Original Article

Aberrant DNA methylation and expression of *EYA4* in gastric cardia intestinal metaplasia

Chenxi Li[1,#], Zhaohui Liu[3,#], Guohua Xu[4,#], Shibin Wu[4,#], Yunhui Peng[4], Ruinuan Wu[5], Shukun Zhao[1], Xiaoqi Liao[1*], Runhua Lin[1,2*]

[1]Department of Pathology, Shantou University Medical College, Shantou, [2]Guangdong Provincial Key Laboratory of Infectious Diseases and Molecular Immunopathology, Shantou, [3]Department of Gastroenterology, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University Health Science Center, Shenzhen, [4]Department of Gastroenterology, Huiyang Sanhe Hospital, Huizhou, [5]Department of Pathology, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University Health Science Center, Shenzhen, P.R. China

[#]These authors contributed equally to this work.

*These authors are co-corresponding authors

Abstract

Background: Intestinal metaplasia (IM) of the gastric cardia is an important premalignant lesion. However, there is limited information concerning its epidemiological and molecular features. Herein, we aimed to provide an overview of the epidemiological data for gastric cardiac IM and evaluate the role of EYA transcriptional coactivator and phosphatase 4 (*EYA4*) as an epigenetic biomarker for gastric cardiac IM.

Methods: The study was conducted in the context of the gastric cardiac precancerous lesion program in southern China, which included 718 non-cancer participants, who undertook endoscopic biopsy and pathological examination in three endoscopy centers, between November 2018 and November 2021. Pyrosequencing and immunohistochemistry were performed to examine the DNA methylation status and protein expression level of *EYA4*.

Results: Gastric cardiac IM presented in 14.1% (101/718) of participants and was more common among older (>50 years; 22.0% [95% CI: 17.8–26.8]) than younger participants (≤50 years; 6.7% [95% CI: 4.5–9.9]; $P < 0.001$). IM was more common in male participants (16.9% [95% CI: 13.2–21.3] vs. 11.3% [95% CI: 8.3–15.1]; $P = 0.04$). Pyrosequencing revealed that IM tissues exhibited significantly higher DNA methylation levels in *EYA4* gene than normal tissues ($P = 0.016$). Further, the protein expression level of *EYA4* was reduced in IM and absent in intraepithelial neoplasia tissues compared to normal tissues ($P < 0.001$).

Conclusions: Detection rates of gastric cardiac IM increase with age and are higher in men. Our findings highlight the important role of promoter hypermethylation and downregulation of *EYA4* in gastric cardiac IM development.

Keywords: DNA methylation, *EYA4,* gastric cardia, intestinal metaplasia

Address for correspondence: Dr. Runhua Lin, Department of Pathology, Shantou University Medical College, No. 22, Xinling Road, Shantou 515041, P.R. China.
E-mail: rhlin2010@163.com
ORCID: 0000-0001-7014-9829

Submitted: 28-May-2022 **Revised:** 29-Aug-2022 **Accepted:** 05-Sep-2022 **Published:** 04-Nov-2022

Access this article online	
Quick Response Code:	Website: www.saudijgastro.com
	DOI: 10.4103/sjg.sjg_228_22

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Li C, Liu Z, Xu G, Wu S, Peng Y, Wu R, *et al*. Aberrant DNA methylation and expression of *EYA4* in gastric cardia intestinal metaplasia. Saudi J Gastroenterol 2022;28:456-65.

INTRODUCTION

Intestinal metaplasia (IM) of the stomach is characterized by the replacement of normal gastric epithelium and gastric glands by intestinal epithelium and intestinal glands. Histologically confirmed gastric IM often confers an increased risk of progression towards gastric cancer.[1-3] Thus, gastric IMs are generally considered to be precursor lesions for the development of dysplasia, and ultimately gastric carcinoma.[4] However, little attention has been given to this premalignant lesion in the gastric cardia. Limited data are available on the prevalence of IM in gastric cardia, especially in cancer-free subjects. Yet, these data may be informative for further molecular investigations. Given the preneoplastic nature of the lesion, it is crucial to improve our understanding of the regulation of genes contributing to the intestinal metaplastic phenotype. Hence, identifying informative biomarkers for gastric cardiac IM could be useful to guide further research regarding gastric cardiac carcinogenesis, that may have preventive implications.

DNA methylation is a key regulator of gene expression that does not alter the DNA sequence. Although it is well known that aberrant DNA methylation is associated with multiple cancer types,[5-7] there is less clarity regarding its role in early neoplastic progression. Indeed, these changes to DNA methylation are critical to understanding whether altered DNA methylation may contribute to premalignant lesion formation. Recent epigenetic studies have shown that altered DNA methylation occurs even in precancerous tissues, and these changes, therefore, may serve as promising early indicators of existing disease, and of risk prediction.[1] More recently, our methylation array-based (Illumina EPIC/850K array) study showed that gastric cardiac IM exhibits distinct DNA methylation profiling and identified some candidate genes that were significantly hypermethylated in their promoter regions in IM tissues. In this work, using a stringent filtering strategy, we found that the *EYA4* promoter region exhibited the largest number of differentially methylated probes (DMPs) in gastric cardiac IM tissue. Thus, we hypothesized that gastric cardia IM presents as a common event in cancer-free subjects, and that promoter hypermethylation of *EYA4* may reduce its expression level in gastric cardiac IM tissues.

For the present study, we aimed to: 1) investigate the detection rate of gastric cardiac IM in cancer-free individuals, 2) verify the DNA methylation level of candidate DMPs in the *EYA4* promoter region, and 3) evaluate the protein expression level of EYA4 in gastric cardiac IM and normal gastric cardiac mucosa.

PATIENTS AND METHODS

Biopsy specimens

Gastric cardiac biopsies were obtained from 718 participants undergoing upper endoscopy for gastrointestinal symptoms, between November 2018 and November 2021, in three centers (Shantou, Huizhou, and Shenzhen) in Guangdong Province, China. All the subjects included in this study were cancer-free at the time of endoscopic examination. Informed written consent was obtained from the participants according to the protocol approved by the Institutional Review Board of Shantou University Medical College (Approval Number: SUMC-2020-23); (Approval date: April 3, 2020). Upper endoscopic examinations were conducted by four experienced endoscopists (attending physician or above, having experiences of gastrointestinal endoscopy for at least 5 years). Biopsies of the endoscopically normal gastric cardiac mucosae were included in this study. Following histopathological assessment, we divided these biopsy samples into four histological categories, including normal gastric cardiac tissue, chronic carditis, chronic atrophic carditis, and IM tissue [Figure 1a and b]. For bisulfite pyrosequencing, the following subjects were included: $n = 10$ (normal tissue, $n = 5$; IM tissue, $n = 5$). For the immunostaining of EYA4, the following samples were included: $n = 88$ (normal tissue, $n = 38$; IM tissue, $n = 47$; and IM with intraepithelial neoplasia [IEN], $n = 3$). A total of four IEN samples were used for histopathological analyses in our study, but finally only three IEN samples were available for immunohistochemistry (IHC).

Histopathological evaluation

Briefly, the endoscopic specimens were fixed with 10% neutral formalin for 24 h, and then specimens were embedded in paraffin. Paraffin-embedded tissues were cut into 4-μm thick sections and stained with hematoxylin and eosin (HE). The pathological diagnosis was independently determined by two pathologists. For the specimens showing IM lesions with routine HE staining on initial examination, two additional tissue sections were stained with Alcian blue/periodic acid-Schiff (AB-PAS; Cat# BA-4121, Lot: C210401, BaSO Biotechnologies, China) staining, and immunohistochemical staining of mucin 2 (MUC2; goblet cell marker) for further validation [Figure 1c].

In this study, we classified all the endoscopic specimens into four categories (normal gastric cardiac tissue, chronic carditis, atrophic carditis, and IM tissue) based on the following histological criteria.[8] Briefly, (a) normal gastric cardiac tissues, represented by normal morphology of the mucosa, namely, foveola, glands, gland necks, and

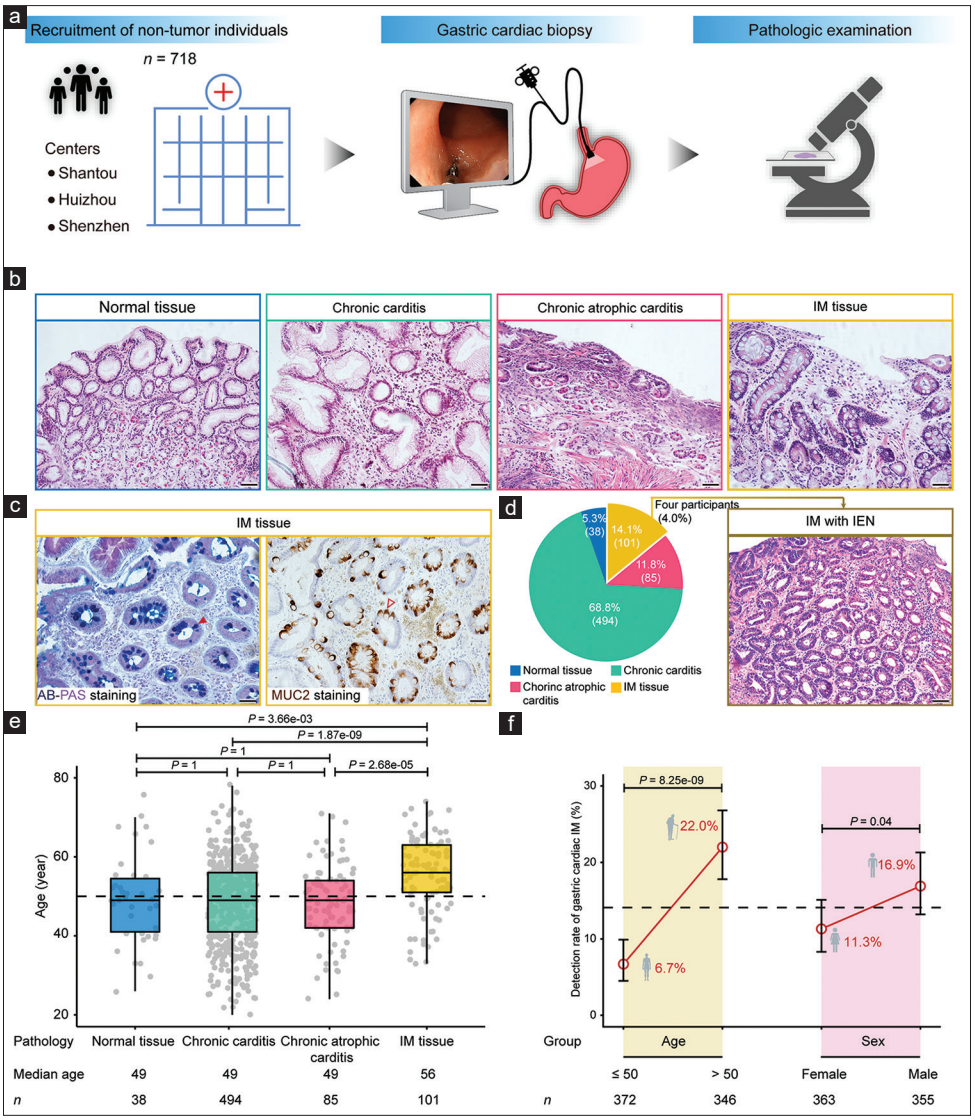


Figure 1: Overview of gastric cardiac biopsy specimens and detection rate of gastric cardiac IM. (a) schematic diagram showing the collection of gastric cardiac tissues from cancer-free individuals. A total of 718 participants underwent upper endoscopy were recruited from three endoscopy centers (Shantou, Huizhou, and Shenzhen) in Guangdong Province (b) hematoxylin and eosin staining of representative gastric cardiac tissues from participants with different pathological changes. Scale bars, 50 μm (c) representative images of Alcian blue-periodic acid-Schiff (AB-PAS) staining and immunostaining of mucin-2 (MUC2) in gastric cardiac IM tissues. The AB staining method stains goblet cell mucus as blue color (as indicated by a closed red arrowhead show AB-positive cells); the open red arrowhead denotes epithelial cells expressing MUC2. Scale bars, 50 μm (d) pie chart depicting the detection rates of 718 individuals in our cohort depending on their pathological changes. Left panel: Histologically, gastric cardiac IM was found in 14.1% (101/718) cancer-free individuals. Right panel: A total of four subjects show low-grade intraepithelial neoplasia of the gastric cardiac mucosa in the context of IM. There is mild irregularity of the glands with crowding of epithelial cells. Most of the glandular cells show mucus depletion, the nuclei are elongated, hyperchromatic, and irregular in size. Scale bar, 50 μm (e) boxplot showing the median age in different histological categories. Statistical significance was analyzed by the Kruskal–Wallis test, followed by Dunnett's post hoc test for multiple comparisons between groups. The black horizontal dashed line indicates the median age of all the study participants. The upper and lower limits of the box represent 25th and 75th quartile age distribution with whiskers extending to 1.5 times the range from top/bottom of the box, the center line within the box corresponds to the median age in each group, outliers are not shown (f) line graph showing detection rates of gastric cardiac IM measured in different age and sex groups. Indicated *P* values were calculated by Pearson's chi-square test. Error bar represents the 95% confidence interval around the detection rate. The overall detection rate of gastric cardiac IM (14.1%) in study participants is depicted by the horizontal dashed black line. Source data (d and f) are provided in Supplementary Table S2

stroma are well-preserved and keep their position and proportions intact. Inflammatory infiltration is minimal or absent. (b) Chronic carditis, characterized by the infiltration of the lamina propria by plasma cells and lymphocytes (with occasional formation of follicles) without glandular atrophy. (c) Chronic atrophic carditis, defined as the reduction or disappearance of native gastric cardiac glands: a reduction in the number of layers of subepithelial glands, often with decrease in the size and number of the glands within the lamina propria. There

is also a chronic inflammation of the lamina propria with abundant lymphocytes, macrophages, and plasma cells. This evaluation is possible in samples that include the muscularis mucosae, being completely represented in the full thickness of the mucosae. (d) IM tissues, recognized as the presence of goblet cells (special staining [AB-PAS] and immunostaining of MUC2 reveal that they secrete specific mucus), columnar absorptive cells with well-defined brush borders, or Paneth cells. Additionally, IEN refers to morphologically dysplastic epithelium (tortuous glandular structures are lined by mucus-depleted epithelial cells with irregular, elongated, and hyperchromatic nuclei) without breaching the basement membrane.[9]

DNA isolation and bisulfite pyrosequencing

Genomic DNA was isolated from fresh-frozen tissues using the AllPrep DNA/RNA Mini Kit (Cat# 80824, Lot: 166018916, QIAGEN, Germany), according to the manufacturer's protocols. The concentration and purity of the extracted genomic DNA were measured with an ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA was stored at –80°C until further analysis.

To quantify the methylation levels of candidate cytosine-phosphate-guanosine (CpG) sites in the promoter of *EYA4*, quantitative bisulfite pyrosequencing was performed. Briefly, 500 ng of total genomic DNA from each of the gastric cardiac tissues was used for bisulfite conversion, using the EZ DNA Methylation Gold Kit (Zymo Research, USA). The primers used were designed using PyroMark Assay Design Software 2.0.2 (QIAGEN) [Supplementary Table S1]. One of the PCR primers was biotin labeled. The sequence for analysis is localized to the promoter region of the *EYA4* gene. The PCR product was checked by 1% agarose gel electrophoresis (showing one clear band). According to the manufacturer's instructions, the biotinylated PCR product was purified as single-stranded DNA to be used as the template in a pyrosequencing reaction, using the Vacuum Prep Workstation (QIAGEN), and pyrosequencing was then performed using PyroMark Gold Q96 reagent (QIAGEN). The methylation percentage for each CpG site was generated automatically using PyroMark Q96 (QIAGEN), and the results were displayed as a pyrogram with the methylation percentage.

IHC

Immunohistochemical staining was conducted in 38 normal gastric cardiac mucosae, 47 IM tissues, and three IEN tissues. Briefly, the paraffin-embedded tissues were sectioned into 4-μm thickness and placed on adhesion glass slides, followed by baking at 65°C for 1 h. Slides were then deparaffinized in xylene (10 min ×3) and rehydrated with descending concentrations of ethanol (100%, 5 min; 95%, 5 min; 85%, 5 min; and 75%, 5 min) and rinsed with phosphate-buffered saline (PBS). Then, antigen retrieval was performed by heating the slides (immersed in 10 mM sodium citrate buffer, pH = 6.0 [for *EYA4*] or EDTA, pH = 9.0 [for MUC2]) for 3 min in a pressure cooker. Slides were washed with PBS (3 min ×3), and the endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min at room temperature, followed by three PBS washes (3 min each). Slides were incubated with EYA4 primary antibody (Cat# ab251675, rabbit polyclonal, Lot: GR3304276-1, Abcam, UK; 1:100 dilution) or MUC2 primary antibody (Cat# ZM-0392, Lot: 21082308, clone Ccp58, ZSGB-Bio, China; ready-to-use) overnight at 4°C. Then, the slides were thoroughly washed in PBS (3 min ×3) and incubated with horseradish peroxidase labeled goat anti-mouse/rabbit secondary antibody (Cat# KIT-5020, Lot: 210224S407c, Maixin Biotechnologies, China) for 30 min at 37°C, followed by an additional three washes in PBS (3 min each). Staining was visualized using the 3,3'-diaminobenzidine (Cat# DAB-0031, Maixin Biotechnologies, China) and counterstained with hematoxylin for 5 min. Finally, slides were dehydrated with series ethanol (75%, 5 min; 85%, 5 min; 95%, 5 min; and 100%, 5 min ×2) and cleared in xyline (5 min ×3). Images were captured on the OLYMPUS B ×53 microscope using Olympus cellSens imaging software (version 1.14).

The protein expression level of EYA4 was estimated semi-quantitatively, based on both staining intensity and proportion of stained cells, according to the following criteria. The intensity of immunostaining was graded from 0 to 3 (0: negative; 1: slightly brown staining; 2: moderately brown staining; and 3: darkly brown staining). The percentage of positively stained cells was graded on a scale of 0–4 (0: negative; 1: 1–10%; 2: 11–50%; 3: 51–80%; and 4: >80%). A final immunostaining score (ranging from 0 to 12) was calculated by multiplying the scores of nuclear staining intensity and percentage of positively stained cells.

Statistical analysis and data visualization

All statistical analyses and data visualization were performed in RStudio (version 2021.09.1.372) (http://www.rstudio.com/) within the R statistical environment (version 4.1.2) (https://www.R-project.org) using the packages dplyr (version 1.0.7), ggplot2 (version 3.3.5), ggrepel (version 0.9.1), factoextra (version 1.0.7), reshape2 (version 1.4.4), pheatmap (version 1.0.12), Gviz (version 1.38.0), GenomicFeatures (version 1.46.1),

BSgenome (version 1.62.0), TxDb.Hsapiens.UCSC.hg19.knownGene (version 3.2.2), and stats (version 4.1.2). All continuous variables with non-normal distribution were presented as median with interquartile range (IQR) for the indicated number of biological replicates, and categorical variables were presented as frequency with proportion. Continuous data were assessed for normality using Shapiro–Walk test (shapiro.test R function) before statistical tests of significance were run. Differences in median age and immunostaining score of EYA4 among multiple groups (did not pass normality test; Figures 1e and 3b) were assessed using the Kruskal–Wallis test, followed by Dunnett's *post hoc* test for multiple comparisons between groups (R package FSA, version 0.9.1). We used Pearson's Chi-square test (chisq.test R function) to compare the detection rates of gastric cardiac IM in each group (stratified by median age [≤50 vs. >50 years] and sex [female vs. male]. For the methylation rate of candidate CpG sites in normal and IM tissues, a Mann–Whitney *U* non-parametric test (wilcox.test R function) was used [Figure 2b]. For hierarchical cluster analysis of DNA methylation, z-scaled values were used to calculate Euclidean distance that was applied for clustering using Ward's method [Supplementary Figure S1b]. The statistical tests used in each experiment are described in their respective figure legends. The exact sample size ("*n*") in each group, where applicable, was provided in the figures or figure legends. Two-tailed *P* values < 0.05 were considered statistically significant. Adobe Illustrator CC2015 (Adobe Systems, San Jose, CA) is used to organize figures. The micrographs (such as those in Figure 3a) are a magnification of a representative area shown adjacent to them.

RESULTS

Higher detection rate of gastric cardiac IM in elder and male participants

The median age of the study participants was 50 (IQR: 42–57) years, there were slightly more female than male participants (50.6% vs. 49.4%). Baseline characteristics of participants are presented in Table 1. The most common pathological findings were chronic carditis (494 participants [68.8%; 95% CI: 65.2–72.2]), followed by IM (101 participants [14.1%; 95% CI: 11.7–16.9]) and chronic atrophic carditis (85 participants [11.8%; 95% CI: 9.6–14.5]; Figure 1d). Of note, foci of IEN occurred in the context of IM in four (4.0%; 95% CI: 1.3–10.4) of the 101 participants with IM. As shown in Figure 1d, the intestinal metaplastic glands are closely packed and lined by irregular cells with elongated and hyperchromatic nuclei.

Table 1: Basic characteristics of the participants enrolled in gastric cardiac biopsy cohort

Characteristics	Study cohort (*n*=718)
Age at diagnosis (years), Median (IQR)	50 (42-57)
Gender, *n* (%)	
Male	355 (49.4)
Female	363 (50.6)

IQR=interquartile range

Among these four histological categories, participants with IM were older than those in other categories ($P < 0.001$; Figure 1e). We then categorized the participants into two age groups using the median age (50 years) of the overall participants in this study as a cutoff. As expected, the detection rates of IM in participants ≤50 years increased from 6.7% (95% CI: 4.5–9.9) to 22.0% (95% CI: 17.8–26.8) in those > 50 years ($P < 0.001$). Subgroup analysis by sex showed a significantly higher detection rate of gastric cardiac IM in male participants compared to that of female participants (16.9% [95% CI: 13.2–21.3] vs. 11.3% [95% CI: 8.3–15.1], $P = 0.04$; Figure 1f). Individual participant characteristics are detailed in Supplementary Table S2.

Hypermethylation of *EYA4* promoter region in gastric cardiac IM tissues

To identify differentially methylated genes in gastric cardiac IM compared with normal gastric cardiac tissue, we reanalyzed a dataset from our group that had originally been generated to assess methylation alterations in gastric cardiac IM.[10] We assigned differentially methylated CpG sites found between normal and IM tissues to candidate genes, which were identified based on a false discovery rate <0.01, the difference in methylation (β value difference) ≥20%, and 19 or more differentially CpG sites located in promoter regions per gene. Through this analysis, we were able to identify five candidate genes showing significant hypermethylation in their promoter regions [Supplementary Figure S1a]. Unsupervised clustering analysis using all CpG sites in the candidate genes confirmed an explicit segregation and a clear epigenetic difference between normal and IM tissues [Supplementary Figure S1b]. Among these candidate genes, *EYA4* has been reported to be hypermethylated in Barrett's esophagus,[11,12] we thus, selected *EYA4* as a promising biomarker for downstream analysis.

Next, we used a quantitative pyrosequencing assay to assess the methylation status of the five candidate CpG sites for the *EYA4* promoter region [Figure 2a, Table 2] on an independent set of gastric cardiac samples with sufficient DNA. As shown in Figure 2b [Supplementary Table S3], IM tissues showed a significant higher DNA methylation level (mean methylation level of five CpG sites) than normal gastric

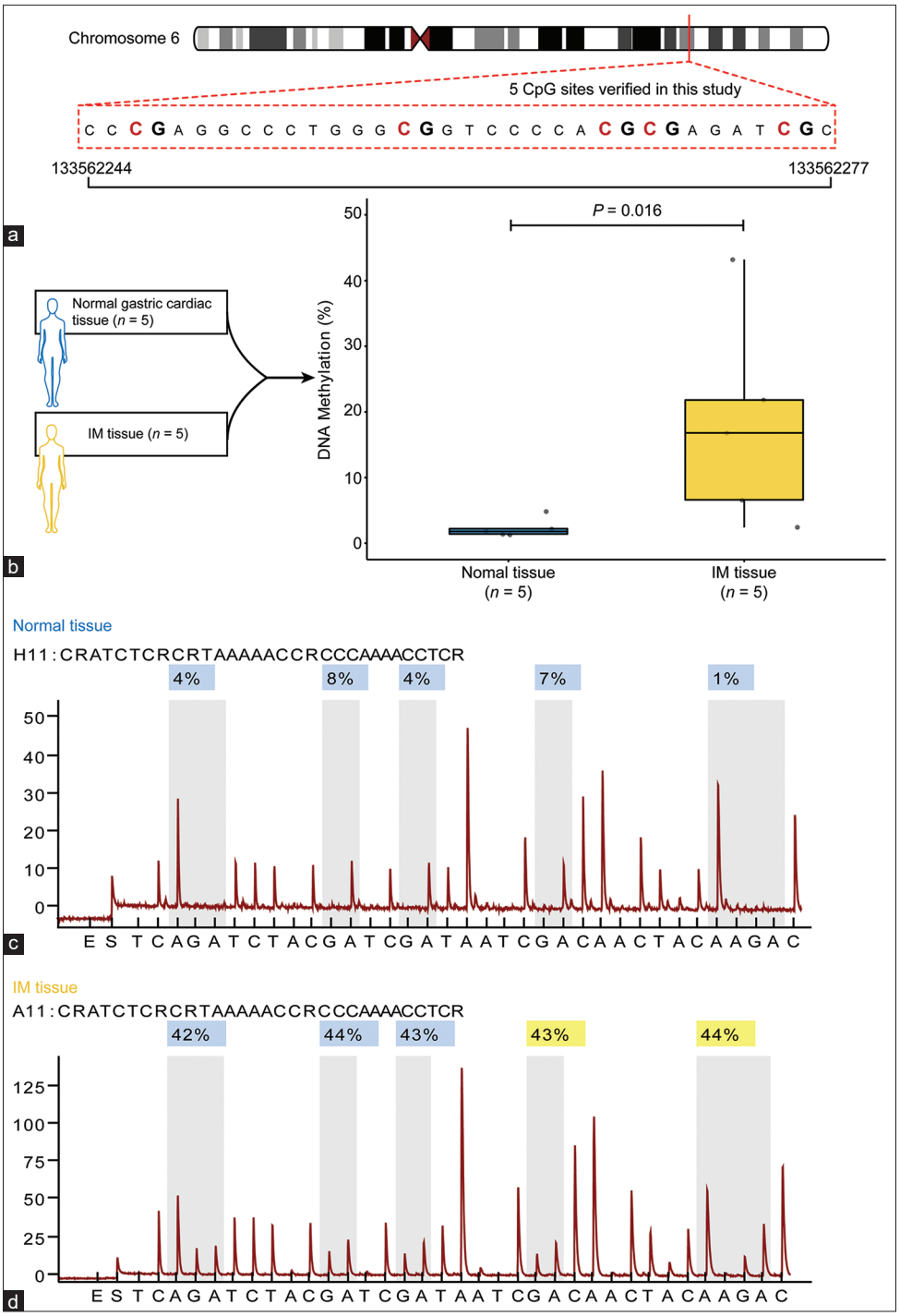


Figure 2: Quantitative pyrosequencing analysis reveals DNA methylation of candidate CpG sites in the *EYA4* promoter region. (a) schematic diagram showing the genomic location of a 34-bp region (Chr: 133562244-133562277) within *EYA4* promoter (indicated by a red vertical bar) and a close-up view of the examined CpG sites (red dashed rectangle) (b) a total of 10 fresh-frozen gastric cardiac tissues (n = 5 for normal and IM tissues, respectively) were used for quantification of DNA methylation levels of five candidate CpG sites (cg11518846, cg20980055, cg20286200, cg05062333, and cg01162672) by pyrosequencing. Box plot showing the median methylation level in normal and IM tissues. Each data dot denotes the average methylation of five candidate CpG sites mentioned above. Indicated *P* value was calculated by the Mann–Whitney *U* non-parametric test (c and d) representative pyrograms showing the methylation level of cg01162672, cg05062333, cg20286200, cg20980055, and cg11518846 in normal (c) and IM tissues (d). The percentage of DNA methylation at each CpG site is indicated by shaded areas, and the quality of the result is shown in blue (good quality) or yellow (pass quality). On the *x-axis*, the nucleotides injected by the pyrosequencer are shown, "E" represents the moment when the enzyme was added to the reaction followed by the substrate "S" that is documented by a small spike. The pyrosequencer added a "T" that is not present in the sequence, thus, no spike was observed. The actual sequence to analyze starts with a "C," followed by the methylated cytosine here marked with "R." On the *y-axis*, luminescence detected by the LCD camera is shown. Source data (b) are provided in Supplementary Table S3

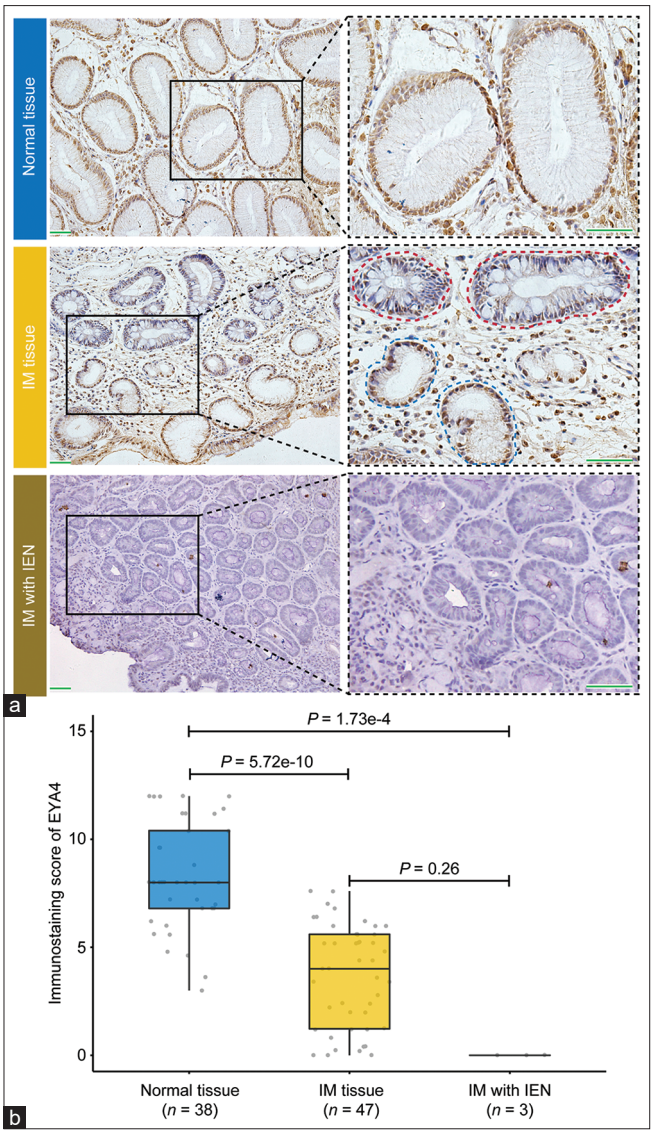


Figure 3: *EYA4* protein expression in normal and IM tissues. (a) representative images of immunohistochemical staining for *EYA4* in normal gastric cardiac tissues and IM tissues. As shown in the upper panel, normal gastric cardiac epithelial cells demonstrate strong nuclear immunostaining for *EYA4*. The middle panel of immunostaining section of IM tissue highlighting areas of intestinal metaplastic glands (outlined by red dashed line) and normal glands (outlined by blue dashed line). The intestinal metaplastic cells show weak to absent immunostaining compared with normal glandular cells. Prominent loss of *EYA4* expression in low-grade intraepithelial neoplasia (IEN) tissue in the context of IM is shown in the lower panel. Scale bars, 50 μm (b) quantitative comparison of immunohistochemical staining for *EYA4* in normal ($n = 38$), IM ($n = 47$), and IM with IEN ($n = 3$) tissues. Gray dots represent the immunostaining score for *EYA4* in each sample. Statistical significance was analyzed by the Kruskal–Wallis test, followed by Dunnett's *post hoc* test for multiple comparisons between groups. The upper and lower limits of the box represent 25th and 75th quartile immunostaining score distribution with whiskers extending to 1.5 times the range from top/bottom of the box, the center line within the box corresponds to the median value of the immunostaining score in each group, outliers are not shown. Source data (b) are provided in Supplementary Table S4

cardiac tissues ($P = 0.016$), demonstrating the association between gastric cardiac IM and hypermethylation in the

Table 2: Candidate hypermethylated CpG sites in the promoter of *EYA4*

Illumina Id†	Delta beta (IM vs. Normal tissues)	Chromosome location (GRCh37/hg19)
cg11518846	0.31	Chr6: 133562246
cg20980055	0.26	Chr6: 133562258
cg20286200	0.36	Chr6: 133562267
cg05062333	0.31	Chr6: 133562269
cg01162672	0.32	Chr6: 133562275

IM = intestinal metaplasia. †Illumina ID is the unique identification number in the HumanEPICBeadChip

EYA4 promoter region. Representative pyrograms for normal and IM tissues are depicted in Figure 2c and d.

Decreased *EYA4* protein expression in gastric cardiac IM tissues

The intriguing observation of significant hypermethylation of the *EYA4* promoter region in gastric cardiac IM tissues led us to hypothesize that these metaplastic lesions may have lower levels of *EYA4* protein compared to the normal gastric cardiac tissues. Formalin-fixed paraffin-embedded biopsy samples were then subjected to immunostaining for *EYA4*. As shown in Figure 3a, *EYA4* was uniformly expressed in the nuclei of normal gastric cardiac epithelial cells (upper panel) and its expression was significantly reduced in IM lesions (middle panel). As expected, loss expression of *EYA4* was noted in all the three IEN samples included in this study (lower panel). Following image quantification, the immunostaining score of *EYA4* was significantly decreased in IM and IEN tissues compared with that in normal gastric cardiac tissues ($P < 0.001$; Figure 3b); [Supplementary Table S4]. These results demonstrated that the downregulated expression of *EYA4* is a promising biomarker for gastric cardiac IM as well as early gastric cardiac carcinogenesis.

DISCUSSION

It is becoming clear that alterations in DNA methylation are associated with precancerous lesions.[13-16] However, the role of DNA methylation in gastric cardiac IM has received relatively less attention. Previously, we reported that DNA methylation profiles of gastric cardiac IMs significantly differed from those of normal gastric cardiac mucosa. In this study, we researched the methylation status of the *EYA4* promoter region and gained insights on epigenetic markers in gastric cardiac IM. In our earlier study, obvious hypermethylation of gene promoter regions was shown in gastric cardiac IM samples. By conducting pyrosequencing in the candidate CpG sites of *EYA4* and IHC analysis of *EYA4* protein, our present study revealed that gastric cardiac IM lesions exhibited hypermethylation in the promoter of *EYA4* and showed a reduced protein

expression. This work provides evidence for promoter hypermethylation of *EYA4* as a promising epigenetic biomarker in gastric cardiac IM.

To date, the data regarding the detection rate of gastric cardiac IM in cancer-free individuals are limited. In this study, we assessed the pathology of the gastric cardiac mucosae covering more than 700 cancer-free individuals in Guangdong Province, China. We specifically focused on the detection rate of gastric cardiac IM to provide baseline data for further investigation of this premalignant lesion. Based on pathological assessment, the overall gastric cardiac IM detection rate in our study population was 14.1% (95% CI: 11.7–16.9). However, there are wide variations in the detection rates of gastric IMs among different studies,[17,18] partly because of the unavoidable sampling bias, because most IMs showed only focal involvement. It deserves to be noted that in our study, the detection rates for gastric cardiac IM increased with age and were higher for men than for women, as shown in Figure 1f. These findings were consistent with that of previous studies on gastric IMs.[19,20] Most likely, this age-dependent rise reflects the natural history of gastric cardiac IM and can be explained by the long duration of exposure to environmental risk factors. Therefore, it is necessary to consider the age and sex for future screening and surveillance for premalignant lesions of gastric cardia. Indeed, not all patients with gastric cardiac IMs will progress to cancer ultimately. Of note, we found IEN in the context of IM in four subjects (4/101; 4.0%) of the study participants. Thus, further work should be done to identify a subset of IM subjects at high risk of neoplastic progression.

DNA methylation changes have been studied in multiple cancer types.[21-24] Furthermore, increasing evidence shows that aberrant DNA methylation events occur even in samples of precancerous lesions.[25] Thus, such epigenetic events are poised to become ideal biomarkers for early stage cancer.[26] However, only a few studies have focused on DNA methylation biomarkers in gastric non-cardiac IMs,[15,27,28] and even less on IMs arising from gastric cardia. We recently conducted array-based genome-wide DNA methylation analysis in gastric cardiac IMs compared with normal gastric cardiac mucosae[10] and found that *EYA4* gene was significantly hypermethylated in gastric cardiac IMs. In this study, we further confirmed that hypermethylation of the *EYA4* gene promoter was evident in gastric cardiac IMs [Figure 2]. This aligns with a previous report highlighting a link between *EYA4* promoter hypermethylation and Barrett's esophagus, a condition of IM in the distal esophagus.[12] Furthermore, several studies reported the hypermethylation of *EYA4* in different cancer types.[29-31] These findings support the claim that promoter DNA methylation of *EYA4* is closely associated with IM development and is a very early event during multistage gastric cardiac carcinogenesis. Therefore, it will be worth investigating how well an *EYA4* methylation test can detect dysplastic and cancerous lesions in future studies.

DNA hypermethylation plays an important role in carcinogenesis because it could cause the silencing of some pivotal genes, especially tumor suppressor genes.[32,33] In this study, we observed that *EYA4* protein was significantly reduced or not present in gastric cardiac IMs and dysplastic lesions, compared with normal tissues [Figure 3a], supporting our hypothesis that EYA4 expression reduction might be, at least partly, because of promoter DNA hypermethylation. These data indicate that EYA4 is a promising biomarker for gastric cardiac IMs. *EYA4* is one of the four members of *EYA* gene family that was initially identified in Drosophila.[34] Of note, interesting clues about the role of *EYA4* protein in tumorigenesis are emerging rapidly, namely, its tumor suppressor role in esophageal squamous cell carcinoma,[31] colorectal carcinoma,[35] pancreatic adenocarcinoma,[36] hepatocellular carcinoma,[37] and bladder cancer.[38] Thus, it is possible that reduced expression of *EYA4* protein contributes to IM development and is an early molecular event during gastric cardiac carcinogenesis. Future investigations should aim to elucidate the functional role and the exact mechanism of *EYA4* in IM development.

The major limitation of this study lies in the small sample size for pyrosequencing validation, for the methylation status of *EYA4* promoter. Therefore, continued efforts to investigate the frequency of *EYA4* methylation and its functional relevance, will improve our understanding of the role of *EYA4* in gastric cardiac IM. Another limitation of the study is the small sample size of IEN ($n = 3$) used for IHC.

In summary, the promoter hypermethylation of *EYA4* may contribute to the downregulation of protein expression in gastric cardiac IM, a precursor lesion of gastric cardiac cancer. This finding highlights the important role of aberrant DNA methylation in the *EYA4* promoter region in the pathogenesis of gastric cardiac IM [Figure 4].

Financial support and sponsorship

This study was supported by Project of Educational Commission of Guangdong Province of China (Grant No. 2017KQNCX068), the research grants from the Shantou Science and Technology Bureau (Grant No. 210712186880511; Grant No. 220507236491772),

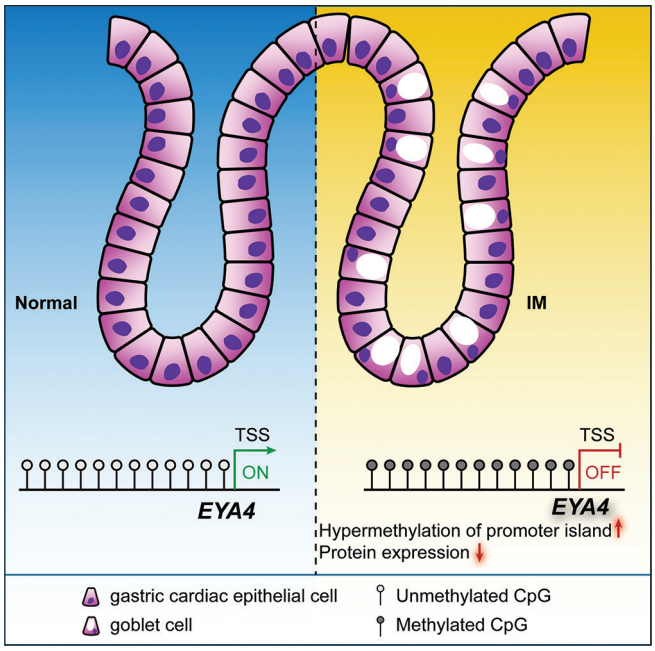


Figure 4: Schematic model depicting the role of hypermethylation in the *EYA4* promoter region in gastric cardiac IM development. Hypermethylation-related reduced expression of the EYA4 protein may contribute to the development of gastric cardiac IM and appears to be a promising biomarker for this premalignant lesion

the Open Fund of Guangdong Provincial Key Laboratory of Infectious Diseases and Molecular Immunopathology (Grant No. GDKL202209), Medical Scientific Research Foundation of Guangdong Province of China (Grant No. A2022329), and the Huizhou Science and Technology Bureau (Grant No. 2021WC0106069).

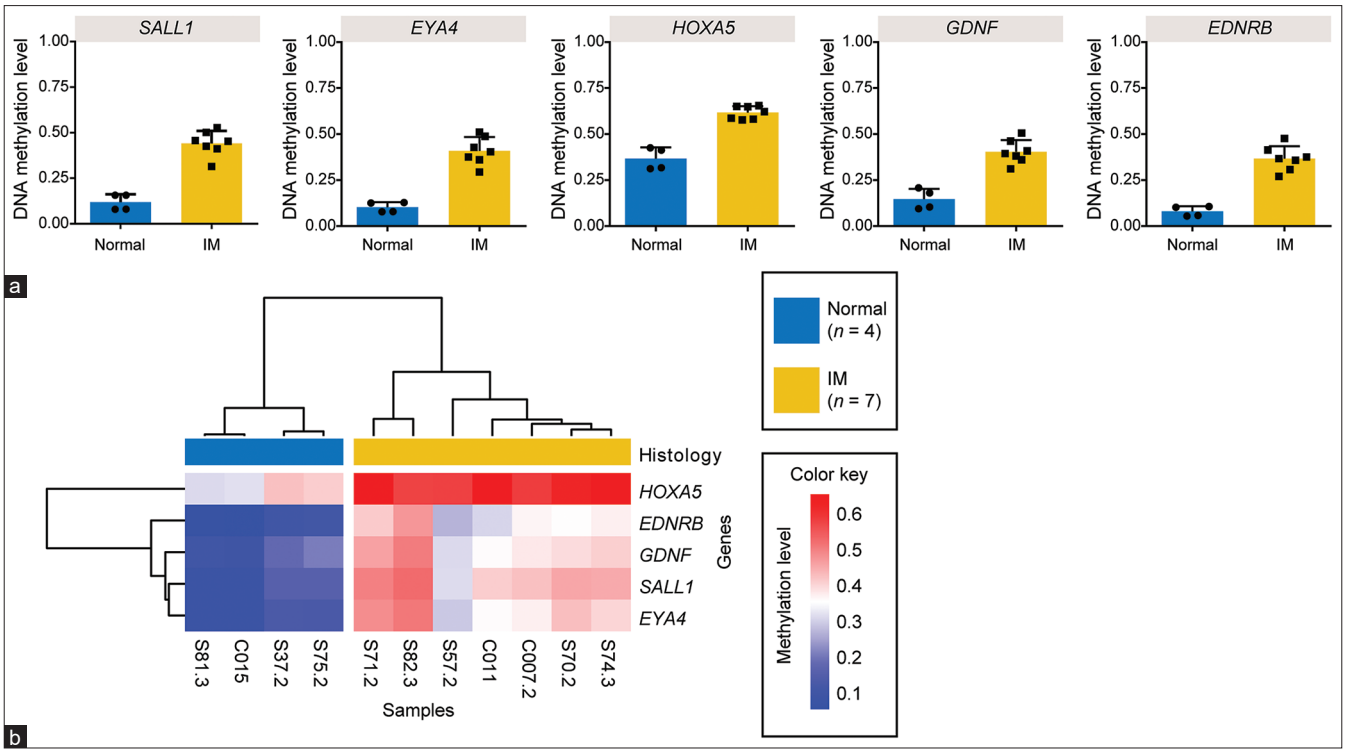
Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Fan J, Li J, Guo S, Tao C, Zhang H, Wang W, *et al.* Genome-wide DNA methylation profiles of low- and high-grade adenoma reveals potential biomarkers for early detection of colorectal carcinoma. Clin Epigenetics 2020;12:56.
2. den Hoed CM, Holster IL, Capelle LG, de Vries AC, den Hartog B, Ter Borg F, *et al.* Follow-up of premalignant lesions in patients at risk for progression to gastric cancer. Endoscopy 2013;45:249-56.
3. Kim N, Park RY, Cho SI, Lim SH, Lee KH, Lee W, *et al.* Helicobacter pylori infection and development of gastric cancer in Korea: Long-term follow-up. J Clin Gastroenterol 2008;42:448-54.
4. Whiting JL, Sigurdsson A, Rowlands DC, Hallissey MT, Fielding JW. The long term results of endoscopic surveillance of premalignant gastric lesions. Gut 2002;50:378-81.
5. Morris MR, Latif F. The epigenetic landscape of renal cancer. Nat Rev Nephrol 2017;13:47-60.
6. Saghafinia S, Mina M, Riggi N, Hanahan D, Ciriello G. Pan-cancer landscape of aberrant DNA methylation across human tumors. Cell Rep 2018;25:1066-80.e8.
7. Natale F, Vivo M, Falco G, Angrisano T. Deciphering DNA methylation signatures of pancreatic cancer and pancreatitis. Clin Epigenetics 2019;11:132.
8. Rosai J.Rosai and Ackerman's Surgical Pathology e-book. Elsevier Health Sciences; 2011.
9. Rugge M, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, *et al.* Gastric dysplasia: The Padova international classification. Am J Surg Pathol 2000;24:167-76.
10. Lin R, Li C, Liu Z, Wu R, Lu J. Genome-wide DNA methylation profiling identifies epigenetic signatures of gastric cardiac intestinal metaplasia. J Transl Med 2020;18:292.
11. Kaz AM, Wong CJ, Luo Y, Virgin JB, Washington MK, Willis JE, *et al.* DNA methylation profiling in Barrett's esophagus and esophageal adenocarcinoma reveals unique methylation signatures and molecular subclasses. Epigenetics 2011;6:1403-12.
12. Zou H, Osborn NK, Harrington JJ, Klatt KK, Molina JR, Burgart LJ, *et al.* Frequent methylation of eyes absent 4 gene in Barrett's esophagus and esophageal adenocarcinoma. Cancer Epidemiol Biomarkers Prev 2005;14:830-4.
13. Schmitz M, Eichelkraut K, Schmidt D, Zeiser I, Hilal Z, Tettenborn Z, *et al.* Performance of a DNA methylation marker panel using liquid-based cervical scrapes to detect cervical cancer and its precancerous stages. BMC Cancer 2018;18:1197.
14. Moinova HR, LaFramboise T, Lutterbaugh JD, Chandar AK, Dumot J, Faulx A, *et al.* Identifying DNA methylation biomarkers for non-endoscopic detection of Barrett's esophagus. Sci Transl Med 2018;10:eaao5848. doi: 10.1126/scitranslmed.aao5848.
15. Huang KK, Ramnarayanan K, Zhu F, Srivastava S, Xu C, Tan ALK, *et al.* Genomic and epigenomic profiling of high-risk intestinal metaplasia reveals molecular determinants of progression to gastric cancer. Cancer Cell 2018;33:137-50.e5.
16. Oh TJ, Oh HI, Seo YY, Jeong D, Kim C, Kang HW, *et al.* Feasibility of quantifying SDC2 methylation in stool DNA for early detection of colorectal cancer. Clin Epigenetics 2017;9:126.
17. Da B, Jani N, Gupta N, Jayaram P, Kankotia R, Yao Yu C, *et al.* High-risk symptoms do not predict gastric cancer precursors. Helicobacter 2019;24:e12548.
18. Kang KP, Lee HS, Kim N, Kang HM, Park YS, Lee DH, *et al.* Role of intestinal metaplasia subtyping in the risk of gastric cancer in Korea. J Gastroenterol Hepatol 2009;24:140-8.
19. McNamara D, Buckley M, Crotty P, Hall W, O'Sullivan M, O'Morain C. Carditis: All Helicobacter pylori or is there a role for gastro-oesophageal reflux?Scand J Gastroenterol 2002;37:772-7.
20. Felley C, Bouzourene H, VanMelle MB, Hadengue A, Michetti P, Dorta G, *et al.* Age, smoking and overweight contribute to the development of intestinal metaplasia of the cardia. World J Gastroenterol 2012;18:2076-83.
21. Baylin SB, Jones PA.A decade of exploring the cancer epigenome-biological and translational implications. Nat Rev Cancer 2011;11:726-34.
22. Zhao SG, Chen WS, Li H, Foye A, Zhang M, Sjöström M, *et al. The* DNA methylation landscape of advanced prostate cancer. Nat Genet 2020;52:778-89.
23. Klughammer J, Kiesel B, Roetzer T, Fortelny N, Nemc A, Nenning KH, *et al.* The DNA methylation landscape of glioblastoma disease progression shows extensive heterogeneity in time and space. Nat Med 2018;24:1611-24.
24. Hovestadt V, Jones DT, Picelli S, Wang W, Kool M, Northcott PA, *et al.* Decoding the regulatory landscape of medulloblastoma using DNA methylation sequencing. Nature 2014;510:537-41.
25. Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S, *et al.* DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. Carcinogenesis 2006;27:1160-8.
26. Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. Lancet 2018;392:777-86.
27. Shin CM, Kim N, Lee HS, Park JH, Ahn S, Kang GH, *et al.* Changes in aberrant DNA methylation after Helicobacter pylori eradication:

A long-term follow-up study. Int J Cancer 2013;133:2034-42.
28. Sugimoto R, Habano W, Yanagawa N, Akasaka R, Toya Y, Sasaki A, *et al*. Molecular alterations in gastric cancer and the surrounding intestinal metaplastic mucosa: An analysis of isolated glands. Gastric Cancer 2021;24:382-91.
29. Emmett RA, Davidson KL, Gould NJ, Arasaradnam RP.DNA methylation patterns in ulcerative colitis-associated cancer: A systematic review. Epigenomics 2017;9:1029-42.
30. Hou X, Peng JX, Hao XY, Cai JP, Liang LJ, Zhai JM, *et al*. DNA methylation profiling identifies EYA4 gene as a prognostic molecular marker in hepatocellular carcinoma. Ann Surg Oncol 2014;21:3891-9.
31. Luo M, Li Y, Shi X, Yang W, Zhou F, Sun N, *et al*. Aberrant methylation of EYA4 promotes epithelial-mesenchymal transition in esophageal squamous cell carcinoma. Cancer Sci 2018;109:1811-24.
32. Sharma G, Mirza S, Parshad R, Srivastava A, Gupta SD, Pandya P, *et al*. Clinical significance of promoter hypermethylation of DNA repair genes in tumor and serum DNA in invasive ductal breast carcinoma patients. Life Sci 2010;87:83-91.
33. Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484-92.
34. Borsani G, DeGrandi A, Ballabio A, Bulfone A, Bernard L, Banfi S, *et al*. EYA4, a novel vertebrate gene related to Drosophila eyes absent. Hum Mol Genet 1999;8:11-23.
35. Kim SJ, Tae CH, Hong SN, Min BH, Chang DK, Rhee PL, *et al*. EYA4 acts as a new tumor suppressor gene in colorectal cancer. Mol Carcinog 2015;54:1748-57.
36. Mo SJ, Liu X, Hao XY, Chen W, Zhang KS, Cai JP, *et al*. EYA4 functions as tumor suppressor gene and prognostic marker in pancreatic ductal adenocarcinoma through β-catenin/ID2 pathway. Cancer Lett 2016;380:403-12.
37. Zhu XX, Li JH, Cai JP, Hou X, Huang CS, Huang XT, *et al*. EYA4 inhibits hepatocellular carcinoma by repressing MYCBP by dephosphorylating β-catenin at Ser552. Cancer Sci 2019;110:3110-21.
38. Dong W, Bi J, Liu H, Yan D, He Q, Zhou Q, *et al*. Circular RNA ACVR2A suppresses bladder cancer cells proliferation and metastasis through miR-626/EYA4 axis. Mol Cancer 2019;18:95.



Supplementary Figure S1: Candidate hypermethylated genes in gastric cardiac IM.These data were from Lin *et al.* 2020 (a) Bar plots displaying the DNA methylation levels of the hypermethylated genes in gastric cardiac IM compared with normal gastric cardiac tissue (b) Heatmap demonstrating unsupervised hierarchical clustering of individuals based on the methylation levels of the candidate hypermethylated genes mentioned in (a)

Supplementary Table S1: List of PCR primers, pyrosequencing primers, and sequence for analysis

Gene	Primer description	Primer sequence	Sequence to analyze
EYA4	PCR_Forward	5'-biotin-TTTTTTTTAGTTTGGAGGTTGTAGT-3'	CRATCTCRCRTAAAAACCRCCC
	PCR_Reverse	5'-TTTATTTAACCTCRAATAACTACCTATTATC-3'	AAAACCTCR
	Pyrosequencing_Reverse	5'-AATAACTACCTATTATCATAATTTA-3'	

Supplementary Table S2: Participant characteristic

Subject ID	Sex	Age	Pathological diagnosis
HY-1	Male	55	Chronic carditis
HY-3	Female	43	Chronic carditis
HY-5	Male	50	Chronic carditis
HY-7	Male	37	Chronic carditis
HY-9	Male	35	Chronic carditis
HY-11	Female	47	Chronic atrophic carditis
HY-13	Female	71	Chronic carditis
HY-15	Female	71	Chronic carditis
HY-17	Female	51	Chronic carditis
HY-19	Female	32	Chronic carditis
HY-21	Female	63	Chronic carditis
HY-23	Male	42	Chronic carditis
HY-25	Male	30	Chronic carditis
HY-29	Female	34	Chronic carditis
HY-31	Female	39	Chronic carditis
HY-33	Male	57	Chronic carditis
HY-35	Male	33	Chronic carditis
HY-37	Male	57	Chronic carditis
HY-39	Male	53	Chronic carditis
HY-41	Male	35	Chronic carditis
HY-43	Male	53	Chronic carditis
HY-45	Male	48	Chronic atrophic carditis
HY-47	Male	54	Chronic carditis
HY-49	Female	48	Chronic carditis
HY-51	Male	56	IM
HY-53	Male	47	Chronic carditis
HY-55	Female	64	IM
HY-57	Male	29	Chronic atrophic carditis
HY-61	Male	31	Chronic carditis
HY-63	Female	69	Chronic carditis
HY-65	Male	26	Chronic carditis
HY-67	Female	45	Chronic carditis
HY-69	Female	25	Chronic carditis
HY-71	Male	47	Normal tissue
HY-73	Male	28	Chronic carditis
HY-75	Male	61	Chronic carditis
HY-77	Male	58	IM
HY-79	Male	49	Chronic carditis
HY-83	Male	58	IM
HY-85	Male	53	Chronic carditis
HY-87	Female	48	Chronic carditis
HY-89	Female	36	Chronic carditis
HY-91	Male	46	Chronic carditis
HY-93	Male	52	Chronic carditis
HY-95	Female	56	Chronic carditis
HY-97	Male	45	Chronic carditis
HY-99	Male	36	Chronic carditis
HY-103	Male	62	Chronic carditis
HY-105	Male	58	Chronic carditis
HY-107	Male	55	IM
HY-109	Male	36	Chronic carditis
HY-113	Female	56	Chronic carditis
HY-115	Male	41	Chronic atrophic carditis
HY-117	Male	41	Chronic carditis
HY-121	Female	43	Chronic carditis
HY-123	Female	38	Chronic carditis
HY-127	Male	47	Chronic atrophic carditis
HY-129	Male	49	Chronic carditis
HY-131	Male	62	Chronic atrophic carditis
HY-133	Male	57	Chronic carditis
HY-137	Male	25	Chronic atrophic carditis
HY-141	Male	51	Chronic carditis
HY-143	Male	39	IM
HY-145	Male	49	Chronic carditis
HY-147	Male	43	Chronic carditis
HY-149	Female	61	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-151	Male	55	Chronic atrophic carditis
HY-153	Female	45	Chronic atrophic carditis
HY-155	Female	33	IM
HY-157	Male	49	Chronic atrophic carditis
HY-159	Male	42	Chronic atrophic carditis
HY-161	Female	62	IM
HY-163	Male	58	Chronic carditis
HY-165	Male	29	Chronic carditis
HY-167	Male	34	Chronic carditis
HY-169	Female	48	IM
HY-171	Male	53	Chronic carditis
HY-173	Female	41	Chronic carditis
HY-175	Male	32	Chronic carditis
HY-177	Female	46	Chronic carditis
HY-181	Male	47	Chronic carditis
HY-183	Female	46	Chronic carditis
HY-187	Male	40	Chronic carditis
HY-189	Female	68	Normal tissue
HY-193	Female	47	Chronic carditis
HY-195	Male	38	Chronic carditis
HY-199	Male	52	IM
HY-201	Female	45	Chronic carditis
HY-203	Male	49	Chronic carditis
HY-205	Female	52	IM
HY-207	Male	45	Chronic carditis
HY-209	Female	54	Chronic carditis
HY-211	Female	45	Chronic carditis
HY-213	Female	55	Chronic carditis
HY-215	Female	59	Chronic carditis
HY-217	Male	60	Chronic carditis
HY-219	Female	24	Chronic atrophic carditis
HY-221	Male	60	Chronic atrophic carditis
HY-223	Female	48	Chronic carditis
HY-225	Male	48	Chronic carditis
HY-227	Female	63	Chronic carditis
HY-229	Female	59	Chronic carditis
HY-231	Female	50	Chronic carditis
HY-233	Male	37	Chronic carditis
HY-237	Male	35	Chronic carditis
HY-239	Male	37	Chronic carditis
HY-241	Male	49	Chronic atrophic carditis
HY-243	Male	64	Normal tissue
HY-245	Male	28	Chronic carditis
HY-247	Male	50	Chronic carditis
HY-253	Male	52	IM
HY-255	Female	44	Chronic carditis
HY-257	Male	47	Chronic carditis
HY-259	Female	43	Chronic carditis
HY-263	Male	64	Chronic atrophic carditis
HY-265	Male	24	Chronic carditis
HY-267	Female	24	Chronic carditis
HY-269	Female	33	Chronic carditis
HY-271	Female	57	Chronic carditis
HY-275	Male	42	Chronic carditis
HY-277	Male	49	Chronic atrophic carditis
HY-279	Female	50	Chronic carditis
HY-281	Male	56	IM
HY-283	Female	46	Chronic carditis
HY-285	Female	44	IM
HY-287	Female	46	Chronic atrophic carditis
HY-289	Male	47	IM
HY-293	Male	58	IM
HY-295	Male	55	Normal tissue
HY-297	Female	34	Chronic carditis
HY-299	Female	36	IM
HY-301	Male	52	IM

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-303	Male	43	Chronic carditis
HY-305	Male	34	Chronic carditis
HY-307	Female	55	Normal tissue
HY-309	Female	48	IM
HY-313	Female	53	Chronic atrophic carditis
HY-315	Male	49	Chronic carditis
HY-319	Male	48	Chronic carditis
HY-321	Male	48	Chronic carditis
HY-323	Male	50	Chronic carditis
HY-325	Female	63	Chronic carditis
HY-327	Female	42	Normal tissue
HY-329	Male	41	Chronic carditis
HY-331	Male	35	Chronic carditis
HY-333	Male	41	Chronic carditis
HY-335	Male	22	Chronic carditis
HY-337	Female	66	Chronic carditis
HY-339	Female	41	IM
HY-341	Female	46	Chronic carditis
HY-343	Female	40	Chronic carditis
HY-345	Male	72	Chronic carditis
HY-347	Male	49	Chronic carditis
HY-349	Female	60	IM
HY-351	Male	54	Chronic carditis
HY-353	Male	49	Normal tissue
HY-355	Female	52	Chronic carditis
HY-361	Male	45	Chronic carditis
HY-365	Female	54	Chronic carditis
HY-367	Male	32	Chronic carditis
HY-369	Female	54	Chronic carditis
HY-371	Male	33	Chronic atrophic carditis
HY-373	Female	68	Normal tissue
HY-375	Male	50	Normal tissue
HY-377	Male	53	Chronic carditis
HY-379	Male	39	Chronic carditis
HY-379	Female	55	Chronic carditis
HY-383	Female	58	Chronic carditis
HY-385	Female	71	Chronic atrophic carditis
HY-387	Female	56	Chronic carditis
HY-389	Male	51	Chronic carditis
HY-391	Male	44	Chronic atrophic carditis
HY-393	Male	66	Normal tissue
HY-395	Male	56	Chronic atrophic carditis
HY-397	Male	58	Chronic carditis
HY-399	Male	47	Chronic atrophic carditis
HY-403	Female	49	Chronic carditis
HY-405	Female	54	Chronic atrophic carditis
HY-407	Female	52	Chronic atrophic carditis
HY-411	Male	52	Chronic carditis
HY-413	Female	41	Chronic carditis
HY-415	Male	40	Chronic carditis
HY-417	Male	49	Chronic atrophic carditis
HY-421	Female	42	Chronic carditis
HY-423	Male	29	Chronic carditis
HY-425	Male	51	Chronic atrophic carditis
HY-427	Male	51	IM
HY-429	Male	49	Chronic carditis
HY-431	Male	37	Chronic carditis
HY-433	Male	52	Chronic carditis
HY-437	Male	51	IM
HY-439	Male	51	Chronic carditis
HY-443	Female	42	Chronic carditis
HY-445	Male	49	Chronic carditis
HY-455	Male	40	Chronic carditis
HY-459	Female	49	Normal tissue
HY-463	Male	47	Chronic carditis
HY-467	Female	51	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-469	Male	48	Chronic carditis
HY-473	Female	71	Chronic carditis
HY-475	Male	46	Chronic carditis
HY-477	Male	39	IM
HY-481	Female	43	Chronic carditis
HY-483	Male	33	Normal tissue
HY-485	Female	47	Chronic carditis
HY-489	Male	48	Chronic carditis
HY-491	Male	52	Chronic carditis
HY-493	Male	58	Chronic carditis
HY-497	Female	53	Chronic carditis
HY-499	Male	64	Chronic atrophic carditis
HY-501	Female	55	Chronic carditis
HY-503	Male	54	Chronic atrophic carditis
HY-505	Female	50	Chronic carditis
HY-507	Male	52	Chronic carditis
HY-509	Male	53	Chronic carditis
HY-511	Male	51	Chronic carditis
HY-513	Male	52	Chronic atrophic carditis
HY-517	Female	62	Chronic carditis
HY-519	Female	59	Chronic carditis
HY-521	Female	47	Chronic carditis
HY-523	Male	38	Chronic carditis
HY-525	Female	57	Chronic atrophic carditis
HY-527	Male	46	Chronic carditis
HY-529	Female	51	Chronic atrophic carditis
HY-531	Male	54	Chronic carditis
HY-537	Male	64	IM
HY-539	Male	52	IM
HY-543	Male	59	Chronic carditis
HY-545	Male	59	Chronic carditis
HY-547	Male	51	Normal tissue
HY-549	Male	55	IM
HY-551	Male	38	Chronic carditis
HY-553	Male	38	Chronic atrophic carditis
HY-557	Female	64	Chronic carditis
HY-559	Female	61	Chronic carditis
HY-561	Female	52	Chronic atrophic carditis
HY-563	Female	36	Chronic carditis
HY-565	Female	54	Chronic carditis
HY-567	Male	46	Chronic carditis
HY-569	Female	47	Normal tissue
HY-571	Male	41	IM
HY-573	Male	40	Normal tissue
HY-575	Male	54	IM
HY-577	Female	58	Chronic carditis
HY-579	Female	45	Chronic carditis
HY-581	Female	59	Chronic carditis
HY-583	Male	58	Chronic carditis
HY-585	Male	36	Chronic carditis
HY-587	Female	37	Chronic carditis
HY-601	Female	49	Normal tissue
HY-607	Male	46	Chronic carditis
HY-609	Female	38	Chronic atrophic carditis
HY-611	Male	45	Chronic carditis
HY-613	Male	51	Chronic carditis
HY-615	Female	51	Chronic carditis
HY-617	Female	40	Chronic carditis
HY-619	Male	58	IM
HY-621	Male	51	Chronic carditis
HY-623	Male	48	Chronic atrophic carditis
HY-625	Female	62	Chronic carditis
HY-627	Female	48	Chronic carditis
HY-629	Female	44	Chronic atrophic carditis
HY-631	Male	76	Chronic carditis
HY-633	Male	49	Chronic atrophic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-635	Female	61	Chronic atrophic carditis
HY-637	Male	59	Chronic carditis
HY-639	Male	46	Chronic carditis
HY-641	Male	46	Chronic atrophic carditis
HY-643	Female	46	Chronic carditis
HY-645	Female	50	Chronic carditis
HY-647	Female	27	Chronic carditis
HY-649	Female	42	Chronic atrophic carditis
HY-651	Female	60	Chronic atrophic carditis
HY-653	Male	61	IM
HY-655	Male	50	Chronic carditis
HY-657	Female	45	Chronic carditis
HY-659	Male	42	Chronic atrophic carditis
HY-661	Female	66	Chronic carditis
HY-663	Male	60	Chronic carditis
HY-665	Female	72	Chronic carditis
HY-667	Male	60	Chronic carditis
HY-669	Female	52	Chronic carditis
HY-671	Female	57	Chronic carditis
HY-673	Female	49	Chronic carditis
HY-675	Male	37	Chronic carditis
HY-677	Male	35	Chronic carditis
HY-679	Female	52	Chronic carditis
HY-681	Male	48	Chronic carditis
HY-683	Female	58	Chronic carditis
HY-685	Female	55	Chronic carditis
HY-687	Male	52	IM
HY-691	Male	61	Chronic carditis
HY-693	Female	50	Chronic carditis
HY-695	Female	71	IM
HY-697	Female	35	Chronic atrophic carditis
HY-699	Male	47	Chronic atrophic carditis
HY-734	Male	64	IM
HY-736	Female	38	Chronic carditis
HY-738	Male	58	Chronic carditis
HY-740	Female	57	Chronic carditis
HY-742	Female	55	Chronic carditis
HY-744	Female	42	Chronic atrophic carditis
HY-746	Female	41	Chronic carditis
HY-749	Male	57	Chronic carditis
HY-751	Female	47	Chronic carditis
HY-753	Female	39	Normal tissue
HY-755	Male	43	Chronic carditis
HY-757	Female	51	Normal tissue
HY-759	Female	48	Normal tissue
HY-761	Female	53	Chronic carditis
HY-765	Female	51	Chronic carditis
HY-767	Female	56	Chronic carditis
HY-769	Female	58	IM
HY-771	Female	40	Chronic carditis
HY-773	Female	57	IM
HY-775	Male	58	Normal tissue
HY-777	Male	65	IM
HY-779	Female	45	Chronic carditis
HY-781	Female	44	IM
HY-783	Female	44	Chronic carditis
HY-785	Female	40	Chronic carditis
HY-787	Male	60	IM
HY-791	Male	23	Chronic carditis
HY-795	Male	57	Chronic carditis
HY-797	Female	55	Chronic carditis
HY-799	Female	49	Chronic carditis
HY-801	Male	62	Chronic carditis
HY-803	Female	49	Chronic carditis
HY-805	Female	45	Normal tissue
HY-806	Male	24	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-809	Male	28	Chronic carditis
HY-811	Female	53	Chronic carditis
HY-813	Female	38	Chronic carditis
HY-815	Male	58	IM
HY-817	Female	53	Chronic carditis
HY-819	Male	38	IM
HY-821	Male	46	Chronic carditis
HY-823	Female	35	Chronic carditis
HY-825	Male	41	Normal tissue
HY-827	Male	76	Normal tissue
HY-829	Male	39	Normal tissue
HY-831	Female	30	Normal tissue
HY-833	Male	50	Chronic carditis
HY-835	Male	38	Chronic carditis
HY-837	Female	41	Normal tissue
HY-839	Female	30	Chronic carditis
HY-841	Male	52	Normal tissue
HY-843	Male	42	Chronic carditis
HY-845	Female	67	Chronic carditis
HY-847	Male	58	Chronic carditis
HY-849	Female	43	Chronic carditis
HY-851	Male	70	Normal tissue
HY-853	Female	72	IM
HY-857	Female	44	Chronic carditis
HY-859	Male	34	Chronic carditis
HY-861	Male	40	Chronic carditis
HY-863	Male	41	Chronic carditis
HY-865	Female	38	Chronic carditis
HY-867	Female	49	Chronic carditis
HY-871	Male	25	Chronic carditis
HY-873	Male	40	Chronic carditis
HY-875	Female	39	IM
HY-877	Male	38	Chronic carditis
HY-879	Male	57	IM
HY-883	Male	33	Chronic carditis
HY-885	Male	53	Chronic carditis
HY-887	Female	53	IM
HY-889	Female	52	Chronic carditis
HY-891	Female	56	IM
HY-893	Male	73	Chronic carditis
HY-895	Male	52	Normal tissue
HY-897	Female	59	Chronic carditis
HY-901	Male	49	Chronic carditis
HY-903	Male	35	Chronic carditis
HY-905	Female	67	Chronic carditis
HY-907	Female	46	Chronic atrophic carditis
HY-909	Female	41	Chronic carditis
HY-911	Female	53	Chronic atrophic carditis
HY-915	Female	69	Chronic carditis
HY-917	Female	55	Normal tissue
HY-919	Male	33	Normal tissue
HY-921	Female	31	Chronic carditis
HY-923	Male	45	Chronic carditis
HY-925	Female	28	Chronic carditis
HY-929	Male	36	Normal tissue
HY-933	Male	56	IM
HY-935	Male	59	Chronic carditis
HY-937	Female	51	Chronic carditis
HY-939	Female	51	Normal tissue
HY-941	Male	50	Chronic carditis
HY-943	Female	40	Normal tissue
HY-945	Male	50	Chronic carditis
HY-947	Male	43	Chronic carditis
HY-949	Female	35	Chronic carditis
HY-951	Male	62	Chronic carditis
HY-955	Male	42	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
HY-957	Female	29	Chronic carditis
HY-961	Male	58	Chronic carditis
HY-963	Female	64	Chronic carditis
HY-967	Female	31	Chronic atrophic carditis
HY-969	Female	63	Chronic carditis
HY-971	Female	52	IM
HY-973	Female	47	Chronic carditis
HY-975	Male	55	Chronic carditis
HY-977	Male	48	Chronic carditis
HY-979	Female	58	Chronic carditis
HY-981	Female	46	Chronic carditis
HY-985	Male	36	Chronic atrophic carditis
HY-987	Female	43	Chronic carditis
HY-989	Female	33	Chronic carditis
HY-991	Female	57	Chronic carditis
HY-993	Female	61	Chronic carditis
HY-995	Female	50	Chronic carditis
HY-997	Female	49	Chronic carditis
HY-999	Female	51	Normal tissue
HY-1001	Female	20	Chronic carditis
HY-1003	Female	48	Chronic carditis
HY-1005	Male	55	Chronic carditis
HY-1007	Female	53	Normal tissue
HY-1009	Female	64	Chronic carditis
HY-1013	Male	56	IM
HY-1015	Male	58	IM
HY-1017	Male	67	Chronic carditis
HY-1021	Male	60	Chronic carditis
HY-1023	Female	69	Chronic atrophic carditis
S323	Female	53	Chronic carditis
S324	Female	64	IM
S325	Female	51	Chronic carditis
S326	Female	46	Chronic carditis
S327	Female	58	Chronic carditis
S328	Female	44	Chronic carditis
S329	Male	60	IM
S414	Male	34	Chronic carditis
S415	Female	53	Chronic carditis
S416	Female	20	Chronic carditis
S417	Female	60	Chronic carditis
S418	Female	58	IM
S419	Male	39	IM
S420	Male	46	IM
S421	Male	27	Chronic carditis
S422	Male	32	Chronic carditis
S423	Male	52	IM
S424	Male	47	IM
C1	Female	71	IM
C10	Male	36	Chronic atrophic carditis
C11	Male	54	IM
C12	Male	46	IM
C13	Male	58	Chronic carditis
C15	Female	25	Chronic carditis
C16	Female	70	Chronic atrophic carditis
C17	Female	59	Chronic carditis
C3	Male	63	IM
C34	Male	66	Chronic carditis
C36	Male	42	Chronic atrophic carditis
C37	Male	45	Chronic atrophic carditis
C38	Female	67	IM
C39	Female	66	Chronic carditis
C4	Female	68	Chronic carditis
C40	Male	62	Chronic carditis
C41	Female	33	Chronic carditis
C42	Female	62	Chronic carditis
C43	Male	42	Chronic atrophic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
C44	Male	62	Chronic atrophic carditis
C45	Male	67	IM
C47	Male	60	IM
C49	Male	54	IM
C5	Male	72	Chronic carditis
C50	Male	74	IM
C6	Male	63	Chronic carditis
C7	Male	66	IM
C8	Female	44	Chronic carditis
S1	Male	46	Chronic carditis
S10	Male	34	IM
S100	Male	62	IM
S101	Male	51	IM
S102	Female	57	Chronic carditis
S103	Female	54	Chronic carditis
S105	Male	42	Chronic carditis
S106	Female	55	Chronic atrophic carditis
S107	Female	42	Chronic carditis
S108	Male	57	Chronic carditis
S109	Male	52	Chronic carditis
S11	Female	55	Chronic carditis
S110	Female	43	Chronic carditis
S111	Female	41	Chronic carditis
S112	Male	54	Chronic carditis
S113	Female	55	Chronic carditis
S114	Male	61	Chronic carditis
S115	Male	46	Chronic carditis
S116	Female	37	Chronic carditis
S117	Female	50	Chronic carditis
S118	Male	51	Chronic carditis
S119	Female	50	Chronic carditis
S12	Female	31	Chronic carditis
S120	Female	63	Chronic carditis
S121	Female	61	Chronic carditis
S122	Female	56	Chronic carditis
S123	Female	50	Chronic carditis
S124	Female	47	Chronic carditis
S125	Male	56	Chronic atrophic carditis
S126	Male	54	Chronic atrophic carditis
S127	Male	40	Chronic carditis
S128	Male	44	Chronic carditis
S129	Male	39	Chronic carditis
S130	Female	37	Chronic carditis
S131	Male	53	Chronic carditis
S132	Male	35	Chronic carditis
S133	Male	51	Chronic carditis
S134	Male	48	Normal tissue
S135	Female	48	Chronic carditis
S136	Female	55	Chronic carditis
S137	Female	51	Chronic carditis
S138	Male	53	Chronic carditis
S139	Male	37	Chronic carditis
S140	Female	56	Chronic carditis
S141	Male	38	Chronic carditis
S143	Male	35	Chronic carditis
S144	Male	55	Chronic carditis
S145	Female	29	Chronic carditis
S146	Male	41	Chronic carditis
S147	Female	35	Chronic atrophic carditis
S148	Female	68	Chronic carditis
S149	Female	47	Chronic carditis
S150	Male	46	Chronic carditis
S151	Female	39	Chronic carditis
S152	Male	65	IM
S153	Female	54	Chronic carditis
S154	Female	32	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
S156	Female	51	Chronic atrophic carditis
S157	Female	56	Chronic atrophic carditis
S158	Female	53	Chronic carditis
S159	Female	54	Chronic atrophic carditis
S160	Female	40	Chronic carditis
S161	Female	59	Chronic atrophic carditis
S162	Female	59	Chronic atrophic carditis
S163	Female	33	Chronic atrophic carditis
S164	Female	40	Chronic carditis
S165	Female	53	Chronic carditis
S166	Female	48	Chronic carditis
S167	Female	45	Chronic carditis
S168	Male	66	IM
S169	Male	57	Chronic carditis
S170	Female	38	Chronic carditis
S171	Female	53	Chronic atrophic carditis
S172	Female	51	IM
S173	Male	50	Chronic carditis
S174	Female	46	Chronic atrophic carditis
S175	Female	55	Chronic carditis
S176	Female	51	Chronic carditis
S177	Female	42	Chronic carditis
S178	Male	54	Chronic atrophic carditis
S179	Female	53	Chronic carditis
S180	Female	33	Chronic carditis
S181	Male	43	Chronic atrophic carditis
S182	Female	64	Chronic atrophic carditis
S183	Male	52	Chronic carditis
S184	Male	62	IM
S185	Male	57	IM
S186	Female	40	Chronic carditis
S187	Male	50	Chronic carditis
S188	Female	40	Chronic atrophic carditis
S189	Male	64	Chronic carditis
S190	Female	36	Chronic carditis
S191	Male	41	Chronic carditis
S192	Male	45	Chronic carditis
S206	Male	51	Chronic carditis
S207	Female	53	Chronic carditis
S208	Male	49	Chronic carditis
S209	Female	49	Chronic carditis
S210	Male	54	Chronic carditis
S211	Female	56	Chronic carditis
S212	Female	57	Chronic carditis
S213	Female	54	Chronic carditis
S214	Female	48	Chronic carditis
S215	Male	43	Chronic carditis
S216	Male	37	Chronic carditis
S217	Female	57	Chronic carditis
S218	Male	61	Chronic carditis
S219	Female	48	Chronic carditis
S220	Female	63	IM
S221	Male	42	Chronic carditis
S222	Male	55	Chronic carditis
S223	Male	61	IM
S224	Male	53	Chronic carditis
S225	Male	44	Chronic carditis
S226	Male	32	Chronic carditis
S227	Female	64	IM
S229	Female	45	Chronic carditis
S231	Female	64	IM
S232	Male	72	IM
S233	Male	35	Chronic carditis
S234	Male	52	Chronic carditis
S235	Female	55	Chronic carditis
S236	Female	57	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
S237	Female	61	IM
S238	Male	45	Chronic carditis
S239	Male	46	Chronic carditis
S240	Female	56	Chronic carditis
S241	Female	51	Chronic carditis
S242	Female	53	Chronic carditis
S243	Female	49	Chronic carditis
S244	Female	52	IM
S245	Female	46	Chronic atrophic carditis
S246	Female	50	IM
S247	Female	50	Chronic carditis
S249	Male	62	Chronic carditis
S250	Male	64	Chronic carditis
S251	Male	56	Chronic carditis
S252	Female	56	Chronic carditis
S253	Male	52	Chronic carditis
S254	Female	33	Chronic carditis
S255	Female	40	Chronic carditis
S256	Female	56	Chronic carditis
S257	Male	53	Chronic carditis
S258	Female	61	Chronic carditis
S259	Female	41	Chronic carditis
S260	Female	43	Chronic carditis
S261	Female	40	Chronic carditis
S262	Male	50	Chronic carditis
S263	Female	58	Chronic carditis
S264	Male	42	Chronic carditis
S265	Female	50	IM
S266	Female	64	Chronic carditis
S267	Female	42	Chronic carditis
S268	Female	41	Chronic carditis
S269	Male	45	Chronic carditis
S271	Female	54	IM
S272	Female	48	IM
S273	Male	53	Chronic carditis
S275	Female	47	Chronic carditis
S276	Female	49	Chronic carditis
S277	Male	65	IM
S278	Female	51	Chronic carditis
S280	Female	63	Chronic carditis
S281	Female	55	Chronic carditis
S282	Female	58	Chronic carditis
S283	Male	40	Chronic carditis
S284	Male	56	Chronic carditis
S285	Male	41	Chronic carditis
S286	Female	49	Chronic carditis
S287	Female	55	Chronic carditis
S288	Male	64	Chronic carditis
S3	Female	52	Chronic carditis
S31	Male	31	Chronic atrophic carditis
S315	Male	45	Chronic carditis
S316	Female	56	Chronic carditis
S317	Male	44	Chronic carditis
S318	Male	57	Chronic carditis
S319	Female	31	Chronic carditis
S32	Female	30	Chronic carditis
S320	Male	62	Chronic carditis
S321	Male	49	Chronic carditis
S322	Male	71	Chronic carditis
S33	Female	51	Chronic carditis
S34	Male	68	IM
S36	Male	62	Chronic carditis
S37	Female	43	Chronic carditis
S38	Female	59	IM
S39	Female	60	Chronic carditis
S4	Female	28	Chronic carditis

Contd...

Supplementary Table S2: Contd...

Subject ID	Sex	Age	Pathological diagnosis
S40	Female	37	Chronic carditis
S42	Male	38	Chronic carditis
S43	Male	49	IM
S44	Male	56	Chronic carditis
S45	Female	66	IM
S47	Female	59	Chronic carditis
S48	Female	45	Chronic carditis
S49	Female	63	IM
S5	Female	29	Chronic carditis
S50	Female	52	Chronic carditis
S51	Male	54	Chronic atrophic carditis
S52	Male	31	Chronic atrophic carditis
S53	Female	62	Chronic carditis
S55	Female	44	Chronic carditis
S56	Male	45	Chronic carditis
S57	Male	63	IM
S58	Male	44	Chronic atrophic carditis
S59	Male	71	IM
S6	Female	22	Chronic carditis
S60	Female	48	Chronic atrophic carditis
S61	Female	33	IM
S62	Male	39	Chronic carditis
S63	Female	27	Chronic carditis
S64	Female	35	Chronic carditis
S65	Male	39	Chronic carditis
S67	Female	35	Chronic carditis
S68	Female	36	Chronic carditis
S69	Female	59	Chronic atrophic carditis
S7	Female	66	IM
S70	Female	37	IM
S71	Female	61	IM
S72	Male	49	Chronic atrophic carditis
S73	Female	45	Chronic atrophic carditis
S74	Male	44	IM
S75	Female	60	Chronic carditis
S76	Female	59	Chronic carditis
S77	Female	60	Chronic carditis
S78	Female	62	Chronic carditis
S79	Female	42	Chronic atrophic carditis
S8	Male	53	Chronic carditis
S80	Female	37	Chronic carditis
S81	Male	43	Chronic carditis
S82	Male	57	IM
S83	Male	68	Chronic carditis
S84	Female	35	Chronic carditis
S85	Male	51	Chronic carditis
S86	Female	27	Chronic carditis
S87	Female	53	Chronic atrophic carditis
S88	Female	40	Chronic carditis
S89	Female	55	IM
S9	Male	26	Normal tissue
S90	Female	36	Chronic carditis
S92	Female	54	Chronic atrophic carditis
S93	Male	78	Chronic carditis
S95	Female	61	Chronic carditis
S96	Male	40	Chronic carditis
S97	Female	65	Chronic carditis
S98	Female	55	Chronic carditis

Supplementary Table S3: DNA mehylation levels detected by pyrosequencing in different histological categories

Histological categories	DNA methylation
Normal	1.8
Normal	1.4
Normal	4.8
Normal	1.2
Normal	2.2
IM	43.2
IM	6.6
IM	21.8
IM	16.8
IM	2.4

Supplementary Table S4: Immunostaining score for *EYA4* in different histological categories

Histological categories	Immunostaining score
Normal	8
Normal	8
Normal	8
Normal	7.2
Normal	8
Normal	12
Normal	11.2
Normal	11.2
Normal	11.2
Normal	12
Normal	8.8
Normal	8
Normal	8
Normal	9.6
Normal	8
Normal	12
Normal	6.8
Normal	8
Normal	10.4
Normal	8
Normal	9.6
Normal	8
Normal	4.6
Normal	6.2
Normal	7
Normal	4.8
Normal	5.6
Normal	6.8
Normal	3.6
Normal	7.2
Normal	3
Normal	12
Normal	12
Normal	11.4
Normal	6
Normal	10.4
Normal	6.8
Normal	5.6
IM	2
IM	4
IM	0.25
IM	1.25
IM	5.2
IM	5.25
IM	7
IM	5.2
IM	6
IM	7.6
IM	5.6
IM	5.6
IM	3.6
IM	6
IM	6.2
IM	5.2
IM	6.4
IM	3.4
IM	6
IM	5.2
IM	2.2
IM	6.8
IM	5.6
IM	7.6
IM	6.4
IM	0
IM	0

Contd...

Supplementary Table S4: Contd...

Histological categories	Immunostaining score
IM	0.4
IM	0
IM	2.4
IM	0.4
IM	1.2
IM	0
IM	0.2
IM	4.8
IM	1.2
IM	5.6
IM	2.4
IM	2
IM	0.8
IM	2.8
IM	3.4
IM	1.2
IM	4
IM	4.4
IM	4.4
IM	5.2
IEN	0
IEN	0
IEN	0