Epigenetic biomarkers for prediction of sensitivity to chemotherapeutic drugs in multiple myeloma

Hiroshi Yasui[1),2)], Tadao Ishida[2)], Masanori Nojima[3)], Yuka Aoki[2)], Hiroshi Ikeda[2)], Hiromu Suzuki[1),2)], Toshiaki Hayashi[2)], Yasuhisa Shinomura[2)], Minoru Toyota[1)]

[1)]Department of Biochemistry,
[2)]First Department of Internal Medicine, and
[3)]Department of Public Health, Sapporo Medical University School of Medicine, Sapporo, Japan

ABSTRACT

Multiple myeloma continues to be a lethal malignancy despite the development of treatments such as high-dose chemotherapy combined with stem cell transplantation. Multiple myeloma arises through an accumulation of multiple genetic changes, including immunoglobulin gene rearrangements involved in Cyclin D. The main difficulties in multiple myeloma treatments are drug-resistance. DNA methylation of the 5' CpG islands of genes is often found in multiple myeloma. To screen for the genes involved in tumorigenesis of multiple myeloma, which are silenced by DNA methylation, we performed cDNA microarray analysis using multiple myeloma cell lines treated with demethylating agent 5-aza-2'-deoxycytidine (DAC), and identified RASD1, a dexamethasone (Dex)-inducible gene, as one of the targets of epigenetic changes. Inactivation of RASD1 was found to correlate with resistance to Dex, and treatment of multiple myeloma cells with DAC restored sensitivity to Dex. These findings suggest the involvement of epigenetic gene silencing in multiple myeloma progression and drug-resistance, and the usefulness of demethylation therapy for multiple myeloma treatment. Furthermore, DNA methylation can be an epigenetic biomarker for multiple myeloma.

Key words : Multiple myeloma, Epigenetics, DNA methylation, Biomarker

INTRODUCTION

Epigenetic gene regulation such as DNA methylation and histone modification is considered to play a significant role in tumor development as well as in tumorigenesis. Under physiological conditions, DNA methylation plays a role in genome imprinting, X-chromosome inactivation, and suppression of repetitive sequences[1)]. DNA methylation of the 5' CpG islands of genes is an epigenetic alteration that leads to heritable changes in gene expression through recruitment of histone deacetylases and histone methyltransferases, which leads to condensation of chromatin. Genome-wide hypomethylation and regional hypermethylation are the common events in tumors[2)].

In the current review, we discuss the role of DNA methylation changes and their potential

CORRESPONDENCE TO : Hiroshi Yasui, M.D., Ph.D.
First Department of Internal Medicine, Sapporo Medical University School of Medicine,
S-1, W-16, Chuo-ku, Sapporo, 060-8543 Japan
E-mail : hiroyasu@sapmed.ac.jp
TEL : 011-611-2111 (ext. 3211)
FAX : 011-611-2282

application for epigenetic biomarkers for prediction of sensitivity to chemotherapeutic drugs in multiple myeloma.

1. Molecular mechanisms involved in tumorigenesis of multiple myeloma.

Multiple myeloma is a plasma cell neoplasia in bone marrow characterized by secreted monoclonal immunoglobulin and clinical features, including lytic bone lesions, anemia, renal function impairment, immune compromise, and hypercalcemia. It can occur de novo or evolve from an asymptomatic premalignant stage of clonal plasma cell proliferation, termed "monoclonal gammopathy of undetermined significance" (MGUS). Approximately 1% of individuals with MGUS evolve to multiple myeloma per year[3]. It is estimated that there will be 20,180 new cases of multiple myeloma diagnosed in the United States and 10,650 deaths attributed to this disease in 2010 alone, which is nearly 2% of all cancer deaths[4]. Multiple myeloma arises through an accumulation of multiple genetic changes, including point mutations, chromosomal gains and losses, and non-random chromosomal translocations such as immunoglobulin gene rearrangements involved in cyclin D, as well as of epigenetic alterations[5,6] (Fig. 1).

Multiple myeloma continues to be a lethal malignancy despite the development of treatments such as high-dose chemotherapy combined with stem cell transplantation due to chemotherapeutic resistance and, therefore, new treatment approaches are needed to improve the patient outcomes[7]. Recently, a bidirectional approach to translational research, moving laboratory discoveries to clinical settings or clinical observations to the laboratory environment, has been established in multiple myeloma[8-10]. Actually, in the past decade, there have been major advances in the treatment of multiple myeloma; new classes of drugs, including proteasome inhibitor bortezomib[11,12], thalidomide[13], and its immunomodulatory derivative lenalidomide[14], have emerged as highly active agents in the treat-

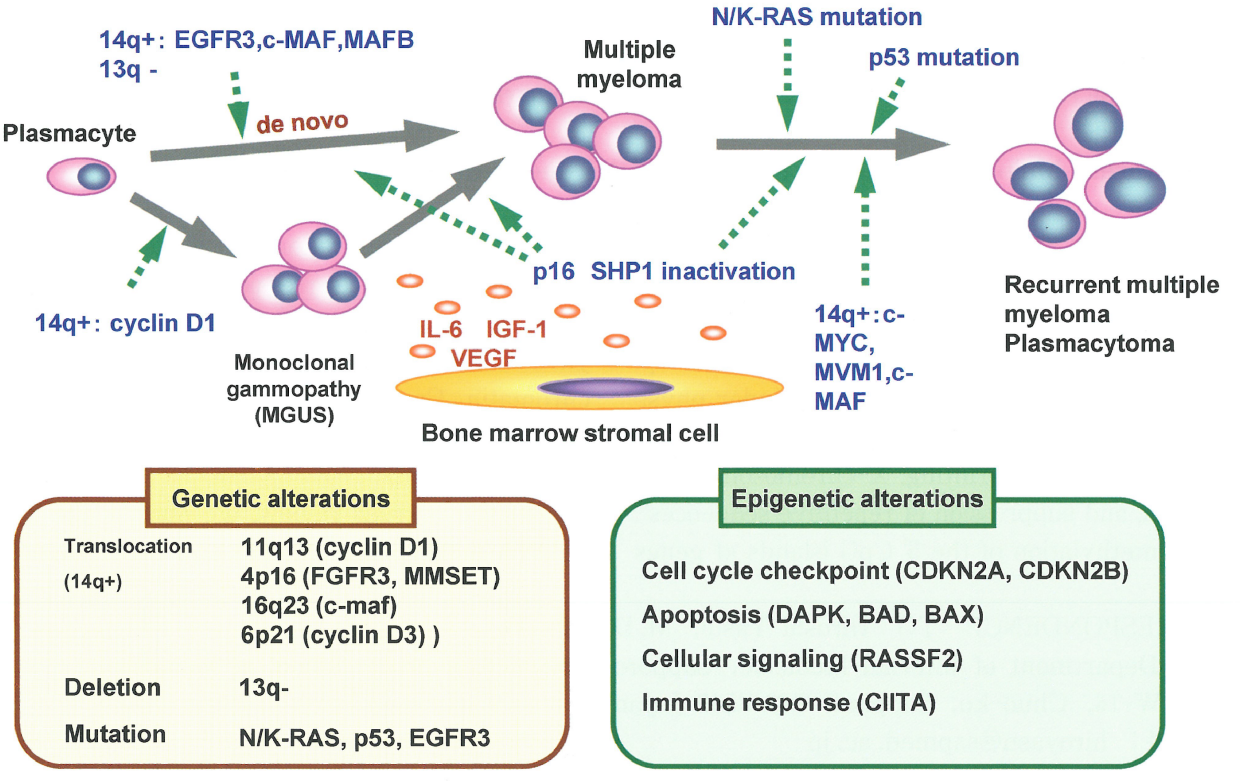

Figure 1 Molecular mechanisms involved in tumorigenesis of multiple myeloma.

ment of multiple myeloma. Dexamethasone (Dex) has long been a key drug due to its efficacy for killing multiple myeloma cells[15,16]. Dex is used even in very new regimens with bortezomib, thalidomide, or lenalidomide[11,12,17,18]. The main difficulties in multiple myeloma treatments are drug-resistance and opportunistic infection due to long-term and high-dose use of Dex. To overcome these problems, attempts have been made to find a new agent that enhances Dex cytotoxicity to multiple myeloma cells[8-11, 19-21].

2. DNA methylation.

Epigenetics is heritable information that does not affect DNA sequences. Among such changes, DNA methylation and modification of histone have been well-studied. In physiological states, DNA methylation plays a role in gene imprinting, X-chromosome inactivation, and silencing of repetitive sequences. DNA methylation, which occurs in cytosine bases located 5' to a guanine, known as CpG or CG dinucleotide, is catalyzed by three DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B, and plays a role in gene silencing (Fig. 2A, 2B). Knockout of DNMT1 together with DNMT3B in a colorectal cancer cell line, HCT116 cells (DKO cells), results in demethylation of 95% of methyl-cytosines[22]. DNA methylation leads to significant changes in chromatin structures, including recruitment of methyl-CpG binding domain proteins, deacetylation, and methylation of histone tails. Treatment of colorectal cancer cells with 5 -aza-2'-deoxycytidine (DAC) together with histone deacetylase inhibitor, trichostatin A (TSA), induces gene expression in a synergistic manner[23]. Analysis of target genes of polycomb repressive complexes in pluripotent embryonic stem cells (ESCs) has shown that patterns of polycomb-based repression are closely associated with target genes of DNA methylation in cancer, indicating a crosstalk between polycomb marks and DNA methylation[24,25]. In fact, EZH2, a histone methyltrasferase, which is a component of polycomb repressive complex 2, is frequently overexpressed in a variety of cancers[26].

Studies of DNA methylation in multiple myeloma have identified certain key genes as targets for epigenetic inactivation, including cell -cycle regulators such as CDKN2A[27], CDKN2B[27], and CHFR[28], and genes involved in cell signaling such as RASSF1[29], and TGFβ receptor II [30], genes involved in apoptosis such as DAPK1[31] and BNIP3[32], and genes involved in antigen presentation such as CIITA[33] (Table 1). Given the fact that more than one thousand genes are silenced by DNA methylation in colorectal cancers[34], the target of epigenetic inactivation in multiple myeloma may be largely unknown. Therefore, identification of novel genes epigenetically inactivated in multiple myeloma is of great importance for better understanding of the pathogenesis of the disease.

Table 1. Genes epigenetically silenced in multiple myeloma

Gene	Chromosomal location	Function	References
CDKN2A	9p21.3	Inhibition of cyclin-dependent kinase	27
CDKN2B	9p21.3	Inhibition of cyclin-dependent kinase	27
CHFR	12q24.33	Mitotic checkpoint	28
RASSF1A	3p21.31	Inhibition of Ras signaling	29
TGF-beta receotor 2	3p24.1	Suppression of cell growth, serine/threonine kinase	30
DAPK1	9q21.33	Apoptosis	31
BNIP3	10q26.3	Apoptosis	32
CIITA	16p13.13	Activtor of class II antigen	33

3. Epigenetic biomarkers for prediction of sensitivity to chemotherapeutic drugs in multiple myeloma and other types of cancer

To screen for tumor-related genes that are silenced by DNA methylation in multiple myeloma cells, we performed cDNA microarray analysis using multiple myeloma cell lines treated with mock or DAC. RASD1 was originally identified as a Dex-inducible gene[35], and has been shown to be a receptor-independent activator of G-protein signaling[36, 37]. RASD1 belongs to the Ras-like gene family (e.g. RIG, ARH 1/NOEY2, RRP22), which has recently been shown to suppress cell growth[38-40]. RASD1 is located in chromosome 17p11.2, in which frequent loss of heterozygosity is detected in various human tumors, and suppresses cell growth[41]. In addition, Furuta et al have reported epigenetic inactivation of RASD1 in a melanoma cell line [42], indicating that inactivation of RASD1 leads to a growth advantage for tumor cells.

While the hypermethylation of RASD1 was observed in approximately 10% of primary multiple myeloma samples, the methylation levels of RASD1 were elevated in all of the multiple myeloma cases that had pair DNAs after repeated anti-tumor therapy, including Dex. In addition, multiple myeloma cells that showed methylation of RASD1 are resistant to dexamethasone, and treatment with Dex with DAC restored the cytotoxicity of Dex to tumor cells (Fig. 3, 4). These findings suggest the involvement of epigenetic gene silencing in multiple myeloma progression and drug-resistance, and the usefulness of demethylation therapy for multiple myeloma treatment.

There is evidence to suggest that epigenetic inactivation of cancer-related genes, including cell-cycle checkpoint and DNA repair,

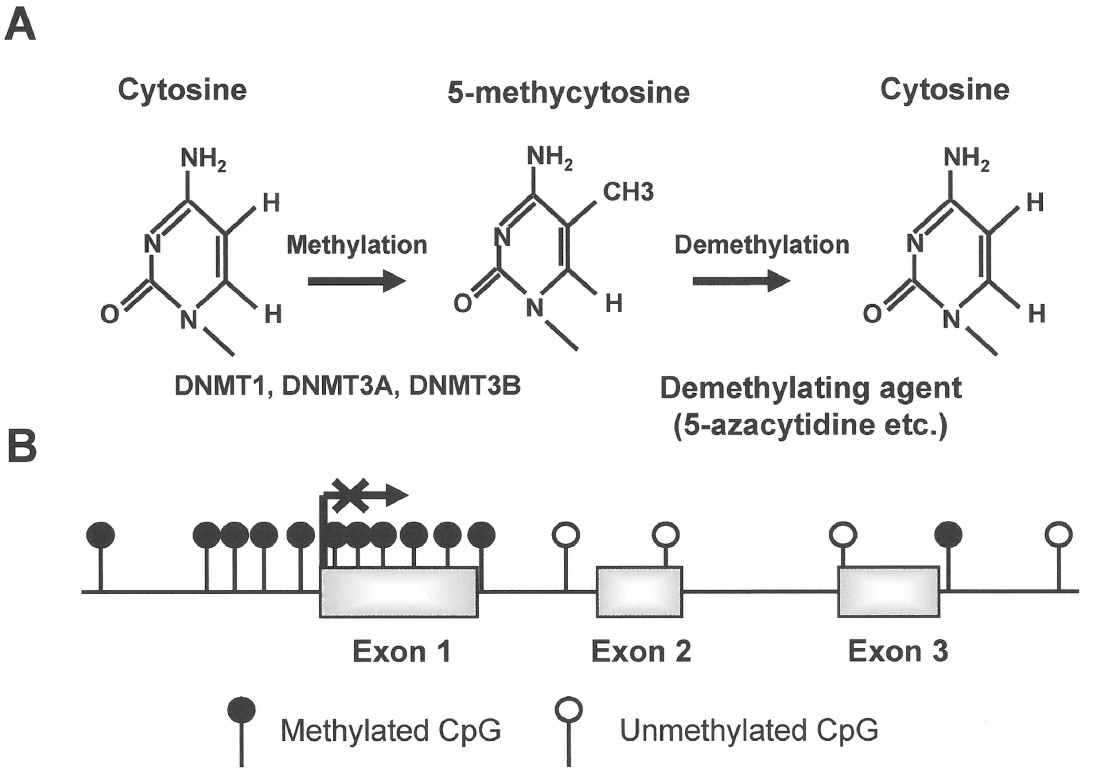

Figure 2. DNA methylation.
(A) In mammals, DNA methylation occurs at the 5' position of cytosine by DNA methyltransferases. DNA methylation is an epigenetic change that can be reversed by DNA methyltransferase inhibitors. (B) In cancer cells, CpG-rich regions, so-called CpG islands, are aberrantly methylated, which leads to a silencing of cancer-related genes.

are associated with sensitivity to chemotherapeutic agents in multiple myeloma and other types of tumors . We have found that a mitotic checkpoint gene, CHFR, is inactivated by DNA methylation in multiple human neoplasia, including multiple myeloma[28]. The microtubule inhibitors induced apoptosis among cancer cells, showing CHFR methylation, and indicated that adenoviral introduction of CHFR into methylated cancer cell lines restores the checkpoint and reduces the incidence of apoptosis[43]. This correlation between CHFR methylation and sensitivity to microtubule inhibitors appears to be specific, as there was no correlation between CHFR methylation and sensitivity to other chemotherapeutic agents or to ultraviolet. These results suggest that CHFR methylation could be used as an epigenetic biomarker marker to predict the sensitivity of tumors to microtubule inhibitors. Consistent with that idea, Koga et al have found that 86% of patients with methylated CHFR tumors showed some regression or no progression of their disease when treated with a microtubule inhibitor, whereas 80% patients with unmethylated CHFR tumor showed progressive deterioration[44]. A correlation between CHFR methylation and sensitivity to microtubule inhibitors has also been noted in oral squamous cell carcinoma[45]. Thus, CHFR methylation may be a clinically useful indicator of the responsiveness of cancers to treatment with microtubule inhibitors. The fact that CHFR is frequently inactivated by genetic or epigenetic alteration in human cancers suggests that this cancer-specific checkpoint defect could also be a useful therapeutic target. Bearing that in mind, we recently established a system to knock down CHFR expression using shRNA[45]. We found that CHFR expression was significantly suppressed in cancer cells transfected with shRNA. The resultant impairment of the prophase checkpoint led to an increased mitotic index in cells treated with microtubule inhibitors, which in turn led to an increased incidence of apoptosis among the cells. This effect was specific to microtubule inhibitors, as no effect was seen when a DNA-damaging agent was used. In addition, the earlier finding that E3 ubiquitin ligases can be targeted using small molecules[46] suggests that drugs that inhibit CHFR's ubiquitin ligase activity could also be useful for enhancing the sensitivity of cancer

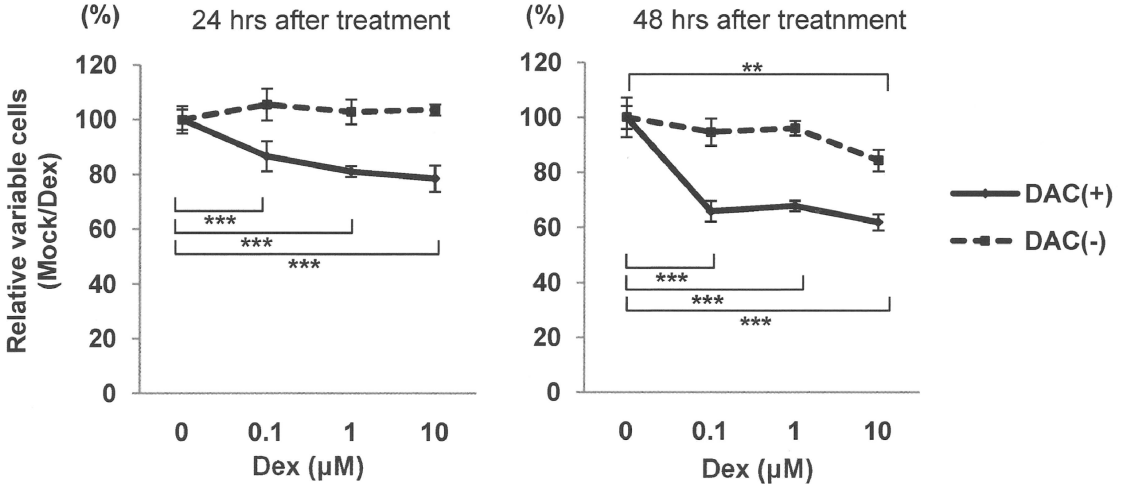

Figure 3. Cytotoxic activity of Dex combined with DAC.
An MST-8 assay was performed to examine the cytotoxic activity of dexamethasone (Dex) combined with 5-Aza-2' -deoxycytidine (DAC). Relative amounts of variable cells are shown on the Y -axis. OPM1 cells show that methylation of RASD1 is resistant to dexamethasone. When cells were treated with Dex with DAC, resistance to Dex was restored. (***P < 0.001 ; one way ANOVA with post hoc Dunnett's test.)

cells to microtubule inhibitors.

Genes involved in DNA repair have also been shown to be associated with the response to chemotherapeutic drugs. The first report on epigenetic alteration associated with sensitivity to chemotherapeutic drugs demonstrated an association between O(6)-methylguanine-DNA-methyltransferase (MGMT) methylation and sensitivity to DNA-alkylating agent[47]. MGMT is a DNA repair enzyme that removes mutagenic adducts from O6-guanine in DNA[48] Epigenetic silencing of MGMT has been reported in human neoplasia, including that in the colon, stomach, and glioma[49]. Methylation of MGMT has been shown to be associated with G:C to A:T transition mutations, indicating that MGMT inactivation leads to genetic instability[50,51]. Alkylating agents are one of the most widely used chemotherapeutic agents in human cancers. MGMT is a DNA repair enzyme that repairs the O6 position of guanine, which is most frequently modified by alkylating agents. Therefore, the toxicity of alkylating agents is reduced in the presence of MGMT[52]. In glioma, an enhanced sensitivity of patients with reduced MGMT expression has been observed[47]. MGMT methylation has been shown to be associated with the response to alkylating agents in glioma, and can be an epigenetic biomarker for glioblastoma patients treated with alkylating agents[53,54].

A subset of colorectal cancers shows methylation of a mismatch repair gene, hMLH1,

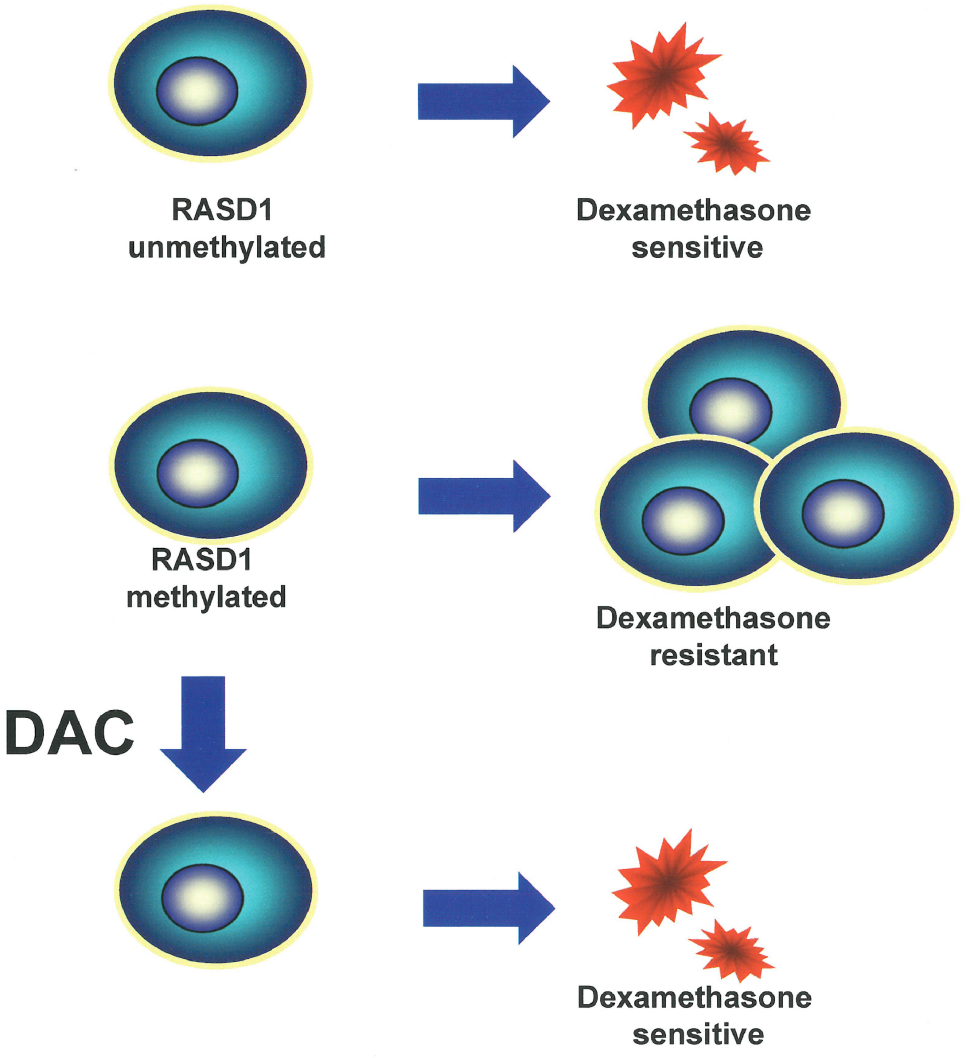

Figure 4. Demethylating agent restores the sensitivity of multiple myeloma cells to dexamethasone.

which is associated with microsatellite instability[55]. Colorectal cancers with microsatellite instability are clinically less aggressive, but respond poorly to 5-fluorouracil[56].The thymidylate synthase, which is necessary for DNA synthesis, and inhibition of thymidylate synthase is an important mechanism for the anticancer effects of 5-fluorouracil. It has been reported that colorectal cancers with hMLH1 methylation express high levels of thymidylate synthase[57]. Colorectal cancer cell lines showing microsatellite instability due to methylation of hMLH1, which is resistant to 5-fluorouracil, become sensitive after treatment treating cells with DAC[58], indicating that hMLH1 methylation can be an epigenetic marker to predict the sensitivity of colorectal cancer to 5-fluorouracil.

In summary, DNA methylation plays an important role in tumorigenesis of multiple myeloma. Drug-resistance of multiple myeloma can be reversed by demethylation therapy for multiple myeloma treatment. Our results also suggest that DNA methylation can be a useful biomarker to predict sensitivity to chemotherapeutic drugs. Further analysis using a genome-wide approach will be necessary for a comprehensive study to clarify the molecular mechanisms of drug-resistance in multiple myeloma.

ACKNOWLEDGMENTS

This study was supported by Aki Horinouchi Research Grant from the International Myeloma Foundation Japan (H.Y.), and by a Grant-in-Aid for Scientific Research (B) (M.T., Y.S.) and a Grant-in-Aid for Young Scientists (B) (H.Y., M. N., H.S.) from the Ministry of Education, Culture, Sports, Science, and Technology. The authors wouid like to dedicate this paper to patients and their family members who battle so bravely against intractable diseases.

REFERENCES

1. Bird A. The essentials of DNA methylation. Cell 1992; 70: 5-8.
2. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007; 128: 683-692.
3. Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G, Kröger N, Einsele H, Vesole DH, Dimopoulos M, San Miguel J, Avet-Loiseau H, Hajek R, Chen WM, Anderson KC, Ludwig H, Sonneveld P, Pavlovsky S, Palumbo A, Richardson PG, Barlogie B, Greipp P, Vescio R, Turesson I, Westin J, Boccadoro M; International Myeloma Working Group. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia 2010; 24: 1121-1127.
4. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; 60: 277-300
5. González D, van der Burg M, Garcáa-Sanz R, Fenton JA, Langerak AW, González M, van Dongen JJ, San Miguel JF, Morgan GJ. Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. Blood 2007; 110: 3112-3121.
6. Hideshima T, Bergsagel, PL, Kuehl, WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004; 104: 607-618.
7. Child JA, Morgan GJ, Davies FE, Owen RG, Bell SE, Hawkins K, Brown J, Drayson MT, Selby PJ; Medical Research Council Adult Leukaemia Working Party. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med 2003; 348: 1875-1883.
8. Yasui H, Hideshima T, Richardson PG, Anderson KC. Recent advances in the treatment of Multiple Myeloma. Curr Pharm Biotechnol 2006; 7: 381-393
9. Yasui H, Imai K. Novel molecular-targeted therapeutics for the treatment of cancer. Anticancer Agents Med Chem 2008; 8: 470-480
10. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC. Multiple myeloma. Lancet 2009; 374: 324-339

11. Hideshima T, Richardson P, Chauhan D, Palombella VJ, Elliott PJ, Adams J, Anderson KC. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001; 61: 3071-3076.
12. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008; 14: 1649-1657.
13. Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, Munshi N, Anaissie E, Wilson C, Dhodapkar M, Zeddis J, Barlogie B. Antitumor activity of thalidomide in refractory multiple myeloma. N Engl J Med 1999; 341: 1565-1571.
14. Dimopoulos M, Spencer A, Attal M, Prince HM, Harousseau JL, Dmoszynska A, San Miguel J, Hellmann A, Facon T, Foà R, Corso A, Masliak Z, Olesnyckyj M, Yu Z, Patin J, Zeldis JB, Knight RD; Multiple Myeloma (010) Study Investigators. Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma. N Engl J Med 2007; 357: 2123-2132
15. Gehring U, Mohit B, Tomkins GM. Glucocorticoid action on hybrid clones derived from cultured myeloma and lymphoma cell lines. Proc Natl Acad Sci U S A 1972; 69: 3124-3127.
16. Smith L, Alexanian R. Treatment strategies for plasma cell myeloma. CA Cancer J Clin 1985; 35: 214-220.
17. Palumbo A, Gay F, Bringhen S, Falcone A, Pescosta N, Callea V, Caravita T, Morabito F, Magarotto V, Ruggeri M, Avonto I, Musto P, Cascavilla N, Bruno B, Boccadoro M. Bortezomib, doxorubicin and dexamethasone in advanced multiple myeloma. Ann Oncol 2008; 19: 1160-1165.
18. Pineda-Roman M, Zangari M, van Rhee F, Anaissie E, Szymonifka J, Hoering A, Petty N, Crowley J, Shaughnessy J, Epstein J, Barlogie B. VTD combination therapy with bortezomib-thalidomide-dexamethasone is highly effective in advanced and refractory multiple myeloma. Leukemia 2008; 22: 1419-1427.
19. Neri P, Yasui H, Hideshima T, Tassone P, Raje N, Catley LP, Ishitsuka K, Blotta S, Kiziltepe T, Ocio EM, Fulciniti M, Kanekal S, Elliott GT, Munshi NC, Anderson KC. In vivo and in vitro cytotoxicity of R-etodolac with dexamethasone in glucocorticoid-resistant multiple myeloma cells. Br J Haematol 2006; 134: 37-44.
20. Yasui H, Hideshima T, Hamasaki M, Roccaro AM, Shiraishi N, Kumar S, Tassone P, Ishitsuka K, Raje N, Tai YT, Podar K, Chauhan D, Leoni LM, Kanekal S, Elliott G, Munshi NC, Anderson KC. SDX-101, the R-enantiomer of etodolac, induces cytotoxicity, overcomes drug resistance, and enhances the activity of dexamethasone in multiple myeloma. Blood 2005; 106: 706-712.
21. Yasui H, Hideshima T, Raje N, Roccaro AM, Shiraishi N, Kumar S, Hamasaki M, Ishitsuka K, Tai YT, Podar K, Catley L, Mitsiades CS, Richardson PG, Albert R, Brinkmann V, Chauhan D, Anderson KC. FTY 720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. Cancer Res 2005; 65: 7478-7484.
22. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Vogelstein B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002; 416: 552-556.
23. Cameron EE, Bachman KE, Myöhänen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999; 21: 103-107.
24. Ohm JE, McGarvey KM, Yu X, Cheng L, Schuebel KE, Cope L, Mohammad HP, Chen W, Daniel VC, Yu W, Berman DM, Jenuwein T, Pruitt K, Sharkis SJ, Watkins DN, Herman JG, Baylin SB. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethyla-

tion and heritable silencing. Nat Genet 2007; 39: 237-242.
25. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, Campan M, Young J, Jacobs I, Laird PW. Epigenetic stem cell signature in cancer. Nat Genet 2007; 39: 157-158.
26. Watanabe Y, Toyota M, Kondo Y, Suzuki H, Imai T, Ohe-Toyota M, Maruyama R, Nojima M, Sasaki Y, Sekido Y, Hiratsuka H, Shinomura Y, Imai K, Itoh F, Tokino T. PRDM5 identified as a target of epigenetic silencing in colorectal and gastric cancer. Clin Cancer Res 2007; 13: 4786-4794.
27. Ng MH, Chung YF, Lo KW, Wickham NW, Lee JC, Huang DP. Frequent hypermethylation of p16 and p15 genes in multiple myeloma. Blood 1997; 89: 2500-2506.
28. Toyota M, Sasaki Y, Satoh A, Ogi K, Kikuchi T, Suzuki H, Mita H, Tanaka N, Itoh F, Issa JP, Jair KW, Schuebel KE, Imai K, Tokino T. Epigenetic inactivation of CHFR in human tumors. Proc Natl Acad Sci U S A 2003; 100: 7818-7823.
29. Ng MH, Lau KM, Wong WS, To KW, Cheng SH, Tsang KS, Chan NP, Kho BC, Lo KW, Tong JH, Lam CW, Chan JC. Alterations of RAS signalling in Chinese multiple myeloma patients: absent BRAF and rare RAS mutations, but frequent inactivation of RASSF1A by transcriptional silencing or expression of a non-functional variant transcript. Br J Haematol 2003; 123: 637-645.
30. de Carvalho F, Colleoni GW, Almeida MS, Carvalho AL, Vettore AL. TGFbetaR2 aberrant methylation is a potential prognostic marker and therapeutic target in multiple myeloma. Int J Cancer 2009; 125: 1985-1991.
31. Ng MH, To KW, Lo KW, Chan S, Tsang KS, Cheng SH, Ng HK. Frequent death-associated protein kinase promoter hypermethylation in multiple myeloma. Clin Cancer Res 2001; 7: 1724-1729.
32. Murai M, Toyota M, Satoh A, Suzuki H, Akino K, Mita H, Sasaki Y, Ishida T, Shen L, Garcia-Manero G, Issa JP, Hinoda Y, Tokino T, Imai K. Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. Br J Cancer 2005; 92: 1165-1172.
33. Morimoto Y, Toyota M, Satoh A, Murai M, Mita H, Suzuki H, Takamura Y, Ikeda H, Ishida T, Sato N, Tokino T, Imai K. Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-gamma in haematopoietic tumour cells. Br J Cancer 2004; 90: 844-852.
34. Schuebel KE, Chen W, Cope L, Glöckner SC, Suzuki H, Yi JM, Chan TA, Van Neste L, Van Criekinge W, van den Bosch S, van Engeland M, Ting AH, Jair K, Yu W, Toyota M, Imai K, Ahuja N, Herman JG, Baylin SB. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genet 2007; 3: 1709-1723.
35. Kemppainen RJ, Behrend EN. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. J Biol Chem 1998; 273: 3129-3131.
36. Cismowski MJ, Takesono A, Ma C, Lizano JS, Xie X, Fuernkranz H, Lanier SM, Duzic E. Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. Nat Biotechnol 1999; 17: 878-883.
37. Takesono A, Cismowski MJ, Ribas C, Bernard M, Chung P, Hazard S 3rd, Duzic E, Lanier SM. Receptor-independent activators of heterotrimeric G-protein signaling pathways. J Biol Chem 1999; 274: 33202-33205.
38. Elam C, Hesson L, Vos MD, Eckfeld K, Ellis CA, Bell A, Krex D, Birrer MJ, Latif F, Clark GJ. RRP22 is a farnesylated, nucleolar, Ras-related protein with tumor suppressor potential. Cancer Res 2005; 65: 3117-3125.
39. Ellis CA, Vos MD, Howell H, Vallecorsa T, Fults DW, Clark GJ. Rig is a novel Ras-related protein and potential neural tumor suppressor. Proc Natl Acad Sci U S A 2002; 99: 9876-9881.

40. Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, Cuevas B, Kuo WL, Gray JW, Siciliano M, Mills GB, Bast RC Jr. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. Proc Natl Acad Sci U S A 1999; 96: 214-219.
41. Vaidyanathan G, Cismowski MJ, Wang G, Vincent TS, Brown KD, Lanier SM. The Ras-related protein AGS1/RASD1 suppresses cell growth. Oncogene 2004; 23: 5858-5863.
42. Furuta J, Nobeyama Y, Umebayashi Y, Otsuka F, Kikuchi K, Ushijima T. Silencing of Peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. Cancer Res 2006; 66: 6080-6086.
43. Satoh A, Toyota M, Itoh F, Sasaki Y, Suzuki H, Ogi K, Kikuchi T, Mita H, Yamashita T, Kojima T, Kusano M, Fujita M, Hosokawa M, Endo T, Tokino T, Imai K. Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res 2003; 63: 8606-8613.
44. Koga Y, Kitajima Y, Miyoshi A, Sato K, Sato S, Miyazaki K. The significance of aberrant CHFR methylation for clinical response to microtubule inhibitors in gastric cancer. J Gastroenterol 2006; 41: 133-139.
45. Ogi K, Toyota M, Mita H, Satoh A, Kashima L, Sasaki Y, Suzuki H, Akino K, Nishikawa N, Noguchi M, Shinomura Y, Imai K, Hiratsuka H, Tokino T. Small interfering RNA-induced CHFR silencing sensitizes oral squamous cell cancer cells to microtubule inhibitors. Cancer Biol Ther 2005; 4: 773-780.
46. Sun Y. Targeting E3 ubiquitin ligases for cancer therapy. Cancer Biol Ther 2003; 2: 623-629.
47. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000; 343: 1350-1354.
48. Pegg AE. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res 1990; 50: 6119-6129.
49. Esteller M, Herman JG. Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. Oncogene 2004; 23: 1-8.
50. Esteller M, Risques RA, Toyota M, Capella G, Moreno V, Peinado MA, Baylin SB, Herman JG. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res 2001; 61: 4689-4692.
51. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 2000; 60: 2368-2371.
52. Ludlum DB. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. Mutat Res 1990; 233: 117-126.
53. Brell M, Tortosa A, Verger E, Gil JM, Viñolas N, Villá S, Acebes JJ, Caral L, Pujol T, Ferrer I, Ribalta T, Graus F. Prognostic significance of O6-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. Clin Cancer Res 2005; 11: 5167-5174.
54. Hegi ME, Diserens AC, Godard S, Dietrich PY, Regli L, Ostermann S, Otten P, Van Melle G, de Tribolet N, Stupp R. Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Can-

cer Res 2004; 10: 1871-1874.
55. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999; 96: 8681-8686.
56. Carethers JM, Chauhan DP, Fink D, Nebel S, Bresalier RS, Howell SB, Boland CR. Mismatch repair proficiency and in vitro response to 5-fluorouracil. Gastroenterology 1999; 117: 123-131.
57. Ricciardiello L, Ceccarelli C, Angiolini G, Pariali M, Chieco P, Paterini P, Biasco G, Martinelli GN, Roda E, Bazzoli F. High thymidylate synthase expression in colorectal cancer with microsatellite instability: implications for chemotherapeutic strategies. Clin Cancer Res 2005; 11: 4234-4240.
58. Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. Int J Cancer 2003; 106: 66-73.

(Accepted for publication, Jan. 31, 2011)