We showed here that IGF-IR blockade enhanced the effect of chemotherapy for esophageal carcinoma. It has been reported that IGF axis is responsible for chemoresistance. IGF-I inhibits 5-FU-induced apoptosis through increasing survivin levels, which prevents Smac/DIABLO release and blocks the activation of caspases [44].

As IGF-IR is closely related to the InsR [5], it is important to avoid adverse effects related to co-inhibition of the InsR and perhaps ideally that any strategy designed to block IGF-IR would have a high degree of specificity for IGF-IR compared to InsR. We show here that ad-IGF-IR/dn does not suppress insulin-induced Akt-phosphorylation, indicating a high degree of receptor selectivity. Thus, our ad-IGF-IR/dn strategy has the distinct potential advantage of blocking both IGF ligand signals, being independent of IGFBPs, interrupting signaling between IGF-IR and Akt-1, and not affecting insulin receptor signaling.

On the other hand, InsR could also work as accelerator of proliferation in cancer cells. Thus, the dual targeting TKI might have some advantages to block cancer progression. However, it was reported that insulin enhances anticancer functions of 5-FU when it is treated before 5-FU for the appropriate time in esophageal and colonic cancer cell lines [45]. As there is discrepancy in the effects of insulin on esophageal cancers, further analysis will be needed.

Several humanized mAbs and TKIs for IGF-IR have been generated, some of which are now in clinical studies [26–28]. This study provides support for testing of these therapies in esophageal cancer. Although some phase III studies for IGF-IR mAbs (but not TKIs) were withdrawn, others including a dual targeting TKI for IGF-IR/InsR, BMS-754807, continue in clinical trials [46].

It is reported that the insensitivity of TE1 to an IGF-IR TKI NVP-AEW541 occurred through maintained ras/ERK activity. Moreover, the transduction of mutant ras reduced the sensitivity of TE-1 cells to NVP-AEW541 [47]. However, these results are different from our reported data that NVP-AEW541 inhibited the cancer progression of four gastrointestinal cancer cell lines, including TE-1 [48]. It would be interesting to analyze the reasons for the differences between these studies.

In addition, we have reported an IGF-IR mAb, figitumumab (CP-751,871), that could suppress gastrointestinal cancers expressing k-ras mutations, including TE-1 [49]. Further studies are needed to assess the effect and mechanism of IGF-IR blockade in k-ras mutated cancers.

In this study, we showed that a dual IGF-IR/InsR TKI is effective for both types of human esophageal carcinomas. Several advantages of dual targeting strategies for esophageal carcinoma have been reported. TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, could suppress Barrett's EAC [50]. The combination of Her2 mAb, trastuzumab, and IGF-IR mAb, α-IR3, was more effective in inhibiting in vitro proliferation of EAC than treatment with either agent alone [42]. Thus, combined targeting of the IGF-IR axis with these other tumor drivers may show significant therapeutic promise.

IGF-IR might therefore be important in the progression of esophageal carcinomas, and IGF-IR target therapies might be candidate options for patients with both types of esophageal cancers.

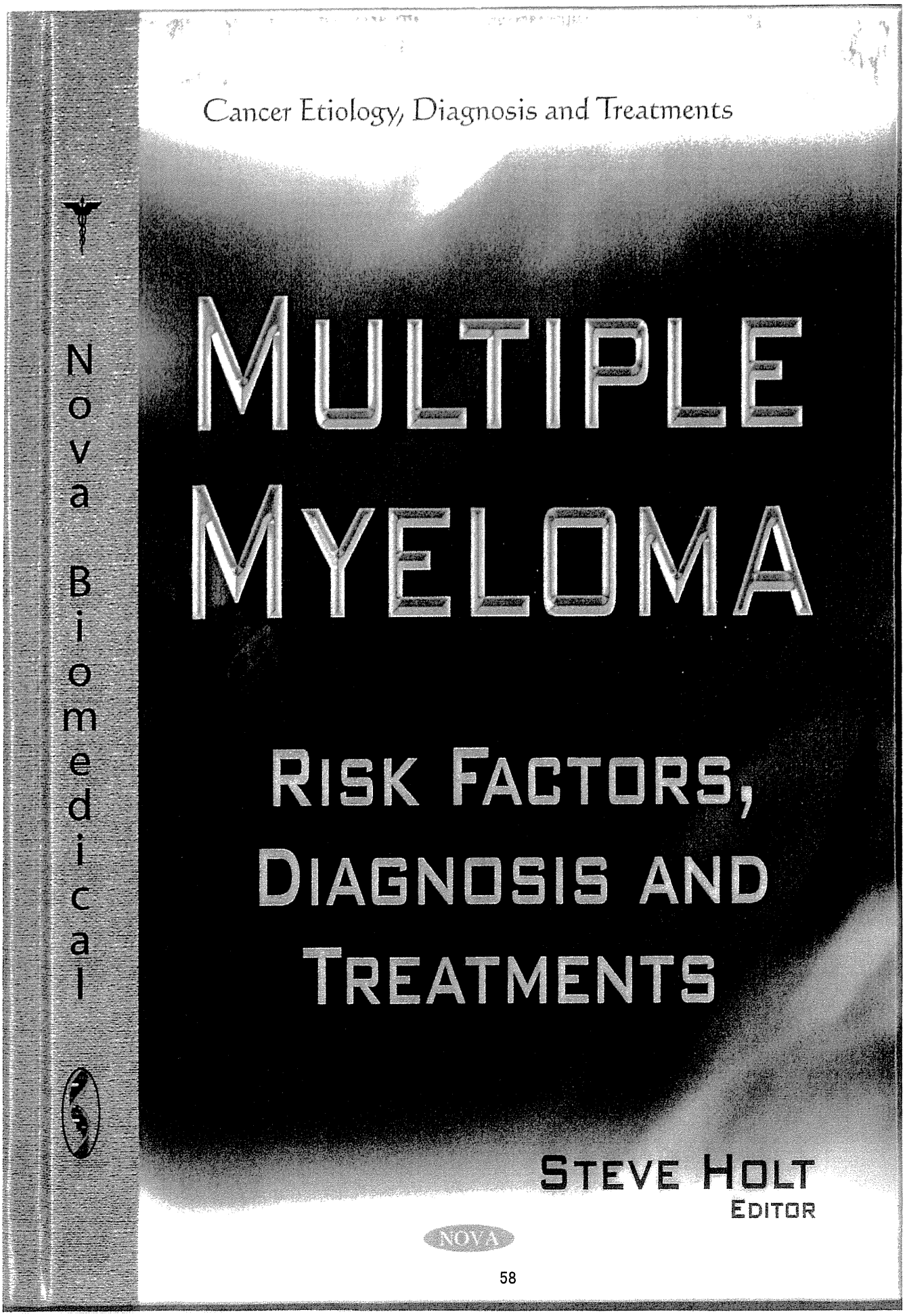
Acknowledgments This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and from the Ministry of Health, Labor and Welfare, Japan. This work was also supported by a grant from the Japanese Society of Strategies for Cancer Research and Therapy.

Conflicts of interest None

References

1. Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med. 2003;349:2241–52.
2. Sagar PM, Gauperaa T, Sue-Ling H, McMahon MJ, Johnston D. An audit of the treatment of cancer of the oesophagus. Gut. 1994;35:941–5.
3. Baserga R. Oncogenes and the strategy of growth factors. Cell. 1994;79:927–30.
4. Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A, Baserga R. Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. Proc Natl Acad Sci U S A. 1993;90:11217–21.
5. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, et al. Insulin-like growth factor i receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. Embo J. 1986;5:2503–12.
6. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst. 2000;92:1472–89.
7. Remacle-Bonnet M, Garrouste F, el Atiq F, Roccabianca M, Marvaldi J, Pommier G. Des-(1–3)-igf-i, an insulin-like growth factor analog used to mimic a potential igf-ii autocrine loop, promotes the differentiation of human colon-carcinoma cells. Int J Cancer. 1992;52:910–7.
8. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (igf)-i and igf-binding protein-3. J Natl Cancer Inst. 1999;91:620–5.
9. Harper J, Burns JL, Foulstone EJ, Pignatelli M, Zaina S, Hassan AB. Soluble igf2 receptor rescues apc(min/+) intestinal adenoma progresion induced by igf2 loss of imprinting. Cancer Res. 2006;66:1940–8.
10. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-i and prostate cancer risk: a prospective study. Science. 1998;279:563–6.
11. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor i (igf-1) and type 1 igf receptor (igf1r). Cell. 1993;75:59–72.
12. Vinayek R, Pichney LS, Tantry U, Dutta SK, Resau J, Vengurlekar S. Characterization of insulin-like growth factor i receptors in human esophageal epithelial cells. Am J Physiol. 1994;267:G105–14.
13. Qureshi FG, Tchorzewski MT, Duncan MD, Harmon JW. Egf and igf-i synergistically stimulate proliferation of human esophageal epithelial cells. J Surg Res. 1997;69:354–8.
14. Tchorzewski MT, Qureshi FG, Duncan MD, Duncan KL, Saini N, Harmon JW. Role of insulin-like growth factor-i in esophageal mucosal healing processes. J Lab Clin Med. 1998;132:134–41.
15. Costigan DC, Guyda HJ, Posner BI. Free insulin-like growth factor i (igf-i) and igf-ii in human saliva. J Clin Endocrinol Metab. 1988;66:1014–8.

16. Mori M, Inoue H, Shiraishi T, Mimori K, Shibuta K, Nakashima H, et al. Relaxation of insulin-like growth factor 2 gene imprinting in esophageal cancer. Int J Cancer. 1996;68:441–6.
17. Liu YC, Leu CM, Wong FH, Fong WS, Chen SC, Chang C, et al. Autocrine stimulation by insulin-like growth factor i is involved in the growth, tumorigenicity and chemoresistance of human esophageal carcinoma cells. J Biomed Sci. 2002;9:665–74.
18. Ouban A, Muraca P, Yeatman T, Coppola D. Expression and distribution of insulin-like growth factor-1 receptor in human carcinomas. Hum Pathol. 2003;34:803–8.
19. Chen SC, Chou CK, Wong FH, Chang CM, Hu CP. Overexpression of epidermal growth factor and insulin-like growth factor-i receptors and autocrine stimulation in human esophageal carcinoma cells. Cancer Res. 1991;51:1898–903.
20. Takaoka M, Harada H, Andl CD, Oyama K, Naomoto Y, Dempsey KL, et al. Epidermal growth factor receptor regulates aberrant expression of insulin-like growth factor-binding protein 3. Cancer Res. 2004;64:7711–23.
21. Imsumran A, Adachi Y, Yamamoto H, Li R, Wang Y, Min Y, et al. Insulin-like growth factor-i receptor as a marker for prognosis and a therapeutic target in human esophageal squamous cell carcinoma. Carcinogenesis. 2007;28:947–56.
22. Doyle SL, Donohoe CL, Finn SP, Howard JM, Lithander FE, Reynolds JV, et al. Igf-1 and its receptor in esophageal cancer: association with adenocarcinoma and visceral obesity. Am J Gastroenterol. 2012;107:196–204.
23. Donohoe CL, Doyle SL, McGarrigle S, Cathcart MC, Daly E, O'Grady A, et al. Role of the insulin-like growth factor 1 axis and visceral adiposity in oesophageal adenocarcinoma. Br J Surg. 2012;99:387–96.
24. Surmacz E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor i receptor. Oncogene. 2003;22:6589–97.
25. Wu JD, Odman A, Higgins LM, Haugk K, Vessella R, Ludwig DL, et al. In vivo effects of the human type i insulin-like growth factor receptor antibody a12 on androgen-dependent and androgen-independent xenograft human prostate tumors. Clin Cancer Res. 2005;11:3065–74.
26. Cohen BD, Baker DA, Soderstrom C, Tkalcevic G, Rossi AM, Miller PE, et al. Combination therapy enhances the inhibition of tumor growth with the fully human anti-type 1 insulin-like growth factor receptor monoclonal antibody cp-751,871. Clin Cancer Res. 2005;11:2063–73.
27. Garcia-Echeverria C, Pearson MA, Marti A, Meyer T, Mestan J, Zimmermann J, et al. In vivo antitumor activity of nvp-aew541-a novel, potent, and selective inhibitor of the igf-ir kinase. Cancer Cell. 2004;5:231–9.
28. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, et al. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell. 2004;5:221–30.
29. Prager D, Li HL, Asa S, Melmed S. Dominant negative inhibition of tumorigenesis in vivo by human insulin- like growth factor i receptor mutant. Proc Natl Acad Sci U S A. 1994;91:2181–5.
30. D'Ambrosio C, Ferber A, Resnicoff M, Baserga R. A soluble insulin-like growth factor i receptor that induces apoptosis of tumor cells in vivo and inhibits tumorigenesis. Cancer Res. 1996;56:4013–20.
31. Adachi Y, Lee CT, Coffee K, Yamagata N, Ohm JE, Park KH, et al. Effects of genetic blockade of the insulin-like growth factor receptor in human colon cancer cell lines. Gastroenterology. 2002;123:1191–204.
32. Min Y, Adachi Y, Yamamoto H, Ito H, Itoh F, Lee CT, et al. Genetic blockade of the insulin-like growth factor-i receptor: a promising strategy for human pancreatic cancer. Cancer Res. 2003;63:6432–41.
33. Lee CT, Park KH, Adachi Y, Seol JY, Yoo CG, Kim YW, et al. Recombinant adenoviruses expressing dominant negative insulin-like growth factor-i receptor demonstrate antitumor effects on lung cancer. Cancer Gene Ther. 2003;10:57–63.
34. Min Y, Adachi Y, Yamamoto H, Imsumran A, Arimura Y, Endo T, et al. Insulin-like growth factor i receptor blockade enhances chemotherapy and radiation responses and inhibits tumour growth in human gastric cancer xenografts. Gut. 2005;54:591–600.
35. Ohashi H, Adachi Y, Yamamoto H, Taniguchi H, Nosho K, Suzuki H, et al. Insulin-like growth factor receptor expression is associated with aggressive phenotypes and has therapeutic activity in biliary tract cancers. Cancer Sci. 2012;103:252–61.
36. Wang Y, Adachi Y, Imsumran A, Yamamoto H, Piao W, Li H, et al. Targeting for insulin-like growth factor-i receptor with short hairpin RNA for human digestive/gastrointestinal cancers. J Gastroenterol. 2010;45:159–70.
37. Wittman M, Carboni J, Attar R, Balasubramanian B, Balimane P, Brassil P, et al. Discovery of a (1h-benzoimidazol-2-yl)-1h-pyridin-2-one (bms-536924) inhibitor of insulin-like growth factor i receptor kinase with in vivo antitumor activity. J Med Chem. 2005;48:5639–43.
38. Lee YJ, Imsumran A, Park MY, Kwon SY, Yoon HI, Lee JH, et al. Adenovirus expressing shRNA to igf-1r enhances the chemosensitivity of lung cancer cell lines by blocking igf-1 pathway. Lung Cancer. 2007;55:279–86.
39. Hana V, Murphy LJ. Expression of insulin-like growth factors and their binding proteins in the estrogen responsive Ishikawa human endometrial cancer cell line. Endocrinology. 1994;135:2511–6.
40. Quinn KA, Treston AM, Unsworth EJ, Miller MJ, Vos M, Grimley C, et al. Insulin-like growth factor expression in human cancer cell lines. J Biol Chem. 1996;271:11477–83.
41. Pennisi PA, Barr V, Nunez NP, Stannard B, Le Roith D. Reduced expression of insulin-like growth factor i receptors in mcf-7 breast cancer cells leads to a more metastatic phenotype. Cancer Res. 2002;62:6529–37.
42. Kalinina T, Bockhorn M, Kaifi JT, Thieltges S, Gungor C, Effenberger KE, et al. Insulin-like growth factor-1 receptor as a novel prognostic marker and its implication as a cotarget in the treatment of human adenocarcinoma of the esophagus. Int J Cancer. 2010;127:1931–40.
43. Kong KL, Kwong DL, Chan TH, Law SY, Chen L, Li Y, et al. Microrna-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. Gut. 2012;61:33–42.
44. Juan HC, Tsai HT, Chang PH, Huang CY, Hu CP, Wong FH. Insulin-like growth factor 1 mediates 5-fluorouracil chemoresistance in esophageal carcinoma cells through increasing survivin stability. Apoptosis. 2011;16:174–83.
45. Zou K, Ju JH, Xie H. Pretreatment with insulin enhances anticancer functions of 5-fluorouracil in human esophageal and colonic cancer cells. Acta Pharmacol Sin. 2007;28:721–30.
46. Carboni JM, Wittman M, Yang Z, Lee F, Greer A, Hurlburt W, et al. Bms-754807, a small molecule inhibitor of insulin-like growth factor-1r/ir. Mol Cancer Ther. 2009;8:3341–9.
47. Bao XH, Takaoka M, Hao HF, Wang ZG, Fukazawa T, Yamatsuji T, et al. Esophageal cancer exhibits resistance to a novel igf-1r inhibitor nvp-aew541 with maintained ras-MAPK activity. Anticancer Res. 2012;32:2827–34.
48. Piao W, Wang Y, Adachi Y, Yamamoto H, Li R, Imsumran A, et al. Insulin-like growth factor-i receptor blockade by a specific tyrosine kinase inhibitor for human gastrointestinal carcinomas. Mol Cancer Ther. 2008;7:1483–93.
49. Ii M, Li H, Adachi Y, Yamamoto H, Ohashi H, Taniguchi H, et al. The efficacy of igf-i receptor monoclonal antibody against human gastrointestinal carcinomas is independent of k-ras mutation status. Clin Cancer Res. 2011;17:5048–59.
50. Watanabe N, Takaoka M, Sakurama K, Tomono Y, Hatakeyama S, Ohmori O, et al. Dual tyrosine kinase inhibitor for focal adhesion kinase and insulin-like growth factor-i receptor exhibits anticancer effect in esophageal adenocarcinoma in vitro and in vivo. Clin Cancer Res. 2008;14:4631–9.


Cancer Etiology, Diagnosis and Treatments
MULTIPLE MYELOMA
RISK FACTORS, DIAGNOSIS AND TREATMENTS
STEVE HOLT
EDITOR
NOVA
Nova Biomedical

CANCER ETIOLOGY, DIAGNOSIS AND TREATMENTS

MULTIPLE MYELOMA

RISK FACTORS, DIAGNOSIS AND TREATMENTS

STEVE HOLT
EDITOR

New York

In: Multiple Myeloma: Risk Factors, Diagnosis and Treatments ISBN: 978-1-63321-514-6
Editor: Steve Holt

Chapter 7

The Role of DNA Methylation in the Genetics and Epigenetics of Multiple Myeloma

Hiroshi Yasui[*1], *Tadao Ishida*[3] *and Kohzoh Imai*[2]
[1]Center for Antibody and Vaccine, Research Hospital,
[2]The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[3]Department of Gastroenterology, Rheumatology,
and Clinical Immunology, Sapporo Medical University, Sapporo, Japan

Abstract

Multiple myeloma (MM) arises through an accumulation of multiple genetic and epigenetic changes, which play a significant role in tumorigenesis and tumor development. DNA methylation is often found in cancers including MM at the 5-carbon on cytosine residues within CpG islands of genes whose products are associated with the promoter regions of protein-coding genes. This methylation is an epigenetic alteration that leads to heritable changes in gene expression through the recruitment of histone deacetylases and histone methyltransferases. We and other researchers have reported the association of global and regional DNA methylation status with MM. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from monoclonal gammopathy of undetermined significance to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression. Thus, DNA methylation could be a useful biomarker of MM tumorigenesis and progression. In the current review, we discuss the role of DNA methylation changes; their potential application as epigenetic biomarkers to facilitate risk assessment, diagnosis, prediction of prognosis, and sensitivity to treatment; and epigenetic therapy in MM.

* Correspondence to: Hiroshi Yasui, M.D., Ph.D. Center for Antibody and Vaccine, Research Hospital, The Institute of Medical Science, The University of Tokyo; 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; TEL: +81-3-3443-8111; FAX: +81-3-6409-2413; E-mail: hiroyasu@ims.u-tokyo.ac.jp

Introduction

Cancers, including multiple myeloma (MM), arise because of an accumulation of multiple genetic changes, which play a significant role in tumorigenesis and tumor development. In addition to genetics, recent studies revealed the role of epigenetics—heritable information that does not affect DNA sequence—in the pathogenesis of cancers, including MM [1-4]. Among epigenetic changes, DNA methylation and histone modification have been well-studied.

DNA methylation occurs at the 5-carbon on cytosine residues in cytosine-guanine pairs known as CpG dinucleotides. DNA methylation is catalyzed by three DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B, and is a crucial regulator in different biological processes, such as embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, genomic instability, and tumorigenesis [5]. Since transcriptionally active regions of the genome are usually CpG rich, methylation of CpG sites is a critical factor affecting gene transcription. DNA hypermethylation of the large clusters of CpG dinucleotides, referred to as CpG islands, at gene promoters and transcription start sites is an epigenetic alteration that can suppress gene expression through the recruitment of methyl-CpG binding domain proteins, histone deacetylases, and histone methyltransferases, thus causing chromatin condensation [6]. Genome-wide hypomethylation and regional hypermethylation are common events in tumors, including hematological malignancies. In MM, DNA hypomethylation was reported as the predominant early change during tumorigenesis that gradually transforms to regional DNA hypermethylation during disease progression [7-9].

In the current review, we discuss the role of alterations in DNA methylation, potential application of epigenetic biomarkers, and target therapeutics in MM.

2. Molecular Mechanism Involved in Tumorigenesis of MM

MM is a neoplastic plasma cell disorder that is characterized by the clonal proliferation of malignant plasma cells in the bone marrow, the presence of monoclonal immunoglobulin in the serum and/or urine in most cases, and associated organ dysfunction, including lytic bone lesions, compromised immunity, anemia, renal failure, and hypercalcemia [10-12]. Recent studies have shown that MM is consistently preceded by a premalignant stage of clonal plasma cell proliferation, termed monoclonal gammopathy of undetermined significance (MGUS) [13,14]. Approximately 1% of MGUS cases evolve to MM per year [15].

MM advances through a multistep transformation process of specific events, including somatic mutations, chromosomal copy-number changes, and non-random chromosomal translocations such as immunoglobulin gene rearrangements involved in cyclin D; furthermore, epigenetic changes drive progression from MGUS, to symptomatic MM, and ultimately to recurrent myeloma, including extramedullary disease and, in some cases, plasma cell leukemia [4,12,16,17] (Figure 1).

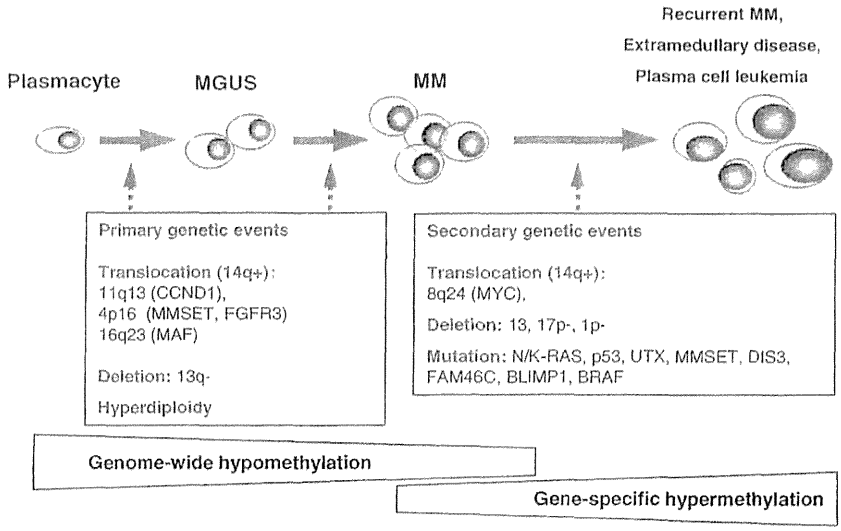


Figure 1. Multiple myeloma (MM) advances through a multistep transformation process due to specific events. These events include somatic mutations, chromosomal copy-number changes, and non-random chromosomal translocations, such as immunoglobulin gene rearrangements involved in cyclin D. Further, epigenetic changes drive progression from monoclonal gammopathy of undetermined significance (MGUS), to symptomatic MM, and ultimately to recurrent myeloma, including extramedullary disease and plasma cell leukemia. Genome-wide hypomethylation occurs during the events indicated by a blue wedge, and gene-specific hypermethylation occurs during the events indicated by a blue wedge.

Recently, several studies using high-throughput sequencing technologies demonstrated heterogeneity of MM genomic evolution and subclonal structure [18-22]. Chapman et al., reported that the analysis of somatic mutations by tumor-genome sequencing in MM cases revealed that, of the numerous genes mutated, identified genes are specifically involved in NFκB activation, protein homeostasis, and histone methylation, which are processes consistent with MM biology [18]. However, the key steps in MM oncogenesis remain unclear [3,4,23,24]. Recent findings also revealed that epigenetics, including DNA methylation and histone modification, is also important in MM pathogenesis. Global methylation analyses in MM have revealed the role of DNA methylation in MM pathogenesis and progress.

2. DNA Hypomethylation in MM

Genome-wide DNA hypomethylation is a common epigenetic alteration in cancer cells. Low levels of DNA methylation in cancer cells is substantially due to the loss of methylation at repetitive sequences such as long interspersed nuclear element-1 (LINE-1; a kind of a retrotransposon), which accounts for 17% of the human genome [19,25]. The following mechanisms have been suggested for DNA hypomethylation in tumorigenesis and tumor development: increased instability of the genome and reactivation of transposable elements (transposons) that can move in DNA [26-28]. Importantly, we and others reported that global methylation levels of DNA repetitive sequences, including LINE-1, progressively decline during the development of MM from MGUS to aggressive myeloma such as plasma cell

leukemia [8,9]. We also reported that there is a significant inverse correlation between the degree of genomic loss and LINE-1 methylation levels, and MM cases with LINE-1 hypomethylation had a significantly poor prognosis [9]. Regarding the pathogenesis of MM plasma cells, microarray data examining genome-wide differences in CpG methylation patterns revealed that genome-wide hypomethylation occurs at the transition from MGUS to MM [7].

3. DNA Hypermethylation in MM

DNA hypermethylation at gene promoters and transcription start sites is an epigenetic alteration that suppresses gene expression. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from MGUS to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression [7]. We and others studied regional DNA hypermethylation in MM and identified certain key genes as targets for epigenetic inactivation (Table 1).

Table 1. Epigenetically silenced genes in multiple myeloma (MM)

Gene	Chromosome	Function	Frequency of DNA hypermethylation of patient MM samples (n > 50)
CDKN2A (p16)	9p21.3	Cell cycle	34% [29]
DAPK1	9q21.33	Apoptosis	52.7% [35]
BNIP3	10q26.3	Apoptosis	5% [36]
RASD1	17p11.2	Cell growth	6–8% [33,40]
SPARC	5q33.1	Cell-extracellular matrix interaction	8–18.2% [36,40]
CD38	4p15	Ectoenzyme	45.9% [40]
GPX3	5q23	Glutathione peroxidase	7.5% [40]
NCAM1 (CD56)	11q23.1	Cell adhesion	5% [40]
PDK4	7q21.3	Regulation of metabolism	15.1% [40]
RBP1	3q23	carrier protein involved in the transport of retinol	16.3% [40]
TGFBI	5q31	Inhibition of cell adhesion	18.2% [40]

These genes include the following: cell-cycle regulators, such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*) [29] and 2B (*CDKN2B*) [30] and checkpoint with fork head and ring finger domains (*CHFR*) [31]; genes involved in cell signaling, such as Ras association

(RalGDS/AF-6) domain family member 1 (*RASSF1*) [32], RAS, dexamethasone-induced 1 (*RASD1*) [33], and transforming growth factor, beta receptor II (*TGFBR2*) [34]; genes involved in apoptosis, such as death-associated protein kinase 1 (*DAPK1*) [35] and BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) [36,37]; genes involved in antigen presentation, such as class II, major histocompatibility complex, transactivator (*CIITA*) [38]; genes involved in cell-extracellular matrix interaction, such as secreted protein, acidic, cysteine-rich (*SPARC*) [36]; and genes involved in polycomb repressive complexes, such as enhancer of zeste, drosophila, homolog 2 (*EZH2*) [39].

Recently, Kaiser et al., investigated the association between DNA methylation and MM prognosis using a genome-wide DNA methylation array of 159 patients treated in the Medical Research Council Myeloma IX trial [40]. They identified the following 8 epigenetically regulated genes with changes in DNA methylation status that were significantly associated with prognosis: *CD38*, *RASD1*, *SPARC*, glutathione peroxidase 3 (*GPX3*), neural cell adhesion molecule 1 (*NCAM1*), pyruvate dehydrogenase kinase 4 (*PDK4*), retinol-binding protein 1 (*RBP1*), and transforming growth factor, β induced (*TGFBI*). Importantly, multivariate analysis confirmed that *GPX3*, *RBP1*, *SPARC*, and *TGFBI* are associated with survival, and methylation of the genes is independent of established risk factors for MM. Methylation levels of these 4 genes is low in MGUS, and then increasing methylation is associated with more aggressive MM cellular phenotypes. Walker et al., investigated DNA methylation patterns associated with MM subtypes [7]. They found specific profiles with increased hypermethylation in clinically aggressive subtypes, such as plasma cell leukemia, and in the prognostically unfavorable t(4;14) cytogenetic subtype with overexpressed *MMSET*, which encodes a histone methyltransferase. These findings suggest that methylation changes affect disease biology.

Recent reports correlated hypermethylation of promoter-associated CpG islands with silencing of microRNAs (miRNAs), which are small 18–22 nucleotide RNAs that regulate many intracellular functions [41]. Dysregulation of miRNA genes has been implicated in MM. Moreover, several reports of MM described the role of hypermethylation of tumor-suppressor miRNA genes, including *miR-34b/c* [42], *miR-194-2-192* [43], and *miR-203* [44]. Combined genome-wide analysis of miRNA methylation and miRNA expression profiling is warranted to clarify the role of epigenetic regulation of miRNA in MM.

4. DNA Methylation As an Epigenetic Biomarker in MM

The current prognostic factors in MM include cytogenetic aberrations, such as the nonhyperdiploid, cytogenetically detected chromosomal 13q deletion, t(4;14), t(14;16), 1q gain, and del(17p) detected by fluorescence *in situ* hybridization [10]. Novel therapeutics, such as the proteasome inhibitor bortezomib, can partially overcome adverse outcomes conferred by these abnormalities [45]. However, there has been much less progress in the development of predictive biomarkers for specific treatments [46]. To identify predictive biomarkers for the effect of myeloma therapeutics, appropriate clinical trial designs are necessary. Since some novel MM therapeutics in development have specific molecular

targets, the identification of biomarkers that also characterize drug sensitivity is a promising therapeutic strategy [45].

As mentioned above, hypermethylation of *TGFB1*, *SPARC*, *RBP1*, and *GPX3* is associated with significantly shorter overall survival, independent of age, international staging system score, and adverse cytogenetics [40]. Future prospective studies will verify these genes as prognostic MM biomarkers.

We identified *RASD1* as a possible biomarker in MM [33]. *RASD1*, located on chromosome 17p11.2 with frequent loss of heterozygosity in various human tumors, encodes a Ras GTPase with tumor suppressor functions induced by dexamethasone [47,48]. Importantly, MM cells that show *RASD1* methylation are resistant to dexamethasone, and combined treatment with dexamethasone and the hypomethylating agent decitabine (5-aza-2′-deoxycytidine), which inhibits DNA methyltransferase, restores the cytotoxicity of dexamethasone to tumor cells. While the hypermethylation of *RASD1* was observed in approximately 10% of primary MM samples, the methylation levels of *RASD1* were elevated in all of the MM cases that had pair DNAs after repeated antimyeloma therapy, including dexamethasone.

Limited studies have addressed the antitumor effects of the hypomethylating agents decitabine and azacitidine (5-azacytidine) in MM, demonstrating significant *in vitro* antimyeloma activity. The mechanisms involve changes in gene expression and induction of DNA damage [49,50]. Recently, a gene expression-based DNA methylation score was reported, which relates the expression of methylation-regulated genes to predict the efficacy of hypomethylating agents—decitabine and azacitidine—in human MM cell lines and in patient MM cells *in vitro* [51,52]. Phase I/II clinical trials are ongoing to study the side effects and best dose of azacitidine in combination with lenalidomide and dexamethasone in MM; therefore, an investigation regarding association of the methylation score and the response of MM patients could provide promising information [53]. Taken together, these findings suggest the involvement of epigenetic gene silencing in MM progression and drug resistance and the usefulness of demethylation therapy for MM treatment.

Conclusion

In summary, DNA methylation functions in MM tumorigenesis and progression. Several reports have suggested that DNA methylation could be a useful biomarker to predict prognosis and sensitivity to treatment. A further comprehensive analysis using a genome-wide approach with high-throughput sequencing technologies will be necessary to clarify the molecular mechanisms of MM oncogenesis and progression. Epigenetics has become to an essential research area where important challenges should be resolved through further investigations of MM.

Acknowledgments

This study was supported by an Aki Horinouchi Research Grant from the International Myeloma Foundation Japan (H.Y.), a Grant-in-Aid for Exploratory Research (K.I., H.Y.), a

Grant-in-Aid for Scientific Research (C) (T.I., H.Y.), and a Scientific Support Programs for Cancer Research, Grant-in-Aid for Scientific Research on Innovative Areas (K.I., H.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology.

References

[1] Dawson MA; Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012,150,12-27.

[2] Toyota M; Suzuki H; Yamashita T, et al.; Cancer epigenomics: implications of DNA methylation in personalized cancer therapy. *Cancer Sci.* 2009,100,787-91.

[3] Yasui H; Ishida T; Maruyama R, et al., Model of translational cancer research in multiple myeloma. *Cancer Sci.* 2012,103,1907-12.

[4] Morgan GJ; Walker BA; Davies FE. The genetic architecture of multiple myeloma. *Nature reviews Cancer* 2012,12,335-48.

[5] Bird A. The essentials of DNA methylation. *Cell* 1992,70,5-8.

[6] Han H; Cortez CC; Yang X; Nichols PW; Jones PA; Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Human molecular genetics* 2011,20,4299-310.

[7] Walker BA; Wardell CP; Chiecchio L, et al., Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood* 2011,117,553-62.

[8] Bollati V; Fabris S; Pegoraro V, et al., Differential repetitive DNA methylation in multiple myeloma molecular subgroups. *Carcinogenesis* 2009,30,1330-5.

[9] Aoki Y; Nojima M; Suzuki H, et al., Genomic vulnerability to LINE-1 hypomethylation is a potential determinant of the clinicogenetic features of multiple myeloma. *Genome medicine* 2012,4,101.

[10] Palumbo A; Anderson K. Multiple myeloma. *N. Engl. J. Med.* 2011,364,1046-60.

[11] Yasui H; Hideshima T; Richardson PG; Anderson KC. Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. *British journal of haematology* 2006,132,385-97.

[12] Hideshima T; Mitsiades C; Tonon G; Richardson PG; Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nature reviews Cancer* 2007,7,585-98.

[13] Landgren O; Kyle RA; Pfeiffer RM, et al., Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood* 2009,113,5412-7.

[14] Weiss BM; Abadie J; Verma P; Howard RS; Kuehl WM. A monoclonal gammopathy precedes multiple myeloma in most patients. *Blood* 2009,113,5418-22.

[15] Kyle RA; Durie BG; Rajkumar SV, et al., 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* 2010,24,1121-7.

[16] Gonzalez D; van der Burg M; Garcia-Sanz R, et al., Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma. *Blood* 2007,110,3112-21.

[17] Schmidt-Hieber M; Gutierrez ML; Perez-Andres M, et al., Cytogenetic profiles in multiple myeloma and monoclonal gammopathy of undetermined significance: a study in highly purified aberrant plasma cells. *Haematologica* 2013,98,279-87.
[18] Chapman MA; Lawrence MS; Keats JJ, et al., Initial genome sequencing and analysis of multiple myeloma. *Nature* 2011,471,467-72.
[19] Bolli N; Avet-Loiseau H; Wedge DC, et al., Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. *Nature communications* 2014,5,2997.
[20] Walker BA; Wardell CP; Melchor L, et al., Intraclonal heterogeneity and distinct molecular mechanisms characterize the development of t(4;14) and t(11;14) myeloma. *Blood* 2012,120,1077-86.
[21] Keats JJ; Chesi M; Egan JB, et al., Clonal competition with alternating dominance in multiple myeloma. *Blood* 2012,120,1067-76.
[22] Egan JB; Shi CX; Tembe W, et al., Whole-genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution, and clonal tides. *Blood* 2012,120,1060-6.
[23] Stratton MR; Campbell PJ; Futreal PA. The cancer genome. *Nature* 2009,458,719-24.
[24] Hanahan D; Weinberg RA. The hallmarks of cancer. *Cell* 2000,100,57-70.
[25] Huang CR; Schneider AM; Lu Y, et al., Mobile interspersed repeats are major structural variants in the human genome. *Cell* 2010,141,1171-82.
[26] Eden A; Gaudet F; Waghmare A; Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 2003,300,455.
[27] Howard G; Eiges R; Gaudet F; Jaenisch R; Eden A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* 2008,27,404-8.
[28] Iskow RC; McCabe MT; Mills RE, et al., Natural mutagenesis of human genomes by endogenous retrotransposons. *Cell* 2010,141,1253-61.
[29] Gonzalez-Paz N; Chng WJ; McClure RF, et al., Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. *Blood* 2007,109,1228-32.
[30] Ng MH; Chung YF; Lo KW; Wickham NW; Lee JC; Huang DP. Frequent hypermethylation of p16 and p15 genes in multiple myeloma. *Blood* 1997,89,2500-6.
[31] Toyota M; Sasaki Y; Satoh A, et al., Epigenetic inactivation of CHFR in human tumors. *Proceedings of the National Academy of Sciences of the United States of America* 2003,100,7818-23.
[32] Ng MH; Lau KM; Wong WS, et al., 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. *British journal of haematology* 2003,123,637-45.
[33] Nojima M; Maruyama R; Yasui H, et al., Genomic screening for genes silenced by DNA methylation revealed an association between RASD1 inactivation and dexamethasone resistance in multiple myeloma. Clinical cancer research : *an official journal of the American Association for Cancer Research* 2009,15,4356-64.
[34] 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. *International journal of cancer Journal international du cancer* 2009,125,1985-91.

[35] Chim CS; Liang R; Fung TK; Choi CL; Kwong YL. Epigenetic dysregulation of the death-associated protein kinase/p14/HDM2/p53/Apaf-1 apoptosis pathway in multiple myeloma. *Journal of clinical pathology* 2007,60,664-9.

[36] Heller G; Schmidt WM; Ziegler B, et al., Genome-wide transcriptional response to 5-aza-2'-deoxycytidine and trichostatin a in multiple myeloma cells. *Cancer research* 2008,68,44-54.

[37] Murai M; Toyota M; Satoh A, et al., Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. *British journal of cancer* 2005,92,1165-72.

[38] Morimoto Y; Toyota M; Satoh A, et al., 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. *British journal of cancer* 2004,90,844-52.

[39] Croonquist PA; Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. *Oncogene* 2005,24,6269-80.

[40] Kaiser MF; Johnson DC; Wu P, et al., Global methylation analysis identifies prognostically important epigenetically inactivated tumor suppressor genes in multiple myeloma. *Blood* 2013,122,219-26.

[41] Wong KY; Huang X; Chim CS. DNA methylation of microRNA genes in multiple myeloma. *Carcinogenesis* 2012,33,1629-38.

[42] Wong KY; Yim RL; So CC; Jin DY; Liang R; Chim CS. Epigenetic inactivation of the MIR34B/C in multiple myeloma. *Blood* 2011,118,5901-4.

[43] Corthals SL; Sun SM; Kuiper R, et al., MicroRNA signatures characterize multiple myeloma patients. *Leukemia* 2011,25,1784-9.

[44] Wong KY; Liang R; So CC; Jin DY; Costello JF; Chim CS. Epigenetic silencing of MIR203 in multiple myeloma. *British journal of haematology* 2011,154,569-78.

[45] Anderson KC. The 39th David A. Karnofsky Lecture: bench-to-bedside translation of targeted therapies in multiple myeloma. *J. Clin. Oncol.* 2012,30,445-52.

[46] Sawyers CL. The cancer biomarker problem. *Nature* 2008,452,548-52.

[47] Kemppainen RJ; Behrend EN. Dexamethasone rapidly induces a novel ras superfamily member-related gene in AtT-20 cells. *The Journal of biological chemistry* 1998,273,3129-31.

[48] Vaidyanathan G; Cismowski MJ; Wang G; Vincent TS; Brown KD; Lanier SM. The Ras-related protein AGS1/RASD1 suppresses cell growth. *Oncogene* 2004,23,5858-63.

[49] Khong T; Sharkey J; Spencer A. The effect of azacitidine on interleukin-6 signaling and nuclear factor-kappaB activation and its in vitro and in vivo activity against multiple myeloma. *Haematologica* 2008,93,860-9.

[50] Kiziltepe T; Hideshima T; Catley L, et al., 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. *Molecular cancer therapeutics* 2007,6,1718-27.

[51] Moreaux J; Reme T; Leonard W, et al., Development of gene expression-based score to predict sensitivity of multiple myeloma cells to DNA methylation inhibitors. *Molecular cancer therapeutics* 2012,11,2685-92.

[52] Moreaux J; Bruyer A; Veyrune JL; Goldschmidt H; Hose D; Klein B. DNA methylation score is predictive of myeloma cell sensitivity to 5-azacitidine. *British journal of haematology* 2014,164,613-6.

[53] Maes K; Menu E; Van Valckenborgh E; Van Riet I; Vanderkerken K; De Bruyne E. Epigenetic modulating agents as a new therapeutic approach in multiple myeloma. *Cancers* 2013,5,430-61.

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

Research Article

Cancer Prevention Research

Analysis of DNA Methylation in Bowel Lavage Fluid for Detection of Colorectal Cancer

Taku Harada[1,2,3], Eiichiro Yamamoto[1,4], Hiro-o Yamano[5], Masanori Nojima[6], Reo Maruyama[1,4], Kohei Kumegawa[1], Masami Ashida[1], Kenjiro Yoshikawa[5], Tomoaki Kimura[5], Eiji Harada[5], Ryo Takagi[5], Yoshihito Tanaka[5], Hironori Aoki[1,5], Masayo Nishizono[5], Michiko Nakaoka[5], Akihiro Tsuyada[1], Takeshi Niinuma[1,4], Masahiro Kai[1], Kazuya Shimoda[2], Yasuhisa Shinomura[4], Tamotsu Sugai[7], Kohzoh Imai[6], and Hiromu Suzuki[1]

Abstract

Aberrant DNA methylation could potentially serve as a biomarker for colorectal neoplasms. In this study, we assessed the feasibility of using DNA methylation detected in bowel lavage fluid (BLF) for colorectal cancer screening. A total of 508 BLF specimens were collected from patients with colorectal cancer ($n = 56$), advanced adenoma ($n = 53$), minor polyp ($n = 209$), and healthy individuals ($n = 190$) undergoing colonoscopy. Methylation of 15 genes (*miR-1-1*, *miR-9-1*, *miR-9-3*, *miR-34b/c*, *miR-124-1*, *miR-124-2*, *miR-124-3*, *miR-137*, *SFRP1*, *SFRP2*, *APC*, *DKK2*, *WIF1*, *LOC386758*, and *ZNF582*) was then analyzed in MethyLight assays, after which receiver operating characteristic (ROC) curves were analyzed to assess the diagnostic performance of BLF methylation. Through analyzing BLF specimens in a training set ($n = 345$), we selected the three genes showing the greatest sensitivity for colorectal cancer detection (*miR-124-3*, 71.8%; *LOC386758*, 79.5%; and *SFRP1*, 74.4%). A scoring system based on the methylation of those three genes (M-score) achieved 82% sensitivity and 79% specificity, and the area under the ROC curve (AUC) was 0.834. The strong performance of this system was then validated in an independent test set ($n = 153$; AUC = 0.808). No significant correlation was found between M-score and the clinicopathologic features of the colorectal cancers. Our results demonstrate that DNA methylation in BLF specimens may be a useful biomarker for the detection of colorectal cancer. *Cancer Prev Res; 7(10); 1002–10. ©2014 AACR.*

Introduction

Colorectal cancer is one of the most commonly occurring malignancies worldwide, and early detection is essential for its successful treatment. Large population studies have shown that the fecal occult blood test (FOBT) is a highly cost-effective screening method that reduces colorectal cancer–related mortality (1). Moreover, the performance of the immunochemical FOBT (iFOBT or Fecal Immunochemical Test, FIT) has been improved (2, 3) and is now widely used for colorectal cancer screening in Japan and Europe. However, the FOBT continues to have limitations, especially for detection of early-stage colorectal cancers. Several other methods, including colonoscopy and barium enema, have been available for years, but none of these methods has been established as a gold standard for colorectal cancer screening.

Fecal DNA tests are a noninvasive and potentially effective means of screening for both early colorectal lesions and advanced colorectal cancers (4, 5). As such, the feasibility of detecting genetic mutation of oncogenes or tumor-suppressor genes, such as *APC*, *KRAS*, *TP53*, and *BAT-26*, has been extensively tested, but the diagnostic performance of these assays remains unsatisfactory (6, 7). Epigenetic alterations are also commonly observed in colorectal cancers. Because of its high frequency and the wide variety of affected genes, aberrant DNA methylation has emerged as a new biomarker for stool-based colorectal cancer screening. For instance, *SFRP2* methylation occurs in approximately 90% of primary colorectal cancers (8), and was one of the first epigenetic markers reported in fecal DNA (9). More recently, a variety of other genes have been identified as potential biomarkers for stool-based methylation testing, including *VIM*, *GATA4*, *TFPI2*, *PHACTR3*, *AGTR1*, *WNT2*, and *miR-34b/c* (10–15).

[1]Department of Molecular Biology, Sapporo Medical University, Sapporo, Japan. [2]Division of Gastroenterology and Hematology, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan. [3]Center for Gastroenterology, Teine-Keijinkai Hospital, Sapporo, Japan. [4]Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo, Japan. [5]Department of Gastroenterology, Akita Red Cross Hospital, Akita, Japan. [6]The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan. [7]Department of Molecular Diagnostic Pathology, Iwate Medical University, Morioka, Japan.

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

T. Harada, E. Yamamoto, and H.-O. Yamano contributed equally to this article.

Corresponding Author: Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, S1, W17, Chuo-Ku, Sapporo 060-8556, Japan. Phone: 81-11-611-2111; Fax: 81-11-622-1918; E-mail: hsuzuki@sapmed.ac.jp

doi: 10.1158/1940-6207.CAPR-14-0162

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

In an earlier study, we demonstrated that DNA methylation is detectable in the mucosal wash fluid from colorectal tumors, which can be collected during colonoscopy (16). Importantly, wash fluid from invasive cancers exhibited significantly higher levels of methylation of tumor-related genes than noninvasive tumors. This prompted us to postulate that wash fluid from invasive tumors contained greater numbers of exfoliated tumor cells, and that the methylation was a potential biomarker predictive of tumor invasiveness. Our results also suggested that a DNA methylation test might complement the diagnostic performance of colonoscopy and that intestinal wash fluid could be a useful source for analysis of tumor-derived DNA methylation. We therefore hypothesized that oral bowel lavage fluid (BLF) might contain tumor-derived DNA, and thus molecular alteration in BLF specimens could be a useful biomarker for colorectal cancer screening. To test that idea, in this study, we analyzed DNA methylation of tumor-related genes in BLF specimens from patients with colorectal tumors and healthy individuals, and examined its clinical utility for cancer detection.

Materials and Methods

Patients and BLF specimens

All samples were collected from Japanese patients who underwent colonoscopy at Akita Red Cross Hospital (Akita, Japan) because of abdominal symptoms or a positive FOBT. 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 (Sapporo, Japan). Before colonoscopy, patients were pretreated with 2 L of polyethylene glycol lavage solution and 10 mL of BLF specimens were collected from the rectum at the beginning of the colonoscopy (Fig. 1A). BLF samples were initially classified into four groups according to the Boston bowel preparation scale (BBPS; Fig. 1B; ref. 17). Then, on the basis of colonoscopic and histologic findings, the BLF samples were divided into four groups: patients with colorectal cancer, patients with advanced adenoma, patients with minor polyp, and individuals without colorectal lesions. Advanced adenomas were defined as being 1 cm or more in diameter, and/or with villous components, and/or with high-grade dysplasia. Minor polyps were defined as being adenomas that did not satisfy the above criteria. A total of 508 BLF samples from 56 patients with colorectal cancer, 53 patients with advanced adenoma, 209 patients with minor polyp, and 190 individuals with a normal colon were collected. In addition, biopsy specimens were collected from 44 of the 56 patients with colorectal cancer. BLF and tissue specimens were suspended in ThinPrep PreservCyt solution (Hologic) and stored at 4°C until DNA extraction. Genomic DNA was extracted using the standard phenol–chloroform procedure. FIT was performed in 349 individuals, including 17 patients with colorectal cancer. Samples were randomly sorted into two groups (training set and test set) for validation analysis (Table 1).

Methylation analysis

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen), after which methylation analysis was carried out as described previously (18). PCR for MethyLight assays was run in a 20-μL volume containing 50 ng of bisulfite-treated DNA, 625 nmol/L each primer, 250 nmol/L TaqMan-MGB probe, and 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Fast real-time PCR was done using a 7500 Fast Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems). The PCR protocol entailed 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. The Alu repetitive element was used as an endogenous control, and the percentage of methylated reference (PMR) was calculated as described previously (19, 20). Sequence information for the primers and probes used for *miR-1-1*, *miR-9-1*, *miR-9-3*, *miR-34b/c*, *miR-124-1*, *miR-124-2*, *miR-124-3*, *miR-137*, *SFRP1*, *SFRP2*, *DKK2*, *WIF1*, *LOC386758*, and *ZNF582* is listed in Supplementary Table S1; those used for *APC* is described elsewhere (20).

Statistical analysis

Quantitative variables were analyzed using the Student *t* test. The Fisher exact test and the χ^2 test were used for analysis of categorical data. The Pearson correlation coefficient was used to evaluate correlations between continuous data. Receiver–operating characteristic (ROC) curves for the diagnosis of colorectal cancer were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cutoff PMR value for each gene was defined as the point on the ROC curve closest to the upper left corner. A diagnostic scoring system using a panel of selected marker genes was constructed by analyzing a training set using the following three-step algorithm: (i) methylation status of marker genes in BLF was assessed; (ii) the number of methylated genes was determined, which we termed the methylation score (M-score); and (iii) the samples were classified into four groups based on the M-score. Values of $P < 0.05$ (two-sided) were regarded as significant. All statistical analyses were performed using the SPSS Statistics 18 (IBM Corporation) and GraphPad Prism ver. 5.0.2 (GraphPad Software).

Results

Detection of DNA methylation in BLF specimens

After collecting 10-mL BLF specimens from the rectums of the study participants at the beginning of their colonoscopy, we successfully extracted sufficient amounts of genomic DNA to perform a methylation analysis (Fig. 1A). To determine the best time to obtain the BLF specimens, we scored the BLF samples using the BBPS (Fig. 1B; ref. 17). Among the 268 BLF samples initially collected, 58 were scored as 3, 154 were scored as 2, 46 were scored as 1, and 10 were scored as 0. BLF samples without residual stool (BBPS scores 2 and 3) contained significantly smaller amounts of genomic DNA than those with residual stool (BBPS scores 0 and 1; Fig. 1C). However, MethyLight assays revealed that the endogenous control Alu element was detected at lower threshold cycle

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

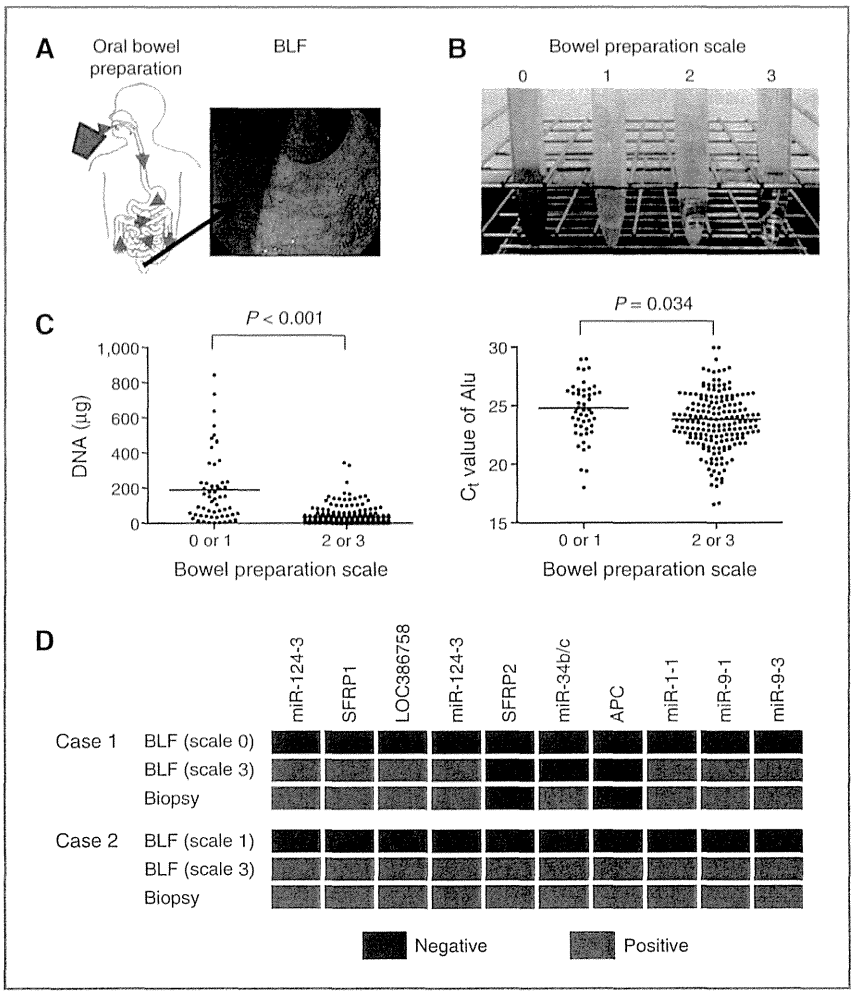


Figure 1. Collection of BLF and detection of DNA methylation. A, after oral bowel preparation, BLF specimens were collected from the rectum of individuals undergoing colonoscopy. B, BLF samples representative of the indicated BBPS scores. C, association between bowel preparation and the amount of extracted DNA (left) and Alu elements in MethyLight assays (right). Note that a larger amount of DNA is obtained from BLF specimens with a lower BBPS score, but human Alu element is more readily detectable in specimens with a higher BBPS score. D, MethyLight assay results for the indicated genes in BLF specimens with low and high BBPS scores and biopsy specimens from 2 representative patients with colorectal cancer.

(C_t) values in BLF specimens with high BBPS scores than in those with residual stool (Fig. 1C). This suggests that the relative fraction of human genomic DNA is larger in higher BBPS score BLF, most likely because of the smaller amount of contaminating bacteria-derived DNA. We then analyzed BLF specimens from selected patients with colorectal cancer, comparing the detectability of DNA methylation between specimens with lower and higher BBPS scores. As shown in Fig. 1D, methylation of representative genes was readily detectable in BLF specimens with a higher BBPS score, whereas it was undetectable in specimens with a lower score (Fig. 1D). For these reasons, we collected BLF specimens after sufficient bowel preparation for the next analysis.

Selection of marker genes for colorectal cancer detection

Our training set consisted of 355 BLF specimens obtained from patients with colorectal cancer ($n = 39$), advanced adenomas ($n = 31$), or minor polyps ($n = 135$), as well as individuals with no colorectal lesions ($n = 150$; Table 1). Using these specimens, we performed quantitative MethyLight assays to assess the methylation status of 15 genes known to be frequent targets of aberrant CpG island methylation in colorectal cancer (*miR-1-1*, *miR-9-1*, *miR-9-3*, *miR-34b/c*, *miR-124-1*, *miR-124-2*, *miR-124-3*, *miR-137*, *SFRP1*, *SFRP2*, *APC*, *DKK2*, *WIF1*, *LOC386758*, and *ZNF582*; refs. 8, 21). The methylation levels of the respective genes were calculated as PMR values, and we generated ROC curves to assess their clinical utility for detection of colorectal cancer (Supplementary Table S2 and Supplementary Fig. S1). Among the candidate marker genes analyzed, we found that *miR-124-3*, *LOC386758*, and *SFRP1* were highly discriminative between patients with colorectal cancer and those without colorectal cancer (Supplementary Table S2). The most discriminating PMR cutoffs for *miR-124-3*, *LOC386758*, and *SFRP1* were 11.1, 0.0003, and 1.1, while the most

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

Table 1. Clinicopathologic features of the subjects in this study

	Training set (*N* = 355)	Test set (*N* = 153)	Total (*N* = 508)
Demographics			
Median age, y (range)	61 (28–93)	61 (33–89)	61 (28–93)
Male	235	108	343
Female	120	45	165
Colorectal cancer			
Total *N*	39	17	56
Location (right/left/rectum)	13/11/15	7/7/3	20/18/18
Median size, cm (range)	4.5 (0.7–11.5)	4.8 (1.5–9.3)	4.6 (0.7–11.5)
Dukes stage (A/B/C/D)	9/16/11/3	4/8/4/1	13/24/15/4
Advanced adenoma[a]			
Total *N*	31	22	53
Location (right/left/rectum)	16/12/3	14/7/1	30/19/4
Median size, cm (range)	1.7 (0.6–4.0)	1.3 (0.6–2.6)	1.5 (0.6–4.0)
Minor polyp[b]			
Total *N*	135	74	209
Location (right/left/rectum)	80/46/9	47/17/9	128/63/18
Median size, cm (range)	0.5 (0.1–0.9)	0.5 (0.2–0.9)	0.5 (0.1–0.9)
Normal colon			
Total *N*	150	40	190

[a]Advanced adenomas were defined as adenomas 1 cm or greater in diameter, and/or containing villous components, and/or with high-grade dysplasia.

[b]Minor polyps were defined as adenomas other than advanced adenomas.

sensitive setting (PMR > 0) also achieved high sensitivity and specificity (Supplementary Table S2).

To develop a more efficient diagnostic system for detection of colorectal cancer, we constructed a scoring system based on the methylation of *miR-124-3*, *LOC386758*, and *SFRP1*. Using the number of methylated genes (PMR > 0), we classified the samples into four groups based on their M-score (Fig. 2A). A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish samples obtained from patients with colorectal cancer by plotting the sensitivity over 1 − specificity at each point (Fig. 2B). We then validated the diagnostic system by analyzing an independent test set (Table 1 and Fig. 2A and B). AUCs in the training and test sets were 0.834 and 0.808, respectively, confirming the accuracy of our system for detecting colorectal cancer.

The association between the clinical characteristics and M-scores is summarized in Table 2 and Supplementary Table S3. Higher M-scores were significantly associated with colorectal cancer, but their association with advanced adenomas or minor polyps was limited (Fig. 2C and Supplementary Table S3). M-scores were not significantly associated with tumor location, size or stage (Table 2). We also did not find a correlation between M-scores and age in patients with colorectal cancer, though we observed a tendency for higher M-scores to be associated with older age in non–colorectal cancer individuals, perhaps due to age-dependent methylation (Supplementary Fig. S2). These results suggest that the M-score system is able to reveal the presence of colorectal cancers, irrespective of the tumor's location, size, or clinical stage, but greater age may increase the false-positive rate.

Although the results summarized above demonstrate the clinical utility of BLF methylation for colorectal cancer screening, the system failed to detect 5 of the 56 patients with colorectal cancer (Supplementary Table S3). We therefore tested whether the apparent absence of methylation in those 5 BLF specimens actually reflects the unmethylated status of the genes in tumor tissues. For this purpose, we analyzed biopsy specimens from 41 patients with colorectal cancer with different M-scores (score 3, $n = 20$; score 2, $n = 10$; score 1, $n = 8$; and score 0, $n = 3$), and found that the majority of the tumors exhibited methylation of all three genes (*miR-124-3*, *LOC386758*, and *SFRP1*), irrespective of the M-score (Supplementary Fig. S3). MethyLight assays revealed that the C_t values for the endogenous Alu tended to be higher in BLF specimens with low M-scores, indicating that the apparent absence of BLF methylation may be result of too little tumor-derived DNA in the sample.

BLF methylation and upper gastrointestinal tract cancer

We next assessed whether BLF methylation could be used to detect upper gastrointestinal tract cancers. Among the individuals enrolled in this study were 294 who underwent upper gastrointestinal endoscopy; of those, 21 were found to have a gastric cancer. BLF methylation was detected in 12

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

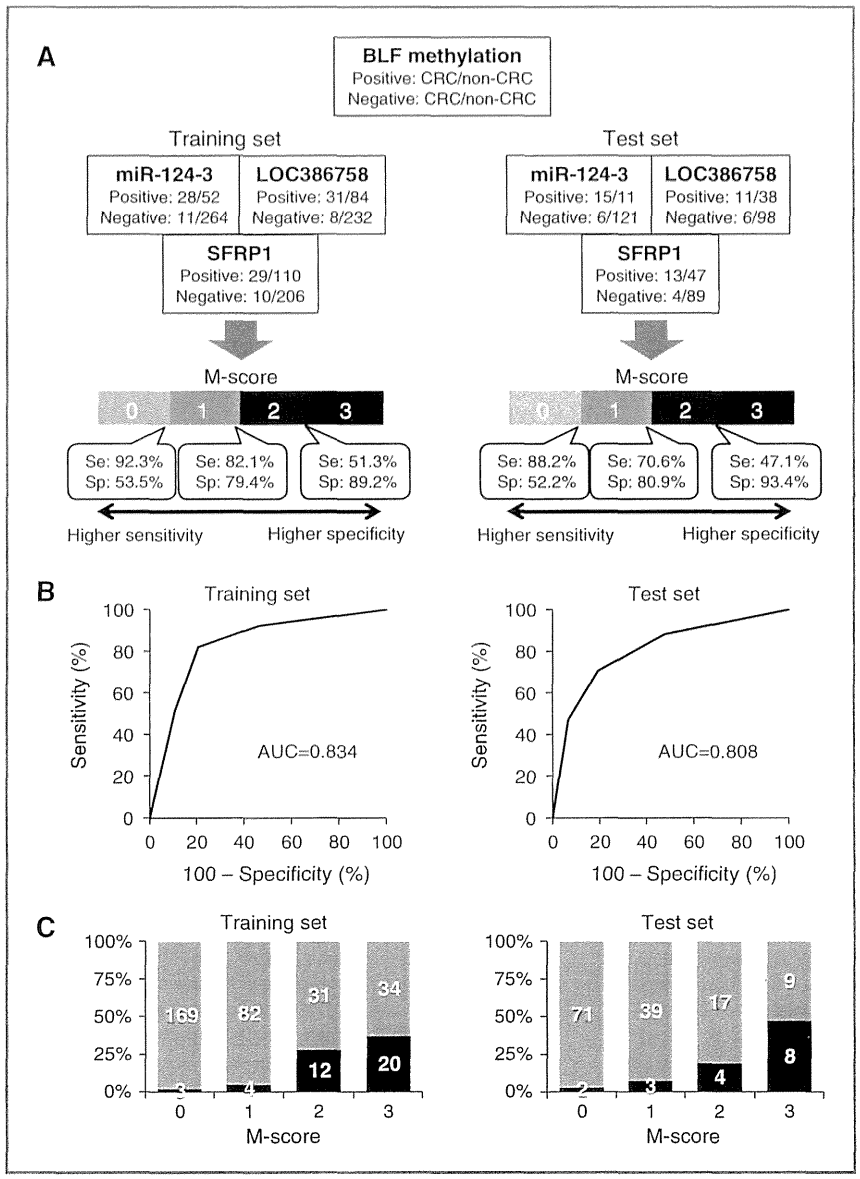


Figure 2. Diagnostic system for detecting colorectal cancer using BLF methylation. A, workflow of a system established on the basis of the ability to distinguish patients with colorectal cancer from colorectal cancer–free individuals. Results of the training set are shown on the left; those of the test set are on the right. A BLF M-score was determined from the number of methylation-positive genes, and samples were classified into four groups based on the M-score. The sensitivity (Se) and specificity (Sp) at each point are indicated below. B, ROC curve analysis of the training and test sets. The AUC is shown in the graphs. C, Percentages of patients with colorectal cancer in the respective M-score groups.

of the 21 patients with gastric cancer, and a majority of the positive cases showed only a minimal number of methylated markers (M-score 0, $n = 9$; score 1, $n = 8$; score 2, $n = 1$; and score 3, $n = 3$). Five of the 8 patients with gastric cancer with minimal methylation (M-score, 1) also had minor colorectal polyps, which could also have been the source of the methylated DNA. Interestingly, 2 of the 4 patients with gastric cancer with high M-scores (>2) were also found to have colorectal cancers, while the remaining 2 patients showed no colorectal lesions. These results suggest it would be difficult to use BLF methylation as a biomarker for upper gastrointestinal cancers.

BLF methylation and FOBT

FIT was performed in 349 of the study participants, including 17 patients with colorectal cancer (Table 3). Most of the patients with colorectal cancer were positive on the FIT (14 of 17), while a significant number of colorectal cancer–free individuals also showed positive results (142 of 332). For that reason, we next tested whether the diagnostic performance of FIT could be improved by combining it with the BLF methylation test. In the FIT-negative group ($n = 193$), which included 3 patients with colorectal cancer, all the patients with colorectal cancer were detected using the M-score system (Table 3). In the FIT-positive group, most of the patients with

Published OnlineFirst August 19, 2014; DOI: 10.1158/1940-6207.CAPR-14-0162

Table 2. Correlation between clinical features and BLF methylation in colorectal cancer

		M-score				
	Total *N*	**0**	**1**	**2**	**3**	***P***
Location						
Proximal colon	20	3	3	4	10	
Distal colon	18	2	2	6	8	
Rectum	18	0	2	6	10	0.720
Tumor size, cm						
−2.0	8	1	0	2	5	
2.1–4.0	18	2	3	4	9	
4.1–6.0	17	1	2	8	6	
6.1–	13	1	2	2	8	0.720
Dukes stage						
A	13	3	1	5	4	
B	24	1	3	6	14	
C+D	19	1	3	5	10	0.410

NOTE: *P* values were calculated using the χ^2 test.

colorectal cancer (12 of 14) exhibited BLF methylation (M-score $\geq$ 1), while a majority of the BLF methylation-negative subjects were colorectal cancer–free (80 of 82). Thus, the combination of FIT and the BLF methylation test significantly improved the positive predictive value (PPV) in both the FIT-negative and FIT-positive groups.

BLF methylation and computed tomographic colonography

Because computed tomographic colonography (CTC) has emerged in recent years as a noninvasive screening method for colorectal cancer (22), we examined the feasibility of using BLF methylation testing to complement the

Table 3. Diagnostic performance of the FIT and BLF methylation test for detection of colorectal cancer

FIT only

Study	Total *N*	CRC	CRC-free	Sensitivity	Specificity	PPV	NPV
Allison et al.	7,493	32	7,461	0.688	0.944	0.050	0.999
Current study	349	17	332	0.824	0.428	0.090	0.984

FIT and BLF methylation test

				FIT-negative group					
M-score	**Total *N***	**CRC**	**CRC-free**	**Cutoff**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	***P***
0	102	0	102						
1	54	0	54	≥1	1.000	0.537	0.000	1.000	0.103
2	25	1	24	≥2	1.000	0.821	0.081	1.000	0.007
3	12	2	10	3	0.667	0.947	0.167	0.994	0.090

				FIT-positive group					
M-score	**Total *N***	**CRC**	**CRC-free**	**Cutoff**	**Sensitivity**	**Specificity**	PPV	**NPV**	***P***
0	82	2	80						
1	43	5	38	≥1	0.857	0.563	0.162	0.976	0.004
2	12	5	7	≥2	0.500	0.831	0.226	0.944	0.008
3	19	2	17	3	0.143	0.880	0.105	0.912	0.681

NOTE: *P* value were calculated using the Fisher exact test.
Abbreviations: CRC, colorectal cancer; NPV, negative predictive value.