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Precision risk stratification of primary gastric cancer after eradication of *H. pylori* by a DNA methylation marker: a multicentre prospective study

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ABSTRACT

Background Precision cancer risk stratification for gastric cancer is urgently needed for the growing number of healthy people after *Helicobacter pylori* eradication. The epimutation burden in non-malignant tissues has been associated with cancer risk in multiple cross-sectional studies.

Objective To confirm the clinical usefulness of a DNA methylation marker for epimutation burden, and to identify a cut-off methylation level for a super-high-risk population.

Design Healthy people after *H. pylori* eradication with open-type atrophy were prospectively recruited. DNA methylation levels of a marker gene, *RIMS1*, were measured in biopsy specimens from gastric antrum and body. The primary endpoint was the incidence rate of gastric cancer in quartiles of the methylation levels.

Results 1624 participants had at least one endoscopic follow-up with a median follow-up of 4.05 years, and a primary gastric cancer developed in 27 participants. The highest quartile of *RIMS1* methylation levels had a higher incidence rate (972.8 per 100 000 person-years) than the lowest quartile (127.1). Cox regression analysis revealed a univariate HR of 7.7 (95% CI 1.8–33.7) and an age- and sex-adjusted HR of 5.7 (95% CI 1.3–25.5). As a secondary objective, a cut-off methylation level of 25.7% (95% CI 1.7–7.7) was obtained to identify a population with a super-high risk based on the number needed to screen of 1000.

Conclusion A DNA methylation marker can risk-stratify healthy people after *H. pylori* eradication even though all of them have clinically high risk. Individuals with super-high risk will need more frequent gastric cancer screening than currently recommended.

Trial registration number UMIN-CTR000016894.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Risk stratification of primary gastric cancer in healthy people after *Helicobacter pylori* eradication is urgently needed since the number of such people is rapidly increasing and gastric cancer can develop in 0.5%–1.2% of them.

⇒ Accumulation levels of epimutations (aberrant DNA methylation), namely epimutation burden, in non-malignant gastric tissues can be measured by a DNA methylation marker, and are correlated with cancer risk by multiple cross-sectional studies.

⇒ However, the utility of a DNA methylation marker for risk prediction of primary cancer in healthy people after *H. pylori* eradication has not yet been confirmed by a prospective study.

WHAT THIS STUDY ADDS

⇒ Epigenetic risk stratification using a DNA methylation marker was confirmed to predict primary gastric cancer risk with a high HR in healthy people after *H. pylori* eradication with open-type gastric atrophy, who clinically have high risk.

⇒ A cut-off value based on the number of 1000 people needed to screen one cancer patient was obtained to identify a super-high-risk population.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The super-high-risk population identified by the DNA methylation marker will need annual gastric cancer screening rather than once every 2 years as in the current guideline.

⇒ The DNA methylation marker may identify people who can be spared from gastric cancer screening even after *H. pylori* eradication.

INTRODUCTION

Precision risk stratification for gastric cancer, the fifth most common cancer (968 000 cases) and the fifth most common cause of cancer death (660 000 deaths) in 2022 worldwide,[1] is important. *Helicobacter pylori* is involved in almost all gastric cancers in Asia,[2 3] and its eradication is effective for cancer prevention.[4–7] The number of people

after eradication exceeded 9 million in Japan after approval of *H. pylori* eradication under national health insurance in 2013.[8] However, even after eradication, primary gastric cancer can develop in 0.5%–1.2% of people,[6] and endoscopic cancer screening every 2 years is recommended for all people after eradication.[9] To identify subpopulations with low or high risk, clinical risk factors, such as age, sex and the degree of endoscopic atrophy, which reflects the degree of histological intestinal metaplasia,[10 11] have been used, but their discriminative capability is insufficient for precision risk stratification.

As a biomarker of a new mode-of-action, accumulation levels of mutations and epimutations (aberrant DNA methylation) in non-malignant tissues, namely mutation and epimutation burdens, are now known to be associated with cancer risk.[12 13] In the stomach, high methylation levels of multiple genes in gastric mucosa were correlated with gastric cancer risk by multiple cross-sectional studies.[14–20] Mechanistically, genome-wide aberrant DNA methylation is induced by *H. pylori* infection via chronic inflammation in gastric mucosa,[13] and leads to silencing of multiple tumour-suppressor genes[21] and activation of retrotransposons.[22 23] The burden of these aberrant epigenetic alterations, namely epimutation burden, is highly correlated with the DNA methylation levels of a single marker gene.[24] In a previous multicentre prospective study using a DNA methylation marker, *miR124a3*, the risk of metachronous gastric cancer in patients with gastric cancer cured by endoscopic treatment was successfully predicted.[25 26] However, the study did not change clinical practice because the study population had extremely high gastric cancer incidence (1.7%/year), even in the subpopulation with the lowest methylation level.

Here, to improve the current clinical practice in healthy people after *H. pylori* eradication, we aimed to confirm the clinical utility of a DNA methylation marker for epimutation burden in this population with open-type gastric atrophy, all of whom have clinically high risk,[27–29] by a multicentre biomarker trial. The primary objective was to assess whether the incidence rate of primary gastric cancer in the quartile with the highest methylation level (quartile 4, Q4) of a pre-selected DNA methylation marker, *RIMS1*,[30] was higher than that in the quartile with the lowest (Q1). A secondary objective was to determine a cut-off methylation level to identify a subpopulation with a super-high risk who will benefit from annual endoscopic cancer screening.

METHODS

Study design

This study is a multicentre prospective cohort study to confirm the clinical utility of a DNA methylation marker to predict the risk of primary gastric cancer in healthy people after *H. pylori* eradication (figure 1A). Adult healthy people after *H. pylori* eradication with open-type gastric atrophy were enrolled at multiple centres in Japan. After enrolment, two biopsy specimens were taken from the antrum and body for DNA methylation analysis, and annual follow-up by endoscopy was started to find a primary gastric cancer for 5 years. This study was prospectively registered with the University Hospital Medical Information Network-Clinical Trials Registry (UMIN-CTR000016894).

Participant and recruitment

The participants were healthy people after *H. pylori* eradication with open-type gastric atrophy, all of whom clinically have high cancer risk. *H. pylori* infection was confirmed by urea breath test, rapid urease test, serum *H. pylori* antibody, *H. pylori* culture, urine *H. pylori* antibody or stool *H. pylori* antigen. Before enrolment, successful *H. pylori* eradication was confirmed by urea breath test or stool *H. pylori* antigen after 2 or more months of eradication therapy. The duration after *H. pylori* eradication was defined as a period between the date of confirmation of successful *H. pylori* eradication and the date of biopsy. Open-type gastric atrophy was defined according to Kimura-Takemoto classification,[31] namely atrophy extending from the pylorus to the fundus and greater curvature beyond the cardia. The high-risk population with open-type atrophy was targeted because this population was expected to develop primary gastric cancer with an annual incidence of 0.67%[28] and more intensive screening might be needed for its subpopulation with the highest risk. The participants were recruited and enrolled at 68 institutions in Japan (the full list is in the list of the collaborating/cooperating institutions in online supplemental material).

Inclusion and exclusion criteria

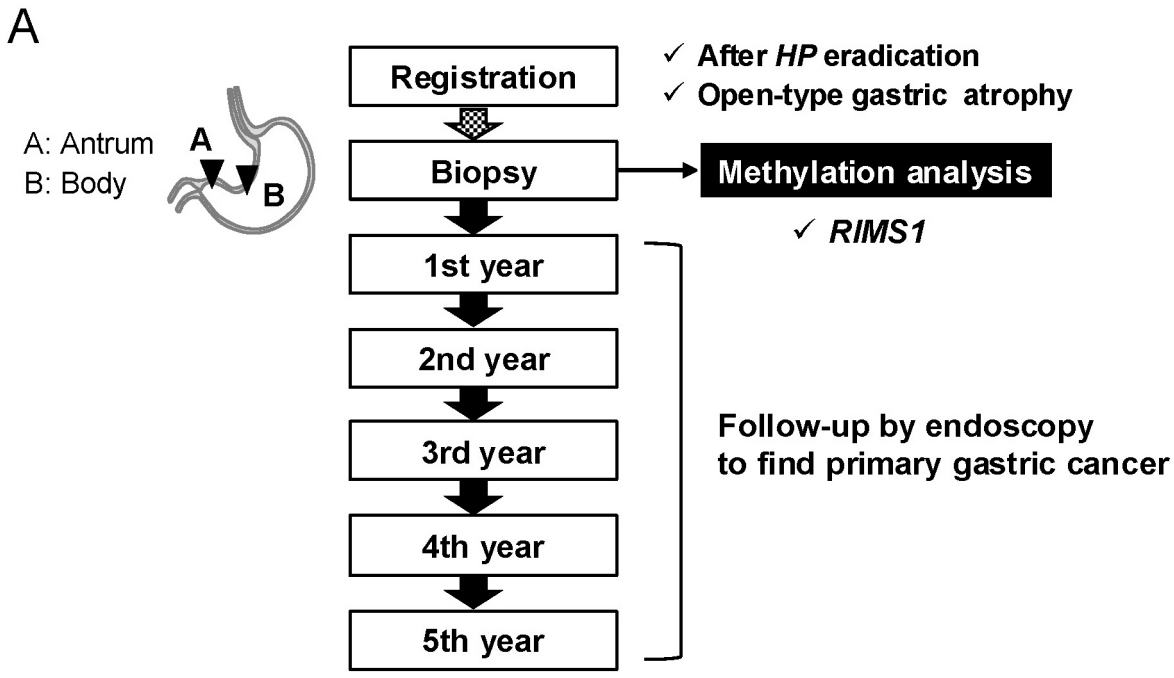
Inclusion criteria were adult healthy people (≥20, ≤75 years of age) (a) after 10 months or more of successful *H. pylori* eradication, (b) who did not have gastric cancer, (c) with open-type gastric atrophy confirmed by endoscopy, (d) who have received eradication therapy after February 2013, when it started to be covered by Japanese national health insurance,[8] (e) whose gastroscopy before eradication showed open-type gastric mucosal atrophy, (f) who were confirmed for *H. pylori* negativity by urea breath or stool *H. pylori* antigen test and (g) from whom written informed consent was obtained to participate in the study.

Exclusion criteria were those (a) who had a history of gastric cancer or gastrectomy, (b) who had a history of chemotherapy or endocrine therapy, or (c) who had a risk of bleeding due to biopsy, (d) who had a history of chemotherapy, endocrine therapy or radiation therapy, including treatments for other cancer types, (e) whose endoscopy at the time of eradication indicated the presence of adenoma or cancer, (f) with psychosis or psychiatric symptoms who were considered to have difficulty in participating in the study, (g) who received continuous systemic administration of steroids (oral or intravenous), (h) who were taking two or more antiplatelet drugs (patients using a single platelet drug are not excluded), taking anticoagulants or with coagulation abnormalities, (i) who had active bleeding of the gastrointestinal tract, (j) for whom biopsy was considered to be dangerous by the examining physician or (k) who had hereditary gastrointestinal neoplastic diseases, such as familial adenomatous polyposis, Lynch syndrome or hereditary non-polyposis colorectal cancer, Peutz-Jeghers syndrome, Cowden syndrome or hereditary diffuse gastric cancer.

Biopsy sample collection and DNA methylation measurement

All the centres followed a protocol of biopsy collection and storage, and after shipping, all the samples were analysed in a single analytical centre. Genomic DNA was extracted from biopsy specimens by the phenol/chloroform method, and quantified using a Qubit Fluorometer (Thermo Fisher, Massachusetts, USA).

The DNA methylation marker used in this study, *RIMS1*, was previously isolated as a marker not influenced by blood cell contamination and highly useful to predict gastric cancer risk in healthy people after *H. pylori* eradication.[30] To minimise the effect of such contamination, all the samples were collected at least ten months after *H. pylori* eradication. It is clinically known that inflammation has already been subdued at this time.[32–34] The *RIMS1* methylation level was quantified by pyrosequencing



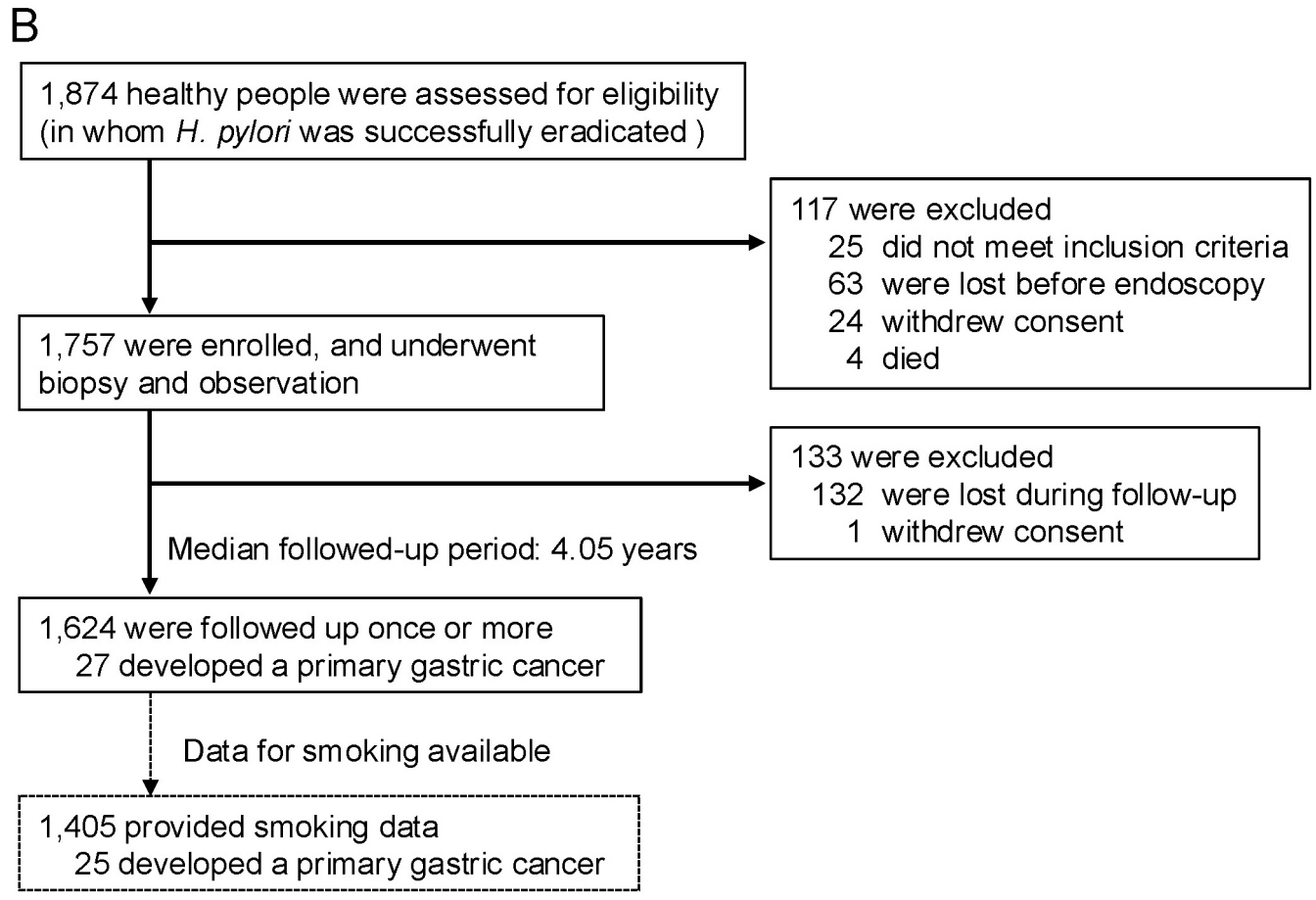


Figure 1 Participant enrolment and outcomes. (A) Schema of the study design. (B) A total of 1874 healthy people after *Helicobacter pylori* eradication with open-type gastric atrophy were assessed for eligibility, and 1757 were enrolled and underwent biopsy. Among them, 1624 participants were followed up by endoscopic examination at least once, and 27 people developed primary gastric cancer. The data on smoking were available for 1405 participants.

in Riken Genesis according to its standard operational procedures. A target CpG site was located at position 72 596 493 on Chr6 in hg19, and the primer sequences and PCR condition are shown in online supplemental table S1. The methylation level of an individual was defined as the higher methylation level in the gastric antrum and body (maximum methylation level) for the primary endpoint since gastric cancer can arise in any position in the stomach. The methylation levels in the antrum and body were also used for auxiliary analyses.

Endpoint and objectives

The study aimed to produce confirmatory results to conclude whether the DNA methylation level of a DNA methylation marker could stratify the primary gastric cancer risk in healthy people after *H. pylori* eradication. The primary endpoint was an incidence rate of primary gastric cancer identified by annual endoscopy, and the primary objective was to compare the hazards in the highest (quartile 4: Q4) versus lowest (Q1) quartile of the *RIMS1* maximum methylation levels.

A secondary objective was to determine a cut-off DNA methylation level to identify a population with a super-high gastric cancer risk.

Sample size and statistical power

The null hypothesis was that there is no significant difference in incidence rates of primary gastric cancer between Q4 and Q1. The high-risk population with open-type atrophy who were expected to develop primary gastric cancer with an annual incidence of 0.67%[28] was targeted because more intensive screening might be needed for its subpopulation with the highest risk. HRs of 3 and 6 were assumed based on the previously obtained HR of 3.0 to predict metachronous gastric cancer among patients with gastric cancer by a DNA methylation marker.[25 26] Based on a gastric cancer incidence of 0.5%/year, and a follow-up period of 5 years, the required number of participants with a power of 80%, with a two-sided type I error rate of 0.05 for rejecting a null hypothesis was calculated as 2052 for HR of 3 and 972 for HR of 6, respectively.

Statistical analysis

For the primary objective, the following analyses were performed. Person-years of follow-up were calculated from the time between the date of observation initiation to the date of gastric cancer diagnosis or the date of last endoscopic follow-up, whichever came first. Cumulative incidences of primary gastric cancer by quartiles of the observed distribution of *RIMS1* maximum methylation level were assessed graphically by Kaplan-Meier analysis. A cumulative incidence was obtained as one minus the Kaplan-Meier estimator of the survival (cumulative incidence=1−survival). Comparison between Q4 and Q1 (reference) was made in terms of the HR associated with Q4 in a univariate Cox regression model. Due to the confirmatory nature of the study and to maintain a two-sided type I error rate of 0.05, the primary analysis was conducted without any interim analysis. A multivariate Cox regression model adjusted for age and sex was also fitted. Auxiliary analyses were conducted using the *RIMS1* methylation level of the antrum and body. In each case, the univariate and multivariate analyses adjusted for age and sex were conducted. Furthermore, in addition to age and sex, smoking status and duration after *H. pylori* eradication were used for additional adjusting covariates. In all models, the proportionality assumption was tested by the Grambsch-Therneau test and no violation was found.

For the secondary objective to determine a cut-off DNA methylation level for a population with a super-high gastric cancer risk, the maximum methylation level was used as a continuous covariate in the Cox regression model. To account for a potentially non-linear effect of the methylation level on the occurrence of gastric cancer, models including various functions of the methylation level (linear, quadratic, quadratic with one knot at the median of the observed methylation level distribution) were fitted, with the most appropriate model selected by the Akaike Information Criterion. Based on the obtained model and the Breslow estimator of the baseline hazard function, the predicted 1-year probability of gastric cancer occurrence as a function of the *RIMS1* methylation level was computed for a population of participants aged 40 years, with 50.8% of them being women. The cut-off methylation level was determined as the 1-year probability of gastric cancer occurrence corresponding to the number needed to screen (NNS), including NNS of 1000 adopted in cancer screening in the Japanese guidelines of cancer screening 2014 edition.[35 36] In addition, analyses were repeated using the *RIMS1* methylation level of the antrum and body instead of the maximum level.

Statistical significance was determined by a p value of less than 0.05 by a two-sided test. All statistical analyses were performed with R statistical software, V.4.4.0 (R Foundation for Statistical Computing).

Epimutation burden analysis

Genome-wide DNA methylation analysis was performed by an Infinium HumanMethylation450 and EPIC BeadChip microarray (Illumina, California, USA) using 500 ng genomic DNA modified by sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, California, USA). 485 512 (Infinium HumanMethylation450)/862 927 (Infinium HumanMethylationEPIC) probes were normalised using the MACON web tool (https://rias.rhelixa.com/macon/human_methylation/new).[37] An epimutation burden of a sample was calculated as the inverse of a correlation coefficient of methylation levels between non-malignant gastric mucosa samples with open-type gastric atrophy and without atrophy (never *H. pylori*-infected), as previously reported.[24]

RESULTS

Participants and gastric cancer development

Between May 2015 and May 2019, 1874 otherwise healthy people after *H. pylori* eradication with open-type gastric atrophy were assessed for eligibility. After exclusion of 118 people, 1757 participants were enrolled, and two biopsy specimens were taken from the antrum (A) and body (B) to assess the DNA methylation level of a marker gene, *RIMS1*, in a large area of the stomach. The characteristics of the participants at baseline are summarised in table 1.

The enrolled participants were then followed up by annual endoscopy with a median follow-up period of 4.05 years. Among the 1757 participants (50.8% women), 1624 (92.4% of all, 50.7% women) were followed up at least once and 27 participants (49.1% women) developed a primary gastric cancer (figure 1). The cancers developed in the upper part of the stomach (19.2%), middle (46.2%) and lower (34.6%), and their pathology was tubular adenocarcinoma (88.5%) and poorly differentiated adenocarcinoma (11.5%). Further details are summarised in online supplemental table S2.

Table 1 Participant characteristics at the baseline

Characteristic	All participants (n=1757)	Participants followed up at least once (n=1624)	
		Cancer-free participants (n=1597)	Cancer cases (n=27)
Age, median (IQR), years	64 (56–69)	64 (56–69)	68 (62–72)
Age distribution, n (%)			
20–49	199 (11.3%)	175 (11.0%)	1 (7.3%)
50–59	385 (21.9%)	350 (21.9%)	4 (14.8%)
60–69	812 (46.2%)	747 (46.8%)	12 (44.4%)
70–75	361 (20.5%)	325 (20.4%)	10 (37.0%)
Gender, n (%)			
Male	865 (49.2%)	787 (49.3%)	14 (51.9%)
Female	892 (50.8%)	810 (50.7%)	13 (49.1%)
RIMS1 methylation level, median (IQR), %			
Maximum (higher A or B)	10.2 (3.5–23.3)	10.3 (3.5–23.3)	25.0 (6.8–34.1)
Antrum (A)	6.6 (2.8–19.6)	6.6 (2.8–19.5)	23.0 (6.2–32.0)
Body (B)	4.4 (2.6–14.2)	4.7 (2.6–14.3)	14.3 (3.8–26.4)
Duration after *H. pylori* eradication*, median (IQR)			
Days	470 (358–738)	475 (359–741)	584 (352–702)

*Period between the date of confirmation of successful *H. pylori* eradication and the date of biopsy.

Biomarker characteristics

The *RIMS1* methylation level was confirmed to reflect the epimutation burden using archived samples, not those from the 1624 participants in this study. Non-malignant gastric mucosa samples with open-type gastric atrophy after *H. pylori* eradication from patients with cancer (n=13), those from healthy people (n=7) and non-malignant gastric mucosa samples without atrophy (never *H. pylori*-infected) from healthy people (n=4) were analysed for genome-wide DNA methylation levels, and their epimutation burdens were calculated. A high correlation (R=0.908) between the epimutation burden and *RIMS1* methylation level obtained by pyrosequencing was observed (online supplemental figure S1), and the *RIMS1* methylation level was confirmed to reflect epimutation burden.

The *RIMS1* methylation levels were measured for the antral and body samples from the 1624 participants by pyrosequencing, and a maximum methylation level in the antrum and body was used as a primary biomarker, and its distribution was examined. A large fraction (n=793; 48.8%) of participants had low methylation levels (<10%) (figure 2A), and it was suggested that the majority of the participants had a low cancer risk. As planned, the 1624 participants were aligned into quartiles by the maximum *RIMS1* methylation level (online supplemental table S3).

Methylation levels in the antrum and body had a moderate but highly significant correlation (R=0.516; p<0.001) (figure 2B). Since the two positions were considered to have the capacity to assess the cancer risk in different areas of the stomach, the methylation levels of the antrum and body were used for auxiliary analysis. The two auxiliary methylation levels showed similar distributions to that of the maximum methylation level (online supplemental figure S4).

Risk stratification by the DNA methylation marker

The incidence rate of gastric cancer for the entire study population was 431.5 (per 100 000 person-years). That for the quartile with the lowest maximum methylation level (Q1) was 127.1 (per 100 000 person-years) and that for the highest (Q4) was 972.8 (table 2). The univariate analysis showed that the hazard in Q4 was significantly higher than that in Q1 with an HR of 7.7 (95% CI 1.8–33.7; p=0.007), achieving the primary objective (figure 3). The multivariate analysis adjusting for age and sex showed that Q4 had an HR of 5.7 compared with Q1 (95% CI 1.3–25.5; p=0.023) (table 2). Subgroup analyses stratified by age and sex (online supplemental table S4) also showed that the hazard in Q4 was significantly higher than that in Q1 in the old and female subgroups, and tended to be higher in males (p=0.087) (online supplemental table S5). For the auxiliary univariate and multivariate analyses, *RIMS1* methylation levels in the antrum and body were used. The characteristics of the

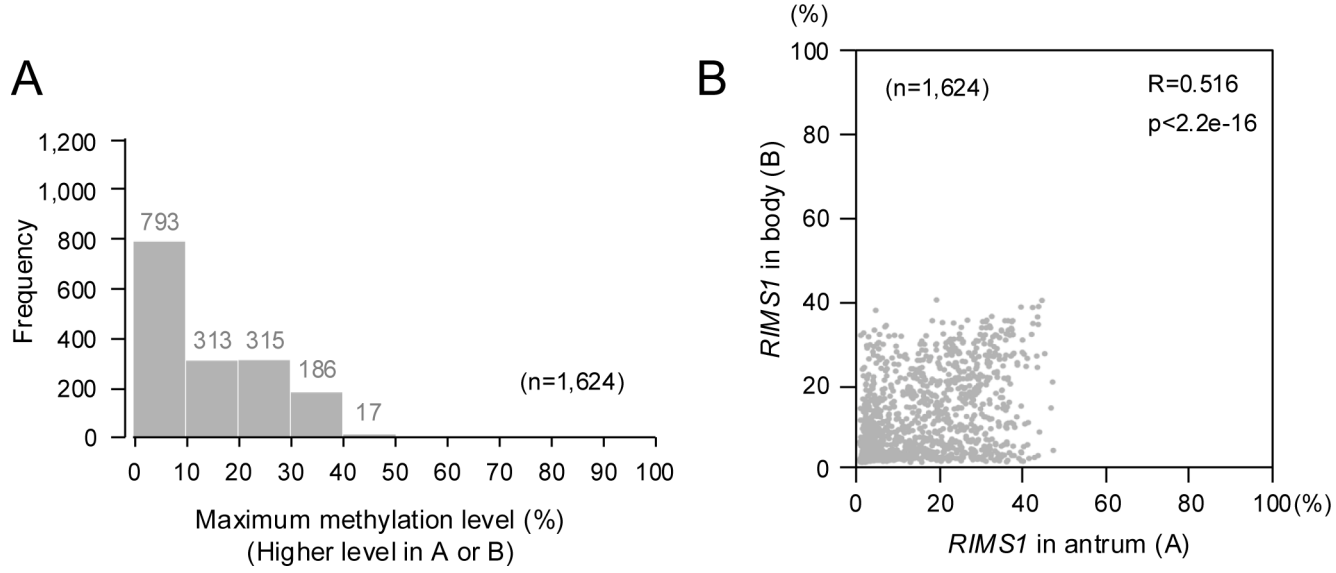


Figure 2 Distribution of *RIMS1* methylation levels. (A) Distribution of maximum *RIMS1* methylation levels in the 1624 participants who were followed up at least once. (B) Correlation between *RIMS1* methylation levels in antrum and that in body. Its correlation coefficient was 0.516 and highly significant (p<0.001).

Table 2 Unadjusted and adjusted HRs for primary gastric cancer by maximum *RIMS1* methylation level

Maximum *RIMS1* methylation level (%)	Number of cases	Person-years at risk	Incidence rate (per 100 000 person-years)	Unadjusted			Adjusted*		
				HR (95% CI)	P value	P for trend	HR (95% CI)	P value	P for trend
Q1 (1.6–3.5)	2	1573.8	127.1	Reference		0.004	Reference		0.015
Q2 (3.6–10.4)	7	1538.6	455.0	3.6 (0.7–17.3)	0.11		2.9 (0.6–14.2)	0.18	
Q3 (10.5–23.7)	3	1603.2	187.1	1.5 (0.2–8.9)	0.67		1.1 (0.2–6.8)	0.91	
Q4 (23.8–45.3)	15	1541.9	972.8	7.7 (1.8–33.7)	0.007		5.7 (1.3–25.5)	0.023	
Entire study population	27	6257.4	431.5						

This analysis was performed in 1624 participants who were followed up at least once.
*Adjusted for age and gender.

quartiles are shown in online supplemental table S4. The results were similar to those obtained using the maximum methylation level (online supplemental figure S3 and table S7).

Furthermore, an ad hoc multivariate analysis was conducted by adjusting smoking status and duration after *H. pylori* eradication in addition to age and sex. Among the 1624 participants who were followed up at least once, the additional information was available for 1405 participants. Their characteristics at baseline are summarised in online supplemental table S8, and the distributions of their *RIMS1* methylation levels are shown in online supplemental figure S4. The analysis also showed that Q4 with the highest maximum methylation level had an HR of 4.7 compared with Q1 (95% CI 1.0–21.6; p=0.047) (online supplemental table S9A) after adjusting for smoking status, duration after *H. pylori* eradication, age and sex. Similar results were obtained using the auxiliary methylation levels in the antrum and body (online supplemental tables S9B,C).

Identification of a cut-off methylation level for super-high-risk subpopulation

As a secondary objective, we determined a cut-off *RIMS1* methylation level to identify people who may benefit from annual endoscopic screening compared with the current practice in Japan of once every 2 years.[38] To this end, we used the NNS, the number of people who must be screened to identify one case,[36 39] used as an index of risk/burden of screening. In the latest Japanese guidelines for gastric cancer screening (2014 edition), an

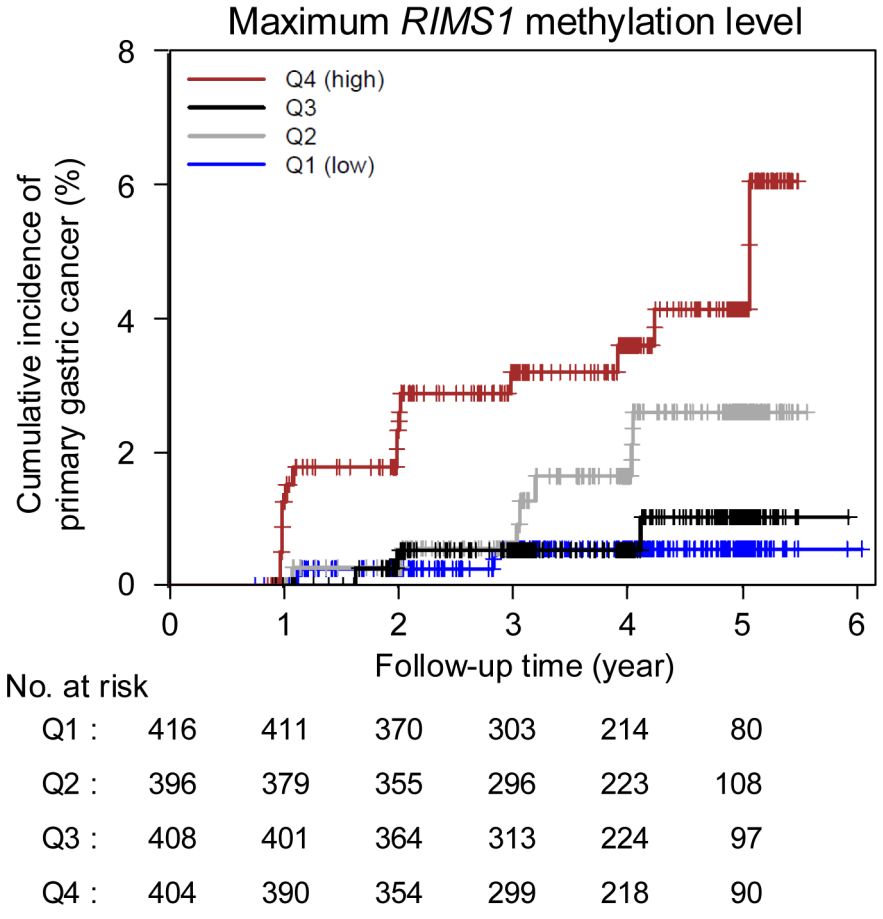


Figure 3 Cumulative incidences of primary gastric cancer in quartiles of the maximum *RIMS1* DNA methylation levels. Estimates of cumulative incidences of primary gastric cancer in the quartiles of the maximum *RIMS1* DNA methylation level. The Kaplan-Meier analysis was conducted in the 1624 patients who were followed up at least once, and the characteristics of the individual quartiles are shown in online supplemental table S1. The red solid line shows quartile 4 (Q4); black line, Q3; light grey line, Q2; and blue line, Q1. Vertical bars show the date of the last endoscopic follow-up.

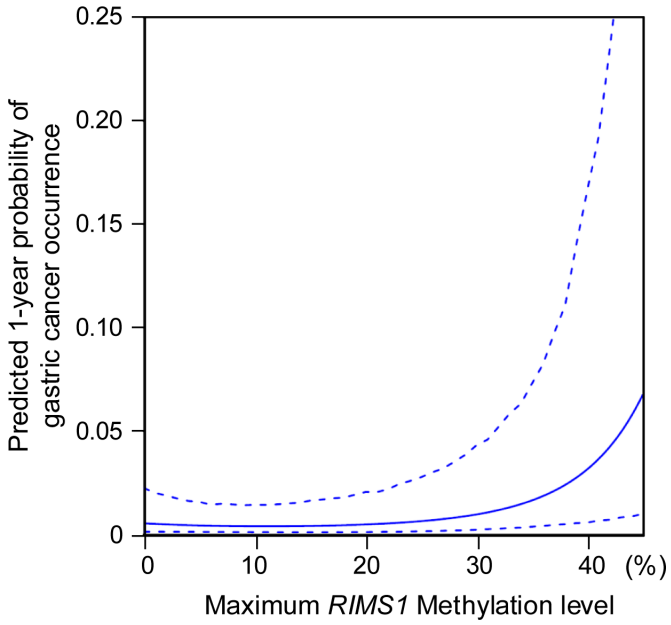


Figure 4 Predicted 1-year probability of primary gastric cancer occurrence according to the maximum *RIMS1* methylation level. The predicted 1-year probability of gastric cancer occurrence as a function of the maximum *RIMS1* methylation level for a population of participants aged 40 years with 50.8% of women was estimated. This analysis was done in the 1624 patients who were followed up at least once. Dotted lines show 95% CI.

NNS of 1000 is adopted.[35 36] Here, first, the effect of the continuous DNA methylation level on the hazard of occurrence of gastric cancer was modelled by a quadratic function (figure 4). Subsequently, cut-off methylation levels for NNS of 750, 1000 and 1250 were obtained based on the estimated 1-year probability of gastric cancer occurrence. Cut-off methylation levels of 29.2%, 25.7% and 22.2% (age- and gender-adjusted HR 3.2, 3.6 and 3.4; 95% CI 1.4–7.1, 1.7–7.7 and 1.6–7.2; p=0.002, p<0.001 and p=0.004, respectively) were obtained for NNS of 750, 1000 and 1250, respectively (table 3). Similar results were obtained using the auxiliary methylation levels in the antrum and body (online supplemental figure S5 and table S10).

DISCUSSION

In this multicentre prospective cohort study, the risk of primary gastric cancer was confirmed to be stratified in healthy people after *H. pylori* eradication with open-type gastric atrophy using a DNA methylation marker gene, *RIMS1*. All the participants in this study had open-type gastric atrophy, and belonged to a population all of whom were clinically considered to have high gastric cancer risk. Nevertheless, the DNA methylation marker was able to stratify the cancer risk with a high HR of 7.7. In addition, a cut-off value to identify a subpopulation with a super-high gastric cancer risk was obtained based on the NNS of 1000 for risk-benefit balance. These results are sufficiently reliable because this study was designed as a trial in which the sample size and follow-up period were predefined by power calculation.

The high reliability of this study can lead to a change in clinical practice in the super-high-risk subpopulation. This well-defined subpopulation is likely to benefit from annual screening of gastric cancer by endoscopy that can reduce the incidence of gastric cancer death,[40] although screening once every 2 years is recommended by the current Japanese guideline for people after *H. pylori* eradication[38] for a cost-benefit balance. This potential for a practice change is a substantial advancement compared with our previous multicentre prospective study, which successfully predicted the risk of metachronous gastric cancer in patients with gastric cancer but could not change clinical practice because the study population had an extremely high gastric cancer risk (1.7%/year).[25 26]

As a next step, the DNA methylation marker has the potential to be applied to healthy people without open-type atrophy, who are known to have a lower risk than those with open-type atrophy[27–29] and are expected to have lower methylation levels.[41] A large-scale study including participants without open-type atrophy or even without gastric atrophy will determine an appropriate cut-off value to identify people with a very low risk who can be spared from the current gastric cancer screening of once every 2 years after *H. pylori* eradication.

Apart from the stomach, an association between aberrant DNA methylation in a non-malignant tissue and cancer risk is reported for multiple types of inflammation-associated cancers such as uterine cervical cancer,[42 43] head and neck cancer[44] and hepatocellular carcinoma.[45] Especially in the uterine cervix, a recent prospective study demonstrated the usefulness of a DNA methylation marker for cancer risk assessment,[43] and the importance of epimutation burden is also suggested for uterine cervical cancer risk. The study and this study support the use of a DNA methylation marker, likely to reflect epimutation burden, in non-malignant tissues, which can be applied to cancers in additional tissues.

Technically, two biopsy specimens were taken from the gastric antrum and body in this study. The DNA methylation levels were moderately correlated, and the obtained HRs were similar (table 2 and online supplemental table S7). Previously, we used surgical specimens and analysed methylation levels in the stomach in multiple locations.[41] Although methylation levels were variable depending on location, the

Table 3 Cut-off methylation levels of maximum *RIMS1*

Predicted 1-year probability of gastric cancer occurrence	Cut-off methylation level (%)	Group (IQR)	Number of cases	Person-years at risk	Incidence rate (per 100 000 person-years)	Unadjusted		Adjusted*	
						HR (95% CI)	P value	HR (95% CI)	P value
1/1250	22.2	Low (3.0–12.6)	12	4560.4	263.1	Reference		Reference	
		High (26.1–33.0)	15	1697.1	883.9	3.4 (1.6–7.2)	0.002	2.9 (1.4–6.3)	0.006
1/1000	25.7	Low (3.1–15.0)	14	4970.0	281.7	Reference		Reference	
		High (28.1–34.5)	13	1287.4	1009.8	3.6 (1.7–7.7)	<0.001	3.1 (1.5–6.7)	0.004
1/750	29.2	Low (3.2–18.0)	18	5399.8	333.3	Reference		Reference	
		High (30.9–35.9)	9	857.6	1049.5	3.2 (1.4–7.1)	0.004	2.7 (1.2–6.0)	0.02

This analysis was performed in 1624 participants who were followed up at least once.
*Adjusted for age and gender.

high tendency was maintained throughout the stomach if a person had high values. Therefore, there is a possibility that the use of only one biopsy specimen is justified for a high-risk population who will not be overlooked using either an antrum or body specimen.

A limitation of this study is the relatively small number of cancer cases (n=27). Although the univariate HR of Q4 against Q1 was very high (HR 7.7), its 95% CI was relatively large (1.8–33.7). To obtain a smaller 95% CI, we plan to conduct a follow-up analysis with more follow-up endoscopies (by 2027). Second, the impact of the DNA methylation level was small in the male subgroup although the unadjusted HR of Q4 against Q1 tended to be high (HR 6.4; p=0.087). It was considered that the small number of cancer cases led to the lack of significance, and our follow-up analysis will confirm the impact of DNA methylation even in males. Third, those who developed gastric cancer in the upper stomach had significantly lower methylation levels than those who developed gastric cancer in the middle or lower stomach (online supplemental figure S6) while the levels were significantly higher than healthy people who had never been infected with *H. pylori* (online supplemental figure S6A). This suggested that gastric cancer in the upper stomach is related, but less related to epimutation burden accumulation than those in the middle and lower stomach. For gastric cancers in the upper stomach, a combination of DNA methylation and mutation may predict cancer risk more accurately, as in the case of the oesophagus.[46]

In conclusion, a DNA methylation marker, *RIMS1*, for epimutation burden can precisely predict primary gastric cancer risk in healthy people after *H. pylori* eradication. The cut-off value will be clinically useful to identify a super-high-risk population who needs annual cancer screening.

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