenetic modification in HCT116 human colorectal cancer cells, we first identified a list of transcriptionally up-regulated genes in DKO cells by comparing microarray expression data sets for HCT116 and DKO cells. By comparing the expression profile of the HCT116 cells with that of the DKO cells, genes regulated by DNA methylation could be identified. Because DKO cells are DNMT1 and DNMT3B double knocked out counterpart of HCT116 cells,³¹ differential gene expression can be inferred as restoration of silenced genes mediated by lack of DNA methylation in DKO cells without functional DNMTs.

We identified 9677 genes that were up-regulated in DKO cells and, among them, 250 genes showing more than twofold or higher gene expression in DKO cells, compared to HCT116 with CpG island gene promoters, were further analyzed. Expression and presence of CpG island promoter of the neighboring genes were evaluated to estimate the length and boundary of the consecutive gene silencing to address the

possibility as LRES (data not shown). Boundaries were defined when two consecutive genes showed no difference in gene expression between the two cells.

On the basis of microarray data (data not shown), we identified chromosome 15q25 harboring *HDGFRP3*, as potential LRES region (Figure 1, A and B). Eight consecutive genes with CpG islands in their promoters (*CPEB1*, *HOMER2*, *BTBD1*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3*) are located within this region, with *RPS17* and *ZSCAN2* classified as the boundary. To validate our microarray data, we evaluated 10 genes by RT-qPCR. Six of these genes (*CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, and *ADAMTSL3*) showed significant differences in gene expression between the two cells, whereas *SH3GL3* showed only a minimal difference, and *BTBD1* along with two boundary genes (*RPS17* and *ZSCAN2*) showed no difference (Figure 1C). We also analyzed the gene expression profile of this region from other colon cancer cells to verify whether coordinated silencing of genes within this region is a common event. Among the 10 colon cancer cells analyzed, LRES was observed in SNUC1, SNU61, LOVO, HT29, and LS174T cells (Supplemental Figure S1A).

Figure 4 Frequency of long-range epigenetic silencing (LRES) on chromosome 15q25 in gastric cancer patients. **A:** Gene expression in the LRES region of primary gastric tumor tissues was determined by real-time quantitative RT-PCR and expressed relative to normal tissue samples. Five primary gastric tissues were compared with their normal counterparts. Gene expression was normalized to 18S ribosomal RNA. **B:** Methylation-specific PCR analysis of CpG island gene promoters within the LRES region in patients 250 and 889. The bisulfite-modified genomic DNA was amplified with unmethylated (U) or methylated (M) primers. **C:** Heat map of percentage of methylated reference (PMR) values for eight genes and 190 advanced gastric cancer cases. Dark brown, PMR ≥ 50 ; brown, $20 \leq \text{PMR} < 50$; light brown, $4 \leq \text{PMR} < 20$; white, PMR < 4 . **D:** Summary of methylation frequency in 190 primary gastric cancer tissue samples. Data are presented as the averages of three independent RNA preparations, with error bars representing $\pm$ SD (**A**).

To verify that concurrent suppression of neighboring genes within the LRES region is mediated by DNA hypermethylation of the CpG island promoters, we performed bisulfite genomic sequencing analysis of the gene promoters (Figure 1D). Down-regulation of *CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3* in HCT116 cells compared with DKO cells was identified to correlate with CpG island hypermethylation. In contrast, these genes were devoid of methylated CpG sites in DKO cells. However, *BTBD1* and the two boundary genes showed no DNA methylation at their promoters, corresponding to their active transcriptional states in both HCT116 and DKO cells. To further investigate if CpG island promoter methylation mediates transcriptional silencing of the LRES region, HCT116 cells were treated with 5-aza-CdR, and

gene restoration after the treatment in methylated genes was confirmed (Supplemental Figure S1C). We also identified that genes with low expression in other colon cancer cells (*HT29* and *SNU C4*) also had DNA methylation at CpG island promoters, corresponding to their gene expression (Supplemental Figure S1B).

Because histone modifications and DNA methylation cooperate to regulate gene expression at multiple levels,^{1,30} we investigated whether transcriptional silencing observed in the LRES region was also associated with typical histone modifications.^{1,37} We compared an active histone mark (H3K4me3) and an inactive histone mark (H3K27me2) in the LRES region by ChIP analysis. Consistent with low gene expression within the LRES region of HCT116 cells, H3K4me3 was barely detectable (Figure 1E), whereas

Table 2 Relationship between LRES Status and Clinicopathological Features

Clinicopathological features	LRES status			P value
	No. of cases	LRES (–)*	LRES (+)*	
Sex				0.082
Male	128	76 (59.4)	52 (40.6)	
Female	61	28 (45.9)	33 (54.1)	
Age (years)				0.002
20–39	12	10 (83.3)	2 (16.7)	
40–69	157	90 (57.3)	67 (42.7)	
70–79	21	5 (23.8)	16 (76.2)	
Location				0.419
Lower	123	69 (56.1)	54 (43.9)	
Middle	38	23 (60.5)	15 (39.5)	
Upper	29	13 (44.8)	16 (55.2)	
Histology grade				0.024
Low	41	29 (70.7)	12 (29.3)	
High	149	76 (51.0)	73 (49.0)	
Lauren classification				0.036
Intestinal	77	49 (63.6)	28 (36.4)	
Diffuse	85	44 (51.8)	41 (48.2)	
Mixed	21	7 (33.3)	14 (66.7)	
TNM stage				0.543
IB	28	18 (64.3)	10 (35.7)	
II	44	27 (61.4)	17 (38.6)	
IIIA	43	23 (53.5)	20 (46.5)	
IIIB	20	9 (45.0)	11 (55.0)	
IV	54	27 (50.0)	27 (50.0)	

*Data are given as number (percentage).

H3K27me2 was enriched (Figure 1F) in this region. However, in DKO cells, the same genes were enriched with both H3K4me3 and H3K27me2 in this region (Figure 1, E and F), consistent with the finding that promoter regions of methylated genes are frequently marked by bivalent histone modification in human cancer cells.^{38,39}

Taken together, we identified a novel LRES region on chromosome 15q25, which is strongly associated with hypermethylation of CpG island promoters and histone modifications.

Identification of LRES Region on Chromosome 15q25 in Gastric Cancer Cells

To determine whether the putative LRES region identified in colon cancer cells also occurs in gastric cancer cells, we evaluated the expression of 10 genes within the LRES region of 11 gastric cancer cells by RT-qPCR (Figure 2A). We found that the gene expression patterns of six cells (SNU16, SNU601, SNU620, SNU638, SNU719, and AGS) were similar to that of HCT116 cells, suggesting that the concurrent silencing of multiple neighboring genes found in colon cancer is also a frequent event in gastric cancer.

To confirm that overall gene suppression of this region in gastric cancer cells is due to epigenetic alteration, we

compared gene expression, methylation status, and chromatin remodeling of SNU601 and SNU484 cells, which showed gene expression patterns similar to those of HCT116 and DKO cells, respectively (Figure 2B). Results of RT-qPCR showed that the expression of seven genes (*CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3*) was barely detectable in SNU601 cells but was strong in SNU484 cells, whereas the expression of boundary genes (*RPS17* and *ZSCAN2*) and *BTBD1* did not differ significantly between these cells (Figure 2B).

By using methylation-specific PCR analysis, we evaluated whether DNA methylation of promoter CpG islands was associated with transcriptional inactivation in SNU601 cells. We determined the methylation status of CpG sites on the basis of sequence differences after bisulfite treatment using two different primer sets specific for methylated (Figure 2C) or unmethylated (Figure 2C) sites, which revealed distinct differences between SNU601 and SNU484 cells. Methylation of *CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3* was associated with low transcription in SNU601 cells, whereas these genes were unmethylated in SNU484 cells. In contrast, boundary genes (*RPS17* and *ZSCAN2*) and *BTBD1* were unmethylated in both cells (Figure 2C). We also identified similar methylation patterns in other gastric cancer cells (SNU719, AGS, and SNU5) (Supplemental Figure S2A).

To gain further insight into the mechanism underlying transcriptional silencing in this region, we used ChIP analysis to evaluate H3K4me3 (Figure 2D) and H3K27me2 (Figure 2E) in SNU601 and SNU484 cells. Active histone marks (H3K4me3) were enriched across the LRES region in SNU484 cells, with lower levels of inactive histone marks (H3K27me2) compared with SNU601 cells, corresponding to active transcription (Figure 2, D and E). In contrast, SNU601 cells were devoid of active histone marks, whereas inactive histone marks were significantly enriched, consistent with the low gene expression (Figure 2, D and E).

Taken together, these results confirm that LRES of chromosome 15q25 in gastric cancer cells is mediated by DNA methylation and histone modifications.

Differential Epigenetic Modification after 5-Aza-CdR Treatment within the LRES Region

To better understand the molecular mechanism underlying epigenetic silencing during tumorigenesis, we examined the dynamics of DNA methylation and histone modification after inducing gene activation by treating SNU601 cells with the demethylating agent, 5-aza-CdR (or 5AZA).³⁵ We found that genes with promoter CpG island methylation (*CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3*) were reactivated after 5-aza-CdR treatment (Figure 3A). Gene restoration after 5-aza-CdR treatment was also confirmed in other gastric cancer cells (SNU719 and AGS) (Supplemental Figure S2B).

Table 3 Univariate and Multivariate Cox Regression Analysis for Disease-Free Survival

Feature	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age (years)		0.096		0.021
≤59				
≥60	1.407 (0.941–2.104)		1.702 (1.082–2.678)	
Histology grade		0.683		0.808
Low				
High	1.110 (0.672–1.834)		1.070 (0.617–1.856)	
Location		0.145		0.634
Lower				
Middle	1.431 (0.892–2.295)		1.083 (0.650–1.805)	
Upper	0.733 (0.385–1.392)		0.739 (0.357–1.531)	
TNM stage		<0.001		<0.001
IB				
II	2.386 (0.778–7.318)		2.444 (0.785–7.603)	
IIIA	3.615 (1.223–10.685)		3.936 (1.302–11.900)	
IIIB	6.401 (2.061–19.880)		6.884 (2.153–22.012)	
IV	16.403 (5.875–45.800)		18.032 (6.319–51.462)	
LRES		0.824		0.027
LRES (–)				
LRES (+)	0.955 (0.637–1.431)		0.598 (0.379–0.943)	

LRES, long-range epigenetic silencing.

To confirm that this regional activation of neighboring genes was due to demethylation of CpG islands, each gene was analyzed by bisulfite genomic sequencing after 5-aza-CdR treatment. Although restoration of expression was observed for genes with hypermethylated CpG islands after 5-aza-CdR treatment, *HDGFRP3* was the only gene showing complete demethylation after treatment (Figure 3B). In contrast, methylation of the six remaining genes remained constant under the same conditions.

Because histone modifications are another layer of epigenetic regulation for gene transcription,⁴ we examined whether changes in histone modification could explain gene restoration after 5-aza-CdR treatment. Results of the ChIP assay showed enrichment of active histone marks (H3K4me3) in SNU601 cells after 5-aza-CdR treatment for all gene promoters tested (Figure 3C). Interestingly, inactive histone marks (H3K27me2) were also significantly enriched after treatment (Figure 3D).

These results suggest that although DNA methylation and histone modification cooperate to maintain gene silencing within the LRES region, their stability and dynamics differ considerably. DNA methylation appears to remain constant to maintain the silenced state, whereas histone modifications are dynamically regulated on 5-aza-CdR treatment.

LRES on Chromosome 15q25 Is Frequently Observed in Gastric Cancer Patients

To determine whether the LRES region of chromosome 15q25 also occurs in primary gastric tumor tissues, we compared mRNA expression and DNA methylation of these genes in primary tumor tissues with their normal counterparts. Results of RT-qPCR analysis of primary gastric tumors from five patients

revealed regional silencing in all tumor tissues compared to normal tissues (Figure 4A). To determine whether gene suppression was mediated by promoter CpG island methylation, we performed methylation-specific PCR with two primary gastric tumors (patients 250 and 889), which showed that lower gene expression in the tumors was associated with promoter CpG island methylation (Figure 4B). In contrast, these genes were not methylated in normal tissues (Figure 4B), suggesting that long-range silencing of chromosome 15q25 is mediated by tumor-specific DNA methylation. Similar to results in primary gastric tumor tissues, tumor-specific gene silencing within the LRES region in primary colon tissues was also observed (Supplemental Figure S3).

To extend our observations demonstrating the existence of LRES in tumors, we determined the methylation status of eight genes within the LRES region in 190 advanced gastric cancer tissue samples using the MethyLight assay (Figure 4C).⁴⁰ We identified that *CPEB1*, *HOMER2*, *TM6SF1*, *HDGFRP3*, *BNC1*, *SH3GL3*, and *ADAMTSL3* were methylated at frequencies >22.6%. However, *BTBD1* was never methylated at all (Figure 4D). Of the 190 tissue specimens, 85 (44.7%) showed methylation of more than six genes, but 11 (5.8%) showed no methylation of any genes within the LRES region (Figure 4, C and D). These results indicate that concordant silencing of multiple genes on chromosome 15q25 is common in gastric cancer.

Clinical Implication of LRES on Chromosome 15q25 in Gastric Cancer

To determine whether clinicopathological features of gastric cancers differed according to LRES status, we first

Table 4 Univariate and Multivariate Cox Regression Analysis for Overall Survival

Feature	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Age (years)		0.415		0.211
≤59				
≥60	1.191 (0.782–1.815)		1.354 (0.8472–2.179)	
Histology grade		0.466		0.361
Low				
High	1.218 (0.717–2.069)		1.308 (0.735–2.331)	
Location		0.441		0.524
Lower				
Middle	1.168 (0.702–1.944)		0.824 (0.469–1.446)	
Upper	0.712 (0.363–1.394)		0.657 (0.300–1.436)	
TNM stage		<0.001		<0.001
IB				
II	4.328 (0.968–19.341)		4.313 (0.952–19.546)	
IIIA	5.651 (1.284–24.870)		5.850 (1.301–26.312)	
IIIB	10.948 (2.424–49.444)		11.438 (2.475–52.863)	
IV	30.320 (7.325–125.494)		31.020 (7.411–129.835)	
LRES		0.859		0.035
LRES (–)				
LRES (+)	0.962 (0.629–1.472)		0.598 (0.469–1.446)	

LRES, long-range epigenetic silencing.

compared the average number of methylated genes in patients with different clinicopathological features. Our results showed that methylated genes were significantly more abundant in female patients, older patients, patients with high-grade cancer, and patients with diffuse or mixed-type gastric cancer (Supplemental Table S1). Patients were subdivided into two groups according to the number of methylated genes. Those with six or more methylated genes were defined as LRES positive, and those with fewer than six methylated genes were defined as LRES negative. Patients in the LRES-positive group were significantly older [20 to 39 years, 2/12 (16.7%); 40 to 69 years, 67/157 (42.7%); 70 to 79 years, 16/21 (76.2%); $P = 0.002$] (Table 2). In addition, the LRES-positive group was associated with high-grade differentiation [73/149 (49.0%); $P = 0.036$], diffuse or mixed type [diffuse type, 41/85 (48.2%); mixed type, 14/21 (66.7%); $P = 0.036$], and female sex [33/61 (54.1%); $P = 0.082$].

To determine whether LRES influences disease-free and overall survival in gastric cancer, Kaplan-Meier survival analysis was performed; however, we did not detect a significant correlation between LRES and disease-free survival ($P = 0.824$) or overall survival ($P = 0.859$) (Supplemental Figure S4, A and B). To determine whether the prognostic value of LRES was independent of other clinicopathological features associated with the clinical outcome of gastric cancer, multivariate analysis was performed using the Cox proportional hazards model. The risk factors examined were age, histology grade, tumor location, and TNM stage, which are known to affect the outcome of gastric cancer.^{13,14} Results of univariate analysis showed that only TNM stage was significantly associated with disease-free and overall survival (Tables 3 and 4). However, multivariate Cox regression

analysis showed that LRES and TNM stage were independent predictors of disease-free and overall survival after adjusting for confounding factors (Figure 5 and Tables 3 and 4). LRES was associated with lower recurrence (hazard ratio, 0.598; 95% CI, 0.379–0.943; $P = 0.027$) and better prognosis (hazard ratio, 0.598; 95% CI, 0.469–1.446; $P = 0.035$). Taken together, LRES could serve as a molecular marker to stratify patients with lower risk of recurrence and better survival among patients with poor prognostic indicators.

Discussion

For many years, the most widely studied epigenetic abrogation in tumorigenesis has been focused on DNA methylation and histone modification of discrete gene promoters interacting to inactivate their normal functions.^{4,41} However, the extent and spread of epigenetic modification and its potential influence on neighboring genes have not been previously addressed. In this context, recent studies are focusing on epigenetic deregulation occurring in adjacent genes spanning large chromosomal regions in a process called LRES.^{28,42} Uncovering the underlying mechanism of LRES and its association with particular clinicopathological features may provide another important marker for patients' stratification.

Herein, we identified a novel example of the LRES region in human gastrointestinal tract cancers affecting 10 neighboring genes on chromosome 15q25. We confirmed the concurrent down-regulation of gene expression within this region by comparing human colon cancer HCT116 cells with their DNMT1 and DNMT3B double-knockout derivative, DKO cells.³¹ Consecutive gene suppression in this region strongly correlated with promoter CpG island

Figure 5 Clinical outcome of the gastric cancer patients with long-range epigenetic silencing (LRES). Disease-free survival (A) and overall survival (B) were analyzed using Cox proportional hazards regression. * $P < 0.05$

hypermethylation (Figure 1D), loss of active histone marks (H3K4me3) (Figure 1E), and gain of inactive histone marks (H3K27me2) (Figure 1F). Down-regulation of this region was also identified in gastric cancer cells, again mediated by CpG island promoter methylation and histone modification, demonstrating that epigenetic silencing in cancer is not always localized to discrete CpG islands or single genes but can span larger chromosomal domains (Figure 2).

The coexistence of both active and inactive histone marks in promoter regions of methylated genes across chromosome

15q25 in HCT116 cells is noteworthy (Figure 1, E and F), because this signature is typical of the bivalent domains initially described in developmentally regulated genes.⁴³ These bivalent histone modifications maintain developmental genes poised for activation until lineage commitment in embryonic stem cells.⁴⁴ But, in cancer cells, genes residing on bivalent chromatin are vulnerable to abnormal DNA methylation, resulting in tighter gene inactivation during tumorigenesis. Thus, we suggest that low gene expression along chromosome 15q25, mediated by bivalent histone modification, is locked into a permanently silenced state by DNA methylation during tumorigenesis.

Interestingly, 5-aza-CdR-induced activation of DNA methylated genes in SNU601 cells was accompanied by an increase of both H3K4me3 and H3K27me2 levels, whereas DNA methylation level was unchanged (Figure 3). We hypothesize that once LRES of a chromosomal domain has occurred, DNA methylation acts as a primary cellular memory mechanism to maintain silencing in the LRES region, whereas the balance between the two bivalent histone marks provides plasticity for temporal gene reactivation. We also noted that although DNA methylation and histone modification are contiguous over the LRES region, the susceptibility of each gene to DNA methylation is different. Although other genes remained methylated after reactivation by 5-aza-CdR treatment, *HDGFRP3* showed complete demethylation of CpG island promoter. Conversely, this observation could support the seeding and spreading model of LRES, in which the *HDGFRP3* sequence triggers DNA methylation, which then spreads to neighboring genes.²²

Our results also indicate that LRES is common in primary gastric tumor tissues (Figure 4). Comparison of the five primary gastric tumor samples with their normal counterparts revealed that gene silencing, mediated by CpG island hypermethylation, is specific to tumor tissue. We also found that 44.7% of the advanced gastric tumor tissues were methylated in more than six genes, which we defined as LRES positive. Therefore, we investigated whether LRES could serve as a molecular marker to stratify patients into distinct subgroups with different clinicopathological features and outcomes. Our results showed that LRES-positive status was significantly associated with older age of onset, high histology grade, and diffuse or mixed-type gastric cancer (Table 2). In addition, LRES-positive status was associated with the female sex; however, this relationship was not significant. Although Kaplan-Meier survival analysis did not reveal significant differences in disease-free or overall survival between the LRES subgroups (Supplemental Figure S4, A and B), Cox proportional hazards regression showed LRES to be an independent prognostic indicator of lower recurrence and better survival (Figure 5 and Tables 2 and 3). Because the clinicopathological features associated with LRES-positive status are poor prognostic indicators, the prognostic impact of LRES for recurrence and survival may have been masked in the Kaplan-Meier survival analysis (Tables 3 and 4). We suggest that LRES could serve as a biomarker to identify patients with

lower risk of recurrence and better survival among patients with poor prognostic indicators. Notably, we observed better outcomes only in patients with six or more methylated genes, when patients were subdivided into smaller groups according to the number of methylated genes (0 to approximately 2, 3 to approximately 5, and 6 to approximately 8 methylated genes) (Supplemental Figure S4, C and D). This again supports that LRES is an independent prognostic marker to stratify patients of different outcome.

Herein, we focused on a defined genomic region on chromosome 15q25 in gastrointestinal cancer and identified a new set of genes with cancer-specific DNA methylation. Several other LRES regions have been identified, but their underlying mechanisms remain unclear. However, our findings emphasize the differential dynamics of DNA methylation and histone modification within the LRES regions, indicating the importance of studying the relationship of each epigenetic modification in the context of chromatin domains rather than isolated genes. We have also demonstrated that patients with LRES are associated with particular clinicopathological features, lower recurrence rate, and better survival, demonstrating that LRES may serve as a novel biomarker for gastric cancer patients. Taken altogether, identification of LRES markers may improve the chance to precisely stratify patients according to clinically relevant subgroups to better predict prognosis.

Acknowledgment

We thank all members of our group for discussions.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.11.022>.

References

- Rodriguez-Paredes M, Esteller M: Cancer epigenetics reaches mainstream oncology. *Nat Med* 2011, 17:330–339
- Baylin SB, Jones PA: A decade of exploring the cancer epigenome: biological and translational implications. *Nat Rev Cancer* 2011, 11: 726–734
- Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002, 3:415–428
- Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007, 8:286–298
- Ghavifekr Fakhr M, Farshdousti Hagh M, Shانهbandi D, Baradaran B: DNA methylation pattern as important epigenetic criterion in cancer. *Genet Res Int* 2013, 2013:317569
- Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002, 21: 5427–5440
- Robertson KD: DNA methylation and chromatin: unraveling the tangled web. *Oncogene* 2002, 21:5361–5379
- Jones PL, Wolffe AP: Relationships between chromatin organization and DNA methylation in determining gene expression. *Semin Cancer Biol* 1999, 9:339–347
- Ohtani-Fujita N, Fujita T, Aoike A, Osifchin NE, Robbins PD, Sakai T: CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* 1993, 8: 1063–1067
- Simpkins SB, Bocker T, Swisher EM, Mutch DG, Gersell DJ, Kovatich AJ, Palazzo JP, Fishel R, Goodfellow PJ: MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. *Hum Mol Genet* 1999, 8: 661–666
- Herman JG, Civin CI, Issa JP, Collector MI, Sharkis SJ, Baylin SB: Distinct patterns of inactivation of p15INK4B and p16INK4A characterize the major types of hematological malignancies. *Cancer Res* 1997, 57:837–841
- Jemal A, Center MM, DeSantis C, Ward EM: Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 2010, 19:1893–1907
- Werner M, Becker KF, Keller G, Hofler H: Gastric adenocarcinoma: pathomorphology and molecular pathology. *J Cancer Res Clin Oncol* 2001, 127:207–216
- Marzouk O, Schofield J: Review of histopathological and molecular prognostic features in colorectal cancer. *Cancers (Basel)* 2011, 3: 2767–2810
- Nalejska E, Maczynska E, Lewandowska MA: Prognostic and predictive biomarkers: tools in personalized oncology. *Mol Diagn Ther* 2014, 18:273–284
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP: CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999, 96:8681–8686
- Lee S, Cho NY, Choi M, Yoo EJ, Kim JH, Kang GH: Clinicopathological features of CpG island methylator phenotype-positive colorectal cancer and its adverse prognosis in relation to KRAS/BRAF mutation. *Pathol Int* 2008, 58:104–113
- Ogino S, Noshio K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS: CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009, 58:90–96
- Park SY, Kook MC, Kim YW, Cho NY, Jung N, Kwon HJ, Kim TY, Kang GH: CpG island hypermethylator phenotype in gastric carcinoma and its clinicopathological features. *Virchows Arch* 2010, 457: 415–422
- Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M: CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. *Clin Cancer Res* 2006, 12:2995–3002
- Enomoto S, Maekita T, Tsukamoto T, Nakajima T, Nakazawa K, Tatematsu M, Ichinose M, Ushijima T: Lack of association between CpG island methylator phenotype in human gastric cancers and methylation in their background non-cancerous gastric mucosae. *Cancer Sci* 2007, 98:1853–1861
- Clark SJ: Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. *Hum Mol Genet* 2007, 16(Spec No. 1):R88–R95
- Frigola J, Song J, Stirzaker C, Hinshelwood RA, Peinado MA, Clark SJ: Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. *Nat Genet* 2006, 38:540–549
- Forn M, Munoz M, Tauriello DV, Merlos-Suarez A, Rodilla V, Bigas A, Batlle E, Jorda M, Peinado MA: Long range epigenetic silencing is a trans-species mechanism that results in cancer specific deregulation by overriding the chromatin domains of normal cells. *Mol Oncol* 2013, 7:1129–1141
- Dallosso AR, Oster B, Greenhough A, Thorsen K, Curry TJ, Owen C, Hancock AL, Szemes M, Paraskeva C, Frank M, Andersen CL, Malik K: Long-range epigenetic silencing of chromosome 5q31 protocadherins is involved in early and late stages of colorectal tumorigenesis through modulation of oncogenic pathways. *Oncogene* 2012, 31:4409–4419

26. Javierre BM, Rodriguez-Ubrea J, Al-Shahrour F, Corominas M, Grana O, Ciudad L, Agirre X, Pisano DG, Valencia A, Roman-Gomez J, Calasanz MJ, Prosper F, Esteller M, Gonzalez-Sarmiento R, Ballestar E: Long-range epigenetic silencing associates with deregulation of Ikaros targets in colorectal cancer cells. *Mol Cancer Res* 2011, 9:1139–1151
27. Devaney J, Stirzaker C, Qu W, Song JZ, Statham AL, Patterson KI, Horvath LG, Tabor B, Coolen MW, Hulf T, Kench JG, Henshall SM, Pe Benito R, Haynes AM, Mayor R, Peinado MA, Sutherland RL, Clark SJ: Epigenetic deregulation across chromosome 2q14.2 differentiates normal from prostate cancer and provides a regional panel of novel DNA methylation cancer biomarkers. *Cancer Epidemiol Biomarkers Prev* 2011, 20:148–159
28. Coolen MW, Stirzaker C, Song JZ, Statham AL, Kassir Z, Moreno CS, Young AN, Varma V, Speed TP, Cowley M, Lacaze P, Kaplan W, Robinson MD, Clark SJ: Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity. *Nat Cell Biol* 2010, 12:235–246
29. Novak P, Jensen T, Oshiro MM, Watts GS, Kim CJ, Futscher BW: Agglomerative epigenetic aberrations are a common event in human breast cancer. *Cancer Res* 2008, 68:8616–8625
30. Dallosso AR, Hancock AL, Szemes M, Moorwood K, Chilukamarri L, Tsai HH, Sarkar A, Barasch J, Vuononvirta R, Jones C, Pritchard-Jones K, Royer-Pokora B, Lee SB, Owen C, Malik S, Feng Y, Frank M, Ward A, Brown KW, Malik K: Frequent long-range epigenetic silencing of protocadherin gene clusters on chromosome 5q31 in Wilms' tumor. *PLoS Genet* 2009, 5:e1000745
31. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Vogelstein B: DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 2002, 416:552–556
32. Ku JL, Park JG: Biology of SNU cell lines. *Cancer Res Treat* 2005, 37:1–19
33. Park J-G, Frucht H, LaRocca RV, Bliss DP Jr, Kurita Y, Chen T-R, Henslee JG, Trepel JB, Jensen RT, Johnson BE, Bang Y-J, Kim J-P, Gazdar AF: Characteristics of cell lines established from human gastric carcinoma. *Cancer Res* 1990, 50:2773–2780
34. Song SH, Kim A, Ragoczy T, Bender MA, Groudine M, Dean A: Multiple functions of Ldb1 required for beta-globin activation during erythroid differentiation. *Blood* 2010, 116:2356–2364
35. Yun J, Song SH, Park J, Kim HP, Yoon YK, Lee KH, Han SW, Oh DY, Im SA, Bang YJ, Kim TY: Gene silencing of EREG mediated by DNA methylation and histone modification in human gastric cancers. *Lab Invest* 2012, 92:1033–1044
36. Song SH, Hou C, Dean A: A positive role for NLI/Ldb1 in long-range beta-globin locus control region function. *Mol Cell* 2007, 28:810–822
37. Fullgrabe J, Kavanagh E, Joseph B: Histone onco-modifications. *Oncogene* 2011, 30:3391–3403
38. McGarvey KM, Van Neste L, Cope L, Ohm JE, Herman JG, Van Criekinge W, Schuebel KE, Baylin SB: Defining a chromatin pattern that characterizes DNA-hypermethylated genes in colon cancer cells. *Cancer Res* 2008, 68:5753–5759
39. Easwaran H, Johnstone SE, Van Neste L, Ohm J, Mosbrugger T, Wang Q, Aryee MJ, Joyce P, Ahuja N, Weisenberger D, Collisson E, Zhu J, Yegnasubramanian S, Matsui W, Baylin SB: A DNA hypermethylation module for the stem/progenitor cell signature of cancer. *Genome Res* 2012, 22:837–849
40. Shin SH, Park SY, Ko JS, Kim N, Kang GH: Aberrant CpG island hypermethylation in pediatric gastric mucosa in association with *Helicobacter pylori* infection. *Arch Pathol Lab Med* 2011, 135:759–765
41. Feinberg AP, Tycko B: The history of cancer epigenetics. *Nat Rev Cancer* 2004, 4:143–153
42. Bae JM, Kim JH, Kang GH: Epigenetic alterations in colorectal cancer: the CpG island methylator phenotype. *Histol Histopathol* 2013, 28:585–595
43. Rodriguez J, Munoz M, Vives L, Frangou CG, Groudine M, Peinado MA: Bivalent domains enforce transcriptional memory of DNA methylated genes in cancer cells. *Proc Natl Acad Sci U S A* 2008, 105:19809–19814
44. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE: Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 2007, 448:553–560