Gastric Cancer (2023) 26:667–676
https://doi.org/10.1007/s10120-023-01399-w

ORIGINAL ARTICLE

The methylation level of a single cancer risk marker gene reflects methylation burden in gastric mucosa

Takahiro Irie[1,2] · Harumi Yamada[1,3] · Chihiro Takeuchi[1] · Yu-Yu Liu[1] · Hadrien Charvat[4] · Taichi Shimazu[5] · Takayuki Ando[6] · Takao Maekita[7] · Seiichiro Abe[8] · Hiroyuki Takamaru[8] · Masaaki Kodama[9] · Kazunari Murakami[9] · Kiichi Sugimoto[2] · Kazuhiro Sakamoto[2] · Toshikazu Ushijima[1]

Received: 31 March 2023 / Accepted: 12 May 2023 / Published online: 23 May 2023

Abstract

Background Gastric cancer risk can be accurately predicted by measuring the methylation level of a single marker gene in gastric mucosa. However, the mechanism is still uncertain. We hypothesized that the methylation level measured reflects methylation alterations in the entire genome (methylation burden), induced by *Helicobacter pylori* (*H. pylori*) infection, and thus cancer risk.

Methods Gastric mucosa of 15 healthy volunteers without *H. pylori* infection (G1), 98 people with atrophic gastritis (G2), and 133 patients with gastric cancer (G3) after *H. pylori* eradication were collected. Methylation burden of an individual was obtained by microarray analysis as an inverse of the correlation coefficient between the methylation levels of 265,552 genomic regions in the person's gastric mucosa and those in an entirely healthy mucosa.

Results The methylation burden significantly increased in the order of G1 (n = 4), G2 (n = 18), and G3 (n = 19) and was well correlated with the methylation level of a single marker gene (r = 0.91 for *miR124a-3*). The average methylation levels of nine driver genes tended to increase according to the risk levels ($P = 0.08$ between G2 vs G3) and was also correlated with the methylation level of a single marker gene (r = 0.94). Analysis of more samples (14 G1, 97 G2, and 131 G3 samples) yielded significant increases of the average methylation levels between risk groups.

Conclusions The methylation level of a single marker gene reflects the methylation burden, which includes driver gene methylation, and thus accurately predicts cancer risk.

Keywords Methylation burden · Driver gene methylation · Gastric cancer · Risk prediction

✉ Toshikazu Ushijima
tushijima142@hoshi.ac.jp

1 Department of Epigenomics, Institute for Advanced Life Sciences, Hoshi University, Tokyo, Japan

2 Department of Coloproctological Surgery, Juntendo University Graduate School of Medicine, Tokyo, Japan

3 Department of Surgery, Kyoto University, Kyoto, Japan

4 Faculty of International Liberal Arts, Juntendo University, Tokyo, Japan

5 Division of Behavioral Sciences, National Cancer Center Institute for Cancer Control, National Cancer Center, Tokyo, Japan

6 Third Department of Internal Medicine, University of Toyama, Toyama, Japan

7 Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan

8 Endoscopy Division, National Cancer Center Hospital, Tokyo, Japan

9 Department of Gastroenterology Faculty of Medicine, Oita University, Oita, Japan

Introduction

Prediction of gastric cancer risk is very important to implement endoscopic screening for those who need it and to remove unnecessary concerns for those with negligible risk, even after eradication of *Helicobacter pylori* (*H. pylori*) [1]. To this end, multiple modalities, such as endoscopic findings and pepsinogen I/II ratio, have been developed [2–4]. At the same time, DNA methylation in gastric mucosa was shown to be highly useful, and multiple marker genes have been isolated after extensive selection in a number of cross-sectional studies [5–8]. Even compared with point mutation accumulations, DNA methylation accumulation in gastric mucosa had a greater impact on cancer risk [9]. Importantly, a multicenter prospective cohort study demonstrated that the methylation level of a single marker gene, such as *miR124a-3*, *EMX1*, and *NKX6-1*, could predict gastric cancer risk accurately [10, 11]. However, it is still unclear why the methylation level of a single marker gene can predict gastric cancer risk accurately.

Biologically, DNA methylation of a promoter CpG island (CGI) consistently represses transcription of its downstream gene, and this mechanism can inactivate tumor-suppressor genes, such as *CDH1*, *CDKN2A* and *MLH1* [12, 13]. Indeed, in gastric cancer cells, cancer-related pathways were more frequently inactivated by aberrant DNA methylation than by mutations [14]. The deep involvement of aberrant DNA methylation in gastric cancer is explained by the fact that *H. pylori* infection, the most important inducer of gastric cancer, potently induces aberrant DNA methylation via induction of chronic inflammation [15–18]. DNA methylation is induced in a large number of genomic regions, which include i) promoter CGIs of driver genes, ii) those of passenger genes, and iii) CGIs in exons [19]. These lead to a hypothesis that methylation alterations in the entire genome (methylation burden), the sum of deviations in the methylation levels of genome-wide loci from an entirely healthy gastric mucosa, is critical for gastric cancer risk, and that a marker gene methylation level, if carefully selected, is correlated with the methylation burden.

In this study, we aimed to demonstrate that the methylation burden and the accumulation of driver gene methylation in gastric mucosae were correlated with the methylation level of a single marker gene.

Materials and methods

Tissue samples and DNA extraction

Gastric mucosa samples, regardless of the presence of atrophy or intestinal metaplasia, were collected from 246 people, who were classified into three groups (Groups 1, 2 and 3). Group 1 (G1) consisted of 15 healthy volunteers who had never been infected with *H. pylori*, who were considered to have a low risk of developing gastric cancer [20]. Group 2 (G2) consisted of 98 healthy individuals who received successful eradication of *H. pylori* and had gastric atrophy, who were considered to have an intermediate risk [21]. Group 3 (G3) consisted of 133 gastric cancer patients who underwent curative endoscopic submucosal dissection and received successful eradication of *H. pylori,* who were considered to have a high risk [22]. Endoscopic gastric mucosal atrophy was determined by Kimura-Takemoto classification [23]. Among all the participants, seven G1 and 27 G2 people were newly recruited, and the others were enrolled in our previous studies [11, 19]. The previous studies and this study were approved by the National Cancer Center Ethics Committee (approval nos. 2008–104, 2015–139) and Hoshi University Research Ethics Committee (approval nos. 2022–10, 2022–12), and written informed consent was obtained from all participants. Characteristics of all the samples are shown in Supplementary Table 1.

All the samples were endoscopically biopsied from antral region and were stored in RNA later (Life Technologies, Carlsbad, CA, USA) or ISOGEN (Nippon Gene, Tokyo, Japan). For *H. pylori*-eradicated individuals, the samples were collected six months or more after eradication. Genomic DNA was extracted by the phenol/chloroform method, and was quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies).

Selection of driver and marker genes

Driver genes were selected based on previous papers in which those genes were reported as driver genes of gastric cancer [14, 24, 25] (Supplementary Table 2). As marker genes, we selected two marker genes, *miR124a-3* and *EMX1*, which were previously identified by a genome-wide analysis and were shown to be useful as a cancer risk marker in a prospective clinical study [10, 11].

Genome-wide and region-specific DNA methylation analysis

Genome-wide DNA methylation analysis was performed by an Infinium HumanMethylation450 BeadChip microarray (Illumina, CA, USA) using 500 or 750 ng genomic DNA modified by sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research, CA, USA). A DNA methylation level was obtained as a β value in the range from 0 (unmethylated) to 1 (fully methylated), as previously described [26]. 485,512 probes were assembled into 276,831 genomic blocks using MACON web tool (https://rias.rhelixa.com/macon/human_methylation/new) [27]. A genomic block was defined as a collection of probes within 500 bp, and annotated by its location against a transcription start site (TSS) and a CpG island (CGI). A single genomic block in a CGI and within 200 bp from a TSS of a driver gene, except for *CDKN2A* (*p16*), was assessed for its methylation level. For *CDKN2A*, a probe immediately downstream of its TSS was analyzed because no probes were located in 200 bp from its TSS although it had a CGI spanning from its promoter region to exon 1.

Region-specific DNA methylation analysis was performed by bisulfite-pyrosequencing using PyroMark Q24 Advanced (Qiagen, CA, USA). Genomic DNA (1 µg) was modified by sodium bisulfite using an EZ DNA Methylation Kit (Zymo Research). A target CpG site of a driver gene was selected in the same CGI analyzed in the genome-wide DNA methylation analysis, except for *CDKN2A*. For *CDKN2A*, its target CpG site was located in 200 bp upstream region from its TSS. DNA methylation levels of the target CpG site were calculated using PSQ Assay Design software (Qiagen). The primer sequences for the target CpG site and PCR conditions are listed in Supplementary Table 3 [28]. Fully unmethylated DNA was prepared by amplifying genomic DNA using an Illustra™ GenomiPhi™ DNA amplification kit (Cytiva, Tokyo, Japan) twice, and fully methylated DNA was prepared by methylating the fully unmethylated DNA using CpG methyltransferase (M. SssI) (New England Bio Labs, Ipswich, MA, USA).

Calculation of methylation burden

The methylation burden was defined as the sum of deviations in the methylation levels in the entire genome from an entirely healthy gastric mucosa. From the 276,831 genomic blocks, 6,582 blocks on sex chromosome and 4,697 blocks whose methylation levels failed to be analyzed in any of the samples were excluded, and 265,552 genomic blocks were used for calculation. The methylation burden of an individual was calculated as an inverse of the correlation coefficient between the methylation levels of the 265,552 genomic blocks in the person's gastric mucosa and those in an entirely healthy gastric mucosa, namely the average of four young healthy individuals (G1_Y1-Y4).

Statistical analysis

Scatter plots were drawn by R (version 4.2.0.). Pearson's correlation coefficients were calculated also by R. The methylation burden and the average methylation levels of driver and marker genes were compared between two of the three groups (G1, G2, and G3) by Student's t test. P values < 0.05 were considered as significant.

Results

Increased methylation burden according to the risk groups

To investigate methylation burden in the three risk groups, we performed genome-wide DNA methylation analysis of four entirely healthy gastric mucosa and four old G1, 18 G2 and 19 G3 samples (Supplementary Table 4). The methylation burdens were calculated by comparing between the average methylation level of four entirely healthy gastric mucosa and the methylation level of an individual sample in three risk groups (Fig. 1a, and Supplementary Fig. 1). The methylation burden significantly increased according to the risk levels of the samples ($P < 0.05$ for G1 vs G2 and G2 vs G3) (Fig. 1b). In addition, the methylation burden was well correlated with the methylation level of a single marker gene ($r = 0.910$ for *miR124a-3,* and 0.931 for *EMX1*) (Fig. 1c). Together, the methylation burden was considered to be highly correlated with gastric cancer risk and could be measured by the methylation level of a single marker gene in gastric mucosa.

Accumulation of driver gene methylation in the three risk groups

The methylation burden was composed of hypermethylation of many genomic blocks and hypomethylation, also of many blocks. In general, hypermethylation of promotor CGI of tumor suppressor genes can silence these genes, and global hypomethylation can lead to chromosomal instability [29]. Hence, the increased methylation burden was expected to lead to methylation of multiple driver genes and increased gastric cancer risk. Therefore, we selected 59 driver genes based on the literature [14, 24, 25] (Supplementary Table 2), and analyzed the accumulation of methylation of the 59 driver genes using their average methylation level. The average level increased according to the risk levels of the samples, but not significantly ($P < 0.05$ for G1 vs G2, and $P = 0.56$ for G2 vs G3) (Fig. 2a). The average level was

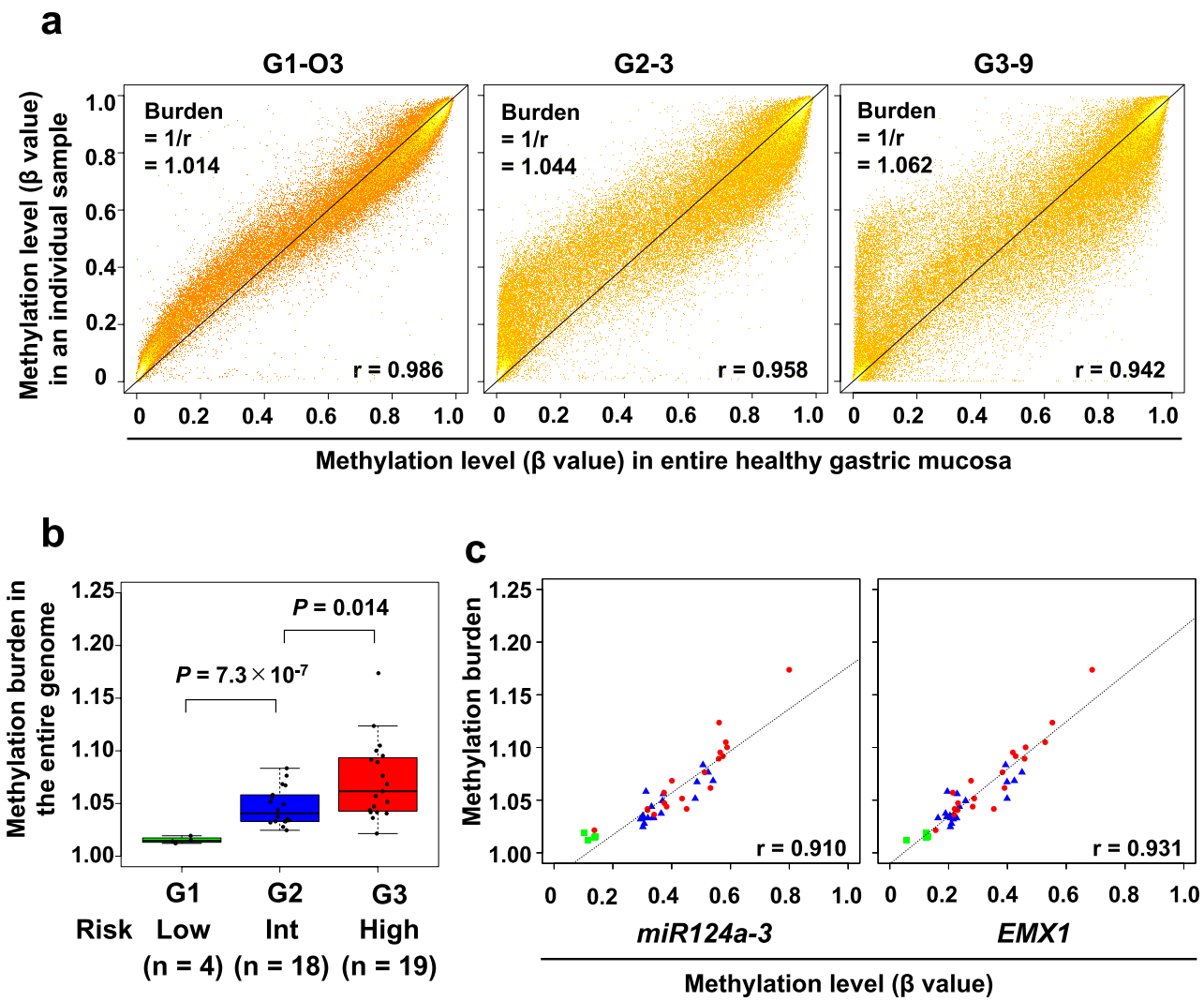


Fig. 1 Methylation burden in the entire genome, which was considered as a deviation of the methylation levels from an entirely healthy gastric mucosa. **a** Representative analysis of the methylation burden in old G1, G2, and G3 samples. Each scatter plot shows the differences of the methylation levels (β values) of 265,552 genomic blocks in the entire genome between an individual sample and an entirely healthy gastric mucosa. **b** The methylation burden in the three risk groups. The methylation burden significantly increased according to the risk levels of samples. **c** A correlation between the methylation burden and the methylation level of a single marker gene. The methylation burden was well correlated with the methylation level of a single marker gene

correlated with the methylation level of a single marker gene (r = 0.89 for *miR124a-3,* and 0.85 for *EMX1*) (Fig. 2b). The lack of significant difference between G2 and G3 suggested that the selection of driver genes needed improvement.

Therefore, we conducted additional literature searches to select genes extensively analyzed by multiple investigators in gastric cancer using the following search terms [(gene name) AND (methylation) AND (gastric cancer) AND ((silencing) OR (silenced) OR (inactivated) OR (inactivating))] in the PubMed database. We selected two genes that had at least three papers from eight major pathways of gastric cancer. Finally, nine driver genes were selected (*CDKN2A*, *CHFR*, *CDH1*, *BNIP3*, *miR34B*, *RPRM*, *MLH1*, *SFRP1*, and *SFRP5*) (Supplementary Table 5). The average methylation levels of the nine driver genes tended to increase according to the risk levels of the samples ($P < 0.05$ for G1 vs G2, and $P = 0.08$ for G2 vs G3) (Fig. 2c). The average level was also well correlated with the methylation level of a single marker gene (r = 0.944 for *miR124a-3*, and 0.92 for *EMX1*) (Fig. 2d). The result indicated that DNA methylation of driver genes was accumulated in gastric mucosa, composing gastric cancer risk.

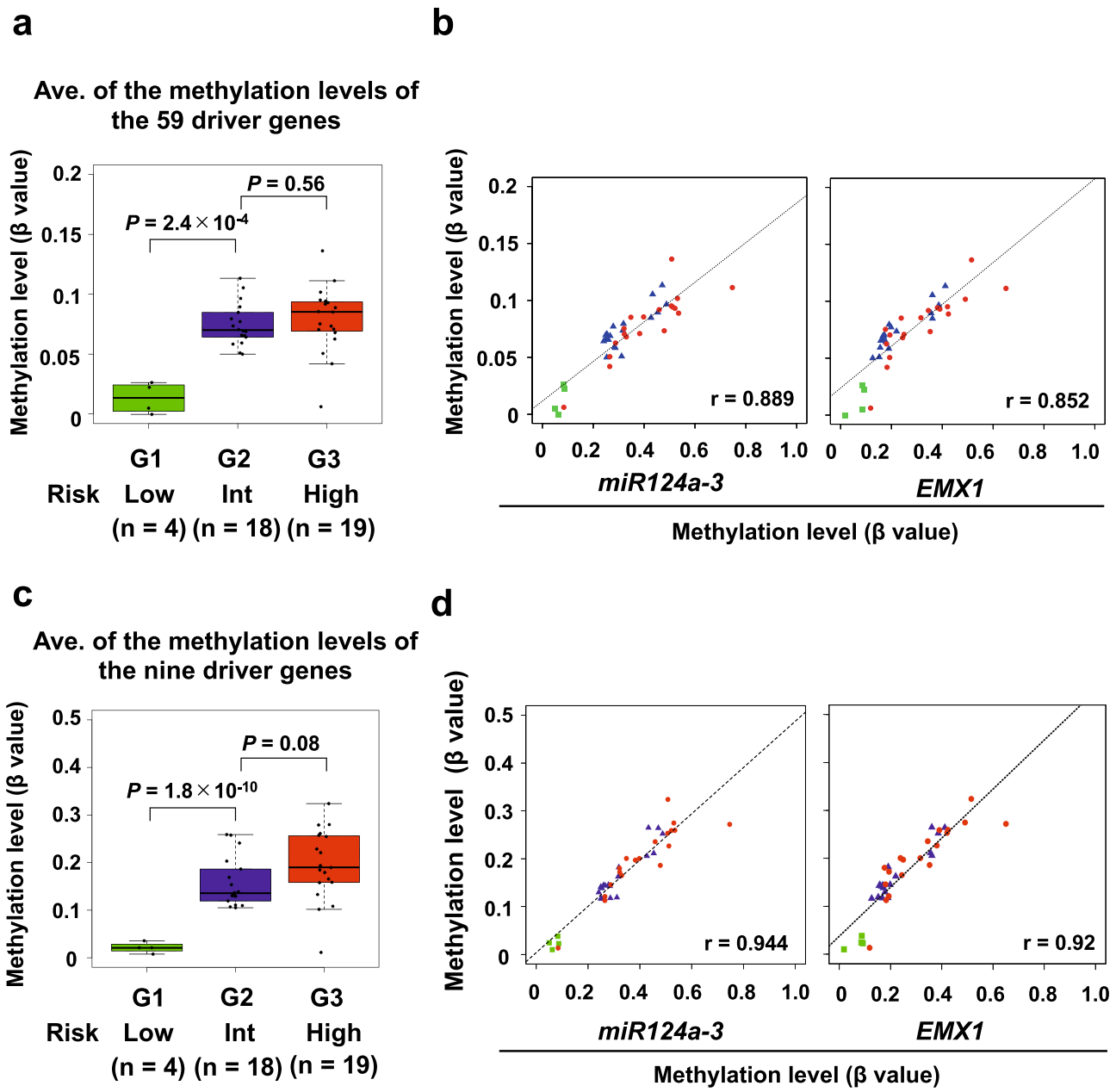
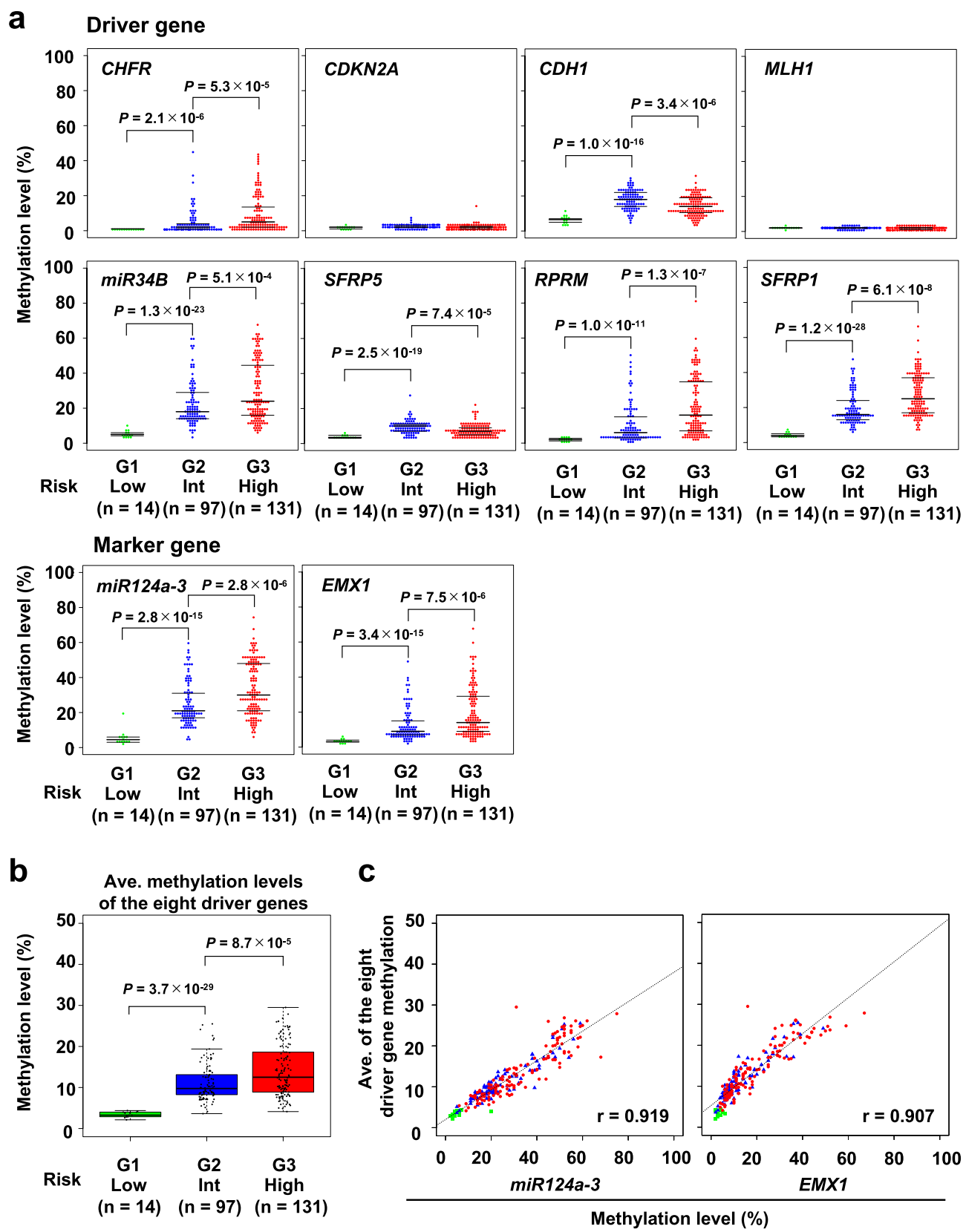


Fig. 2 Accumulation tendency of driver gene methylation. **a** Accumulation levels of 59 driver genes in the three risk groups were measured as their average methylation levels. The average level increased according to the risk levels of the samples, but not significantly. **b** A high correlation between the average methylation levels of the 59 driver genes and the methylation level of a single marker gene, *miR124a-3* or *EMX1*. **c** The average methylation levels of nine driver genes in the three risk groups. The average level tended to increase according to the risk levels of the samples ($P < 0.05$ for G1 vs G2, and $P = 0.08$ for G2 vs G3). **d** A high correlation between the average methylation levels of the nine driver genes and the methylation level of a single marker gene

Driver gene methylation in a large-scale sample

To confirm the accumulation of driver gene methylation according to gastric cancer risk, we increased the number of samples by adopting a cost-effective method, namely pyrosequencing (Supplementary Table 6). We successfully designed high-quality primers for pyrosequencing of eight of the nine driver genes (*CDKN2A*, *CHFR*, *CDH1*, *miR34B*, *RPRM*, *MLH1*, *SFRP1*, and *SFRP5*). The high quality of the primers was confirmed from two aspects. First, linearity was evaluated by analyzing the mixture of fully unmethylated DNA and fully methylated DNA. The primers for the eight driver genes and the two marker genes had good linearity, especially at low DNA methylation levels (Supplementary


a
Driver gene
CHFR
P = 5.3 × 10-5
P = 2.1 × 10-6
CDKN2A
CDH1
P = 3.4 × 10-6
P = 1.0 × 10-16
MLH1
miR34B
P = 5.1 × 10-4
P = 1.3 × 10-23
SFRP5
P = 7.4 × 10-5
P = 2.5 × 10-19
RPRM
P = 1.3 × 10-7
P = 1.0 × 10-11
SFRP1
P = 6.1 × 10-8
P = 1.2 × 10-28
Methylation level (%)
Risk
G1 Low (n = 14)
G2 Int (n = 97)
G3 High (n = 131)
Marker gene
miR124a-3
P = 2.8 × 10-6
P = 2.8 × 10-15
EMX1
P = 7.5 × 10-6
P = 3.4 × 10-15
b
Ave. methylation levels of the eight driver genes
P = 8.7 × 10-5
P = 3.7 × 10-29
c
Ave. of the eight driver gene methylation
r = 0.919
r = 0.907
miR124a-3
EMX1
Methylation level (%)

◂**Fig. 3** Significant increases of the accumulation levels of driver gene methylation using a large number of samples. **a** The methylation levels of eight driver genes and two marker genes in the three risk groups. In *CHFR*, *miR34B*, *RPRM* and *SFRP1*, their methylation levels increased according to the risk levels. *CDKN2A* and *MLH1* were not methylated at detectable levels in almost any of the samples. *CDH1* and *SFRP5* were more methylated in G2 and G3 samples than in G1 samples, but their methylation levels were lower in G3 samples than in G2 samples. **b** The average methylation levels of the eight driver genes. The average level significantly increased according to the risk levels of the samples. **c** A high correlation between the average methylation levels of the eight driver genes and the methylation level of a single marker gene

Fig. 2a). Second, we analyzed the correlation between the result obtained by DNA methylation microarray and that by pyrosequencing using the same samples analyzed by the DNA methylation microarray. There was a high correlation between the two measurement methods for the eight driver genes (Supplementary Fig. 2b). *CDKN2A* did not show such correlation due to different locations of target CpG sites (Supplementary Fig. 2c; see discussion).

The methylation levels of the eight driver genes were analyzed in 14 G1, 97 G2, and 131 G3 samples (Supplementary Table 6). *CHFR*, *miR34B*, *RPRM* and *SFRP1* were methylated and their methylation levels increased according to the risk levels of the samples. *CDKN2A* and *MLH1* were not methylated at detectable levels in almost any of the samples. *CDH1* and *SFRP5* were more methylated in G2 and G3 samples than in G1 samples, but their methylation levels did not increase more in G3 than in G2 samples. The two marker genes showed increased methylation levels according to the risk levels of the samples (Fig. 3a). The average methylation levels of the eight driver genes significantly increased according to the risk levels of the samples ($P < 0.05$ for G1 vs G2 and G2 vs G3) (Fig. 3b). The average level was correlated with the methylation level of a single marker gene (Fig. 3c).

Subgroup analysis by age, gender, and gastric atrophy

Finally, the independence of methylation level from known risk factors of gastric cancer, namely age [30, 31], gender [32], and the extent of gastric atrophy [33], was assessed by subgroup analyses. In the age subgroups with 65 years old as a cutoff point, the methylation level of *miR124a-3* was significantly higher in G3 than in G2. Similarly, in males and females, the methylation level of *miR124a-3* was significantly higher in G3 than in G2. In the subgroups with closed-type and open-type atrophy, the methylation level of *miR124a-3* was significantly higher in G3 than in G2. On the other hand, within the same risk group, there were no significant differences in the methylation level of *miR124a-3* by age, gender, and the extent of gastric atrophy (Fig. 4, Supplementary Fig. 3 for *EMX1* and the eight driver genes). These findings showed that the methylation level of a single marker gene and the average methylation levels of the eight driver genes were associated with gastric cancer risk, even after stratifying for age, gender, and gastric atrophy.

Discussion

In the current study, we demonstrated that methylation burden, along with the accumulation of driver gene methylation, increased according to the risk levels of gastric mucosa samples, and that they were strongly correlated with the methylation level of a single marker gene. This result explained the reason why the methylation level of a single marker gene can accurately predict cancer risk. It provides a rationale for the clinical use of measuring the methylation level of a single marker gene for cancer risk prediction. The use of a single marker gene is convenient and inexpensive, and will bring great benefits to patients and healthcare burden. Our finding is expected to be applicable to other cancers such as colitic, liver, and uterine cervical cancer, which are closely related to chronic inflammation. Indeed, several marker genes have been identified for these cancers [34–36].

The methylation burden was defined as the sum of deviations of methylation levels of genomic loci throughout the genome in an individual sample from those in an entirely healthy gastric mucosa, including hypermethylation and hypomethylation. As for the hypermethylation, DNA methylation of a promoter CGI is known to inactivate tumor-suppressor genes and promote cancer development, and the methylation accumulation levels of eight driver genes significantly increased according to the risk levels of the samples. However, its weaker association with cancer risk than methylation burden indicated that methylation burden is more directly involved in cancer risk. As for the hypomethylation, global hypomethylation is considered to be mainly due to hypomethylation of repetitive elements, and can lead to chromosomal instability, which are involved in carcinogenesis [29, 37]. The probes in the DNA methylation microarray used here are distributed mainly in gene promoters and CpG islands [38], and may be insufficient to analyze global hypomethylation. However, hypomethylation of repetitive elements has been shown to be reflected in methylation statuses of probes in a DNA methylation microarray [39].

The *CDKN2A* methylation level analyzed by pyrosequencing did not show a high correlation with that analyzed by DNA methylation microarray. This was because target CpG sites analyzed by pyrosequencing were located in 200 bp upstream region from its TSS and those by DNA methylation microarray were located in the 1st exon. The CpG sites analyzed by pyrosequencing for *CDKN2A* were located in 200 bp region upstream its TSS overlapping a

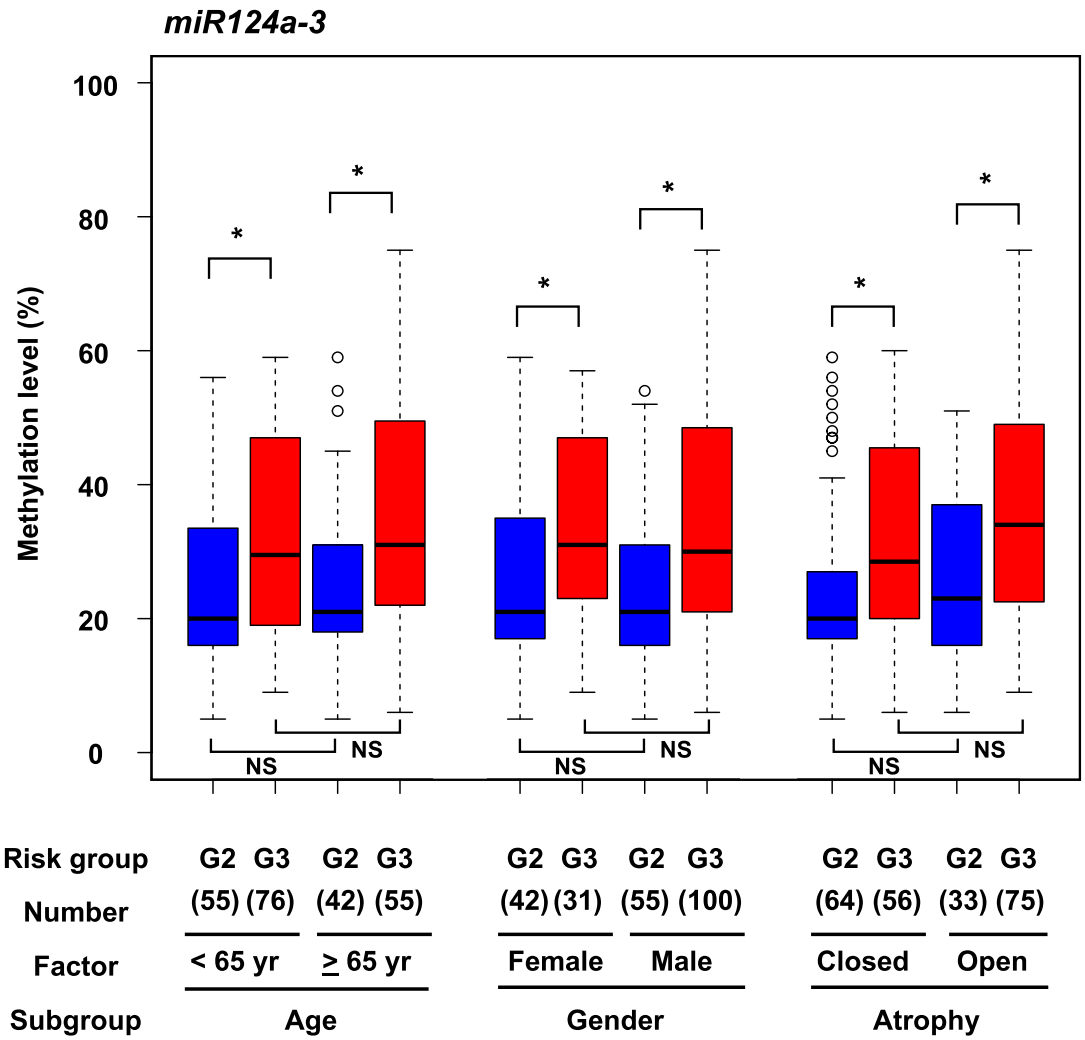


Fig. 4 Subgroup analyses by age, gender and gastric atrophy. There were significant differences in the methylation levels of *miR124a-3* between G2 and G3 in all the three subgroup analyses. At the same time, within one risk group, there were no significant differences in the methylation levels of *miR124a-3* by age, gender, or gastric atrophy. yr, year; Closed, Closed-type atrophy; Open, Open-type atrophy; $^*P < 0.05$

CpG island, and were reported to be a valid target in terms of methylation-silencing [40]. Another issue that needs explanation is that *CDH1* and *SFRP5* methylation levels were lower in G3 than in G2. This could be due to the larger fraction of cells, for example, metaplastic cells, that need *CDH1* and *SFRP5* expression in G3 samples.

The accumulation level of driver gene methylation was calculated as an average methylation level of selected driver genes. This method at least enabled us to obtain a correlation between the accumulation level of driver gene methylation and the methylation level of a single marker gene. However, the impacts of individual driver genes on carcinogenesis are considered to be variable, and it might be better to take their relative importance into account. In addition, some driver genes may work in parallel and need to coexist, and others may work in upstream or downstream and be alternative. Since our current knowledge is far less than complete, we adopted here the simplest equation to avoid overfitting due to multiple hypotheses.

The limitation includes the small number of samples, especially G1 samples for genome-wide analysis ($n = 4$). Fortunately, variation within the G1 samples was small ($r \geq 0.981$ for any two of the four samples), and we considered that the number is tolerable. At the same time, even within the same risk level, some samples may show extraordinary values, such as a few G2 samples with high methylation burden and high average methylation levels of the nine driver genes. These patients may develop gastric cancer in

the future, and to minimize the influence of such samples, it would be better to have a large number of samples. In addition, in the subgroup analyses, we assessed the effects of age, gender, and gastric atrophy, but were unable to assess that of intestinal metaplasia (IM), an established risk factor of gastric cancer [41] because we did not have information on IM status for all the samples.

In conclusion, we revealed the mechanism of why the methylation level of a single marker gene can accurately predict cancer risk.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10120-023-01399-w.

Acknowledgements The authors are grateful to Ms. Mika Wakabayashi for her technical assistance.

Author contributions Conception and design: TI, HY, CT, and TU. Development of methodology: TI, HY, and CT. Acquisition of materials: TS, TA, TM, SA, IO, MK, and KM. Analysis and interpretation of data: TI, HY, CT, YL, HC, TS, and TU. Writing, review, and/or revision of the manuscript: TI, KS (Kiichi Sugimoto), KS (Kazuhiro Sakamoto), and TU.

Funding This study is funded by AMED (grant number 22ck0106552h9903).

Data availability DNA methylation data are available at the Gene Expression Omnibus (GEO) database with the accession number GSE226818.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Consent to participate Informed consent was obtained from all patients for being included in the study.

Consent for publication Not applicable.

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