alterations, can generate more malignant cancers and even determine patient outcome (FIGURE 2C).

Even though high-throughput detection technologies have recently been developed, the dynamics of DNA methylation at nonunique sequences, such as repetitive sequences and gene bodies, still remain to be determined [7]. However, our BAC array-based methods do not focus only on specific promoter regions and CpG islands, and have successfully identified the BAC regions including nonunique sequences in which coordinated DNA methylation alterations have clinicopathological impact. Evaluation of the correlation between the DNA methylation status of identified nonunique sequences and the clinicopathological diversity of cancers may provide new insights into the roles of DNA methylation during multistage carcinogenesis.

Carcinogenetic risk estimation based on DNA methylation profiles

With respect to HCCs, the results of analysis of DNA methylation status of CpG islands of multiple genes have been reported. Some groups have attempted to use DNA methylation profiles as molecular markers of early HCCs or as prognosic indicators for patients with HCCs [63,64]. Since BAMCA detects DNA methylation alterations that are regulated in a coordinated manner at multiple CpG sites in individual large regions of chromosomes, BAMCA may be able to identify unique diagnostic indicators that may be overlooked by other technologies. We then applied the BAMCA method to multistage hepatocarcinogenesis. HCCs are known to be medullary tumors without a fibrous stroma. Therefore, we were able to obtain cancer cells of high purity from fresh surgical specimens, avoiding any contamination with stromal cells. In samples of noncancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hyper-methylation in comparison with normal liver tissue from patients without HCCs. Patients showing DNA hypo- or hyper-methylation on more BAC clones in their samples of noncancerous liver tissue frequently developed metachronous or recurrent HCCs after hepatectomy [65], suggesting that DNA methylation alterations at precancerous stages may not occur randomly but tend to lead to the development of more malignant HCCs.

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at precancerous stages is a priority. To reveal the baseline liver histology, microscopy examination of liver biopsy specimens is performed for patients with hepatitis virus infection prior to interferon therapy. Therefore, carcinogenetic risk estimation using such liver biopsy specimens would be advantageous for close follow-up of patients who are at high risk of HCC development.

Inflammation itself can induce drastic DNA methylation alterations at the chronic hepatitis stage. Although a proportion of such alterations do participate in progression to HCC, the remaining inflammation-associated DNA methylation alterations may diminish after the hepatitis stage has passed and as HCC develops. DNA methylation alterations associated only with inflammation and not with carcinogenesis cannot be regarded as indicators for carcinogenetic risk estimation in patients who are being followed up owing to chronic hepatitis. Therefore, to estimate the degree of carcinogenetic risk based on DNA methylation profiles, we focused on BAC clones for which DNA methylation status was altered at the chronic hepatitis and liver cirrhosis stages and were inherited by HCCs from such precancerous stages. Among them, a bioinformatics approach identified the top 25 BAC clones for which DNA methylation status was able to discriminate 15 samples of noncancerous liver tissue from patients with HCCs in the learning cohort from 10 samples of normal liver tissue with sufficient sensitivity and specificity. We established the criteria for carcinogenetic risk estimation by combining the cutoff values of signal ratios for the 25 BAC clones (FIGURE 3A). Based on these criteria, the sensitivity and specificity for diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the learning cohort as being at high risk of carcinogenesis were both 100% [65]. Our criteria enabled diagnosis of additional noncancerous liver tissue samples obtained from patients with HCCs in the validation cohort (n = 50) as being at high risk of carcinogenesis with a sensitivity and specificity of 96% [65].

The number of BAC clones satisfying the criteria in noncancerous liver tissue samples showing chronic hepatitis obtained from patients with HCCs was not significantly different from that in noncancerous liver tissue samples showing cirrhosis obtained from patients with HCCs. In addition, the average number of BAC clones satisfying the criteria was significantly lower in samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs, than that in noncancerous liver tissue samples obtained from

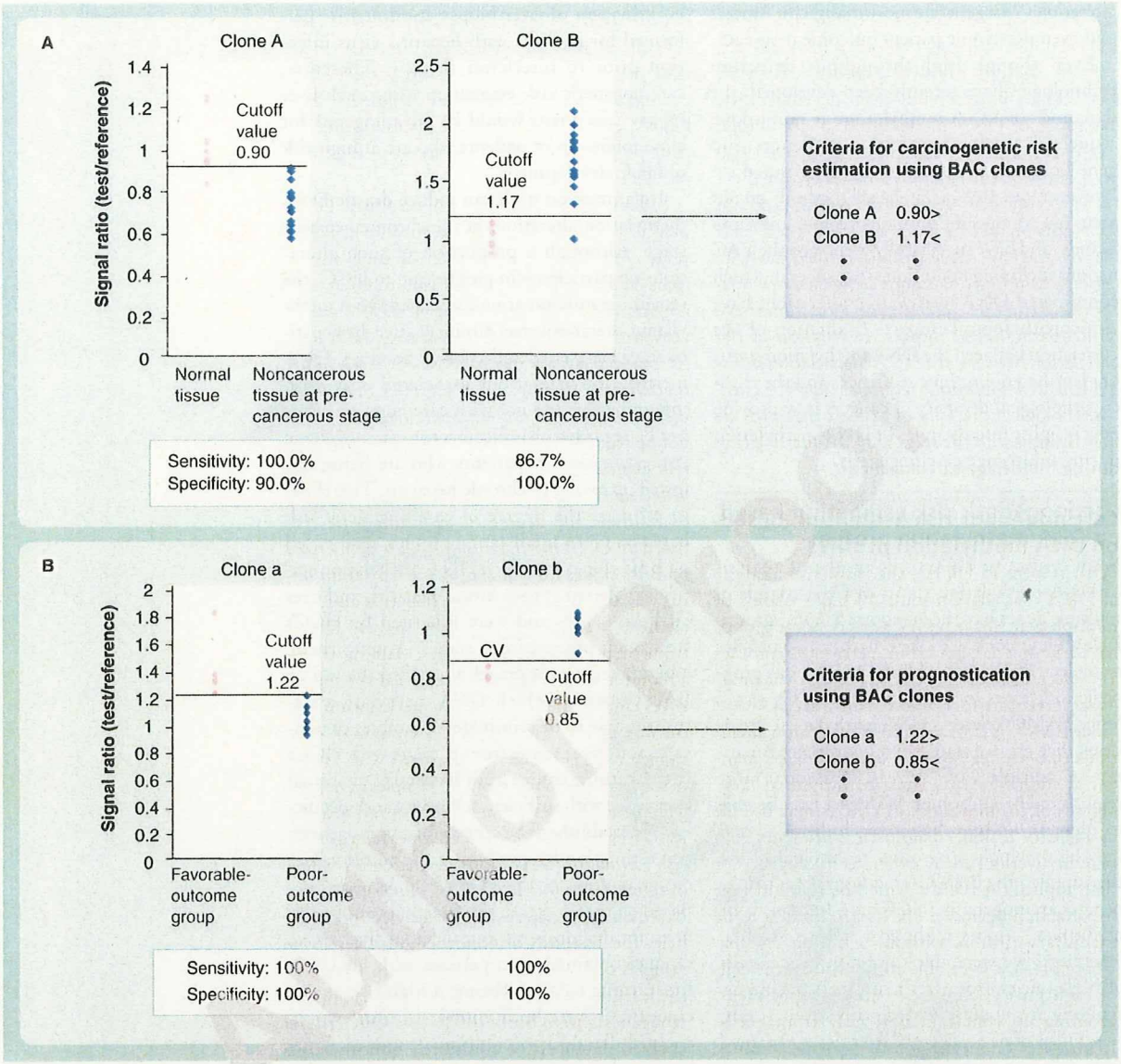

Figure 3. Carcinogenetic risk estimation and prognostication of patients with cancers based on DNA methylation status. (A) Carcinogenetic risk estimation based on DNA methylation status. Examples of scattergrams of the signal ratios in normal tissue samples and noncancerous tissue samples at precancerous stages for representative bacterial artificial chromosome (BAC) clones, clone A and clone B. Using the cutoff values in each panel, noncancerous tissue samples at precancerous stages were discriminated from normal tissue samples with sufficient sensitivity and specificity. Based on a combination of the cutoff values for the selected BAC clones, the criteria for carcinogenetic risk estimation were established. **(B)** Prognostication of patients with cancers based on DNA methylation status. Examples of scattergrams of the signal ratios in the favorable-outcome group and poor-outcome group for representative BAC clones, clone A and clone B. Using the cutoff values in each panel, patients belonging to the poor-outcome group were discriminated from those belonging to the favorable-outcome group. Based on a combination of the cutoff values for the selected BAC clones, the criteria for prognostication were established.

patients with HCCs. Therefore, our criteria may be applicable for classifying liver tissue obtained from patients who are followed up because of hepatitis virus infection, chronic hepatitis or liver cirrhosis into that which may generate HCCs and that which will not [65].

Next, we quantitatively examined the DNA methylation status at each *XmaI*/*SmaI* site on the 25 BAC clones by pyrosequencing. The sensitivity and specificity of the criteria revised by pyrosequencing have been successfully improved by using CpG sites having the largest diagnostic

impact on each BAC clone. It has been validated that screening by BAMCA, which has an ability for detecting any tendency for coordinated regulation of DNA methylation at multiple CpG sites in the entire BAC region, followed by revision using pyrosequencing, is a promising approach for carcinogenetic risk estimation. Pyrosequencing can be performed using a very small amount of degraded DNA extracted from liver biopsy specimens. In other words, unless another approach such as pyrosequencing is used to validate BAMCA data, risk assessment of liver biopsy specimens based only on BAMCA is premature. We now intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients with HBV or HCV infection.

Urothelila carcinomas are clinically remarkable because of their multicentricity owing to the 'field effect', whereby carcinogenic agents in the urine cause malignant transformation of multiple urothelial cells [66]. 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 (FIGURE 4A) [66]. 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 carcinoma is poor. In our previous study, accumulation of DNA methylation on C-type CpG islands associated with DNMT1 overexpression was observed even in noncancerous urothelia obtained from patients with UCs, and was further increased especially in nodular invasive carcinomas [67,68]. These previous data suggest that carcinogenetic risk estimation of UCs based on DNA methylation status might be a promising strategy.

We carefully took the tissue specimens from the surface of elevated UC lesions to avoid contamination with noncancerous urothelial and stromal cells. Principal component analysis based on BAMCA data revealed that stepwise DNA methylation alterations from 17 samples of noncancerous urothelia obtained from patients with UCs to 40 samples of UCs, in comparison with 18 samples of normal urothelia, occurred in a genome-wide manner [55]. We then performed unsupervised 2D hierarchical clustering analysis based on BAMCA data for noncancerous urothelia. The examined patients with UCs were clustered into two subclasses, clusters A_{NU} and B_{NU}. The incidence of invasive UCs (pT2 or more) was significantly higher in patients belonging to cluster B_{NU} defined on the basis of DNA methylation status in their noncancerous urothelia in comparison to cluster A_{NU} [55]. Moreover, Wilcoxon test identified the BAC clones whose signal ratios 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). DNA methylation profiles on such BAC clones of noncancerous urothelia obtained from patients with invasive UCs were inherited by the invasive UCs themselves (FIGURE 4B) [55]. DNA methylation alterations that were correlated with the development of more malignant invasive cancers were already accumulated in noncancerous urothelia.

To estimate the degree of carcinogenetic risk based on DNA methylation profiles in noncancerous urothelia, 83 BAC clones whose signal ratios discriminated noncancerous urothelia obtained from patients with UCs from normal urothelia with a sensitivity and specificity of 75% or more than 75% were identified. We established the criteria for carcinogenetic risk estimation by combining the cutoff values of the signal ratios for these 83 BAC clones [55]. We are currently attempting to develop a methodology for assessing the tendency of 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.

Approximately 10–30% of patients with UCs of the renal pelvis and ureter develop intravesical metachronous UCs after nephroureterectomy [69]. Therefore, such patients need 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. Since such metachronous UC originates from the noncancerous urothelium of the urinary bladder, we focused on the DNA methylation status of noncancerous urothelia, which may be exposed to the same carcinogens

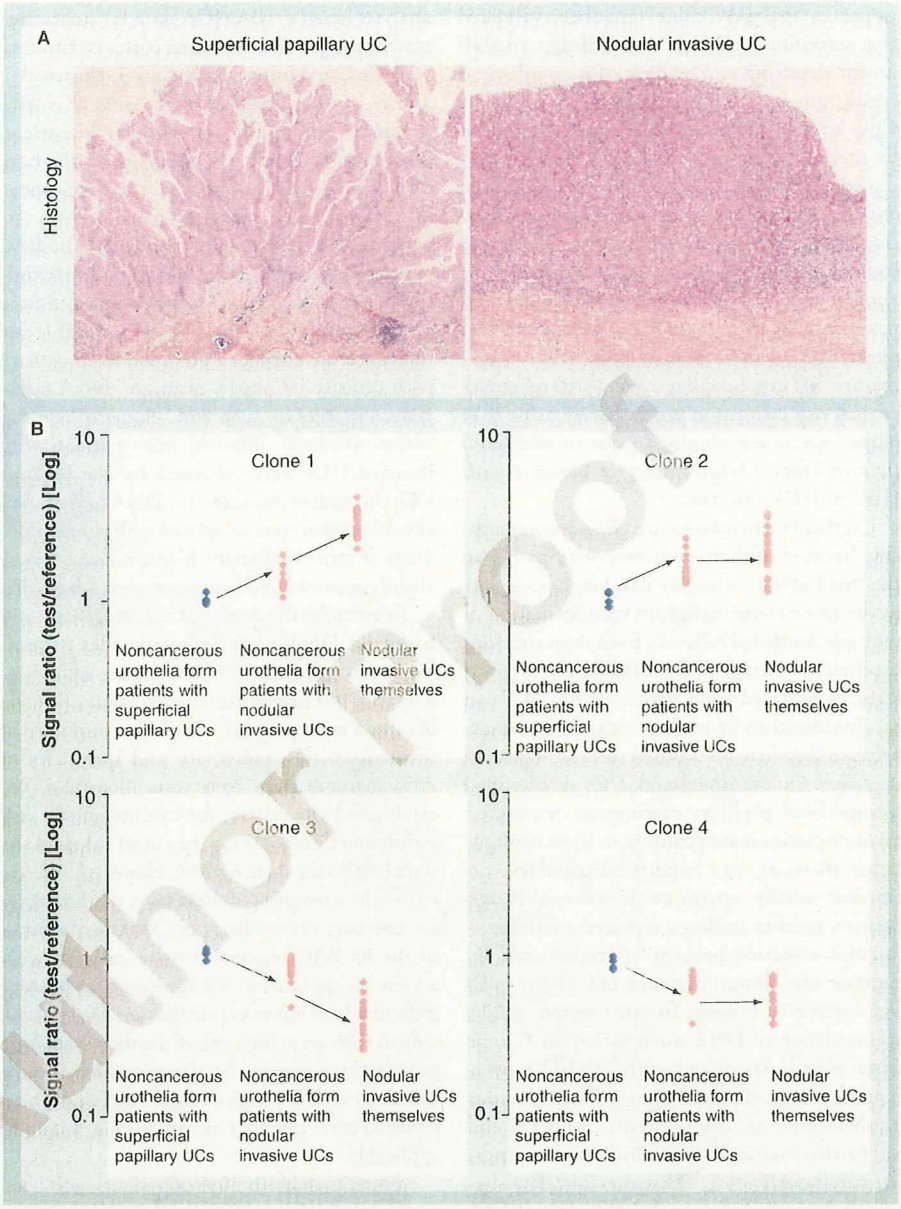

Figure 4. Significance of DNA methylation alterations at precancerous stages during urothelial carcinogenesis. For legend please see facing page..

in the urine, obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter. Unsupervised 2D hierarchical clustering analysis based on BAMCA data for noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter was able to group the examined patients into two subclasses, clusters A_{NP} and B_{NP}. The patients in cluster B_{NP} frequently developed intravesical metachronous UCs, whereas none belonging to cluster A_{NP} did so [55], indicating that DNA methylation profiles of noncancerous urothelia obtained by nephroureterectomy from patients with UCs of the renal pelvis or ureter are correlated with the risk of intravesical metachronous UC development. We identified nine BAC clones whose signal ratios discriminated noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter who developed intravesical metachronous UC after nephroureterectomy from noncancerous urothelia obtained from patients with UCs of the renal pelvis or ureter who did

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

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 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 urothelila 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 urothelila 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.

profiles of each cluster and the identification of target molecules for prevention and therapy in 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.

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