厚生労働科学研究補助金（第３次対がん総合戦略研究事業）

分担研究報告書

DNA メチル化の分子機構の解析およびがんにおいて不活化される新規遺伝子の同定

研究分担者　豊田　実　札幌医科大学生化学講座　教授

研究要旨
本研究では、ゲノムワイドなメチル化解析を行い、癌における DNA メチル化の役割を明らかにすることを目的とする。本年度は胃癌および乳癌における mir-34b/c の異常メチル化異常を明らかにした。また、乳癌におけるエピジェネティックな異常の網羅的解析により、DNA メチル化により不活化される新規癌抑制遺伝子候補 NTN4 を同定した。さらに、ChIP シークエンス法による遺伝子転写開始点の網羅的解析を行い、従来のプロモーターアレイでは解析出来なかった、新たな DNA メチル化の標的遺伝子、non-coding RNA を同定した。

A．研究目的

本研究では、がんにおける DNA メチル化の網羅的解析により、発がんに関与する新規遺伝子を同定し、がん化における役割を明らかにすることを目的とする。H20 年度までの研究で、大腸癌における遺伝子変異と DNA メチル化異常の関連、mir-34b/c のエピジェネティックな異常に関して明らかにしてきた。本年度は胃癌における mir-34b/c の異常メチル化、乳癌におけるエピジェネティックな異常の網羅的解析、ChIP シークエンス法による遺伝子転写開始点の網羅的解析を行った。

B．研究方法

DNA メチル化に関しては、Bisulfite sequencing 法および Bisulfite-pyrosequence 法により、遺伝子発現に関しては、Microarray 法、RT-PCR 法および real-time PCR 法により解析した。ヒストン修飾に関しては、クロマチン免疫沈降(ChIP)後、次世代シークエンサーにより解析した。

（倫理面への配慮）

平成 17 年厚生労働省告示第 255 号「臨床研究に関する倫理指針」に従い、倫理面に充分配慮して研究を進める。手術材料の残余の組織などの研究利用につき、患者に説明し文書で同意を得、連結可能匿名化して解析を行い、患者のプライバシーを遵守し、札幌医科大学の倫理委員会の承認を得て使用する。

C．研究結果

これまで、癌において異常メチル化により不活化される microRNA のスクリーニングにより、microRNA-34b/c が大腸癌において異常メチル化により不活化されることを報告してきた(Toyota et al, Cancer Res, 2008)。本年度は、胃癌および乳癌における mir-34b/c の異常メチル化について検討した。mir-34b/c の異常メチル化は胃癌症例 68 例中 48 例(48%)に認められた。mir-34b/c を胃癌細胞株に導入することにより、細胞増殖を抑制すること、mir-34b/c が MET の発現を抑制することを明らかにした。さらに、RNA FISH 法により、正常胃組織における mir-34b/c の発現について in situ で解析し、mir-34b/c は胃粘膜の底部、固有腺にあたる領域に強く発現し、胃腺の上部では発現が低いことを明らかにした。これらの結果より、mir-34b/c の異常メチル化は、大腸癌だけでなく、多くの腫瘍の発生進展に関与し、癌診断や治療の標的として重要である可能性が示唆された。

乳癌の発生と進展における DNA メチル化異常の役割を明らかにするため、乳癌細胞株をメチル化阻害剤処理することにより、発現誘導される遺伝子群を cDNA マイクロアレイを用いて網羅的に解析した。その結果、288 遺伝子が脱メチル化により発現誘導されることを明らかにした。これら遺伝子中、DFNA5、SFRP1、DKK3、FKBP6、LOXL4、OSBPL3、NTN4、PGP9.5、PON1、TRIM50 の 10 遺伝子に関して、real-time PCR 法により、乳癌細胞株において、遺伝子発現が DNA メチル化阻害剤処理により誘導されることを確認した。また、bisulfite-pyrosequencing 法により、発現抑制がプロモーター領域の DNA メチル化により起こることを明らかにした。同定した新規メチル化の標的遺伝子 NTN4 に関して、癌抑制機能を有するか、コロニーフォーメーションアッセイにより検討したところ、NTN4 は細胞増殖を抑制する作用を有することが明らかとなった。NTN4 のメチル化は正常乳腺組織に比べ、乳癌組織で明らかに高値を示し(感度 98.6%, 95%CI: 92.6-100、特異度 76.5%,

95%CI: 50.1-93.2)、乳癌の検出のマーカーとして有用である可能性が示唆された(Breast Cancer Res Treat, in press)。

microRNA の前駆体は、しばしば成熟型 microRNA の数 10kB 上流から転写され、エピジェネティックな異常の解析において、転写開始点を正確に把握して、DNA メチル化解析を行うことが重要である。ゲノムワイドな転写開始点のマッピングを行うため、ヒストン H3 リジン 4 トリメチル化(H3K4me3)を認識する抗体で免疫沈降後、次世代シークエンサーを用いて転写開始点の網羅的解析を行った。その結果、DNMT1 および DNMT3B において、HCT116 細胞と比べ、25,000 程度の H3K4me3 のピークを認めた。これらのピークの中には、従来のプロモーターアレイでは同定出来なかった、新たな DNA メチル化の標的遺伝子、non-coding RNA が存在した。

D．考察

mir-34b/c の異常メチル化について胃癌および乳癌における検討を行った。癌の種類により、メチル化される microRNA には差があると考えられ、各種腫瘍の発生に関与する microRNA の同定が重要と考えられる。また、microRNA のメチル化解析には転写開始点を正確に決定することが重要である。ChIP シークエンス法を用いて、転写開始点のマッピングを行い、microRNA 前駆 RNA の新規の転写開始点を多数同定した。NTN4 は netrin ファミリーに属する遺伝子で血管新生の抑制に関与する。NTN4 の異常メチル化は乳癌臨床例において高率に認められ、血清中のメチル化検出により乳癌の早期発見や再発予測に利用出来る可能性が示唆された。

E．結論

胃癌および乳癌において mir-34b/c がエピジェネティックな異常により不活化されることを明らかにした。また、乳癌において DNA メチル化により不活化される新規癌抑制遺伝子候補 NTN4 を同定した。次世代シークエンサーを用いた H3K4me3 の網羅的解析は、microRNA など non-coding RNA の転写開始点の決定に重要な役割を果たすことが示唆された。

F．研究発表

1.論文発表

本研究費に謝辞があるもの

1. Suzuki, H, Igarashi, S, Nojima, M, Maruyama, R, Yamamoto, E, Kai, M, Akashi, H, Watanabe, Y, Yamamoto, H, Sasaki, Y, Itoh, F, Imai, K, Sugai, T, Shen, L, Issa, JPJ, Shinomura, Y, Tokino, T and Toyota M. IGFBP7 is p53 responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. **Carcinogenesis**, 31: 342-349, 2010.
2. Yamashita, M, Toyota, M, Suzuki, H, Nojima, M, Yamamoto, E, Kamimae, S, Watanabe, Y, Kai, M, Akashi, H, Maruyama, R, Sasaki, Y, Yamano, H, Sugai, T, Shinomura, Y, Imai, K, Tokino, T and Itoh, F. Epigenetic inactivation of interferon regulatory factor genes in gastric cancer. **Cancer Sci**, in press.
3. Fujikane, T, Nishikawa, N, Toyota, M, Suzuki, H, Nojima, M, Maruyama, R, Ashida, M, Ohe-Toyota, M, Kai, M, Nishidate, T, Sasaki, Y, Ohmura, T, Hirata, K and Tokino, T. Genomic Screening for genes upregulated by demethylation identified novel targets of epigenetic silencing in breast cancer. **Breast Cancer Res Treat**, in press.
4. Nojima, M, Maruyama, R, Yasui, H, Suzuki, H, Maruyama, Y, Tarasawa, I, Sasaki, Y, Asaoku, H, Sakai, H, Hayashi, T, Mori, M, Imai, K, Tokino, T, Ishida, T, Toyota, M and Shinomura, Y. Genomic Screening for Genes Silenced by DNA Methylation Revealed an Association between RASD1 Inactivation and Dexamethasone Resistance in Multiple Myeloma. **Clin Cancer Res**, 15: 4356-4364, 2009.
5. Toyota, M, Suzuki, H, Yamashita, T, Hirata, K, Shinomura, Y, Tokino, T and Imai, K. Cancer Epigenomics : Implications of DNA methylation in personalized therapy. **Cancer Sci**, 100: 787-791, 2009.
6. Toyota, M, Suzuki, H and Shinomura, Y. The Epigenome of colorectal cancer. **Current Colorectal Cancer Reports**, 5: 84-89, 2009.
7. Toyota, M, Suzuki, H, Yamamoto, E, Yamano, H, Imai, K and Shinomura, Y. Integrated analysis of genetic and epigenetic alterations in cancer. **Epigenomics**, 1: 291-299, 2009.

本研究費に密接に関係するもの

1. Watanabe, Y, Kim, HS, Castoro, RJ, Chyng, W, Estecio, MR, Kondo, K, Guo, Y, Ahmed, SS, Toyota, M, Itoh, F, Suk, KT, Cho, MY, Shen, L, Jelinek, J and Issa, JP. Sensitive and specific detection of early gastric cancer using DNA methylation analysis of gastric washing. **Gastroenterol**, 136: 2149-2158, 2009.
2. Samuel, MS, Suzuki, H, Buchert, M, Putoczki, TL, Tebbutt, NC, Lundgren-May, T, Christou, A, Inglese, M, Toyota, M, Heath, JK, Ward, RL, Waring, PM and Ernst, M. Elevated Dnmt3a activity promotes polyposis in ApcMin mice by relaxing extracellular restrains on Wnt signaling. **Gastroenterol**,137: 902-913, 2009.
3. Kashima, L, Toyota, M, Mita, H, Suzuki, H,

Idogawa, M, Ogi, K, Sasaki, Y and Tokino, T. CHFR, a potent tumor suppressor, downregulates interleukin-8 via inhibition of NF-kB. **Oncogene**, 28: 2643-2653, 2009.

4. Tomita, M, Toyota, M, Ishikawa, C, Nakazato, T, Okudaira, T, Matsuda, T, Uchihara, J, Taira, N, Ohshiro, K, Senba, M, Tanaka, Y, Ohshima, K, Saya, H, Tokino, T and Mori, N. Overexpression of Aurora a by loss of CHFR gene expresion increases the growth of HTLV-1-infected T cells through enhanced NF-kB activity. **Int J Cancer**, 124: 2607-2615, 2009.
5. Goto, Y, Shinjo, K, Kondo, Y, Shen, L, Toyota, M, Suzuki, H, Gao, Y, An, B, Fujii, M, Murakami, H, Osada, H, Taniguchi, T, Usami, N, Kondo, M, Hasegawa, Y, Shimokata, K, Matsuo, K, Hida, T, Fujimoto, N, Kishimoto, T, Issa, JP and Sekido, Y. Characteristic epigenetic profiles of malignant pleural mesothelioma. **Cancer Res**, 69: 9073-9082, 2009.
6. Suzuki, H, Toyota, M, Kondo, Y and Shinomura, Y. Inflammation-related aberrant patterns of DNA methylation: detection and role in epigenetic deregulation of cancer cell transcriptome. **Methods Mol Biol**, 512: 55-69, 2009.
7. Sugai, T, Habano, W, Jiao, YF, Toyota, M, Suzuki, H, Tsukahara, M, Koizuka, H, Akasaka, R, Koeda, K, Wakabayashi, G and Suzuki, K. Molecular analysis of single isolated glands in gastric cancers and their surrounding gastric intestinal metaplastic mucosa. **Oncol Rep**, 23: 25-33, 2010.
8. Yamashita, Y, Yuan, J, Suetake, I, Suzuki, H, Ishikawa, Y, Choi, YL, Ueno, T, Soda, M, Hamada, T, Haruta, H, Takada, S, Miyazaki, Y, Kiyoi, H, Ito, E, Naoe, T, Tomonaga, M, Toyota, M, Tajima, S, Iwama, A and Mano, H. Array-based genomic resequencing of human leukemia. **Oncogene**, in press.
9. Sugai, T, Habano, W, Endoh, M, Konishi, Y, Akasaka, R, Toyota, M, Yamano, H, Koeda, K, Wakabayashi, G and Suzuki, K. Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers. **Int J Cancer**, in press.

2.学会発表

1. Toyota, M. Colorectal cancer epigenetics. 37th Meeting of International Society for Oncology and Biomarkers. Amsterdam, September, 2009.
2. Toyota, M, Suzuki, H, Yamamoto, E, Kamimae, S, Nijima, M, Shinomura, Y and Imai, K. Colorectal cancer epigenome and clinical implication. First Guangzhou Symposium of Cancer Epigenetics and Kick-off Meeting of A3 Forsight Program and 973 Project. Guangzhou, November, 2009.
3. Toyota, M. CpG island methylator phenotype in colorectal cancer. The 14th Korean-Japan Cancer Research Workshop. Kanazawa, December, 2009.
4. Toyota, M, Suzuki, H, Akashi, H, Maruyama, R, Yamamoto, E, Nojima, M, Shinomura, Y and Imai, K. Genome wide analysis of transcription start sites of epigenetically silenced genes using next-generation sequencer. AACR Special Conference on Cancer Epigenetics. Puerto Rico, January, 2010.
5. 豊田　実、篠村恭久、今井浩三　エピゲノム解析による消化器癌の診断と発癌リスク予測　シンポジウム:第46回日本臨床分子医学会学術総会　2009年4月
6. 豊田　実　癌抑制遺伝子のDNAメチル化とRNAi　エピジェネティクス:ゲノムを管理し活用する戦略　千里ライフサイエンスセミナー　2009年4月
7. 豊田　実、今井浩三、篠村恭久　エピゲノム解析による炎症を介した胃発癌の分子機構解析と診断・発癌リスク予測への応用　第51回日本消化器病学会大会　2009年10月

G．知的財産権の出願・登録状況（予定を含む）

1. 特許取得

豊田 実、山本英一郎、神前正幸、鈴木 拓、山野泰穂. Method for detection of diseases using colonic mucosa. 出願先：米国、出願番号：61/238,106、平成21年8月28日出願.

2. 実用新案登録

該当無し

3. その他

該当無し

厚生労働科学研究補助金（第３次対がん総合戦略研究事業）

分担研究報告書

胃癌におけるエピジェネティック異常に基づいた高精度がん化予測診断

研究分担者　伊東文生　聖マリアンナ医科大学消化器・肝臓内科　教授

研究要旨

内視鏡診断技術の発達によりStageI胃癌症例が50%以上を占めるわが国において、胃癌治療もより低侵襲に行われることが求められている。裏返すと、今後わが国のハイリスク残胃症例が急増することは明らかであり、効果的なフォローアッププログラムを構築することが重要となる。現在、残胃に対するフォローは内視鏡医による経験に基づいた診断および、スポット生検による病理診断のみである。我々は、残胃を低侵襲かつ効率的に診断すべく方法を発明した。すなわち胃洗浄廃液による遺伝子診断である。胃の発癌機構にエピジェネティックな異常が大きく関与することを臨床診断へ応用し、胃洗浄廃液により回収された胃粘膜細胞由来のgDNAをMINT25遺伝子のメチル化異常を分子マーカーとすることにより、癌存在診断、予測診断ができる可能性を今までに報告してきた。さらに昨年度はMINT25以外の新たな候補遺伝子を網羅的に探索し、選出することに成功した。

これにより、本年度は術後残胃患者（開腹術、内視鏡治療を含む）の効果的なフォローアッププログラムを、より高感度に構築できる可能性がでてきた。

A．研究目的

通常内視鏡検査・治療時に発生する胃洗浄廃液からgDNAを抽出、DNAのメチル化を網羅的に調べることにより、胃がん診断に応用可能な既知のメチル化遺伝子だけでなく、新たな候補遺伝子の選出を可能とし、高感度な再発の予測診断プログラムを構築する。

B．研究方法

1．自主試験に対する倫理委員会の承諾後、低侵襲治療（腹腔鏡下部分胃切除術、開腹部分胃切除術、経内視鏡的粘膜切除術、経内視鏡的切開剥離術）予定胃がん患者へ説明。承諾の得られた100症例の治療前、治療後経過観察時（最低1回）の2点で胃洗浄サンプルを得る（計200サンプル）。検体管理センターを介して連結可能匿名化とした後、gDNAを抽出、バイサルファイト処理を行い、-20度にて管理する。

2．MCA-microarray法を用いることにより網羅的な遺伝子メチル化解析を行い、新たな候補遺伝子を選出する。さらに、すべてのデータを統計解析し再発予測、同定診断プログラムを構築する。

（倫理面への配慮）

研究に必要な検体は通常破棄される胃洗浄廃液であり、大学施設生命倫理委員会への承諾を行った後、患者様への十分なインフォームドコンセントのもと同意を得た症例にのみ実施されるものである。また、試料については、連結可能匿名化を行い、医療情報管理を厳重に行うこととする。

C．研究結果

我々はまず、既知のマーカー候補遺伝子に加えMCAM（MCA-Microarray法）により得られた18候補遺伝子のなかから最も有意差のあるSOX17遺伝子を選出。検証セット（40症例、80サンプル）を用いて既に選出したMINT25および新たに選出したSOX17の遺伝子メチル化レベルを解析した。

結果、内視鏡治療前後における2つの候補遺伝子（MINT25, SOX17）は治療後で有意にメチル化レベルの低下を示した。

D．考察

通常の内視鏡検査時に破棄している胃洗浄廃液を用い、エピジェネティックな異常を診断に応用することは非常に有用であることが証明され、さらに新たな候補遺伝子を追加することで診断能の向上に

つなげることが出来る可能性が示唆された。さらに今後は臨床への応用を目指すべく、多施設による臨床自主試験が早急に望まれる。

E．結論

胃洗浄液を用いたエピジェネティック診断は感度、特異度に優れ、かつ侵襲度の非常に低い検査法として有用である。

内視鏡治療前後の胃洗浄廃液を回収し、メチル化異常を調べることにより完全切除診断、異時再発予測診断に応用できる可能性が示唆された。

F．研究発表

1.論文発表

本研究費に謝辞があるもの

1. Suzuki, H, Igarashi, S, Nojima, M, Maruyama, R, Yamamoto, E, Kai, M, Akashi, H, Watanabe, Y, Yamamoto, H, Sasaki, Y, Itoh, F, Imai, K, Sugai, T, Shen, L, Issa, JP, Shinomura, Y, Tokino, T and Toyota, M. IGFBP7 is a p53-responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. **Carcinogenesis**, 31: 342-349, 2010.
2. Watanabe, Y, Kim, HS, Castoro, RJ, Chung, W, Estecio, MR, Kondo, K, Guo, Y, Ahmed, SS, Toyota, M, Itoh, F, Suk, KT, Cho, MY, Shen, L, Jelinek, J and Issa, JP. Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes. **Gastroenterology**, 136: 2149-2158, 2009.

2.学会発表

1. Watanabe, Y, Maehata, T, Toyota, M and Itoh, F. Usefulness of DNA Methylation Analysis Using Gastric Washes as a Treatment. AACR Cancer Epigenetics. PuertoRico, January, 2010.
2. 伊東文生　胃洗浄液を用いたメチル化解析の胃癌診断への応用　第 68 回日本癌学会学術集会 2009 年 10 月
3. 渡邊嘉行、山下真幸、伊東文生　新たな胃癌特異的メチル化遺伝子の網羅的解析　第 68 回日本癌学会学術集会　2009 年 10 月
4. 渡邊嘉行、前畑忠輝、伊東文生　胃洗浄液 MCAM による胃がん分子内視鏡診断 JDDW2009　2009 年 10 月

G．知的財産権の出願・登録状況（予定を含む）

1. 特許取得

Date of Filing: 15.05.07
Priority: JP/15.05.06/ JPA 2006134878
Title: Method for Detecting Disease-related Marker Using Gastric Mucosal Lavage Fluid
Designated States: AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SK TR

2. 実用新案登録

該当無し。

3. その他

該当無し。

厚生労働科学研究補助金（第３次対がん総合戦略研究事業）

分担研究報告書

膵胆道領域の癌診断における epigenetic molecular marker の有用性

研究分担者　松林宏行　静岡県立静岡がんセンター内視鏡科　医長

研究要旨
膵胆道系腫瘍の早期診断を目的として、そのハイリスク群或は前駆病変から発癌までの過程における各種遺伝子のメチル化を幅広く検索し、膵癌診断用・胆道癌診断用のメチル化マーカーprofile を作成する。また実際に、膵液・胆汁あるいは組織検体における DNA メチル化の検索が臨床的にハイリスク群の絞り込みや癌診断に寄与するかを検討する。

A．研究目的

膵液や胆汁を用いた DNA メチル化検索がヒト膵胆道癌のハイリスク群の絞り込みあるいは診断のためのマーカーになるかを検討する。

5.

B．研究方法

GeneChip を用いて、ヒト膵胆道癌細胞株に脱メチル化剤を付加した後でｘ５～x10倍の遺伝子発現の回復を認めた遺伝子群を絞り込む。これら遺伝子群および既知の膵胆道癌の発癌に関与する癌抑制遺伝子・癌関連遺伝子の promoter 領域のメチル化を切除癌検体に応用し、癌部で特異的にメチル化が認められるかを確認する。次に、膵液・胆汁検体に応用し、癌例に対して感度・特異度高く検出されるかを検索する。

（倫理面への配慮）

当院倫理審査委員会に研究計画書の承認を得て、患者様に膵液・胆汁の研究利用への同意を得た後に研究に用いる。

C．研究結果

現在、研究検体の集積過程である。

D．考察

現在、研究検体の集積過程である。

E．結論

現在、研究検体の集積過程である。

F．研究発表

1.論文発表

なし

2.学会発表

なし

G．知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

本年度中に胆汁中 DNA メチル化の有無の解析結果の中間あるいは最終結果を提示する予定。

厚生労働科学研究補助金（第３次対がん総合戦略研究事業）

分担研究報告書

DNA 低メチル化の消化管腫瘍発生への影響

研究分担者　山田泰広　京都大学 iPS 細胞研究センター　特定拠点教授

研究要旨
DNA メチル化を伴うがん抑制遺伝子のサイレンシングは、様々ながんで報告され、脱メチル化剤の臨床応用が注目されている。昨年度までの研究において、グローバルな DNA 低メチル化状態が、舌から大腸までの全消化管において腫瘍形成抑制的に作用することを確認した。本年度は、DNA 低メチル化による腫瘍抑制作用機序の検討を行った。家族性大腸腺腫症のモデルマウスである Apc Min マウス大腸発がんにおける DNA 低メチル化による大腸発がん抑制作用をモデルとし、腫瘍細胞に対して分子病理的な検討を行った。DNA 低メチル化によって大腸腫瘍発生は強く抑制されたが、その抑制作用は発がん後期に特に強く見られた。DNA メチル化レベルの低い大腸腫瘍細胞には、杯細胞への分化誘導が観察された。一方で神経内分泌細胞への分化には明らかな影響は確認されなかった。DNA 低メチル化状態にある腫瘍細胞では有意に細胞増殖能の低下が見られた。細胞増殖活性の低下は、杯細胞への分化を示す腫瘍細胞でより顕著に認められた。これらの結果は、DNA メチル化修飾が大腸腫瘍細胞の未分化状態および増殖状態の維持に働くことを示し、DNA メチル化状態の修飾は、消化管腫瘍発生の予防および治療に応用可能であることを示唆するものと考えられた。

A．研究目的

マウスモデルを用いて、グローバルな DNA 低メチル化による消化管発がん抑制作用のメカニズムを検索し、消化器がんにおける脱メチル化剤の臨床応用への可能性を検討する。

B．研究方法

腫瘍モデルとして、家族性大腸腺腫症のモデルマウスである Apc Min/+マウス大腸発がんモデルを用いた。DNA 低メチル化のモデルマウスとして、DNA methyltransferase である Dnmt1 の低発現マウスを用いた。Dnmt1 knock-out allele である c allele と低発現 allele である chip allele を Apc Min/+マウスと組み合わせることにより、Apc Min/+; Dnmt1 chip/c マウスを作出した。対照コントロールとして Apc Min/+; Dnmt1 chip/+マウスを用いた。Dnmt1 chip/c マウスおよび Dnmt1 chip/+マウスの DNA メチル化レベルは、腺管分離法により大腸上皮細胞のみを選択的に回収し、DNA を抽出後、メチル化レベルを比較検討した。通常高度にメチル化されていることが知られる pericentromeric repeat に対して、DNA メチル化感受性制限酵素を用いたサザンブロットにて比較検討した。

我々の過去の研究により（PNAS 2005）、Apc Min/+; Dnmt1 chip/c マウスには大腸腫瘍が全く発生しないことが分かっている。そこで Apc Min/+; Dnmt1 chip/c マウスに、強力な大腸発がんプロモーターである dextran sodium sulfate (DSS)を投与することで大腸腫瘍誘発を試みた。DSS(2%)は 7 週齢マウスに 1 週間飲水投与し、投与終了後 3 週後（短期）、および 13 週後（長期）に屠殺し、大腸を摘出した。大腸腫瘍およびその周囲組織は24時間緩衝ホルマリンにて固定し、大腸遠位側 3 cm を長軸方向に 3 等分し、パラフィンに包埋後、組織切片を作製、病理組織学的に検討した。

杯細胞への分化は PAS-AB 染色を用いて、神経内分泌細胞への分化はクロモグラニン A に対する免疫染色にて行った。また、細胞増殖活性の検討は Ki-67 免疫染色により、陽性細胞率の比較により検討した。β-catenin 染色により Apc 遺伝子が不活化した病変を同定した。

腫瘍性病変の定量は HE 染色およびβ-catenin 染色により行った。Longitudinal 組織切片上の粘膜長あたりに占める病変長を各組織切片上計測し、病変の広がりを検討した。

（倫理面への配慮）

全ての動物実験は、動物実験実施機関（岐阜大学）の動物実験委員会の承認を得た。動物愛護の精神に

配慮して実験を施行した。

C．研究結果

Pericentromeric repeat の DNA メチル化レベルの比較検討により、Dnmt1 chip/c マウス大腸粘膜において実際に DNA メチル化レベルが低下していることを確認した。

DSS 投与により、DNA メチル化レベルの低下した Apc Min/+; Dnmt1 chip/c マウスにも大腸腫瘍が発生した。DSS 投与後短期（3 週後）での腫瘍性病変の発生は Dnmt1 chip/c マウスの方が、Dnmt1 chip/+マウスに比して抑制される傾向が見られたが、統計学的に優位な差は見られなかった（Dnmt1 chip/c マウス 10.9 ± 2.14%, Dnmt1 chip/+ マウス 17.1 ± 2.79% p=0.0859）。一方で、DSS 投与後長期（13 週後）での腫瘍発生は、Dnmt1 chip/c マウスにおいて強く抑制された（Dnmt1 chip/c マウス 15.8±1.92%, Dnmt1 chip/+ マウス 30.2±3.30% p<0.001）。DNA 低メチル化における腫瘍抑制効果は腫瘍発生後期により顕著であることが示唆された。

Dnmt1 chip/c マウスおよび Dnmt1 chip/+マウスに発生した腫瘍の比較検討において、腫瘍腺管あたりの PAS-AB 陽性粘液細胞率（mean±s.d.）は Dnmt1 chip/c マウスにおいて有意に増加した（Dnmt1 chip/c マウス 9.45±0.878%, Dnmt1 chip/+マウス 6.95±0.789% p<0.04）。DNA メチル化レベル低下により、腫瘍細胞の分化が誘導されることが分かった。一方で神経内分泌細胞を示唆するクロモグラニン A 陽性細胞の出現率（mean±s.d.）は Dnmt1 chip/c マウスおよび Dnmt1 chip/+マウスにおいて有意な差は検出されなかった（Dnmt1 chip/c マウス 0.06±0.04%, Dnmt1 chip/+マウス 0.17±0.17% p=0.388）。DNA 低メチル化による腫瘍細胞での分化誘導の方向性は杯細胞選択的に起こることが示唆された。

Ki67 染色により Dnmt1 chip/c マウスおよび Dnmt1 chip/+マウスに発生した腫瘍細胞の細胞増殖活性を比較検討した。Dnmt1 chip/c マウスでは Ki67 陽性細胞率（mean±s.d.）が、Dnmt1 chip/+マウスに比して有意に減少していた（Dnmt1 chip/c マウス 31.8±2.31%, Dnmt1 chip/+マウス 44.5±1.62% p<0.001）。DNA 低メチル化による細胞増殖活性の抑制が示唆された。

連続切片を用いた、PAS-AB 染色、Ki67 染色による細胞分化と細胞増殖活性の比較により、杯細胞への分化を伴う腫瘍細胞において、Ki67 陽性細胞の著しい減少を確認した。腫瘍細胞での杯細胞分化誘導が、細胞増殖能低下の原因となった可能性が示唆された。

D．考察

DNA メチル化レベルの低下は、短期的な大腸腫瘍病変形成には影響が少ない一方で、長期的な腫瘤形成に対して強い抑制作用が見られた。昨年度までの研究結果により、DNA 低メチル化における、舌発がんおよび胃発がん抑制作用は、浸潤がん発生により強く見られることを明らかにした。今回の結果からも、DNA メチル化が発がんのプロモーション期およびプログレッション期に関与していることが示唆された。

抑制メカニズムには、細胞増殖活性の抑制、未分化細胞に対する分化促進作用の関与が示唆された。腫瘍において分化誘導が確認された腺管と、細胞増殖活性が低下した部位が一致し、腫瘍細胞での分化誘導が細胞増殖能抑制の原因として重要であることが示された。

E．結論

グローバルな DNA 低メチル化による大腸腫瘍抑制メカニズムは、腫瘍細胞での分化誘導とそれに伴う細胞増殖活性の抑制が関与していることが示唆された。

F．研究発表

1.論文発表

本研究費に謝辞があるもの

1. Baba, S, Yamada, Y, Hatano, Y, Miyazaki, Y, Mori, H, Shibata, T and Hara, A. Global DNA hypomethylation suppresses squamous carcinogenesis in the tongue and esophagus. **Cancer Sci**, 100: 1186-1191, 2009.
2. Yamada, Y, Aoki, H, Kunisada, T and Hara, A. Rest promotes the early differentiation of mouse ESCs but is not required for their maintenance. **Cell Stem Cell**, 6: 10-5, 2010.

2.学会発表

1. 山田泰広、波多野裕一郎、平田暁大、原　明 Role of epigenetic modifications in colon tumorigenesis. 第 68 回日本癌学会学術総会シンポジウム　2009 年 10 月
2. 山田泰広、波多野裕一郎、廣瀬善信、原　明 発がんにおけるエピジェネティック異常の意義 第 98 回日本病理学会総会ワークショップ 2009 年 5 月
3. Yamada, Y, Aoki, H, Kunisada, T and Hara, A. Tumor suppressor Rest promotes the early differentiation of embryonic stem cell. The 14th Korea–Japan Cancer Research Workshop "Translational cancer research and progress in colorectal cancer". Kanazawa, December, 2009.

G．知的財産権の出願・登録状況（予定を含む）

該当無し

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Kanai, Y and Arai, E.	DNA methylation status in chronic liver disease and hepatocellular carcinoma.	Wang, XW, Grisham, J and Thorgeirsson, S.	Molecular Genetics of Liver Neoplasia	Springer		in press	

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
Niwa, T, Ushijima, T, et al.	Inflammatory processes triggered by H. pylori infection cause aberrant DNA methylation in gastric epithelial cells.	Cancer Res	70	1430 -1440	2010
Takeshima, H and Ushijima, T.	Methylation Destiny: Moira Takes Account of Histones and RNA Polymerase II.	Epigenetics	5	89 -95	2010
Ushijima, T and Asada, K.	Aberrant DNA methylation in contrast with mutations.	Cancer Sci	101	300 -305	2010
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
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	2009
Takeshima, H, 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
Hosoya, K, Itoh, F, 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
Terada, K, Ushijima, T, et al.	Association between frequent CpG island methylation and HER2 amplification in human breast cancers.	Carcinogenesis	30	466 -471	2009
Yamashita, S, Ushijima, T, et al.	Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis.	DNA Res	16	275 -286	2009
Ushijima, T and Yamashita, S.	Methylation-sensitive representational difference analysis (MS-RDA).	Methods Mol Biol	507	117 -130	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
Arai, E and Kanai, Y.	DNA methylation profiles in precancerous tissue and cancers: Carcinogenetic risk estimation and prognostication based on DNA methylation status.	Epigenomics			in press

Kanai, Y.	Genome-wide DNA methylation profiles in precancerous conditions and cancers.	Cancer Sci	101	36 -45	2010
Nishiyama, N, Kanai, Y, et al.	Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage.	Cancer Sci	101	231 -240	2010
Okamura, J, Kanai, Y, et al.	Intraductal carcinosarcoma with a heterologous mesenchymal component originating in intraductal papillary-mucinous carcinoma (IPMC) of the pancreas with both carcinoma and osteosarcoma cells arising from IPMC cells.	J Clin Pathol	63	266 -269	2010
Arai, E, Kanai, Y, et al.	Genome-wide DNA methylation profiles in both precancerous conditions and clear cell renal cell carcinomas are correlated with malignant potential and patient outcome.	Carcinogenesis	30	214 -221	2009
Arai, E, Kanai, Y, et al.	Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma.	Int J Cancer	125	2854 -2862	2009
Sekine, S, Kanai, Y, et al.	Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis.	Gastroenterology	5	2304 -2315	2009
Sekine, S, Kanai, Y, et al.	Dicer is required for proper liver zonation.	J Pathol	219	365 -372	2009
Sekine, S, Kanai, Y, et al.	Esophageal melanomas harbor frequent NRAS mutations unlike melanomas of other mucosal sites.	Virchows Arch	454	513 -517	2009
Akishima-Fukasawa, Y, Kanai, Y, et al.	Prognostic significance of CXCL12 expression in patients with colorectal carcinoma.	Am J Clin Pathol	132	202 -210	2009
Yamanashi, T, Kanai, Y, et al.	Podoplanin expression identified in stromal fibroblasts as a favorable prognostic marker in patients with colorectal carcinoma.	Oncology	77	53 -62	2009
Yamashita, M, Toyota, M, Itoh, F, et al.	DNA methylation of interferon regulatory factors in gastric cancer anc noncancerous gastric mucosae.	Cancer Sci			in press
Fujikane, T, Toyota, M, et al.	Genomic screening for genes upregulated by demethylation identified novel targets of epigenetic silencing in breast cancer.	Breast Cancer Res Treat			in press
Suzuki, H, Itoh, F, Toyota M, et al.	IGFBP7 is p53 responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype.	Carcinogenesis	31	342 -349	2010
Nojima, M, Toyota, M, et al.	Genomic Screening for Genes Silenced by DNA Methylation Revealed an Association between RASD1 Inactivation and Dexamethasone Resistance in Multiple Myeloma.	Clin Cancer Res	15	4356 -4364	2009
Toyota, M, et al.	Cancer Epigenomics: Implications of DNA methylation in personalized therapy.	Cancer Sci	100	787 -791	2009
Toyota, M, et al.	The Epigenome of colorectal cancer.	Current Colorectal Cancer Reports	5	84 -89	2009
Toyota, M, et al.	Integrated analysis of genetic and epigenetic alterations in cancer.	Epigenomics		291 -299	2009

Watanabe, Y, Toyota, M, Itoh, F, et al.	Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes.	Gastroenterol	136	2149 -2158	2009
Yamada, Y, et al.	Rest promotes the early differentiation of mouse ESCs but is not required for their maintenance.	Cell Stem Cell	6	10 -15	2010
Baba, S, Yamada, Y, et al.	Global DNA hypomethylation suppresses squamous carcinogenesis in the tongue and esophagus.	Cancer Sci	100	1186 -1191	2009
Saito, Y, Kanai, Y, et al.	Chromatin remodeling at Alu repeats by epigenetic treatment activates silenced microRNA-512-5p with downregulation of Mcl-1 in human gastric cancer cells.	Oncogene	28	2738 -2744	2009
Yamashita, Y, Toyota, M, et al.	Array-based genomic resequencing of human leukemia.	Oncogene			in press
Sugai, T, Toyota, M, et al.	Molecular analysis of gastric differentiated-type intramucosal and submucosal cancers.	Int J Cancer			in press
Sugai, T, Toyota, M, et al.	Molecular analysis of single isolated glands in gastric cancers and their surrounding gastric intestinal metaplastic mucosa.	Oncol Rep	23	25 -33	2010
Samuel, MS, Toyota, M, et al.	Elevated Dnmt3a activity promotes polyposis in ApcMin mice by relaxing extracellular restrains on Wnt signaling.	Gastroenterol	137	902 -913	2009
Kashima, L, Toyota, M, et al.	CHFR, a potent tumor suppressor, downregulates interleukin-8 via inhibition of NF-kB.	Oncogene	28	2643 -2653	2009
Tomita, M, Toyota, M, et al.	Overexpression of Aurora a by loss of CHFR gene expresion increases the growth of HTLV-1-infected T cells through enhanced NF-kB activity.	Int J Cancer	124	2607 -2615	2009
Goto, Y, Toyota, M, et al.	Characteristic epigenetic profiles of malignant pleural mesothelioma.	Cancer Res	69	9073 -9082	2009
Suzuki, H, Toyota, M, et al.	Inflammation-related aberrant patterns of DNA methylation: detection and role in epigenetic deregulation of cancer cell transcriptome.	Methods Mol Biol	512	55 -69	2009

200924002A（2/3）

厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

ヒト多段階発がん過程におけるエピジェネティックな異常の網羅的解明と臨床応用に関する研究

平成２１年度　総括・分担研究報告書
-研究成果の刊行物・別刷①-

研究代表者　牛島　俊和

平成２２年（２０１０）年　４月

２／３冊

Molecular and Cellular Pathobiology

Cancer Research

Inflammatory Processes Triggered by *Helicobacter pylori* Infection Cause Aberrant DNA Methylation in Gastric Epithelial Cells

Tohru Niwa[1], Tetsuya Tsukamoto[2], Takeshi Toyoda[2], Akiko Mori[1], Harunari Tanaka[2], Takao Maekita[3], Masao Ichinose[3], Masae Tatematsu[2], and Toshikazu Ushijima[1]

Abstract

Altered patterns of DNA methylation associated with *Helicobacter pylori* (*HP*) infection of gastric epithelial cells are thought to contribute to gastric cancer risk. However, it is unclear whether this increased risk reflects an infection-associated inflammatory response or the infection itself. In this study, we sought to clarify mechanisms in a gerbil model of gastric cancer where we showed that *HP* infection is causally involved in induction of aberrant DNA methylation. By genome-wide screening, CpG islands that were aberrantly methylated in gerbil gastric cancer cell lines were isolated, and 10 islands were shown to be specifically methylated only in gastric mucosae infected with *HP*. By temporal analysis, methylation levels in gastric epithelial cells started to increase at 5 to 10 weeks after infection and reached high levels by 50 weeks. When *HP* was eradicated, methylation levels markedly decreased 10 and 20 weeks later, but they remained higher than those in gerbils that were not infected by *HP*. Expression levels of several inflammation-related genes (*CXCL2, IL-1β, NOS2*, and *TNF-α*) paralleled the temporal changes of methylation levels. Significantly suppressing inflammation with the immunosuppressive drug cyclosporin A did not affect colonization by *HP* but blocked the induction of altered DNA methylation. Our findings argue that DNA methylation alterations that occur in gastric mucosae after *HP* infection are composed of transient components and permanent components, and that it is the infection-associated inflammatory response, rather than *HP* itself, which is responsible for inducing the altered DNA methylation. *Cancer Res; 70(4); 1430–40. ©2010 AACR.*

Introduction

Aberrant DNA methylation of promoter CpG islands (CGI) is one of the major inactivating mechanisms of tumor-suppressor genes and is deeply involved in human carcinogenesis (1). Nevertheless, there is only limited information on its inducers and induction mechanisms. Chronic inflammation, known to promote certain types of cancers (2), is one of the possible inducers of aberrant methylation. The presence of aberrant methylation is frequently observed in noncancerous tissues of patients with inflammation-associated cancers, such as liver cancers, ulcerative colitis–associated colon cancers, and gastric cancers (3–7). However, a causal role of chronic inflammation in methylation induction remains to be established.

Authors' Affiliations: [1]Carcinogenesis Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan; [2]Division of Oncological Pathology, Aichi Cancer Center Research Institute, Chikusa, Nagoya, Japan; and [3]Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Toshikazu Ushijima, Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1-Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511; Fax: 81-3-5565-1753; E-mail: tushijim@ncc.go.jp.

doi: 10.1158/0008-5472.CAN-09-2755

In human gastric mucosae, the presence of *Helicobacter pylori* (*HP*) infection, a well-known inducer of chronic inflammation and gastric cancers (8, 9), is associated with high methylation levels or high incidences of methylation (5, 10–12). In addition, among individuals without *HP* infection, noncancerous gastric mucosae of gastric cancer patients have higher methylation levels than gastric mucosae of healthy individuals (5, 10). In addition, eradication of *HP* leads to a decreased incidence of *CDH1* (*E-cadherin*) promoter methylation (11, 13, 14). These findings suggest that *HP* infection induces aberrant methylation in gastric mucosae and indicate that levels of accumulated methylation are associated with gastric cancer risk. However, because infection experiments are impossible in humans, it needs to be clarified in animal models whether or not *HP* infection induces methylation and what mechanisms are involved.

HP infection in humans is best modeled in Mongolian gerbils (*Meriones unguiculatus*). As in man, *HP* infection induces severe inflammation in gerbil gastric mucosae and promotes gastric carcinogenesis induced by administration of *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N'*-nitrosoguanidine (15). The incidence of gastric cancers in gerbils depends on the duration of *HP* infection, and eradication of *HP* significantly reduces the incidence (16), as in man (17, 18). Thus, we can expect that the gerbil model is also useful in analyzing whether *HP* infection induces aberrant methylation and what mechanisms are involved *in vivo*. However, unfortunately,

little information is available for the gerbil genome, and the genetic and molecular analysis of this model has been hampered.

In this study, we aimed to show that *HP* infection is causally involved in induction of aberrant DNA methylation and to clarify a critical factor involved. For this, we first isolated CGIs that could be methylated in gerbil gastric cancers by a genome-wide screening method, methylation-sensitive representational difference analysis (MS-RDA). Using the CGIs isolated, we then showed that methylation was induced specifically in gerbils with *HP* infection and that inflammation induced by *HP* infection, not *HP* itself, was critically involved in methylation induction.

Materials and Methods

Cell lines. Two gerbil gastric cancer cell lines, MGC1 and MGC2, were established from a single gastric cancer induced in a gerbil by MNU and *HP* infection (19). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum on a type I collagen–coated dish (Asahi Techno Glass). Although we did not check the cross-contamination of cell lines biochemically or genetically just before use, they had the same morphology and growth rates as described previously (19).

Animal experiments and sample preparation. Male Mongolian gerbils (MGS/Sea) were purchased from Kyudo. To induce gastric cancers, male gerbils were administrated with 30 ppm of MNU (Sigma-Aldrich) in drinking water for a week at 7, 9, 11, 13, and 15 wk of age, and then inoculated with *HP* (ATCC 43504, American Type Culture Collection) by gavage at 17 wk of age (20). At 57 wk, gerbils were sacrificed and stomachs were resected. Because it was difficult to identify cancers macroscopically in gastric mucosae with severe hyperplasia, we dissected an area of gastric cancer tissue by an apparatus for laser microdissection (ASLMD, Leica Microsystems) after histologic confirmation. For temporal analysis of methylation levels, male gerbils were inoculated with *HP* (ATCC 43504) at 5 wk of age. Eradication therapy was done at 55 wk of age by administering amoxicillin, clarithromycin, and lansoprazole by gavage (20). Gerbils that had *HP* after the eradication therapy were excluded from analysis. As a vehicle control, 0.5% of carboxymethyl cellulose was given by gavage. To suppress gastritis, gerbils were administered with 250 μg/mL cyclosporin A (CsA; Neoral, Novartis Pharma) in drinking water for 20 wk. The stomach was resected and cut along the greater curvature. From the posterior wall of the pyloric region (pyloric antrum), which contains the pyloric glands, gastric epithelial cells (GEC) were isolated by the gland isolation technique (21). The anterior wall of the pyloric region was further cut into two pieces: one for RNA and DNA extraction from a sample with mucus and mucosal and submucosal layers and the other for histologic analysis. Whole blood was obtained from the inferior vena cava. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Human clinical samples. Human gastric mucosae were obtained by endoscopic biopsy from 10 *HP*-negative (five men and five women; average age 42.4 y, ranging from 29 to 56 y) and 10 *HP*-positive (four men and six women; average age 42.4 y, ranging from 23 to 53 y) healthy volunteers, whose *HP* status had been judged by a serum anti-*HP* antibody test (SBS). Gastric cancer samples were obtained from surgical specimens from 14 patients who underwent gastrectomy due to early gastric cancers (seven men and seven women; average age 65.9 y, ranging from 47 to 79 y). Sampling was conducted under the approval of Institutional Review Boards.

Nucleic acid extraction. From tissue sections, DNA was extracted by heating the dissected sections at 100°C for 20 min at pH 12, followed by phenol/chloroform extraction (22). From isolated glands, DNA was extracted by proteinase K digestion and the phenol/chloroform method. From the whole blood, DNA was extracted with a QuickGene DNA whole blood kit (Fujifilm). RNA was isolated with Isogen (Wako).

Quantitative PCR for gene expression analyses and* HP *detection. To analyze gene expression levels, cDNA was synthesized from 2 μg of DNase-treated RNA with an oligo-d $(T)_{12-18}$ primer. Real-time PCR using gene-specific primers (Supplementary Table S1) and SYBR Green Real-time PCR Master Mix (TOYOBO) was done, and the amplification curve of a sample was compared with curves of standard DNA samples with known copy numbers. Standard DNA samples were prepared by serial dilution of a PCR product or a plasmid containing a cloned PCR fragment after its quantification. Gene expression levels were normalized to that of *Gapdh*. To measure the amount of *HP*, real-time PCR using specific primers for the *jhpr3* gene of *HP* was carried out and normalized to the gerbil *Il4* gene (Supplementary Table S1).

Methylation-sensitive representational difference analysis. MS-RDA is a subtraction method that can identify differentially methylated loci between two genomes independent of genomic information (23) and was done using *Hpa*II or *Sac*II methylation-sensitive restriction enzyme as described previously (24). The final PCR product was cloned into pGEM T-Easy (Promega) and sequenced. If a DNA fragment had a CpG score ≥0.65 and G + C content ≥55%, the fragment was considered to be derived from a CGI. To identify homologous regions in mice and men, database searches were carried out at a GenBank web site.

Methylation analysis. Fully methylated and fully unmethylated controls were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs) and amplifying genomic DNA with ϕ29 DNA polymerase (GenomiPhi DNA Amplification Kit, GE Healthcare), respectively (25). One microgram of DNA digested with *Bam*HI was treated with sodium bisulfite and suspended in 80 μL of Tris-EDTA (TE) buffer as described previously (22). In the case of paraffin-embedded samples, DNA was treated with sodium bisulfite without *Bam*HI digestion and suspended in 20 μL of TE buffer. One microliter of aliquot was used as a template for methylation-specific PCR (MSP) and bisulfite sequencing. Conventional MSP and bisulfite sequencing were done with specific primer sets (Supplementary Table S2) as described previously (22). Quantitative MSP (qMSP) was done

Table 1. CGIs methylated in gerbil gastric cancer cell lines and *HP*-infected GECs

Clone name	GenBank accession no.	Genomic location deduced from analyses using human or mouse genome database	Nucleotide position in human or mouse sequences
HE6	AB429514	Exon 2 of *Ntrk2* gene*	16,449,514–16,449,840 bp in NT_023935.17 (human chr. 9)
HG2	AB429515	Exon 1 of *Gpr37* gene*	49,589,571–49,589,704 bp in NT_007933.14 (human chr. 7)
SA9	AB429516	Exon 1 of *Nol4* gene*	13,292,105–13,292,430 bp in NT_010966.13 (human chr. 18)
SB1	AB429517	Intergenic region between *Sp4* and *Sp8* genes*	20,698,454–20,698,697 bp in NT_007819.16 (human chr. 7)
SB5	AB429513	Not identified	Not identified
SC3	AB429518	Promoter region of *Rnf152* gene*	7,352,575–7,352,875 bp in NT_025028.13 (human chr. 18)
SD2	AB429519	Promoter region of *Nptx2* gene*	23,480,374–23,480,422 bp in NT_007933.14 (human chr. 7)
SE3	AB429520	Intron 1 of *Slc35f1* gene*	39,311,942–39,312,270 bp in NT_001838990.2 (human chr. 6)
SF12	AB429521	Intergenic region between *Cntn1* and *Pdzm4* genes	53,513,634–53,513,936 bp in NT_039621.7 (mouse chr. 15)
SH6	AB429522	Intergenic region between *Sox1* and *Loc729095* gene*	213,253–213,298 bp in NT_027140.6 (human chr. 13)

*Conserved regions identified in the human database.

by real-time PCR using primers specific to DNA molecules methylated at a locus and to a repeat sequence. Methylation levels were expressed as a percentage of the methylated reference, which was obtained as [(number of methylated fragments of a target CGI in sample) / (number of repeat sequences in sample)] / [(number of methylated fragments of a target CGI in *SssI*-treated DNA) / (number of repeat sequences in *SssI*-treated DNA)] × 100. As a repeat sequence, the B2 repeat was used for gerbil DNA (ref. 26; Supplementary Table S2 and Supplementary Fig. S1) and the *Alu* repeat was used for human DNA (27).

Statistical analysis. Statistical analyses were conducted with SPSS 13.0J (SPSS Japan, Inc.). To evaluate significant difference between two independent groups of sample data, the Mann-Whitney *U* test was used. Spearman's rank correlation coefficient (*r*) was used to measure correlation.

Results

Identification of CGIs specifically methylated by* HP *infection in GECs of Mongolian gerbils. To identify CGIs methylated in GECs of gerbils with *HP* infection, we adopted the strategy of a genome-wide screening in cancers and high-sensitivity analysis in GECs. The genome-wide screening was done by MS-RDA using a pool of two gerbil gastric cancer cell lines (MGC1 and MGC2) as the driver and GECs of noninfected gerbils as the tester. The final products of two series of MS-RDA using *Hpa*II and *Sac*II were cloned and 180 DNA fragments were sequenced. One hundred three of them were nonredundant, and 56 of them contained a sequence likely to have originated from a CGI. Due to the lack of information on the gerbil genome, we first analyzed the methylation statuses of CpG sites within the DNA fragments isolated using MSP. MSP primers were successfully designed for 27 of the 56 DNA fragments, and we analyzed the two gastric cancer cell lines, five samples of GECs from gerbils infected with *HP* for 50 weeks, and five samples of GECs from age-matched gerbils without infection. Ten (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6) of the 27 DNA fragments were methylated in the cell lines and GECs of *HP*-infected gerbils, but not in any GECs of gerbils without infection (Table 1; Fig. 1). The others were methylated only in the cell lines or methylated even in GECs of gerbils without infection.

Methylation in primary gastric cancers was analyzed for three randomly selected CGIs (HE6, SA9, and SB5). The methylation levels of HE6 and SB5 in eight primary cancer samples were similar to or below the mean methylation levels in GECs with *HP* infection for 50 weeks. In contrast, the methylation level of SA9 in most cancer samples was 2.1- to 19.1-fold higher than the mean methylation level in GECs from *HP*-infected gerbils (Supplementary Fig. S2). These results suggested that *HP* infection induced aberrant methylation of multiple but specific CGIs in gerbil GECs, and that methylation of some of these CGIs was associated with growth advantage of the cells.

Methylation of the corresponding CGIs in human samples. To examine whether or not these CGIs are also methylated in humans by *HP* infection, conserved regions of the

10 gerbil CGIs in humans were searched for. Eight of the 10 CGIs were found to be conserved between gerbils and humans (marked in Table 1), and five were located in the vicinities of genes (Fig. 2A, left). When the methylation levels of these five CGIs were quantified in human gastric mucosal biopsies, all of them had 5- to 48-fold higher methylation levels in individuals with *HP* infection (n = 10) than in those without (n = 10; right). Their methylation levels had close correlation with each other (correlation coefficient = 0.70–0.88; Supplementary Table S3).

The methylation levels of the five CGIs were then analyzed in primary human gastric cancers. *NTRK2, GPR37, NOL4,* and *NPTX2* had methylation in seven, three, four, and five, respectively, of 14 cancers analyzed, using the average methylation level of mucosal biopsies of *HP*-infected healthy volunteers as a threshold. There was no case with methylation of *RNF152* (Fig. 2B). These results showed that some of these CGIs were also methylated in human gastric cancers.

Induction of DNA methylation by chronic* HP *infection. Using the 10 CGIs isolated by MS-RDA, the effect of *HP* infection on methylation induction was analyzed at 1, 5, 10, and 50 weeks after *HP* infection (Fig. 3A). The methylation levels of HG2, SB5, and SD2 started to increase at 5 weeks after infection. At 10 weeks, CGIs other than SE3 and SH6 showed significantly higher methylation levels than those of the noninfected gerbils (3.2- to 85.0-fold). At 50 weeks, all the CGIs showed significantly higher methylation levels (14.3- to 215-fold; Fig. 3B; Supplementary Fig. S3). These results suggested that chronic *HP* infection, not acute *HP* infection, was responsible for methylation induction.

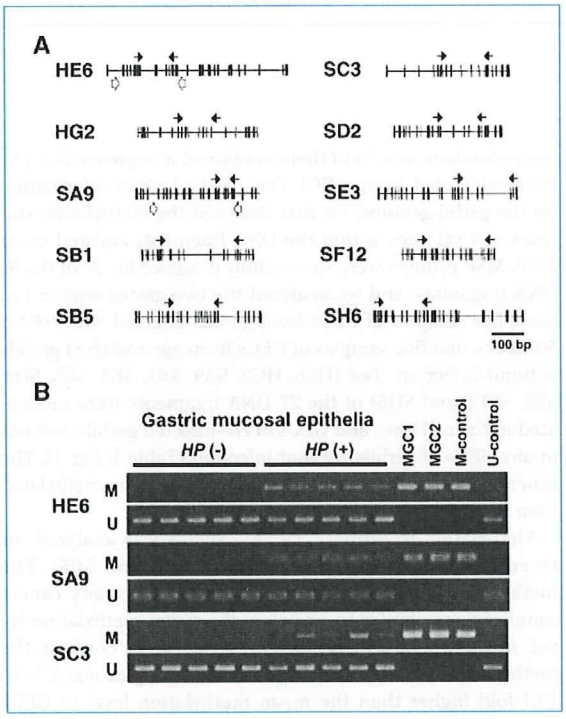

Figure 1. Isolation of CGIs that were aberrantly methylated in gerbil gastric cancers and GECs. A, a CpG map of the fragment isolated by MS-RDA. Vertical lines, individual CpG sites; arrows, positions of MSP primers; open arrows (HE6 and SA9), positions of bisulfite sequencing primers. B, representative results of MSP analyses in GECs from gerbils with and without *HP* infection for 50 wk and gastric cancer cell lines. M, MSP using a primer pair specific to methylated DNA; U, MSP using a primer pair specific to unmethylated DNA; M-control, genomic DNA treated with *SssI* methylase; U-control, DNA amplified with GenomiPhi.

The presence of dense methylation (methylation of a majority of CpG sites on a single DNA molecule) was confirmed by bisulfite sequencing of HE6 and SA9 in GECs of two gerbils with *HP* infection and two without. Densely methylated DNA molecules were detected only in *HP*-infected gerbils (Fig. 3C). The vast majority of DNA molecules were either largely unmethylated or largely methylated, and the fraction of methylated DNA molecules was in accordance with methylation levels measured by qMSP. The methylation levels of the 10 CGIs closely correlated with each other (average correlation coefficient = 0.87; range 0.70–0.95; Fig. 3D; Supplementary Table S4).

Decrease in methylation levels after* HP *eradication. *HP* was eradicated at 50 weeks after infection, and the methylation levels of the 10 CGIs were measured in GECs of the gerbils before and 1, 10, and 20 weeks after the eradication (Fig. 3A). Complete absence of *HP* was confirmed by PCR of *HP* genomic DNA (Fig. 4C). At 1 week after eradication, no decrease in methylation was observed (Fig. 3B; Supplementary Fig. S3). At 10 weeks after eradication, in contrast, the methylation levels of the 10 CGIs decreased to 9% to 32% of those before the eradication (significant for 9 of the 10 CGIs, except for SH6). An additional 10 weeks (20 weeks after eradication) did not lead to a further decrease in methylation levels. Importantly, the methylation levels after the decrease due to eradication were still significantly ($P < 0.01$ for two CGIs, and $P < 0.05$ for seven CGIs) higher than those in gerbils without any *HP* infection in their life.

Close association between methylation induction and inflammation, and not* HP *itself. *HP* infection is known to induce severe inflammation in gastric mucosae in gerbils, as in humans. Histologic analysis revealed that infiltration of polymorphonuclear cells and mononuclear cells started at 5 to 10 weeks after *HP* infection, and it became severe at 50 weeks (Fig. 4A; Supplementary Fig. S4). After eradication, a decrease in infiltration was not clear at 1 week, but was marked by 10 and 20 weeks (Fig. 4A). These histologic findings were paralleled by expression of inflammatory cell markers [*Cd3g, Cd14, Ela2,* and *Ms4a1* (*Cd20*) for T cell, macrophage, neutrophil, and B cell, respectively] in gastric tissues containing both mucosal and submucosal layers (Fig. 4B). Although *Ms4a1* expression decreased after eradication, gerbils without eradication (continuous infection) also showed a similar decrease, indicating that the decrease in *Ms4a1* expression (B-cell infiltration) was independent of *HP* eradication.

To explore the components of inflammation associated with methylation induction, the expression of inflammation-related genes [*Cox2, Cxcl2* (*MIP-2*), *Ifng, Il1b, Il2, Il4,*

Il6, Il7, Nos2 (*iNos*), and *Tnf* (*Tnf-α*)] was also quantified (Fig. 4B). A marked increase after *HP* infection and a decrease after eradication were observed for *Cxcl2, Il1b, Nos2*, and *Tnf*, paralleling inflammatory cell markers (Fig. 4B). The *Cox2, Ifng, Il2, Il4*, and *Il6* expression did not parallel the methylation levels after *HP* eradication, and the *Il7* expression showed a paradoxical increase compared with the group of continuous infection (Fig. 4B). Regarding the amount of *HP* in gastric mucosae, it had no association with methylation levels (Fig. 4C).

There remained a possibility that inflammatory cells had methylation of the CGIs analyzed, and that their contamination into GECs led to an apparent increase in methylation levels. To exclude this possibility, we analyzed the methylation levels of the 10 CGIs in DNA extracted from the whole blood of *HP*-infected gerbils. With the exception of SB1 and SB5, which showed relatively high methylation levels in the blood, 8 of the 10 CGIs showed almost no methylation (Supplementary Fig. S5). This excluded the possibility that methylation detected in the GECs was due to methylation in inflammatory cells contaminating the GECs.

Suppression of methylation induction by suppression of inflammation. To conclude that inflammation is indispensable for methylation induction, we suppressed *HP*-induced inflammation by administration of CsA, which blocks T-cell activation through inhibition of the calcineurin signal

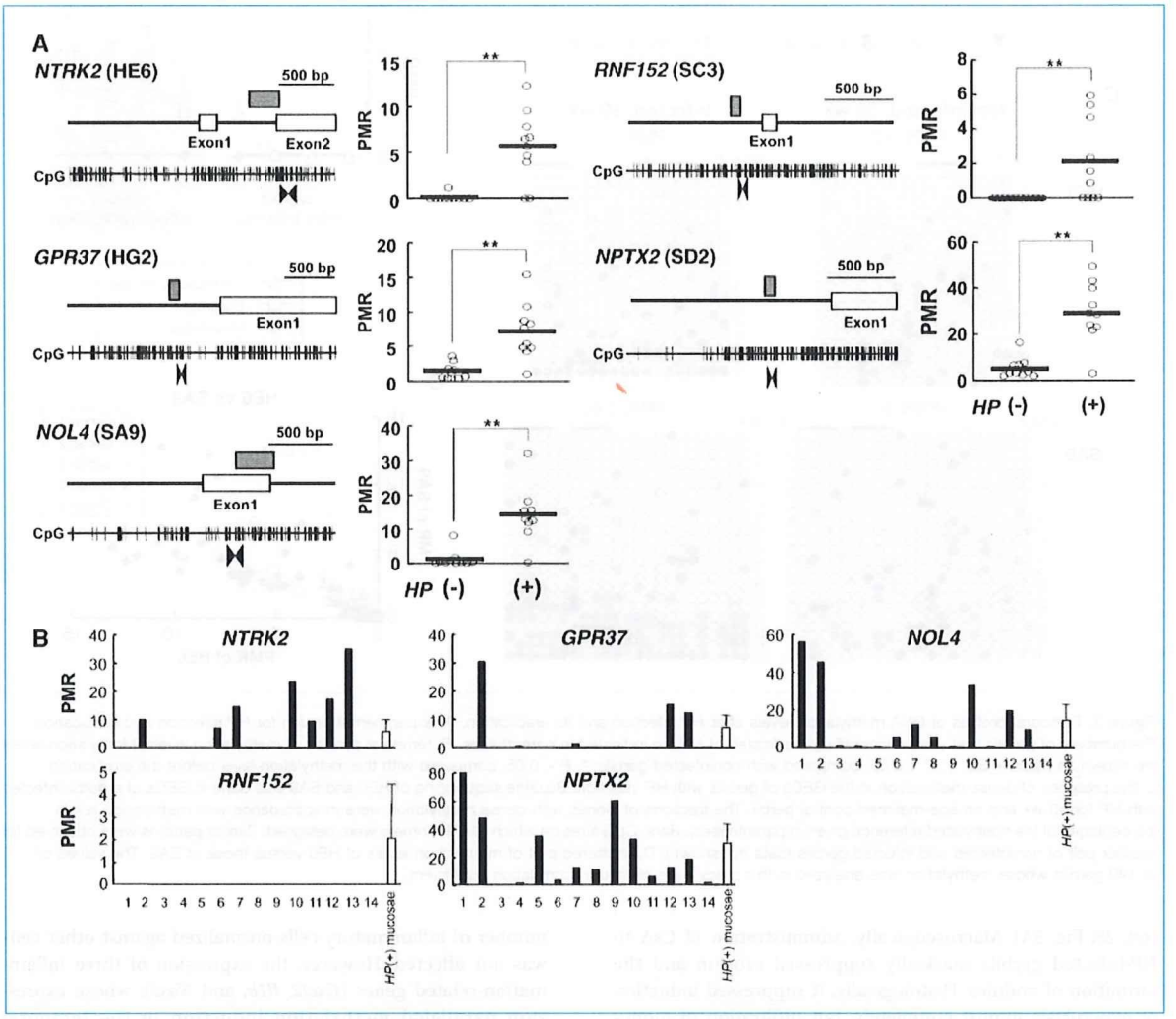

Figure 2. Methylation of homologous regions in human gastric mucosae. A, methylation levels in human gastric mucosal biopsies. Left, genomic structures and the regions analyzed by qMSP. Vertical lines, individual CpG sites; gray box, regions with homology between gerbil and man; open boxes, exons; faced arrowheads, positions of primers for qMSP. Right, result of qMSP analyses. Methylation levels were quantified in 10 healthy volunteers without *HP* infection and 10 with *HP* infection. Bold horizontal bars, average. **, $P < 0.01$. B, methylation levels in primary gastric cancers. Fourteen primary gastric cancer samples and a pool of 10 mucosal biopsies of *HP*-infected healthy volunteers were analyzed. For the gastric mucosae, their mean methylation level and SD are shown. PMR, percentage of the methylated reference.

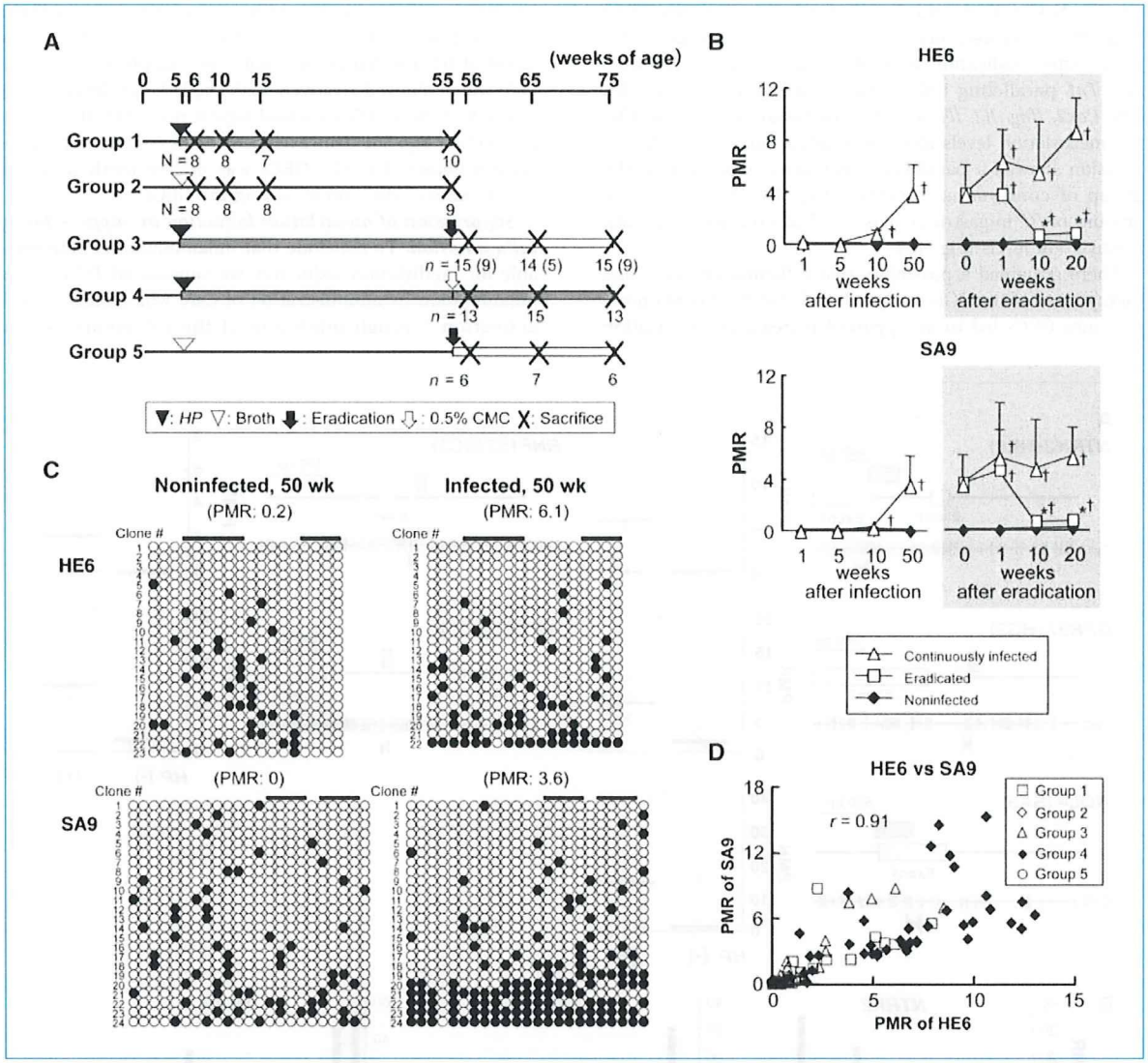

Figure 3. Temporal profiles of DNA methylation levels after *HP* infection and its eradication. A, experimental design for *HP* infection and eradication. The numbers of gerbils that were successfully eradicated of *HP* are indicated in parentheses. B, temporal profiles of methylation levels. Methylation levels are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the methylation level before the eradication. C, the presence of dense methylation in the GECs of gerbils with *HP* infection. Bisulfite sequencing of HE6 and SA9 was done in GECs of a gerbil infected with *HP* for 50 wk and an age-matched control gerbil. The fractions of clones with dense methylation were in accordance with methylation levels (percentages of the methylated reference given in parentheses). Bars, CpG sites on which qMSP primers were designed. Similar patterns were observed for another pair of noninfected and infected gerbils (data not shown). D, scattered plot of methylation levels of HE6 versus those of SA9. The values of all 149 gerbils whose methylation was analyzed in this study were plotted. *r*, correlation coefficient.

(ref. 28; Fig. 5A). Macroscopically, administration of CsA to *HP*-infected gerbils markedly suppressed erosion and the formation of nodules. Histologically, it suppressed induction of hyperplasia almost completely, but infiltration of mononuclear and polymorphonuclear cells remained (Fig. 5B). Importantly, the number of *HP* colonized in the stomach was not affected by the CsA treatment (Supplementary Fig. S6). The expression levels of inflammatory cell markers (*Cd3g, Cd14*, and *Ela2*) were not reduced, indicating that the number of inflammatory cells normalized against other cells was not affected. However, the expression of three inflammation-related genes (*Cxcl2, Il1b*, and *Nos2*), whose expression paralleled methylation induction in the temporal analysis, was significantly reduced by the CsA treatment (Fig. 5C).

The DNA methylation levels of the 10 CGIs were markedly reduced in GECs of CsA-treated gerbils (0% to 28% of methylation levels of GECs from *HP*-infected gerbil without the

CsA treatment; Fig. 5D; Supplementary Fig. S7). These results showed that the CsA treatment suppressed inflammatory responses but not *HP* colonization, and that the suppression of inflammatory responses markedly repressed methylation induction.

Expression analysis of genes with promoter methylation in* HP*-infected GECs. HG2, SC3, and SD2 were located in the promoter regions of *Gpr37, Rnf152*, and *Nptx2*, respectively. Promoter CGIs are generally resistant to DNA methylation (29), and only when genes are transcribed at low levels are they susceptible to DNA methylation (30–32). To confirm the low expression and the effect of methylation on gene expression, we analyzed their expression levels in GECs isolated from gerbils with and without *HP* infection (10 and 50 weeks

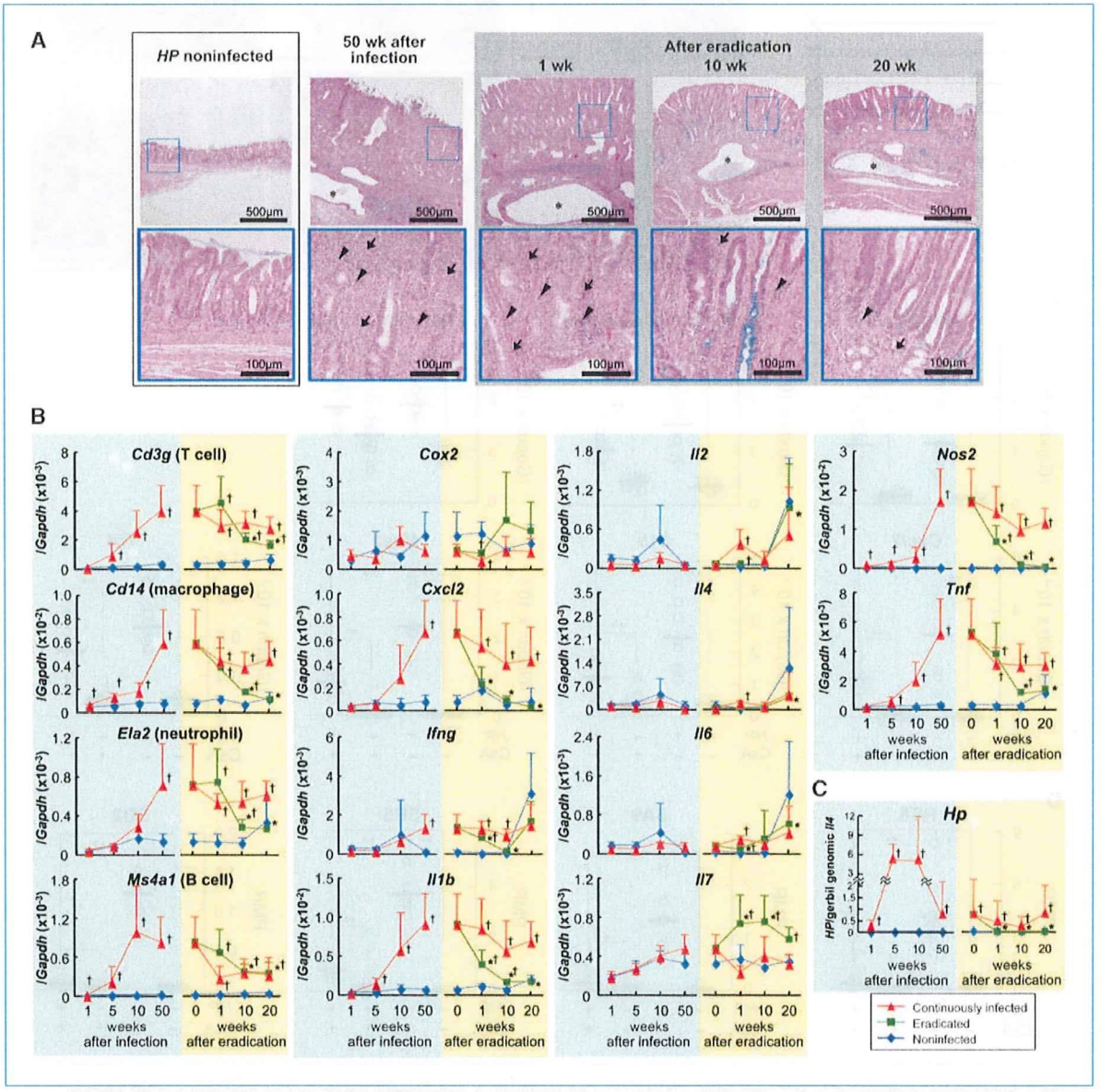

Figure 4. Changes in inflammation after *HP* infection and its eradication. A, histologic changes in gastric mucosa before and after *HP* eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) did not change at 1 wk after eradication but markedly decreased at 10 and 20 wk. However, the presence of fibrosis and heterotopic proliferative glands (*) did not differ. B, temporal profiles of expression of inflammatory cell markers and inflammation-related genes. Red, green, and blue lines, gerbils with continued infection, gerbils with eradication, and those without any *HP* infection, respectively. C, numbers of *HP* in the gerbil stomach. Real-time PCR of *HP*-specific DNA using DNA extracted from gastric tissues containing mucus was done. Values are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the expression level before eradication.