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創薬基盤推進研究事業

胃粘膜に蓄積したエピジェネティック異常の定量による多発胃がん発生予測に関する前向き研究

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研究代表者　牛島　俊和

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研究要旨

がん患者の非がん部には、既にDNAメチル化異常が蓄積しており、その量が発がんリスクと相関することがある (エピジェネティックな発がんの素地)。胃がんでは、早期胃がんに対する内視鏡的粘膜下層剥離術 (ESD) 後に、異時性多発胃がんの発生が多く、多数の患者の高頻度の経過観察が負担となっている。そこで、本研究では、ESD 後の異時性多発胃がんの発生予測に非がん部胃粘膜生検組織におけるDNAメチル化量の測定が有用であるか否かを前向き研究により明らかにすることを目的とした。平成20年度に登録を開始し、平成22年度までにESD患者829例の登録を完了し、追跡を開始した。また、新規マーカー遺伝子として *miR-124a-3*、及び *NKX6-1* を開発し、単発胃がん131例、多発胃がん22例を用いた横断的メチル化解析により、オッズ比 6.1、3.6 で多発胃がん患者を同定できることを見出した。症例の登録・追跡は順調に進んでおり、世界初の「組織に蓄積したDNAメチル化異常を利用した疾患リスク診断」として成果が得られる可能性が高い。

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A．研究目的

早期胃がんに対する内視鏡的粘膜下層剥離術(ESD)後、高頻度に異時性多発胃がんが発生する (年率 2.5%)ことが問題となっている。高頻度の多発の原因として、胃がん患者の非がん部胃粘膜には、*H. pylori* 感染により誘発されたDNAメチル化異常が既に蓄積していること、*H. pylori* 感染陰性者では胃粘膜 DNA メチル化レベルは胃がんリスクと相関することを、申請者は見出した [Clin Cancer Res, 16:989, 2006; CEBP, 15:2317, 2006]。

エピジェネティックな発がんの素地は、胃がんのみならず、乳がん、大腸がん、食道がん、肝がん、腎がん等でも認められることが、複数の研究者によりこれまでに示された。したがって、エピジェネティックな発がんの素地の程度の測定により発がんリスク診断が実現できると予測される。また、このような方法が実用化されれば、非常に応用範囲が広い。そこで、本研究では、非がん部胃粘膜での DNA メチル化異常の定量により、ESD 後の異時性多発胃がんの発生が予測可能か否か、前向き研究により明らかにすることを、主たる目的とした。更に、臨床で利用しうる程度に高いオッズ比を示す新規マーカー遺伝子を分離することも目的とした。

B．研究方法

(1) 対象

早期胃がんに対して ESD を施行予定または施行後の症例を登録 (一次登録)し、ピロリ菌感染がある場合は除菌した。除菌後胃粘膜の DNA メチル化レベルが一定化した時点で、DNA メチル化レベルを測定し、追跡を開始した (二次登録、目標 1000 例)。二次登録した患者について 5 年間の予定で、追跡調査を行っている。

(2) 定量的 DNA メチル化解析

胃がんリスクとの相関が示されている *FLNc*、*THBD*、及び *miR-124a-3* のプロモーター領域 CpG アイランドについて、定量的メチル化特異的 PCR 法により、メチル化された DNA 分子と、メチル化されていない DNA 分子の絶対数を測定した。全 DNA 分子数に対するメチル化された DNA 分子数を求めることにより、胃粘膜の「DNA メチル化レベル」を算出、蓄積した DNA メチル化異常の量の指標とした。

(3) 新規マーカー遺伝子の探索

個別遺伝子の評価と CpG アイランドアレイを用いた網羅的解析の両方を行った。個別遺伝子として、近年重要性に注目が集まる microRNA (miRNA)遺伝子を対象に、リスクマーカーとしての有用性を検討した。

網羅的解析は、胃がん患者の非がん部胃粘膜と健常者の胃粘膜とを用いて抗メチル化 DNA 抗体による濃縮後、CpG アイランドアレイを用いた網羅的解析を行った。

（倫理面への配慮）

本研究は、各施設の倫理審査委員会による承認を得て実施している。DNA メチル化は、動物では生殖細胞系列で初期化されるものであり、子孫に対する影響がある遺伝情報の解析は、本研究には含まれない。

C．研究結果

(1) 症例の登録状況

国立がん研究センターでは平成 20 年 4 月 17 日に倫理審査委員会の承認を取得、専任者を配置し、登録を開始した。また、和歌山県立医科大学では平成 20 年 11 月 14 日、東京大学では 11 月 21 日に、各施設の倫理審査委員会の承認を取得、支援者を配置し、登録を開始した。平成 22 年 7 月 31 日までで、3 施設で ESD 患者 958 例 (国立がん研究センター中央病院 716 例、和歌山医大付属病院 96 例、東大病院 146 例)の症例登録 (一次登録)を終了した。外科切除追加症例、他臓器がん合併症例等を除外した後、829 例 (国立がん研究センター中央病院 633 例、和歌山医大付属病院 75 例、東大病院 121 例)を二次登録し、追跡を開始した。

当初の計画では、脱落症例の発生を考慮し、新規分担研究施設の追加 (静岡県立静岡がんセンター)を行い、合計 1,000 例の二次登録を目指す予定であった。しかしながら、静岡がんセンターでは倫理審査委員会での審査が、平成 22 年 5 月時点でも開始されず、症例登録を行わなかった。

本研究期間の終了時点で、平成 21 年度までに開発した *miR-124a-3*、及び、平成 22 年度に開発した *EMX1*、*NKX6-1* について、観察開始時の横断的メチル化解析を完了した。

(2) 新規マーカー遺伝子

本研究の開始時に得られていた *FLNc* や *THBD* に加え、平成 21 年度には microRNA (miRNA)遺伝子を対象にリスクマーカーとしての有用性を検討し、*miR-124a-3* 遺伝子を同定した。

平成 22 年度には、胃がん患者の非がん部胃粘膜と健常者の胃粘膜とを用いて DNA メチル化アレイ解析を行い、胃がん患者の非がん部胃粘膜で高度にメチル化される 7 遺伝子 (*EMX1*、*miR-663*、*NKX6-1*、*OTP*、*OPLAH*、*CYP1B1*、*NEFM*)を同定した。

単発胃がん 131 例、多発胃がん 22 例を用いて横断的メチル化解析を行い、上記の *miR-124a-3*、及び、*NKX6-1* はオッズ比 6.1、3.6 で多発胃がん患者を単発胃がん患者から区別できることを見出した。

(3) 測定方法の高度化

数%程度の DNA メチル化の正確な定量には定量 MSP 法が最適とされている。しかし、発がんリスク診断では、異なる時間・場所・実施者による高度の再現性が必要である。平成 21 年度までに、細胞株由来のコントロール DNA を用いて複数回、DNA メチル化を定量し、変動係数 0.03 とほぼ同一の値が得られる測定手順を確立した。

D．考察

本研究期間内に、3 施設において順調に症例の登録が完了し、829 例の追跡を開始した。除菌後多発胃がんの発生率は年間 1.1-2.5%と報告されており、ハザード比 2.5 のマーカーが得られた場合、有意差を得るために最小限必要な症例数、720 例を超えている。今後、脱落症例を可能な限り少なくし、追跡を継続する。

本研究期間の終了時点で、*miR-124a-3*、*EMX1*、及び *NKX6-1* について観察開始時の横断的メチル化解析を完了した。これにより、発がんリスク予測の基礎データを構築するのみならず、生活歴等との相関も解析可能となる。今後も、各種研究費の支援を求め、5 年間の追跡を完遂する予定である。

新規マーカー遺伝子として、本研究の開始時に得られていた *FLNc* や *THBD* に加え、平成 21 年度までに *miR-124a-3*、平成 22 年度には 7 遺伝子 (*EMX1*、*miR-663*、*NKX6-1*、*OTP*、*OPLAH*、*CYP1B1*、*NEFM*)を同定した。

単発胃がん 131 例、多発胃がん 22 例を用いた横断的メチル化解析により、*miR-124a-3*、及び、*NKX6-1* はオッズ比 6.1、3.6 で多発胃がん患者を単発胃がん患者から区別できることを見出した。この結果から、前向き研究においても有用性が示される可能性は十分に高いと考えている。単に有意差を示すのみでなく、臨床で利用しうる程度に高い感度と特異度を達

成するために、さらに高いオッズ比を示す新規マーカーの分離を目指す。

定量的MSP法は、平成21年度までに、細胞株由来のコントロールDNAを用いて複数回DNAメチル化を定量し、ほぼ同一の値が得られる測定手順を確立した。

E．結論

非がん部胃粘膜に蓄積したDNAメチル化異常定量による発がんリスク診断前向き試験のための症例の登録・追跡を予定通り開始した。世界初の「組織に蓄積したDNAメチル化異常を利用した疾患リスク診断」として成果が得られる可能性が高い。

F．研究発表

1．論文発表

本研究費に謝辞があるもの

1. Yoshida T, Yamashita S, Takamura-Enya T, Niwa T, Ando T, Enomoto S, Maekita T, Nakazawa K, Tatematsu M, Ichinose M and Ushijima T. Alu and Satalpha hypomethylation in *Helicobacter pylori*-infected gastric mucosae. **Int J Cancer**, 128: 33-39, 2011.
2. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, Oda I, Gotoda T and Ushijima T. Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication. **J Gastroenterol**, 45: 37-44, 2010.
3. Hosoya K, Yamashita S, Ando T, Nakajima T, Itoh F and Ushijima T. Adenomatous polyposis coli 1A is likely to be methylated as a passenger in human gastric carcinogenesis. **Cancer Lett**, 285: 182-189, 2009.
4. Oka D, Yamashita S, Tomioka T, Nakanishi Y, Kato H, Kaminishi M and Ushijima T. The presence of aberrant DNA methylation in noncancerous esophageal mucosae in association with smoking history: a target for risk diagnosis and prevention of esophageal cancers. **Cancer**, 115: 3412-3426, 2009.
5. Terada K, Okochi-Takada E, Akashi-Tanaka S, Miyamoto K, Taniyama K, Tsuda H, Asada K, Kaminishi M and Ushijima T. Association between frequent CpG island methylation and HER2 amplification in human breast cancers. **Carcinogenesis**, 30: 466-471, 2009.
6. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T and Ushijima T. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. **Int J Cancer**, 124: 2367-2374, 2009.
7. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K and Ushijima T. The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae. **Int J Cancer**, 124: 905-910, 2009.
8. Yanaoka K, Oka M, Mukoubayashi C, Yoshimura N, Enomoto S, Iguchi M, Magari H, Utsunomiya H, Tamai H, Arii K, Ohata H, Fujishiro M, Takeshita T, Mohara O and Ichinose M. Cancer high-risk subjects identified by serum pepsinogen tests –outcomes after 10-year follow-up in asymptomatic middle-aged males. **Cancer Epidemiol Biomarkers Prev**, 17: 838-845, 2008.
9. Yanaoka K, Oka M, Yoshimura N, Mukoubayashi C, Enomoto S, Iguchi M, Magari H, Utsunomiya H, Tamai H, Arii K, Yamamichi N, Fujishiro M, Takeshita T, Mohara O and Ichinose M. Risk of gastric cancer development in asymptomatic middle-aged Japanese subjects based on serum pepsinogen and Helicobacter pylori antibody levels. **Int J Cancer**, 123: 917-926, 2008.

本研究費に密接に関係するもの

1. Hur K, Niwa T, Toyoda T, Tsukamoto T, Tatematsu M, Yang HK and Ushijima T. Insufficient role of cell proliferation in aberrant DNA methylation induction, and involvement of specific types of inflammation. **Carcinogenesis**, 32: 35-41, 2011.
2. Ushijima T and Asada K. Aberrant DNA methylation in contrast with mutations. **Cancer Sci**, 101: 300-305, 2010.
3. Takeshima H and Ushijima T. Methylation destiny: Moira takes account of histones and RNA polymerase II. **Epigenetics**, 5: 89-95, 2010.
4. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M and Ushijima T. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. **Cancer Res**, 70: 1430-1440, 2010.
5. Takachi R, Inoue M, Shimazu T, Sasazuki S, Ishihara J, Sawada N, Yamaji T, Iwasaki M, Iso H, Tsubono Y and Tsugane S. Consumption of sodium and salted foods in relation to cancer and cardiovascular disease: the Japan Public Health Center-based Prospective Study. **Am J Clin Nutr**, 91: 456-464, 2010.
6. Sasazuki S, Inoue M, Sawada N, Iwasaki M, Shimazu T, Yamaji T and Tsugane S. Plasma levels of C-reactive protein and serum amyloid A

and gastric cancer in a nested case-control study: Japan Public Health Center-based prospective study. **Carcinogenesis**, 31: 712-718, 2010.

7. Inoue I, Mukoubayashi C, Yoshimura N, Niwa T, Deguchi H, Watanabe M, Enomoto S, Maekita T, Ueda K, Iguchi M, Yanaoka K, Tamai H, Arii K, Oka M, Fujishiro M, Takeshita T, Iwane M, Mohara O and Ichinose M. Elevated risk of colorectal adenoma with *Helicobacter pylori*-related chronic gastritis: a population-based case-control study. **Int J Cancer**, in press.
8. Yanaoka K, Oka M, Yoshimura N, Deguchi H, Mukoubayashi C, Enomoto S, Maekita T, Inoue I, Ueda K, Utsunomiya H, Iguchi M, Tamai H, Fujishiro M, Nakamura Y, Tsukamoto T, Inada K, Takeshita T and Ichinose M. Preventive effects of etodolac, a selective cyclooxygenase-2 inhibitor, on cancer development in extensive metaplastic gastritis, a *Helicobacter pylori*-negative precancerous lesion. **Int J Cancer**, 126: 1467-1473, 2010.
9. Enomoto S, Yanaoka K, Utsunomiya H, Niwa T, Inada K, Deguchi H, Ueda K, Mukoubayashi C, Inoue I, Maekita T, Nakazawa K, Iguchi M, Arii K, Tamai H, Yoshimura N, Fujishiro M, Oka M and Ichinose M. Inhibitory effects of Japanese apricot (Prunus mume Siebold et Zucc.; Ume) on *Helicobacter pylori*-related chronic gastritis. **Eur J Clin Nutr**, 64: 714-719, 2010.
10. Ono S, Fujishiro M, Kodashima S, Minatsuki C, Hirano K, Niimi K, Goto O, Yamamichi N and Koike K. Controversy on the management of anticoagulants and antiplatelet agents for scheduled endoscopy. **Dig Endosc**, 23: 1-4, 2011.
11. Goto O, Fujishiro M, Kodashima S, Ono S, Niimi K, Hirano K, Yamamichi N and Koike K. A second-look endoscopy after endoscopic submucosal dissection for gastric epithelial neoplasm may be unnecessary: a retrospective analysis of postendoscopic submucosal dissection bleeding. **Gastrointest Endosc**, 71: 241-248, 2010.
12. Goto O, Fujishiro M, Kodashima S, Ono S, Niimi K, Yamamichi N and Omata M. Feasibility of endoscopic submucosal dissection for patients with chronic renal failure on hemodialysis. **Dig Endosc**, 22: 45-48, 2010.
13. Niimi K, Fujishiro M, Kodashima S, Goto O, Ono S, Hirano K, Minatsuki C, Yamamichi N and Koike K. Long-term outcomes of endoscopic submucosal dissection for colorectal epithelial neoplasms. **Endoscopy**, 42: 723-729, 2010.
14. Takeshima H, Yamashita S, Shimazu T, Niwa T and Ushijima T. The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. **Genome Res**, 19: 1974-1982, 2009.
15. Yanaoka K, Oka M, Ohata H, Yoshimura N, Deguchi H, Mukoubayashi C, Enomoto S, Inoue I, Iguchi M, Maekita T, Ueda K, Utsunomiya H, Tamai H, Fujishiro M, Iwane M, Takeshita T, Mohara O and Ichinose M. Eradication of *Helicobacter pylori* prevents cancer development in subjects with mild gastric atrophy identified by serum pepsinogen levels. **Int J Cancer**, 125: 2697–2703, 2009.
16. Ono S, Fujishiro M, Goto O, Kodashima S and Omata M. En bloc resection of cardia cancer and lipoma with endoscopic submucosal dissection. **Dig Liver Dis**, 41: 237, 2009.
17. Goto O, Fujishiro M, Kodashima S, Ono S and Omata M. Is it possible to predict the procedural time of endoscopic submucosal dissection for early gastric cancer? **J Gastroenterol Hepatol**, 24: 379-383, 2009.
18. Ono S, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N and Omata M. Technical feasibility of endoscopic submucosal dissection for early gastric cancer in patients taking anti-coagulants or anti-platelet agents. **Dig Liver Dis**, 41: 725-728, 2009.
19. Goto O, Fujishiro M, Kodashima S, Ono S and Omata M. Outcomes of endoscopic submucosal dissection for early gastric cancer with special reference to validation for curability criteria. **Endoscopy**, 41: 118-122, 2009.
20. Miki K, Fujishiro M, Kodashima S and Yahagi N. Long-term results of gastric cancer screening using the serum pepsinogen test method among an asymptomatic middle-aged Japanese population. **Dig Endosc**, 21: 78-81, 2009.
21. Miki K and Fujishiro M. Cautious comparison between East and West is necessary in terms of the serum pepsinogen test. **Dig Endosc**, 21: 134-135, 2009.
22. Yamamichi N, Inada K, Furukawa C, Sakurai K, Tando T, Ishizaka A, Haraguchi T, Mizutani T, Fujishiro M, Shimomura R, Oka M, Ichinose M, Tsutsumi Y, Omata M and Iba H. Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate villin expression in gastrointestinal cells. **Exp Cell Res**, 315: 1779-1789, 2009.
23. Yamamichi N, Shimomura R, Inada K, Sakurai K, Haraguchi T, Ozaki Y, Fujita S, Mizutani T, Furukawa C, Fujishiro M, Ichinose M, Shiogama K, Tsutsumi Y, Omata M and Iba H. Locked nucleic

acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. **Clin Cancer Res**, 15: 4009-4016, 2009.
24. Fujishiro M, Oda I, Yamamoto Y, Akiyama J, Ishii N, Kakushima N, Fujiwara J, Morishita S, Kawachi H, Taniguchi H and Gotoda T. Multi-center survey regarding the management of anticoagulation and antiplatelet therapy for endoscopic procedures in Japan. **J Gastroenterol Hepatol**, 24: 214–218, 2009.
25. Goto O, Fujishiro M, Kodashima S, Kakushima N, Ono S, Yahagi N and Omata M. Feasibility of electrocautery snaring as the final step of endoscopic submucosal dissection for stomach epithelial neoplasms. **Dig Liver Dis**, 41: 26-30, 2009.
26. Nakajima T, Enomoto S and Ushijima T. DNA methylation: a marker for carcinogen exposure and cancer risk. **Environ Health Prev Med**, 13: 8-15, 2008.
27. Fujishiro M, Kodashima S, Takubo K, Kakushima N and Omata M. Detailed comparison between endo-cytoscopy and horizontal histology of an esophageal intraepithelial squamous cell carcinoma. **Dis Esophagus**, 21: 181-185, 2008.
28. Fujishiro M. Endoscopic submucosal dissection for gastric cancer. **Curr Treat Options Gastroenterol**, 11: 119-124, 2008.
29. Goto O, Fujishiro M, Kakushima N, Kodashima S, Ono S, Yamaguchi H, Nomura S, Kaminishi M and Omata M. Endoscopic submucosal dissection as a staging measure may not lead to worse prognosis in early gastric cancer patients with additional gastrectomy. **Dig Liver Dis**, 40: 293-297, 2008.
30. Ogura K, Okamoto M, Sugimoto T, Yahagi N, Fujishiro M, Kakushima N, Kodashima S, Kawabe T and Omata M. Efficacy and safety of endoscopic submucosal dissection for gastric cancer in patients with liver cirrhosis. **Endoscopy**, 40: 443-445, 2008.
31. Fujishiro M, Kodashima S, Goto O, Ono S, Muraki Y, Kakushima N and Omata M. Successful en bloc resection of superficial esophageal cancer treated by endoscopic submucosal dissection with a splash-needle (with video). **Endoscopy**, 40: E81-82, 2008.
32. Kakushima N and Fujishiro M. Endoscopic submucosal dissection for gastrointestinal neoplasms. **World J Gastroenterol**, 14: 2362-2367, 2008.
33. Otani K, Kitayama J, Kaisaki S, Ishigami H, Hidemura A, Fujishiro M, Omata M and Nagawa H. Early gastric cancer shows different associations with adipose tissue volume depending on histological type. **Gastric Cancer**, 11: 86-95, 2008.
34. Fujishiro M. Perspective on the practical indications of endoscopic submucosal dissection of gastrointestinal neoplasms. **World J Gastroenterol**, 14: 4289-4295, 2008.
35. Fujishiro M, Kodashima S, Goto O, Ono S, Muraki Y, Kakushima N and Omata M. Technical feasibility of endoscopic submucosal dissection for the gastrointestinal epithelial neoplasms with a splash-needle. **Surg Laparosc Endosc Percutan Tech**, 18: 592-597, 2008.
36. Fujishiro M, Kodashima S, Ono S, Goto O, Yamamichi N, Yahagi N, Kashimura K, Matsuura T, Iguchi M, Oka M, Ichinose M and Omata M. Submucosal injection of normal saline may prevent unexpected deep thermal injury of argon plasma coagulation in an in vivo porcine stomach. **Gut and Liver**, 2: 95-98, 2008.
37. Ono S, Fujishiro M, Kodashima S, Goto O and Omata M. Submerging endoscopic submucosal dissection leads to successful en bloc resection of colonic laterally spreading tumor with submucosal fat. **Gut and Liver**, 2: 209-212, 2008.

2．学会発表

1. Shigematsu Y, Niwa T, Ichinose M and Ushijima T. Current status of our research to identify CpG islands whose DNA methylation status is associated with the presence of lymph node metastases of gastric cancers. NSFC A3 Foresight Program 2010 Seminar. Beijin, October, 2010.
2. Ando T, Asada K, Yoshida T, Sugiyama T and Ushijima T. Involvement of microRNA silencing in the formation of field defect for gastric carcinogenesis. 18th United European Gastroenterology Week. Barcelona, October, 2010.
3. Takeshima H, Yamashita S, Shimazu T and Ushijima T. The presence of repetitive elements LINE and SINE confers susceptibility to aberrant DNA methylation induction. 5th Asian Epigenomics Meeting and A3 Symposium. Jeju, June, 2010.
4. Yamashita S, Gyobu K and Ushijima T. Identification of DNA methylation markers to predict metastasis of esophageal cancer by MeDIP-CpG island microarray analysis. 5th Asian Epigenomics Meeting and A3 Symposium. Jeju, June, 2010.
5. Takeshima H, Yamashita S, Niwa T, Shimazu T and Ushijima T. The presence of RNA polymerase II,

active or stalled, predicts epigenetic fate of promoter CpG islands. 101st Annual Meeting of American Association for Cancer Research. Washington DC, April, 2010.

6. Ushijima T. The Role of Epigenetics in the Development of Gastric Cancer. SIDDS 2009. Seoul, November, 2009.
7. Ushijima T. Epigenetic Field Defect for Gastric Cancers. Guangzhou First International Symposium of Cancer Epigenetics. Guangzhou, November, 2009.
8. Ushijima T. Induction of aberrant DNA methylation as a major carcinogenic pathway by *Helicobacter pylori* infection. 15th International Workshop on Campylobacter, Helicobacter, and related-organisms. Niigata, September, 2009.
9. Ushijima T. Induction of aberrant DNA methylation by inflammation caused by *H. pylori* infection. 29th Sapporo Cancer Seminar on "*Helicobacter pylori* and gastric cancer". Sapporo, July, 2009.
10. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T and Ushijima T. Involvement of microRNA silencing in the formation of field defect for gastric carcinogenesis. AACR 100th annual meeting. Colorado, April, 2009.
11. Ushijima T, Niwa T and Takeshima H. Epigenetic Field for Cancerization: As a Target for Risk Diagnosis, Cancer Prevention, and Marker for Molecular Epidemiology. Avison Symposium at Yonsei University. Seoul, January, 2009.
12. Goto O, Fujishiro M, Kodashima S, Ono S, Niimi K, Hirano K, Yamamichi N and Koike K. A second-look endoscopy after endoscopic submucosal dissection (ESD) for gastric epithelial neoplasm may be unnecessary: a retrospective analysis of post-ESD bleeding. GASTRO2009 UEGW/WCOG. London, November, 2009.
13. Goto O, Fujishiro M, Kodashima S, Ono S, Niimi K, Yamamichi N and Omata M. Usefulness of magnifying endoscopy with narrow band imaging (NBI) for endoscopic submucosal dissection (ESD) of early gastric cancer. The 8th Korea-Japan Joint Symposium on Gastrointestinal Endoscopy. Seoul, March, 2009.
14. Ushijima T, Niwa T and Ando T. Epigenetic field for gastric cancerization, and its induction by Helicobacter pylori infection. ISOBM 2008. Tokyo, October, 2008.
15. Nakajima T, Yamashita S, Maekita T, Niwa T and Ushijima T. The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae. 16th UEGW. Vienna, October, 2008.
16. Ushijima T, Nakajima T, Ando T and Niwa T. Epigenetic carcinogenic factors in the environment. 38th Annual Meeting of European Environmental Mutagen Society. Dubrovnik, September, 2008.
17. Ushijima T. Formation of epigenetic field for cancerization by environmental factors. The 2nd Shanghai Symposium of Epigenetics in Development and Diseases. Shanghai, July, 2008.
18. Ushijima T. Epigenetic field defect, and cancer risk diagnosis. AACR Special Conference "Cancer Epigenetics", Boston, May 2008.
19. Goto O, Fujishiro M, Kodashima S, Ono S and Omata M. Fasibiloity of electrocautery snaring as the final step of endoscopic submucosal dissection for stomach epithelial neoplasms. DDW. San Diego, May, 2008.
20. Goto O, Fujishiro M, Kodashima S, Ono S and Omata M. Is it possible to predict procedural time of endoscopic submucosal dissection for early gastric cancer? UEGW. Vienna, October, 2008.
21. Ono S, Fujishiro M, Kodashima S, Goto O and Omata M. Treatment outcomes of endoscopic submucosal dissection for early gastric cancer in patients with anti-coagulants or anti-platelet agents. UEGW. Vienna, October, 2008.
22. 山道信毅、稲田健一、一瀬雅夫、藤城光弘、小俣政男、伊庭英夫　クロマチン構造変換因子 SWI/SNF 複合体の胃癌における発現異常 触媒サブユニット Brm の発現欠失、組織型・分化マーカーとの関係　日本消化器関連学会週間 2009 年 10 月　京都
23. 中島　健、安藤孝将、牛島俊和　胃癌リスクマーカーとしての DNA メチル化異常－臨床応用に向けて　日本消化器関連学会週間　シンポジウム 6　消化器癌におけるエピジェネティクス 2009 年 10 月　京都
24. 前北隆雄、一瀬雅夫　DNA メチル化異常を用いた新規胃がんリスクマーカーの開発　日本消化器関連学会週間　シンポジウム 6　消化器癌におけるエピジェネティクス　2009 年 10 月　京都
25. 中島　健、山下　聡、牛島俊和　ヘリコバクター・ピロリ感染による特定遺伝子のメチル化と低転写の関与　第94回消化器病学会総会　2008 年 5 月　福岡
26. 中島　健、山下　聡、前北隆雄、丹羽　透、牛島俊和　ヘリコバクター・ピロリ感染による胃粘膜での DNA メチル化異常と標的遺伝子特異性　第 67 回日本癌学会学術総会　2008 年 10 月

名古屋

27. 前北隆雄、一瀬雅夫　胃炎進展・胃がんリスクと DNA メチル化異常　第７回消化器病フォーラム　2008 年 11 月

G．知的財産権の取得状況

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

別紙４

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoshida T, Ushijima T, et al.	Alu and Satalpha hypomethylation in *Helicobacter pylori*-infected gastric mucosae.	Int J Cancer	128	33-39	2011
Nakajima T, Ushijima T, et al.	Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication.	J Gastroenterol	45	37-44	2010
Hosoya K, Ushijima T, et al.	Adenomatous polyposis coli 1A is likely to be methylated as a passenger in human gastric carcinogenesis.	Cancer Lett	285	182-189	2009
Oka D, Ushijima T, et al.	The presence of aberrant DNA methylation in noncancerous esophageal mucosae in association with smoking history: a target for risk diagnosis and prevention of esophageal cancers.	Cancer	115	3412-3426	2009
Terada K, Ushijima T, et al.	Association between frequent CpG island methylation and HER2 amplification in human breast cancers.	Carcinogenesis	30	466-471	2009
Ando T, Ushijima T, et al.	DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect.	Int J Cancer	124	2367-2374	2009
Nakajima T, Ushijima T, et al.	The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae.	Int J Cancer	124	905-910	2009
Yanaoka K, Fujishiro M, Ichinose M, et al.	Cancer high-risk subjects identified by serum pepsinogen tests –outcomes after 10-year follow-up in asymptomatic middle-aged males.	Cancer Epidemiol Biomarkers Prev	17	838-845	2008
Yanaoka K, Fujishiro M, Ichinose M, et al.	Risk of gastric cancer development in asymptomatic middle-aged Japanese subjects based on serum pepsinogen and Helicobacter pylori antibody levels.	Int J Cancer	123	917-926	2008
Hur K, Ushijima T, et al.	Insufficient role of cell proliferation in aberrant DNA methylation induction, and involvement of specific types of inflammation.	Carcinogenesis	32	35-41	2011
Ushijima T and Asada K.	Aberrant DNA methylation in contrast with mutations.	Cancer Sci	101	300-305	2010
Takeshima H and Ushijima T.	Methylation destiny: Moira takes account of histones and RNA polymerase II.	Epigenetics	5	89-95	2010
Niwa T, Ichinose M, Ushijima T, et al.	Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells.	Cancer Res	70	1430-1440	2010
Takachi R, Shimazu T, et al.	Consumption of sodium and salted foods in relation to cancer and cardiovascular disease: the Japan Public Health Center-based Prospective Study.	Am J Clin Nutr	91	456-464	2010
Sasazuki S, Shimazu T, et al.	Plasma levels of C-reactive protein and serum amyloid A and gastric cancer in a nested case-control study: Japan Public Health Center-based prospective study.	Carcinogenesis	31	712-718	2010

Inoue I, Ichinose M, et al.	Elevated risk of colorectal adenoma with *Helicobacter pylori*-related chronic gastritis: a population-based case-control study.	Int J Cancer			in press
Yanaoka K, Ichinose M, et al.	Preventive effects of etodolac, a selective cyclooxygenase-2 inhibitor, on cancer development in extensive metaplastic gastritis, a *Helicobacter pylori*-negative precancerous lesion.	Int J Cancer	126	1467-1473	2010
Enomoto S, Ichinose M, et al.	Inhibitory effects of Japanese apricot (Prunus mume Siebold et Zucc.; Ume) on *Helicobacter pylori*-related chronic gastritis.	Eur J Clin Nutr	64	714-719	2010
Ono S, Yamamichi N, et al.	Controversy on the management of anticoagulants and antiplatelet agents for scheduled endoscopy.	Dig Endosc	23	1-4	2011
Goto O, Yamamichi N, et al.	A second-look endoscopy after endoscopic submucosal dissection for gastric epithelial neoplasm may be unnecessary: a retrospective analysis of postendoscopic submucosal dissection bleeding.	Gastrointest Endosc	71	241-248	2010
Goto O, Yamamichi N, et al.	Feasibility of endoscopic submucosal dissection for patients with chronic renal failure on hemodialysis.	Dig Endosc	22	45-48	2010
Niimi K, Yamamichi N, et al.	Long-term outcomes of endoscopic submucosal dissection for colorectal epithelial neoplasms.	Endoscopy	42	723-729	2010
Takeshima H, Shimazu T, Ushijima T, et al.	The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands.	Genome Res	19	1974-1982	2009
Yanaoka K, Fujishiro M, Ichinose M, et al.	Eradication of *Helicobacter pylori* prevents cancer development in subjects with mild gastric atrophy identified by serum pepsinogen levels.	Int J Cancer	125	2697–2703	2009
Ono S, Fujishiro M, et al.	En bloc resection of cardia cancer and lipoma with endoscopic submucosal dissection.	Dig Liver Dis	41	237	2009
Goto O, Fujishiro M, Kodashima S, Ono S and Omata M.	Is it possible to predict the procedural time of endoscopic submucosal dissection for early gastric cancer?	J Gastroenterol Hepatol	24	379-383	2009
Ono S, Fujishiro M, et al.	Technical feasibility of endoscopic submucosal dissection for early gastric cancer in patients taking anti-coagulants or anti-platelet agents.	Dig Liver Dis	41	725-728	2009
Goto O, Fujishiro M, et al.	Outcomes of endoscopic submucosal dissection for early gastric cancer with special reference to validation for curability criteria.	Endoscopy	41	118-122	2009
Miki K, Fujishiro M, et al.	Long-term results of gastric cancer screening using the serum pepsinogen test method among an asymptomatic middle-aged Japanese population.	Dig Endosc	21	78-81	2009
Miki K and Fujishiro M.	Cautious comparison between East and West is necessary in terms of the serum pepsinogen test.	Dig Endosc	21	134-135	2009
Yamamichi N, Fujishiro M, et al.	Cdx2 and the Brm-type SWI/SNF complex cooperatively regulate villin expression in gastrointestinal cells.	Exp Cell Res	315	1779-1789	2009
Yamamichi N, Fujishiro M, Ichinose M, et al.	Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development.	Clin Cancer Res	15	4009-4016	2009
Fujishiro M, et al.	Multi-center survey regarding the management of anticoagulation and antiplatelet therapy for endoscopic procedures in Japan.	J Gastroenterol Hepatol	24	214–218	2009
Goto O, Fujishiro M, et al.	Feasibility of electrocautery snaring as the final step of endoscopic submucosal dissection for stomach epithelial neoplasms.	Dig Liver Dis	41	26-30	2009
Nakajima T, Ushijima T, et al.	DNA methylation: a marker for carcinogen exposure and cancer risk.	Environ Health Prev Med	13	8-15	2008

Fujishiro M, et al.	Detailed comparison between endo-cytoscopy and horizontal histology of an esophageal intraepithelial squamous cell carcinoma.	Dis Esophagus	21	181-185	2008
Fujishiro M.	Endoscopic submucosal dissection for gastric cancer.	Curr Treat Options Gastroenterol	11	119-124	2008
Goto O, Fujishiro M, et al.	Endoscopic submucosal dissection as a staging measure may not lead to worse prognosis in early gastric cancer patients with additional gastrectomy.	Dig Liver Dis	40	293-297	2008
Ogura K, Fujishiro M, et al.	Efficacy and safety of endoscopic submucosal dissection for gastric cancer in patients with liver cirrhosis.	Endoscopy	40	443-445	2008
Fujishiro M, et al.	Successful en bloc resection of superficial esophageal cancer treated by endoscopic submucosal dissection with a splash-needle (with video).	Endoscopy	40	E81-82	2008
Kakushima N and Fujishiro M.	Endoscopic submucosal dissection for gastrointestinal neoplasms.	World J Gastroenterol	14	2362-2367	2008
Otani K, Fujishiro M, et al.	Early gastric cancer shows different associations with adipose tissue volume depending on histological type.	Gastric Cancer	11	86-95	2008
Fujishiro M.	Perspective on the practical indications of endoscopic submucosal dissection of gastrointestinal neoplasms.	World J Gastroenterol	14	4289-4295	2008
Fujishiro M, et al.	Technical feasibility of endoscopic submucosal dissection for the gastrointestinal epithelial neoplasms with a splash-needle.	Surg Laparosc Endosc Percutan Tech	18	592-597	2008
Fujishiro M, Ichinose M, et al.	Submucosal injection of normal saline may prevent unexpected deep thermal injury of argon plasma coagulation in an in vivo porcine stomach.	Gut and Liver	2	95-98	2008
Ono S, Fujishiro M, et al.	Submerging endoscopic submucosal dissection leads to successful en bloc resection of colonic laterally spreading tumor with submucosal fat.	Gut and Liver	2	209-212	2008

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Alu and Satα hypomethylation in *Helicobacter pylori*-infected gastric mucosae

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Global hypomethylation and regional hypermethylation are supposed to be hallmarks of cancer cells. During gastric carcinogenesis, in which *Helicobacter pylori* infection is causally involved, aberrant hypermethylation is already present in *H. pylori*-infected gastric mucosae. In contrast, little is known about global hypomethylation, which can be caused by hypomethylation of individual repetitive elements and other sequences. We, therefore, investigated hypomethylation of individual repetitive elements and the global 5-methylcytosine content in four groups of gastric mucosal samples that represented the time course of *H. pylori* infection and gastric carcinogenesis [gastric mucosae of *H. pylori*-negative healthy volunteers (G1, n = 34), *H. pylori*-positive healthy volunteers (G2, n = 42), *H. pylori*-positive gastric cancer patients (G3, n = 34) and *H. pylori*-negative gastric cancer patients (G4, n = 20)] and 52 primary gastric cancers. Major variants of Alu, LINE1 and Satα were identified, and their methylation levels were quantified by bisulfite pyrosequencing. Compared with G1, the Alu methylation level was decreased in G2, G3, G4 and cancers (89.2–97.1% of that in G1, $p < 0.05$). The Satα methylation level was decreased in G2 (91.6%, $p < 0.05$) and G3 (94.3%, $p = 0.08$) but not in G4 and cancers. The LINE1 methylation level was decreased only in cancers. The 5-methylcytosine content was at similar levels in G2, G3 and G4 and highly variable in cancers. These results showed that Alu and Satα hypomethylation is induced in gastric mucosae by *H. pylori* infection during gastric carcinogenesis, possibly in different target cells, and that global hypomethylation is not always present in human gastric cancers.

Carcinogenesis

Global hypomethylation and regional hypermethylation are supposed to be hallmarks of cancer cells.[1] Global hypomethylation, defined as the content of 5-methylcytosine in the genome,[2] is considered to be due to hypomethylation of repetitive elements, which are normally heavily methylated,[3] and other sequences. Global hypomethylation is known to cause chromatin decondensation that results in chromosomal instability and cancer development.[4–7] In addition, hypomethylation of repetitive elements is associated with its elevated transcription,[8] and that of normally methylated promoter CpG islands can lead to elevated expression of tumor antigens and possible oncogenes.[8–10] On the other hand, hypermethylation is observed in normally unmethylated promoter CpG islands and silences downstream genes, including tumor suppressor and other passenger genes.[11]

Hypermethylation of CpG islands can be present not only in cancers but also in noncancerous tissues.[12] Especially in gastric mucosae, aberrant DNA hypermethylation is induced by *Helicobacter pylori* infection, a major cause of gastric cancers.[13,14] The methylation levels of CpG islands are very low in gastric mucosae of *H. pylori*-negative healthy individuals (G1; incidence of gastric cancers = 0.03% per year or less[15]). They are very high in gastric mucosae of *H. pylori*-positive healthy individuals (G2; incidence = 0.14%[16]) and in noncancerous gastric mucosae of *H. pylori*-positive gastric cancer patients (G3; incidence of secondary gastric cancer = 4.1%[17]). They are high but lower than in G2 and G3 in noncancerous gastric mucosae of *H. pylori*-negative gastric cancer patients (G4; incidence = 6.2%[18]). *H. pylori* infection is known to disappear when severe gastric atrophy is induced as a result of chronic *H. pylori* infection,[19–22] and the four groups, G1–G4, are considered to represent the natural history of *H. pylori* infection. Methylation levels correlate with gastric cancer risk only in *H. pylori*-negative individuals,[13,14] suggesting that methylation levels in these individuals reflect the degree of epigenetic damage in stem cells.[23,24]

In contrast, global hypomethylation during gastric carcinogenesis remains unclear, not only when but also where in the genome it takes place. Major normally methylated repetitive elements consist of Alu, LINE1 and Satα, which

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constitute 10, 17 and 4% of the genome, respectively,[25–27] and collectively cover over 30% of the total CpG sites in the genome.[28,29] Alu and LINE1 belong to interspersed elements,[25] and Satα is a tandem repeat element[30,31] confined to the centromeres.[32] Hypomethylation of Satα is known to be induced by loss-of-function mutations of DNA methyltransferase 3B.[33] As a fundamental basis to understand gastric carcinogenesis, we have to clarify whether or not hypomethylation is present in *H. pylori*-infected gastric mucosae and, if present, which repetitive elements or global 5-methylcytosine content are mainly affected.

In this study, we aimed to clarify these issues. To this end, we first identified major variants of Alu, LINE1 and Satα, and then measured their methylation levels by bisulfite pyrosequencing of DNA from gastric mucosal samples of G1, G2, G3 and G4 and gastric cancer tissues.

Material and Methods

Tissue samples

Gastric mucosae were collected by endoscopic biopsy of the antral region in 34 *H. pylori*-negative (G1: 16 male and 18 female; average age = 51 years, range = 25–91 years) and 42 *H. pylori*-positive healthy volunteers (G2: 21 male and 21 female; average age = 57 years, range = 23–86 years; 19 with gastric atrophy and 23 without; nine with gastric ulcers, eight with duodenal ulcers and three with hyperplastic polyps) and noncancerous gastric mucosae from 34 *H. pylori*-positive (G3: 26 male and 8 female; average age = 68 years, range = 39–87 years; 23 early cancers and 11 advanced cancers) and 20 *H. pylori*-negative gastric cancer patients (G4: 15 male and 5 female; average age = 69 years, range = 38–84 years; 17 early cancers and three advanced cancers). Gastric cancer tissues were obtained from 52 gastric cancer patients (cancers: 52 male; average age = 60 years, range = 29–84 years) who underwent gastrectomy. Informed consents were obtained from all the individuals. Gastric mucosae, noncancerous gastric mucosae and cancer tissues were frozen in liquid nitrogen immediately after biopsy or resection and stored at −80°C until extraction of genomic DNA.

All cancer tissues were histologically diagnosed according to the Japanese classification of gastric carcinoma[34] and were classified according to the Lauren classification system (11 intestinal and 41 diffuse types).[35] *H. pylori* infection status was detected by use of a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan).

Cell lines and DNA extraction

Six gastric cancer cell lines, AGS, KATOIII, MKN28, MKN45, MKN74 and NUGC3, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44 and HSC57, were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan. High molecular weight DNA was extracted by the phenol/chloroform method.

Sequencing analysis of repetitive DNA elements

Genomic DNA of a human gastric cancer cell line (AGS) was amplified by PCR with the primers for the three repetitive DNA elements (Supporting Information Table 1): Alu (AluSp from the database of the Genetic Information Research Institute: http://www.girinst.org/), LINE1 (X58075) and Satα (M38468). The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI), and 12–41 clones for each repetitive DNA element were cycle sequenced. Sequencing was performed using a DYEnamic ET Terminator (GE Healthcare, Buckinghamshire, United Kingdom) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sodium bisulfite modification and bisulfite pyrosequencing

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described.[36] The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for bisulfite pyrosequencing. An annealing temperature that could amplify both unmethylated and methylated DNAs was determined by comparing amplification of DNA from peripheral leukocytes (mixture of unmethylated and methylated DNA) and DNA that was fully methylated by *Sss*I methylase (New England Biolabs, Beverly, MA) (Supporting Information Table 2). The PCR product was annealed to 0.2 μM pyrosequencing primers, and pyrosequencing was carried out using the PSQ 96 Pyrosequencing System (Qiagen, Valencia, CA). A methylation level was obtained using PSQ Assay Design software (Qiagen). Two CpG sites (ALU1 and ALU2) were measured for Alu, three for LINE1 (LINE1-1, LINE1-2 and LINE1-3) and one for Satα (SATα).

Analysis of the global 5-methylcytosine content

Genomic DNA (2.5 μg) was incubated with five units of DNase I (Sigma, St. Louis, MO) and 4 mM $MgCl_2$ at 37°C for 18 hr. The sample was further treated with three units of nuclease P1 in 10 mM NaAc (pH 5.2) and 50 μg/ml $ZnSO_4$ at 37°C for 7 hr and then with 2.5 units of *Escherichia coli* alkaline phosphatase in 0.1 M NH_4HCO_3 at 37°C for 16 hr. After purification, the samples were subjected to liquid chromatography equipped with a photodiode array detector and an electrospray ionization time-of-flight mass spectrometry (LCMS; LCT premier XE, Waters). Peaks of the four deoxyribonucleotides (2′-deoxyguansine, 2′-deoxyadenosine, 2′-deoxycytidine (dC) and thymidine) were monitored with UV 260 nm, whereas that of 5-methyl-2′-deoxycytidine (5mdC) was detected by a molecular ion of 242 [M+1], retention times of which were compared with that of the authentic sample. Global 5-methylcytosine content was quantified as the fraction of 5mdC quantity in the total 5mdC and dC quantity. The LCMS analysis was performed three times for each sample, and the mean coefficient of variation was confirmed to be less than 3%. Eight of the samples were also subjected to high-performance liquid chromatography (HPLC)-UV.

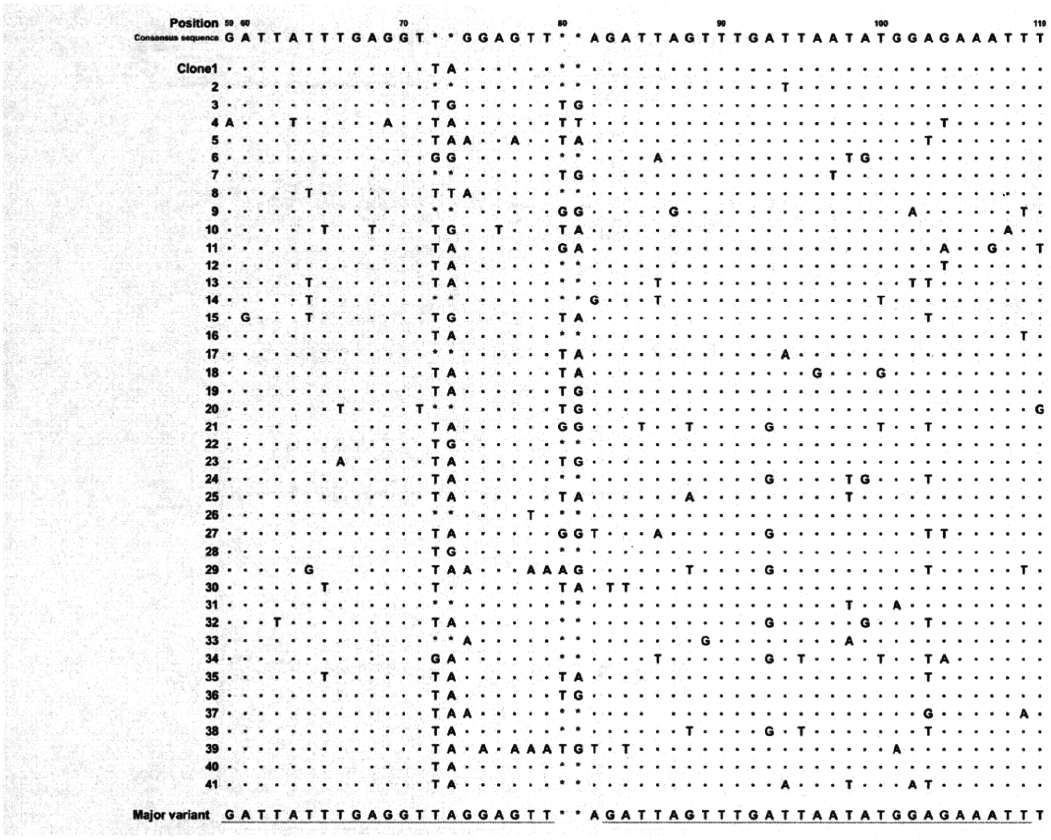

Figure 1. The most frequent Alu sequences after bisulfite conversion and location of CpG sites analyzed by pyrosequencing. The sequence obtained by virtual bisulfite conversion of a consensus sequence in the database is shown at the top. The 41 sequences are obtained by virtual bisulfite treatment of the sequences obtained. A dot shows no variation from the sequence at the top. Two consecutive asterisks show a CpG site. The most frequent sequence (shown at the bottom) was identified as the major variant. The sequences used for bisulfite pyrosequencing are underlined.

Statistical analysis

A difference in mean methylation levels or mean global 5-methylcytosine content was analyzed by the Welch *t* test. Correlation between the global 5-methylcytosine content by LCMS and that by HPLC-UV, correlation of methylation levels among repetitive DNA elements and correlation between age and methylation level were analyzed using Pearson's product-moment correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL), and the results were considered significant when p values less than 0.05 were obtained by a two-sided test.

Results

Identification of the major variants of individual repetitive DNA elements

A major variant was identified for each of the three repetitive DNA elements to measure methylation levels of as many repeat units as possible. Based on the consensus sequence in the database, each element was amplified by PCR with low stringency, and clones obtained were sequenced (Supporting Information Fig. 1). Since Alu and LINE1 were more variable than Satα, more clones were sequenced for Alu (41 clones) and LINE1 (19 clones) than for Satα (12 clones). After virtual conversion by bisulfite treatment of the sequences obtained, the most frequent sequence was identified as the major variant for each repetitive element (Supporting Information Fig. 2).

Primers for bisulfite pyrosequencing (Alu in Fig. 1; and Supporting Information Table 2) were designed based on the major variant, covering the most frequent sequences at the CpG site. The Alu methylation level was measured at two CpG sites, ALU1 (position +80 in the consensus sequence) and ALU2 (+197), which represented five and six, respectively, of the 41 sequences. The LINE1 methylation level was measured at three CpG sites, LINE1-1 (+138), LINE1-2 (+206) and LINE1-3 (+270), which represented seven, eight and 11, respectively, of the 19 sequences. The SATα methylation level was measured at one CpG site (+360) that was common to all the 12 sequences.

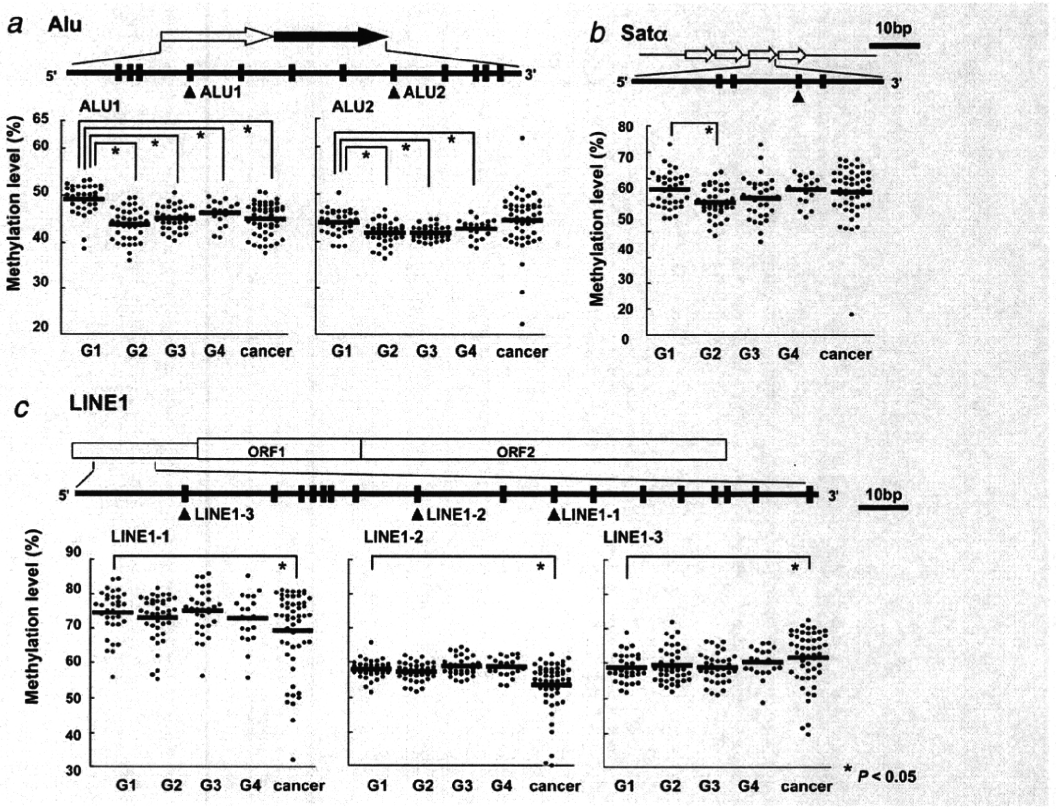

Figure 2. Methylation levels of the three repetitive DNA elements in gastric mucosae of G1-G4 and gastric cancers. Vertical ticks, individual CpG sites; arrowheads, locations of the measured CpG site. A horizontal line in a chart represents a mean methylation level for each group. (*a*) Distribution of methylation levels at two CpG sites of Alu. Compared with G1, the methylation level was decreased in G2, G3 and G4. The methylation level was decreased in cancers at ALU1 but not at ALU2. The top arrows show two duplicated arms of an Alu unit. (*b*) Distribution of methylation levels at one CpG site of Satα. Compared with G1, the methylation level was decreased in G2 and tended to be decreased in G3. The methylation level was not decreased in G4 and cancers. (*c*) Distribution of methylation levels at three CpG sites of LINE1. Compared with G1, the methylation level was not decreased in G2, G3 and G4 at LINE1-1, LINE1-2 and LINE1-3. The methylation level was decreased in cancers at LINE1-1 and LINE1-2 but was increased at LINE1-3. ORF: open reading frame.

The presence of Alu and Satα hypomethylation in *H. pylori*-infected gastric mucosae

The Alu methylation level was measured by bisulfite pyrosequencing in gastric mucosae (G1, G2, G3 and G4) and cancers (Fig. 2*a*; Supporting Information Table 3). In the normal control group (G1), the methylation level was 49.2 ± 3.2% (mean ± SD) at ALU1 and 44.1 ± 2.5% at ALU2. The mean methylation level at ALU1 was decreased in G2 (decreased to 89.2% of that in G1, $p < 0.05$), G3 (decreased to 91.9%, $p < 0.05$) and G4 (decreased to 94.1%, $p < 0.05$), and remained low in cancers (90.9% of that in G1, $p < 0.05$). Similarly, the mean methylation level at ALU2 was decreased in G2, G3 and G4 (decreased to 94.8, 95.0 and 97.1% of that in G1; $p < 0.05$, < 0.05 and < 0.05, respectively). However, a decrease was not observed in cancers (100.9% of that in G1, $p = 0.61$).

The methylation level of SATα was 55.9 ± 6.8% in G1 (Fig. 2*b*; Supporting Information Table 3). The mean methylation level was decreased in G2 (decreased to 91.6% of that in G1; $p < 0.05$) and had a tendency to be decreased in G3 (decreased to 94.3%, $p = 0.08$). However, in contrast with Alu, it was not decreased in G4 (100.0% of that in G1, $p = 0.98$) or cancers (98.2% of that in G1, $p = 0.63$).

The absence of LINE1 hypomethylation in *H. pylori*-infected gastric mucosae

The methylation levels at LINE1-1, LINE1-2 and LINE1-3 were 74.5 ± 6.6%, 58.0 ± 2.6% and 58.7 ± 3.9%, respectively, in G1 (Fig. 2*c*; Supporting Information Table 3). The mean methylation levels at LINE1-1, LINE1-2 and LINE1-3 were not decreased in G2, G3 or G4, respectively. The methylation levels at these CpG sites were highly variable in cancers, but the mean methylation levels at LINE1-1 and LINE1-2 were significantly decreased and that at LINE1-3 was significantly increased (LINE1-1, 93.2% of that in G1, $p < 0.05$; LINE1-2, 92.4% of that in G1, $p < 0.05$; LINE1-3, 105.1% of that in G1, $p < 0.05$).

No decrease of global 5-methylcytosine content in *H. pylori*-infected gastric mucosae and its high variability in gastric cancers

The global 5-methylcytosine content was measured by LCMS for five samples of G1, 17 of G2, 18 of G3, six of G4 and 27 cancer samples, because of the large amount of DNA necessary for the analysis. The global 5-methylcytosine content in G1 was 4.6 ± 0.8% and was not decreased in G2, G3 and G4 (100.3%, 92.5% and 100.7% of that in G1; $p = 0.96$, 0.31 and 0.94, respectively). In cancers, the global 5-methylcytosine content was highly variable (4.3 ± 1.4%), some showing no decrease and the others showing marked decrease (Fig. 3).

To confirm that the high variability observed was due to that of the 5-methylcytosine content in cancer cells and not due to high variability of the population of cancer cells in cancer tissues, the global 5-methylcytosine content was analyzed in gastric cancer cell lines and was again shown to have high variability (2.8–7.5%). The global 5-methylcytosine content obtained by the current LCMS method was in good accordance with that obtained by the conventional HPLC-UV method (n = 8, correlation coefficient = 0.95, $p < 0.05$).

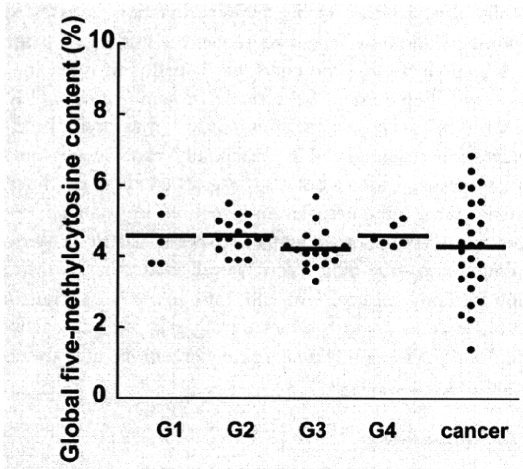

Figure 3. Distribution of global 5-methylcytosine content in G1-G4 and cancers. A horizontal line represents the mean global 5-methylcytosine content for each group. The global 5-methylcytosine content was at a similar level among G1, G2, G3 and G4. In cancers, the global 5-methylcytosine content was highly variable, some showing no decrease and others showing marked decease.

Correlation of hypomethylation among individual repetitive elements and the lack of correlation between hypomethylation and age

Correlations of the methylation levels among individual repetitive DNA elements were significant but weak (correlation coefficients = 0.15 - 0.55, $p < 0.05$; Supporting Information Table 4). Correlation between the global 5-methylcytosine content and the methylation level of individual repetitive elements was not significant, except for ALU2 (Supporting Information Table 5).

Hypermethylation of some CpG islands is known to be observed in an age-dependent manner.[37] Therefore, we analyzed association between hypomethylation of the three repetitive DNA elements and age within G1, whose members had no influence of *H. pylori* infection (Table 1). However, we observed no association. We also analyzed association between hypomethylation and gender, but again there was no association. Hypomethylation of LINE1 (LINE1-2 and LINE1-3) was marginally associated with intestinal-type histology, but that of Alu and Satα was not (Supporting Information Table 6).

Discussion

Our study showed that Alu and Satα hypomethylation was already present in *H. pylori*-infected gastric mucosae and that Alu, but not Satα, hypomethylation persisted after *H. pylori* infection discontinued and was also present in cancers. In contrast, LINE1 hypomethylation was present only in cancers. It was strongly indicated that hypomethylation is induced in gastric mucosae by *H. pylori* infection at Alu and Satα repetitive elements as an early event during gastric carcinogenesis whereas LINE1 hypomethylation is induced as a result of cellular transformation. To our knowledge, the presence of Alu and Satα hypomethylation in *H. pylori*-infected gastric mucosae during gastric carcinogenesis is shown here for the first time. Regarding hypomethylation of a specific repetitive element in noncancerous tissues that in liver, tissues exposed to hepatitis B virus[38] has been reported. Because hypomethylation is

Table 1. Lack of association between hypomethylation of the three repetitive DNA elements and age (or gender)

	ALU1	ALU2	LINE1–1	LINE1–2	LINE1–3	SATα
Age						
r	0.14	−0.30	−0.24	0.13	0.02	0.07
p	0.45	0.08	0.17	0.48	0.93	0.71
Gender						
Male (n = 16)	48.2 ± 4.1	44.0 ± 2.2	74.2 ± 7.5	58.5 ± 1.4	59.3 ± 4.1	58.4 ± 7.8
Female (n = 18)	50.1 ± 2.0	44.3 ± 2.8	74.8 ± 5.8	57.5 ± 3.3	58.3 ± 3.8	53.7 ± 5.0
p	0.12	0.74	0.79	0.25	0.47	0.05

r, correlation coefficient. To avoid confounding effects of *H. pylori* infection, the analyses were conducted in *H. pylori*-negative healthy volunteers (G1).

known to lead to genomic instability,[5] precise understanding of the timing of occurrence of hypomethylation is important as a fundamental basis to understand gastric carcinogenesis.

Alu and Satα hypomethylation showed different profiles in G1–G4, which are considered to represent the time course of gastric carcinogenesis.[19–22] Satα methylation levels were significantly decreased in G2 and tended to be decreased in G3, but not after disappearance of *H. pylori* infection (G4), whereas Alu hypomethylation persisted. The dynamics of Satα were reminiscent of hypermethylation of many protein-coding genes, which is potently induced by *H. pylori* infection and decreases after eradication of *H. pylori*.[13,39] As a mechanism for the different profiles of Alu and Satα, we can hypothesize that their hypomethylations are induced in different cell types. If methylation is induced in stem cells, it is expected to persist even after *H. pylori* infection discontinues whereas methylation induced in progenitor cells can disappear.[14] There is a possibility that Alu hypomethylation is relatively more easily induced in gastric stem cells than Satα hypomethylation. As a mechanism of how *H. pylori* infection induces hypomethylation of Alu and Satα, insufficiency of maintenance DNA methylation can be considered. It is known that expression levels of DNA methyltransferases are lower in gastric epithelial cells with *H. pylori* infection than those without in humans and gerbils.[40,41]

The finding here is important as a fundamental basis of gastric carcinogenesis associated with *H. pylori* infection. Alu is distributed throughout the genome,[40] and its hypomethylation could possibly lead to chromosomal instability as an early event during gastric carcinogenesis, as is known in mice.[4–6] From a clinical viewpoint, we initially expected that hypomethylation could be used as a cancer risk marker such as hypermethylation of CpG islands.[13,14] However, Alu hypomethylation had only low sensitivity and specificity in distinguishing healthy volunteers and gastric cancer patients among *H. pylori*-negative individuals (Fig. 2*a*), and use of hypomethylation as a risk marker was considered not to be realistic. Nevertheless, the early occurrence of Alu hypomethylation and its possible involvement in chromosomal instability suggested a possibility that suppression of hypomethylation induction can be used as a novel target of cancer prevention.

In cancers, LINE1 methylation level, which is often used as a surrogate for global hypomethylation,[42,43] was highly variable. The high variability of the LINE1 methylation level in gastric cancers was in good accordance with that reported in bladder and colon cancers.[44,45] It was considered that, because a cancer tissue is monoclonal, its methylation level reflects that of its single precursor cell and, thus, stochastically shows a low or high level. When methylation levels of the three CpG sites analyzed for LINE1 were compared in gastric cancers, methylation levels were decreased at two CpG sites while increased at another site. This suggested that there is a difference in susceptibility to hypomethylation among CpG sites. The difference of susceptibility could be related on the location of a CpG site within LINE1 because the CpG site whose methylation level was increased was located at an edge of LINE1.

The global 5-methylcytosine content in gastric cancers was also highly variable. In contrast, global hypomethylation is generally considered as one of the hallmarks of cancer cells.[1,3] In most studies, global hypomethylation is assessed by hypomethylation of repetitive sequences and not by the global 5-methylcytosine content. The 5-methylcytosine content is already reported to be variable in some cancers.[46] In gastric cancers, only our previous study[39] measured the 5-methylcytosine content, and it was highly variable not only in primary gastric cancers but also in gastric cancer cell lines. Therefore, global hypomethylation measured by the global 5-methylcytosine content was highly variable in gastric cancers.

In conclusion, our data strongly indicated that *H. pylori* infection potently induces Alu and Satα hypomethylation in gastric mucosae as an early event during gastric carcinogenesis and that global 5-methylcytosine content is not always decreased in gastric cancers.

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References

1. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007;128:683–92.
2. Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res* 1982;10:2709–21.
3. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006;7:21–33.
4. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature* 1998;395:89–93.
5. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003;300:455.
6. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. *Science* 2003; 300:489–92.
7. Holm TM, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout WM III, Jaenisch R. Global loss of imprinting leads to widespread tumorigenesis in adult mice. *Cancer Cell* 2005;8:275–85.
8. Howard G, Eiges R, Gaudet F, Jaenisch R, Eden A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* 2008;27:404–8.
9. Shen L, Kondo Y, Guo Y, Zhang J, Zhang L, Ahmed S, Shu J, Chen X, Waterland RA, Issa JP. Genome-wide profiling of DNA methylation reveals a class of normally methylated CpG island promoters. *PLoS Genet* 2007;3:2023–36.
10. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, Barrios M, Castillejo JA, Navarro G, Colomer D, Prosper F, Heiniger A, et al.

Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene* 2005;24:7213–23.
11. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer* 2005;5:223–31.
12. Ushijima T. Epigenetic field for cancerization. *J Biochem Mol Biol* 2007;40:142–50.
13. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, Arii K, Kaneda A, Tsukamoto T, Tatematsu M, Tamura G, Saito D, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res* 2006;12:989–95.
14. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T, Ushijima T. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. *Int J Cancer* 2009;124:2367–74.
15. Ohata H, Kitauchi S, Yoshimura N, Mugitani K, Iwane M, Nakamura H, Yoshikawa A, Yanaoka K, Arii K, Tamai H, Shimizu Y, Takeshita T, et al. Progression of chronic atrophic gastritis associated with *Helicobacter pylori* infection increases risk of gastric cancer. *Int J Cancer* 2004;109:138–43.
16. Lee KJ, Inoue M, Otani T, Iwasaki M, Sasazuki S, Tsugane S. Gastric cancer screening and subsequent risk of gastric cancer: a large-scale population-based cohort study, with a 13-year follow-up in Japan. *Int J Cancer* 2006;118:2315–21.
17. Fukase K, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, Terao S, Amagai K, Hayashi S, Asaka M. Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* 2008;372:392–7.
18. Yanaoka K, Oka M, Ohata H, Yoshimura N, Deguchi H, Mukoubayashi C, Enomoto S, Inoue I, Iguchi M, Maekita T, Ueda K, Utsunomiya H, et al. Eradication of *Helicobacter pylori* prevents cancer development in subjects with mild gastric atrophy identified by serum pepsinogen levels. *Int J Cancer* 2009;125:2697–703.
19. Karnes WE Jr, Samloff IM, Siurala M, Kekki M, Sipponen P, Kim SW, Walsh JH. Positive serum antibody and negative tissue staining for *Helicobacter pylori* in subjects with atrophic body gastritis. *Gastroenterology* 1991;101:167–74.
20. Sipponen P, Kosunen TU, Valle J, Riihela M, Seppala K. *Helicobacter pylori* infection and chronic gastritis in gastric cancer. *J Clin Pathol* 1992;45:319–23.
21. Forman D, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, Sitas F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991;302:1302–5.
22. Forman D, Webb P, Parsonnet J. *H pylori* and gastric cancer. *Lancet* 1994;343:243–4.
23. Ushijima T, Nakajima T, Maekita T. DNA methylation as a marker for the past and future. *J Gastroenterol* 2006;41:401–7.
24. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, Oda I, Gotoda T, Ushijima T. Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication. *J Gastroenterol* 2010;45:37–44.
25. Weiner AM. SINEs and LINEs: the art of biting the hand that feeds you. *Curr Opin Cell Biol* 2002;14:343–50.
26. Deininger PL, Moran JV, Batzer MA, Kazazian HH Jr. Mobile elements and mammalian genome evolution. *Curr Opin Genet Dev* 2003;13:651–8.
27. Lee C, Wevrick R, Fisher RB, Ferguson-Smith MA, Lin CC. Human centromeric DNAs. *Hum Genet* 1997;100:291–304.
28. Rollins RA, Haghighi F, Edwards JR, Das R, Zhang MQ, Ju J, Bestor TH. Large-scale structure of genomic methylation patterns. *Genome Res* 2006;16:157–63.
29. Schmid CW. Human Alu subfamilies and their methylation revealed by blot hybridization. *Nucleic Acids Res* 1991;19:5613–7.
30. Jordan IK, Rogozin IB, Glazko GV, Koonin EV. Origin of a substantial fraction of human regulatory sequences from transposable elements. *Trends Genet* 2003;19:68–72.
31. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, et al. Initial sequencing and analysis of the human genome. *Nature* 2001;409:860–921.
32. Jeanpierre M. Human satellites 2 and 3. *Ann Genet* 1994;37:163–71.
33. Schuffenhauer S, Bartsch O, Stumm M, Buchholz T, Petropoulou T, Kraft S, Belohradsky B, Hinkel GK, Meitinger T, Wegner RD. DNA, FISH and complementation studies in ICF syndrome: DNA hypomethylation of repetitive and single copy loci and evidence for a trans acting factor. *Hum Genet* 1995;96:562–71.
34. Japanese Gastric Cancer A. Japanese Classification of Gastric Carcinoma—2nd English Edition. *Gastric Cancer* 1998;1:10–24.
35. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965;64:31–49.
36. Kaneda A, Kaminishi M, Sugimura T, Ushijima T. Decreased expression of the seven ARP2/3 complex genes in human gastric cancers. *Cancer Lett* 2004;212:203–10.
37. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001;61:3573–7.
38. Lee HS, Kim BH, Cho NY, Yoo EJ, Choi M, Shin SH, Jang JJ, Suh KS, Kim YS, Kang GH. Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin Cancer Res* 2009;15:812–20.
39. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T. Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. *Cancer Sci* 2004;95:58–64.
40. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, Ichinose M, Tatematsu M, Ushijima T. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* 2010;70:1430–40.
41. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T. The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae. *Int J Cancer* 2009;124:905–10.
42. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 2005;33:6823–36.
43. Yamamoto E, Toyota M, Suzuki H, Kondo Y, Sanomura T, Murayama Y, Ohe-Toyota M, Maruyama R, Nojima M, Ashida M, Fujii K, Sasaki Y, et al. LINE-1 hypomethylation is associated with increased CpG island methylation in *Helicobacter pylori*-related enlarged-fold gastritis. *Cancer Epidemiol Biomarkers Prev* 2008;17:2555–64.
44. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang G, Yang AS. Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *Int J Cancer* 2009;125:723–9.
45. Schernhammer ES, Giovannucci E, Kawasaki T, Rosner B, Fuchs C, Ogino S. Dietary Folate, Alcohol, and B Vitamins in Relation to LINE-1 Hypomethylation in Colon Cancer. *Gut* 2010;59:794–9.
46. Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res* 1983;11:6883–94.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication

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Abstract
Background *Helicobacter pylori* (*HP*) infection potently induces aberrant DNA methylation in gastric mucosae, and its accumulation is associated with gastric cancer risk. Cross-sectional analysis of methylation levels (fraction of methylated DNA molecules) and temporal analysis of methylation incidence suggested that methylation levels decrease after *HP* infection discontinues. We aimed to demonstrate the decrease in methylation levels.
Methods Thirty-five patients with *HP* infection who had undergone curative endoscopic resection and 11 healthy volunteers were recruited. Methylation levels were quantified by real-time methylation-specific PCR. Histology was evaluated according to the updated Sydney System.
Results In the 20 patients with successful eradication, the *FLNc* methylation level, along with infiltration of inflammatory cells, decreased from 0.6 to 0.4% at 6 weeks ($P = 0.049$) and remained low at 1 year. The *THBD* methylation level (30.1%) remained high at 6 weeks, but decreased to 19.0% at 1 year ($P = 0.0032$). Nine healthy volunteers with successful eradication tended to show a decrease of both *FLNc* and *THBD* at 6 weeks. However, the methylation levels after the decrease were still higher than those of healthy individuals without *HP* infection. In the 15 patients with persistent infection, the methylation levels remained the same. Before eradication, the *THBD* methylation level correlated with the degree of inflammatory cell infiltration ($P < 0.05$).
Conclusions Methylation levels in gastric mucosae decreased to certain levels after *HP* eradication in profiles unique to individual markers. Involvement of chronic inflammation in methylation induction was suggested.

Keywords DNA methylation · Epigenetics · *Helicobacter pylori* · Eradication · Molecular epidemiology

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Introduction

Gastric cancer is one of the most common cancers in the world, especially in eastern Asia [1]. To reduce its morbidity and mortality by early detection and early treatment, identification of individuals with high risk is important. Also, endoscopic resection (ER) is becoming a standard treatment for early gastric cancer [2, 3], and this