Figure 4. Significance of DNA methylation alterations at precancerous stages during urothelial carcinogenesis. (A) Histological features of superficial papillary UC and nodular invasive UC. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethrocystoscopic resection because of recurrences. By contrast, the clinical outcome of nodular invasive carcinomas is poor. **(B)** Scattergrams of the signal ratios in noncancerous urothelia obtained from patients with superficial UCs, noncancerous urothelia obtained from patients with invasive UCs, and invasive UCs themselves. Wilcoxon test revealed that the signal ratios of 131 bacterial artificial chromosome (BAC) clones differed significantly between noncancerous urothelia obtained from patients with superficial UCs and noncancerous urothelia obtained from patients with invasive UCs. If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly higher than those in noncancerous urothelia obtained from patients with superficial UCs (67 BAC clones), the average signal ratios in invasive UCs themselves were even higher than (42 BAC clones such as clone 1), or not significantly different from (25 BAC clones such as clone 2), those in noncancerous urothelia obtained from patients with invasive UCs without exception. If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly lower than those in noncancerous urothelia obtained from patients with superficial UCs (64 BAC clones), the average signal ratios in invasive UCs themselves were even lower than (38 BAC clones such as clone 3), or not significantly different from (26 BAC clones such as clone 4), those in noncancerous urothelia obtained from patients with invasive UCs without exception. Therefore, DNA methylation profiles of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves.
UC: Urothelial carcinoma.

not with a sensitivity and specificity of 100% [55]. Thus, after validation using other technologies such as pyrosequencing, a combination of CpG sites on the present nine BAC clones may provide an optimal indicator for the development of intravesical metachronous UC.

Prognostication of patients with cancers based on DNA methylation profiles

Some RCCs relapse and metastasize to distant organs, even if resection has been considered complete. Recently, immunotherapy and novel targeting agents have been developed for treatment of RCC. However, unless relapsed or metastasized tumors are diagnosed early by close follow-up, the effectiveness of any therapy is very restricted. Therefore, to assist close follow-up of patients who have undergone nephrectomy and are still at risk of recurrence and metastasis, prognostic indicators have been explored. Among the examined patients in the abovementioned cluster B_{TK}, 38% died owing to recurrent RCCs, whereas only 2.3% of the patients in cluster A_{TK} died. Multivariate analysis revealed that our clustering was a predictor of recurrence and was independent of histological grade, macroscopic configuration, vascular involvement and renal vein tumor thrombi [60]. We were able to set the cutoff values of the signal ratios for 14 BAC clones to determine whether or not patients in this cohort belonged to cluster B_{TK} with a sensitivity and specificity of 100% [60].

To establish criteria for prognostication of patients with HCCs, in the learning cohort, HCC samples obtained from patients who had survived more than 4 years after hepatectomy and HCC samples obtained from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test revealed that the signal ratios of 41 BAC clones differed significantly between the two groups (n = 19). We established the criteria for prognostication by combining the cutoff values of signal ratios for the 41 BAC clones (Figure 3B) [65]. Multivariate analysis revealed that satisfying the criteria for 32 or more BAC clones was a predictor of recurrence, and was independent of histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity [65]. The cancer-free and overall survival rates of patients with HCCs in the validation cohort (n = 44) satisfying the criteria for 32 or more BAC clones were significantly lower than those of patients with HCCs satisfying the criteria for less than 32 BAC clones [65]. Such prognostication using biopsy or hepatectomy specimens may be able to assist clinicians in devising therapeutic strategies for patients with insufficient liver function.

Recently, new forms of systemic chemotherapy and targeted therapy have been developed for treatment of UCs. In order to start adjuvant systemic chemotherapy immediately in patients who have undergone surgery and are still at high risk of recurrence and metastasis, prognostic indicators have been explored. It is expected that a combination of several CpG islands of tumor-related genes would be useful as epigenetic markers for prognostication of UCs [70]. In addition, when we applied BAMCA to UCs, unsupervised 2D hierarchical clustering analysis based on BAMCA data for UCs was

able to group the examined patients into two subclasses, clusters A_{TU} and B_{TU}. Among the patients belonging to cluster B_{TU}, 19% suffered recurrence after surgery, whereas none belonging to cluster A_{TU} did so [55]. Wilcoxon test revealed that the signal ratios of 20 BAC clones in UCs differed significantly between the patients who suffered recurrence after surgery and the patients who did not. The criteria for a combination of the 20 BAC clones were able to discriminate patients who suffered recurrence after surgery from patients who did not with a sensitivity and specificity of 100%, whereas a high histological grade, invasive growth (pT2 or more) and vascular or lymphatic involvement were incapable of such complete discrimination [55]. The reliability of such prognostication will need to be validated in a prospective study.

Future perspective

The incidence of DNA methylation alterations is generally high in various organs during multistage carcinogenesis. Since even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, and these can be detected using highly sensitive methodology. Therefore, they may be better diagnostic indicators than mRNA and protein-expression profiles, which can be easily affected by the microenvironment of cancer cells or precursor cells. Genome-wide DNA methylation profiling can provide indicators for carcinogenetic risk estimation and prognostication using samples of urine, sputum and other body fluids, and also biopsy and surgically resected specimens. However, exploitation of diagnostic indicators can never be regarded as optimal, and it is expected that ongoing technical innovation and prospective validation will lead to further improvements of diagnostic sensitivity and specificity.

Patients with cancers are frequently clustered into subclasses showing both distinct genome-wide DNA methylation profiles and distinct clinicopathological characteristics (FIGURE 1B). Such clustering of cancers may provide clues for clarification of the molecular mechanisms establishing the distinct DNA methylation profiles of each cluster and the identification of target molecules for prevention and therapy in

Executive summary

Introduction

- Human cancer cells show a drastic change in DNA methylation status, that is overall DNA hypomethylation and regional DNA hypermethylation.
- DNA methylation alterations are known to result in altered expression of tumor-related genes and chromosomal instability in human cancers.

DNA methylation alterations during multistage carcinogenesis

- DNA methylation alterations play a significant role even at the precancerous stage, especially in association with chronic inflammation and persistent infection with viruses, such as hepatitis B virus or hepatitis C virus.
- DNA methyltransferase 1 overexpression in cancers is frequently correlated with accumulation of DNA methylation of tumor-related genes and poorer patient outcome.

Genome-wide DNA methylation analysis

- For genome-wide analysis, microarray platforms are used in combination with DNA methylation-sensitive restriction enzyme-based or antimethyl-cytosine antibody affinity techniques, and new generation sequencing technologies are also being introduced.
- Bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) may be suitable for overviewing the DNA methylation tendency of individual large regions among all chromosomes.

Genome-wide DNA methylation profiles at precancerous stages are inherited by cancers & determine tumor aggressiveness

- Distinct DNA methylation profiles in noncancerous tissue at the precancerous stage is basically inherited by the cancer developing in each individual patient.
- DNA methylation alterations at the precancerous stage, which may not occur randomly but may foster further epigenetic and genetic alterations, can generate more malignant cancers and even determine patient outcome.

Carcinogenetic risk estimation based on DNA methylation profiles

- On the basis of BAMCA data, criteria for estimation of the risk of hepatocellular carcinoma and urothelial carcinoma development have been established.

Prognostication of patients with cancers based on DNA methylation profiles

- On the basis of BAMCA data, criteria for the prognostication of patients with renal cell carcinomas, hepatocellular carcinomas and urothelial carcinomas have been established.

Future perspective

- Genome-wide DNA methylation profiling can provide indicators for carcinogenetic risk estimation and prognostication using samples of body fluids and tissue specimens.
- Based upon genome-wide DNA methylation profiling, translational epigenetics has come of age.

patients belonging to each cluster. Based upon of genome-wide DNA methylation profiling, translational epigenetics has clearly come of age.

Financial & competing interests disclosure

This study was supported by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, a Grant from the New Energy and Industrial Technology Development Organization (NEDO), and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio). The authors have no other relevant affiliations or financial involvement with any organization or entity with financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Bibliography

Papers of special note have been highlighted as:
- ▪ of interest

1 Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* 3, 415–428 (2002).

2 Jones PA, Baylin SB: The epigenomics of cancer. *Cell* 128, 683–692 (2007).

3 Sharma S, Kelly TK, Jones PA: Epigenetics in cancer. *Carcinogenesis* 31, 27–36 (2009).

4 Cedar H, Bergman Y: Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* 10, 295–304 (2009).

5 Delcuve GP, Rastegar M, Davie JR: Epigenetic control. *J. Cell Physiol.* 219, 243–250 (2009).

6 Illingworth RS, Bird AP: CpG islands – 'a rough guide'. *FEBS Lett.* 583, 1713–1720 (2009).

7 Mohn F, Schubeler D: Genetics and epigenetics: stability and plasticity during cellular differentiation. *Trends. Genet.* 25, 129–136 (2009).

8 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.* USA 92, 7416–7419 (1995).

▪ **E-cadherin tumor suppressor gene was shown to be silenced by DNA methylation, and the universality of a 'two-hit' mechanism involving DNA hypermethylation and loss of heterozygosity during carcinogenesis has been proposed.**

9 Baylin SB, Ohm JE: Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? *Nat. Rev. Cancer* 6, 107–116 (2006).

10 Shibata D: Inferring human stem cell behaviour from epigenetic drift. *J. Pathol.* 217, 199–205 (2009).

11 Ohm JE, McGarvey KM, Yu X *et al.*: A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat. Genet.* 39, 237–242 (2007).

12 Pogribny IP, Beland FA: DNA hypomethylation in the origin and pathogenesis of human diseases. *Cell Mol. Life Sci.* 66, 2249–2261 (2009).

13 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.* 159, 465–471 (2001).

14 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.* 173, 243–246 (2005).

15 Okano M, Bell DW, Haber DA, Li E: DNA methyltransferases DNMT3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99, 247–257 (1999).

16 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. USA* 99, 10060–10065 (2002).

▪ **Overexpression of an inactive splice variant of DNMT3b, which competes with the major splice variant for targeting to pericentromeric satellite regions, has been proposed as one of the molecular mechanisms for chromosomal instability during hepatocarcinogenesis.**

17 Laird PW: The power and the promise of DNA methylation markers. *Nat. Rev. Cancer* 3, 253–266 (2003).

18 Issa JP, Kantarjian HM: Targeting DNA methylation. *Clin. Cancer Res.* 15, 3938–3946 (2009).

19 Swanton C, Caldas C: Molecular classification of solid tumours: towards pathway-driven therapeutics. *Br. J. Cancer* 100, 1517–1522 (2009).

20 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* 28, 2434–2442 (2007).

▪ **Results of empirical analysis of DNA methylation status and DNA methyltransferase abnormalities in clinical tissue samples in connection with the clinicopathological parameters of human cancers have been reviewed.**

21 Kanai Y: Alterations of DNA methylation and clinicopathological diversity of human cancers. *Pathol. Int.* 58, 544–558 (2008).

22 Kanai Y: Genome-wide DNA methylation profiles in precancerous conditions and cancers. *Cancer Sci.* 101, 36–45 (2010).

23 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.* 87, 1210–1217 (1996).

▪ **One of the earliest reports of DNA methylation alterations at the precancerous stage, especially in noncancerous liver tissue showing chronic hepatitis or cirrhosis, which are widely considered to be precancerous conditions for hepatocellular carcinomas.**

24 Kanai Y, Hui AM, Sun L *et al.*: DNA hypermethylation at the D17S5 locus and reduced HIC-1 mRNA expression are associated with hepatocarcinogenesis. *Hepatology* 29, 703–709 (1999).

25 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* 32, 970–979 (2000).

26 Etoh T, Kanai Y, Ushijima S *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.* 164, 689–699 (2004).

27 Wentzensen N, Sherman ME, Schiffman M, Wang SS: Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol. Oncol.* 112, 293–299 (2009).

28 Ushijima T: Epigenetic field for cancerization. *J. Biochem. Mol. Biol.* 40, 142–150 (2007).

29 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.* 57, 4913–4915 (1997).

30 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP: CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA* 96, 8681–8686 (1999).

31 Kanai Y, Ushijima S, Hui AM *et al.*: The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int. J. Cancer* 71, 355–359 (1997).

32 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* 33, 561–568 (2001).

33 Saito Y, Kanai Y, Nakagawa T *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* 105, 527–532 (2003).

34 Peng DF, Kanai Y, Sawada M *et al.*: Increased DNA methyltransferase 1 (DNMT1) protein expression in precancerous conditions and ductal carcinomas of the pancreas. *Cancer Sci.* 96, 403–408 (2005).

35 Peng DF, Kanai Y, Sawada M *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* 27, 1160–1168 (2006).

36 Ooi SK, O'Donnell AH, Bestor TH: Mammalian cytosine methylation at a glance. *J. Cell Sci.* 122, 2787–2791 (2009).

37 Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF: Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277, 1996–2000 (1997).

38 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* 91, 205–212 (2001).

39 Damiani LA, Yingling CM, Leng S, Romo PE, Nakamura J, Belinsky SA: Carcinogen-induced gene promoter hypermethylation is mediated by DNMT1 and causal for transformation of immortalized bronchial epithelial cells. *Cancer Res.* 68, 9005–9014 (2008).

40 Liao X, Siu MK, Chan KY *et al.*: Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. *Int. J. Cancer* 123, 296–302 (2008).

41 Hino R, Uozaki H, Murakami N *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.* 69, 2766–2774 (2009).

42 Gao P, Yang X, Xue YW *et al.*: Promoter methylation of glutathione *S*-transferase pi1 and multidrug resistance gene 1 in bronchioloalveolar carcinoma and its correlation with DNA methyltransferase 1 expression. *Cancer* 115, 3222–3232 (2009).

43 Lin RK, Hsieh YS, Lin P *et al.*: The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation and tumor suppressor gene hypermethylation in mice and lung cancer patients. *J. Clin. Invest.* 120, 521–532 (2010).

44 Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW: CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. *Cancer Res.* 59, 2302–2306 (1999).

45 Park HJ, Yu E, Shim YH: DNA methyltransferase expression and DNA hypermethylation in human hepatocellular carcinoma. *Cancer Lett.* 233, 271–278 (2006).

46 Kondo Y, Shen L, Suzuki S *et al.*: Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. *Hepatol. Res.* 37, 974–983 (2007).

47 Estecio MR, Yan PS, Ibrahim AE *et al.*: High-throughput methylation profiling by MCA coupled to CpG island microarray. *Genome Res.* 17, 1529–1536 (2007).

48 Estecio MR, Issa JP: Tackling the methylome: recent methodological advances in genome-wide methylation profiling. *Genome Med.* 1, 106 (2009).

49 Bibikova M, Fan JB: GoldenGate assay for DNA methylation profiling. *Methods Mol. Biol.* 507, 149–163 (2009).

50 Gunderson KL: Whole-genome genotyping on bead arrays. *Methods Mol. Biol.* 529, 197–213 (2009).

51 Eid J, Fehr A, Gray J *et al.*: Real-time DNA sequencing from single polymerase molecules. *Science* 323, 133–138 (2009).

52 Maekita T, Nakazawa K, Mihara 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.* 12, 989–995 (2006).

53 Clark SJ: Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis. *Hum. Mol. Genet.* 16(Spec. No. 1), R88–R95 (2007).

54 Inazawa J, Inoue J, Imoto I: Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. *Cancer Sci.* 95, 559–563 (2004).

55 Nishiyama N, Arai E, Chihara Y *et al.*: Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. *Cancer Sci.* 101, 231–240 (2010).

▪ **Indicators for risk estimation of the development of urothelial carcinomas and prognostication of patients with urothelial carcinomas have been established based on bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) data.**

56 Misawa A, Inoue J, Sugino Y *et al.*: Methylation-associated silencing of the nuclear receptor *1I2* gene in advanced-type neuroblastomas, identified by bacterial artificial chromosome array-based methylated CpG island amplification. *Cancer Res.* 65, 10233–10242 (2005).

57 Tanaka K, Imoto I, Inoue J *et al.*: Frequent methylation-associated silencing of a candidate tumor-suppressor, CRABP1, in esophageal squamous-cell carcinoma. *Oncogene* 26, 6456–6468 (2007).

58 Sugino Y, Misawa A, Inoue J *et al.*: Epigenetic silencing of prostaglandin E receptor 2 (PTGER2) is associated with progression of neuroblastomas. *Oncogene* 26, 7401–7413 (2007).

59 McRonald FE, Morris MR, Gentle D *et al.*: CpG methylation profiling in VHL related and VHL unrelated renal cell carcinoma. *Mol. Cancer* 8, 31–41 (2009).

60 Arai E, Ushijima S, Fujimoto H *et al.*: Genome-wide DNA methylation profiles in both precancerous conditions and clear cell renal cell carcinomas are correlated with malignant potential and patient outcome. *Carcinogenesis* 30, 214–221 (2009).

▪ **Genome-wide DNA methylation profiles in precancerous conditions of the kidney have been shown to be inherited by clear cell renal cell carcinomas developing in each individual patient, and to determine tumor aggressiveness and patient outcome.**

61 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* 119, 288–296 (2006).

62 Arai E, Ushijima S, Tsuda H *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.* 14, 5531–5539 (2008).

63 Lee HS, Kim BH, Cho NY *et al.*: Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin. Cancer Res.* 15, 812–820 (2009).

64 Moribe T, Iizuka N, Miura T *et al.*: Methylation of multiple genes as molecular markers for diagnosis of a small, well-differentiated hepatocellular carcinoma. *Int. J. Cancer* 125, 388–397 (2009).

65 Arai E, Ushijima S, Gotoh M *et al.*: Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma. *Int. J. Cancer* 125, 2854–2862 (2009).

▪ **Indicators for carcinogenetic risk estimation in chronically diseased liver, and prognostication of patients with hepatocellular carcinomas, have been established based on BAMCA data.**

66 Kakizoe T: Development and progression of urothelial carcinoma. *Cancer Sci.* 97, 821–828 (2006).

67 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.* 173, 1767–1771 (2005).

68 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.* 170, 2463–2466 (2003).

69 Manabe D, Saika T, Ebara S *et al.*: Comparative study of oncologic outcome of laparoscopic nephroureterectomy and standard nephroureterectomy for upper urinary tract transitional cell carcinoma. *Urology* 69, 457–461 (2007).

70 Kim WJ, Kim YJ: Epigenetic markers as promising prognosticators for bladder cancer. *Int. J. Urol.* 16, 17–22 (2009).

Cancer Science
The official journal of the Japanese Cancer Association JCA

Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage

Naotaka Nishiyama,[1,2] Eri Arai,[1] Yoshitomo Chihara,[1] Hiroyuki Fujimoto,[3] Fumie Hosoda,[4] Tatsuhiro Shibata,[4] Tadashi Kondo,[5] Taiji Tsukamoto,[2] Sana Yokoi,[6] Issei Imoto,[6] Johji Inazawa,[6] Setsuo Hirohashi[1] and Yae Kanai[1,7]

[1]Pathology Division, National Cancer Center Research Institute, Tokyo; [2]Urology Division, Sapporo Medical University, Sapporo; [3]Urology Division, National Cancer Center Hospital, Tokyo; [4]Cancer Genomics Project, National Cancer Center Research Institute, Tokyo; [5]Proteome Bioinformatics Project, National Cancer Center Research Institute, Tokyo; [6]Department of Molecular Cytogenetics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan

(Received June 16, 2009/Revised August 13, 2009/Accepted August 19, 2009/Online publication September 22, 2009)

To clarify genome-wide DNA methylation profiles during multistage urothelial carcinogenesis, bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) was performed in 18 normal urothelia obtained from patients without urothelial carcinomas (UCs) (C), 17 noncancerous urothelia obtained from patients with UCs (N), and 40 UCs. DNA hypo- and hypermethylation on multiple BAC clones was observed even in N compared to C. Principal component analysis revealed progressive DNA methylation alterations from C to N, and to UCs. DNA methylation profiles in N obtained from patients with invasive UCs were inherited by the invasive UCs themselves, that is DNA methylation alterations in N were correlated with the development of more malignant UCs. The combination of DNA methylation status on 83 BAC clones selected by Wilcoxon test was able to completely discriminate N from C, and diagnose N as having a high risk of carcinogenesis, with 100% sensitivity and specificity. The combination of DNA methylation status on 20 BAC clones selected by Wilcoxon test was able to completely discriminate patients who suffered from recurrence after surgery from patients who did not. The combination of DNA methylation status for 11 BAC clones selected by Wilcoxon test was able to completely discriminate patients with UCs of the renal pelvis or ureter who suffered from intravesical metachronous UC development from patients who did not. Genome-wide alterations of DNA methylation may participate in urothelial carcinogenesis from the precancerous stage to UC, and DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication. (*Cancer Sci* 2010; 101: 231–240)

It is known that DNA hypomethylation results in chromosomal instability as a result of changes in chromatin structure, and that DNA hypermethylation of CpG islands silences tumor-related genes in cooperation with histone modification in human cancers.[1–5] Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and the precancerous stages.[6,7] On the other hand, in patients with cancers, aberrant DNA methylation is significantly associated with poorer tumor differentiation, tumor aggressiveness, and poorer patient outcome.[6,7] Therefore, alterations of DNA methylation may play a significant role in multistage carcinogenesis.

With respect to urothelial carcinogenesis, we have reported accumulation of DNA methylation on C-type CpG islands in a cancer-specific but not age-dependent manner, and protein overexpression of DNA methyltransferase (DNMT) 1, a major DNMT, even in noncancerous urothelia with no apparent histological changes obtained from patients with urothelial carcinomas (UCs).[8,9] Moreover, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 protein overexpression was more frequently evident in aggressive nodular invasive UCs[8–10] resulting in poorer patient outcome than in superficial papillary UCs, which usually remain noninvasive even after repeated urethrocystoscopic resection.[11,12] Since aberrant DNA methylation is one of the earliest molecular events during urothelial carcinogenesis and also participates in tumor aggressiveness, it may be possible to estimate the future risk of developing more malignant UCs. However, only a few previous studies focusing on UCs[13] have employed recently developed array-based technology for assessing genome-wide DNA methylation status,[14–16] and such studies have focused on identification of tumor-related genes that are silenced by DNA methylation.[13] DNA methylation profiles, which could become the optimum indicators for carcinogenetic risk estimation and prognostication of UCs, should therefore be explored using array-based approaches.

In this study, in order to clarify genome-wide DNA methylation profiles during multistage urothelial carcinogenesis, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA)[17–19] using a microarray of 4361 BAC clones[20] in normal urothelia obtained from patients without UCs, noncancerous urothelia obtained from patients with UCs, and UCs themselves.

Materials and Methods

Patients and tissue samples. Seventeen samples of noncancerous urothelia (N1–N17) and 40 samples of UCs (T1–T40) of the urinary bladder, ureter, and renal pelvis were obtained from specimens that had been surgically resected by radical cystectomy (12 patients) or nephroureterectomy (28 patients) at the National Cancer Center Hospital, Tokyo, Japan. The patients comprised 31 men and nine women whose mean age was 69.03 ± 9.77 (mean ± SD) years (range, 49–85 years). Microscopic examination revealed no remarkable histological changes in the noncancerous urothelia. The patients from whom noncancerous urothelia were obtained comprised 11 men and six women with a mean age of 70.41 ± 9.33 (mean ± SD) years (range, 49–85 years). There were 17 superficial UCs (two pTa and 15 pT1 tumors) and 23 invasive UCs (six pT2, 16 pT3, and one pT4 tumor) according to the criteria proposed by World Health Organization classification.[21] For comparison, 18 samples of normal urothelia obtained from patients without UCs (C1–C18) were used. Fourteen, three, and one patient underwent nephrectomy for renal cell carcinoma, nephrectomy for retroperitoneal sarcoma around the kidney, and partial cystectomy for urachal carcinoma, respectively. The patients from whom normal urothelia were obtained comprised 13 men and five women with a mean age of 61.17 ± 15.16 (mean ± SD) years (range, 27–82 years). This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan and has

[7]To whom correspondence should be addressed. E-mail: ykanai@ncc.go.jp

doi: 10.1111/j.1349-7006.2009.01330.x

been performed in accordance with the Declaration of Helsinki in 1995. All patients gave their informed consent prior to their inclusion in this study.

BAMCA. High-molecular-weight DNA from fresh frozen tissue samples was extracted using phenol-chloroform, followed by dialysis. Because DNA methylation status is known to be organ-specific,[22] the reference DNA for analysis of the developmental stages of UCs should be obtained from the urothelium, and not from other organs or peripheral blood. Therefore, a mixture of normal urothelial DNA obtained from 11 male patients (C19–C29) and six female patients (C30–C35) without UCs was used as a reference for analyses of male and female test DNA samples, respectively. DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4361 BAC clones located throughout chromosomes 1–22, X and Y,[20] as described previously.[17–19] Briefly, 5-μg aliquots of test or reference DNA were first digested with 100 units of the methylation-sensitive restriction enzyme Sma I and subsequently with 20 units of the methylation-insensitive Xma I. Adapters were ligated to the Xma I-digested sticky ends, and PCR was performed with an adapter primer set. Test and reference PCR products were labeled by random priming with Cy3- and Cy5-dCTP (GE Healthcare, Buckinghamshire, UK), respectively, and precipitated together with ethanol in the presence of Cot-I DNA. The mixture was applied to array slides and incubated at 43°C for 72 h. Arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, CA, USA) and analyzed using GenePix Pro 5.0 imaging software (Axon Instruments) and Acue 2 software (Mitsui Knowledge Industry, Tokyo, Japan). The signal ratios were normalized in each sample to make the mean signal ratios of all BAC clones 1.0.

Statistics. Differences in the average number of BAC clones that showed DNA methylation alterations (DNA hypo- and hypermethylation) between groups of samples were analyzed using the Mann–Whitney *U*-test. Differences at $P < 0.05$ were considered significant. Principal component analysis based on BAMCA data was performed using the Expressionist software program (Gene Data, Basel, Switzerland). Unsupervised two-dimensional hierarchical clustering analysis of tissue samples and the BAC clones were performed using the Expressionist software program. Correlations between the subclassification of patients yielded by unsupervised hierarchical clustering analysis and clinicopathological parameters of UCs were analyzed using the χ^2-test. Differences at $P < 0.05$ were considered significant. BAC clones whose signal ratios yielded by BAMCA were significantly different between groups of samples were identified by Wilcoxon test ($P < 0.01$).

Results

Genome-wide DNA methylation alterations during multistage urothelial carcinogenesis. Figure 1(b,c) shows examples of scanned array images and scattergrams of the signal ratios (test signal/reference signal), respectively, for normal urothelium from a patient without UC (panel C), and both noncancerous urothelium (panel N) and cancerous tissue (panel T) from a patient with UC. In all normal urothelia (C1–C18), the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars in Fig. 1c). Therefore, in noncancerous urothelia obtained from patients with UCs and UCs, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared to normal urothelia, respectively, as in our previous study.[23] In noncancerous urothelia obtained from patients with UCs, many BAC clones showed DNA hypo- or hypermethylation (panel N of Fig. 1c). In UCs themselves, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, that is deviation of the signal ratio from 0.67 or 1.5, was increased (panel T of Fig. 1c) in comparison with noncancerous urothelia obtained from patients with UCs. The average number of BAC clones showing DNA hypomethylation increased significantly from noncancerous urothelia obtained from patients with UCs (24.53 ± 31.48) to UCs (236.78 ± 92.78, $P = 4.37e\text{-}9$). The average number of BAC clones showing DNA hypermethylation increased significantly from noncancerous urothelia obtained from patients with UCs (29.18 ± 39.84) to UCs (289.13 ± 82.42, $P = 7.35e\text{-}9$). Principal component analysis based on BAMCA data (signal ratios) revealed progressive DNA methylation alterations from normal urothelia, to noncancerous urothelia obtained from patients with UCs, and to UCs (Fig. 1d).

Clinicopathological significance of DNA methylation alterations in noncancerous urothelia obtained from patients with UCs. In order to clarify the clinicopathological significance of DNA methylation alterations in noncancerous urothelia obtained from patients with UCs, unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia was performed. Seventeen patients with UCs were clustered into two subclasses, Clusters A_N and B_N, which contained nine and eight patients, respectively, based on the DNA methylation status of the noncancerous urothelia (Fig. 2a). All eight patients (100%) belonging to Cluster B_N suffered from invasive UCs (pT2 or more), whereas five (55.6%) of the patients belonging to Cluster A_N did so ($P = 0.0311$).

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios of 131 BAC clones differed significantly between noncancerous urothelia obtained from patients with superficial UCs (pTa and pT1) and noncancerous urothelia obtained from patients with invasive UCs (pT2 or more). If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly higher than those in noncancerous urothelia obtained from patients with superficial UCs (67 BAC clones), the average signal ratios in the invasive UCs themselves were even higher than (42 BAC clones, e.g. RP11-79K14 and RP11-29C11 in Fig. 2b) or not significantly different from (25 BAC clones, e.g. RP11-3A9 and RP11-73G16 in Fig. 2b) those in noncancerous urothelia obtained from patients with invasive UCs, without exception. If the average signal ratios in noncancerous urothelia obtained from patients with invasive UCs were significantly lower than those in noncancerous urothelia obtained from patients with superficial UCs (64 BAC clones), the average signal ratios in the invasive UCs themselves were even lower than (38 BAC clones, e.g. RP11-210F15 and RP11-368O13 in Fig. 2b) or not significantly different from (26 BAC clones, e.g. RP11-442N24 and RP11-65C22 in Fig. 2b) those in noncancerous urothelia obtained from patients with invasive UCs, without exception, that is DNA methylation status of the 131 BAC clones in noncancerous urothelia obtained from patients with invasive UCs was inherited by the invasive UCs themselves.

DNA methylation profiles discriminating noncancerous urothelia obtained from patients with UCs from normal urothelia. Our finding that DNA methylation alterations in noncancerous urothelia were correlated with the development of UCs, as described above, prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles in noncancerous urothelia. We attempted to establish criteria for indicating that noncancerous urothelia obtained from patients with UCs, and not normal urothelia, were at high risk of carcinogenesis.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 201 BAC clones differed significantly between normal urothelia obtained from patients without UCs and noncancerous urothelia obtained from patients with UCs. Figure 3(a) shows

doi: 10.1111/j.1349-7006.2009.01330.x

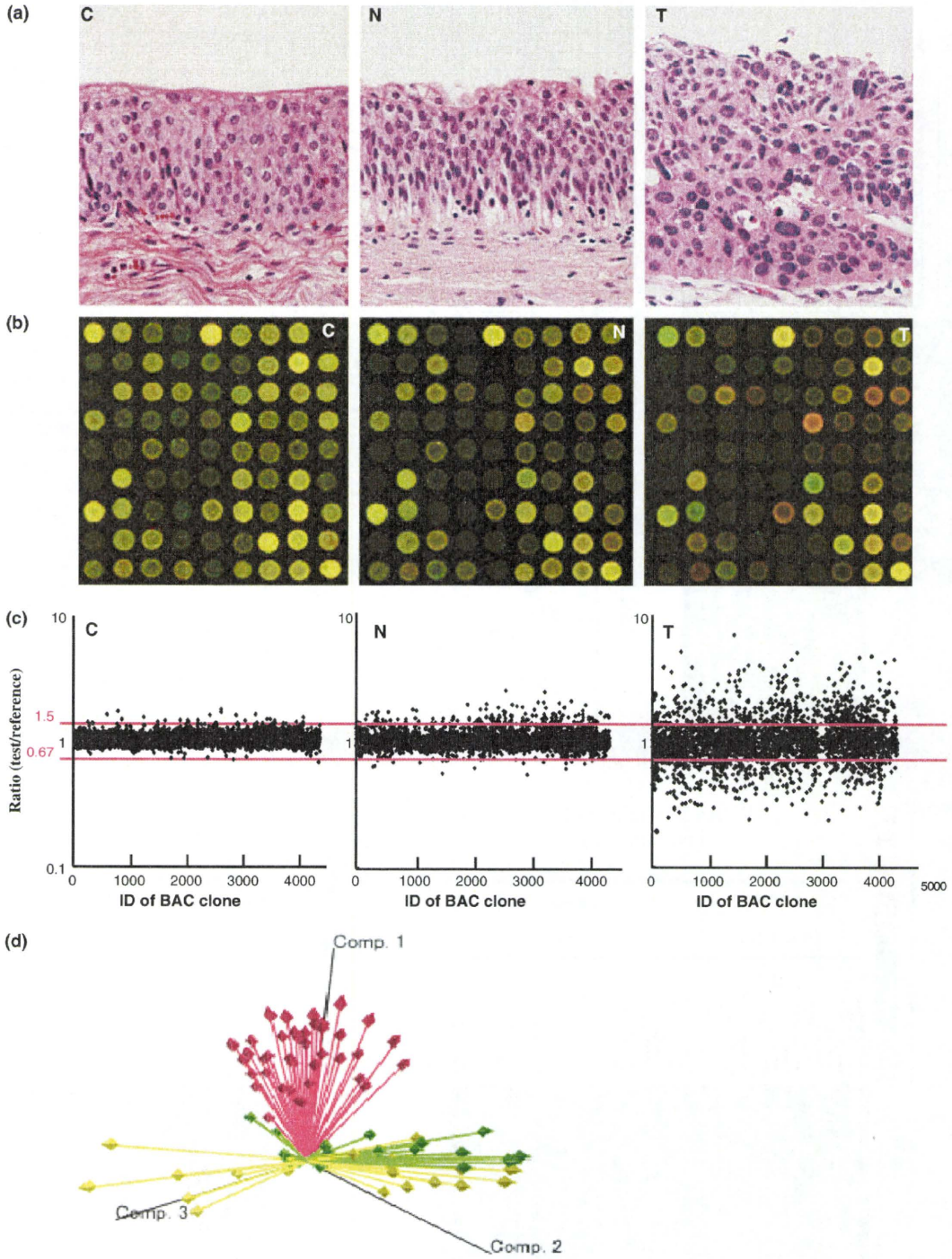
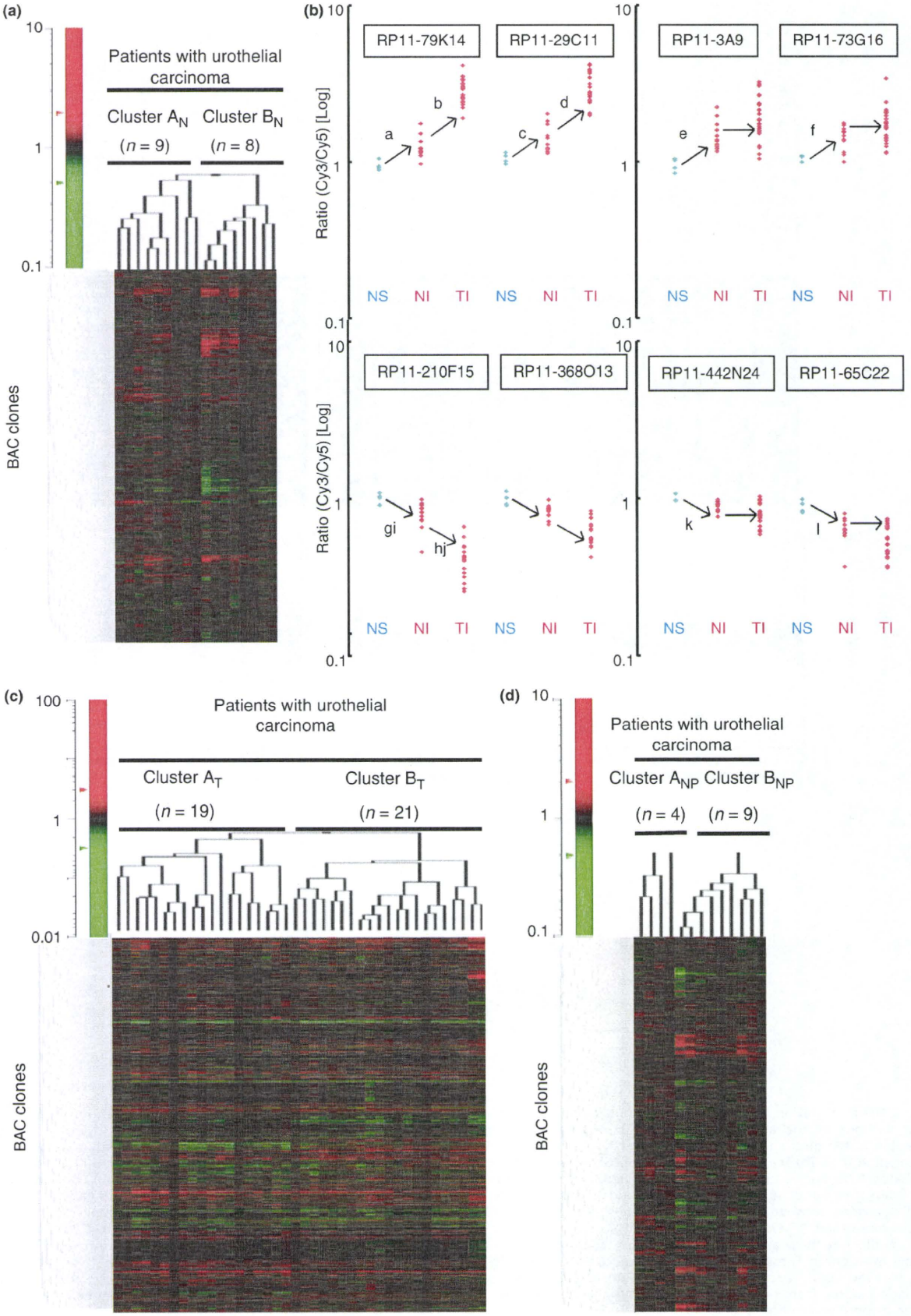


Fig. 1. DNA methylation alterations during multistage urothelial carcinogenesis. (a) Microscopic view of normal urothelium obtained from a patient without urothelial carcinoma (UC) (C), noncancerous urothelium obtained from a patient with UC (N), and UC (T). N shows no remarkable histological changes in comparison to C, that is no cytological or structural atypia is evident. Hematoxylin–eosin staining. Original magnification, ×20. (b) Scanned array images obtained by bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) in C, N, and T. Co-hybridization was done with test and reference DNA labeled with Cy3 and Cy5, respectively. (c) Scattergrams of the signal ratios (test signal/reference signal) obtained by BAMCA in C, N, and T. In all 18 normal urothelia (C1–C18), the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars). Therefore, in N and T, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation on each BAC clone compared to C, respectively. Even though N did not show any marked histological changes in comparison to C (panels C and N in [a]), many BAC clones showed DNA hypo- or hypermethylation. In T, more BAC clones showed DNA hypo- or hypermethylation, whose degree, that is deviation of the signal ratio from 0.67 or 1.5, was increased in comparison to N. (d) Principal component analysis based on BAMCA data (signal ratios). Progressive alterations of DNA methylation status from normal urothelia (yellow arrows) to noncancerous urothelia obtained from patients with UCs (green arrows), and to UCs (red arrows) were observed.



doi: 10.1111/j.1349-7006.2009.01330.x

Fig. 2. Correlations between DNA methylation status and clinicopathological parameters. (a) Unsupervised two-dimensional hierarchical clustering analysis based on bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data (signal ratios) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs). The signal ratio is shown in the color range map. Seventeen patients with UCs were hierarchically clustered into two subclasses, Clusters A_N (n = 9) and B_N (n = 8). Eight patients (100%) belonging to Cluster B_N developed invasive UCs (pT2 or more), whereas five patients (55.6%) belonging to Cluster A_N did so (P = 0.0311). (b) Scattergrams of the signal ratios in tissue samples. NS, noncancerous urothelia obtained from patients with superficial UCs. NI, noncancerous urothelia obtained from patients with invasive UCs. TI, invasive UCs. If the average signal ratios in NI were significantly higher than those in NS, the average signal ratios in TI themselves were even higher than (BAC clones RP11-79K14 and RP11-29C11), or not significantly different from (BAC clones RP11-3A9 and RP11-73G16), those in NI without exception. If the average signal ratios in NI were significantly lower than those in NS, the average signal ratios in TI themselves were even lower than (BAC clones RP11-210F15 and RP11-368O13), or not significantly different from (BAC clones RP11-442N24 and RP11-65C22), those in NI without exception. [a]P = 0.001680673, [b]P = 9.23504e-7, [c]P = 0.002197802, [d]P = 3.64223e-6, [e]P = 0.000840336, [f]P = 0.007692306, [g]P = 0.004395604, [h]P = 8.31509e-6, [i]P = 0.004395604, [j]P = 1.10173e-5, [k]P = 0.005882353, [l]P = 0.001098901. (c) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) in UCs. Forty patients with UCs were hierarchically clustered into two subclasses, Clusters A_T (n = 19) and B_T (n = 21). All four patients with recurrence belonged to Cluster B_T. (d) Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter. Thirteen patients with UCs of the renal pelvis or ureter were hierarchically clustered into two subclasses, Clusters A_{NP} (n = 4) and B_{NP} (n = 9). All four patients who developed intravesical metachronous UC belonged to Cluster B_{NP}.

scattergrams of the signal ratios in normal urothelia and noncancerous urothelia obtained from patients with UCs for representative examples of the 201 BAC clones. Using the cut-off values described in Figure 3(a), noncancerous urothelia obtained from patients with UCs were discriminated from normal urothelia with sufficient sensitivity and specificity (Fig. 3a). From the 201 BAC clones, 83 for which such discrimination was performed with a sensitivity and specificity of 75% or more than 75% were selected (Table S1). The cut-off values of the signal ratios for the 83 BAC clones, and their sensitivity and specificity, are shown in Table S1.

A histogram showing the number of BAC clones satisfying the criteria listed in Table S1 for 18 normal urothelia (C1–C18) and 17 noncancerous urothelia obtained from patients

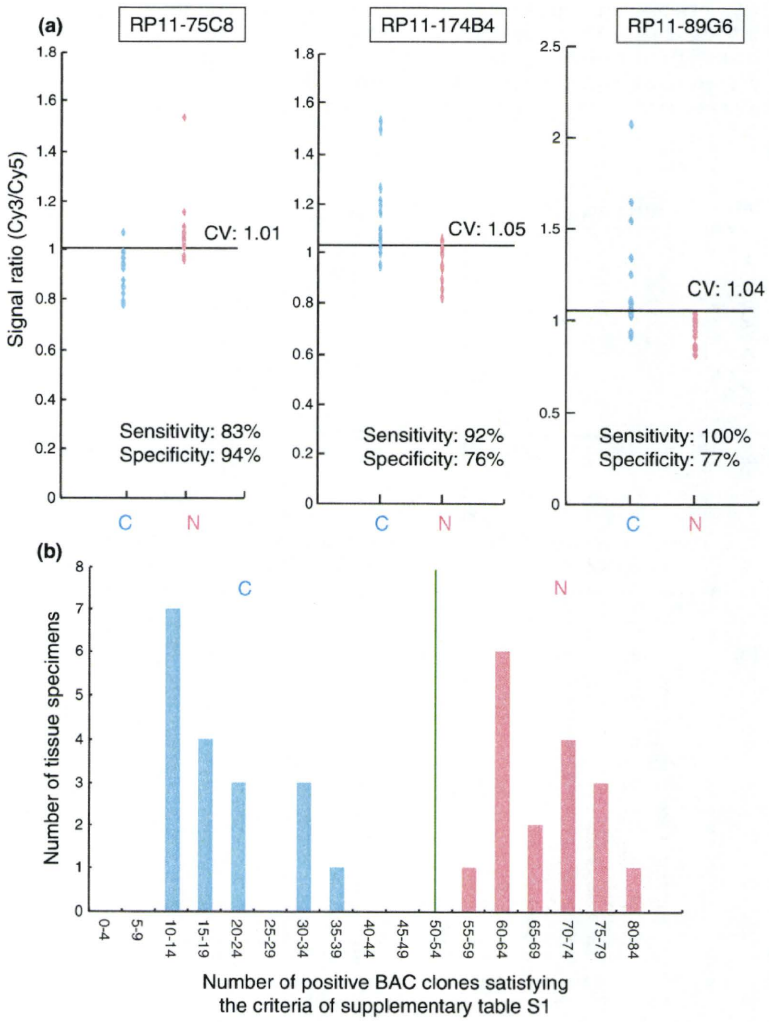


Fig. 3. DNA methylation profiles discriminating noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C). (a) Scattergrams of the signal ratios in C and N on representative bacterial artificial chromosome (BAC) clones, RP11-75C8, RP11-174B4, and RP11-89G6. Using the cut-off values (CV) described in each panel, N in this cohort were discriminated from C with sufficient sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S1 in samples C1–C18 and N1–N17. Based on this histogram, we established a criterion that when the noncancerous urothelia satisfied the criteria in Table S1 for 50 (green bar) or more than 50 BAC clones, they were judged to be at high risk of carcinogenesis.

with UCs (N1–N17) is shown in Figure 3(b). Based on this figure, we finally established the following criteria: when noncancerous urothelia satisfied the criteria in Table S1 for 50 or more BAC clones (green bar in Fig. 3b), they were judged to be at high risk of carcinogenesis, and when noncancerous urothelia satisfied the criteria in Table S1 for less than 50 BAC clones, they were judged not to be at high risk of carcinogenesis. Based on these criteria, both the sensitivity and specificity for diagnosis of noncancerous urothelia obtained from patients with UCs in this cohort as being at high risk of carcinogenesis were 100%.

Association of DNA methylation profiles in UCs with recurrence. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for UCs was able to group 40 patients into two subclasses, Clusters A_T and B_T, which contained 19 and 21 patients, respectively (Fig. 2c). Four patients (19.0%) belonging to Cluster B_T suffered recurrence after surgery (metastasis to the pelvic lymph nodes in three, and metastasis to the lung and bone in one), whereas none (0%) belonging to Cluster A_T did so ($P = 0.0449$). The mean observation period was 29.8 ± 28.0 months (mean ± SD). These data prompted us to establish criteria for predicting recurrence of UCs based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 20 BAC clones in UCs differed significantly between the patients who suffered recurrence after surgery and patients who did not. Figure 4(a) shows scattergrams of the signal ratios in UCs obtained from patients who suffered recurrence and those in UCs obtained from patients who did not. DNA methylation status of the 20 BAC clones was able to discriminate patients who suffered recurrence from patients who did not with a sensitivity of 100% using the cut-off values shown in Figure 4(a) and Table S2. A histogram showing the number of BAC clones satisfying the criteria listed in Table S2 for all 40 UCs is shown in Figure 4(b). Satisfying the criteria in Table S2 for 17 or more BAC clones (green bar in Fig. 4b) discriminated patients who suffered recurrence from patients who did not with a sensitivity and specificity of 100%, whereas high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

Association of DNA methylation profiles in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter with intravesical metachronous UC development. It is well known that patients with UCs of the renal pelvis and ureter frequently suffer from metachronous UC development in the urinary bladder after nephroureterectomy.[24,25] Since such metachronous UC originates from the noncancerous urothelium of the urinary bladder, we focused on the DNA methylation status of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter. Unsupervised two-dimensional hierarchical clustering analysis based on BAMCA data (signal ratios) for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter was able to group 13 patients into two subclasses, Clusters A_{NP} and B_{NP}, which contained four and nine patients, respectively (Fig. 2d). Four (44%) of the patients in Cluster B_{NP} developed intravesical metachronous UCs, whereas none (0%) belonging to Cluster A_{NP} did so. These data prompted us to establish criteria that could predict the development of intravesical metachronous UC based on DNA methylation status.

The Wilcoxon test ($P < 0.01$) revealed that the signal ratios on 11 BAC clones in noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter differed

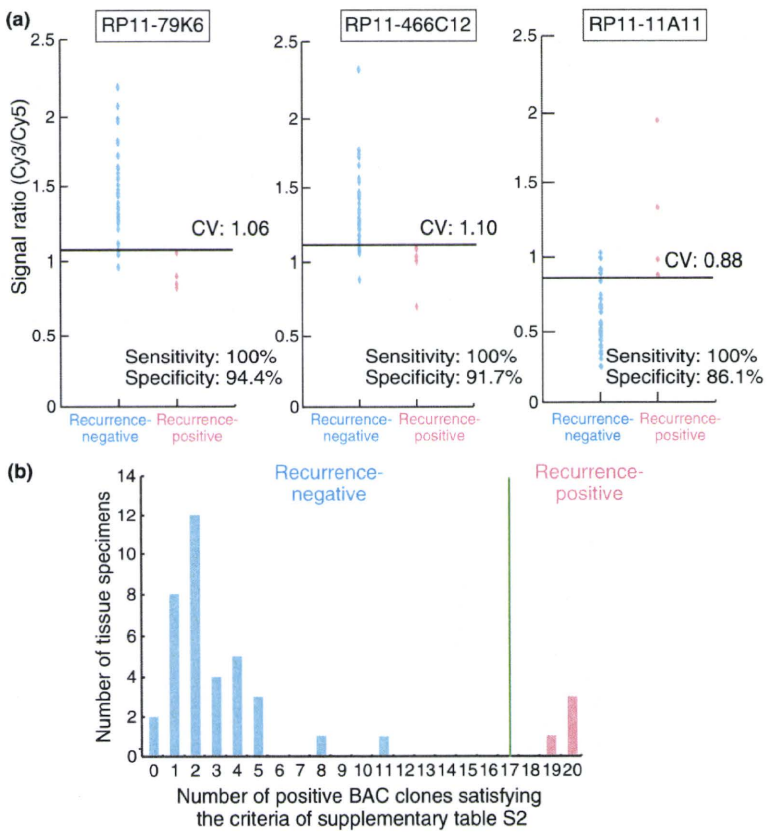


Fig. 4. DNA methylation profiles in urothelial carcinomas (UCs) associated with recurrence. (a) Scattergrams of the signal ratios in UCs from patients who did not develop recurrence ($n = 36$) and UCs from patients who developed recurrence ($n = 4$) on representative bacterial artificial chromosome (BAC), clones, RP11-79K6, RP11-466C12, and RP11-11A11. Using the cut-off values (CV) described in each panel, recurrence-positive patients were discriminated from recurrence-negative patients with 100% sensitivity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S2 in all 40 patients with UCs. Satisfying the criteria in Table S2 for 17 (green bar) or more than 17 BAC clones discriminated recurrence-positive patients from recurrence-negative patients with a sensitivity and specificity of 100%, whereas high histological grade (21), invasive growth (pT2 or more), and vascular or lymphatic involvement were unable to achieve such complete discrimination (data not shown).

doi: 10.1111/j.1349-7006.2009.01330.x

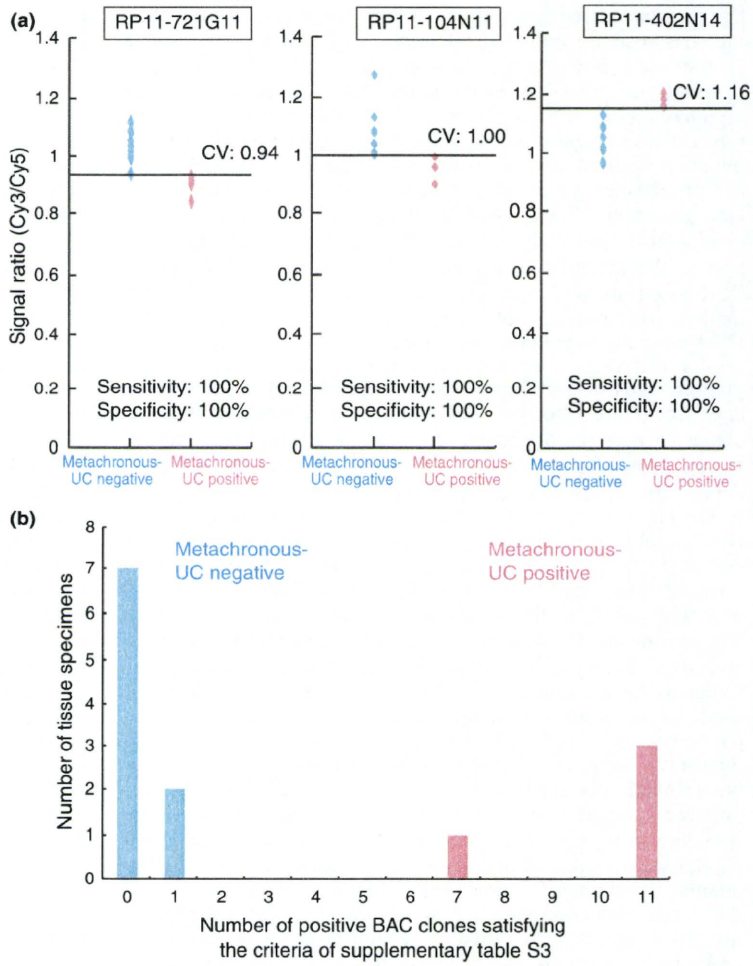


Fig. 5. DNA methylation profiles in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) of the renal pelvis or ureter associated with intravesical metachronous UC development. (a) Scattergrams of the signal ratios in noncancerous urothelia obtained from patients who did not develop intravesical metachronous UCs ($n = 9$) and noncancerous urothelia obtained from patients who developed intravesical metachronous UCs ($n = 4$) on representative bacterial artificial chromosome (BAC) clones, RP11-721G11, RP11-104N11, and RP11-402N14. Using the cut-off values (CV) described in each panel, metachronous UC-positive patients were discriminated from metachronous UC-negative patients with 100% sensitivity and specificity. (b) Histogram showing the number of BAC clones satisfying the criteria listed in Table S3 in all 13 patients with UCs of the renal pelvis or ureter from whom noncancerous urothelia were obtained. Patients who were negative and positive for metachronous UC were confirmed to show a marked difference in the DNA methylation status of the 11 BAC clones.

significantly between patients who developed intravesical metachronous UC after nephroureterectomy and patients who did not. DNA methylation status of nine of the 11 BAC clones was able to discriminate patients who suffered from intravesical metachronous UC development from patients who did not with a sensitivity and specificity of 100% using the cut-off values shown in Figure 5(a) and Table S3. A histogram showing the number of BAC clones satisfying the criteria listed in Table S3 for 13 noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter is shown in Figure 5(b).

Discussion

Urothelial carcinomas are clinically remarkable because of their multicentricity: synchronously or metachronously multifocal UCs often develop in individual patients. A possible mechanism for such multiplicity is the "field effect," whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells.[26] Even noncancerous urothelia showing no remarkable histological features obtained from patients with UCs can be considered to be at the precancerous stage, because they may be exposed to carcinogens in the urine. On the other hand, UCs are classified as superficial papillary carcinomas or nodular invasive carcinomas according to their configuration. Superficial papillary carcinomas usually remain noninvasive, although patients need to undergo repeated urethrocystoscopic resections because of recurrences. In contrast, the clinical outcome of nodular invasive carcinomas is poor.[11,12]

In our previous study, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 protein overexpression was observed even in noncancerous urothelia obtained from patients with UCs.[8,9] Aberrant DNA methylation was further increased, especially in nodular invasive carcinomas.[8–10] These previous data suggested that carcinogenetic risk estimation and prognostication of UCs based on DNA methylation status might be a promising strategy. Although optimal diagnostic indicators have never been explored using array-based genome-wide DNA methylation analysis, alterations of DNA methylation on several CpG islands in UCs have been reported separately.[27–31]

Many researchers in the field of cancer epigenetics have used promoter arrays to identify the genes that are methylated in cancer cells.[14–16] However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions that do not directly participate in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur.[32] Genomic regions in which DNA hypomethylation affects chromosomal instability may not be contained in promoter arrays. Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in

human cancers due to a process of long-range epigenetic silencing, has recently attracted attention.[33] Therefore, we used a custom-made BAC array[20] that may be suitable for gaining an overview of the DNA methylation status of individual large regions among all chromosomes (Table S4), and for obtaining reproducible diagnostic indicators. In fact we have successfully obtained optimal indicators for carcinogenetic risk estimation and prognostication of renal cell carcinomas[23] and hepatocellular carcinomas[34] by BAMCA using the same array as that employed in this study. On the other hand, we must pay attention to the quantitative accuracy of BAMCA, because it is a PCR-based method differing from other genome-wide DNA methylation analyses not using PCR, such as the methylated DNA immunoprecipitation-microarray. In order to validate the results of BAMCA, we quantitatively evaluated the DNA methylation status of each Xma I/Sma I site yielding labeled products which are effective in BAMCA on representative BAC clones, by pyrosequencing. As shown in the example in Figure S1 and Table S5, pyrosequencing validated the BAMCA data on the representative BAC clone.

The present DNA methylation analysis revealed that stepwise DNA methylation alterations during urothelial carcinogenesis occurred in a genome-wide manner (Fig. 1). We then performed unsupervised hierarchical clustering analysis based on the genome-wide DNA methylation status of noncancerous urothelia, and as a result, 17 patients were subclassified into Clusters A_N and B_N. Corresponding UCs showing deeper invasion were found to be accumulated in Cluster B_N. Genome-wide DNA methylation profiles of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves (Fig. 2b). DNA methylation profiles of noncancerous urothelia obtained from patients with superficial UCs were not always inherited by superficial UCs (data not shown), corresponding to the alternative malignant progression of superficial papillary carcinoma to nodular invasive carcinoma, via papillonodular carcinoma. Genome-wide DNA methylation alterations that were correlated with the development of more malignant invasive cancers were already accumulated in noncancerous urothelia, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to further accumulation of genetic and epigenetic alterations and generate more malignant cancers.

The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate noncancerous urothelia obtained from patients with UCs from normal urothelia and diagnose them as having a high risk of urothelial carcinogenesis with a sensitivity and specificity of 100%. We are currently attempting to develop methodology for assessing the tendency for DNA methylation in the 83 BAC regions in urine samples with a view to application for screening of healthy individuals. If it proves possible to identify individuals who are at high risk of urothelial carcinogenesis, then strategies for the prevention or early detection of UCs, such as smoking cessation or repeated urine cytology examinations, might be applicable.

Even after surgery with curative intent, some UCs relapse and metastasize to lymph nodes or distant organs.[35] Recently, new systemic chemotherapy and targeted therapy have been developed for treatment for UCs.[36] In order to start adjuvant systemic chemotherapy immediately in patients who have undergone surgery and are still at high risk of recurrence and metastasis, prognostic indicators have been explored. The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate patients who suffered recurrence after surgery from patients who did not with a sensitivity and specificity of 100% (Fig. 4b), whereas a high histological grade,[21] invasive growth (pT2 or more), and vascular or lymphatic involvement, which are known to have a prognostic impact,[37,38] were incapable of such complete discrimination (data not shown). Therefore, a combination of the 20 BAC clones can have significant prognostic value for patients with UCs. Since a sufficient quantity of good-quality DNA can be obtained from each surgical specimen, our array-based analysis that overviews aberrant DNA methylation of each BAC region is immediately applicable to routine laboratory examinations for prognostication after surgery. The reliability of such prognostication will need to be validated in a prospective study.

As mentioned above, UCs are remarkable because of their multicentricity. Approximately 10–30% of patients with UCs of the renal pelvis and ureter develop intravesical metachronous UCs after nephroureterectomy.[24,25] Therefore, such patients have to undergo repeated urethrocystoscopic examinations to detect intravesical metachronous UCs. To decrease the need for invasive urethrocystoscopic examinations and assist close follow-up of such patients after nephroureterectomy, indicators for intravesical metachronous UCs have been needed. All of our patients who developed intravesical metachronous UCs after nephroureterectomy belonged to Cluster B_{NP}, indicating that DNA methylation profiles of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter, which may be exposed to the same carcinogens in the urine as noncancerous urothelia from which metachronous UCs originate, are correlated with the risk of intravesical metachronous UC development. The present genome-wide analysis revealed DNA methylation profiles that were able to completely discriminate patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UCs from patients who did not, in noncancerous urothelia from nephroureterectomy specimens. A combination of the present 11 BAC clones may be an optimal indicator for the development of intravesical metachronous UC. The reliability of such prognostication will again need to be validated in a prospective study.

With respect to background factors of genome-wide DNA methylation alterations during urothelial carcinogenesis, smoking history did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S6). In addition, immunohistochemically examined DNMT1 protein expression levels did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S7), indicating that expression levels of DNMT1 did not by themselves simply determine DNA methylation profiles. However, our previous study revealed remarkable protein overexpression of DNMT1 in noncancerous urothelia obtained from patients with UCs as compared to normal urothelia.[8] Therefore, undefined cofactors may recruit DNMT1 or other proteins regulating DNA methylation status to aberrant target sequences and may participate in DNA methylation alterations in noncancerous urothelia obtained from patients with UCs. Further studies are needed to elucidate molecular mechanisms of DNA methylation alterations in such noncancerous urothelia.

Moreover, when the DNA methylation status for CpG islands of *p16*, human MutL homologue 1 (*hMLH1*), thrombospondin-1 (*THBS-1*), and death-associated protein kinase (*DAPK*) genes and the methylated in tumor (MINT)-1, -2, -12, -25, and -31 clones were examined in noncancerous urothelia obtained from patients with UCs and in UCs by methylation-specific PCR and combined bisulfite restriction enzyme analysis as in our previous study,[9,39] the incidence of DNA

doi: 10.1111/j.1349-7006.2009.01330.x

methylation on each CpG island and the average number of methylated CpG islands did not correlate significantly with the numbers of BAC clones showing DNA hypo- or hypermethylation in noncancerous urothelia obtained from patients with UCs and in UCs, or with clustering (Cluster A_N *vs* Cluster B_N and Cluster A_T *vs* Cluster B_T) (Table S8). Therefore, molecular mechanisms for alterations of genome-wide DNA methylation profiles may differ from those for regional DNA hypermethylation on CpG islands.

Although BAMCA mainly provides an overview of the DNA methylation status of individual large regions among all chromosomes as mentioned above, it may also be able to identify genes for which expressions are regulated by DNA methylation, since there are promoter regions of specific genes including CpG islands on BAC clones showing clinicopathologically significant DNA hypo- or hypermethylation (Table S4). Expression levels and the DNA methylation status of these genes, as well as the functions of the proteins coded by such genes, will be examined in a future investigation. If further studies identify tumor-related genes for which expression levels are regulated by DNA methylation among such candidates, these tumor-related genes may serves as targets for epigenetic prevention and therapy, along with the molecules causing alterations of genome-wide DNA methylation profiles.

Acknowledgments

This study was supported by a Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan; a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan; a Grant from the New Energy and Industrial Technology Development Organization (NEDO); and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio). N. Nishiyama is an awardee of a research resident fellowship from the Foundation for Promotion of Cancer Research in Japan.

References

1 Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415–28.
2 Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003; **300**: 455.
3 Baylin SB, Ohm JE. Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006; **6**: 107–16.
4 Gronbaek K, Hother C, Jones PA. Epigenetic changes in cancer. *Apmis* 2007; **115**: 1039–59.
5 Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. *Nature* 2007; **447**: 433–40.
6 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.
7 Kanai Y. Alterations of DNA methylation and clinicopathological diversity of human cancers. *Pathol Int* 2008; **58**: 544–8.
8 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.
9 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.
10 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.
11 Kakizoe T, Tobisu K, Takai K, Tanaka Y, Kishi K, Teshima S. Relationship between papillary and nodular transitional cell carcinoma in the human urinary bladder. *Cancer Res* 1998; **48**: 2299–303.
12 Kakizoe T. Development and progression of urothelial carcinoma. *Cancer Sci* 2006; **97**: 821–8.
13 Aleman A, Adrien L, Lopez-Serra L *et al.* Identification of DNA hypermethylation of SOX9 in association with bladder cancer progression using CpG microarrays. *Br J Cancer* 2008; **98**: 466–73.
14 Estecio MR, Yan PS, Ibrahim AE *et al.* High-throughput methylation profiling by MCA coupled to CpG island microarray. *Genome Res* 2007; **17**: 1529–36.
15 Jacinto FV, Ballestar E, Ropero S, Esteller M. Discovery of epigenetically silenced genes by methylated DNA immunoprecipitation in colon cancer cells. *Cancer Res* 2007; **67**: 11481–6.
16 Nielander I, Bug S, Richter J, Giefing M, Martin-Subero JI, Siebert R. Combining array-based approaches for the identification of candidate tumor suppressor loci in mature lymphoid neoplasms. *Apmis* 2007; **115**: 1107–34.
17 Misawa A, Inoue J, Sugino Y *et al.* Methylation-associated silencing of the nuclear receptor 12 gene in advanced-type neuroblastomas, identified by bacterial artificial chromosome array-based methylated CpG island amplification. *Cancer Res* 2005; **65**: 10233–42.
18 Sugino Y, Misawa A, Inoue J *et al.* Epigenetic silencing of prostaglandin E receptor 2 (PTGER2) is associated with progression of neuroblastomas. *Oncogene* 2007; **26**: 7401–13.
19 Tanaka K, Imoto I, Inoue J *et al.* Frequent methylation-associated silencing of a candidate tumor-suppressor, CRABP1, in esophageal squamous-cell carcinoma. *Oncogene* 2007; **26**: 6456–68.
20 Inazawa J, Inoue J, Imoto I. Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. *Cancer Sci* 2004; **95**: 559–63.
21 Lopez-Beltran A, Sauter G, Gasser T *et al.* Tumours of the urinary system. In: ed. Eble JN, Sauter G, Epstein JI, Sesterhenn IA. *World Health Organization classification of tumours. Pathology and genetics. Tumours of the urinary system and male genital organs.* Lyon: IARC Press, 2004; 89–157.
22 Illingworth R, Kerr A, Desousa D *et al.* A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biol* 2008; **6**: e22.
23 Arai E, Ushijima S, Fujimoto H *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.
24 Manabe D, Saika T, Ebara S *et al.* Comparative study of oncologic outcome of laparoscopic nephroureterectomy and standard nephroureterectomy for upper urinary tract transitional cell carcinoma. *Urology* 2007; **69**: 457–61.
25 Hall MC, Womack S, Sagalowsky AI, Carmody T, Erickstad MD, Roehrborn CG. Prognostic factors, recurrence, and survival in transitional cell carcinoma of the upper urinary tract: a 30-year experience in 252 patients. *Urology* 1998; **52**: 594–601.
26 Harris AL, Neal DE. Bladder cancer – field versus clonal origin. *N Engl J Med* 1992; **326**: 759–61.
27 Maruyama R, Toyooka S, Toyooka KO *et al.* Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res* 2001; **15**: 8659–63.
28 Sathyanarayana UG, Maruyama R, Padar A *et al.* Molecular detection of noninvasive and invasive bladder tumor tissues and exfoliated cells by aberrant promoter methylation of laminin-5 encoding genes. *Cancer Res* 2004; **15**: 1425–30.
29 Catto JW, Azzouzi AR, Rehman I *et al.* Promoter hypermethylation is associated with tumor location, stage, and subsequent progression in transitional cell carcinoma. *J Clin Oncol* 2005; **23**: 2903–10.
30 Yates DR, Rehman I, Abbod MF *et al.* Promoter hypermethylation identifies progression risk in bladder cancer. *Clin Cancer Res* 2007; **13**: 2046–2053.
31 Kim EJ, Kim YJ, Jeong P, Ha YS, Bae SC, Kim WJ. Methylation of the RUNX3 promoter as a potential prognostic marker for bladder tumor. *J Urol* 2008; **180**: 1141–5.
32 Maekita T, Nakazawa K, Mihara 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.
33 Frigola J, Song J, Stirzaker C, Hinshelwood RA, Peinado MA, Clark SJ. Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. *Nat Genet* 2006; **38**: 540–9.
34 Arai E, Ushijima S, Gotoh M *et al.* Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma. *Int J Cancer* 2009; (in press).

35 Stein JP, Lieskovsky G, Cote R *et al.* Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J Clin Oncol* 2001; **19**: 666–75.
36 Gallagher DJ, Milowsky MI, Bajorin DF. Advanced bladder cancer: status of first-line chemotherapy and the search for active agents in the second-line setting. *Cancer* 2008; **113**: 1284–93.
37 Lipponen PK. The prognostic value of basement membrane morphology, tumour histology and morphometry in superficial bladder cancer. *J Cancer Res Clin Oncol* 1993; **119**: 295–300.
38 Scrimger RA, Murtha AD, Parliament MB *et al.* Muscle-invasive transitional cell carcinoma of the urinary bladder: a population-based study of patterns of care and prognostic factors. *Int J Radiat Oncol Biol Phys* 2001; **51**: 23–30.
39 Etoh T, Kanai Y, Ushijima S *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.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Examples of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) data validation by pyrosequencing.

Table S1. Eighty-three bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) (N) from normal urothelia (C) with a sensitivity and specificity of 75% or more.

Table S2. Twenty bacterial artificial chromosome (BAC) clones that were able to discriminate urothelial carcinomas (UCs) in patients who developed recurrence (Pos) from those in patients who did not (Neg).

Table S3. Eleven bacterial artificial chromosome (BAC) clones that were able to discriminate noncancerous urothelia in patients with urothelial carcinomas (UCs) of the renal pelvis or ureter who developed intravesical metachronous UC (Pos) from those in patients who did not (Neg).

Table S4. Genes, CpG islands in the promoter regions, and repeat elements of bacterial artificial chromosome (BAC) clones in Tables S1, S2, and S3.

Table S5. Primer sets for validation study by pyrosequencing.

Table S6. Correlation between smoking history and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S7. Correlation between protein expression levels of DNA methyltransferase (DNMT) 1 and DNA methylation status in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Table S8. Correlation between regional DNA hypermethylation on CpG islands and the results of bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA) in noncancerous urothelia obtained from patients with urothelial carcinomas (UCs) and UCs.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi: 10.1111/j.1349-7006.2009.01330.x

Research Article

Cancer Prevention Research

Epigenetic Alteration of DNA in Mucosal Wash Fluid Predicts Invasiveness of Colorectal Tumors

AU Seiko Kamimae[1], Eiichiro Yamamoto[1,2], Hiro-o Yamano[5], Masanori Nojima[3], Hiromu Suzuki[1,2], Masami Ashida[1], Tomo Hatahira[1], Akiko Sato[1], Tomoaki Kimura[6], Kenjiro Yoshikawa[6], Taku Harada[6], Seiko Hayashi[6], Hiroyuki Takamaru[2], Reo Maruyama[1,2], Masahiro Kai[1], Morie Nishiwaki[5], Tamotsu Sugai[7], Yasushi Sasaki[4], Takashi Tokino[4], Yasuhisa Shinomura[2], Kohzoh Imai[8], and Minoru Toyota[1]

Abstract

Although conventional colonoscopy is considered the gold standard for detecting colorectal tumors, accurate staging is often difficult because advanced histology may be present in small colorectal lesions. We collected DNA present in mucosal wash fluid from patients undergoing colonoscopy and then assessed the methylation levels of four genes frequently methylated in colorectal cancers to detect invasive tumors. We found that methylation levels in wash fluid were significantly higher in patients with invasive than those with noninvasive tumors. Cytologic and *K-ras* mutation analyses suggested that mucosal wash fluid from invasive tumors contained greater numbers of tumor cells than wash fluid from noninvasive tumors. Among the four genes, levels of *mir-34b/c* methylation had the greatest correlation with the invasion and showed the largest area under the receiver operating characteristic curve (AUC = 0.796). Using cutoff points of *mir-34b/c* methylation determined by efficiency considerations, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) and 0.765/0.833 for the 17.8% (well-balanced) cutoffs. In the validation test set, the AUC was also very high (0.915), the sensitivity/specificity were 0.870/0.875 for 13.0% and 0.565/0.958 for 17.8%. Using the diagnostic tree constructed by an objective algorithm, the diagnostic accuracy of the invasiveness of colorectal cancer was 91.3% for the training set and 85.1% for the test set. Our results suggest that analysis of the methylation of DNA in mucosal wash fluid may be a good molecular marker for predicting the invasiveness of colorectal tumors. *Cancer Prev Res; 4(5); 1–9. ©2011 AACR.*

Introduction

Colorectal cancer is one of the most common neoplasias worldwide (1), and its early detection and accurate preoperative staging are essential for reducing the incidences of invasion and metastasis. The fecal occult blood test is widely used to screen for colorectal tumors, though its sensitivity and specificity are not high (2, 3). Conventional colonoscopy is considered the gold standard for detecting colorectal cancers and adenomas, whereas several other methods, including computed tomography, ultrasonography, and 3D magnetic resonance, have been used for staging (4, 5). Generally, tumor size is used as the marker for invasion and lymph node metastasis; however, accurate staging is often difficult because advanced histology may be present in as much as 10% of small (5–10 mm) colorectal adenomas (6–8).

Magnified endoscopy is a highly useful method for diagnosing invasive colorectal cancer (9, 10). Although conventional endoscopic examination with indigo carmine dye is not sufficient to determine whether or not a colorectal cancer is invasive, pit pattern analysis, using high-magnification observation with crystal violet, reportedly enables the diagnosis of invasive colorectal cancers. Recently, narrow-band imaging magnification endoscopy has also been used to predict the invasiveness of colorectal tumors (11). However, it has been suggested that the skills required for pit pattern analysis will limit the number of endoscopists who use the technique.

DNA methylation plays a critical role in the tumorigenesis of colorectal cancer (12, 13). For example, promoter hypermethylation is associated with the silencing of various cancer-related genes (14, 15), and aberrant methylation of the CpG islands of genes in stool and serum/plasma can be used as a molecular marker for detection of colorectal tumors (16–20). On the contrary, because DNA

Authors' Affiliations: [1]Department of Biochemistry, [2]First Department of Internal Medicine, [3]Department of Public Health, and [4]Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University; [5]GeneticLab Co. Ltd., Sapporo; [6]Akita Red Cross Hospital, Akita; [7]Department of Pathology, Iwate Medical University, Morioka; and [8]The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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

S. Kamimae, E. Yamamoto, and H. Yamano contributed equally to this work.

Corresponding Author: Minoru Toyota, Department of Biochemistry, Sapporo Medical University, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan. Phone: 81-11-611-2111 (Ext. 2680); Fax: 81-11-622-1918. E-mail: mtoyota@sapmed.ac.jp

doi: 10.1158/1940-6207.CAPR-10-0214

methylation is an early event (21–23), it does not enable one to distinguish between premalignant lesions and invasive tumors. As yet, there is no study describing a molecular test for predicting the invasiveness of colorectal tumors.

In this study, we examined the methylation levels of 4 genes frequently methylated in colorectal cancers by using DNA obtained from mucosal wash fluid. We found that the methylation level of DNA in the wash fluid was significantly higher in patients with invasive than those with noninvasive tumors. Our results suggest that methylation of DNA in mucosal wash fluid could be a good molecular marker for predicting the invasiveness of colorectal tumors.

Materials and Methods

Specimens and sample preparation

Colorectal tumor tissues and washing fluid were collected from Japanese patients who underwent endoscopic mucosal resection (EMR) or surgical resection of colorectal tumors at Akita Red Cross Hospital. Informed consent was obtained from all patients before collection of the specimens. Approval for this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University.

We used 2 methods to obtain DNA from mucosal washing fluid. When a colorectal tumor was detected during colonoscopy, the tumor's surface mucus was either washed away by using 20 mL of water, which was aspirated through the suction channel of the endoscope, and suspended in ThinPrep PreservCyt solution (Hologic, Inc.; method 1) or washed away with 20 mL of normal saline by using an NT tube and collected (method 2). Each sample of collected washing fluid was placed in a Non-GYN PreservCyt vial (Hologic, Inc.) for a minimum of 15 minutes, after which the solution was transferred to a disposable centrifuge tube and centrifuged at 500 × *g* for 20 minutes. The resultant supernatant was discarded, and the cell pellet was suspended in ThinPrep solution until DNA extraction. After collection of the washing fluid and endoscopic observation, biopsies of the colorectal tumor and corresponding normal colonic mucosa were carried out by using biopsy forceps under endoscopic guidance. For cytology analysis, a ThinPrep slide was prepared by a T2000 ThinPrep processor (Hologic, Inc.) and a nongynecologic ThinPrep Filter with 5-μm pores. The slide was then Papanicolaou stained and examined by GLab cytotechnologists and a pathologist (GeneticLab Co. Ltd.), and atypical cells were identified. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope (Olympus) as described previously (24).

A total of 337 biopsy specimens including 150 colorectal tumors and 187 normal colonic mucosa specimens were examined. We also examined 88 samples of washing fluid from 70 colorectal tumor patients and 18 healthy patients. For testing the clinical usefulness of the study, we examined an additional 47 colorectal tumor biopsy samples as the test set, which were independently obtained several months after the collection of prior samples. On the basis of histologic examination after any type of resection, biopsy specimens and wash fluid samples from a colorectal tumor were divided into 2 groups: invasive tumors and noninvasive tumors. Invasive tumors were defined as submucosal invasive tumors.

Bisulfite pyrosequencing

DNA was extracted from biopsy specimens and washing fluid by using the standard phenol-chloroform procedure, after which 1-μg samples of genomic DNA were modified with sodium bisulfite by an EpiTect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was then carried out as described previously (25). Primers for pyrosequencing were designed by PSQ Assay Design software (Qiagen). Following PCR, the biotinylated PCR product was purified, made single-stranded, and then used as a template in a pyrosequencing reaction run according to the manufacturer's instructions. The PCR products were bound to streptavidin Sepharose beads HP (Amersham Biosciences), after which beads with the immobilized PCR product were purified, washed, and denatured by using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified PCR product, pyrosequencing was carried out by a PSQ96MA system (Qiagen) and Pyro Q-CpG software (Qiagen). The primer sequences are listed in Supplementary Table S1.

K-ras mutation analysis

Mutation of codons 12 and 13 of *K-ras* was examined by using direct sequencing and pyrosequencing, as described previously (26). Pyrosequencing was done by a *K-ras* mutation detection kit (Qiagen) as suggested by the supplier.

Statistical analysis

All statistical analyses were carried out by SPSSJ 15.0 (SPSS Japan Inc.). To compare differences in methylation levels or other continuous values between groups, *t* tests or ANOVA with a post hoc Tukey test were carried out. Fisher's exact test was used for analysis of categorical data. To evaluate correlations between continuous values, Pearson's correlation coefficients were calculated. Receiver operating characteristic (ROC) curves for the diagnosis of invasive tumors were constructed on the basis of methylation levels, followed by area under the curve (AUC) calculation. A diagnostic tree to discriminate invasive tumors was constructed by using the training set based on the following objective algorithm. Step 1: classify the samples based on the most efficient cutoff of tumor size. Step 2: classify the samples based on the most efficient cutoffs of methylation levels in 4 sequences under the classification of the previous step. Step 3: repeat step 2 until no additional efficacy is observed. $P < 0.05$ (2-sided) were considered significant.

Results

Preparation of specimens

We first compared the 2 methods used to obtain tumor cells from colonoscopy wash fluid. When tumors were

Q3 **Table 1.** Amounts of DNA and quality of cytology obtained by using the 2 collection methods tested

	Biopsy (n = 11)	Wash fluid (method 1)			Biopsy (n = 37)	Wash fluid (method 2)		
		Total (n = 11)	IT (n = 8)	NI (n = 3)		Total (n = 37)	IT (n = 18)	NI (n = 19)
DNA, μg	21.28 ± 15.25	15.00 ± 11.12			17.08 ± 13.00	16.53 ± 16.88		
Cytology								
Diagnosable		2	1	1		17	11	6
Not diagnosable		9	7	2		20	7	13
		18.2%	12.5%	33.3%		45.9%	61.1%	31.6%

Abbreviations: IT: invasive tumors, NI: noninvasive tumors.

found during colonoscopy, the tumor surfaces were washed with water (method 1) or saline (method 2; Supplementary Fig. S1A and B). Both methods enabled us to obtain enough DNA for molecular analysis (Table 1), but comparison of the cytology revealed that wash fluid obtained with saline (method 2) enabled more accurate detection of anaplastic cells (Supplementary Fig. S1B; Supplementary Table 1). We therefore used method 2 for subsequent analyses.

DNA methylation in biopsy specimens

The clinical features of the patients examined in this study are summarized in Table 2. There was no statistically significant difference in age or gender between patients with invasive and noninvasive tumors. We selected 4 genes for analysis, *mir-34b/c*, *SFRP1*, *SFRP2*, and *DKK2*, which are frequently methylated in colorectal cancer (25, 27, 28). Methylation analysis was carried out by using bisulfite pyrosequencing with DNA from 337 specimens, including 52 invasive tumors, 98 noninvasive tumors, and 187 specimens of normal colon tissue. We found that the methylation levels of the 4 genes were significantly higher in cancerous tissue than in normal colorectal mucosa (Fig. 1); however, we found no difference in tissue methylation levels between invasive and noninvasive tumors.

DNA methylation in mucosal washing fluid

We next examined gene methylation in 76 samples of mucosal wash fluid from 36 patients with invasive tumors and 34 with noninvasive tumors, and from 18 patients without colorectal lesions (Fig. 1). When we compared the invasive and noninvasive tumors, we found no differences for *SFRP2* and *DKK2*. However, *mir-34b/c* and *SFRP1* showed higher levels of methylation in wash fluid from patients with invasive than with noninvasive tumors.

We then used ROC analysis to further compare mucosal wash fluid from invasive and noninvasive tumors (Fig. 3A and B). The odds ratios (OR) for the risk of invasion associated with the methylation levels of the 4 genes tested are shown in Table 3. High levels of methylation were significantly associated with an increased risk of invasion. Among the 4 genes, levels of *mir-34b/c* methylation had the greatest impact on the risk of invasion and showed the largest AUC (0.796). Using various cutoff points of *mir-34b/c* methylation determined on the basis of efficiency considerations for clinical use, the sensitivity/specificity were 0.861/0.657 for the 13.0% (high sensitivity) cutoff point and 0.765/0.833 for the 17.8% (well balanced) cutoff. We also subdivided the tumors on the basis of whether they were ≥25 mm or <25 mm in size. In tumors 25 mm or larger, *mir-34b/c* showed the highest AUC (0.816); its sensitivity/specificity was 0.862/0.667 and its OR was 12.5. In tumors smaller than 25 mm, *SFRP1* showed the highest AUC (0.810), with a sensitivity/specificity of 0.821/0.833 and an OR of 23.0.

We further verified the utility of DNA methylation in mucosal washing, using an independent set of specimens, which was established as the test set (Figs. 2, 3C and D; Table 4). All of these ROC analyses were considered the training set. In the test set, the methylation levels of *mir-34b/c* showed very high AUC again (0.915), sensitivity/specificity was 0.870/0.875 for the 13.0% cutoff, and 0.565/0.958 for the 17.8% cutoff. In tumors 25 mm or larger, the AUC of the methylation levels of *mir-34b/c* was 0.778. In tumors smaller than 25 mm, the AUC of the methylation levels of *SFRP1* was 0.695.

To make a more efficient diagnostic method suitable for clinical situations, we then constructed a diagnostic tree to classify invasive and noninvasive tumors on the basis of the combination of methylation levels detected in wash fluid (Fig. 4A). First, because most endoscopists make an endoscopic diagnosis according to the size of lesions, we defined the most efficient cutoff of tumor size as the first node of the diagnostic tree. As the next nodes, we used the most efficient cutoffs of the methylation levels of the 4 genes (*mir-34*, *SFRP1*, *SFRP2*, and *DKK2*). For example, as shown in Figure 4A, if the tumor size is more than 25 mm and the methylation level of *mir-34b/c* is more than 15%, this lesion is diagnosed as an invasive tumor. In the training set, the sensitivity and specificity were 0.943 (33/35) and 0.882 (30/34), respectively. The total accuracy of the diagnosis was 91.3% (63/69). For use in clinical situations, we validated this diagnostic tree by using an independent test

Table 2. Clinicopathologic features of the patients

	Training set								Test set					
	Biopsy sample				Washing fluid				Biopsy sample			Washing fluid		
	IT ($n = 52$)	NI ($n = 98$)	*P*	Normal ($n = 187$)	IT ($n = 36$)	NI ($n = 34$)	*P*	Normal ($n = 18$)	IT ($n = 20$)	NI ($n = 21$)	*P*	IT ($n = 23$)	NI ($n = 24$)	*P*
Demographics														
Age	67.4	66.7	0.720	67.2	68.8	64.7	0.101	59.6	69.3	69.6	0.553	70.3	69.3	0.707
Male	16 (30.8%)	24 (24.5%)		126 (67.4%)	22 (61.1%)	25 (73.5%)		9 (50.0%)	14 (70%)	14 (66.7%)		14 (60.9%)	17 (70.8%)	
Female	36 (69.2%)	74 (75.5%)	0.408	61 (32.6%)	14 (38.9%)	9 (26.5%)	0.269	9 (50.0%)	6 (30%)	7 (33.3%)	0.332	9 (39.1%)	7 (29.2%)	0.213
Tumor size														
≥25 mm	31 (59.6%)	12 (12.2%)			27 (75%)	5 (14.7%)			15 (75%)	3 (14.3%)		18 (78.3%)	3 (12.5%)	
<25 mm	21 (40.4%)	86 (87.8%)	<0.001		9 (25%)	29 (85.3%)	<0.001		5 (25%)	18 (85.7%)	<0.001	5 (21.7%)	21 (87.5%)	<0.001
Location														
Right	21 (40.4%)	44 (44.9%)			12 (33.3%)	17 (50%)			11 (55%)	12 (57.1%)		11 (47.8%)	13 (54.2%)	
Left	15 (28.8%)	25 (25.5%)			14 (38.9%)	7 (20.6%)			7 (35%)	7 (33.3%)		8 (34.8%)	8 (33.3%)	
Rectum	16 (30.8%)	29 (29.6%)	0.853		10 (27.8%)	10 (29.4%)	0.332		2 (10%)	2 (9.5%)	0.883	4 (17.4%)	3 (12.5%)	0.980
Histology														
Hyper/inflammatory		15 (15.3%)				3 (8.8%)				0 (0.0%)			0 (0.0%)	
Tubular adenoma		29 (29.6%)				10 (29.4%)				9 (42.9%)			12 (50%)	
Tubulovillous adenoma		28 (28.6%)				7 (20.6%)				7 (33.3%)			7 (29.2%)	
Severe dysplasia		26 (26.5%)				14 (41.2%)				5 (23.8%)			5 (20.8%)	
Cancer	52 (100.0%)				36 (100.0%)				20 (100.0%)			23 (100.0%)		

Abbreviations: IT: invasive tumors, NI: noninvasive tumors.

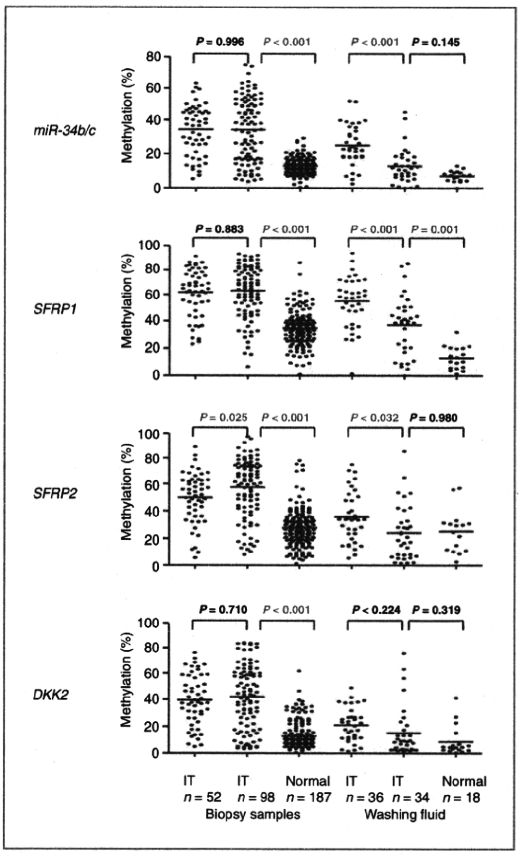


Figure 1. Levels of *mir-34b/c*, *SFRP1*, *SFRP2*, and *DKK2* methylation in invasive and noninvasive colorectal tumors. Methylation levels detected with DNA from biopsy and wash fluid. The genes analyzed are shown on the left.

set ($n = 47$). The application of the diagnostic tree to the test set is shown in Figure 4B. Although a slight reduction in sensitivity was observed (0.740, 17/23), the specificity was very high (0.958, 21/24). The total accuracy of the diagnosis was 85.1% (40/47) for the test set.

We also assessed the correlation between methylation levels detected in biopsy specimens and in wash fluid (Supplementary Fig. S2). We found that overall methylation levels in biopsy tissues and washing fluid were well correlated. When we divided the data for invasive and noninvasive tumors, however, only invasive tumors showed a significant correlation between methylation levels in biopsy tissue and washing fluid.

Detection of *K-ras* mutation by mucosal washing fluid

Finally, we tested for mutation of *K-ras* codons 12 and 13 by using DNA obtained from biopsy tissue or wash fluid (Supplementary Table S2). With invasive tumors, mutations of *K-ras* were found in 9 of 27 (33.3%) biopsy specimens. Among the 9 positive tumors, we were able to also detect mutations in 7 (77.7%) wash fluid samples. With noninvasive tumors, mutations were detected in 6 of 24 (25%) biopsy specimens, but in only 2 (33.3%) of the corresponding wash fluid samples. Addition of *K-ras* mutation did not improve the accuracy of diagnosis of invasiveness by the diagnostic tree (data not shown). Consistent with this finding, nuclear staining showed more intact nuclei in wash fluid from invasive tumors than from noninvasive tumors (Supplementary Fig. S1C). Thus, samples from invasive tumors seem to contain higher concentrations of tumor-derived DNA than samples from noninvasive tumors.

Discussion

Small colorectal tumors are usually removed by endoscopic mucosal dissection, but if the tumor is invasive, surgical treatment is required because of the higher risk of lymph node metastasis. Consequently, precise preoperative diagnosis is critical for appropriate treatment of colorectal tumors. Magnifying colonoscopy is a useful means of distinguishing invasive from noninvasive tumors (9, 10). However, invasive colorectal tumors show a heterogeneous pit pattern, making it difficult to determine a therapeutic

Table 3. Results of ROC analyses of the methylation levels in 4 genes in the training set.

Tumor size	Training set					
	Genes	AUC Estimate (95% CI)	Cutoff (%)	Sensitivity Estimate (95% CI)	Specificity Estimate (95% CI)	ORs Estimate (95% CI)
Total	*miR34b/c*	0.796 (0.686–0.906)	13.0	0.861 (0.705–0.953)	0.647 (0.465–0.803)	11.4 (3.5–36.9)
			17.8	0.833 (0.609–0.899)	0.765 (0.588–0.893)	16.3 (5.0–53.0)
			21.0	0.611 (0.435–0.769)	0.882 (0.726–0.967)	11.8 (3.4–40.7)
	SFRP1	0.736 (0.616–0.857)	45.0	0.750 (0.578–0.879)	0.706 (0.525–0.849)	7.2 (2.5–20.7)
	SFRP2	0.688 (0.562–0.814)	33.0	0.583 (0.408–0.745)	0.765 (0.588–0.893)	4.5 (1.6–12.8)
	DKK2	0.702 (0.572–0.831)	11.0	0.806 (0.64–0.918)	0.647 (0.465–0.803)	7.6 (2.6–22.5)
≥25mm	*miR34b/c*	0.816 (0.665–0.967)	15.0	0.862 (0.683–0.961)	0.667 (0.223–0.957)	12.5 (1.7–92.3)
<25mm	*SFRP1*	0.810 (0.594–1.000)	51.0	0.821 (0.631–0.939)	0.833 (0.359–0.996)	23.0 (2.2–242.1)