

# The accumulation of DNA demethylation in Sat $\alpha$ in normal gastric tissues with *Helicobacter pylori* infection renders susceptibility to gastric cancer in some individuals

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**Abstract.** *Helicobacter pylori* (HP) infection is widely recognized as a risk factor for gastric cancer, but only a minority of infected individuals develop gastric cancer. The aim of this study was to determine whether DNA demethylation in non-cancerous gastric mucosa (NGM) significantly enhances susceptibility to gastric cancer. A total of 165 healthy volunteers, including 83 HP-positive and 82-negative individuals, as well as 83 patients with single and 18 with synchronous double gastric cancer (GC) were enrolled in this study. The relative demethylation levels (RDLs) of repetitive sequences, including Alu, LINE-1 and Sat  $\alpha$ , were quantified by real-time methylation-specific polymerase chain reaction. The Alu RDL did not exhibit any differences within each respective group, whereas LINE-1 RDL was significantly elevated in cancer tissues compared with the NGM in the other groups ( $P < 0.001$ ). Our results indicated that a gradual increase in Sat  $\alpha$  RDL correlated with HP infection and cancer development. Sat  $\alpha$  RDL was significantly elevated in the NGM in HP-positive compared with HP-negative ( $P < 0.001$ ), and significantly elevated in cancer tissues ( $P < 0.001$ ). Although the Sat  $\alpha$  RDL of the NGM in the total population increased in an age-dependent manner, it was significantly increased in a fraction of younger GC patients ( $< 45$  years) compared with all of the others (45 years or older,  $P = 0.0391$ ). In addition, double GC exhibited a significantly higher Sat  $\alpha$  RDL in the NGM compared with single GC ( $P = 0.0014$ ). In these two fractions, Sat  $\alpha$  RDL in the NGM exhibited an inverse correlation

with age. In conclusion, the present study demonstrated that the accumulation of DNA demethylation in Sat  $\alpha$  RDL in the NGM with HP infection potentially renders susceptibility to gastric cancer in a fraction of GC patients younger than 45 years or in patients with multiple cancers.

## Introduction

Gastric cancer is the second leading cause of cancer-related death in the world. Epidemiological studies have demonstrated that there is a multistep and multifocal process in gastric carcinogenesis (1). In this process, a sequential series of precursor lesions, namely, chronic superficial gastritis, atrophic gastritis, and intestinal metaplasia are involved in gastric carcinogenesis (2). The initial stage of gastritis has been previously demonstrated to be linked to infection with *Helicobacter pylori* (HP). HP infection is widely recognized as a risk factor for gastric cancer, but only a minority of infected individuals develop gastric cancer (3), suggesting that another accelerator, such as genetic and epigenetic alteration, is implicated in the susceptibility to gastric cancer mediated by HP infection.

In several diseases characterized by chronic inflammation, such as chronic hepatitis, ulcerative colitis, and HP infected chronic gastritis, hypermethylation of the promoter region CpG islands, epigenetic modification of the DNA, is associated with transcriptional inactivation of tumor suppressor genes (4). Epigenetic alterations are commonly observed not only in cancer tissues but also in non-cancerous tissues (5,6), whereas genetic alterations are found in isolated cases and in only a minor fraction of normal tissue cells (7,8). Thus, hypermethylation is considered to be a hallmark of cancer development initiation.

Global hypomethylation is another epigenetic alteration that has been identified in many human cancers (9,10). In contrast to the pivotal role of regional hypermethylation, the role of global hypomethylation remains unclear. Recent experimental studies in mouse models have provided new insight into cancer development by demonstrating a link between global hypomethylation and chromosomal instability (11,12). We previously demonstrated that global hypomethylation

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**Key words:** DNA demethylation, *Helicobacter pylori*, Alu, LINE-1 and Sat  $\alpha$ , repetitive sequence, gastric cancer, age, multiple cancers

Table I. Clinicopathological features of patients.

## A, Clinicopathological features of 165 healthy volunteers and 101 patients with gastric cancer

Parameter	Healthy volunteers		Gastric cancer patients	P-value
	HP (-)	HP (+)		
Mean age ( $\pm$ SD)	56.3 $\pm$ 12.4	56.9 $\pm$ 11.0	65.3 $\pm$ 12.0	<0.001 <sup>a</sup>
Gender (Male/female)	44/39	53/29	74/27	0.002 <sup>a</sup>

HP (-), *H. pylori*-negative, HP (+), *H. pylori*-positive. <sup>a</sup>Healthy volunteers vs. gastric cancer patients (ANOVA with post hoc test).

## B, Clinicopathological features of 83 patients with single gastric cancer patients and 18 patients with double gastric cancer patients

Parameter	Single gastric cancer patients (n=83)	Double gastric cancer patients (n=18)	P-value
Mean age ( $\pm$ SD)	64.1 $\pm$ 12.4	71.2 $\pm$ 7.8	<b>0.022</b>
Gender (Male/female)	59/24	15/3	0.290
Diff (Intestinal/diffuse)	63/20	7/11	<b>0.002</b>
Depth (T1/T2/T3/T4)	14/34/33/2	3/11/4/0	0.625
Location (U/M/L)	20/21/42	5/6/7	0.652
LN meta			
(Negative/positive)	22/61	8/10	0.131
ly (0/1/2/3)	11/24/34/14	2/7/7/2	0.836
v (0/1/2/3)	15/27/25/16	1/8/7/2	0.395
Mean Satellite $\alpha$ RDL ( $\pm$ SD)	1.02 $\pm$ 0.07	1.55 $\pm$ 0.24	<b>0.025</b>

Depth, depth of invasion; T1, mucosal or submucosal; T2, muscularis propria; T3, subserosa; T4, serosa exposed; U, upper body of the stomach; M, middle body; L, lower body; Diff, types of differentiation; LN meta, lymph node metastasis; ly, degree of lymphatic infiltration; v, degree of venous infiltration.

in human gastrointestinal cancers correlated with genomic damage in an age-dependent manner (13,14). This led to the proposal of a 'wear and tear' model for cancer development that links aging and cancer through the accumulation of DNA demethylation and its causal relationship with genomic damage.

The aim of this study was to determine the significance of DNA demethylation in non-cancerous gastric mucosa (NGM) in terms of susceptibility to gastric cancer development in individuals infected with HP by investigating the extent of DNA demethylation in the NGM in HP-negative healthy individuals and HP-positive healthy individuals, and in patients with single and multiple gastric carcinoma.

## Materials and methods

**Patients and specimens.** Samples of the non-cancerous gastric mucosa (NGM) analyzed in this study were obtained from 165 healthy volunteers (83 *Helicobacter pylori*-positive and 82-negative individuals) who underwent upper gastrointestinal endoscopy at an affiliated hospital of the Saitama Medical Center, Jichi Medical University, Japan. Samples of the NGM were collected by endoscopic biopsy of the pyloric region of the stomach. Tumor tissues and corresponding normal gastric

mucosae were also obtained from 83 patients with single gastric cancer (GC) and 18 patients with synchronous double GC (Table I) who underwent curative surgery from May 2001 to December 2010 at the Saitama Medical Center, Jichi Medical University, Japan. All GC patients were recruited from those with *Helicobacter pylori* infection. Corresponding normal gastric mucosa was obtained from the surgical margin of each resected specimen prior to sampling of tumor tissue to avoid contamination by tumor cells. Tissue specimens were immediately soaked in RNAlater (Ambion, Austin, TX) and stored at -80°C after the RNAlater solution was removed. This study was approved by the Research Ethics Committee at Jichi Medical University. Written informed consent was obtained from each study participant.

**Detection of *Helicobacter pylori* infection.** A rapid urease test (PyloriTek Test; Serim, Elkhart, IN, USA) was performed on the remaining two specimens (one from the greater curvature of the antrum and one from the body), as previously described (15). Blood samples obtained from the subjects were evaluated for anti-HP immunoglobulin IgG levels. Patients with blood and biopsy samples that scored negative in these two tests (Campylobacter-like organism test and HP IgG test) were regarded as HP-negative.

Table II. PCR primers and TaqMan probes for MethyLight.

	Sequences (5'-3')
Alu-C	Forward: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA Reverse: ATTAACATAAATAATCTTAACTCCTAACCTCA Probe: CCTACCTTAACCTCCC-MGB
Alu	Forward: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA Reverse: ATTAACATAAATAATCTTAACTCCTAACCTCA Probe: FAM-CCTACCTTAACCTCCC-MGB
LINE-1	Forward: TTTATTAGGGAGTGTTAGATAGTGGGTG Reverse: CCTTACACTTCCCAAATAAAACAATACC Probe: FAM-TACTTCAACTCATACACAATAC-MGB
Satellite $\alpha$	Forward: TTGATGGAGTATTTTAAATATATGTTTGTAGT Reverse: AAATTCTAAAAATATTCCTCTTCAATTACATAAA Probe: FAM-TTTATCCCATTTCCAACAAA-MGB

**DNA extraction and bisulfite modification.** The dissected tissue was placed in buffered proteinase K solution at 56°C for 3 h. Genomic DNA was isolated and purified using a BioRobot EZ1 Workstation and an EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA purity was assessed by spectrophotometry (Nanodrop, ND1000; PeqLab, Erlangen, Germany), at absorbances of 260 and 280 nm. The 260/280 ratio exceeded 1.8 in all instances. The sodium bisulfite conversion of genomic DNA was performed using an EpiTect Bisulfite Kit (Qiagen). DNA quantities of 1  $\mu$ g in a volume of up to 40  $\mu$ l were processed using this standard protocol. The treatment of genomic DNA with sodium bisulfite converted unmethylated (not methylated) cytosine to uracil, which was then converted to thymidine during subsequent PCR steps. This process revealed the sequence differences between the methylated and unmethylated DNA.

**MethyLight methods.** After bisulfite modification, each sample was examined using MethyLight technology for duplicate Alu, LINE-1, and satellite- $\alpha$  (Sat  $\alpha$ ) sequences. Two sets of primers and probes specifically designed to bind to bisulfite-converted DNA were used in the reaction: one set of Alu, LINE-1, and Sat  $\alpha$  primers and a probe for unmethylated target analyses (unmethylated reaction) and another set of primers for the reference locus, ALU-C (normalization control reaction), as previously described (16). The primer and probe sequences are summarized in Table II. MethyLight data are reported as a relative level between the values derived from the real-time PCR standard curve, and plotted as log (quantity) vs. threshold cycle (Ct) value for the unmethylated reaction as well as for a methylation-independent control reaction. Whole genome amplification method provided us fully unmethylated DNA obtained from peripheral blood leukocyte (PBL) DNA, which served as the demethylation constant reference that enabled determination of the relative demethylation level. We defined the RDL as (Alu, LINE-1 or Sat  $\alpha$  reaction/ALU-C reaction) sample/(Alu, LINE-1 or Sat  $\alpha$  reaction/ALU-C reaction) fully unmethylated control DNA. In each MethyLight reaction, 1  $\mu$ l of bisulfite-modified DNA solution was used. Thermal cycling

was initiated with a denaturation step at 95°C for 10 sec, followed by 50 cycles of 95°C for 5 sec and 60°C for 30 sec. The PCR was performed on an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Carlsbad, CA, USA) with a final reaction volume of 25  $\mu$ l containing Premix Ex Taq (Takara Bio Inc., Otsu, Japan), 600 nM of each primer, and 200 nM probe.

**Statistical analyses.** All statistical analyses were performed using the StatView version 5.0 software program (SAS Institute, Cary, NC) and the statistical software SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). Continuous variations were expressed as the mean  $\pm$  standard error. When necessary, the differences in qualitative variables were evaluated using either the  $\chi^2$  test or Fisher's exact test. Continuous variables were compared using analyses of variance (ANOVA) with a post hoc test and Student's t-test. All reported P-values were two-sided, and P-values <0.05 were considered to represent a statistically significant result.

## Results

**Clinicopathological features.** The average age of GC patients was significantly higher compared with healthy volunteers (Table IA; P<0.001). The male to female ratio in the gastric cancer patient group was significantly higher compared with the healthy volunteer group (Table IA; P=0.002). Among the GC patients, the average age of patients with synchronous double GC was significantly higher compared with single GC patients (Table IB; P=0.022). Diffuse type gastric cancer was significantly more frequent in the double GC group compared with the single GC group (Table IB; P=0.002).

**Relative demethylation levels of Alu, LINE-1, and Sat  $\alpha$  in the non-cancerous gastric mucosa and cancer tissues.** We determined the significance of DNA demethylation in enhancement of susceptibility to gastric cancer in the non-cancerous gastric mucosa (NGM) most likely initiated by HP infection. We evaluated the relative demethylation levels (RDLs) of Alu, LINE-1, and Sat  $\alpha$  in the NGM from healthy volunteers

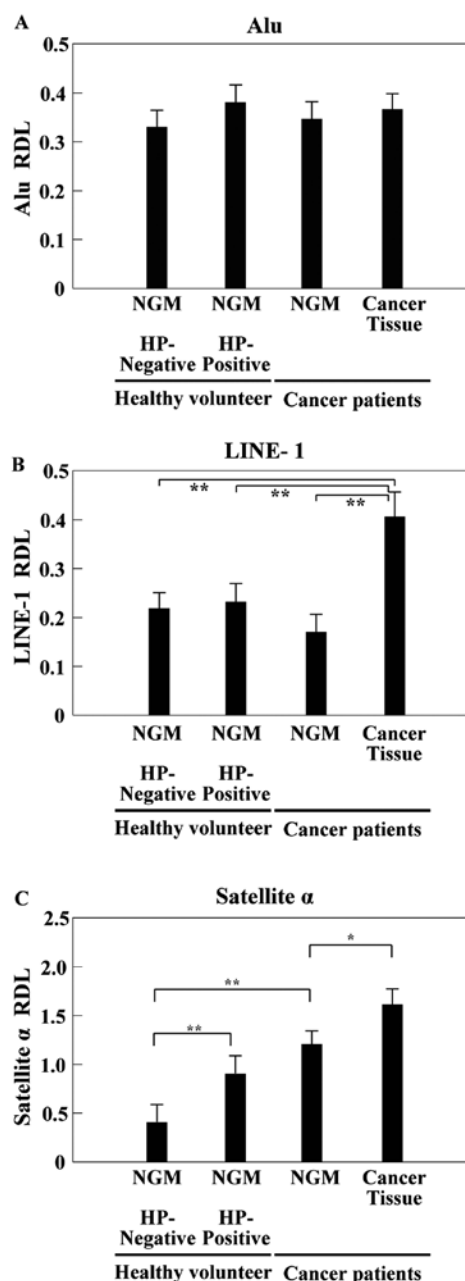


Figure 1. Comparison of LINE-1 (A), ALU (B) and Sat  $\alpha$  (C) RDLs, in non-cancerous gastric mucosa (NGM) from HP-negative volunteers, HP-positive volunteers, and the NGM and cancer tissues from GC patients. Alu RDL did not exhibit any differences between study groups (A), whereas LINE-1 RDL was significantly increased in cancer tissues compared with the NGM in other groups (B). A gradual increase was observed in Sat  $\alpha$  RDL in correlation with HP infection and cancer development. Sat  $\alpha$  RDL significantly increased in the NGM from HP-positive individuals compared with the HP-negative individuals ( $P<0.001$ ), and a further increase was observed in the NGM of individuals exhibiting single GC ( $P=0.067$ ). Sat  $\alpha$  RDL in cancer tissues, significant enhancement was noted (C). All GC patients were recruited from those with *Helicobacter pylori* infection. \* $P<0.05$ , \*\* $P<0.001$ .

with or without HP infection, and in the NGM from patients with single and double GC, and matched cancer tissues were quantified by real-time methylation-specific polymerase chain reaction (MSP). No differences were exhibited between Alu RDL within each group (Fig. 1A;  $0.383\pm0.023$  in HP-positive vs.  $0.337\pm0.057$  in HP-negative,  $0.359\pm0.072$  in the NGM

from CG patients vs.  $0.389\pm0.055$  in cancer tissues). LINE-1 RDL was significantly elevated in cancer tissues compared with the NGM from other groups (Fig. 1B,  $0.232\pm0.162$  in HP-positive vs.  $0.214\pm0.065$  in HP-negative,  $0.227\pm0.153$  in the NGM from CG patients vs.  $0.389\pm0.055$  in cancer tissues). A gradual increase was observed in Sat  $\alpha$  RDL in the NGM in correlation with HP infection and cancer development. Sat  $\alpha$  RDL significantly increased in the NGM from HP-positive compared with the NGM from HP-negative healthy volunteers (Fig. 1C;  $0.864\pm0.560$  in HP-positive vs.  $0.359\pm0.028$  in HP-negative,  $P<0.001$ ) and were elevated in the NGM from single GC patients (Fig. 1C;  $1.018\pm0.071$  in the NGM from CG patients vs.  $0.864\pm0.560$  in HP-positive,  $P=0.067$ ). Sat  $\alpha$  RDL in cancer tissues, a significant enhancement was noted (Fig. 1C;  $0.864\pm0.560$  in HP-positive vs.  $1.786\pm0.071$  in cancer tissues,  $P<0.001$ ,  $1.018\pm0.071$  in the NGM from CG patients vs.  $1.786\pm0.071$  in cancer tissues,  $P=0.002$ ). These data suggested that a Sat  $\alpha$  repetitive sequence exhibited a gradual increase during the development of cancer and could potentially serve as a surrogate marker for gastric cancer susceptibility.

*Correlation between Sat  $\alpha$  RDL in the NGM and age.* We previously demonstrated that DNA demethylation increases with age in human gastrointestinal cancers (13), which led us to postulate that a similar correlation would be seen in the NGM from patients with gastric cancer. In the present study, we investigated whether there was correlation between Sat  $\alpha$  RDL in the NGM and age. Regardless of cancer status, our findings indicated that Sat  $\alpha$  RDL in the NGM increased in an age-dependent manner (Fig. 2A;  $R^2=0.020$ ,  $P=0.029$ ), whereas Sat  $\alpha$  RDL in the NGM from patients with GC displayed a parabolic distribution (Fig. 2B;  $R^2=0.126$ ,  $P=0.005$ ), which led us to note the fraction of young GC patients with high Sat  $\alpha$  RDL in the NGM. Comparison within each age range revealed that Sat  $\alpha$  RDL in the NGM of individuals without HP infection remains low at any age range, whereas Sat  $\alpha$  RDL in the NGM of those with HP infection increased, suggesting that HP infection accelerated DNA demethylation in the stomach (Fig. 2C). While no difference of Sat  $\alpha$  RDL in the NGM from HP-positive and GC patients was observed in a fraction of individuals 45 years or older, a higher Sat  $\alpha$  RDL in the NGM from GC patients was exhibited in a fraction of individuals younger than 45 years of age (Fig. 2C;  $0.340\pm0.038$  in HP-negative vs.  $1.434\pm0.354$  in the NGM from CG patients,  $P=0.001$ ,  $0.500\pm0.138$  in HP-positive vs.  $1.434\pm0.354$  in the NGM from CG patients,  $P=0.059$ ). In addition, in GC patients, a significant difference in Sat  $\alpha$  RDL of the NGM was seen between younger ( $<45$  years) and the others (45 years or older) (Fig. 2D,  $1.143\pm0.354$  in  $<45$  years vs.  $0.967\pm0.066$  in  $\geq 45$  years,  $P=0.039$ ). Once we excluded GC patients younger than 45 years of age, a linear correlation between Sat  $\alpha$  RDL in the NGM and age was seen but an inverse correlation with age was apparent in GC patients younger than 45 years of age (Fig. 2E). Sat  $\alpha$  RDL in the NGM from GC patients younger than 45 years of age were significantly elevated compared with healthy volunteers, regardless of HP infection status or age (Fig. 2F;  $0.359\pm0.028$  in negative vs.  $0.846\pm0.056$  in positive,  $P<0.0001$ ,  $0.846\pm0.056$  in positive vs.  $1.143\pm0.354$  in  $\geq 45$  years with CG,  $P=0.039$ ).

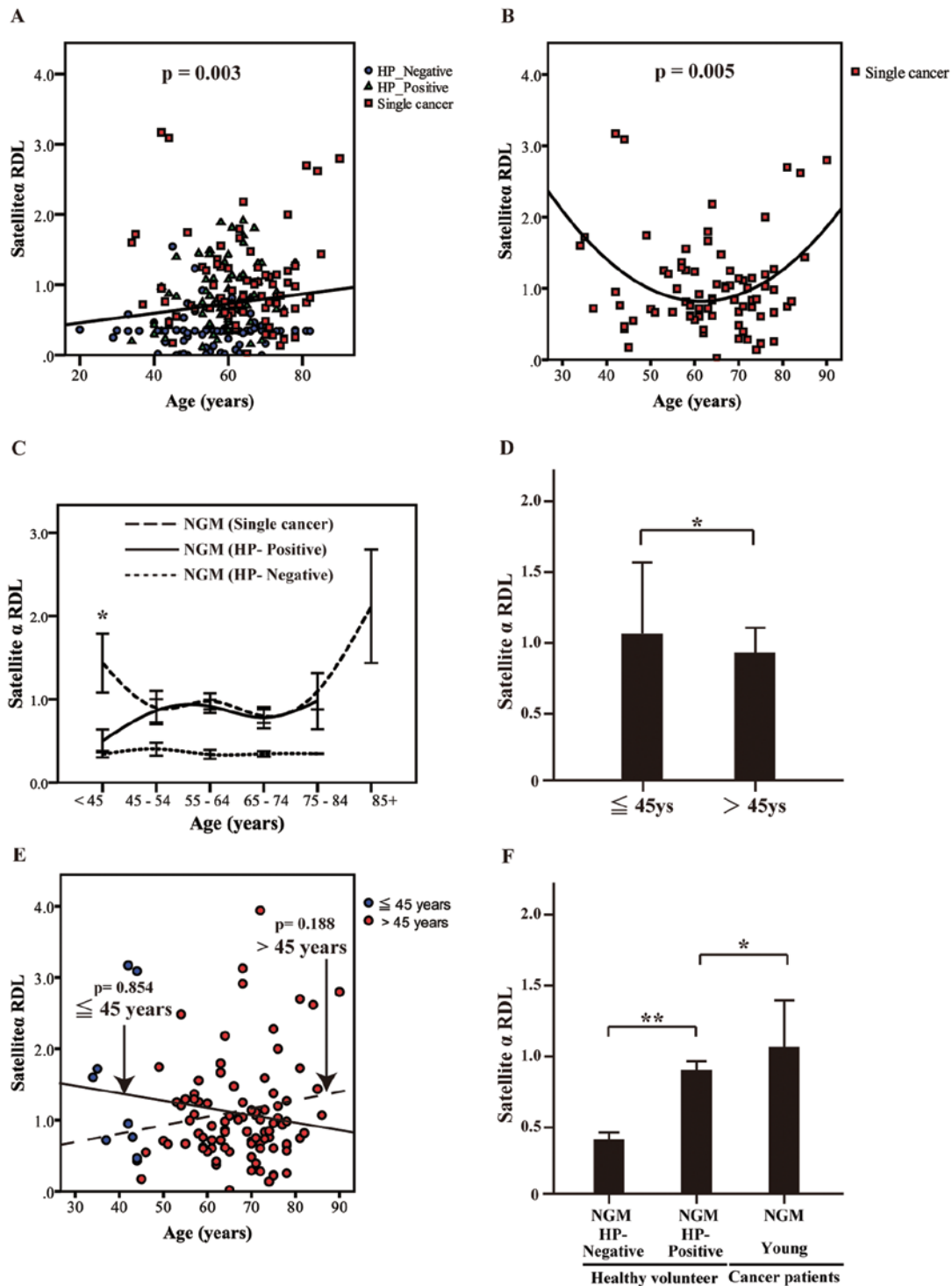


Figure 2. (A) Sat  $\alpha$  RDL in the NGM of HP-negative volunteers, HP-positive volunteers, and GC patients, and their correlation to age. Regardless of cancer status, Sat  $\alpha$  RDL in the NGM increased in an age dependent manner. All GC patients were recruited from those with *Helicobacter pylori* infection. Regression line:  $y = 0.30x + 0.11$ ,  $R^2 = 0.237$ . (B) Sat  $\alpha$  RDL in the NGM from GC patients and the relationship to age. Once we excluded healthy volunteers from the analyses, a bimodal distribution of Sat  $\alpha$  RDL in the NGM was seen, which led us to observe the fraction of young patients with high Sat  $\alpha$  RDL in the NGM. Regression curve:  $y = 0.001x^2 - 0.157x + 5.656$ ,  $R^2 = 0.134$ . (C) Sat  $\alpha$  RDL in the NGM within each age group among HP-negative volunteers, HP-positive volunteers, and single GC patients. Sat  $\alpha$  RDL in the NGM of individuals without HP infection remains low at any age, whereas Sat  $\alpha$  RDL in the NGM of those with HP infection increased, suggesting that HP infection accelerated DNA demethylation in the stomach. While no difference of Sat  $\alpha$  RDL in the NGM from HP-positive and GC patients was observed in a fraction of individuals at 45 years or older, a higher Sat  $\alpha$  RDL in the NGM from GC patients was exhibited in a fraction of individuals younger than 45 years of age (\*HP-negative vs. CG patients,  $P = 0.001$ , HP-positive vs. CG patients,  $P = 0.059$ ). (D) Comparison of Sat  $\alpha$  RDL in the NGM between GC patients 45 years or older, and those younger than 45 years of age. Younger patients under 45 years of age exhibited a significantly higher Sat  $\alpha$  RDL in the NGM compared with those 45 years or older. \* $P < 0.05$ . (E) Sat  $\alpha$  RDL in the NGM from GC patients and their relationship to age in two groups, younger GC patients ( $< 45$  years) and the other GC patients (45 years or older). Once we excluded GC patients younger than 45 years of age, a linear correlation between Sat  $\alpha$  RDL in the NGM and age was seen (Regression line:  $y = 0.04x - 0.64$ ,  $R^2 = 0.058$ ) but an inverse correlation with age was apparent in GC patients younger than 45 years of age (Regression line:  $y = -0.008x + 1.76$ ,  $R^2 = 0.001$ ). (F) Comparison of Sat  $\alpha$  RDL in the NGM between HP-negative healthy volunteers, HP-positive healthy volunteers, and GC patients younger than 45 years of age. Sat  $\alpha$  RDL in the NGM from GC patients younger than 45 years of age significantly increased compared with healthy volunteers regardless of HP infection or age. \* $P < 0.05$ , \*\* $P < 0.001$ .

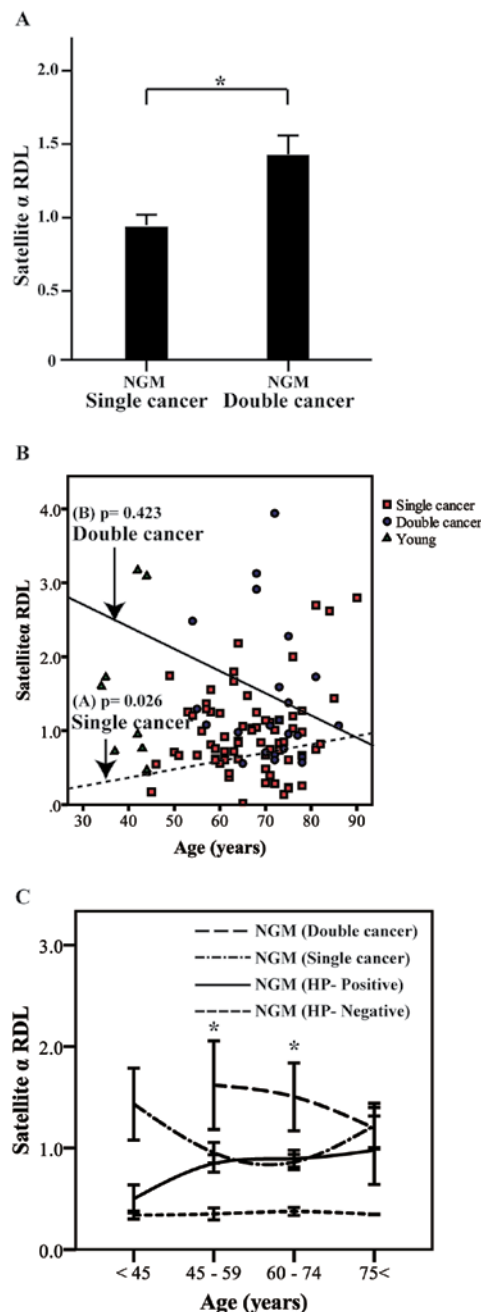


Figure 3. (A) Comparison of Sat  $\alpha$  RDL in the NGM between single and synchronous double GC patients. Sat  $\alpha$  RDL in the NGM was significantly higher in patients with synchronous double GC compared with those with single GC. All GC patients were recruited from those with *Helicobacter pylori* infection. \* $P < 0.05$ . (B) Sat  $\alpha$  RDL in the NGM from GC patients with single and synchronous double GC and the relationship to age. Sat  $\alpha$  RDL in the NGM from single GC expressed a parabolic distribution in connection with age, whereas Sat  $\alpha$  RDL in the NGM from synchronous double GC exhibited an inverse correlation with age. Single cancer includes single GC and young GC patients. (C) Sat  $\alpha$  RDL in the NGM within each age range among HP-negative volunteers, HP-positive volunteers, the NGM of single GC patients and synchronous double cancer. In individuals included in an age range from 45 to 74 years of age, the Sat  $\alpha$  RDL in the NGM were significantly elevated in patients with synchronous double GC compared with others, including single GC patients, HP-negative volunteers, and HP-positive volunteers. \* $P < 0.05$  (difference between single and synchronous double GC).

Enhancement in Sat  $\alpha$  RDL in the NGM from patients with synchronous double gastric cancer. Multiple cancers may

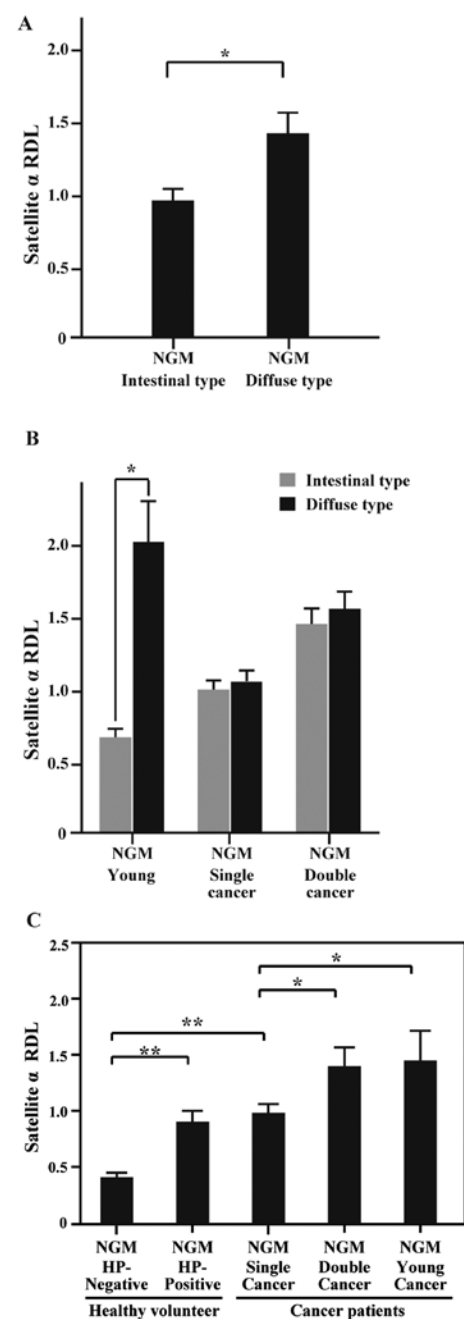


Figure 4. (A) Comparison of Sat  $\alpha$  RDL in NGM between intestinal type and diffuse type GC patients. Diffuse-type GC patients exhibited significantly higher Sat  $\alpha$  RDL in the NGM. All GC patients were recruited from those with *Helicobacter pylori* infection. \* $P < 0.05$ . (B) Comparison of Sat  $\alpha$  RDL in NGM between intestinal type and diffuse type GC patients in single GC patients younger than 45 years of age, single GC, and synchronous double GC patients. In a fraction of patients younger than 45 years of age, Sat  $\alpha$  RDL of the NGM in diffuse type GC exhibited a 3-fold increase compared with that in intestinal type GC, whereas no difference in types of differentiation was observed in both single and synchronous double GC patients. \* $P < 0.05$ . (C) Comparison of Sat  $\alpha$  RDL in NGM from HP-negative volunteers, HP-positive volunteers, single GC patients 45 years or older, synchronous double GC patients, and single GC patients younger than 45 years of age. The accumulation of DNA demethylation in Sat  $\alpha$  RDL in the NGM was significantly increased upon HP infection. There was significantly elevated accumulation in GC patients younger than 45 years of age and in multiple GC patients \* $P < 0.05$ , \*\* $P < 0.001$ .

arise simultaneously from regions of normal tissue that contain certain genomic alterations. This phenomenon is



Table III. Multivariate analyses of gastric cancers.

A, Multivariate analyses to predict the existence of multiple gastric cancers				
Factors	Variables	Odds ratio	95% Confidence limits	P-Value
Age		1.080	1.020-1.144	<b>0.008</b>
Gender	Female <sup>a</sup> vs. Male	2.440	0.569-10.462	0.230
Depth	(m/sm/mp) <sup>a</sup> vs. (ss/se)	0.667	0.161-2.761	0.576
LN meta	Negative <sup>a</sup> vs. positive	0.299	0.082-1.089	0.067
Diff	Intestinal <sup>a</sup> vs. diffuse	<b>9.321</b>	2.374-36.597	<b>0.001</b>
RDL	≤1.5 <sup>a</sup> vs. >1.5	<b>5.690</b>	1.559-20.776	<b>0.008</b>

B, Multivariate analyses to predict the existence of multiple intestinal type gastric cancer				
Factors	Variables	Odds ratio	95% Confidence limits	P-Value
Age		<b>1.203</b>	1.018-1.442	<b>0.030</b>
Gender	Female <sup>a</sup> vs. male	7.241	0.306-171.071	0.220
Depth	(m/sm/mp) <sup>a</sup> vs. (ss/se)	1.038	0.152-7.074	0.970
LN meta	Negative <sup>a</sup> vs. positive	0.453	0.064-4.400	3.222
RDL	≤1.5 <sup>a</sup> vs. >1.5	<b>7.899</b>	1.211-51.508	<b>0.003</b>

<sup>a</sup>Reference category (odds, 1). Differentiation and RDL were significant variables to predict the presence of double cancers among several clinical, pathological and genetic factors. Depth, depth of invasion; m, mucosa; sm, submucosa; mp, muscularis propria; subserosa or serosa exposed; LN, lymph node metastasis; RDL, relative demethylation level with cut-off of 1.5.

exemplified by what is commonly referred to as 'field cancerization' that is caused by carcinogen exposure (7,17). It has been proposed that aberrant methylation serves as an indicator of epigenetic field cancerization (18,19). In our earlier study, we demonstrated that the relative demethylation levels (RDL) in non-cancerous colonic mucosa from synchronous double colon cancer patients were significantly enhanced (unpublished data). Herein, we postulated that multi-cancer development of the stomach could also be affected by accelerated demethylation in the region of the stomach. To verify this hypothesis, we investigated whether there was a difference in Sat  $\alpha$  RDL in the NGM between patients harboring synchronous double GC and those with single GC. Our results indicated that the Sat  $\alpha$  RDL in the NGM were significantly elevated in patients with synchronous double GC compared with single GC (Fig. 3A;  $1.545 \pm 0.236$  in double vs.  $1.018 \pm 0.071$  in single,  $P=0.006$ ). In addition, Sat  $\alpha$  RDL in the NGM of synchronous double GC patients exhibited an inverse correlation with age (Fig. 3B;  $P=0.423$ ). Because the average age of patients with synchronous double GC was significantly higher compared with single GC patients (Table IB), we compared the Sat  $\alpha$  RDL in the NGM within respective age matched groups, and the results indicated that the Sat  $\alpha$  RDL in the NGM in GC patients included in an age range from 45 to 74 years of age were significantly elevated in patients with synchronous double GC compared with single GC (Fig. 3C;  $1.782 \pm 0.702$  in double vs.  $0.994 \pm 0.101$  in single in the age range from 45 to 64 years,  $P=0.038$ ;  $1.622 \pm 0.392$  in double vs.  $0.860 \pm 0.071$  in single in the age range from 65 to 74 years,  $P=0.003$ ).

Multivariate analyses revealed that Sat  $\alpha$  RDL in the NGM was an independent factor to predict the existence of multiple tumors. Next, we determined whether measurement of Sat  $\alpha$  RDL in the NGM could serve as an independent predictive biomarker for the existence of multiple tumors in the stomach. Since gastric cancer consists of two distinct pathophysiological features according to the type of differentiation such as diffuse and intestinal type, we initially investigated the correlations between Sat  $\alpha$  RDL in the NGM and the type of differentiation. Diffuse-type GC patients exhibited significantly high Sat  $\alpha$  RDL in the NGM (Fig. 4A;  $1.423 \pm 0.180$  in diffuse vs.  $0.986 \pm 0.070$  in intestinal  $P=0.007$ ), whereas this feature was not observed when Sat  $\alpha$  RDL in cancer tissues were analyzed (data not shown), suggesting that Sat  $\alpha$  RDL displayed a divergence during cancer progression. In a fraction of patients younger than 45 years of age, Sat  $\alpha$  RDL of the NGM in diffuse type GC exhibited a 3-fold increase compared with that in intestinal type GC (Fig. 4B;  $2.06 \pm 0.470$  in diffuse vs.  $0.652 \pm 0.124$  in intestinal,  $P=0.036$ ). In contrast, no difference according to type of differentiation was observed in either single or double GC patients (Fig. 4B;  $P=0.554$  in single and  $P=0.904$ ). These results indicated that an increase of Sat  $\alpha$  RDL in the NGM in diffuse-type GC patients were likely affected by a fraction of patients younger than 45 years of age. Next, to verify the independence of Sat  $\alpha$  RDL in the NGM as a biomarker able to predict the existence of multiple cancers, we performed multivariate analyses which revealed that the age, the diffuse type of differentiation, and Sat  $\alpha$  RDL in the NGM were independent factors to predict the existence of multiple tumors (Table IIIA, age odds ratio, 1.080,  $P=0.008$ ,

differentiation odds ratio, 9.321,  $P=0.001$  Sat  $\alpha$  RDL in the NGM odds ratio, 5.690,  $P=0.008$ ). In further analyses that excluded the diffuse-type, the age and Sat  $\alpha$  RDL in the NGM were independent factors to predict the existence of multiple tumors in intestinal type GC (Table IIIB; age odds ratio, 1.203,  $P=0.030$ ,  $P=0.001$  Sat  $\alpha$  RDL in the NGM odds ratio, 7.899,  $P=0.003$ ).

*The accumulation of DNA demethylation in Sat  $\alpha$  RDL in the NGM with HP infection potentially renders a fraction of GC patients less than 45 years of age, or with multiple cancers, susceptible to gastric cancer.* Finally, we compared all fractions of NGMs from healthy volunteers with or without HP infection, and patients with single and synchronous double GC. Sat  $\alpha$  RDL significantly increased in the NGM from HP-positive compared with the NGM from HP-negative healthy volunteers and were elevated in the NGM from single GC patients, furthermore, its significant enhancement was noted in both fractions of GC patients younger than 45 years of age and GC patients with synchronous double GC (Fig. 4C,  $0.358\pm0.029$  in negative,  $0.846\pm0.056$  in positive,  $0.967\pm0.066$  in single,  $1.545\pm0.236$  in double, and  $1.434\pm0.354$  in young individuals).

## Discussion

The present study demonstrated that Sat  $\alpha$  RDL in the NGM were affected by HP infection; this finding was further enhanced in cancer tissues from patients with single GC in conjunction with increasing age. No significant differences in Sat  $\alpha$  RDL were found in the NGM in patients with HP infection, regardless of cancer status, whereas a portion of individuals, including single GC patients younger than 45 years of age and double cancer patients, exhibited a significant increase in Sat  $\alpha$  RDL in the NGM.

With respect to other repetitive sequences, Alu RDL did not exhibit any differences between individuals, regardless of HP infection or the presence of cancer, whereas LINE-1 RDL significantly increased only in cancer tissues. The divergence of DNA demethylation patterns in various types of repetitive sequences that our findings indicate is in concordance with the results reported by Yoshida *et al* (20). Yoshida *et al* reported that the DNA demethylation observed in LINE-1 sequences was induced as a result of cellular transformation, whereas the DNA demethylation in Sat  $\alpha$  repetitive sequences was induced in the NGM by HP infection as an early event during gastric carcinogenesis.

A gradual increase of Sat  $\alpha$  RDL in the NGM was observed in correlation to age among individuals recruited in this study. This age-dependent accumulation of the DNA demethylation strongly supports the 'wear and tear' model that we previously proposed (13,14) in which gastric and colon cancer development was demonstrated through the accumulation of DNA demethylation in connection with aging and its causal role on genomic damage. We hypothesize that a gradual and accumulative age-dependent failure to preserve methylation replication fidelity leads to epigenetic errors in the continuously replicating stem cells of the stomach or colon, which increases the risk of mitotic mistakes and eventually results in chromosomal instability and cancer. Our data clearly

demonstrated that this age-dependent accumulation of DNA demethylation was initiated by HP infection in the stomach because this feature was not recognized in individuals without the presence of HP infection (Fig. 2C).

A bimodal distribution of Sat  $\alpha$  RDL in the NGM from patients with single GC was recognized in the present study that was not found in colon cancer (unpublished data). This feature led us to notify the fraction of young patients with high Sat  $\alpha$  RDL in the NGM in which an inverse correlation of risk with age was found. Fig. 2C clearly demonstrates the marked difference in Sat  $\alpha$  RDL in the NGM of individuals with single GC and healthy volunteers regardless of HP infection in the population younger than 45 years of age. Patients harboring synchronous double GC cancer, in which Sat  $\alpha$  RDL in the NGM was also significantly enhanced, exhibited an inverse correlation between Sat  $\alpha$  RDL in the NGM and age as well. These data suggest that aging was not likely among the mechanisms underlying the accumulation of DNA demethylation exhibited in this fraction of individuals compared with the others in which a gradual increase of Sat  $\alpha$  RDL in the NGM was observed in an age-dependent manner; however, the exact mechanism underlying the trigger to enhance DNA demethylation in this fraction of individuals remains unclear. One potential mechanism is hereditary, or an acquired defect of DNA methyltransferase.

Another novel finding presented in this study is that the Sat  $\alpha$  RDL in the NGM in patients exhibiting double GC were significantly elevated (Fig. 4C). Patients with multiple gastric cancer exhibit a much higher chance of developing another gastric cancer compared with cases with a single gastric cancer (21), indicating that certain individuals are at very high risk compared with others. Cases of multiple cancer may arise simultaneously from regions of normal tissue containing certain genomic alterations. This phenomenon is exemplified by the 'field cancerization' caused by carcinogen exposure (7,17). Recent studies have demonstrated that the involvement of epigenetic alterations in field cancerization can be observed in the stomach (6,22), liver (5), colon (4,23,24), Barrett's esophagus (25), lung (26), breast (27) and kidney (28). In our previous study, we demonstrated that the relative demethylation levels (RDL) in the non-cancerous colonic mucosa from synchronous double cancer patients was significantly elevated whereas no RDL difference was found in the NCM of single GC patients and healthy volunteers (unpublished data). The present study verified that DNA demethylation is also enhanced in the NGM from GC patients with double cancer (Fig. 4C) as confirmed in the NCM of synchronous double colon cancer patients. Furthermore, we explored whether Sat  $\alpha$  RDL in the NGM could serve as an independent predictive biomarker for the existence of multiple tumors in the stomach. Multivariate analyses revealed that the age, the diffuse type of differentiation and Sat  $\alpha$  RDL in the NGM were the independent factors to predict the existence of multiple tumors in accordance with several clinicopathological factors (Table IIIA). Further analyses, excluding diffuse-type, indicated that Sat  $\alpha$  RDL in the NGM as well as the age remained as an independent factor to predict the existence of multiple cancers in intestinal type GC (Table IIIB). Considering that the Sat  $\alpha$  RDL in the NGM were significantly elevated in patients with double GC compared with single GC in an age range from 45 to 74 years of age



(Fig. 3C), GC patients with a major type of GC, intestinal type, harboring high Sat  $\alpha$  RDL in the NGM, and patients included in this age range, are potentially at risk for double GC.

In conclusion, our results demonstrate the biological significance of DNA demethylation of Sat  $\alpha$  RDL in the NGM with HP infection and the mechanism that renders susceptibility to gastric cancer in a fraction of individuals under the age of 45 years, or those with multiple GC. Because gastric cancer occurs in young patients, it behaves aggressively, and the evaluation of demethylation levels in the NGM using screening biopsies may contribute to reduce the advanced gastric cancer rate in patients at a young age. In addition, expanding the indications for endoscopic mucosal resection for early cases of GC reduced the need for extensive stomach resection, but also led to increased opportunities to conserve the stomach mucosa, which might retain a malignant potential for the development of GC. Our data herein is potentially beneficial to the diagnostic and therapeutic strategies for predisposition and treatment of gastric cancer.

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