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a proapoptotic gene, confers chemoresistance to melanoma and leukemia cells by mediating resistance to cytochrome c-dependent apoptosis [114]. These findings demonstrate the potential clinical utility of DNA methylation markers for individualized therapy of cancer patients.

s0065 3.12 NEW TECHNOLOGIES FOR DNA METHYLATION ANALYSIS AND FUTURE DIRECTIONS

p0270 Currently available forms of screening technology, such as single-base-pair resolution whole-genome DNA methylation analysis using second-generation sequencers, and international efforts aimed at determining reference epigenome profiles, are now opening new avenues of epigenome therapy for cancer patients. Although broad DNA methylation profiling was initially performed on the basis of two-dimensional gel electrophoresis, adaptation of microarray hybridization techniques used in gene expression and genome studies to the profiling of DNA methylation patterns opened the door to the era of the epigenome. Enzyme-based and affinity enrichment-based DNA methylation analysis techniques have been proved suitable for examination of human tissue samples using hybridization arrays [115]. Currently available high-throughput DNA sequencing technologies using second-generation sequencers are now capable of single-base-pair resolution for whole-genome DNA methylation analysis. Although projects involving analysis of large numbers of human tissue samples will still rely on array-based approaches for several more years, the trend will be towards bisulfite shotgun sequencing [94]. Nanopore sequencing provides single-molecule detection and avoids any bias introduced by differential amplification of methylation-derived states [116]. Moreover, third-generation sequencers for real-time sequencing can directly detect 5-methylcytosine without bisulfite conversion [117]. In addition, genome-wide analysis of histone modification and non-coding RNA is also being robustly performed. Thus, high-throughput mapping of the epigenome, i.e. an overview of DNA methylation, histone modification, non-coding RNA, and chromatin accessibility in normal, precursor and cancer cells, is now highly reproducible and standardized.

p0275 Importantly, changes in the epigenome are potentially reversible by drug treatments. This has significant implications for the prevention and therapy of human cancers. Indeed, several inhibitors of chromatin-modifying enzymes, including DNMT inhibitors, as well as HDAC inhibitors, have been approved by the US Food and Drug Administration and the EU, and are now being used in clinical practice [118,119]. However, to maximize the potential of such therapeutic approaches, a more comprehensive characterization of the epigenome changes that occur during normal development and adult cell renewal should be accomplished by international consortia.

p0280 Scientists and representatives of major funding agencies have decided to launch the International Human Epigenome Consortium (IHEC) [120]. Just as the Human Genome Project provided a reference "normal" sequence for studying human disease, high-resolution reference epigenome maps consisting of the various epigenetic layers of detailed DNA methylation as well as histone modification, nucleosome occupancy and corresponding coding, and non-coding RNA expression in different normal cell types will be provided by IHEC. Such a reference human epigenome will be available to the worldwide research community. Information on the methods utilized by IHEC members will be useful for producing large epigenomic datasets related to health and diseases in humans. It will become possible to compare profiles of different human populations, thereby helping to evaluate the impact of environment and nutrition on the epigenome. Epigenome reference maps will have an immediate impact on our understanding of cancers as well as diabetes, cardiopulmonary diseases, neuropsychiatric disorders, imprinting disorders, inflammation, and autoimmune diseases, and will hopefully lead to breakthroughs in the prevention, diagnosis, and therapy of human cancers.

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References

[1] Issa JP, Just W. Epigenetics. FEBS Lett 2011;585:1993.

[2] Skinner MK. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. Epigenetics 2011;6:838–42.

[3] Siedlecki P, Zielenkiewicz P. Mammalian DNA methyltransferases. Acta Biochim Pol 2006;53:245–56.

[4] Cheng X, Blumenthal RM. Mammalian DNA methyltransferases: a structural perspective. Structure 2008;16:341–50.

[5] Hermann A, Gowher H, Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. Cell Mol Life Sci 2004;61:2571–87.

[6] Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247–57.

[7] Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH. Dnmt3L and the establishment of maternal genomic imprints. Science 2001;294:2536–9.

[8] Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev 2011;25:1010–22.

[9] Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 2007;39:457–66.

[10] Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 2009;41:178–86.

[11] Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 2009;324:930–5.

[12] Veron N, Peters AH. Epigenetics: Tet proteins in the limelight. Nature 2011;473:293–4.

[13] Burmeister T, Meyer C, Schwartz S, Hofmann J, Molkentin M, Kowarz E, et al. The MLL recombinome of adult CD10-negative B-cell precursor acute lymphoblastic leukemia: results from the GMALL study group. Blood 2009;113:4011–5.

[14] Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med 2009;360:2289–301.

[15] Langemeijer SM, Aslanyan MG, Jansen JH. TET proteins in malignant hematopoiesis. Cell Cycle 2009;8:4044–8.

[16] Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, et al. Suppression of intestinal neoplasia by DNA hypomethylation. Cell 1995;81:197–205.

[17] Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science 2003;300:455.

[18] Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300:489–92.

[19] 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.

[20] Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis 2010;31:27–36.

[21] Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.

[22] Lewandowska J, Bartoszek A. DNA methylation in cancer development, diagnosis and therapy—multiple opportunities for g enotoxic agents to act as methylome disruptors or remediators. Mutagenesis 2011;26:475–87.

[23] Tsai HC, Baylin SB. Cancer epigenetics: linking basic biology to clinical medicine. Cell Res 2011;21:502–17.

[24] Clark SJ. Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. Hum Mol Genet 2007;16(Spec No 1):R88–95.

[25] Saito Y, Jones PA. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. Cell Cycle 2006;5:2220–2.

[26] Li M, Marin-Muller C, Bharadwaj U, Chow KH, Yao Q, Chen C. MicroRNAs: control and loss of control in human physiology and disease. World J Surg 2009;33:667–84.

[27] Kanai Y, Hirohashi S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. Carcinogenesis 2007;28:2434–42.

[28] Kanai Y. Alterations of DNA methylation and clinicopathological diversity of human cancers. Pathol Int 2008;58:544–58.

[29] Kanai Y, Ushijima S, Tsuda H, Sakamoto M, Sugimura T, Hirohashi S. Aberrant DNA methylation on chromosome 16 is an early event in hepatocarcinogenesis. Jpn J Cancer Res 1996;87:1210–7.

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[30] Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 2000;32:970–9.

[31] Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A 1995;92:7416–9.

[32] Hirohashi S, Kanai Y. Cell adhesion system and human cancer morphogenesis. Cancer Sci 2003;94:575–81.

[33] Kanai Y, Ushijima S, Hui AM, Ochiai A, Tsuda H, Sakamoto M, et al. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. Int J Cancer 1997;71:355–9.

[34] Kanai Y, Hui AM, Sun L, Ushijima S, Sakamoto M, Tsuda H, et al. DNA hypermethylation at the D17S5 locus and reduced HIC-1 mRNA expression are associated with hepatocarcinogenesis. Hepatology 1999;29:703–9.

[35] Kanai Y, Ushijima S, Tsuda H, Sakamoto M, Hirohashi S. Aberrant DNA methylation precedes loss of heterozygosity on chromosome 16 in chronic hepatitis and liver cirrhosis. Cancer Lett 2000;148:73–80.

[36] Etoh T, Kanai Y, Ushijima S, Nakagawa T, Nakanishi Y, Sasako M, et al. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol 2004;164:689–99.

[37] Ryan JL, Jones RJ, Kenney SC, Rivenbark AG, Tang W, Knight ER, et al. Epstein-Barr virus-specific methylation of human genes in gastric cancer cells. Infect Agent Cancer 2010;5:27.

[38] Hino R, Uozaki H, Murakami N, Ushiku T, Shinozaki A, Ishikawa S, et al. Activation of DNA methyltransferase 1 by EBV latent membrane protein 2A leads to promoter hypermethylation of PTEN gene in gastric carcinoma. Cancer Res 2009;69:2766–74.

[39] Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, 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.

[40] Sawada M, Kanai Y, Arai E, Ushijima S, Ojima H, Hirohashi S. Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion. Cancer Lett 2007;251:211–9.

[41] Laurson J, Khan S, Chung R, Cross K, Raj K. Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein. Carcinogenesis 2010;31:918–26.

[42] Eguchi K, Kanai Y, Kobayashi K, Hirohashi S. DNA hypermethylation at the D17S5 locus in non-small cell lung cancers: its association with smoking history. Cancer Res 1997;57:4913–5.

[43] Chen W, Cooper TK, Zahnow CA, Overholtzer M, Zhao Z, Ladanyi M, et al. Epigenetic and genetic loss of Hic1 function accentuates the role of p53 in tumorigenesis. Cancer Cell 2004;6:387–98.

[44] Wales MM, Biel MA, el Deiry W, Nelkin BD, Issa JP, Cavenee WK, et al. p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. Nat Med 1995;1:570–7.

[45] Sun L, Hui AM, Kanai Y, Sakamoto M, Hirohashi S. Increased DNA methyltransferase expression is associated with an early stage of human hepatocarcinogenesis. Jpn J Cancer Res 1997;88:1165–70.

[46] Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology 2001;33:561–8.

[47] Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, et al. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 2003;105:527–32.

[48] Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kosuge T, et al. Increased DNA methyltransferase 1 (DNMT1) protein expression in precancerous conditions and ductal carcinomas of the pancreas. Cancer Sci 2005;96:403–8.

[49] Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S, et al. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. Carcinogenesis 2006;27:1160–8.

[50] Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, et al. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 1999;27:2291–8.

[51] Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.

[52] Issa JP, Shen L, Toyota M. CIMP, at last. Gastroenterology 2005;129:1121–4.

[53] Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, Hirohashi S. DNA methyltransferase expression and DNA methylation of CPG islands and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer 2001;91:205–12.

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[54] Nakagawa T, Kanai Y, Saito Y, Kitamura T, Kakizoe T, Hirohashi S. Increased DNA methyltransferase 1 protein expression in human transitional cell carcinoma of the bladder. J Urol 2003;170:2463–6.

[55] Nakagawa T, Kanai Y, Ushijima S, Kitamura T, Kakizoe T, Hirohashi S. DNA hypermethylation on multiple CpG islands associated with increased DNA methyltransferase DNMT1 protein expression during multistage urothelial carcinogenesis. J Urol 2005;173:1767–71.

[56] Denis H, Ndlovu MN, Fuks F. Regulation of mammalian DNA methyltransferases: a route to new mechanisms. EMBO Rep 2011;12:647–56.

[57] Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A 2007;104:15805–10.

[58] Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 2009;113:6411–8.

[59] Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. Rna 2008;14:872–7.

[60] Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 2010;51:881–90.

[61] Zhao S, Wang Y, Liang Y, Zhao M, Long H, Ding S, et al. MicroRNA-126 regulates DNA methylation in CD4+ T cells and contributes to systemic lupus erythematosus by targeting DNA methyltransferase 1. Arthritis Rheum 2011;63:1376–86.

[62] Huang J, Wang Y, Guo Y, Sun S. Down-regulated microRNA-152 induces aberrant DNA methylation in hepatitis B virus-related hepatocellular carcinoma by targeting DNA methyltransferase 1. Hepatology 2010;52:60–70.

[63] Lopez de Silanes I, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, et al. The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. Nucleic Acids Res 2009;37:2658–71.

[64] Kanai Y, Ushijima S, Nakanishi Y, Sakamoto M, Hirohashi S. Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. Cancer Lett 2003;192:75–82.

[65] Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med 2010;363:2424–33.

[66] Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet 2011;43:309–15.

[67] Fan H, Liu D, Qiu X, Qiao F, Wu Q, Su X, et al. A functional polymorphism in the DNA methyltransferase-3A promoter modifies the susceptibility in gastric cancer but not in esophageal carcinoma. BMC Med 2010;8:12.

[68] Wang L, Rodriguez M, Kim ES, Xu Y, Bekele N, El-Naggar AK, et al. A novel C/T polymorphism in the core promoter of human de novo cytosine DNA methyltransferase 3B6 is associated with prognosis in head and neck cancer. Int J Oncol 2004;25:993–9.

[69] Hansen RS, Wijmenga C, Luo P, Stanek AM, Canfield TK, Weemaes CM, et al. The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc Natl Acad Sci U S A 1999;96:14412–7.

[70] Wong N, Lam WC, Lai PB, Pang E, Lau WY, Johnson PJ. Hypomethylation of chromosome 1 heterochromatin DNA correlates with q-arm copy gain in human hepatocellular carcinoma. Am J Pathol 2001;159:465–71.

[71] Nakagawa T, Kanai Y, Ushijima S, Kitamura T, Kakizoe T, Hirohashi S. DNA hypomethylation on pericentromeric satellite regions significantly correlates with loss of heterozygosity on chromosome 9 in urothelial carcinomas. J Urol 2005;173:243–6.

[72] Soejima K, Fang W, Rollins BJ. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. Oncogene 2003;22:4723–33.

[73] Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci U S A 2002;99:10060–5.

[74] Hervouet E, Vallette FM, Cartron PF. Dnmt3/transcription factor interactions as crucial players in targeted DNA methylation. Epigenetics 2009;4:487–99.

[75] Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res 2008;647:21–9.

[76] Hou P, Ji M, Xing M. Association of PTEN gene methylation with genetic alterations in the phosphatidylinositol 3-kinase/AKT signaling pathway in thyroid tumors. Cancer 2008;113:2440–7.

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[77] Maddodi N, Bhat KM, Devi S, Zhang SC, Setaluri V. Oncogenic BRAFV600E induces expression of neuronal differentiation marker MAP2 in melanoma cells by promoter demethylation and down-regulation of transcription repressor HES1. J Biol Chem 2010;285:242–54.

[78] Hou P, Liu D, Xing M. Genome-wide alterations in gene methylation by the BRAF V600E mutation in papillary thyroid cancer cells. Endocr Relat Cancer 2011;18:687–97.

[79] MacLeod AR, Rouleau J, Szyf M. Regulation of DNA methylation by the Ras signaling pathway. J Biol Chem 1995;270:11327–37.

[80] Deng C, Yang J, Scott J, Hanash S, Richardson BC. Role of the ras-MAPK signaling pathway in the DNA methyltransferase response to DNA hypomethylation. Biol Chem 1998;379:1113–20.

[81] Pruitt K, Ulkü AS, Frantz K, Rojas RJ, Muniz-Medina VM, Rangnekar VM, et al. Ras-mediated loss of the pro-apoptotic response protein Par-4 is mediated by DNA hypermethylation through Raf-independent and Raf-dependent signaling cascades in epithelial cells. J Biol Chem 2005;280:23363–70.

[82] Young NL, Dimaggio PA, Garcia BA. The significance, development and progress of high-throughput combinatorial histone code analysis. Cell Mol Life Sci 2010;67:3983–4000.

[83] Gardner KE, Allis CD, Strahl BD. Operating on chromatin, a colorful language where context matters. J Mol Biol 2011;409:36–46.

[84] Bogdanovic O, Veenstra GJ. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma 2009;118:549–65.

[85] Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Hum Mol Genet 2007;16(Spec No 1):R28–49.

[86] Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. Nat Genet 2007;39:237–42.

[87] Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature 2006;439:871–4.

[88] Ding L, Kleer CG. Enhancer of Zeste 2 as a marker of preneoplastic progression in the breast. Cancer Res 2006;66:9352–5.

[89] Bryant RJ, Cross NA, Eaton CL, Hamdy FC, Cunliffe VT. EZH2 promotes proliferation and invasiveness of prostate cancer cells. Prostate 2007;67:547–56.

[90] Fussbroich B, Wagener N, Macher-Goeppinger S, Benner A, Falth M, Sultmann H, et al. EZH2 depletion blocks the proliferation of colon cancer cells. PLoS One 2011;6:e21651.

[91] Mohammad HP, Cai Y, McGarvey KM, Easwaran H, Van Neste L, Ohm JE, et al. Polycomb CBX7 promotes initiation of heritable repression of genes frequently silenced with cancer-specific DNA hypermethylation. Cancer Res 2009;69:6322–30.

[92] Kanai Y. Genome-wide DNA methylation profiles in precancerous conditions and cancers. Cancer Sci 2010;101:36–45.

[93] Arai E, Kanai Y. DNA methylation profiles in precancerous tissue and cancers: carcinogenetic risk estimation and prognostication based on DNA methylation status. Epigenomics 2010;2:467–81.

[94] Laird PW. Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet 2010;11:191–203.

[95] Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A 2007;104:18654–9.

[96] Arai E, Kanai Y, Ushijima S, Fujimoto H, Mukai K, Hirohashi S. Regional DNA hypermethylation and DNA methyltransferase (DNMT) 1 protein overexpression in both renal tumors and corresponding nontumorous renal tissues. Int J Cancer 2006;119:288–96.

[97] Arai E, Wakai-Ushijima S, Fujimoto H, Hosoda F, Shibata T, Kondo T, et al. Genome-wide DNA methylation profiles in renal tumors of various histological subtypes and non-tumorous renal tissues. Pathobiology 2011;78:1–9.

[98] Arai E, Ushijima S, Fujimoto H, Hosoda F, Shibata T, Kondo T, 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 2009;30:214–21.

[99] Arai E, Kanai Y. Genetic and epigenetic alterations during renal carcinogenesis. Int J Clin Exp Pathol 2011;4:58–73.

[100] Arai E, Ushijima S, Tsuda H, Fujimoto H, Hosoda F, Shibata T, et al. Genetic clustering of clear cell renal cell carcinoma based on array-comparative genomic hybridization: its association with DNA methylation alteration and patient outcome. Clin Cancer Res 2008;14:5531–9.

[101] Nishiyama N, Arai E, Nagashio R, Fujimoto H, Hosoda F, Shibata T, et al. Copy number alterations in urothelial carcinomas: their clinicopathological significance and correlation with DNA methylation alterations. Carcinogenesis 2011;32:462–9.

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[102] Sepulveda AR, Jones D, Ogino S, Samowitz W, Gulley ML, Edwards R, et al. CpG methylation analysis—current status of clinical assays and potential applications in molecular diagnostics: a report of the Association for Molecular Pathology. J Mol Diagn 2009;11:266—78.

[103] Cairns P, Esteller M, Herman JG, Schoenberg M, Jeronimo C, Sanchez-Cespedes M, et al. Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. Clin Cancer Res 2001;7:2727—30.

[104] Hoque MO, Begum S, Topaloglu O, Chatterjee A, Rosenbaum E, Van Criekinge W, et al. Quantitation of promoter methylation of multiple genes in urine DNA and bladder cancer detection. J Natl Cancer Inst 2006;98:996—1004.

[105] Watanabe Y, Kim HS, Castoro RJ, Chung W, Estecio MR, Kondo K, et al. Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes. Gastroenterology 2009;136:2149—58.

[106] Gotoh M, Arai E, Wakai-Ushijima S, Hiraoka N, Kosuge T, Hosoda F, et al. Diagnosis and prognostication of ductal adenocarcinomas of the pancreas based on genome-wide DNA methylation profiling by bacterial artificial chromosome array-based methylated CpG island amplification. J Biomed Biotechnol 2011;2011:780836.

[107] Arai E, Ushijima S, Gotoh M, Ojima H, Kosuge T, Hosoda F, et al. Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma. Int J Cancer 2009;125:2854—62.

[108] Nagashio R, Arai E, Ojima H, Kosuge T, Kondo Y, Kanai Y. Carcinogenetic risk estimation based on quantification of DNA methylation levels in liver tissue at the precancerous stage. Int J Cancer 2011;129:1170—9.

[109] Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, et al. Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. Cancer Sci 2010;101:231—40.

[110] Marsit CJ, Houseman EA, Christensen BC, Gagne L, Wrensch MR, Nelson HH, et al. Identification of methylated genes associated with aggressive bladder cancer. PLoS One 2010;5:e12334.

[111] Jarmalaite S, Jankevicius F, Kurgonaite K, Suziedelis K, Mutanen P, Husgafvel-Pursiainen K. Promoter hypermethylation in tumour suppressor genes shows association with stage, grade and invasiveness of bladder cancer. Oncology 2008;75:145—51.

[112] Satoh A, Toyota M, Itoh F, Sasaki Y, Suzuki H, Ogi K, et al. Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res 2003;63:8606—13.

[113] Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, et al. Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. J Clin Oncol 2008;26:4189—99.

[114] Campioni M, Santini D, Tonini G, Murace R, Dragonetti E, Spugnini EP, et al. Role of Apaf-1, a key regulator of apoptosis, in melanoma progression and chemoresistance. Exp Dermatol 2005;14:811—8.

[115] Estecio MR, Issa JP. Tackling the methylome: recent methodological advances in genome-wide methylation profiling. Genome Med 2009;1:106.

[116] Branton D, Deamer DW, Marziali A, Bayley H, Benner SA, Butler T, et al. The potential and challenges of nanopore sequencing. Nat Biotechnol 2008;26:1146—53.

[117] Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, et al. Real-time DNA sequencing from single polymerase molecules. Science 2009;323:133—8.

[118] Kelly TK, De Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. Nat Biotechnol 2010;28:1069—78.

[119] Boumber Y, Issa JP. Epigenetics in cancer: what's the future? Oncology (Williston Park) 2011;25:220—6, 228.

[120] International Human Epigenome Consortium [homepage on the internet] Cited. Available from: http://www.ihec-epigenomes.org/; February 2012.

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ORIGINAL ARTICLE

Identification of gastric cancer risk markers that are informative in individuals with past *H. pylori* infection

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Abstract
Background Epigenomic damage induced by *Helicobacter pylori* infection is accumulated in gastric mucosae before the development of malignancy. In individuals without current *H. pylori* infection, DNA methylation levels of specific CpG islands (CGIs) are associated with gastric cancer risk. Because risk estimation in individuals with past infection is clinically important, we here aimed to identify the risk markers that reflect epigenomic damage induced by *H. pylori* infection, and that are informative in these individuals.
Methods Gastric mucosae were obtained from 55 gastric cancer patients (GC-Pt) (21 with current infection and 34 with past infection) and 55 healthy volunteers (HV) (7 never-infected, 21 with current infection, and 27 with past infection). Hypermethylated CGIs were searched for by methylated DNA immunoprecipitation-CGI microarray, and methylation levels were analyzed by quantitative methylation-specific polymerase chain reaction (PCR).
Results By microarray analysis of a pool of three samples from GC-Pt with past infection and another pool of samples from HV with past infection, 15 hypermethylated CGIs in the former pool were isolated. Seven of them had significantly higher methylation levels in GC-Pt with past infection ($n = 10$) than in HV with past infection ($n = 10$) ($P < 0.001$). In a validation cohort (21 GC-Pt with past infection and 14 HV with past infection), the seven new markers had large areas under the receiver-operating characteristic curves (0.78–0.84) and high odds ratios (12.7–36.0) compared with two currently available markers (0.60–0.65, 5.0–5.7).
Conclusions We identified seven novel gastric cancer risk markers that are highly informative in individuals with past infection.

Keywords Carcinogenesis · DNA methylation · Gastric cancer · *Helicobacter pylori*

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Introduction

Early detection of cancer is critically important to reduce its morbidity and mortality, and early detection can be achieved by identifying individuals at high risk of developing cancers. In the risk estimation of gastric cancers, a history of *Helicobacter pylori* infection, which increases gastric cancer risk 2.2- to 21-fold [1–4], plays the major role, but the vast majority of individuals with a history of *H. pylori* infection do not develop gastric cancers. Also, gene polymorphisms associated with gastric cancers have been identified, and they have been shown to confer odds ratios (ORs) mostly between 1.0 and 2.0 [5, 6]. To obtain

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clinically useful risk markers, we have to develop markers that are informative even in individuals with a history of *H. pylori* infection and that confer higher ORs.

Recently, we showed that *H. pylori* infection induces epigenomic damage, especially aberrant DNA methylation, in gastric mucosae [7]. DNA methylation levels of specific CpG islands (CGIs) were very high in the gastric mucosae of individuals with active *H. pylori* infection irrespective of gastric cancer risk, and decreased to certain levels after *H. pylori* was eradicated [8]. Importantly, these methylation levels in individuals without active *H. pylori* infection were correlated with gastric cancer risk [7, 9]. It is considered that aberrant DNA methylation is induced both in gastric stem cells and in non-stem cells, that methylation induced in stem cells will remain even after *H. pylori* eradication, and that methylation levels in individuals without current *H. pylori* infection reflect gastric cancer risk (degree of the epigenetic field defect) [10].

The correlation between methylation levels and gastric cancer risk has been analyzed in individuals without current *H. pylori* infection [7, 9]. Based on the data in our previous study [7], currently available methylation risk markers, *FLNc* and *THBD*, have ORs of 4.2–7.0 to detect gastric cancer patients (GC-Pt) among such individuals. However, individuals without current *H. pylori* infection indeed consist of never-infected individuals and those with past infection, and risk estimation is important in individuals with past infection.

In this study, we aimed to identify gastric cancer risk markers that reflect epigenomic damage induced by *H. pylori* infection, and that are informative in individuals with past infection.

Materials and methods

Tissue samples and determination of *H. pylori* infection status

Fifty-five healthy volunteers (HV) with endoscopic findings of no malignancy were recruited, with written informed consents, on the occasion of a gastric cancer screening program, with the approval of the institutional review board. Fifty-five GC-Pt who had undergone curative endoscopic submucosal dissection (ESD) of a well-differentiated adenocarcinoma in the non-cardia according to the Japanese classification of gastric carcinoma [11] were also recruited, with written informed consents, with the approval of the Institutional Review Board. Gastric mucosae were collected by endoscopic biopsy of the antrum. The biopsy specimens were frozen in liquid nitrogen immediately after biopsy, and stored at −80°C until DNA extraction. High molecular weight DNA was extracted by the phenol/chloroform method.

Current *H. pylori* infection was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan) in HV and by urea breath test (Otsuka Pharmaceutical, Tokushima, Japan) in GC-Pt. Also, the presence of current or past *H. pylori* infection was detected by the endoscopic presence of atrophic gastritis in the antrum, because atrophic change induced by *H. pylori* infection arises in the antrum in 83% of individuals with *H. pylori* infection [12] and remains in all individuals who have had *H. pylori* eradication therapy [13]. "Never-infected individuals" were defined as those who were negative for *H. pylori* analysis and did not have atrophic gastritis in the antrum. "Individuals with current infection" were defined as those who were positive for *H. pylori* analysis. "Individuals with past infection" were defined as those who were negative for *H. pylori* analysis and had atrophic gastritis in the antrum.

Methylated DNA immunoprecipitation-CGI microarray analysis

Methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis was performed as previously described [14, 15]. Briefly, 5 μg of genomic DNA was immunoprecipitated with an anti-5-methylcytidine antibody (Diagnode, Liége, Belgium), and the precipitated DNA and the input DNA were labeled with cyanin (Cy) 5 and Cy3, respectively. A human CGI oligonucleotide microarray (Agilent Technologies, Santa Clara, CA, USA) was hybridized with the labeled probes and scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). Scanned data were processed with Feature Extraction Software Version 9.1 (Agilent Technology) and Agilent G4477AA ChIP Analytics 1.3 software. The signal of a probe was converted into a "Me value", which represented the methylation level as a value from 0 (unmethylated) to 1 (methylated). Differentially methylated regions were detected by comparison between the Me values of two samples, and data were visualized in the UCSC Genome Browser (http://genome.ucsc.edu/) on NCBI36/hg18 assembly (National Center for Biotechnology Information, Bethesda, MD, USA).

Sodium bisulfite modification and quantitative methylation-specific polymerase chain reaction

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *SssI* methylase (New England Biolabs, Beverly, MA, USA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA, and the modified

DNA was suspended in 40 μl of Tris–ethylenediamine tetraacetic acid (EDTA) buffer [16]. An aliquot of 2 μl of sodium bisulfite-treated DNA was used in one reaction of quantitative methylation-specific polymerase chain reaction (PCR; qMSP).

qMSP was performed using primer sets specific to methylated and unmethylated sequences (Supplementary Table 1), SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME, USA), and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA that contained known numbers of molecules (10^1–10^9 molecules). Standard DNA was prepared by purifying the PCR products using the Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA). The methylation level was calculated as the fraction of methylated (M) molecules in the total number of DNA molecules (number of M molecules + number of unmethylated molecules). The percentage of methylated reference (PMR) was calculated as the fraction of the methylated reference {(number of M molecules in a sample)/(number of *Alu* repeat sequences in a sample)}/{(number of M molecules in *SssI*-treated DNA)/(number of *Alu* repeat sequences in *SssI*-treated DNA)} [17].

Statistical analysis

Differences in mean methylation levels or PMR were analyzed by the Student's *t*-test. The receiver-operating characteristic (ROC) curve was drawn, and the area under the curve (AUC) and OR were analyzed by binomial distribution and binomial logistic regression analysis, respectively. All the analysis was performed using PASW statistics (SPSS, Chicago, IL, USA), and the results were considered significant when *P* values of less than 0.05 were obtained by two-sided tests.

Results

Isolation of hypermethylated CGIs in GC-Pt compared with HV in individuals with past *H. pylori* infection

A pool of three samples from HV with past infection and another pool of three samples from GC-Pt with past infection were analyzed by MeDIP-CGI microarray analysis. CGIs that were hypermethylated in the latter group compared with the former group were selected as follows: (1) Me value in the latter pool was higher than that in the former pool by 0.2 or more, (2) Me value in the former pool was lower than 0.4, and (3) criteria (1) and (2) were satisfied in three consecutive probes. A total of 15 CGIs were isolated by these criteria (Table 1), and representative data around CGI #5 are shown in Fig. 1.

From the 15 CGIs, those differentially methylated in a screening set, which consisted of 10 HV with past infection and 10 GC-Pt with past infection, were searched for by evaluating PMRs by qMSP (Supplementary Table 2). Seven CGIs (#1 to #7; Table 1), distributed on various chromosomes, were methylated at significantly higher

Table 1 CGIs identified by MeDIP-CGI microarray

CGI no.	Gene symbol	Name	Chromosomal position	Location around a gene
#1	*EMX1*	Empty spiracles, homeobox 1	2p13.2	Intron 1
#2	*miR663*	MicroRNA 663	20p11.1	Overlap
#3	*NKX6-1*	NK6, homeobox 1	4q21.23	Intron 1
#4	*OTP*	Orthopedia homeobox	5q13.3	Downstream
#5	*OPLAH*	5-Oxoprolinase (ATP-hydrolysing)	8q24.3	Downstream
#6	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1	2p22.2	Exon 1
#7	*NEFM*	Neurofilament, medium polypeptide	8p21	Exon 1
#8	*PMF1*	Polyamine-modulated factor 1	1q22	Intron 1
#9	*BDNF*	Brain-derived neurotrophic factor	11p14.1	Intron 1
#10	*SSTR5*	Somatostatin receptor 5	16p13.3	Promoter
#11	*MYO1D*	Myosin ID	17q11.2	Intron 1
#12	*CAMK2N2*	Calcium/calmodulin-dependent protein kinase II inhibitor 2	3q27.1	Promoter
#13	*GATA4*	GATA binding protein 4	8p23.1	Promoter
#14	*NFATC1*	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	18q23	Promoter
#15	*ANKRD9*	Ankyrin repeat domain 9	14q32.31	Exon 1

CGI CpG island, *MeDIP* methylated DNA immunoprecipitation

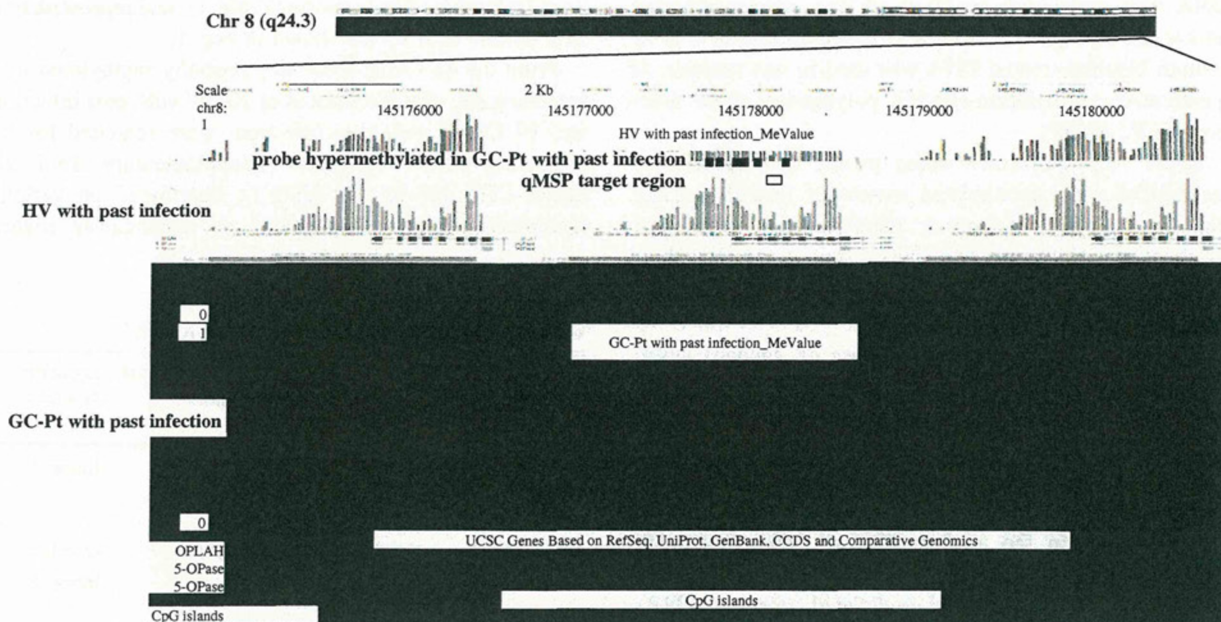

Fig. 1 Data of methylated DNA immunoprecipitation-CpG island (MeDIP-CGI) microarray analysis in the genomic region around CGI #5. Methylation levels were assessed by Me values, and the Me values of the two pools were visualized by the UCSC Genome Browser (http://genome.ucsc.edu/) for a genomic region (from nt. 145,174,733 to nt. 145,180,586 on chromosome 8 in NCBI36/hg18 assembly). *Vertical bars* show Me values of individual probes. *Closed boxes* above the Me values indicate the differentially methylated probes. Quantitative methylation-specific polymerase chain reaction (*qMSP*) primers were designed in the area shown by the *open box*. *HV* healthy volunteers, *GC-Pt* gastric cancer patients

levels in GC-Pt than in HV ($P < 0.05$). Relative positions against a gene also varied—two CGIs being located in exon 1, two in intron 1, two 300 bp downstream of the annotated end, and one overlapping with *pre-microRNA 663*.

Validation of the usefulness of the seven markers

The usefulness of the seven CGIs was validated by qMSP analysis of an independent set of samples (Fig. 2). The validation set consisted of seven never-infected HV (Group [G] 1), 21 HV with current infection (G2), 14 HV with past infection (G3), 21 GC-Pt with current infection (G4), and 21 GC-Pt with past infection (G5) (Supplementary Table 3). For comparison, two currently available markers (*FLNc* and *THBD*) were also analyzed. In the individuals with past infection (G3 and G5), the seven CGIs had levels that were 2.8-, 1.5-, 3.8-, 2.3-, 2.5-, 1.8-, and 3.8-fold, respectively, higher in G5 than in G3 ($P < 0.01$). *FLNc* tended to have a higher level in G5 than in G3 ($P = 0.087$), but *THBD* did not show any significant difference ($P = 0.341$). These data showed that the methylation levels of all the seven CGIs had the power of cancer risk estimation even in individuals with past infection.

In the HV, methylation levels in G2 were much higher than those in G1 ($P < 0.05$), but those in G3 were lower than those in G2. This observation supported the model that active infection by *H. pylori* induces methylation potently in non-stem cells, in addition to stem cells, and that methylation levels will eventually decrease after *H. pylori* infection has been eradicated. Also, methylation levels in G3 were significantly higher (four of the seven CGIs, $P < 0.05$) or tended to be higher than those in G1. This observation again supported the model that methylation induced in stem cells will remain even after *H. pylori* infection is eradicated.

Power of the seven CGIs as gastric cancer risk markers

AUCs to detect individuals in G5 were calculated using individuals in G3 and G5 (Table 2; Fig. 3). AUCs for the seven CGIs ranged between 0.78 and 0.84 and were significantly larger than 0.5 ($P < 0.01$). In contrast, the AUCs for the two currently available markers were 0.69 (95% CI 0.51–0.87) and 0.65 (95% CI 0.45–0.84), respectively, and were not significantly different from 0.5. Using optimal cut-off values obtained by the ROCs, ORs for the seven CGIs were calculated to be 12.7–36.0 (Table 2). ORs for

are associated with gastric cancer risk even in individuals with past infection. These seven CGIs are promising candidate markers to estimate gastric cancer risk.

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References

1. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
2. Forman D, Webb P, Parsonnet J. *H. pylori* and gastric cancer. Lancet. 1994;343:243–4.
3. Suzuki H, Iwasaki E, Hibi T. *Helicobacter pylori* and gastric cancer. Gastric Cancer. 2009;12:79–87.
4. Ekström AM, Held M, Hansson LE, Engstrand L, Nyrén O. *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. Gastroenterology. 2001;121:784–91.
5. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature. 2000;404:398–402.
6. Loh M, Koh KX, Yeo BH, Song CM, Chia KS, Zhu F, et al. Meta-analysis of genetic polymorphisms and gastric cancer risk: variability in associations according to race. Eur J Cancer. 2009;45:2562–8.
7. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, 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.
8. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, et al. Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication. J Gastroenterol. 2010;45:37–44.
9. Nakajima T, Maekita T, Oda I, Gotoda T, Yamamoto S, Umemura S, et al. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. Cancer Epidemiol Biomarkers Prev. 2006;15:2317–21.
10. Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol. 2007;40:142–50.
11. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma—2nd English edition. Gastric Cancer. 1998;1:10–24.
12. Asaka M, Sugiyama T, Nobuta A, Kato M, Takeda H, Graham DY. Atrophic gastritis and intestinal metaplasia in Japan: results of a large multicenter study. Helicobacter. 2001;6:294–9.
13. Ohkusa T, Fujiki K, Takashimizu I, Kumagai J, Tanizawa T, Eishi Y, et al. Improvement in atrophic gastritis and intestinal metaplasia in patients in whom *Helicobacter pylori* was eradicated. Ann Intern Med. 2001;134:380–6.
14. Yamashita S, Hosoya K, Gyobu K, Takeshima H, Ushijima T. Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. DNA Res. 2009;16:275–86.
15. Takeshima H, Yamashita S, Shimazu T, Niwa T, Ushijima T. The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. Genome Res. 2009;16:275–86.
16. 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.
17. Weisenberger DJ, Campan M, Long TI, Kim MJ, Woods C, Fiala E, et al. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res. 2005;33:6823–36.
18. Selgrad M, Bomschein J, Rokkas T, Malfertheiner P. Clinical aspects of gastric cancer and *Helicobacter pylori*—screening, prevention, and treatment. Helicobacter. 2010;15:40–5.
19. Kim N, Jung HC. The role of serum pepsinogen in the detection of gastric cancer. Gut Liver. 2010;4:307–19.
20. Mizuno S, Kobayashi M, Tomita S, Miki I, Masuda A, Onoyama M, et al. Validation of the pepsinogen test method for gastric cancer screening using a follow-up study. Gastric Cancer. 2009;12:158–63.
21. Hellman A, Chess A. Gene body-specific methylation on the active X chromosome. Science. 2007;315:1141–3.
22. Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP. A human B cell methylome at 100-base pair resolution. Proc Natl Acad Sci USA. 2009;106:671–8.
23. Rashid A, Issa JP. CpG island methylation in gastroenterologic neoplasia: a maturing field. Gastroenterology. 2004;127:1578–88.
24. Oue N, Motoshida J, Yokozaki H, Hayashi K, Tahara E, Taniyama K, et al. Distinct promoter hypermethylation of p16INK4a, CDH1, and RAR-beta in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol. 2002;198:55–9.
25. Shin CM, Kim N, Jung Y, Park JH, Kang GH, Kim JS, et al. Role of *Helicobacter pylori* infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci. 2011;101:1337–46.
26. Suzuki H, Tokino T, Shinomura Y, Imai K, Toyota M. DNA methylation and cancer pathways in gastrointestinal tumors. Pharmacogenomics. 2008;9:1917–28.
27. World Cancer Research Fund/American Institute for Cancer Research. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington, DC: AICR. 2007.
28. Nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser. 2003;916:1–149.
29. Hur K, Niwa T, Toyoda T, Tsukamoto T, Tatematsu M, Yang HK, et al. Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation. Carcinogenesis. 2011;32:35–41.
30. Tatematsu M, Nozaki K, Tsukamoto T. *Helicobacter pylori* infection and gastric carcinogenesis in animal models. Gastric Cancer. 2003;6:1–7.
31. Nozaki K, Shimizu N, Inaba K, Tsukamoto T, Inoue M, Kumagai T, et al. Synergistic promoting effects of *Helicobacter pylori* infection and high-salt diet on gastric carcinogenesis in Mongolian gerbils. Jpn J Cancer Res. 2002;93:1083–9.

Table 2 AUC and OR for new and currently available markers

CGI no.	Gene symbol	AUC	95% CI	*P* value	OR	95% CI	*P* value
#1	*EMX1*	0.84	0.70–0.97	<0.001	23.8	3.7–153	<0.001
#2	*miR663*	0.78	0.62–0.94	0.006	26.7	2.8–258	0.005
#3	*NKX6-1*	0.84	0.69–0.99	<0.001	15.0	2.8–80.1	0.002
#4	*OTP*	0.83	0.69–0.97	0.001	36.0	3.7–354	0.002
#5	*OPLAH*	0.83	0.69–0.98	0.001	15.6	2.9–83.5	0.001
#6	*CYP1B1*	0.78	0.62–0.94	0.006	12.7	2.1–76.7	0.006
#7	*NEFM*	0.84	0.71–0.98	<0.001	23.8	3.7–153	<0.001
–	*FLNc*	0.69	0.51–0.87	0.055	5.7	1.2–25.9	0.025
–	*THBD*	0.65	0.45–0.84	0.152	5.0	1.1–21.8	0.032

CGI CpG island, *AUC* area under the curve, *CI* confidence interval, *OR* odds ratio

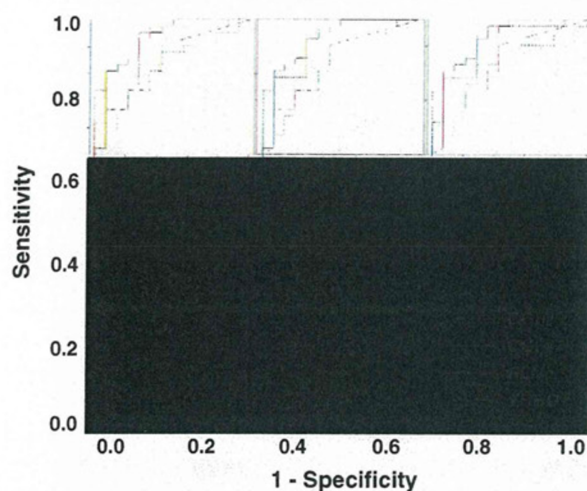

Fig. 3 Receiver-operating characteristic (ROC) curves of CGI #3 and #7, whose AUC values were the largest in the seven CGIs, are shown with those of two currently available markers, *FLNc* and *THBD*. *Black line*, *dotted line*, *dot-and-dash line*, and *dashed line* show ROC curves of CGI #3, #7, *FLNc*, and *THBD*, respectively. The AUC values of CGI #3 and #7 were larger than those of *FLNc* and *THBD*

the two currently available markers, *FLNc* and *THBD*, were 5.7 (95% CI 1.2–25.9) and 5.0 (95% CI 1.1–21.8), respectively. These results clearly showed that the methylation levels of the seven CGIs had greater power than the two currently available markers to estimate gastric cancer risk in individuals with past infection.

Discussion

In the present study, by carrying out genome-wide methylation analysis of gastric cancer patients (GC-Pt) and healthy volunteers (HV), both with past infection, we screened seven gastric cancer risk markers that are highly informative in individuals with past infection. Their usefulness was validated in 35 individuals (21 GC-Pt and 14 age-matched HV). To our knowledge, this is the first study that has evaluated epigenetic gastric cancer risk markers in individuals with past infection, and these markers are expected to be especially useful. This is because the number of individuals with past infection is increasing as more and more people receive *H. pylori* eradication therapy [18], but the usefulness of the current methods for gastric cancer risk estimation, i.e., a combination of the detection of *H. pylori* infection and the serum pepsinogen test, in this population has not been established [18–20].

None of the seven CGIs were located in promoter regions. We analyzed the association between the methylation levels of the seven CGIs and the expression levels of genes close to them, but no association was observed for any of the seven CGIs (data not shown). This was in line with the current knowledge that DNA methylation of only promoter CGIs consistently causes gene silencing, but that methylation of gene bodies may or may not be associated with increased expression [14, 21, 22]. The lack of association between methylation and gene expression supported the hypothesis that the methylation of these seven CGIs reflects the degree of overall epigenomic damage in gastric stem cells, and that the degree of epigenomic damage, and not the change of expression of individual genes, is associated with gastric cancer risk.

Epigenomic damage induced by *H. pylori* infection is one of the major causes of gastric cancer [23–26], but it is not known whether the epigenomic damage is independent of other risk factors. For example, salt intake is a risk factor for gastric cancer [27, 28], and although it does not induce methylation in gastric mucosae by itself in a Mongolian gerbil model [29, 30], it shows synergistic effects with *H. pylori* on cancer development [31]. It is not known yet whether epigenomic damage in the gastric mucosa provides independent information from past salt exposure or whether the exposure is already reflected in methylation levels. Multivariate analysis in a large cohort with a reliable record of history of salt intake will clarify this issue, and might provide a risk marker that complements the epigenetic gastric cancer risk markers.

In conclusion, we identified seven CGIs whose methylation levels are increased after *H. pylori* infection, and

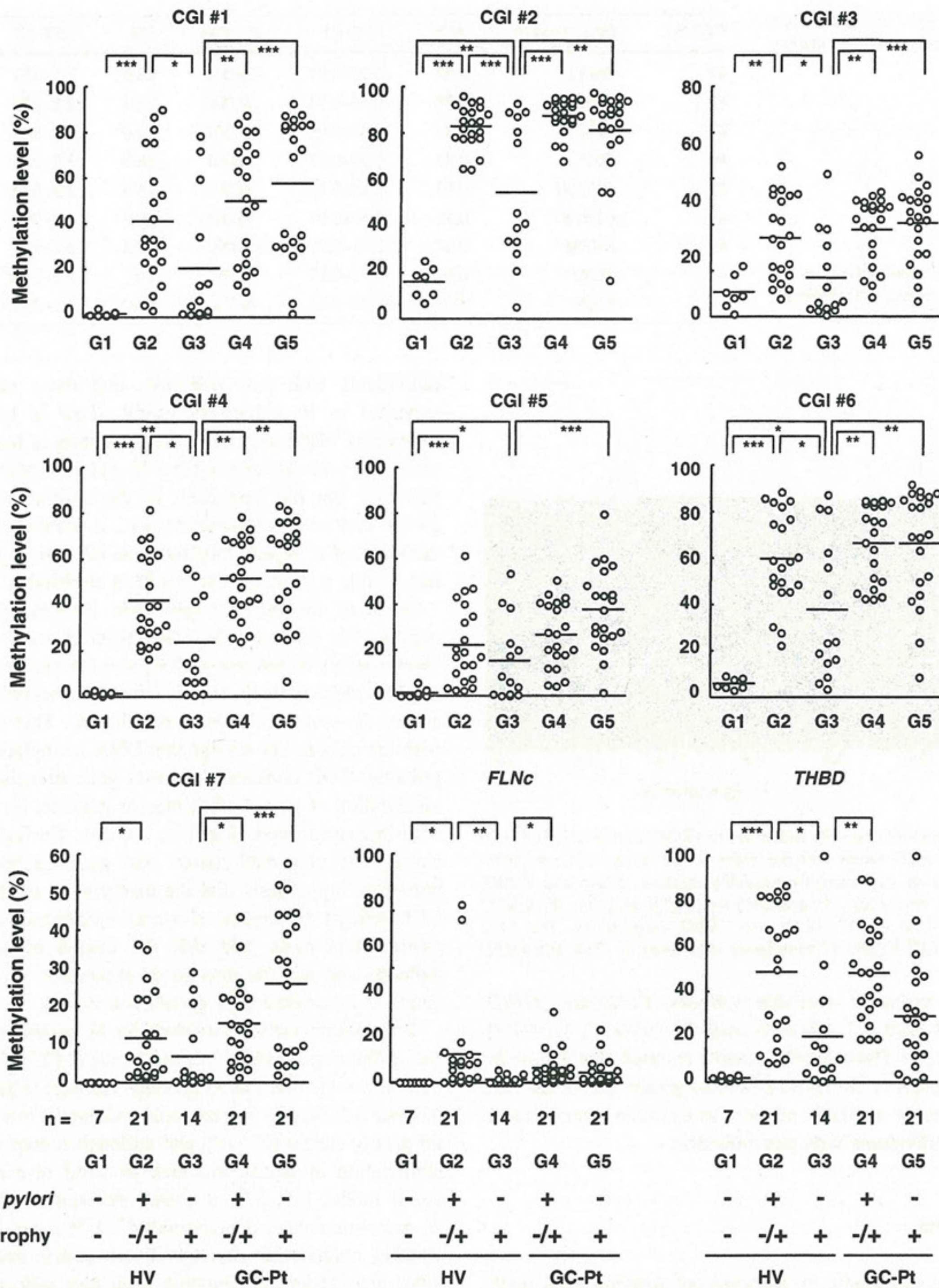

Fig. 2 Methylation levels of the seven CGIs and two currently available markers, *FLNc* and *THBD*, in the validation set. The *horizontal line* represents the mean methylation level in each group. Methylation levels of the seven CGIs in Group 5 (G5) were significantly higher than those in G3 ($P < 0.01$), but there were no significant differences for the two currently available markers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

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Clinical Cancer Research

Molecular Pathways: Involvement of *Helicobacter pylori*–Triggered Inflammation in the Formation of an Epigenetic Field Defect, and Its Usefulness as Cancer Risk and Exposure Markers

Toshikazu Ushijima and Naoko Hattori

Abstract

Infection-associated cancers account for a large proportion of human cancers, and gastric cancer, the vast majority of which is associated with *Helicobacter pylori* infection, is a typical example of such cancers. Epigenetic alterations are known to occur frequently in gastric cancers, and *H. pylori* infection has now been shown to induce aberrant DNA methylation in gastric mucosae. Accumulation of aberrant methylation in gastric mucosae produces a field for cancerization, and methylation levels correlate with gastric cancer risk. *H. pylori* infection induces methylation of specific genes, and such specificity is determined by the epigenetic status in normal cells, including the presence of H3K27me3 and RNA polymerase II (active or stalled). Specific types of inflammation, such as that induced by *H. pylori* infection, are important for methylation induction, and infiltration of monocytes appears to be involved. The presence of an epigenetic field defect is not limited to gastric cancers and is observed in various types of cancers. It provides translational opportunities for cancer risk diagnosis incorporating life history, assessment of past exposure to carcinogenic factors, and cancer prevention. *Clin Cancer Res; 18(4); 923–9. ©2011 AACR.*

Background

Infection-associated cancers account for a large proportion of human cancers. These include gastric cancers induced by *Helicobacter pylori* (1), hepatocellular carcinomas induced by hepatitis C virus (HCV) and hepatitis B virus [HBV (2–4)], cervical cancers induced by human papilloma virus [HPV (5, 6)], and lymphomas and nasopharyngeal cancers associated with Epstein-Barr virus [EBV (7, 8)]. The carcinogenic mechanisms of these infection-associated cancers have been extensively investigated, and although multiple contributing mechanisms have been clarified, they are not yet completely understood.

General mechanisms of infection-associated cancers

Virus-associated cancers have complex mechanisms of carcinogenesis. Viral oncogenes, such as E6 and E7 of HPV and X protein of HBV, can be integrated into host cells and produce aberrant growth signals and inactivate tumor-suppressor genes (6). Also, integration of virus genes into the host genome can alter the expression of nearby tumor-related genes and induce a genomic instability that will eventually contribute to cancer development (4). Even if the virus genes are not integrated, they can be persistently expressed and perturb important cellular signaling, such as cell proliferation, apoptosis, and cytokine expression, as in the case of HCV and EBV (7).

Both bacterial and viral infections can be associated with severe tissue damage and resultant chronic inflammation (4, 6, 7). Tissue damage itself activates cell proliferation and increases the chance that mutations will occur. In addition, chronic inflammation is considered to be deeply involved in cancer development and progression by multiple mechanisms, such as increased production of active oxygen species, induction of inflammation-mediated cell proliferation, and increased cytokine production (9, 10). In addition to this, induction of epigenetic alterations is now recognized as one of the mechanisms underlying induction of cancer by chronic inflammation.

H. pylori infection and epigenetic alterations in gastric cancers

Gastric cancer is still the third-leading cause of death from cancer in men and the fifth-leading cause in women worldwide, although its incidence is gradually decreasing (11). The vast majority of gastric cancers are caused by *H. pylori* infection (12), which is a Gram-negative bacterium (13). A minor percentage (~10%) of gastric cancers are associated with EBV infection (14). It is known that when *H. pylori* infects the human stomach, it induces severe inflammation, including ulcers, then chronic inflammation, and finally gastric cancers within tens of years. Investigators have mainly discussed the carcinogenic mechanisms of *H. pylori* from

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the standpoint of induction of cell proliferation, mutations, and direct activation of cellular signaling (15–17).

However, tumor-suppressor genes such as *CDKN2A*, *CDH1*, *MLH1*, and *RUNX3* are inactivated more frequently by aberrant DNA methylation than by mutations, indicating that gastric cancer is an epigenetic disease (18). In addition to methylation silencing of driver tumor-suppressor genes, recent genome-wide analyses have revealed that hundreds of passenger genes are also methylated in gastric cancers (19). The fact that *H. pylori* infection induces epigenetic alterations provides the missing link between the causal role of *H. pylori* infection in gastric carcinogenesis and the deep involvement of epigenetic alterations in gastric cancers. Gastric cancer is a typical example of a disease in which infection, chronic inflammation, and epigenetic alterations are interconnected.

Induction of epigenetic alterations by *H. pylori* and the formation of field defects

The first hint that the presence of aberrant DNA methylation might be associated with *H. pylori* infection came from the observation that promoter methylation of *CDH1* was detected more frequently in the gastric mucosae of individuals with *H. pylori* infection than in those without the infection (20). By quantifying the methylation levels of 8 marker CpG islands, Maekita and colleagues (21) convincingly showed that individuals with *H. pylori* infection have much higher methylation levels in their gastric mucosae (5.4- to 303-fold) than those without ($P < 0.0001$). In addition to the 8 marker CpG islands associated with protein-coding genes, CpG islands of microRNA genes are also methylated in association with *H. pylori* infection (22, 23).

In one study, patients with gastric cancer who had previously had an *H. pylori* infection but were currently not infected had lower methylation levels of the 8 marker CpG islands in the gastric mucosae compared with patients who were currently infected with *H. pylori* (21). This suggests that the methylation level is very high when active *H. pylori* infection is present in the stomach and decreases to certain levels when the infection is discontinued. In other studies, various degrees of decrease were observed in individuals who received eradication therapy for *H. pylori* (24, 25), and the methylation level after the decrease was considered to represent the degree of epigenomic damage to the individual. This decrease of methylation could be due to a turnover of gastric epithelial cells with methylation or to the removal of 5-methylcytosine, which is present in individuals with active *H. pylori* infection.

Of importance, among individuals without current *H. pylori* infection, the methylation levels of the 8 marker CpG islands in gastric mucosae were shown to correlate with gastric cancer risk (21, 26). Patients with gastric cancers had 2.2- to 32-fold higher methylation levels in gastric mucosae compared with healthy individuals (21), and patients with multiple gastric cancers had significantly higher methylation levels than those with a single gastric cancer (26). This correlation strongly supports the notion that the accumulation of aberrant methylation in gastric mucosae produces an epigenetic field for cancerization, i.e., a field defect (Fig. 1; ref. 27).

Epigenetic field for cancerization

In the epigenetic field for gastric cancers, tumor-suppressor genes that are causally involved in gastric cancer development (i.e., driver genes), such as *CDKN2A*, *CDH1*, *MLH1*, and *RUNX3*, are methylated only at very low levels, showing that such events are present only in a very small fraction of cells (21). In contrast, many other genes that are unlikely to be causally involved in gastric carcinogenesis (i.e., passenger genes), such as *HAND1* (a transcription factor involved in heart morphogenesis), are methylated at high levels, showing that their methylation is present in a large fraction of cells. Most of the genes that are highly methylated in gastric cancers are either unexpressed or expressed only at low levels in normal cells (28). Generally, genes with low expression are susceptible to methylation induction (29), and it is considered that most of the genes that are methylated in the epigenetic field were methylated as a consequence of gastric carcinogenesis. In addition to accumulation of aberrant methylation, an epigenetic field involves hypomethylation of the Alu and Satα repeat sequences (30), which potentially can be involved in genomic instability.

Epigenetic field defects are present not only in gastric cancers but in other cancers as well (27). In the case of hepatocellular carcinoma, aberrant DNA methylation was frequently observed in noncancerous tissues of cancer patients compared with normal livers of patients with metastatic liver tumors (31). A quantitative analysis revealed increased methylation of multiple tumor-suppressor genes, such as *SOCS1*, *RASSF1A*, and *CDH1*, in HCV-infected, noncancerous liver tissues (32), suggesting the importance of an epigenetic field for HCV-associated hepatocarcinogenesis. In the case of esophageal adenocarcinoma, the presence of *APC* and *CDKN2A* methylation in Barrett's metaplasia has been reported (33), and such methylation was shown to be associated with progression of Barrett's metaplasia (34). Also in the case of esophageal squamous cell carcinoma, methylation of specific genes, such as *UCHL1* and *HOXA9*, in esophageal mucosae was associated with the risk of developing esophageal squamous cell carcinoma (35, 36). In ulcerative colitis, the driver gene *CDKN2A* and passenger genes such as *MYOD* and *ESR* were shown to be methylated in colonic mucosae, which are predisposed to colon cancers (37, 38). In addition, in the case of sporadic colorectal cancers, *MGMT* methylation in cancer tissues was associated with high levels of *MGMT* methylation in the background colonic mucosae (39). The presence of epigenetic field defects has also been indicated for breast (40), renal (41), and bladder cancers (42, 43).

Critical roles of specific types of inflammation in methylation induction

The association between *H. pylori* infection and high levels of DNA methylation in gastric mucosae in humans strongly indicates that *H. pylori* infection induces aberrant

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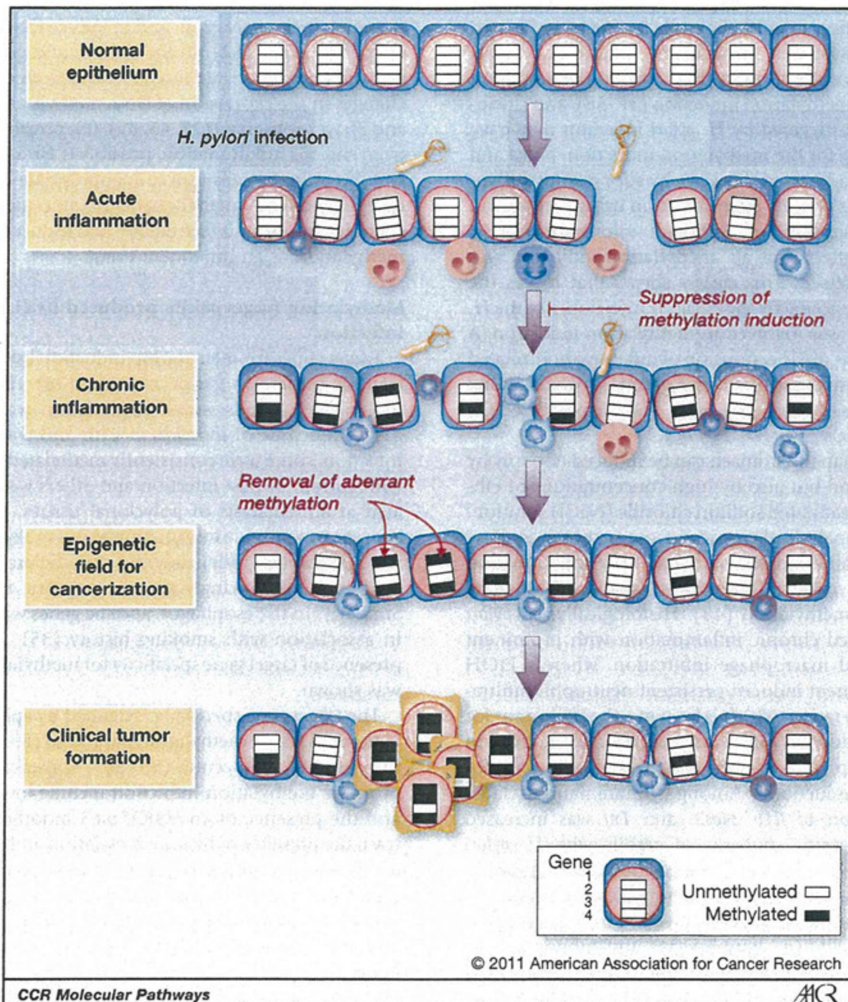

Figure 1. Formation of epigenetic field for cancerization by chronic inflammation triggered by *H. pylori* infection. *H. pylori* infection induces acute inflammation, followed by chronic inflammation, formation of an epigenetic field for cancerization, and development of gastric cancers. Aberrant methylation is induced in driver genes (schematically represented by gene 1) and passenger genes (genes 3 and 4). Specific genes are methylated in gastric mucosae with *H. pylori* infection, and driver genes usually have very low methylation levels. On the other hand, passenger genes that have low or no expression in normal cells usually have high methylation levels. The methylation level of some passenger genes reflects the degree of accumulation of epigenomic damage, and correlates with gastric cancer risk. Chronic inflammation triggered by *H. pylori* infection is critical for methylation induction, and if data from a mouse colitis model are combined, the importance of monocytes can be speculated. As translational targets, methylation levels of specific genes in normal-appearing tissues can be used as a cancer risk marker that reflects a person's life. The methylation signature has potential as a marker for past exposure to specific environmental factors. Suppression of induction of aberrant DNA methylation, and possibly removal of accumulated aberrant methylation can be used for cancer prevention (shown in red).

DNA methylation. This cause-consequence relationship was shown with the use of Mongolian gerbils, in which *H. pylori* infection-induced gastritis and gastric cancers can be recapitulated (44). Gerbils infected with *H. pylori* developed severe gastritis and had markedly increased methylation levels, showing the causal role of *H. pylori* infection in methylation induction (45). The methylation levels were clearly decreased after eradication of the *H. pylori*, in agreement with the decreased methylation levels observed in patients who received eradication therapy.

In the attempt to determine how *H. pylori* induces methylation, investigators have considered both direct and indirect actions of *H. pylori*. First, because *H. pylori* possesses multiple DNA methyltransferases (46) and a type IV secretion system [a syringe-like structure capable of delivering bacterial materials into a host cell (47)], *H. pylori* itself may

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induce methylation in epithelial cells by injecting its own DNA methyltransferases. Alternatively, studies in patients with ulcerative colitis showed that chronic inflammation played a role in methylation induction (37, 38), and chronic inflammation triggered by *H. pylori* infection may have been responsible for the methylation induction. Niwa and colleagues (45) addressed this issue by suppressing inflammation in gerbils with *H. pylori* infection using cyclosporin A, an immunosuppressant. Although colonization of *H. pylori* was not affected at all, methylation induction was markedly suppressed. This clearly shows that it was the inflammation triggered by the *H. pylori* infection, not the *H. pylori* itself, that was involved in methylation induction. A temporal analysis of the expression of inflammation-related genes in gastric mucosae of infected gerbils showed that the expression levels of *Cxcl2*, *Il1b*, *Nos2*, and *Tnf* paralleled the methylation levels.

Inflammation in the stomach can be induced not only by *H. pylori* infection but also by high concentrations of ethanol (EtOH) or saturated sodium chloride (NaCl) solution. A methylation analysis of gastric mucosae exposed to these kinds of inflammation showed that only inflammation triggered by *H. pylori* infection was capable of inducing aberrant DNA methylation (48). Histologically, *H. pylori* infection induced chronic inflammation with prominent lymphocyte and macrophage infiltration, whereas EtOH and NaCl treatment induced persistent neutrophil infiltration. Cell proliferation, which is known to be important for methylation induction (38), was most strongly induced in the NaCl group and was shown to be insufficient for methylation induction. Among inflammation-related genes, expression of *Il1b*, *Nos2*, and *Tnf* was increased specifically in gastric mucosae of gerbils with *H. pylori* infection. Therefore, it is considered that specific types of inflammation are necessary for methylation induction.

Chronic inflammation is characterized by infiltration of mononuclear cells, i.e., lymphocytes and monocytes. To clarify which cell type(s) plays the major role in methylation induction, Katsurano and colleagues (49) examined SCID mice, which lack both B and T lymphocytes. Because *H. pylori* cannot infect mice efficiently, they used a colitis model induced by dextran sulfate sodium (DSS). Even in SCID mice, DNA methylation and colon tumors could be induced at the same levels as in wild-type mice. This shows that lymphocytes are dispensable for methylation induction, and strongly suggests that monocytes are important. Expression of *Ifng*, *Il1b*, and *Nos2* was induced in both wild-type and SCID mice by DSS treatment.

If we hypothesize that the same effectors are working in gerbil stomachs infected by *H. pylori* and mouse colons treated with DSS, we can conclude that expression of *Il1b* and *Nos2* may be involved in methylation induction. Promoter polymorphisms of *IL1B* are reported to be associated with human gastric cancer susceptibility by increasing or decreasing IL1β production in response to *H. pylori* infection and thus the progression of gastric atrophy (50, 51). Increased production of NO *in vitro* is reported to increase the enzyme activity of DNA methyltransferases without changing their expression, and to induce DNA methylation of specific genes (52). In the human and gerbil stomachs infected by *H. pylori* and mouse colons treated with DSS, no changes in the expression of DNA methyltransferases 1, 3a, and 3b were observed (28, 45, 49). It is possible that a signal from chronic inflammation, possibly IL1β, and elevation of NO in epithelial cells lead to inappropriate localization of deregulated DNA methyltransferase(s) to methylation-susceptible CpG islands (see below) and induce aberrant DNA methylation as an infrequent event.

Methylation fingerprints produced by *H. pylori* infection

Target genes for methylation induction by *H. pylori* infection are present in gastric mucosae (28). Among 48 promoter CpG islands whose methylation was analyzed in gastric mucosae of individuals with and without *H. pylori* infection, some were consistently methylated in individuals with current or past infection and others were not methylated at all. Analysis of polyclonal tissues, unlike that of cancers, can provide information about multiple events that have taken place independently, and the presence of target genes was convincingly shown in gastric mucosae (29). Similarly, in the esophagus, specific genes were methylated in association with smoking history (35), and again the presence of target gene specificity for methylation induction was shown.

The target gene specificity is defined by epigenetic factors in the cells where methylation is induced (29, 53, 54) and in the genome architecture (55, 56). Epigenetic factors that promote methylation induction include low transcription and the presence of an H3K27me3 modification. In contrast, the presence of histone acetylation and RNA polymerase II (active or stalled) protects CpG islands from becoming methylated. A multivariate analysis revealed that the most influential factors are the promoting effect of H3K27me3 and the protective effect of RNA polymerase II (54). A genomic factor that promotes methylation induction is a distant location from repetitive elements (55, 56). It is currently speculated that infection by *H. pylori* induces H3K27me3 and removes RNA polymerase II at its target genes, and that these genes then become methylated.

Clinical-Translational Advances

Cancer risk marker that reflects life history

The importance of predicting cancer risks has been repeatedly emphasized because the ability to select high-risk individuals enables efficient cancer screening and reduces social costs (57–59). To this end, a massive effort has been made in association studies, and many cancer risk alleles for common cancers have been identified. Most of these risk alleles give odds ratios between 1.5 and 2.0 (51, 58, 59), and can be used to estimate cancer risk when a person is born.

At the same time, a person is exposed to various environmental carcinogenic factors, and the cancer risk of an adult will differ depending on what sort of life he or she has

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spent. Therefore, a cancer risk marker that incorporates information about life-to-date is important. At least some of a person's life history, such as smoking and infection by *H. pylori*, is imprinted on the epigenome and produces an epigenetic field for cancerization. The severity of the field can be measured as methylation levels of specific marker genes, and correlates with cancer risk. The odds ratios obtained by DNA methylation markers of gastric cancers, such as *THBD*, *FLNc*, and *miR-124a*, range from 2.4 to 22.1 [calculated based on our previous reports (21, 22)]. Passenger genes can be useful as marker genes because they are consistently methylated and have high methylation levels in noncancerous tissues (21, 27), whereas driver genes are only stochastically methylated and have low methylation levels (Fig. 1). Therefore, for evaluating the degree of epigenomic damage that has been done in the past, methylation of passenger genes is often superior to that of driver genes.

The presence of an epigenetic field defect is also known for other types of cancers, as mentioned above (27). Therefore, investigators are now developing methods to estimate epigenetic cancer risk, taking life history into account, in various types of cancer. For example, a multicenter study was conducted to evaluate the validity of methylation markers to predict progression of Barrett's esophagus, and methylation of *HPP1*, *CDKN2A*, and *RUNX3* were shown to be informative (34).

Marker for past exposure to specific environmental factors

H. pylori infection is associated with methylation of a specific set of genes, most of which are considered as passengers, in gastric mucosae (28). A history of smoking is associated with methylation of *UCHL1* and *HOXA9*, which are also considered to be passengers, in esophageal mucosae (35, 36). Once the specificity of methylation signatures to various carcinogenic agents is clarified, past exposure to such carcinogenic factors can be estimated by the methylation signature. The methylation signature has advantages over other exposure markers because it persists for a long time and does not require any record by humans. For example, past exposure to *H. pylori* infection can be estimated by serum antibody, but it persists only up to several years after *H. pylori* infection discontinues (60). The ability to estimate past exposure using a methylation signature would be very helpful from an epidemiological viewpoint.

Epigenetic cancer prevention

The presence of an epigenetic field for cancerization and the deep involvement of chronic inflammation in its formation provide targets for cancer prevention. Suppression of induction of aberrant DNA methylation is expected to lead to a decreased incidence of cancers. This concept is supported by animal models for macroscopic colon tumors (61, 62), lung tumors (63), and prostate cancers (64, 65). It was shown that in gerbil stomachs, administration of a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), decreased the incidence of gastric cancers induced by *H. pylori* infection and a mutagen, *N*-methyl-*N*-nitrosourea (unpublished). In addition to suppression of DNA methylation induction, suppression of H3K27me3, a premarker for DNA methylation induction, is also an attractive target. The histone methyltransferase that is responsible for this modification, EZH2, is known to be overexpressed in aggressive tumors (66) and precancerous lesions (67), and therefore inhibitors of EZH2, such as 3-deazaneplanocin A (66), may have preventive applications.

Anti-inflammatory drugs, especially nonsteroidal anti-inflammatory drugs (NSAIDs), are effective for prevention of at least some cancers, but their use is still limited to individuals with high risk (68). The use of NSAIDS is limited in part because of possible side-effects, such as peptic ulcer. To avoid such side-effects and suppress the pathways that are responsible for cancer development, researchers are actively investigating the mechanisms of cancer induction by chronic inflammation (69). Because induction of epigenetic alterations is one of these important mechanisms, suppression of specific components of inflammation that are responsible for induction of epigenetic alterations is expected to provide a good target for cancer prevention.

Lastly, DNA methylation is reversible by DNA demethylating agents, such as 5-aza-dC and 5-azacytidine (70). Currently available demethylating agents do not have a high specificity for aberrantly methylated genes, and can demethylate normally methylated sequences. Such sequences include normally methylated CpG islands and repetitive sequences originating from retrotransposons, and it is feared that DNA demethylating agents might induce demethylation of these retrotransposons. Therefore, for cancer prevention using current demethylating agents, we must carefully balance risk and benefit, and probably such agents are not widely indicated. However, many epigenetic drugs are being developed, and it is possible that some of the new demethylating agents will have a specificity or preference for aberrantly methylated promoter CpG islands, and can be used in a wider range of individuals in the future.

Conclusions

The fact that *H. pylori* infection induces aberrant DNA methylation in gastric mucosae provides the missing link between the major role of *H. pylori* infection in gastric cancers and the deep involvement of epigenetic alterations in gastric cancers. The severity of infection correlates with gastric cancer risk and can provide a unique cancer risk marker that reflects a person's life. *H. pylori* infection has been shown to induce methylation of specific genes, and there are underlying mechanisms. The methylation signature has potential as a marker for past exposure to *H. pylori* infection. Specific types of inflammation, such as that induced by *H. pylori* infection, are capable of inducing aberrant methylation, and monocytes appear to be involved in the induction. Suppression of methylation induction,

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specific inflammatory processes, and reversal of epigenetic alterations are targets for cancer prevention.

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References

1. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
2. Kremsdorf D, Soussan P, Paterlini-Brechot P, Brechot C. Hepatitis B virus-related hepatocellular carcinoma: paradigms for viral-related human carcinogenesis. Oncogene 2006;25:3823–33.
3. Levrero M. Viral hepatitis and liver cancer: the case of hepatitis C. Oncogene 2006;25:3834–47.
4. Feitelson MA, Lee J. Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. Cancer Lett 2007;252:157–70.
5. Whiteside MA, Siegel EM, Unger ER. Human papillomavirus and molecular considerations for cancer risk. Cancer 2008;113[Suppl]: 2981–94.
6. Martin D, Gutkind JS. Human tumor-associated viruses and new insights into the molecular mechanisms of cancer. Oncogene 2008;27[Suppl 2]:S31–42.
7. Saha A, Robertson ES. Epstein-Barr virus-associated B-cell lymphomas: pathogenesis and clinical outcomes. Clin Cancer Res 2011;17: 3056–63.
8. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
9. Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. Int J Cancer 2007;121:2373–80.
10. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883–99.
11. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
12. Correa P. Bacterial infections as a cause of cancer. J Natl Cancer Inst 2003;95:E3.
13. Asaka M, Kato M, Takahashi S, Fukuda Y, Sugiyama T, Ota H, et al. Japanese Society for *Helicobacter* Research. Guidelines for the management of *Helicobacter pylori* infection in Japan: 2009 revised edition. Helicobacter 2010;15:1–20.
14. Fukayama M. Epstein-Barr virus and gastric carcinoma. Pathol Int 2010;60:337–50.
15. Peek RM Jr, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2002;2:28–37.
16. Matsumoto Y, Marusawa H, Kinoshita K, Endo Y, Kou T, Morisawa T, et al. *Helicobacter pylori* infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. Nat Med 2007;13:470–6.
17. Hatakeyama M. *Helicobacter pylori* CagA—a bacterial intruder conspiring gastric carcinogenesis. Int J Cancer 2006;119:1217–23.
18. Ushijima T, Sasako M. Focus on gastric cancer. Cancer Cell 2004; 5:121–5.
19. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2¢-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci 2006;97:64–71.
20. Chan AO, Lam SK, Wong BC, Wong WM, Yuen MF, Yeung YH, et al. Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer. Gut 2003;52:502–6.
21. Maekita T, Nakazawa K, Mihara M, Nakajima T, Yanaoka K, Iguchi M, 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.
22. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, 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 2009;124:2367–74.
23. Suzuki H, Yamamoto E, Nojima M, Kai M, Yamano HO, Yoshikawa K, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 2010;31:2066–73.
24. Miyazaki T, Murayama Y, Shinomura Y, Yamamoto T, Watabe K, Tsutsui S, et al. E-cadherin gene promoter hypermethylation in *H. pylori*-induced enlarged fold gastritis. Helicobacter 2007;12:523–31.
25. Nakajima T, Enomoto S, Yamashita S, Ando T, Nakanishi Y, Nakazawa K, et al. Persistence of a component of DNA methylation in gastric mucosae after *Helicobacter pylori* eradication. J Gastroenterol 2010; 45:37–44.
26. Nakajima T, Maekita T, Oda I, Gotoda T, Yamamoto S, Umemura S, et al. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. Cancer Epidemiol Biomarkers Prev 2006;15:2317–21.
27. Ushijima T. Epigenetic field for cancerization. J Biochem Mol Biol 2007;40:142–50.
28. 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.
29. Takeshima H, Ushijima T. Methylation destiny: Moira takes account of histones and RNA polymerase II. Epigenetics 2010;5:89–95.
30. Yoshida T, Yamashita S, Takamura-Enya T, Niwa T, Ando T, Enomoto S, et al. Alu and Sata hypomethylation in *Helicobacter pylori*-infected gastric mucosae. Int J Cancer 2011;128:33–9.
31. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 2000;32:970–9.
32. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. Hepatology 2008;47:908–18.
33. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, et al. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 2000;60:5021–6.
34. Jin Z, Cheng Y, Gu W, Zheng Y, Sato F, Mori Y, et al. A multicenter, double-blinded validation study of methylation biomarkers for progression prediction in Barrett's esophagus. Cancer Res 2009;69:4112–5.
35. Oka D, Yamashita S, Tomioka T, Nakanishi Y, Kato H, Kaminishi M, 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 2009;115:3412–26.
36. Lee YC, Wang HP, Wang CP, Ko JY, Lee JM, Chiu HM, et al. Revisit of field cancerization in squamous cell carcinoma of upper aerodigestive tract: better risk assessment with epigenetic markers. Cancer Prev Res 2011;4:1982.
37. Hsieh CJ, Klump B, Holzmann K, Borchard F, Gregor M, Porschen R. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. Cancer Res 1998;58:3942–5.
38. 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.

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Published OnlineFirst December 28, 2011; DOI:10.1158/1078-0432.CCR-11-2011

39. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, et al. MGMT promoter methylation and field defect in sporadic colorectal cancer. J Natl Cancer Inst 2005;97:1330–8.
40. Yan PS, Venkataramu C, Ibrahim A, Liu JC, Shen RZ, Diaz NM, et al. Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. Clin Cancer Res 2006;12:6626–36.
41. Arai E, Kanai Y, Ushijima S, Fujimoto H, Mukai K, Hirohashi S. Regional DNA hypermethylation and DNA methyltransferase (DNMT) 1 protein overexpression in both renal tumors and corresponding nontumorous renal tissues. Int J Cancer 2006;119:288–96.
42. Wolff EM, Chihara Y, Pan F, Weisenberger DJ, Siegmund KD, Sugano K, et al. Unique DNA methylation patterns distinguish noninvasive and invasive urothelial cancers and establish an epigenetic field defect in premalignant tissue. Cancer Res 2010;70:8169–78.
43. Nishiyama N, Arai E, Chihara Y, Fujimoto H, Hosoda F, Shibata T, et al. Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. Cancer Sci 2010;101:231–40.
44. Tatematsu M, Nozaki K, Tsukamoto T. *Helicobacter pylori* infection and gastric carcinogenesis in animal models. Gastric Cancer 2003; 6:1–7.
45. Niwa T, Tsukamoto T, Toyoda T, Mori A, Tanaka H, Maekita T, et al. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res 2010;70:1430–40.
46. Vitkute J, Stankevicius K, Tamulaitiene G, Maneliene Z, Timinskas A, Berg DE, et al. Specificities of eleven different DNA methyltransferases of *Helicobacter pylori* strain 26695. J Bacteriol 2001;183:443–50.
47. Hatakeyama M. *Helicobacter pylori* and gastric carcinogenesis. J Gastroenterol 2009;44:239–48.
48. Hur K, Niwa T, Toyoda T, Tsukamoto T, Tatematsu M, Yang HK, et al. Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation. Carcinogenesis 2011;32:35–41.
49. Katsurano M, Niwa T, Yasui Y, Shigematsu Y, Yamashita S, Takeshima H, et al. Early-stage formation of an epigenetic field defect in a mouse colitis model, and non-essential roles of T- and B-cells in DNA methylation induction. Oncogene 2011. Jun 20 [Epub ahead of print].
50. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402.
51. Loh M, Koh KX, Yeo BH, Song CM, Chia KS, Zhu F, et al. Meta-analysis of genetic polymorphisms and gastric cancer risk: variability in associations according to race. Eur J Cancer 2009;45:2562–8.
52. Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. J Exp Med 1999;190:1595–604.
53. McCabe MT, Lee EK, Vertino PM. A multifactorial signature of DNA sequence and polycomb binding predicts aberrant CpG island methylation. Cancer Res 2009;69:282–91.
54. Takeshima H, Yamashita S, Shimazu T, Niwa T, Ushijima T. The presence of RNA polymerase II, active or stalled, predicts epigenetic fate of promoter CpG islands. Genome Res 2009;19:1974–82.
55. Estécio MR, Gallegos J, Vallot C, Castoro RJ, Chung W, Maegawa S, et al. Genome architecture marked by retrotransposons modulates predisposition to DNA methylation in cancer. Genome Res 2010;20: 1369–82.
56. Takeshima H, Yamashita S, Shimazu T, Ushijima T. Effects of genome architecture and epigenetic factors on susceptibility of promoter CpG islands to aberrant DNA methylation induction. Genomics 2011;98: 182–8.
57. Ponder BA. Cancer genetics. Nature 2001;411:336–41.
58. Amir E, Freedman OC, Seruga B, Evans DG. Assessing women at high risk of breast cancer: a review of risk assessment models. J Natl Cancer Inst 2010;102:680–91.
59. Kim ST, Cheng Y, Hsu FC, Jin T, Kader AK, Zheng SL, et al. Prostate cancer risk-associated variants reported from genome-wide association studies: meta-analysis and their contribution to genetic variation. Prostate 2010;70:1729–38.
60. Ekström AM, Held M, Hansson LE, Engstrand L, Nyrén O. *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. Gastroenterology 2001;121:784–91.
61. Eads CA, Nickel AE, Laird PW. Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. Cancer Res 2002;62:1296–9.
62. Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, et al. Suppression of intestinal neoplasia by DNA hypomethylation. Cell 1995;81:197–205.
63. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, et al. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 2003;63:7089–93.
64. McCabe MT, Low JA, Daignault S, Imperiale MJ, Wojno KJ, Day ML. Inhibition of DNA methyltransferase activity prevents tumorigenesis in a mouse model of prostate cancer. Cancer Res 2006; 66:385–92.
65. Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML. 5-aza-2¢-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clin Cancer Res 2007;13: 2136–43.
66. Chase A, Cross NC. Aberrations of EZH2 in cancer. Clin Cancer Res 2011;17:2613–8.
67. Ding L, Erdmann C, Chinnaiyan AM, Merajver SD, Kleer CG. Identification of EZH2 as a molecular marker for a precancerous state in morphologically normal breast tissues. Cancer Res 2006;66: 4095–9.
68. Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. Lancet Oncol 2009;10: 501–7.
69. Aggarwal BB, Vijayalekshmi RV, Sung B. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res 2009;15:425–30.
70. Issa JP, Kantarjian HM. Targeting DNA methylation. Clin Cancer Res 2009;15:3938–46.

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