Journal of Human Genetics (2013) 58, 428–433

www.nature.com/jhg

REVIEW

Clinical application of the CpG island methylator phenotype to prognostic diagnosis in neuroblastomas

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Clinical applications of aberrant DNA methylation to cancer diagnostics and therapeutics are accelerating. Especially, the CpG island methylator phenotype (CIMP), simultaneous methylation of multiple genes, provides information that cannot be obtained by other diagnostic methods and therapeutic opportunities. CIMP is known to be associated with poor or good prognosis depending upon cancer types. We identified that CIMP in neuroblastomas (NBLs) is strongly associated with poor prognosis in Japanese NBL cases (hazard ratio (HR) = 22). Almost all NBLs with *MYCN* amplification displayed CIMP, and even among NBLs without *MYCN* amplification, NBLs with CIMP had worse prognosis (HR = 12). The prognostic power was faithfully reproduced in German NBL cases by the same methods, and also in Italian and Swedish NBL cases with different analytical methods. Mechanistically, methylation silencing of different sets of tumor-suppressor genes is involved in poor prognosis of NBLs with CIMP, and the presence of CIMP is most sensitively detected by methylation of the *PCDHB* family. For therapeutic purposes, a combination of 5-aza-2′-deoxycytidine, a DNA-demethylating drug, with 13-*cis*-retinoic acid, a differentiating drug, has been shown to be effective for NBLs *in vitro*, and further development of a better combination(s) is awaited. Now, epigenetic diagnosis and therapeutics are becoming or have become an important choice for cancer patients.
Journal of Human Genetics (2013) **58,** 428–433; doi:10.1038/jhg.2013.64; published online 6 June 2013

Keywords: CIMP; neuroblastoma; poor prognosis

INTRODUCTION

Aberrant DNA methylation is inherited over cell divisions and is deeply involved in carcinogenesis. Increasing numbers of cancer-specific aberrant methylation have been identified, and their usefulness in cancer diagnostics is becoming clear. DNA-demethylating drugs have been developed, and their usefulness in cancer therapeutics is also becoming clear. In cancer diagnostics, methylation of the Septin-9 gene in plasma is now being clinically evaluated as a detection marker for colorectal cancers.[1] Methylation of O^6-methylguanine-DNA methyltransferase in glioblastoma tissues is also used to predict the response of tumors to alkylating drugs.[2] Methylation of glutathione *S*-transferase pi1 specific to prostate cancer cells is used to detect the presence of prostate cancer in prostatectomy or biopsy tissue.[3] In addition to methylation of specific genes, the CpG island methylator phenotype (CIMP), methylation of multiple genes, has been reported to be associated with prognosis in various cancers.[4]

The therapeutic application of DNA methylation has already been brought into practice. Two demethylating drugs, 5-aza-2′-deoxycytidine (5-aza-CdR) and 5-azacytidine (5-aza-CR), have been approved by the US Food and Drug Administration for treatment of myelodysplastic syndrome. Application of these demethylating drugs to treatment of solid tumors is being tested in clinical trials.[5] In addition to these clinically used drugs, a new generation of DNA-demethylating drugs, such as SGI-110 and CP-4200, are also being developed.[6] Combinations of DNA-demethylating drugs with other drugs, such as histone deacetylase inhibitors,[7] cellular differentiating drugs[8] and cytotoxic drugs,[9] have produced some promising results.

In this review, we will introduce CIMP in various cancers and then focus on the high usefulness of CIMP in prognostic diagnosis of neuroblastomas (NBLs) and the potential in their treatment.

CIMP IN VARIOUS CANCERS OTHER THAN NBLS

CIMP was originally established in colorectal cancers by the pioneering work of Toyota *et al*[10]. Some colorectal cancers had frequent DNA methylation of specific CpG islands and were associated with microsatellite instability. Colorectal cancers are now classified into three groups, CIMP-high (CIMP1, HME), CIMP-low (CIMP2, IME) and CIMP-0 (CIMP-negative, LME), using tumor-specific methylation markers.[11–13] CIMP-high cases show low mortality compared with CIMP-0 (hazard ratio (HR) = 0.44; 95% confidence interval (CI) = 0.22–0.88; $n = 649$)[11] (Table 1). CIMP-high, -low and -0 are strongly associated with mutations of *BRAF*, *KRAS* and *TP53*, respectively.[11,12] Although the cause–consequence relationship between oncogene mutations and CIMP has not been established, some investigators propose that methylation silencing of senescence pathways is necessary to suppress oncogene-induced senescence and for a cell with an oncogene mutation to survive.[14,15]

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Received 24 April 2013; revised 6 May 2013; accepted 11 May 2013; published online 6 June 2013

Table 1 CIMP in various cancers and association with prognosis

	CIMP markers			
Cancer type	*Original markers*	*Other markers*	*Prognosis of patients with CIMP*	*Reference*
Colorectal cancer	MINT27, MINT2, MINT1, MINT12, MINT17, MINT31, MINT25	None	NA	10
Colorectal cancer	None	*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1*	NA	50
Colorectal cancer	MINT27, MINT2, MINT1, MINT12, MINT17, MINT31	*p16, p16ex1, hMLH1, RASSF1A, DAPK, MGMT, TIMP3, ER, sFRP1, MyoD1, HPP1, hTERT, RIZ1, p14, Megalin, COX2, THBS1, THBS2, SOCS1, RUNX3, Neurog1*	NA	12
Colorectal cancer	None	*p16, hMLH1, CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CRABP1*	Good CIMP-high vs -0 (HR = 0.44; 95% CI = 0.22–0.88; $n = 649$)	11
Colorectal cancer	MINT2, MINT1, MINT17, MINT31,	*p16, hMLH1, RASSF1A, MGMT, TIMP3, p14, CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, ALX4, RASSF5, ABTB2, C4orf31, CHFR, COL4A2, DUSP26, EFEMP1, IGFBP3, IGFBP7, IRF8, LOX, PPP1R3C, SCAM1, STOX2, TLE4, TMEFF2, UCHL1, ADAMTS1, AOX1, CDO1, CLDN23, EDIL3, EFHD1, ELMO1, EPHB1, FBN2, HAND1, ID4, KIAA0495, PENK, PPP1R14A, SSFRP1, SLC30A10, SPON1, THBD, TSPYL5, ZNF447, BNIP3, CIDEB, DFNA5, GRHL2, HLTF, OVOL1, RASSF2, TOLLIP*	NA	13
Gastric cancer	MINT2, MINT1, MINT12, MINT31, MINT25	None	Good CIMP-high vs -negative, $P = 0.004$; CIMP-low vs -negative, $P = 0.012$; $n = 78$[25]	16,17,25
Lung cancer	MINT1, MINT31, MINT32	*p16, MLH1, RASSF1A, RARβ, APC, DAPK, MGMT, GSTP1, CDH1, CDH13, sFRP1, sFRP2, sFRP4, sFRP5, TMS1, LAMC2*	NA	18
Liver cancer	MINT2, MINT1, MINT27, MINT31	*p16, CACNA1G, COX2, ER*	NA	19
Ovarian cancer	None	182 CGIs on a CGI microarray	Poor $P < 0.001$, $n = 19$	24
Leukemia	None	*p16, DAPK, CDH1, CDH13, sFRP1, sFRP2, sFRP4, sFRP5, TMS1, FHIT, ADAMTS1, ADAMTS5, APAF1, ASPP1, DBC1, DIABLO, DKK3, HDPR1, hRFC, LATS1, LATS2, NES1, p14, p15, p57, p73, PACRG, PARK2, PTEN, REPRIMO, RIZ, SHP1, SMC1L1, SMC1L2, SYK, WIF1*	Poor $P = 0.04$, $n = 54$	21
Bladder cancer	None	*p16, RASSF1A, RARβ, APC, DAPK, MGMT, GSTP1, CDH1, CDH13, FHIT*	Poor $P = 0.01$, $n = 98$	22
Esophageal adenocarcinoma	None	*p16, APC, DAPK, MGMT, CDH1, TIMP3, ER*	Poor HR = 2.7; 95% CI = 1.1–6.5; $P = 0.02$; $n = 41$	23
Glioblastoma	None	1503 CpG sites on Infinium HumanMethylation450 bead array	Good $P = 0.017$, $n = 253$	26
Glioblastoma	None	9711 CpG sites on Infinium HumanMethylation450 bead array	Good $P < 0.001$, $n = 72$	27

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; HR, hazard ratio; NA, not applicable.

The presence of CIMP has been reported in several other cancers, such as gastric,[16,17] lung,[18] liver,[19] ovarian cancers[20] and leukemias[21] (Table 1). The definition of CIMP is different in each cancer and the relationship between CIMP and prognosis is also different. Poor survival is associated with CIMP in bladder cancers ($P = 0.01$, $n = 98$),[22] esophageal adenocarcinomas (HR = 2.7; 95% CI = 1.1–6.5; $P = 0.02$; $n = 41$),[23] ovarian tumors ($P < 0.001$, $n = 19$)[24] and leukemias ($P = 0.04$, $n = 54$).[21] On the other hand, better survival is associated with CIMP in gastric cancers (CIMP-high vs -negative, $P = 0.004$, CIMP-low vs -negative, $P = 0.012$, $n = 78$).[25]

CIMP in glioblastomas is exceptionally well-characterized from a molecular viewpoint, and designated as G-CIMP. G-CIMP is associated with good prognosis ($P = 0.017$, $n = 253$),[26] and is known to be caused by isocitrate dehydrogenase 1 (*IDH1*) mutation.[27] Functionally, *IDH1* mutation has been shown as a gain-of-function mutation and to catalyze the NADPH-dependent reduction of α-ketoglutarate to 2-hydroxyglutarate.[28] 2-Hydroxyglutarate inhibits TET methylcytosine dioxygenase 2 that catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine,[29] and the inhibition of TET is considered to lead to methylation of multiple genes. Similar to the association of *IDH1* mutation and G-CIMP, *IDH1/2* mutation is also associated with methylation patterns in AML.[30]

IDENTIFICATION OF CIMP IN NBLS AS A STRONG PROGNOSTIC MARKER

NBL, derived from primitive cells of the sympathetic nervous system, is the most frequent extracranial cancer in childhood,[31] and is characterized by two extreme outcomes, spontaneous regression and

rapid progression. Accurate risk prediction is important for NBL in order to implement a necessary and sufficient level of treatment, and the International Neuroblastoma Risk Group classification system has been established for this purpose.[32] In this system, seven prognostic factors (stage, age, histologic category, grade of tumor differentiation, DNA ploidy and copy-number status at *MYCN* and at chromosome 11q) are used as the most clinically relevant factors. However, especially in the NBLs without *MYCN* amplification, it is still difficult to predict accurate prognosis and to decide on the therapeutic strategy.

Considering the major involvement of epigenetic machinery in embryonic development,[33,34] we searched for CGIs specifically methylated in NBLs with poor prognosis, not in those with good prognosis, using methylation-sensitive representational difference analysis.[35] Five CGI (or CGI groups), namely the *PCDHB* family, the *PCDHA* family, *HLP, DKFZp451I127* and *CYP26C1*, were found to be specifically methylated in NBLs with poor prognosis. Methylation of these five CGI (groups) was dependent upon each other, and conformed to the concept of CIMP. Methylation levels of the *PCDHB* family showed a clear bimodal distribution (Figure 1a), and NBL cases with high methylation levels of the *PCDHB* family showed poor overall survival with a HR of 22 (95% CI = 5.3–93) in 140 Japanese NBL cases.[35] Therefore, we decided to use the methylation levels of the *PCDHB* family as a marker of CIMP in NBLs.

To avoid false 'too good' results likely to be obtained by a genome-wide screening, we analyzed the prognostic power of CIMP in 152 German NBL cases in collaboration with Dr Schwab and Dr Westermann[36] (Figure 1b). DNA of German NBL cases was sent to our laboratory in Tokyo without clinical information, and methylation levels of the *PCDHB* family were analyzed. Regarding the cutoff value, as values between 40 and 60% gave high HRs in Japanese NBL cases, cases with methylation levels lower than 40% and higher than 60% were classified as NBLs without and with CIMP, respectively. The strong association between CIMP and poor overall survival was faithfully reproduced in German NBL cases with a HR of 9.5 (95% CI = 3.2–28). In addition, German NBL cases had information on disease-free survival, which was not available in Japanese NBL cases, and CIMP was shown to have prognostic significance also for disease-free survival with a HR of 5.4 (95% CI = 2.9–10). In addition to our studies, the strong prognostic power of CIMP was further confirmed in Italian and Swedish NBL cases.[37,38] Currently, in order to evaluate the clinical utility of CIMP as a prognostic marker, a prospective study is being conducted.

COMPARISON OF CIMP WITH *MYCN* AMPLIFICATION

MYCN amplification is known as the strongest prognostic marker for NBLs and is one of the first molecular markers used in practice.[39–41] The presence of *MYCN* amplification is therefore used as a biomarker for stratification of NBLs in practice and trials. Importantly, almost all NBLs with *MYCN* amplification displayed CIMP (37/38 in Japanese and 23/23 in German NBL cases) while some NBLs without *MYCN* amplification also displayed CIMP[35,36] (Figure 2a). The cases with *MYCN* amplification showed poor overall survival with HR of 9.5

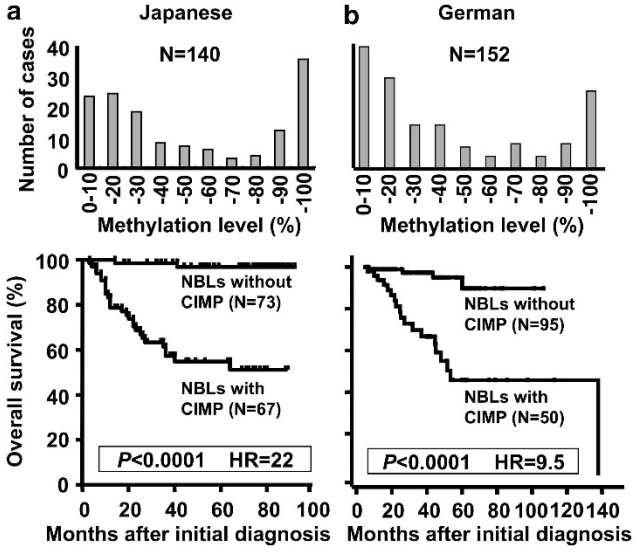

Figure 1 Bimodal distribution of methylation levels of the *PCDHB* family and its association with survival. (**a**) In 140 Japanese neuroblastoma (NBL) cases, the methylation level of the *PCDHB* family showed bimodal distribution (modified from Abe *et al.*[35]). The cutoff value for *PCDHB* family was set at 40%. NBLs with CpG island methylator phenotype (CIMP) had significantly and markedly worse overall survival, analyzed by the Kaplan–Meier method. (**b**) In 152 German NBL cases, a similar bimodal distribution of the *PCDHB* family methylation level was observed. As cutoff values between 40 and 60% gave high HRs in Japanese NBL cases, before the analysis, cutoff values of 40 and 60% were set for cases without and with CIMP, respectively, and cases with intermediate values were classified as intermediate. It was reproduced that NBLs with CIMP had significantly and markedly worse overall survival (modified from Abe *et al.*[36]).

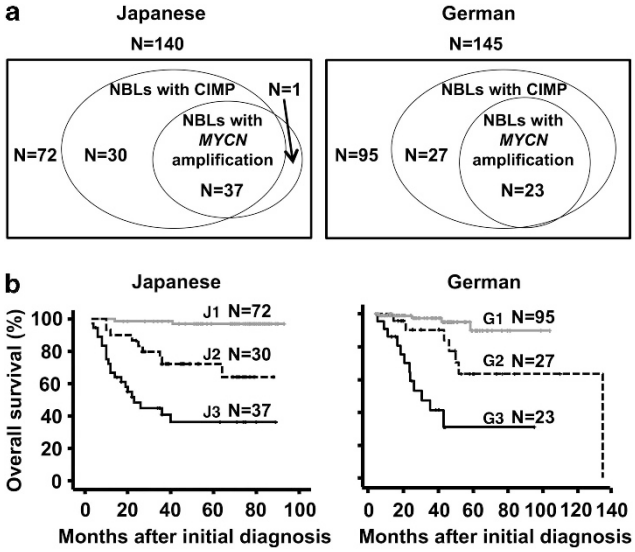

Figure 2 Comparison of CpG island methylator phenotype (CIMP) and *MYCN* amplification. (**a**) The relationship between neuroblastomas (NBLs) with CIMP and those with *MYCN* amplification are shown by Venn diagrams. Almost all NBLs with *MYCN* amplification (37/38 in Japanese and 23/23 in German NBL cases) displayed CIMP, and some additional cases had CIMP only. (**b**) Kaplan–Meier analysis of (J1, G1) NBL cases without CIMP or *MYCN* amplification ($N=72$ in Japanese and $N=95$ in German NBL cases), (J2, G2) NBL cases with CIMP without *MYCN* amplification ($N=30$ in Japanese and $N=27$ in German NBL cases), (J3, G3) NBL cases with both CIMP and *MYCN* amplification ($N=37$ in Japanese and $N=23$ in German NBL cases). Among NBLs without *MYCN* amplification (J1, J2 in Japanese and G1, G2 in German NBL cases), CIMP also had a significant and strong prognostic marker with a hazard ratio of 12 (95% confidence interval (CI) = 2.6–59; $P=0.002$) in Japanese and 4.5 (95% CI = 1.3–16; $P=0.02$) in German NBL cases.

(95% CI = 4.4–21) and 12 (95% CI = 4.9–29) in Japanese and German NBL cases, respectively.[35,36] Therefore, NBL cases were classified into three groups: (J1) NBL cases without CIMP nor *MYCN* amplification ($N = 72$), (J2) NBL cases with CIMP without *MYCN* amplification ($N = 30$) and (J3) NBL cases with both CIMP and *MYCN* amplification ($N = 37$) in Japanese NBL cases. German NBL cases were also classified into three groups (G1–G3) in the same manner (Figure 2b). The three groups, respectively, showed step-wise increases of risk, and notably, among NBLs without *MYCN* amplification (J1, J2 in Japanese and G1, G2 in German NBL cases), NBLs with CIMP showed worse prognosis with a HR of 12 (95% CI = 2.6–59) in Japanese and 4.5 (95% CI = 1.3–16) in German NBL cases. The almost complete inclusion of NBLs with *MYCN* amplification in those with CIMP indicates that these two abnormalities are very closely associated with each other. However, it is still unknown whether CIMP causes *MYCN* amplification, *MYCN* amplification causes CIMP, these two must coexist for development of NBLs, or if there is a shared upstream event.

MECHANISM FOR THE ASSOCIATION BETWEEN CIMP AND POOR PROGNOSIS

CGIs of the *PCDHB* family are located in their gene body, and their methylation was not associated with gene expression levels.[35] This suggested that, although methylation of the *PCDHB* family was closely associated with poor survival, simultaneous methylation of promoter CGIs was mechanistically involved in the poor survival (Figure 3). Indeed, in our study of Japanese NBL cases, methylation of promoter CGIs of the *RASSF1A*, *BLU*, *CYP26C1*, *FERD3L*, *CRYBA2* and *PCDHGC4* were methylated at significantly higher incidences in NBLs with CIMP, indicating that methylation silencing of tumor-suppressor genes was indeed associated with CIMP.[8,35]

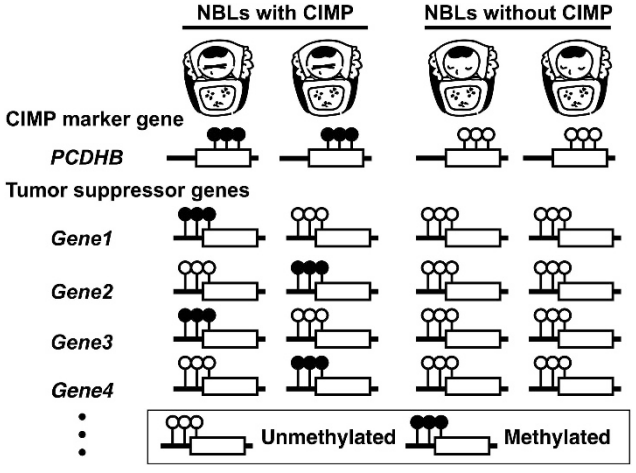

Figure 3 A likely mechanism for the association between CpG island methylator phenotype (CIMP) and poor prognosis. In neuroblastomas (NBLs) with and without CIMP, the exonic CGIs of the *PCDHB* family and promoter CGIs of tumor-suppressor genes typically showed methylation statuses as in this scheme. NBLs with CIMP had methylation of the exonic CGIs of the *PCDHB* family consistently, and that of promoter CGIs of multiple tumor-suppressor genes with lower frequencies. Although methylation silencing of tumor-suppressor genes was considered to be responsible for the poor prognosis of NBLs with CIMP, methylation of individual genes had less sensitivity of CIMP than methylation of the *PCDHB* family. On the other hand, NBLs without CIMP did not have methylation of the exonic CGIs of the *PCDHB* family or that of promoter CGIs of tumor-suppressor genes, and thus were considered to have a good prognosis.

To strengthen this hypothesis, we further analyzed methylation of promoter CGIs of genes whose silencing was reported to be involved in development or progression of NBLs, namely *CASP8, EMP3*, *HOXA9*, *NR1I2* and *CD44*. *CASP8* is an anti-apoptotic gene, and its methylation was reported to be associated with poor survival with HR of 5.3 ($P = 0.008$).[42] Also, methylation of *EMP3*, *HOXA9*, *NR1I2* and *CD44* were associated with poor survival with a *P*-value of 0.014, 0.03, 0.04 and 0.049, respectively.[42–45] We found that CIMP was associated with methylation of multiple promoter CGIs, mainly *CASP8* and *NR1I2*, but had stronger prognostic power than methylation of individual genes.[46] These results strengthened the hypothesis that CIMP leads to poor prognosis by induction of methylation of promoter CGIs of various tumor-suppressor genes at low incidences.

CIMP AS A POTENTIAL TARGET OF EPIGENETIC THERAPY

Poor prognosis of NBLs with CIMP is likely to be caused by silencing of multiple genes due to methylation of their promoter CGIs. As silenced genes are multiple and variable among individual NBLs, it was hypothesized that simultaneous demethylation of multiple genes could be effective for treatment of NBLs (Figure 4). Indeed, treatment of NBL cell lines with a demethylating drug, 5-aza-CdR, enhanced the sensitivity to the differentiation effect by 13-*cis*-retinoic acid.[8]

In addition to CIMP, aberrant histone modifications or modifying enzymes are also emerging as potential therapeutic targets of NBLs. For example, lysine-specific demethylase 1 (LSD1), a histone H3 lysine4 (H3K4) demethylase, is highly expressed in poorly differentiated NBLs, and inhibition of LSD1 using a monoamine oxidase inhibitor, tranylcypromine, resulted in growth suppression of NBLs *in vitro* and *in vivo*.[47] To improve selectivity for LSD1 over monoamine oxidase inhibitor, LSD1-selective inhibitors were developed,[48] and they are expected to show high anticancer efficacy and low toxicity in normal cells.

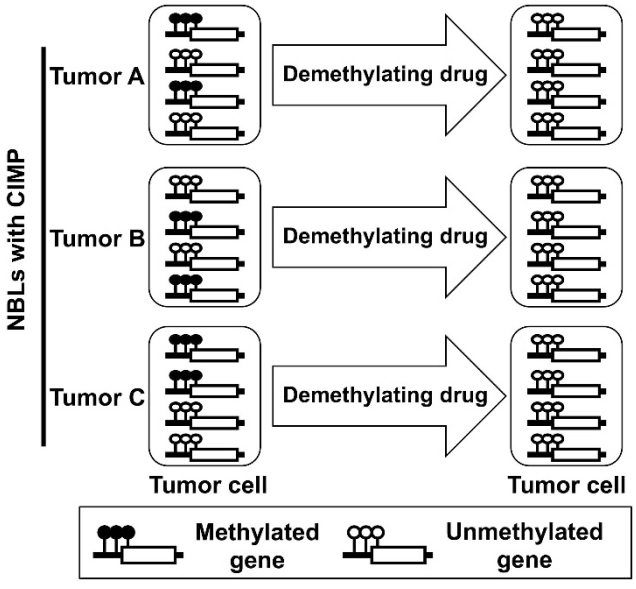

Figure 4 The concept of demethylating drug treatment for neuroblastomas (NBLs). In individual NBLs (tumor A–C), multiple but different tumor-suppressor genes are methylation-silenced, and reversal of these individual genes is difficult. By the use of a demethylating drug, multiple tumor-suppressor genes become simultaneously demethylated, and NBL cells are expected to show better responses to differentiation and cytotoxic agents.

Clinically, a phase I study of 5-aza-CdR with doxorubicin and cyclophosphamide in children with NBLs and other solid tumors was conducted in the United States,[49] and low-dose 5-aza-CdR ($5\,mg\,m^{-2}$) turned out to have tolerable toxicity in children. However, doses of 5-aza-CdR capable of producing clinically relevant biologic effects were not well tolerated. Different combinations between 5-aza-CdR and other drugs, such as differentiating drugs (13-*cis*-retinoic acid) or other epigenetic drugs (LSD1 inhibitors and histone deacetylase inhibitors), could improve the effectiveness of the demethylating drug for NBLs.

CONCLUSIONS

The usefulness of aberrant DNA methylation in cancer diagnostics and therapeutics is now coming into practice. In NBLs, CIMP has prognostic power in cases without *MYCN* amplification, and its strong prognostic power was validated in German, Italian and Swedish NBL cases. Combinations of a DNA-demethylating drug with a differentiating drug has been shown to be effective for NBLs with CIMP *in vitro*, and the appropriate dose and appropriate combination is expected to improve survival of NBL cases. Epigenetic diagnosis and therapeutics are becoming or have become an important choice for cancer patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by Grants-in-Aid for the Third-Term Cancer Control Strategy Program from the Ministry of Health, Labour and Welfare, Japan, and by the National Cancer Center Research and Development Fund, Japan.

1 Church, T. R., Wandell, M., Lofton-Day, C., Mongin, S. J., Burger, M., Payne, S. R. *et al.* Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* in press (2013).
2 Hegi, M. E., Diserens, A. C., Gorlia, T., Hamou, M. F., de Tribolet, N., Weller, M. *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* **352,** 997–1003 (2005).
3 Van Neste, L., Herman, J. G., Otto, G., Bigley, J. W., Epstein, J. I. & Van Criekinge, W. The epigenetic promise for prostate cancer diagnosis. *Prostate* **72,** 1248–1261 (2011).
4 Teodoridis, J. M., Hardie, C. & Brown, R. CpG island methylator phenotype (CIMP) in cancer: causes and implications. *Cancer Lett.* **268,** 177–186 (2008).
5 Cowan, L. A., Talwar, S., Yang, A. S. & Will, D. N. A. Methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics* **2,** 71–86 (2010).
6 Foulks, J. M., Parnell, K. M., Nix, R. N., Chau, S., Swierczek, K., Saunders, M. *et al.* Epigenetic drug discovery: targeting DNA methyltransferases. *J. Biomol. Screen.* **17,** 2–17 (2012).
7 Juergens, R. A., Wrangle, J., Vendetti, F. P., Murphy, S. C., Zhao, M., Coleman, B. *et al.* Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov.* **1,** 598–607 (2011).
8 Abe, M., Watanabe, N., McDonell, N., Takato, T., Ohira, M., Nakagawara, A. *et al.* Identification of genes targeted by CpG island methylator phenotype in neuroblastomas, and their possible integrative involvement in poor prognosis. *Oncology* **74,** 50–60 (2008).
9 Matei, D., Fang, F., Shen, C., Schilder, J., Arnold, A., Zeng, Y. *et al.* Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res.* **72,** 2197–2205 (2012).
10 Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B. & Issa, J. P. CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA* **96,** 8681–8686 (1999).
11 Ogino, S., Nosho, K., Kirkner, G. J., Kawasaki, T., Meyerhardt, J. A., Loda, M. *et al.* CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* **58,** 90–96 (2009).
12 Shen, L., Toyota, M., Kondo, Y., Lin, E., Zhang, L., Guo, Y. *et al.* Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc. Natl Acad. Sci. USA* **104,** 18654–18659 (2007).
13 Yagi, K., Akagi, K., Hayashi, H., Nagae, G., Tsuji, S., Isagawa, T. *et al.* Three DNA methylation epigenotypes in human colorectal cancer. *Clin. Cancer Res.* **16,** 21–33 (2010).
14 Suzuki, H., Igarashi, S., Nojima, M., Maruyama, R., Yamamoto, E., Kai, M. *et al.* IGFBP7 is a p53-responsive gene specifically silenced in colorectal cancer with CpG island methylator phenotype. *Carcinogenesis* **31,** 342–349 (2010).
15 Hinoue, T., Weisenberger, D. J., Pan, F., Campan, M., Kim, M., Young, J. *et al.* Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. *PLoS One* **4,** e8357 (2009).
16 Kim, H., Kim, Y. H., Kim, S. E., Kim, N. G. & Noh, S. H. Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability. *J. Pathol.* **200,** 23–31 (2003).
17 Toyota, M., Ahuja, N., Suzuki, H., Itoh, F., Ohe-Toyota, M., Imai, K. *et al.* Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res.* **59,** 5438–5442 (1999).
18 Marsit, C. J., Houseman, E. A., Christensen, B. C., Eddy, K., Bueno, R., Sugarbaker, D. J. *et al.* Examination of a CpG island methylator phenotype and implications of methylation profiles in solid tumors. *Cancer Res.* **66,** 10621–10629 (2006).
19 Shen, L., Ahuja, N., Shen, Y., Habib, N. A., Toyota, M., Rashid, A. *et al.* DNA methylation and environmental exposures in human hepatocellular carcinoma. *J. Natl Cancer Inst.* **94,** 755–761 (2002).
20 Strathdee, G., Appleton, K., Illand, M., Millan, D. W., Sargent, J., Paul, J. *et al.* Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. *Am. J. Pathol.* **158,** 1121–1127 (2001).
21 Roman-Gomez, J., Jimenez-Velasco, A., Agirre, X., Castillejo, J. A., Navarro, G., Calasanz, M. J. *et al.* CpG island methylator phenotype redefines the prognostic effect of t(12;21) in childhood acute lymphoblastic leukemia. *Clin. Cancer Res.* **12,** 4845–4850 (2006).
22 Maruyama, R., Toyooka, S., Toyooka, K. O., Harada, K., Virmani, A. K., Zochbauer-Muller, S. *et al.* Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res.* **61,** 8659–8663 (2001).
23 Brock, M. V., Gou, M., Akiyama, Y., Muller, A., Wu, T. T., Montgomery, E. *et al.* Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. *Clin. Cancer Res.* **9,** 2912–2919 (2003).
24 Wei, S. H., Chen, C. M., Strathdee, G., Harnsomburana, J., Shyu, C. R., Rahmatpanah, F. *et al.* Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers. *Clin. Cancer Res.* **8,** 2246–2252 (2002).
25 Kusano, M., Toyota, M., Suzuki, H., Akino, K., Aoki, F., Fujita, M. *et al.* Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. *Cancer* **106,** 1467–1479 (2006).
26 Noushmehr, H., Weisenberger, D. J., Diefes, K., Phillips, H. S., Pujara, K., Berman, B. P. *et al.* Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell* **17,** 510–522 (2010).
27 Turcan, S., Rohle, D., Goenka, A., Walsh, L. A., Fang, F., Yilmaz, E. *et al.* IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* **483,** 479–483 (2012).
28 Dang, L., White, D. W., Gross, S., Bennett, B. D., Bittinger, M. A., Driggers, E. M. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462,** 739–744 (2009).
29 Xu, W., Yang, H., Liu, Y., Yang, Y., Wang, P., Kim, S. H. *et al.* Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19,** 17–30 (2011).
30 Figueroa, M. E., Abdel-Wahab, O., Lu, C., Ward, P. S., Patel, J., Shih, A. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18,** 553–567 (2010).
31 Maris, J. M. Recent advances in neuroblastoma. *N. Engl. J. Med.* **362,** 2202–2211 (2010).
32 Cohn, S. L., Pearson, A. D., London, W. B., Monclair, T., Ambros, P. F., Brodeur, G. M. *et al.* The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J. Clin. Oncol.* **27,** 289–297 (2009).
33 Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33** (Suppl), 245–254 (2003).
34 Li, E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat. Rev. Genet.* **3,** 662–673 (2002).
35 Abe, M., Ohira, M., Kaneda, A., Yagi, Y., Yamamoto, S., Kitano, Y. *et al.* CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res.* **65,** 828–834 (2005).
36 Abe, M., Westermann, F., Nakagawara, A., Takato, T., Schwab, M. & Ushijima, T. Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett.* **247,** 253–258 (2007).
37 Banelli, B., Brigati, C., Di Vinci, A., Casciano, I., Forlani, A., Borzi, L. *et al.* A pyrosequencing assay for the quantitative methylation analysis of the PCDHB gene cluster, the major factor in neuroblastoma methylator phenotype. *Lab. Invest.* **92,** 458–465 (2011).
38 Kiss, N. B., Kogner, P., Johnsen, J. I., Martinsson, T., Larsson, C. & Geli, J. Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. *BMC Med. Genet.* **13,** 83 (2012).
39 Schwab, M., Alitalo, K., Klempnauer, K. H., Varmus, H. E., Bishop, J. M., Gilbert, F. *et al.* Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* **305,** 245–248 (1983).

40 Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. & Bishop, J. M. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* **224,** 1121–1124 (1984).
41 Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y. *et al.* Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N. Engl. J. Med.* **313,** 1111–1116 (1985).
42 Grau, E., Martinez, F., Orellana, C., Canete, A., Yanez, Y., Oltra, S. *et al.* Hypermethylation of apoptotic genes as independent prognostic factor in neuroblastoma disease. *Mol. Carcinog.* **50,** 153–162 (2011).
43 Alaminos, M., Davalos, V., Ropero, S., Setien, F., Paz, M. F., Herranz, M. *et al.* EMP3, a myelin-related gene located in the critical 19q13.3 region, is epigenetically silenced and exhibits features of a candidate tumor suppressor in glioma and neuroblastoma. *Cancer Res.* **65,** 2565–2571 (2005).
44 Alaminos, M., Davalos, V., Cheung, N. K., Gerald, W. L. & Esteller, M. Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma. *J. Natl Cancer Inst.* **96,** 1208–1219 (2004).
45 Misawa, A., Inoue, J., Sugino, Y., Hosoi, H., Sugimoto, T., Hosoda, F. *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).
46 Asada, K., Watanabe, N., Nakamura, Y., Ohira, M., Westermann, F., Scwab, M. *et al.* Stronger prognostic power of the CpG island methylator phenotype than methylation of individual genes in neuroblastomas. *Jpn J. Clin. Oncol.* in press (2013).
47 Schulte, J. H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R. *et al.* Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. *Cancer Res.* **69,** 2065–2071 (2009).
48 Ueda, R., Suzuki, T., Mino, K., Tsumoto, H., Nakagawa, H., Hasegawa, M. *et al.* Identification of cell-active lysine specific demethylase 1-selective inhibitors. *J. Am. Chem. Soc.* **131,** 17536–17537 (2009).
49 George, R. E., Lahti, J. M., Adamson, P. C., Zhu, K., Finkelstein, D., Ingle, A. M. *et al.* Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. *Pediatr. Blood Cancer* **55,** 629–638 (2010).
50 Weisenberger, D. J., Siegmund, K. D., Campan, M., Young, J., Long, T. I., Faasse, M. A. *et al.* CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat. Genet.* **38,** 787–793 (2006).