STK31 upregulation is associated with chromatin remodeling in gastric cancer and induction of tumorigenicity in a xenograft mouse model

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Abstract. Pathological changes in the epigenetic landscape of chromatin are hallmarks of cancer. Our previous study showed that global methylation of promoters may increase or decrease during the transition from gastric mucosa to intestinal metaplasia (IM) to gastric cancer (GC). Here, CpG hypomethylation of the serine/threonine kinase STK31 promoter in IM and GC was detected in a reduced representation bisulfite sequencing database. STK31 hypomethylation, which resulted in its upregulation in 120 cases of primary GC, was confirmed. Using public genome-wide histone modification data, upregulation of STK31 promoter activity was detected in primary GC but not in normal mucosae, suggesting that STK31 may be repressed in gastric mucosa but activated in GC as a consequence of hypomethylation-associated chromatin remodeling. STK31 knockdown suppressed the proliferation, colony formation, and migration activities of GC cells in vitro, whereas stable overexpression of STK31 promoted the proliferation, colony formation, and migration activities of GC cells in vitro and tumorigenesis in nude mice. Patients with GC in which STK31 was upregulated exhibited significantly shorter survival times in a combined cohort. Thus, activation of STK31 by chromatin remodeling may be associated with gastric carcinogenesis and also may help predict GC prognosis.

Introduction

Gastric cancer (GC) is one of the most common types of malignancy, more than one million cases are diagnosed each year worldwide and survival rate decreases as cancer progresses (1,2). Many patients with GC are diagnosed at an advanced stage because GC is initially asymptomatic and biomarkers are lacking (2). Therefore, the identification of biomarkers for early-stage detection and prediction of prognosis may improve the efficacy of GC treatment strategies (3).

Gastric carcinogenesis is a multistep process that arises from superficial gastritis and progresses to chronic atrophic gastritis, intestinal metaplasia (IM), dysplasia and carcinoma (4). Similar to other types of cancer, gastric carcinogenesis exhibits a multifactorial etiology involving environmental, genetic and epigenetic components. Among epigenetic alterations, there has been interest in hypermethylation/repression of tumor-suppressor genes (5). Moreover, it is hypothesized that DNA hypomethylation promotes cancer development via activation of proto-oncogenes (6), although examples of this are lacking. However, advances in global methylation profiling suggest that aberrant expression of STK31 contributes to tumorigenicity in somatic cancer cells, and thus STK31...
and STK31 may be potential therapeutic targets in human somatic cancer. In colorectal cancer, for example, STK31 expression can be reactivated by treating diseased tissue with 5-aza-2-deoxycytidine (5-aza-dC) (13). The kinase domain of STK31 regulates tumorigenicity via control of differentiation state, suggesting that STK31 may be regulated by an epigenetic mechanism (15). However, the role of STK31 in GC and the mechanism by which STK31 transcription is controlled in GC is not clear.

The aim of the present study was to determine whether the regulation of STK31 expression is associated with chromatin remodeling, including DNA methylation and histone modification at the promoter region, and to elucidate the role of STK31 in gastric carcinogenesis. In order to reveal the epigenetic alteration in IM and/or GC, methylome data of the RRBS and 450K HumanMethylation BeadChip data and publicly available genome-wide histone modification data were used. In order to identify the role of STK31 in GC cells, in vitro experiments, such as proliferation, colony forming and migration assays in stable GC cells, and in vivo experiments with xenograft mice were performed. Moreover, patients for which STK31 was upregulated were investigated for survival times in a combined cohort. These results may provide insight into the role of for STK31 in the development of GC and as a potential therapeutic and prognostic target for patients with GC.

Materials and methods

Cell lines and tissue samples. A total of 16 GC cell lines, namely SNU001, SNU005, SNU016, SNU216, SNU484, SNU520, SNU601, SNU620, SNU638, SNU668, SNU719, AGS, KATOIII, MNK01, MNK45 and MNK74, were obtained from the Korean Cell Line Bank (cellbank.snu.ac.kr/main/index.html) and cultured in RPMI-1640 medium (Welgene, Inc.) to yield a 228-bp product and reverse, 5'-ATTGCTCTTGGCATCAAG-3' to yield a 228-bp product and reverse, 5'-TAAGAGATGGCCACGGCTGTCT-3' and reverse, 5'-TCCCTTCTGCATCTGTCGCA-3' to yield a 275-bp product. RT-qPCR for STK31 was performed using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). cDNA (100 ng) was amplified as aforementioned for 45 cycles with 2X SYBR-Green Supermix (Bio-Rad Laboratories, Inc.). β-actin was used as a control. The relative amount of target mRNA was quantified using comparative threshold cycle (Cq) method (16).

Pyrosequencing. Two CpG sites, namely CpG#23 and #24, in Region 2 were selected for quantification of the extent of methylation. Bisulfite-modified DNA (100 ng) was amplified by PCR in a 20 μl reaction using 2X Dye Mix polymerase (Enzymics Co., Ltd.) to yield a 221-bp product using the following primers: Forward, 5'-TTGTGGGGGTTGTAGTAGTGGTAG-3' and reverse, 5'-CCCTAACACCATACACATTACAC-3'. PCR was performed using an initial melting step of 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec, with a final step of 72°C for 7 min. β-actin served as the PCR control. The PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. The primer sequences for RT-qPCR were as follows: STK31 forward, 5'-AACCTGCTCTCCAGGTTCACA-3' and reverse, 5'-ATTGCTCTTGGCATCAAG-3'.

Treatment with 5-aza-dC and trichostatin A (TSA). SNU638, SNU719 and MKN74 cells were maintained as aforementioned. Cells were seeded in 100-mm dishes at a density of 1x10⁶ cells per dish, then treated with 10 μM DNA methylation inhibitor 5-aza-dC (Sigma-Aldrich; Merck KGaA) every 24 h for 3 days and harvested. Another group of these cells at the same cell density was treated with 0.5 μM histone deacetylase inhibitor TSA (Sigma-Aldrich; Merck KGaA) for 3 days and harvested. In
order to test the combined effect of 5-aza-dC and TSA, cells were treated with 10 µM 5-aza-dC every 24 h for 3 days and then with 0.5 µM TSA for 1 day. After 2-5 days, cells were washed with phosphate-buffered saline, and total RNA was extracted using a RNeasy Mini kit (QiaGen, Inc.). All experiments were performed at 37°C. RT-qPCR for STK31 was performed as aforementioned. A total of three independent experiments was performed.

Establishment of stable cell lines. STK31-knockdown (KD) cells were established using TRCN0000368917 and TRCN0000003276 (STK31-sh1 and STK31-sh4, respectively; Sigma-Aldrich; Merck KGaA) targeting STK31 mRNA: pLKO.1-puro (Sigma-Aldrich; Merck KGaA) was used as a control. For lentivirus construction, 293T cells were obtained from Koram Biotech Corp. and co-transfected with 2 µg MISSION Lentiviral Packaging Mix and 2 µg control or STK31 short hairpin (sh) RNA using a 2nd generation lentiviral vector and Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). In order to establish STK31-expressing cell lines, 2 µg full-length STK31 cDNA was cloned into vector pCDH-CMV-MCS-EF1-Puro (System Biosciences, LLC). For lentivirus construction, 293T cells were co-transfected using a 2nd generation lentiviral vector with 2 µg MISSION Lentiviral Packaging Mix plus empty vector or STK31-expressing vector using Lipofectamine 2000. After 48 and 72 h incubation at 37°C, 5% CO₂, supernatant containing the lentivirus was collected from 293T cells and centrifuged at 250 x g for 3 min at room temperature, then filtered and applied to target cells.

Cell growth analysis. MKN1 cells were treated 0.05% trypsin for 3 min at 37°C and then harvested at a density of 2x10⁵ per ml. Cells were washed with ice-cold PBS and fixed with 70% ethanol for 24 h at 4°C. Prior to analysis, cells were stained with 50 µg/ml propidium iodide solution for 2 min at room temperature. Cell cycle analysis was performed using a flow cytometer (FacsCalibur; BD Biosciences) with blue laser 488 nm and FlowJo 10.7.1 and Cell Quest Software (BD BioSciences).

Cell migration assay. Transwell migration assays were performed in a 24-well Transwell chamber (Corning, Inc.) fitted with a polycarbonate membrane (pore size, 8 mm). Cells were suspended in 100 µl serum-free RPMI-1640 medium (Welgene, Inc.), and 2x10⁴ cells were seeded in the upper chamber. The lower chamber was filled with RPMI-1640 medium containing 10% fetal bovine serum. After 16-24 h incubated at 37°C, migrated cells were stained for 2 h at room temperature with 0.5% crystal violet solution. A total of three independent fields of view were observed using a fluorescence microscope (magnification, x10) for each membrane, and migrated cells were manually counted in each field.

Gap closure assay. Mobility of STK31-expressing MKN74 cells was measured using gap closure assay (Ibidi Gmbh). Cell suspension at a density of 1x10⁶ per ml in 70 µl volume was detached from the well using forceps and filled in a 24-well Transwell system (Bio-Rad Laboratories, Inc.) Proteins were transferred to a polyvinylidene fluoride membrane (Sigma-Aldrich; Merck KGaA) and blocked in 5% skimmed milk in Tris-buffered saline (0.1% Tween-20) for 30 min at room temperature. The membranes were incubated with primary antibodies (1:1,000) at 4°C overnight. The antibodies were as follows: Anti-STK31 (cat. no. ab155172; Abcam), anti-Caspase3 (cat. no. 9662; Cell Signaling Technology, Inc.), anti-PARP (cat. no. 9542; Cell Signaling Technology, Inc.) and anti-Tubulin (cat. no. T5168; Sigma-Aldrich; Merck KGaA), then Probed with mouse anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.). Immunopositive bands were visualized using an enhanced luminescence image analyzer LAS-4000 (FUJIFILM Wako Pure Chemical Corporation) and the intensity for each band was estimated by ImageJ version 1 software (National Institutes of Health).
was calculated according to the following formula: Volume
\( \text{mm}^3 = \text{width}^2 \times \text{length} / 2 \). All mice were euthanized using
30-70% volume/min CO\(_2\) in chamber at day 53. The humane
endpoints were when the largest tumor size exceeded 20 mm
or feed intake or drinking water were affected due to necrosis,
infection or ulcer. None of the mice died before endpoints of
the study. Experiments using mouse were conducted under
the Institutional Animal Care and Use Committee-approved
protocols at KRIBB in accordance with institutional guidelines
(approval no. KRIBB-AEC-15102).

Public data. The 450K HumanMethylation BeadChip data
(asscession number GSE103186) for 39 gastric mucosas and
76 IM tissue samples from GC-free patients (18) were down‑
loaded to compare methylation status in the STK31 promoter.
Gene expression and 450K HumanMethylation BeadChip data for 230 primary GCs and 450K HumanMethylation
Beadchip data for two normal tissue samples (19) were downloaded from The Cancer Genome Atlas (TCGA)
portal (portal.gdc.cancer.gov/) and the National Center for
Biotechnology Information Gene Expression Omnibus (ncbi.
.nlm.nih.gov/geo/). Because methylation data for normal
tissue are limited in the TCGA database, additional data for 10
gastric mucosa samples were obtained public data (acces‑
sion nos. GSE50192 (n=4) and GSE31848 (n=6)) (20,21) produced
by the same platform. In order to assess the activation status
of the STK31 promoter in primary GCs, public data (acces‑
sion no. GSE51776) for histone modifications produced by
nano-scale chromatin immunoprecipitation-sequencing (Nano‑ChiP-seq) of paired GC and non-tumor tissue was
downloaded (22). The downloaded sequence reads were quality
controlled using cut-adapt (v1.1) on a public site
(github.com/marcelm/cutadapt/). Quality-control parameters
were read for quality >30 (Phred score), read length >20 bp
and replicated level <40%. The sequence reads were mapped
with bowtie2 (v2.2.2) using default parameters (n=1). Duplicatae reads were removed using the picard (v.2.18.14)
markduplicates function. Unique reads were identified by
peak calling using maqc2 (v.2.0.5) with default parameters.
For predicting patient outcomes, gene expression data (acces‑
sion no. GSE26253) for GC (n=432) in the Samsung Medical
Center cohort (SMC) (23) was downloaded from the Gene
Expression Omnibus (NCBI).

Results

STK31 is an early-onset target for hypomethylation in GC development. Our previous study (8) identified 174
hypomethylated promoters in GC cells (91 GC-specific and
83 early-onset) via methylene analysis with laser-capture
microdissected cells of a single patient with intestinal-type
GC (IGC). Early-onset hypomethylation was defined as a
methylation difference >2-fold in IM and GC compared with
gastric mucosa cells. STK31 was an early-onset hypomethyl‑
ated targets (Fig. 1B). RRBS data from the UCSC Genome
Browser (hg19) revealed that CpG methylation signatures
(purple vertical lines) at the STK31 promoter were predomin‑
ant in GM cells but mostly absent in IM and completely
absent in GC (Fig. 1B).

CpG hypomethylation of the STK31 promoter is associated
with STK31 upregulation in primary GC. RT-qPCR analysis
was performed with four paired gastric tumor and adjacent
non-tumor tissues, revealing that STK31 was silenced in
non-tumors but expressed in all tumor samples tested
(Fig. 2A). Bisulfite sequencing analysis with three of four
paired clinical tissues revealed 51.4-69.4% methylation in
Region 1 of non-tumors but 16.7-30.3% in paired tumors
(Fig. 2B). For Region 2, the difference was also significant
(non-tumor, 92.7-96.4%; tumor, 64.8-86.1%; Fig. 2B), although
not as large as for Region 1. These data suggested that STK31
expression levels in tumors may be a consequence of CpG
hypomethylation at the promoter comprising Regions 1 and 2.

STK31 expression levels and promoter methylation
were analyzed using 145 paired clinical tissue samples of
the CNUH cohort. RT-qPCR analysis of STK31 revealed
significant upregulation in tumor (16.70±3.60%) compared
with non-tumor samples (13.29±5.49%; Fig. 2C; P=0.02). A
a significant increase in expression was defined as >2-fold
with respect to the values for tumors compared with paired
non-tumors; increased STK31 expression was apparent in
approximately half (72/145) of tumors. Methylation at two
CpG sites, namely CpG#23 and #24, within Region 2 was
quantified by pyrosequencing (Fig. 1C) of 120 paired clinical
tissues and compared with the corresponding RT-qPCR
data. Pyrosequencing of these two sites revealed 92.1±6.4%
methylation in non-tumors and 79.7±13.0% in tumors,
the difference for which was significant (Fig. 2D; P<2.2x10^{-10}).
Finally, there was a negative correlation between methylation
at the two CpG sites and STK31 expression in 120 matched
tumors (Fig. 2E; r=-0.34; P=1.1x10^{-4}).
From the public data for 450K HumanMethylation BeadChip of the TCGA, methylation status between GC and non-tumors at CG sites proximal to the STK31 promoter was compared; the promoter and upstream region were hypomethylated in primary GC, whereas there was no difference in CpG methylation on the gene body (Fig. 3A). For example, methylation at cg05000488 within the STK31 promoter was significantly decreased in GC, especially in IGC (Fig. 3B), whereas STK31 mRNA expression levels were significantly increased in GC (Fig. 3C), revealing a negative correlation between CpG methylation and STK31 mRNA expression levels (Fig. 3D). In addition, methylation status was compared at the same CG sites proximal to the STK31 promoter from the public data (GSE103186) for gastric mucosae and IM tissue from GC-free patients (17). Methylation at cg05000488 within the STK31 promoter was significantly decreased in IM compared with that in gastric mucosae (Fig. 3E).

Change in promoter methylation in GC cell lines alters STK31 expression. In order to investigate the association between STK31 expression and methylation of its promoter in GC cell lines, RT-qPCR and bisulfite sequencing analysis of GC cell lines were performed. Based on the RT-qPCR results, cell lines were divided into two groups according to the median relative STK31 expression level. Figure 4A and B; the STK31-expressing group (+; >median value) included SNU484, SNU638, SNU719, and MKN74. For Region 1, bisulfite sequencing revealed that members of the STK31 (+) group, except for SNU484, exhibited low mean methylation (12.1-69.1%), whereas the STK31 (-) group had relatively high methylation (81.2-92.6%; Fig. 4B). In Region 2, however, members of both groups were highly methylated (86.8-99.1%; Fig. 4B). The association between methylation status at two CG sites (CG#23 and #24) and STK31 expression was examined in both groups; methylation status tended to be decreased in STK31 (+) group compared with the STK31 (-) group and STK31 expression levels tended to be increased in STK31 (+) group compared with the STK31 (-) group (Fig. 4C) in both bisulfite sequencing and pyrosequencing analyses. In order to determine whether STK31 expression is controlled epigenetically, cells were treated with 5-aza-dC and/or TSA. Treatment with 5-aza-dC significantly restored STK31 expression levels in SNU-638, SNU-719, and MKN74 cells (Fig. 4D). Treatment of GC cells with both 5-aza-dC and TSA also restored STK31 expression levels (Fig. 4D), suggesting that its expression in GC cells may be regulated epigenetically.

Genome-wide histone modification data reveal upregulation of the STK31 promoter in primary GC. From the public data (GSE51776), unique reads were identified by peak calling using macs2 with default parameters. This yielded peak regions for
H3K4me3 (chromatin mark for active promoters), H3K4me1 (chromatin mark for active enhancers and promoters) and H3K27ac (chromatin mark for active regulatory elements), for which five paired normal and gastric tumor tissue samples were merged. Then, signatures for promoter activity were examined near the STK31 promoter. Gain of STK31 promoter activity (increased H3K4me3 and H3K27ac) was evident in primary GC but not in normal mucosae (Fig. 5).

**Figure 2.** STK31 expression and bisulfite sequencing analysis of clinical tissue samples. (A) RT-qPCR analysis of STK31 in four paired gastric T and N tissue samples. β-actin was used as an internal control. (B) Bisulfite sequencing analysis was performed with three paired GC and N tissue samples. Black and white circles indicate methylated and non-methylated CpG sites, respectively. Each row represents a single clone. Mean percentages of CpG sites methylated in each sample are shown. Asterisks indicate CpG sites (CpG#23 and #24) used for pyrosequencing. Arrows indicate CpG probes from 450K BeadChip. (C) STK31 expression in 145 paired N and GC samples from the CNUH cohort. RT-qPCR was performed and expression levels were normalized to β-actin. (D) STK31 methylation in paired samples from the CNUH cohort. Pyrosequencing was performed at two CpG sites. The average value for methylation was calculated for each sample. (E) Pearson's correlation analysis between STK31 methylation and expression levels in the CNUH cohort. STK, serine/threonine kinase; RT-q, reverse transcription-quantitative; T, tumor; N, non-tumor; CNUH, Chungnam National University Hospital.

**STK31 KD inhibits cell proliferation and migration and induces G1 arrest in vitro.** It was next investigated whether STK31 expression in SNU484 or MKN1 cells, in which STK31 was highly expressed (Fig. 6A), could be knocked down by two shRNAs. RT-qPCR and western blotting analysis confirmed that STK31 expression levels significantly decreased in STK31-KD SNU484 and MKN01 cells (Fig. 6A). Each shRNA significantly decreased colony formation (Fig. 6B),
Figure 3. Correlation between STK31 promoter methylation and expression levels in primary gastric tumors or STK31 promoter methylation in IM from the public database. (A) Methylation status at CpG sites proximal to the STK31 promoter. β-values at 14 CpG sites from TSS500, TSS200, TSS100, exon 1 and the gene body were retrieved from 450K HumanMethylation BeadChip data for 29 gastric mucosa samples (normal) and 214 gastric tumors including IGC (n=140), DGC (n=57) and mixed-type GC (n=17) from the TCGA database. Red arrow indicates cg05000488, the CpG site in TSS100 at which correlation with STK31 expression levels was examined. (B) STK31 methylation status at cg05000488 was examined in normal tissue, IGC, DGC and mixed-type GC. P-values were determined using the Wilcoxon rank-sum test and corrected for multiple comparisons by Bonferroni method (n=3). (C) STK31 expression was examined. Pairwise P-values were calculated using Student's t-test and corrected for multiple comparisons by Bonferroni method (n=3). (D) Pearson's correlation analysis between methylation at cg05000488 and STK31 expression levels in the TCGA cohort. (E) Methylation status at CpG sites proximal to the STK31 promoter in IM. β-values at 13 CpG sites were retrieved from 450K BeadChip data for 39 normal and 76 IM samples from public data GSE103186 (17). P-values were determined using Student's t-test. *P<0.05, **P<0.01, **P<0.001. STK, serine/threonine kinase; IM, intestinal metaplasia; TSS, transcription start site; IGC, intestinal-type gastric cancer; DGC, diffuse-type gastric cancer; TCGA, The Cancer Genome Atlas.
Figure 4. *STK31* expression and bisulfite sequencing analysis of GC cell lines. (A) RT-qPCR analysis of 16 GC cell lines. (B) Analysis of bisulfite sequencing. A total of eight GC cell lines were categorized based on relative *STK31* expression (determined by RT-qPCR) as strong (+) or weak/silenced (−). (C) Association between *STK31* expression and mean methylation at CpG#23 and #24. Methylation status was based on bisulfite sequencing and pyrosequencing analysis. (D) Restoration of *STK31* mRNA levels following treatment with 5-aza and/or TSA. *STK31* expression levels were assessed by RT-qPCR and normalized to β-actin. Data are presented as the mean ± SD of three independent experiments. Pairwise P-values were calculated using Student’s t-test and corrected for multiple comparison by Bonferroni method. *P<0.05. STK, serine/threonine kinase; GC, gastric cancer; RT-q, reverse transcription-quantitative; 5-aza, 5-aza-2-deoxycytidine; TSA, trichostatin A.
proliferation (Fig. 6C) and migration (Fig. 6D) of both STK31-KD cell lines compared with cells treated with control shRNA. In order to investigate the underlying mechanisms for the cell proliferation of STK31 suppression, the effect of STK31 silencing on cell cycle progression in MKN1 cells was assessed by flow cytometry. STK31-KD MKN1 cells was increased in Figure 5. Histone modifications at STK31 promoters in paired normal and GC tissue. Public data for histone modifications in paired tissue, as determined by nano-scale chromatin immunoprecipitation-sequencing (22), were downloaded and processed. H3K4me3 and H3K27ac peak regions from five paired normal and gastric tumor tissue samples were merged and visualized with the UCSC Genome Browser (hg19). Red rectangle highlights gain of promoter activity with increased H3K27ac and H3K4me3 at each promoter in primary GC. STK, serine/threonine kinase; GC, gastric cancer.

Figure 6. In vitro assay of STK31 expression levels in STK31-KD cells. (A) Establishment of STK31-KD cells by expression of shRNA. SNU484 and MKN01 cells were transfected with either of two lentiviral STK31 shRNAs (sh#1, sh#4) or scrambled-sequence shCon and cultured for 2 weeks. KD and control cells were compared by reverse transcription-quantitative PCR and western blotting. Tubulin was used as an internal control. (B) Colony formation assay. Transfected cells were plated on 6-well plates at 1x10^3 cells per well. After 2 weeks, colonies were stained with crystal violet and counted. (C) Relative viability of STK31-KD cells over 4 days was measured using EZ-Cytox Cell Viability Assay kit and compared with empty vector control (PLKO). (D) Migration assay. Transfected cells were plated on Transwell chambers at 2x10^4 cells per well. After 18-22 h, Transwell membranes were stained with crystal violet and cells were counted. (E) Cell cycle analysis of STK31-KD MKN1 cells. Following PI staining, cells were assessed by flow cytometry. (F) Western blot analysis of caspase-3 and PARP cleavage in MKN1 cells. Two membranes were used for PARP and Caspase-3. Pairwise P-values were calculated using Student’s t-test and corrected for multiple comparison by Bonferroni method (n=2). *P<0.05, **P<0.01, ***P<0.001. STK, serine/threonine kinase; KD, knockdown; sh, short hairpin; Con, control.
the G0/G1 phase but decreased in the G2/M phase compared with the control (Fig. 6E). Next, the expression levels of apoptosis regulatory proteins such as Caspase3 and PARP, were assessed. Western blotting revealed that Cleaved caspase-3 and Cleaved PARP were increased in STK31-KD cells compared with the control (Fig. 6F). These results suggest that shRNAs targeting STK31 mRNA modulated the oncogenic potential of STK31 by inducing G1 arrest and apoptosis in GC cells.

STK31 overexpression promotes cell proliferation, migration and tumorigenesis. The effect of ectopic STK31 expression was assessed in MKN74 or AGS cells, in which STK31 was repressed or weakly expressed (Fig. 7A). RT-qPCR and western blotting confirmed that STK31 mRNA was stably expressed in STK31-transfected MKN74 (STK31-MKN74) and AGS (STK31-AGS) cells (Fig. 7A). Moreover, ectopic STK31 expression significantly induced colony formation in each of STK31-MKN74 and STK31-AGS cells (Fig. 7B) compared with control cells. Ectopic STK31 expression also significantly induced the proliferation (Fig. 7C) and cell migration (Fig. 7D-F) in both STK31-transfected cell lines. Finally, in vivo experiments revealed that tumors in mice engrafted with STK31-MKN74 cells were significantly larger than those of control cells (Fig. 7G and H), demonstrating that tumorigenicity was promoted by ectopic STK31 expression in a xenograft mouse model.
Molecular signature of STK31 is informative regarding prognosis of patients with GC. In order to assess the prognostic value of STK31, clinical data from the CNU1H (n=145) and SMC (n=432) cohorts were combined to improve predictability. Using the mean cut-off risk score, each cohort was divided into two groups based on the mean expression value of STK31, and the upper and lower groups were combined and analyzed. Kaplan-Meier survival analysis revealed a significant difference in survival rate between the two groups in the combined cohort (Fig. 8; log-rank test; P=0.02), indicating that patient outcome was significantly poorer in the STK31 high expression group compared with the STK31 low expression group.

Discussion

The present results demonstrate that hypomethylation of CpG sites in the STK31 promoter in GC is correlated with disease progression. The present study demonstrated that DNA hypomethylation occurs in IM as well as GC cells isolated by laser-captured microdissection, suggesting that STK31 expression may be induced during the pre-cancer IM stage. A previous study provided extensive information for 450K BeadChip of gastric mucosae and IM tissue from GC-free patients, showing that CpG methylation at the STK31 promoter was significantly decreased in IM compared with gastric mucosae (18). TCGA Research Network has produced transcriptome and methylation data for primary GC and non-tumor tissues as a part of a study to develop a molecular classification of GC (19). Here, TCGA public data was used to show that the promoter and upstream region of STK31 were hypomethylated in primary GC and that promoter methylation was negatively correlated with STK31 mRNA expression levels. Thus, the public data regarding DNA hypomethylation at the STK31 promoter correspond well with the present results.

The STK31 promoter in GC cell lines may be key for regulating its transcription because the promoter was heavily methylated in the few GC cell lines in which STK31 was silenced. However, the association between promoter methylation and transcriptional efficiency in GC cell lines is unclear. In SNU484 cells, for example, the STK31 promoter was heavily methylated but STK31 was strongly expressed. The present data explain the association between STK31 promoter methylation and its transcription in the majority of GC cell lines, with the exception of SNU484. Further investigation is required to elucidate the association between STK31 promoter methylation and its transcription in SNU484 cells. Our results reveal that STK31 mRNA expression levels were restored in GC cell lines following treatment with 5-aza-dC and/or TSA, indicating that STK31 transcription is activated in GC cells as a consequence of drug-induced chromatin remodeling. Previously, Nano-ChIP-seq has been performed to characterize the landscapes of promoters that undergo changes in methylation in primary GC and matched normal tissue (22). Based on that data, signatures for promoter activity proximal to STK31 were analyzed, which demonstrated gain of promoter activity (increased H3K4me3 and H3K27ac) at regions upstream of STK31 in primary GC but not in normal mucosae. Notably, in the present study, the regions in which promoter activity increased (chr7:23,748,702-23,749,708) overlapped partially with those in which STK31 promoter methylation decreased (chr7:23,749,662-23,749,795) in primary GC. This result suggests that the STK31 promoter may be repressed in gastric mucosae but activated in primary GC as a consequence of chromatin remodeling, i.e., altered DNA methylation and histone modifications.

It has been proposed that, during multistep development, human tumors acquire six hallmarks of cancer, namely sustained proliferative signaling, growth suppressor evasion, resistance to cell death, replicative immortality, induced angiogenesis and invasion and metastasis (24). Two emerging hallmarks have been added to this list, namely energy metabolism reprogramming and immune evasion (25). Genome instability and inflammation have been posited to constitute the underlying bases for these latter two hallmarks. It has been suggested that chromatin structure may be altered in response to certain hallmarks (26). The present results demonstrated that STK31 acquired aberrant gain of function in GC as a consequence of specific epigenetic alterations that promote GC cell proliferation and tumor growth both in vitro and in vivo, suggesting that STK31 serves an important role during the acquisition of certain hallmarks in numerous types of human cancer, including GC. In order to clarify the role of STK31 in vivo, however, further studies are required using a mouse model, such as tail vein injection to determine whether it causes metastasis. The downstream pathways of STK31 in the regulation of cancer cell behavior are not clear. Proteins, such as DEAD-Box helicase 4, Cullin 3 (CUL3) and Heat Shock Protein 70 superfamily, have been identified as interacting partners with STK31 in mouse testis tissue by liquid chromatography-mass spectrometry (9). CUL3 directly binds to BTB-domain containing speckle-type POZ protein (SPOP) (27). Furthermore, binding of CUL3 to SPOP, which is a candidate tumor suppressor gene in several types of cancer including GC, downregulates SPOP and thus enhances the proliferation and migration of human GC cells (28). Further investigation is required to determine whether the oncogenic potential of STK31 is achieved via interaction with CUL3 and SPOP.
Gastric carcinogenesis proceeds through a series of precursor lesions in the GM called Correa's cascade, comprising multi-atrophic gastritis, IM, dysplasia and GC (29). In this process, IM represents a trans-differentiation of the gastric epithelium to yield an IGC, primarily induced by Helicobacter pylori infection and expression of homeobox genes, including caudal type homeobox 2 (CDX2) (30). Epidemiological evidence suggests that IM may be reversible with long-term follow up. For example, a study (31) showed that Helicobacter pylori eradication may reverse IM and that reversibility may be associated with a decrease in CDX2 mRNA levels in patients with dysplasia as well as GC. However, the results of earlier studies on the effects of Helicobacter pylori eradication for improving IM have been inconsistent (32-35). Another study reported that selumetinib, an inhibitor of mitogen-activated protein kinase, may reverse IM in a mouse model based on tamoxifen injection and lead to re-establishment of normal gastric lineage (36). STK31 may be silenced in GM but activated in IM by chromatin remodelling but it is not clear whether STK31 may be a target for reversing IM in the stomach.

Taken together, the present data suggested that STK31 may be a novel IM marker that is hypomethylated longitudinally in GC and its pre-cancer lesion, IM. Furthermore, STK31 may be used as an early detection biomarker to prevent gastric carcinogenesis and predict the prognosis of patients with GC. These findings may contribute to the Pre-Cancer Atlas, a concerted initiative to characterize the molecular alterations in premalignant lesions (37). Further studies are required to clarify the exact role of STK31 in gastric carcinogenesis and to evaluate whether a small molecule or epigenetic editing could be used to modulate STK31 expression levels.

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Availability of data and materials

The data generated as a part of this study are available at the Gene Expression Omnibus (accession no. GSE55159).

Authors' contributions

YSK conceptualized and designed the study, DHB and HJK performed the experiments. YSK, DHB and HJK authenticated all the raw data. BHY and JLP operated the software. DHB and MK analyzed the data. SIL and KSS collected clinical tissue samples and pathological information. SKK and SYK interpreted data. DHB wrote the manuscript. YSK reviewed and edited the manuscript, supervised the study and obtained funding. DHB and HJK visualized the data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All clinical samples were obtained with informed consent and their use was approved by the Internal Review Board at Chungnam National University Hospital (approval no. CNUH201801056006-EH001). All animal experiments were approved by the Internal Animal Care and Use Committee at Korea Research Institute of Bioscience and Biotechnology (approval no. KRIIBB-AEC-16158).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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