protein-coding genes are targets of DNA methylation in BCa, and their urinary methylation appears to be a useful biomarker [28,29]. For instance, methylation of 11 protein-coding genes found in urine sediments revealed the presence of BCa with a high sensitivity and specificity [30], and in another study a panel of three genes (growth differentiation factor 15 [*GDF15*], transmembrane protein with EGF-like and two follistatin-like domains 2 [*TMEFF2*], and vimentin [*VIM*]) in urine could be used to accurately detect BCa [31]. In the present study, we show for the first time that methylation of miRNA genes could serve as a biomarker for detection of BCa. Methylation of miRNA genes was readily detectable in voided urine from cancer patients, and its levels were dramatically reduced after tumor resection, confirming its tumor specificity. We also showed that a combination of multiple miRNA genes could accurately distinguish between preoperative and postoperative urine samples.

Our study has several limitations. The prognostic value of miRNA gene methylation remains unclear, because the prognosis of the patients in this study is not yet available. A follow-up study in post-treatment patients will be needed to test whether urinary methylation can predict outcome or detect BCa recurrence. In addition, urinary methylation in non-BCa patients (eg, patients with other types of cancer) should be tested to evaluate the specificity of our method. Further studies to address these issues would contribute to overcoming the difficulties in translating our present findings into clinical practice.

5. Conclusions

We identified four miRNA genes that are frequent targets of epigenetic silencing in BCa. Although their specific functions in bladder carcinogenesis remain unknown, it is evident that restoration of these miRNAs may be an effective anticancer therapy. Furthermore, methylation of these miRNA genes in urine specimens could serve as a useful and noninvasive biomarker for accurate detection of BCa.

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Study concept and design: Suzuki, Toyota.
Acquisition of data: Shimizu, Ashida, Hatahira, Yamamoto, Maruyama, Kai.
Analysis and interpretation of data: Shimizu, Suzuki, Nojima.
Drafting of the manuscript: Shimizu, Suzuki.
Critical revision of the manuscript for important intellectual content: Taiji Tsukamoto.
Statistical analysis: Nojima.
Obtaining funding: Suzuki, Toyota, Tsukamoto.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2012.11.030.

References

[1] He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
[2] Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704–14.
[3] Chen Q, Chen X, Zhang M, Fan Q, Luo S, Cao X. miR-137 is frequently down-regulated in gastric cancer and is a negative regulator of Cdc42. Dig Dis Sci 2011;56:2009–16.
[4] Catto JW, Miah S, Owen HC, et al. Distinct microRNA alterations characterize high- and low-grade bladder cancer. Cancer Res 2009;69:8472–81.
[5] Neely LA, Rieger-Christ KM, Neto BS, et al. A microRNA expression ratio defining the invasive phenotype in bladder tumors. Urol Oncol 2010;28:39–48.
[6] Adam L, Zhong M, Choi W, et al. miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. Clin Cancer Res 2009;15:5060–72.
[7] Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 2006;9:435–43.
[8] Suzuki H, Takatsuka S, Akashi H, et al. Genome-wide profiling of chromatin signatures reveals epigenetic regulation of microRNA genes in colorectal cancer. Cancer Res 2011;71:5646–58.
[9] Suzuki H, Yamamoto E, Nojima M, et al. Methylation-associated silencing of microRNA-34b/c in gastric cancer and its involvement in an epigenetic field defect. Carcinogenesis 2010;31:2066–73.
[10] Maruyama R, Suzuki H, Yamamoto E, Imai K, Shinomura Y. Emerging links between epigenetic alterations and dysregulation of non-coding RNAs in cancer. Tumour Biol 2012;33:277–85.
[11] Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011;12:861–74.
[12] Wiklund ED, Bramsen JB, Hulf T, et al. Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. Int J Cancer 2011;128:1327–34.
[13] Dudziec E, Miah S, Choudhry HM, et al. Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. Clin Cancer Res 2011;17:1287–96.

[14] Balaguer F, Link A, Lozano JJ, et al. Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis. Cancer Res 2010;70: 6609–18.
[15] Liu M, Lang N, Qiu M, et al. miR-137 targets Cdc42 expression, induces cell cycle G1 arrest and inhibits invasion in colorectal cancer cells. Int J Cancer 2011;128:1269–79.
[16] Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 2008;68:2094–105.
[17] Szulwach KE, Li X, Smrt RD, et al. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J Cell Biol 2010;189: 127–41.
[18] Smrt RD, Szulwach KE, Pfeiffer RL, et al. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. Stem Cells 2010;28:1060–70.
[19] Lujambio A, Ropero S, Ballestar E, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 2007;67:1424–9.
[20] Ando T, Yoshida T, Enomoto S, et al. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 2009;124:2367–74.
[21] Agirre X, Vilas-Zornoza A, Jimenez-Velasco A, et al. Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res 2009;69:4443–53.
[22] Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 2010;31:766–76.
[23] Lujambio A, Calin GA, Villanueva A, et al. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A 2008;105:13556–61.
[24] Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle 2008;7:2591–600.
[25] Hildebrandt MA, Gu J, Lin J, et al. Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma. Oncogene 2010;29: 5724–8.
[26] Yamada Y, Enokida H, Kojima S, et al. MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology. Cancer Sci 2011;102:522–9.
[27] Miah S, Dudziec E, Drayton RM, et al. An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer. Br J Cancer 2012;107:123–8.
[28] Nishiyama N, Arai E, Chihara Y, et al. Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. Cancer Sci 2010;101:231–40.
[29] Cairns P. Gene methylation and early detection of genitourinary cancer: the road ahead. Nat Rev Cancer 2007;7:531–43.
[30] Yu J, Zhu T, Wang Z, et al. A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer. Clin Cancer Res 2007;13:7296–304.
[31] Costa VL, Henrique R, Danielsen SA, et al. Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clin Cancer Res 2010;16:5842–51.

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Review Article

Model of translational cancer research in multiple myeloma

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Recently, intensive laboratory and preclinical studies have identified and validated therapeutic molecular targets in multiple myeloma (MM). The introduction of novel agents such as the proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide and lenalidomide, which were rapidly translated from preclinical studies at the Dana-Farber Cancer Institute into clinical trials, has changed the treatment paradigm and markedly extended overall survival; MM has therefore become a remarkable example of translational cancer research in new drug development. In this article, with the aim of determining the key factors underlying success in translational research, we focus on our studies of MM at Dana-Farber Cancer Institute as well as at our institutes. The identification of these key factors will help to promote translational cancer research not only in MM but also in other hematologic malignancies and solid tumors, to develop novel therapies, to overcome drug resistance, and to thereby improve the prognosis of cancer patients. (*Cancer Sci* 2012; 103: 1907–1912)

Current approaches in multiple myeloma

Multiple myeloma (MM) is a neoplastic plasma cell disorder that is characterized by the clonal proliferation of malignant plasma cells in the bone marrow (BM), 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.[1] The combined use of melphalan and prednisone since the 1960s provided a median survival of 2–3 years for patients with MM. High-dose melphalan with autologous stem cell transplantation was established in the 1990s and this combination further increased the patient median survival to 3–4 years. However, MM was largely incurable, and therefore, novel biological treatment approaches were urgently required. In the last decade, the introduction of novel agents such as the proteasome inhibitor bortezomib and the immunomodulatory drugs (IMiDs) thalidomide and lenalidomide, which were rapidly translated from preclinical studies into clinical trials carried out by Anderson *et al.* at the Dana-Farber Cancer Institute (DFCI) of Harvard Medical School (Boston, MA, USA), has changed the treatment paradigm and markedly extended the overall survival.[1–5] Multiple myeloma has therefore become a remarkable example of translational cancer research in new drug development.[2] In this review, with the aim of determining the key factors underlying the success of translational research, we focused the several dozen studies we were engaged in at DFCI as well as our institutes.[2,3,6–8] This review consists of the following parts: (i) bases for translational research in oncology; (ii) novel targets and drugs; and (iii) biomarkers.

Bases for translational research in oncology

Research in medical science is traditionally divided into two categories, basic and clinical research. Clinical research, as defined by the US National Institutes of Health (NIH), includes: patient-oriented research carried out on human subjects in which an investigator directly interacts with human subjects; epidemiological and behavioral studies; and research on outcomes and health services.[9] In contrast, basic research is carried out without considering practical ends and provides general knowledge.[9] To improve the prognosis of cancer patients, including those afflicted with MM, basic research and clinical research must be translated into practical applications. Translational research is a term that is used to describe the process by which the results of research carried out in the laboratory, in individuals (clinical), or in populations are used to develop new methods of diagnosis and treatment of a disease (clinical practice). The Translational Research Working Group of the National Cancer Institute (USA) also stated that the goal of translational research in oncology is to transform scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality.[10] Bridging the gap between basic research and clinical practice is a key factor for effective translational research. The NIH also concluded that barriers between clinical and basic research render translation of new knowledge to the clinic and back again to the laboratory bench difficult.[11] Therefore, translational research requires a close collaboration between basic scientists and clinical researchers, as well as between academia and industry.[2] Other key factors underlying the success of translational research include an understanding of the process of oncogenesis and the disease status as well as the identification of biological indicators for diagnosis, prognosis, and stratification.[12] In MM, the elucidation of tumor biology through clinical observations and genomic analyses accompanying the introduction of particular targeted drugs provide an example of bedside-back-to-bench research.[2] Ongoing translational research in MM includes genetic and epigenetic studies to evaluate myelomagenesis, identify targeted hallmarks of MM, and identify biomarkers to develop improved classification and personalized medicine;

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translational research also includes the development of novel therapies that target MM cells in the BM microenvironment.

Novel targets and drugs

In 2011, Hanahan and Weinberg updated their proposition of the hallmarks of cancer that enable tumor cell growth and progression.[13] The proposed 10 hallmarks listed in Table 1 provide a framework for understanding cancer biology and therapeutic targets, and offer an effective way to organize and describe MM biology and therapeutic targets. For example, because nuclear factor κB (NFκB) activation in MM cells results in proliferative signaling and resistance to cell death, targeting the NFκB pathway is a promising therapeutic strategy in MM.[3,14] The concept of specific molecular targeting has been applied to the development of cancer therapies, and the two main approaches discussed here are the use of small-molecule agents and the use of therapeutic mAbs.[7] In Table 1, we list candidate small-molecule compounds that target proposed hallmarks in MM, most of which we and our colleagues have studied at DFCI and our institutes.[14–33] These small-molecule compounds that interfere with certain hallmarks of cancer are under development and are being investigated in clinical trials; in some cases, they have been approved for clinical use in the treatment of cancer, including MM.[2,8]

In addition, mAb-based therapies for MM are currently being developed.[7,8,34] Figure 1 presents a list of therapeutic antibodies in MM, which can be divided into three classes based on their mechanisms of action.[35] Class I antibodies recognize and bind to cell-bound antigens to kill target cells through crystallizable fragment-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity, and antibody-dependent apoptosis.[36] The anti-CD38 mAb daratumumab[37] and anti-CD40 mAbs lucatumumab and dacetuzumab[38] are currently in preclinical and clinical development for the treatment of MM.[7,8,34] Our studies show that IMiDs can augment ADCC triggered by mAbs including elotuzumab,[30,36] and this finding provides the rationale for a combination clinical trial.

Class II antibodies also recognize cell-bound antigens, but their proposed mechanism of action does not involve crystallizable fragment effector functions. The main functions of this class of antibodies are to block ligand–receptor interactions and to act as immunoconjugates to convey intracellular toxins or radioactive isotopes. In MM research, novel mAb–maytansinoid immunoconjugates (huN901-DM1, which binds to CD56,[39] and BT062, which binds to CD138[40]) are being developed.

Class III antibodies bind to and neutralize soluble antigens, and their mechanism of action often involves blocking of the soluble ligand from binding to its receptor. A human mAb 1339 targeting interleukin-6 (IL-6) is being developed for MM.[41] The targeting of soluble ligands that affect bone biology by using mAbs such as the anti-RANKL mAb denosumab[42] and the anti-DKK1 mAb BHQ880[43] is promising, not only for preserving bone integrity but also for treating MM.[34] In January 2012, denosumab was approved in Japan for the treatment of bone complications caused by MM.[42]

The biological diversity of tumor cells within the BM microenvironment may influence the number of targets for MM therapies.[2,3,6,8] Candidate agents and approved novel drugs can show significant antitumor activity in MM *in vitro*, but treatment with single agents may not provide sufficient clinical efficacy because of drug resistance. Successful treatments can thus be achieved using other hallmarks and by addressing drug resistance. Therefore, we envisage that the use of functionally multitargeting drugs, as listed in Table 2, will provide effective MM therapies.[2,8]

Proteasome inhibitors. The ubiquitin proteasome pathway regulates the turnover of many intracellular proteins that are tagged with multiple ubiquitin molecules for transport to the 26S proteasome for subsequent degradation. Bortezomib is a prototype 26S proteasome inhibitor that selectively binds to and reversibly inhibits chymotrypsin-like and caspase-like activity.[28,44] In 2001, Hideshima *et al.* reported that bortezomib regulates cell cycle proteins in MM cells and targets intrinsic and extrinsic apoptotic pathways. It also inhibits the secretion of IL-6 and vascular endothelial growth factor

Table 1. Candidate small-molecule compounds targeting hallmarks in multiple myeloma

Hallmark	Candidate agent	Description	References
Sustainment of proliferative signaling	Perifosine	Inhibition of Akt	15
	Adaphostin	Abl cleavage	16
	CAL-101	Inhibition of PI3Kδ	17
	PKF115-584	Inhibition of β-catenin/TCF pathway	18
	SDX-308	Inhibition of β-catenin/TCF pathway	19
Evasion of growth suppressors	Seliciclib	Inhibition of cyclin-dependent kinase	20
Activation of invasion and metastasis	MLN3897	Inhibition of CCR1	21
Enabling replicative immortality	Imetelstat	Inhibition of telomerase	22
Induction of angiogenesis	Pazopanib	Inhibition of VEGFR	23
	IMiDs	Inhibtion of VEGF secretion	24
	Bortezomib	Inhibtion of VEGF secretion	25
Resistance to cell death	ABT-737	Inhibition of Bcl-2/Bcl-XL/Bcl-w	26
	R-etodolac	Upregulation of proapoptotic Mcl-1s	27
	MLN120B	Inhibition of IKKβ	14
	Bortezomib	Inhibition of NFκB	3,29
	IMiDs	Inhibtion of cytokine secretion	3,29
Prevention of immune destruction	IMiDs	Inhibtion of IL-2 secretion	30,60
Deregulation of cellular energetics	Cerulenin	Inhibition of fatty-acid synthase	31
Genome instability and mutation	ABT-888	Inhibition of PARP	32
Tumor-promoting inflammation	MLN120B	Inhibition of IKKβ	14
	BIRB 796	Inhibition of p38 MAPK	33
	IMiDs	Inhibtion of cytokine secretion	3,29

CCR1, chemokine (c-c motif) receptor-1; IKKβ, IKB kinase β; IL-2, interleukin-2; IMiDs, immunomodulatory drugs; NFκB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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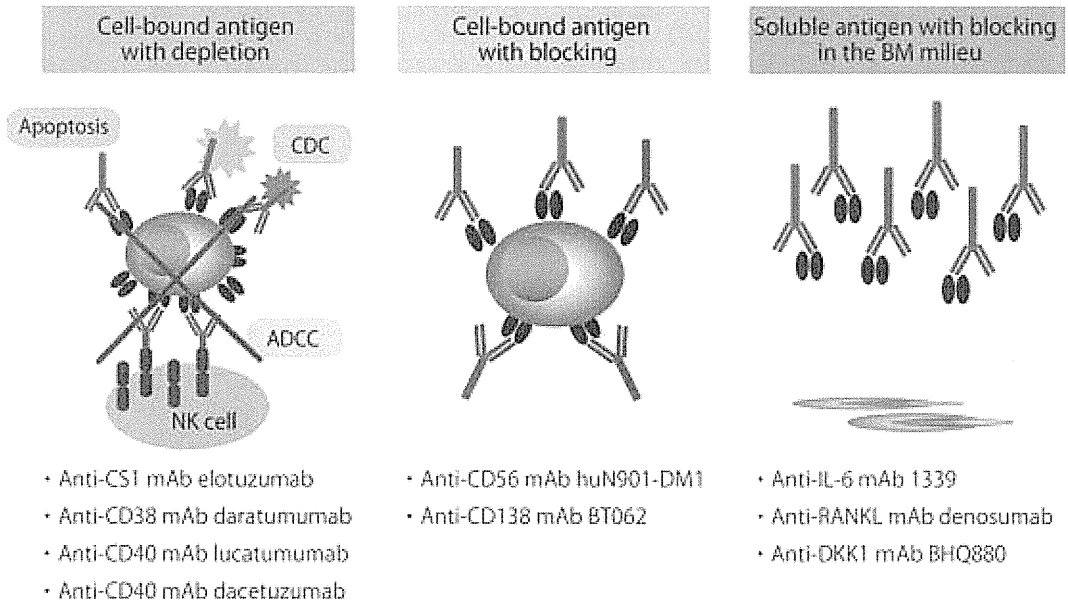

Fig. 1. Classification of therapeutic antibodies based on their mechanisms of action. Class I antibodies recognize and bind to cell-bound antigens. The Fc effector functions are part of the mechanism of action of antibodies. Class II antibodies also recognize and bind to cell-bound antigens but their proposed mechanism of action does not involve Fc effector functions. Class III antibodies bind to and neutralize soluble antigens, and their mechanism of action often involves blocking the soluble ligand from binding to its receptor. ADCC, antibody-dependent cell-mediated cytotoxicity; BM, bone marrow; CDC, complement-dependent cytotoxicity; NK, natural killer.

Table 2. Functionally multitargeting agents in multiple myeloma

Classification	Candidate agent	Description	References
Proteasome inhibitor	Bortezomib	Inhibition of chymotryptic-like and caspase-like activities	28,44
	MLN9708	Inhibition of chymotryptic-like and caspase-like activities	46
	Carfilzomib	Inhibition of chymotryptic-like activity	47
	Marizomib (NPI-0052)	Inhibition of chymotryptic-like, trypsin-like, and caspase-like activities	48
HDAC inhibitor	Vorinostat (SAHA)	Pan-HDAC inhibitor	49
	Panobinostat (LBH-589)	Pan-HDAC inhibitor	50
	Tubacin	HDAC6 selective inhibitor	51
	ACY-1215	HDAC6 selective inhibitor	52
HSP inhibitor	IPI-504 (tanespimycin)	Inhibition of hsp90	54
	SNX-2112	Inhibition of hsp90	55
Drugs influencing	Perifosine	Inhibition of Akt	15
lysophospholipid signaling	FTY720	Sphingosine 1-phosphate agonist	57
	LPAATβ inhibitor	Inhibition of LPAATβ	58
IMiD	Thalidomide	Inhibtion of cytokine secretion	29,59
	Lenalidomide (CC-5013)	Inhibtion of cytokine secretion; induction of apoptosis	29
	Pomalidomide (CC-4047)	Inhibtion of cytokine secretion; induction of apoptosis	29,59
DNA methyltransferase inhibitor	Decitabine	DNA methyltransferase inhibitor	69

HDAC, histone deacetylase; HSP, heat shock protein; IMiDs, immunomodulatory drugs; LPAATβ, lysophosphatidic acid acyltransferase β.

triggered by the binding of MM cells to BM stromal cells and inhibits BM angiogenesis by exerting a direct inhibitory effect on endothelial cells.[25] Bortezomib has undergone a remarkable transition from bench to bedside; a phase II study of bortezomib revealed a 35% response rate with manageable toxicity, and bortezomib was then approved by the US Food and Drug Administration (FDA) for the treatment of relapsed/refractory MM in 2003.[45]

Recently, the orally active agent MLN9708,[46] carfilzomib which selectively inhibits chymotrypsin-like activity,[47] and the broad-based proteasome inhibitor marizomib[48] have been developed in preclinical and clinical studies. Marizomib inhibits chymotrypsin-like, trypsin-like, and caspase-like activity and induces apoptosis in MM cells resistant to conventional agents and bortezomib.[48]

Histone deacetylase inhibitors. Histone deacetylases (HDACs) are enzymes involved in the remodeling of chromatin and play a key role in the epigenetic regulation of gene expression, which ultimately mediates cellular differentiation and survival.[44] The combination of bortezomib with HDAC inhibitors has yielded promising results in preclinical MM models and will thus be applied to clinical trials.

The HDAC inhibitors can be divided into two groups: non-selective pan-HDAC inhibitors such as vorinostat[49] and

panobinostat[50] that predominately target Class I (HDAC1, HDAC2, and HDAC3) and Class IIb (HDAC6) HDACs; and Class I HDAC inhibitors such as romidepsin and entinostat that target only Class I HDACs.[44] Although the mechanism underlying the synergistic activity of HDAC inhibitors with bortezomib is not fully understood, it may involve the role played by HDAC6 in the aggresomal degradation of ubiquitinated proteins.[44,51] The preclinical activity of a novel HDAC6 inhibitor, ACY-1215, alone and in combination with bortezomib, was recently reported[52] and transformed into a clinical study.

Heat-shock protein inhibitors. Heat-shock proteins (HSPs) constitute a class of molecular chaperones that, under normal conditions, facilitate protein folding and regulate the turnover of proteins involved in cell growth and survival. Under conditions of environmental stress, HSP expression increases as an adaptive means to maintain cell homeostasis and enhance cell survival. Because bortezomib induces the expression of stress response-related proteins such as hsp27, hsp70, and hsp90, these proteins are molecular targets for overcoming bortezomib resistance.[53] Inhibition of p38MAPK, which is an upstream molecule of hsp27, enhances the cytotoxicity of bortezomib in MM cells, thereby providing evidence that hsp27 confers bortezomib resistance.[33] Hsp90 inhibitors such as 17-AAG (tanespimycin),[53] IPI-504 (retaspimycin hydrochloride, which is a water-soluble analog of tanespimycin),[54] and SNX-2112[55] enhance bortezomib-induced cytotoxicity in preclinical models. IPI-504 has been translated into a clinical study in MM.[56]

Drugs influencing lysophospholipid signaling. We evaluated several drugs that influence lysophospholipid signaling, such as the sphingosine 1-phosphate analogue FTY720,[57] an LPAATβ inhibitor,[58] and perifosine.[15] Perifosine, which is an alkyl-phosphocholine compound, has been shown to inhibit Akt activation without affecting the activity of PI3K or phosphoinositide-dependent kinase 1. Because perifosine inhibits the Akt activation triggered by bortezomib to enhance MM cytotoxicity *in vitro*, combined therapy with bortezomib and drugs that inhibit Akt signaling is promising. Perifosine in combination with bortezomib is being evaluated in clinical trials.

Immunomodulatory drugs. The IMiDs have several anti-MM effects, including direct cytotoxicity, inhibition of angiogenesis, and induction of tumor immunity, and provide a remarkable example of translational cancer research in MM. In 2000, Hideshima *et al.*[29] reported the mechanism of anti-MM activity of the IMiDs lenalidomide (IMiD3, CC5013) and pomalidomide (IMiD1, CC4047), which potently induce apoptosis or growth arrest in MM cells. The IMiDs also reduce the secretion of IL-6 and vascular endothelial growth factor triggered by the binding of MM cells to BM stromal cells, and they inhibit angiogenesis.[59] Lenalidomide was rapidly applied to clinical trials and was approved by the FDA in 2006 for use in patients who have received prior therapy.[1] The IMiDs also stimulate a T cell co-stimulatory mechanism to induce IL-2 expression and T-cell proliferation.[60] Moreover, IMiDs induce natural killer (NK) cell-mediated cytotoxicity because the proliferation and ADCC of NK cells are induced by IL-2 production.[30] These data provide the cellular and molecular basis for the use of IMiDs as an adjuvant in immunotherapeutic treatment strategies for MM.

Biomarkers

A biomarker, as defined by NIH, is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers can be classified based on their application, such as diagnostic biomarkers, biomarkers for the staging of diseases, biomarkers for disease prognosis, and biomarkers for monitoring the clinical response to an intervention. Genetic heterogeneity has been indicated in MM, and has important implications for tumor pathogenesis, prognosis, and treatment. Importantly, cytogenetic aberrations, including the non-hyperdiploid, cytogenetically detected chromosomal 13q deletion as well as *t*(4,14), *t*(14,16), 1q gain, and del(17p), as detected by FISH, are indicators of high-risk MM associated with a poor outcome.[1] Novel therapies such as bortezomib can overcome, at least in part, the adverse outcome conferred by these abnormalities.[2] However, there has been much less progress in the development of predictive biomarkers for specific treatments.[12] To identify biomarkers to predict the effect of particular targeted therapies, appropriate clinical trial designs are necessary. Phase I studies are needed to establish that the drug inhibits the targeted pathway in the tumor. Phase II studies are required to obtain data for determining predictive biomarkers that identify patients whose tumors are driven by the inhibition of the target molecule so that therapy-specific diagnostic tests can be developed for phase III trials. Because some novel drugs in development in MM have specific molecular targets, the identification of biomarkers that also define drug sensitivity is a promising therapeutic strategy. Examples include the use of PI3K inhibitors in patients who show PI3K activation and IκB inhibitors in patients who show activation of the NFκB pathway. Efforts to examine patient samples by genetic, cytogenetic, and epigenetic methods are important to identify biomarkers to improve patient classification and, if possible, introduce personalized therapy for MM.[2] In this review, we focus on genetic studies that have recently been facilitated by next-generation sequencing technologies, as well as focus on DNA methylation studies that we are engaged in at our institute.

Genetics in MM. Major tumor-genome sequencing projects have been undertaken to identify the numerous genes mutated in cancer.[61] However, the key steps in oncogenesis in human tumors remain unclear. In MM, genomic studies are currently being carried out for the definition of heterogeneity, new target discovery, and development of personalized therapy. The analysis of somatic mutations by sequencing of the tumor genomes in 38 MM cases revealed that the mutated genes involved in NFκB activation, protein homeostasis, and histone methylation are consistent with MM biology.[62] Moreover, activating mutations of *BRAF* were observed in 4% of patients; this finding has immediate clinical translational implications for the use of BRAF inhibitors. It is important to distinguish the driver mutations from the passenger mutations; a driver mutation is defined as a mutation that is causally implicated in oncogenesis, whereas a passenger mutation is defined as a mutation that has no effect on the fitness of a clone but is present in the same genome with a driver mutation.[61] The existence of several driver mutations in individual cancer is consistent with the hallmarks of cancer.[13]

DNA methylation in MM. DNA methylation, which occurs in cytosine bases located 5′ to a guanine in which the cytosine–guanine pairs are known as CpG or CG dinucleotides, is catalyzed by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B).[63] Various cancers are characterized by promoter hypermethylation and consequent epigenetic silencing of multiple genes, and this process can be reversed during DNA synthesis, which renders it a potential therapeutic target.[63] The DNA methyltransferase inhibitors azacitidine and decitabine (5-aza-2′-deoxycytidine) have remarkable activity in the treatment of myelodysplastic syndrome (MDS), and both were approved by the FDA for the treatment of patients with MDS.[8] We and others studied DNA methylation in MM and identified certain key genes, including *RAS*, *dexamethasone-*

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Table 3. Genes epigenetically silenced in multiple myeloma

Gene	Chromosomal location	Function	References
CDKN2A ($p16^{INK4A}$)	9p21.3	Inhibition of cyclin-dependent kinase	64
CDKN2B ($p15^{INK4B}$)	9p21.3	Inhibition of cyclin-dependent kinase	64
CHFR	12q24.33	Mitotic checkpoint	65
RASSF1A	3p21.31	Inhibition of Ras signaling	66
DAPK1	9q21.33	Induction of programmed cell death	67
BNIP3	10q26.3	Induction of apoptosis	68
RASD1	17p11.2	Modulation of coregulator activity of NONO	69

induced 1 (*RASD1*), listed in Table 3.[64–69] Interestingly, MM cells that showed methylation of RASD1 were resistant to dexamethasone, and treatment with decitabine restored RASD1 expression and enhanced the cytotoxicity of dexamethasone in tumor cells. The methylation levels of RASD1 in clinical samples were elevated after repeated chemotherapy, including therapy with dexamethasone. The goal of our ongoing studies is to define RASD1 methylation as a predictive indicator of steroid resistance in MM. Our findings suggest that epigenetic gene silencing is involved in MM progression and drug resistance, and DNA methylation can therefore be a potential biomarker for MM. We are also engaging in genome-wide methylation analyses to determine the molecular mechanisms underlying MM, including oncogenesis, drug resistance, and the heterogeneity of genetic, cytogenetic, and epigenetic aberrations, thereby identifying biomarkers in MM.

Perspectives and conclusions

Ongoing translational cancer studies in MM include: genetic and epigenetic studies to evaluate myelomagenesis, identify targeted hallmarks of MM, and develop improved classification and personalized medicine; the development of next-generation novel therapies targeting MM cells in the BM milieu; and the development of rationally based combination therapies.[2,3,8] To date, many preclinical studies have hinted at the myriad of pathways that can be targeted for a synergistic and multitargeted approach. To identify these areas of molecular synergism, close collaboration between basic researchers and clinical staff is critical. These efforts will help to develop novel therapies, overcome drug resistance, and improve the prognosis of patients with MM.

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Disclosure statement

The authors have no conflicts of interest.

References

1 Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med* 2011; **364**: 1046–60.
2 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.
3 Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer* 2007; **7**: 585–98.
4 Kumar SK, Rajkumar SV, Dispenzieri A *et al.* Improved survival in multiple myeloma and the impact of novel therapies. *Blood* 2008; **111**: 2516–20.
5 Gay F, Larocca A, Wijermans P *et al.* Complete response correlates with long-term progression-free and overall survival in elderly myeloma treated with novel agents: analysis of 1175 patients. *Blood* 2011; **117**: 3025–31.
6 Yasui H, Hideshima T, Richardson PG, Anderson KC. Novel therapeutic strategies targeting growth factor signalling cascades in multiple myeloma. *Br J Haematol* 2006; **132**: 385–97.
7 Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* 2006; **6**: 714–27.
8 Yasui H, Imai K. Novel molecular-targeted therapeutics for the treatment of cancer. *Anticancer Agents Med Chem* 2008; **8**: 470–80.
9 Rubio DM, Schoenbaum EE, Lee LS *et al.* Defining translational research: implications for training. *Acad Med* 2010; **85**: 470–5.
10 Simon R. Translational research in oncology: key bottlenecks and new paradigms. *Expert Rev Mol Med* 2010; **12**: e32.
11 Yu D. Translational research: current status, challenges and future strategies. *Am J Transl Res* 2011; **3**: 422–33.
12 Sawyers CL. The cancer biomarker problem. *Nature* 2008; **452**: 548–52.
13 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–74.
14 Hideshima T, Neri P, Tassone P *et al.* MLN120B, a novel IkappaB kinase beta inhibitor, blocks multiple myeloma cell growth in vitro and in vivo. *Clin Cancer Res* 2006; **12**: 5887–94.
15 Hideshima T, Catley L, Yasui H *et al.* Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces in vitro and in vivo cytotoxicity in human multiple myeloma cells. *Blood* 2006; **107**: 4053–62.
16 Podar K, Raab MS, Tonon G *et al.* Up-regulation of c-Jun inhibits proliferation and induces apoptosis via caspase-triggered c-Abl cleavage in human multiple myeloma. *Cancer Res* 2007; **67**: 1680–8.
17 Ikeda H, Hideshima T, Fulciniti M *et al.* PI3K/p110{delta} is a novel therapeutic target in multiple myeloma. *Blood* 2010; **116**: 1460–8.
18 Sukhdeo K, Mani M, Zhang Y *et al.* Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. *Proc Natl Acad Sci U S A* 2007; **104**: 7516–21.
19 Yasui H, Hideshima T, Ikeda H *et al.* Novel etodolac analog SDX-308 (CEP-18082) induces cytotoxicity in multiple myeloma cells associated with inhibition of beta-catenin/TCF pathway. *Leukemia* 2007; **21**: 535–40.
20 Raje N, Kumar S, Hideshima T *et al.* Seliciclib (CYC202 or R-roscovitine), a small-molecule cyclin-dependent kinase inhibitor, mediates activity via down-regulation of Mcl-1 in multiple myeloma. *Blood* 2005; **106**: 1042–7.
21 Vallet S, Raje N, Ishitsuka K *et al.* MLN3897, a novel CCR1 inhibitor, impairs osteoclastogenesis and inhibits the interaction of multiple myeloma cells and osteoclasts. *Blood* 2007; **110**: 3744–52.
22 Brennan SK, Wang Q, Tressler R *et al.* Telomerase inhibition targets clonogenic multiple myeloma cells through telomere length-dependent and independent mechanisms. *PLoS ONE* 2010; **5**: e12487.
23 Podar K, Tonon G, Sattler M *et al.* The small-molecule VEGF receptor inhibitor pazopanib (GW786034B) targets both tumor and endothelial cells in multiple myeloma. *Proc Natl Acad Sci U S A* 2006; **103**: 19478–83.

24 Kumar S, Raje N, Hideshima T *et al.* Antimyeloma activity of two novel N-substituted and tetraflourinated thalidomide analogs. *Leukemia* 2005; **19**: 1253–61.
25 Roccaro AM, Hideshima T, Raje N *et al.* Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. *Cancer Res* 2006; **66**: 184–91.
26 Chauhan D, Velankar M, Brahmandam M *et al.* A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. *Oncogene* 2007; **26**: 2374–80.
27 Yasui H, Hideshima T, Hamasaki M *et al.* SDX-101, the R-enantiomer of etodolac, induces cytotoxicity, overcomes drug resistance, and enhances the activity of dexamethasone in multiple myeloma. *Blood* 2005; **106**: 706–12.
28 Hideshima T, Richardson P, Chauhan D *et al.* The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 2001; **61**: 3071–6.
29 Hideshima T, Chauhan D, Shima Y *et al.* Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. *Blood* 2000; **96**: 2943–50.
30 Hayashi T, Hideshima T, Akiyama M *et al.* Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. *Br J Haematol* 2005; **128**: 192–203.
31 Okawa Y, Hideshima T, Ikeda H *et al.* Fatty acid synthase is a novel therapeutic target in multiple myeloma. *Br J Haematol* 2008; **141**: 659–71.
32 Neri P, Ren L, Gratton K *et al.* Bortezomib-induced "BRCAness" sensitizes multiple myeloma cells to PARP inhibitors. *Blood* 2011; **118**: 6368–79.
33 Yasui H, Hideshima T, Ikeda H *et al.* BIRB 796 enhances cytotoxicity triggered by bortezomib, heat shock protein (Hsp) 90 inhibitor, and dexamethasone via inhibition of p38 mitogen-activated protein kinase/Hsp27 pathway in multiple myeloma cell lines and inhibits paracrine tumour growth. *Br J Haematol* 2007; **136**: 414–23.
34 Richardson PG, Lonial S, Jakubowiak AJ, Harousseau JL, Anderson KC. Monoclonal antibodies in the treatment of multiple myeloma. *Br J Haematol* 2011; **154**: 745–54.
35 Jiang XR, Song A, Bergelson S *et al.* Advances in the assessment and control of the effector functions of therapeutic antibodies. *Nat Rev Drug Discov* 2011; **10**: 101–11.
36 Tai YT, Dillon M, Song W *et al.* Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood* 2008; **112**: 1329–37.
37 de Weers M, Tai YT, van der Veer MS *et al.* Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol* 2011; **186**: 1840–8.
38 Tai YT, Catley LP, Mitsiades CS *et al.* Mechanisms by which SGN-40, a humanized anti-CD40 antibody, induces cytotoxicity in human multiple myeloma cells: clinical implications. *Cancer Res* 2004; **64**: 2846–52.
39 Tassone P, Gozzini A, Goldmacher V *et al.* In vitro and in vivo activity of the maytansinoid immunoconjugate huN901-N2′-deacetyl-N2′-(3-mercapto-1-oxopropyl)-maytansine against CD56+ multiple myeloma cells. *Cancer Res* 2004; **64**: 4629–36.
40 Ikeda H, Hideshima T, Fulciniti M *et al.* The monoclonal antibody nBT062 conjugated to cytotoxic Maytansinoids has selective cytotoxicity against CD138-positive multiple myeloma cells in vitro and in vivo. *Clin Cancer Res* 2009; **15**: 4028–37.
41 Fulciniti M, Hideshima T, Vermot-Desroches C *et al.* A high-affinity fully human anti-IL-6 mAb, 1339, for the treatment of multiple myeloma. *Clin Cancer Res* 2009; **15**: 7144–52.
42 Henry DH, Costa L, Goldwasser F *et al.* Randomized, double-blind study of denosumab versus zoledronic acid in the treatment of bone metastases in patients with advanced cancer (excluding breast and prostate cancer) or multiple myeloma. *J Clin Oncol* 2011; **29**: 1125–32.
43 Fulciniti M, Tassone P, Hideshima T *et al.* Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. *Blood* 2009; **114**: 371–9.
44 Hideshima T, Richardson PG, Anderson KC. Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. *Mol Cancer Ther* 2011; **10**: 2034–42.
45 Richardson PG, Barlogie B, Berenson J *et al.* A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 2003; **348**: 2609–17.
46 Chauhan D, Tian Z, Zhou B *et al.* In vitro and in vivo selective antitumor activity of a novel orally bioavailable proteasome inhibitor MLN9708 against multiple myeloma cells. *Clin Cancer Res* 2011; **17**: 5311–21.
47 Kuhn DJ, Chen Q, Voorhees PM *et al.* Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood* 2007; **110**: 3281–90.
48 Chauhan D, Catley L, Li G *et al.* A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. *Cancer Cell* 2005; **8**: 407–19.
49 Mitsiades CS, Mitsiades NS, McMullan CJ *et al.* Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proc Natl Acad Sci U S A* 2004; **101**: 540–5.
50 Catley L, Weisberg E, Kiziltepe T *et al.* Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. *Blood* 2006; **108**: 3441–9.
51 Hideshima T, Bradner JE, Wong J *et al.* Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. *Proc Natl Acad Sci U S A* 2005; **102**: 8567–72.
52 Santo L, Hideshima T, Kung AL *et al.* Preclinical activity, pharmacodynamic and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma. *Blood* 2012; **119**: 2579–89.
53 Mitsiades CS, Mitsiades NS, McMullan CJ *et al.* Antimyeloma activity of heat shock protein-90 inhibition. *Blood* 2006; **107**: 1092–100.
54 Sydor JR, Normant E, Pien CS *et al.* Development of 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504), an anti-cancer agent directed against Hsp90. *Proc Natl Acad Sci U S A* 2006; **103**: 17408–13.
55 Okawa Y, Hideshima T, Steed P *et al.* SNX-2112, a selective Hsp90 inhibitor, potently inhibits tumor cell growth, angiogenesis, and osteoclastogenesis in multiple myeloma and other hematologic tumors by abrogating signaling via Akt and ERK. *Blood* 2009; **113**: 846–55.
56 Siegel D, Jagannath S, Vesole DH *et al.* A phase 1 study of IPI-504 (retaspimycin hydrochloride) in patients with relapsed or relapsed and refractory multiple myeloma. *Leuk Lymphoma* 2011; **52**: 2308–15.
57 Yasui H, Hideshima T, Raje N *et al.* FTY720 induces apoptosis in multiple myeloma cells and overcomes drug resistance. *Cancer Res* 2005; **65**: 7478–84.
58 Hideshima T, Chauhan D, Ishitsuka K *et al.* Molecular characterization of PS-341 (bortezomib) resistance: implications for overcoming resistance using lysophosphatidic acid acyltransferase (LPAAT)-beta inhibitors. *Oncogene* 2005; **24**: 3121–9.
59 Gupta D, Treon SP, Shima Y *et al.* Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. *Leukemia* 2001; **15**: 1950–61.
60 LeBlanc R, Hideshima T, Catley LP *et al.* Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. *Blood* 2004; **103**: 1787–90.
61 Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature* 2009; **458**: 719–24.
62 Chapman MA, Lawrence MS, Keats JJ *et al.* Initial genome sequencing and analysis of multiple myeloma. *Nature* 2011; **471**: 467–72.
63 Toyota M, Suzuki H, Yamashita T *et al.* Cancer epigenomics: implications of DNA methylation in personalized cancer therapy. *Cancer Sci* 2009; **100**: 787–91.
64 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.
65 Toyota M, Sasaki Y, Satoh A *et al.* Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci U S A* 2003; **100**: 7818–23.
66 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. *Br J Haematol* 2003; **123**: 637–45.
67 Ng MH, To KW, Lo KW *et al.* Frequent death-associated protein kinase promoter hypermethylation in multiple myeloma. *Clin Cancer Res* 2001; **7**: 1724–9.
68 Murai M, Toyota M, Satoh A *et al.* Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. *Br J Cancer* 2005; **92**: 1165–72.
69 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. *Clin Cancer Res* 2009; **15**: 4356–64.

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Cancer Research

Upregulation of miR-196a and *HOTAIR* Drive Malignant Character in Gastrointestinal Stromal Tumors

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Abstract

Large intergenic noncoding RNAs (lincRNA) have been less studied than miRNAs in cancer, although both offer considerable theranostic potential. In this study, we identified frequent upregulation of miR-196a and lincRNA *HOTAIR* in high-risk gastrointestinal stromal tumors (GIST). Overexpression of miR-196a was associated with high-risk grade, metastasis and poor survival among GIST specimens. miR-196a genes are located within the *HOX* gene clusters and microarray expression analysis revealed that the *HOXC* and *HOTAIR* gene were also coordinately upregulated in GISTs which overexpress miR-196a. In like manner, overexpression of *HOTAIR* was also strongly associated with high-risk grade and metastasis among GIST specimens. RNA interference–mediated knockdown of *HOTAIR* altered the expression of reported *HOTAIR* target genes and suppressed GIST cell invasiveness. These findings reveal concurrent overexpression of *HOX* genes with noncoding RNAs in human cancer in this setting, revealing miR-196a and *HOTAIR* as potentially useful biomarkers and therapeutic targets in malignant GISTs. *Cancer Res; 72(5); 1126–36. ©2012 AACR.*

Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract (1–3). GISTs arise predominantly in the stomach (60%) and small intestine (25%) but also occur in colon and rectum (5%), esophagus (2%), and other organs (3). Immunohistochemically, GISTs are positive for KIT and CD34 and are negative or variably positive for other neural and smooth muscle cell markers. The expression of KIT and CD34 is a characteristic feature of the intestinal cells of Cajal (ICC), which are located in the intestinal wall and regulate gastrointestinal motility. GISTs are thus thought to originate from ICCs or ICC precursors. Activating *KIT* mutations have been identified in 80% to 90% of GISTs, and mutation of the platelet-derived growth factor receptor alpha gene (*PDGFRA*) is observed in approximately 5% of GISTs (1–3). In that context, imatinib mesylate (formerly STI571) was developed as a tyrosine kinase inhibitor and has been shown to inhibit the activities of BCR-ABL, KIT, and PDGFR. Imatinib mesylate is currently being used for the treatment of both chronic myeloid leukemia and metastatic GISTs.

Predicting the biologic potential of GISTs is often difficult, and considerable effort has been made to define the variables that could enable more accurate identification of tumors with malignant potential. In most classification systems, the key prognostic factors for estimating malignant potential are tumor size and mitotic rate, and to a more variable degree, the proliferation index or tumor site (4). Other potential and promising markers of GIST malignancy are molecular alterations. As mentioned, a large majority of GISTs exhibit activating *KIT* or *PDGFRA* mutations. By itself, however, mutation status does not fully explain the diverse biology of GISTs, and it is believed that additional molecular alterations are required for the progression of high-risk GISTs. For instance, expression of CD26 (encoded by *DPP4*) is strongly associated with poor survival among patients with gastric GISTs, suggesting its involvement in the malignant progression of the disease (5).

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In addition, we recently showed that hypomethylation of repetitive DNA elements is predominantly observed in malignant GISTs, and that global hypomethylation correlates with increased chromosomal aberration (6).

miRNAs are a class of small noncoding RNAs that regulate gene expression by inducing translational inhibition or direct degradation of target mRNAs through base pairing to partially complementary sites (7). miRNAs are highly conserved among species and play critical roles in a variety of biologic processes, including development, differentiation, cell proliferation, and apoptosis. Consistent with their role in these processes, a number of studies have shown widespread alteration of miRNA expression patterns in cancer (8, 9). It has also been shown that in cancer global miRNA expression profiles, as well as expression of specific miRNAs, correlate with disease prognosis and clinical outcome (10). To date, however, only a few groups have studied miRNA expression in GISTs (11, 12), and no specific miRNAs that could serve as prognostic markers have yet been identified.

In this study, we investigated the global pattern of miRNA expression in GISTs. Our aim was to evaluate the contribution made by miRNAs to the malignant potential of GISTs and to identify predictive biomarkers. We determined that upregulation of miR-196a is strongly associated with high risk and poor prognosis in GIST patients. Furthermore, we provide evidence that overexpression of miR-196a is accompanied by upregulation of *HOXC* cluster genes and a metastasis-associated noncoding RNA in GISTs.

Materials and Methods

Tumor samples

A total of 56 fresh frozen GIST specimens were obtained from Sapporo Medical University Hospital, Keiyukai Sapporo Hospital, and Osaka University Hospital, as described (6). In addition, formalin-fixed paraffin-embedded (FFPE) tissue sections of 100 GIST specimens were obtained from Niigata University Hospital. Informed consent was obtained from all patients before collection of the specimens, and this study was approved by the respective Institutional Review Boards. Risk grade was assessed according to the risk definition system proposed by Fletcher and colleagues (4). Tumors that were less than 2 cm in diameter with a mitotic count of less than 5/50 high-power fields (HPF) were categorized as very low risk. Tumors that were 2 to 5 cm in diameter with a mitotic count of less than 5/50 HPF were considered to be low risk. Tumors that were less than 5 cm in diameter with a mitotic count of 6 to 10/50 HPF, or were 5 to 10 cm with a mitotic count of less than 5/50 HPF were considered to be intermediate risk. Tumors that were more than 5 cm in diameter with a mitotic count of more than 5/50 HPF, more than 10 cm in diameter with any mitotic count, or any size with a mitotic count of more than 10/50 HPF were considered to be high risk. Total RNA was extracted from fresh frozen tissue specimens using a mirVana miRNA Isolation Kit (Ambion). Total RNA was extracted from FFPE tissue specimens using a RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion). Tumor tissues were reviewed by pathologists and were macrodissected; laser capture microdissection was not carried out in this study.

miRNA microarray analysis

One-color microarray-based miRNA expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies). Briefly, 100 ng of total RNA from fresh frozen GIST tissues was labeled using miRNA Labeling Reagent (Agilent Technologies), after which the labeled RNA was hybridized to a Human miRNA Microarray V3 (Rel 12.0, G4470C; Agilent Technologies), which covers 859 human miRNAs and 80 viral miRNAs. The microarray data were analyzed using GeneSpring GX version 11 (Agilent Technologies). The normalized microarray data were then compared with the TaqMan assay results using GraphPad PRISM version 5 (GraphPad Software Inc.). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE31741.

Quantitative RT-PCR of miRNA

miR-196a expression was analyzed using TaqMan microRNA Assays (Applied Biosystems). Briefly, 5 ng of total RNA were reverse transcribed using specific stem-loop RT primers, after which they were amplified and detected using PCR with specific primers and TaqMan probes. The PCR was run in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems), and SDS v1.4 software (Applied Biosystems) was used for comparative ΔC_t analysis. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control.

Gene expression microarray analysis

One-color microarray-based gene expression analysis was carried out according to the manufacturer's instructions (Agilent Technologies). Briefly, 700 ng of total RNA were amplified and labeled using a Quick Amp Labeling Kit One-Color (Agilent Technologies), after which the synthesized cRNA was hybridized to the Whole Human Genome Oligo DNA microarray, which includes 41,000 probe sets covering 19,416 genes (G4112F; Agilent Technologies). The microarray data were analyzed using GeneSpring GX version 11 (Agilent Technologies). The Gene Expression Omnibus accession numbers for the microarray data are GSE31802 and GSE32064.

Quantitative RT-PCR of *HOTAIR*

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative reverse transcriptase PCR (RT-PCR) of *HOTAIR* was carried out using a TaqMan Gene Expression Assay (Assay ID, Hs03296631_m1; Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems). *GAPDH* (Assay ID, Hs99999905_m1; Applied Biosystems) served as an endogenous control.

DNA copy number and chromatin signature analysis

DNA copy number was analyzed using array-based comparative genome hybridization (CGH) as described previously (6). Trimethylated Histone H3 lysine 4 (H3K4me3) was analyzed using chromatin immunoprecipitation (ChIP) as described previously (13, 14). Details of the experimental procedures are provided in the Supplementary Methods.

Transfection of miRNA inhibitors and siRNA molecules

GIST-T1 cells were described previously (15). For inhibition of miR-196a, cells (3×10^5 cells in 6-well plates) were transfected with 100 pmol of Anti-miR miRNA Inhibitors (Ambion) or Anti-miR miRNA Inhibitors Negative Control #1 (Ambion) using Lipofectamine2000 (Invitrogen). For RNA interference (RNAi)-mediated knockdown of *HOTAIR*, 3 different Stealth siRNAs against *HOTAIR* were generated by Invitrogen, after which a mixture of the 3 was used for transfection. Cells (3×10^5 cells in 6-well plates) were transfected with 100 pmol of siRNA or with Stealth RNAi Negative Control Medium GC (Invitrogen) using Lipofectamine2000 (Invitrogen). Total RNA extraction, cell viability assays, and Matrigel invasion assays were carried out 48 hours after transfection as described in the Supplementary Methods.

Statistical analysis

All gene expression levels were log transformed for subsequent statistical analysis because the distribution of expression data seemed to follow a log-normal distribution. Geometric means were therefore calculated as summary statistics for expression levels. Comparisons of continuous variables were made using *t* tests or one-way ANOVA with post hoc multiple comparisons (Games–Howell test). Pearson's correlation coefficients were calculated to describe the strength of the correlation between 2 variables. Comparisons of categorical variables were made using Fisher exact test. To assess the association between prognostic factors and gene expression levels, logistic or Cox regression analyses were carried out. For these regression analyses, the most optimal cutoff points were employed to calculate ORs and HRs, with or without adjustment for clinical factors. Kaplan–Meier curves were plotted to compare 2 groups stratified by gene expression status. All statistical analyses were done using SPSS Statistics 18 (IBM Corporation).

Results

Detection of upregulated miR-196a expression in high-risk GISTs

To examine the miRNA expression signature in GISTs, we carried out miRNA microarray analysis with 32 fresh frozen GIST specimens (10 low-risk, 8 intermediate-risk, and 14 high-risk GISTs). The clinicopathologic features of the 32 patients are listed in Supplementary Table S1. Of 939 probe sets, 470 were excluded because of the absence of a detectable signal in any of the samples tested. Unsupervised hierarchical clustering using the remaining 469 probe sets revealed that GISTs in which there was abundant expression of miRNAs encoded on chromosome 14q32.31 form a separate cluster (Supplementary Figs. S1 and S2). Moreover, by comparing the miRNA expression profiles with array CGH results, we found that this cluster is enriched in tumors without 14q loss. These results are consistent with recent reports showing an inverse relationship between 14q loss and expression of miRNAs located on 14q in GISTs (11, 12), which is indicative of the reliability of our microarray analysis. We next carried out a scatter plot analysis and found that miR-196a is markedly upregulated in high-risk GISTs, as compared with low- or intermediate-risk GISTs (Fig. 1A). As shown in Fig. 1B, miR-196a was undetectable in all but one of the low- and intermediate-risk GISTs tested, whereas it was upregulated in more than half of the high-risk tumors. The elevated expression of miR-196a was observed in both gastric and small intestinal GISTs (Supplementary Table S1).

Upregulation of miR-196a is associated with GIST malignancy

To assess the clinical importance of miR-196a upregulation in GISTs, we next carried out TaqMan assay with 56 fresh frozen GIST specimens (discovery cohort), including the 32 specimens initially analyzed by microarray. The clinicopathologic features of the patients are summarized in Table 1. The TaqMan assay results were highly consistent with the microarray data, though the TaqMan assay did reveal low levels of miR-196a expression in samples in which there was no detectable signal from the microarray (Supplementary Fig. S3).

Also consistent with the microarray results was the finding that miR-196a was markedly upregulated in high-risk GISTs, as compared with the other groups ($P = 0.004$, one-way ANOVA; Fig. 1C, Supplementary Table S2). In addition, logistic regression analysis revealed that the association between miR-196a upregulation and the high-risk category was highest when we employed a cutoff value of miR-196a/U6 0.4 or more (OR = 13.7; 95% CI: 3.4–54.6; $P < 0.001$; Supplementary Table S3). Survival data were obtained for 32 patients, and Cox hazard analysis revealed the highest HR for patients with elevated miR-196a expression when a cutoff value of 1.4 was employed (Table 2). Kaplan–Meier analysis showed poor overall survival among patients with GISTs expressing high levels of miR-196a, though the effect was not statistically significant (Fig. 1D).

We next used TaqMan assay to analyze an independent validation cohort consisting of 100 FFPE GIST specimens (Table 1). Consistent with the findings summarized above, we observed that upregulation of miR-196a was associated with high-risk GISTs (Fig. 1C, Supplementary Tables S2 and S3). By using the same cutoff value (miR-196a/U6 $\geq$ 1.4), Cox hazard analysis revealed an elevated HR for patients exhibiting high levels of miR-196a expression (Table 2), and Kaplan–Meier analysis showed shorter survival times for the same patients (Fig. 1D). These results confirmed the prognostic value of miR-196a expression in both fresh frozen and FFPE GIST specimens.

Finally, we combined the GIST samples in the discovery and validation cohorts to examine the clinicopathologic significance of miR-196a. Expression of miR-196a correlated positively with high-risk grade (Fig. 1C, Supplementary Tables S2 and S3), poor clinical outcome (Fig. 1D, Table 2), tumor size, mitotic count, and metastasis (Table 3). Interestingly, although expression of miR-196a was not associated with age or gender, it was strongly associated with tumor location (Table 3). The median level of miR-196a expression was lowest in specimens from esophageal GISTs and then increased as the GIST site moved from the oral side toward the anal side of the gastrointestinal tract ($P < 0.001$; Table 3, Supplementary Fig. S4). Importantly, although the average level of miR-196a expression was higher in small intestine than in stomach, it was positively

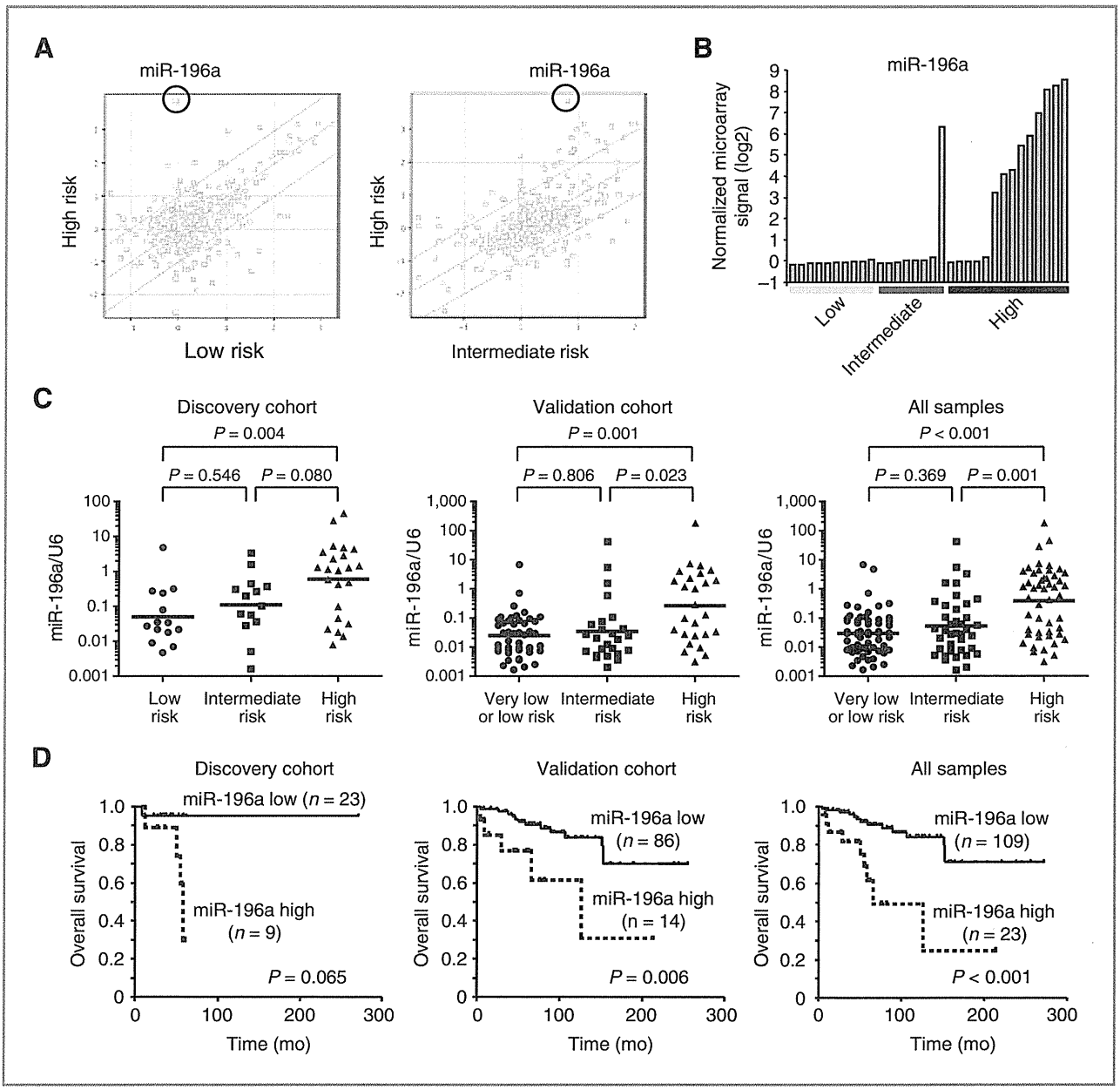

Figure 1. Upregulation of miR-196a expression in GISTs is associated with a high-risk grade and poor prognosis. A, scatter plots analyses: plotting low-risk ($n = 10$) versus high-risk GISTs ($n = 14$; left) and intermediate-risk ($n = 8$) versus high-risk GISTs ($n = 14$; right) revealed overexpression of miR-196a in high-risk GISTs. Microarray data are normalized and log transformed (base 2). Expression of miR-196a is highlighted by a circle. B, levels of miR-196a expression obtained from microarray analysis of 32 GIST specimens. Risk categories are indicated below. C, comparison of miR-196a expression using TaqMan assay in low- ($n = 14$), intermediate- ($n = 14$), and high-risk GISTs ($n = 23$) in a discovery cohort (left), very low- or low-risk ($n = 46$), intermediate-risk ($n = 25$), and high-risk GISTs ($n = 26$) in a validation cohort (middle) and all GIST specimens (right). Results are normalized to internal U6 snRNA. D, Kaplan–Meier curves showing the effect of miR-196a expression (high, miR-196a/U6 $\geq$ 1.4; low, miR-196a/U6 < 1.4) on overall survival in the discovery cohort (left), validation cohort (middle), and all GIST patients (right).

correlated with high-risk grade in both organs (Supplementary Table S4).

Concurrent upregulation of miR-196a and *HOX* cluster genes in GISTs

To analyze the relationship between miR-196a upregulation and the global gene expression profiles in GISTs, we selected age-, gender-, and tumor location–matched GIST specimens showing either low ($n = 7$; average miR-196a/U6 = 0.1) or high miR-196a expression ($n = 7$; average miR-196a/U6 = 15.7) and subjected them to gene expression microarray analysis (Supplementary Table S5). We found that for 4,947 probe sets (corresponding to 3,206 unique genes), there was more than a 2-fold difference in expression between GISTs with miR-196a

Table 1. Clinical features of the GIST samples used in this study

Discovery cohort	
Age (y, median ± SD)	68.0 ± 15.2
Gender	
Male	32 (57.1%)
Female	24 (42.9%)
Tumor location	
Stomach	40 (71.4%)
Small intestine	14 (25.0%)
Omentum	1 (1.8%)
Colorectum	1 (1.8%)
Risk category ($n = 51$)	
Low	14 (27.5%)
Intermediate	14 (27.5%)
High	23 (45.0%)
Validation cohort	
Age (y, median ± SD)	64.0 ± 12.4
Gender	
Male	44 (44.0%)
Female	56 (56.0%)
Tumor location	
Esophagus	5 (5.2%)
Stomach	84 (84.0%)
Small intestine	8 (8.0%)
Colorectum	3 (3.0%)
Risk category ($n = 97$)	
Very low	1 (1.0%)
Low	45 (46.4%)
Intermediate	25 (25.8%)
High	26 (26.8%)

overexpression and those without it. Hierarchical clustering analysis using the 4,947 probe sets clearly distinguished between tumors on the basis of their miR-196a expression status (Fig. 2A), and Gene Ontology analysis suggested that genes related to "immune system," "plasma membrane," and "cell communication" are strongly overrepresented among the differentially expressed genes (Supplementary Table S6).

To further characterize the differentially expressed genes, we carried out a gene set analysis and obtained the highest enrichment score with the *HOX* gene set (Supplementary Table S7). We found miR-196a to be encoded at 2 paralogous loci, *miR-196a-1* and *miR-196a-2*, which are located within the *HOXB* and *HOXC* clusters, respectively (Fig. 2B; ref. 16). Hierarchical clustering analysis using the expression data for *HOXC* genes clearly differentiated the GIST samples into 2 groups, and we observed perfect correspondence between higher expression of multiple *HOXC* genes and upregulation of miR-196a (Fig. 2C). By contrast, genes in other *HOX* clusters did not show such obvious correlations with miR-196a (Fig. 2C, Supplementary Fig. S5). We next compared the microarray signal for each *HOX* gene with the miR-196a expression and found strong positive correlations between the expression levels of a number of *HOXC* genes and those of miR-196a (Fig. 3D, Supplementary Fig. S6). Notably, we also found that expression of *HOTAIR*, which encodes a large intergenic noncoding RNA (lincRNA) and is located in an antisense orientation relative to the *HOXC* genes, is concurrently upregulated with miR-196a (Fig. 2C and D). Levels of miR-196a expression also correlated moderately with those of the *HOXB* genes neighboring *miR-196a-1* (*HOXB13* and *HOXB9*), but the correlations were less significant than those with *HOXC* genes (Supplementary Fig. S7).

The similarity between the expression patterns of *HOXC* genes and those of the noncoding RNAs encoded in the same locus is indicative of a common regulatory mechanism involved in the activation of these genes in GISTs. However, array CGH analysis of 27 GIST specimens failed to detect either gain or loss in any *HOX* loci, irrespective of *miR-196a* or *HOX* gene expression, which makes it unlikely that genomic amplification is the cause of their overexpression (Supplementary Fig. S8).

Upregulation of *HOTAIR* is associated with GIST malignancy

A recent study showed that *HOTAIR* is overexpressed in primary breast cancer and is associated with metastasis (17). To examine its clinical significance in GISTs, we carried out TaqMan assays of *HOTAIR* with the discovery cohort samples. We found that the microarray signals for *HOTAIR* were highly

Table 2. miR-196a expression is associated with poor clinical outcome in GIST patients

		Outcome					
	miR-196a/U6	Survival	Death	HR (95% CI)	*P*	HR[a] (95% CI)	*P*
Discovery cohort	<1.4	22	1				
	≥1.4	5	4	6.3 (0.7–57.5)	0.104	32.9 (2.0–551.3)	0.015
Validation cohort	<1.4	73	13				
	≥1.4	9	5	3.9 (1.4–11.1)	0.011	8.4 (2.6–26.9)	<0.001
All samples	<1.4	95	14				
	≥1.4	14	9	4.9 (2.1–11.7)	<0.001	9.1 (3.5–23.7)	<0.001

[a]Age and gender adjusted HR.

Table 3. Correlation between miR-196a expression and clinicopathologic features of GISTs

		miR-196a/U6				
	n	Geometric mean	95% CI	*P*		
Age (y)						
<65	76	0.093	0.051–0.168			
≥65	81	0.074	0.044–0.127	0.581		
Gender						
M	76	0.104	0.059–0.186			
F	80	0.069	0.040–0.119	0.297		
Location						
Esophagus	5	0.019	0.001–0.417	Ref		
Stomach	124	0.061	0.039–0.094	0.741	Ref	
Small intestine	22	0.395	0.163–0.957	0.161	0.002	Ref
Colorectum	4	4.936	2.564–9.502	0.023	<0.001	<0.001
				<0.001		
Tumor size (cm)						
≤5.0	70	0.045	0.026–0.075			
>5.0	81	0.118	0.066–0.210	0.016		
Mitotic count (/50 HPF)						
≤5	105	0.036	0.025–0.053			
>5	35	0.539	0.215–1.353	<0.001		
Metastasis						
Yes	28	0.747	0.307–1.819			
No	108	0.041	0.027–0.063	<0.001		

consistent with the TaqMan assay results (Supplementary Fig. S9). *HOTAIR* was upregulated exclusively in high-risk GISTs, as compared with low- or intermediate-risk GISTs ($P = 0.018$; Fig. 3A), and its expression correlated positively with the expression of miR-196a (Fig. 3B) and *HOXC* genes (Fig. 3C, Supplementary Fig. S10). In addition, logistic regression analysis revealed that high levels of *HOTAIR* expression in GISTs (*HOTAIR*/*GAPDH* ≥ 0.0002) were strongly associated with metastasis (age and gender adjusted OR = 8.2; 95% CI: 1.4–48.4; $P = 0.021$). Cox hazard analysis suggested an elevated HR for patients with high *HOTAIR* expression (Table 4), and Kaplan–Meier analysis showed poor overall survival for the same patients, though the effect was not statistically significant (Fig. 3D). We also tried to analyze *HOTAIR* expression in the FFPE specimens; however, we failed to detect expression of either *HOTAIR* or *GAPDH* in these samples, most likely due to an inadequate quality of the RNA.

Reduced expression of miR-196a and *HOTAIR* target genes in GISTs

To examine the functional role of miR-196a in GISTs, we interrogated our gene expression microarray data for miR-196a target genes computationally predicted by TargetScan. Of the 2,248 genes whose expression was reduced in GISTs overexpressing miR-196a, 95 corresponded to predicted targets (Supplementary Fig. S11, Supplementary Table S8). This gene list included *ANXA1* (Annexin A1), which is an experimentally validated miR-196a target gene (18). In addition, expression of several *HOX* genes, including *HOXB8*, was reduced in GISTs overexpressing miR-196a, which is consistent with an earlier finding of miR-196a–directed cleavage of *HOXB8* mRNA (Supplementary Fig. S11; ref. 19).

In normal human fibroblasts, *HOTAIR* represses *HOXD* gene expression by interacting with polycomb repressive complex 2 (PRC2; ref. 20). In breast cancer cells, overexpression of *HOTAIR* was shown to recruit PRC2 to more than 800 gene promoters, leading to histone H3K27 methylation and epigenetic silencing of the target genes (17). We therefore examined our microarray data for the reported *HOTAIR*-induced PRC2 target genes. Among 14 GISTs analyzed with the microarray, all 7 tumors strongly expressing miR-196a showed elevated *HOTAIR* expression (average *HOTAIR*/*GAPDH* = 0.00254), whereas all tumors only weakly expressing miR-196a showed little or no *HOTAIR* expression (average *HOTAIR*/*GAPDH* = 0.00001). We found that expression of 144 *HOTAIR* target genes was reduced in GISTs overexpressing *HOTAIR* (Supplementary Fig. S11, Supplementary Table S9). These results indicated that overexpression of miR-196a and *HOTAIR* may contribute to the malignant progression of GISTs by modulating expression of their target genes.

Inhibition of *miR-196a* and *HOTAIR* suppresses GIST cell invasion

We next utilized a cultured GIST cell line to determine whether upregulation of miR-196a and *HOTAIR* is responsible for the malignant potential of GISTs. We found that both

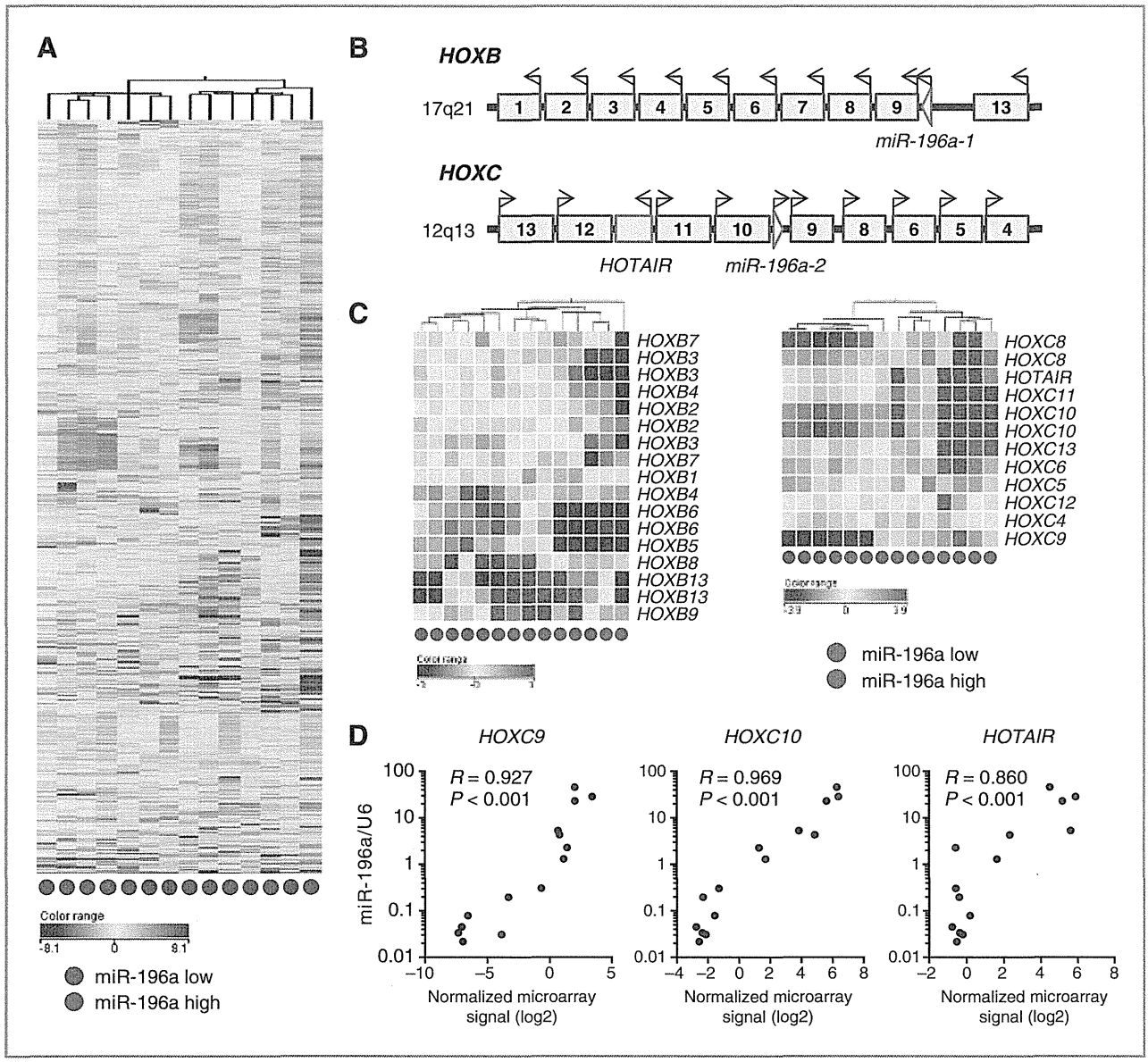

Figure 2. *GIST* gene expression signatures reveal a strong correlation between the *miR-196a* and *HOXC* genes. A, heat map of the gene expression signatures correlated with miR-196a expression. Rows represent probe sets and columns represent patients. A total of 4,947 probe sets differentially expressed (>2-fold change) between GISTs with ($n = 7$) and without ($n = 7$) miR-196a overexpression were selected, after which hierarchical clustering was carried out. The miR-196a expression status is indicated below. B, schematic representations of the *miR-196a* family locations within the *HOX* gene clusters. C, hierarchical clustering analysis using *HOXB* (left) and *HOXC* (right) expression data. miR-196a expression status is indicated below. D, correlations between the expression levels of miR-196a and *HOXC* genes or *HOTAIR*. Expression of miR-196a was analyzed using TaqMan assay and was normalized to internal U6 snRNA. Microarray data for *HOXC* and *HOTAIR* were normalized and log transformed (base 2). The Pearson correlation coefficients and *P* values are shown.

miR-196a and *HOTAIR* are expressed in GIST-T1 cells (Supplementary Fig. S12). We then carried out cell viability and Matrigel invasion assays after transfecting GIST-T1 cells with an anti–miR-196a inhibitor molecule. Gene expression microarray analysis revealed that a number of predicted miR-196a target genes, including *ANXA1* and *HOXA5*, were upregulated by inhibition of miR-196a (Supplementary Table S10), and although we observed no effects on cell viability, inhibition of miR-196a moderately suppressed cell invasion (Supplementary Fig. S13). We next disrupted *HOTAIR* expression by transfecting the cells with siRNAs targeting it (Fig. 3E). Although knockdown of *HOTAIR* did not significantly affect cell viability, it suppressed the invasiveness of GIST-T1 cells (Fig. 3E and F). Moreover, gene expression microarray analysis revealed that a number of reported *HOTAIR* target genes, including *PCHD10*, *SEMA6A*, and *STK17B*, were upregulated upon knockdown of *HOTAIR* (Supplementary Table S11). In all, we found that 1,424 genes were upregulated by siHOT (>2-fold), and Gene Ontology analysis revealed enrichment of genes related to "nucleus," "chromosome," and "membrane-bounded

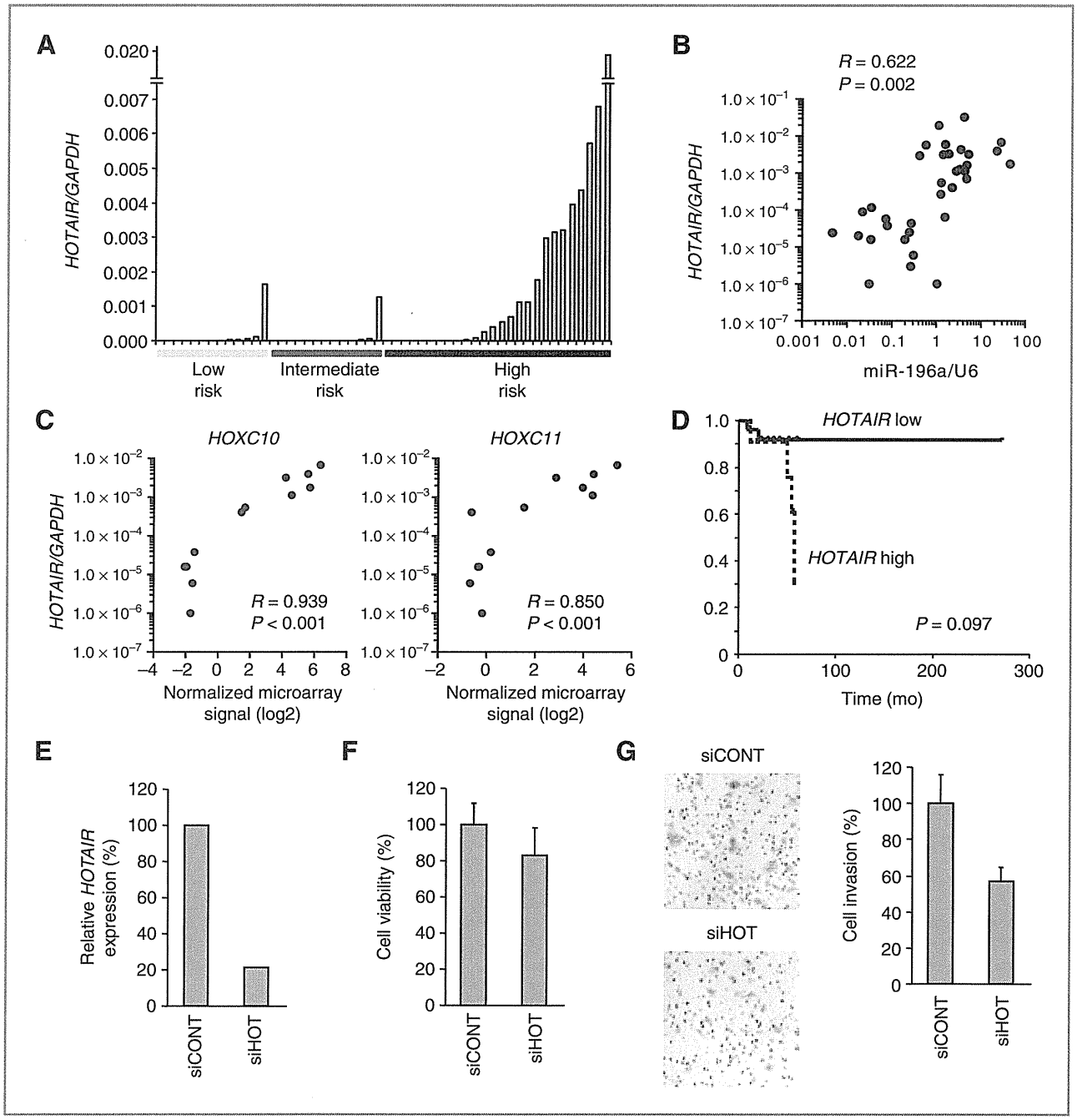

Figure 3. Upregulation of *HOTAIR* in malignant GISTs. A, TaqMan assay of *HOTAIR* in a panel of GIST specimens ($n = 52$). Results are normalized to internal *GAPDH* expression. Risk categories are indicated below. B, correlation between levels of *HOTAIR* and miR-196a expression detected using TaqMan assay. The Pearson correlation coefficient and *P* value are shown. C, correlations between levels of *HOTAIR* expression detected using TaqMan assay and those of *HOXC* genes detected from microarrays. The Pearson correlation coefficients and *P* values are shown. D, Kaplan–Meier curves showing the effect of *HOTAIR* expression (high, *HOTAIR/GAPDH* $\geq$ 0.0002; low, *HOTAIR/GAPDH* < 0.0002) on overall survival among GIST patients. E, TaqMan assay for *HOTAIR* in GIST-T1 cells transfected with control siRNA (siCONT) or siRNA targeting *HOTAIR* (siHOT). F, cell viability assay using GIST-T1 cells transfected with siCONT or siHOT. Cell viabilities were determined 48 hours after transfection. Shown are the means of 8 replications; error bars represent SDs. G, Matrigel invasion assay using GIST-T1 cells transfected with siCONT or siHOT. Shown on the right are the means of 3 random microscopic fields per membrane; error bars represent SDs.

organelle" (Supplementary Tables S12 and S13). These results suggested that *HOTAIR* may modulate transcription of a large number of genes and may have previously unidentified roles in GIST cells.

Finally, we sought to clarify the biologic relationship between miR-196a, *HOTAIR*, and *HOXC* genes. We first tested whether upregulation of miR-196a is a downstream effect of *HOTAIR* dysregulation, or vice versa. We found that inhibition

Table 4. HOTAIR expression is associated with poor clinical outcome in GIST patients

	Outcome					
HOTAIR/GAPDH	**Survival**	**Death**	**HR (95% CI)**	***P***	**HR[a] (95% CI)**	***P***
<0.0002	26	2				
≥0.0002	7	4	3.8 (0.7–21.2)	0.123	9.0 (1.2–68.9)	0.034

[a]Age and gender adjusted HR.

of miR-196a had no effect on *HOTAIR* expression in GIST-T1 cells, nor did knockdown of *HOTAIR* affect miR-196a expression. This suggested that overexpression of miR-196a or *HOTAIR* is not a simple downstream effect of their dysregulation (Supplementary Fig. S12). By contrast, analysis of the chromatin status in GIST-T1 cells using ChIP-PCR revealed enrichment of trimethylated histone H3 lysine 4 (H3K4me3), a hallmark of active gene transcription, at the transcription start sites of multiple *HOXC* genes and *HOTAIR* (Supplementary Fig. S14). Moreover, we found concurrent overexpression of miR-196a, *HOTAIR*, and *HOXC* genes in other cancer cells, including the KatoIII gastric cancer cell line. By carrying out high-resolution ChIP-seq analysis with the KatoIII cells, we observed significant enrichment of H3K4me3 over a wide range (more than 50 kb) of the *HOXC* cluster, which suggested that an epigenetic mechanism is involved in the dysregulation of this genomic region (Supplementary Fig. S15).

Discussion

Although the results of recent studies suggest that the gene expression signatures of GISTs are predictive of malignancy and drug sensitivity of the tumors (5, 21), the clinical significance of the miRNA expression signature is not yet fully understood. In this study, we found that upregulation of miR-196a is strongly associated with a high-risk grade, metastasis, and poor prognosis in GIST patients. Furthermore, overexpression of miR-196a is accompanied by upregulation of multiple *HOXC* genes and the metastasis-related lincRNA *HOTAIR*. To our knowledge, this is the first article to show concurrent overexpression of collinear *HOX* genes and noncoding RNAs in human malignancy.

A number of studies have implicated miR-196a in malignancy, but its role may differ among tumor types. Upregulation of miR-196a is observed in esophageal adenocarcinomas and their precancerous lesions, Barrett's esophagus and dysplasia, which suggests miR-196a is a potential marker of the malignant progression of Barrett's esophagus (22). Strong expression of miR-196a is also associated with a poor prognosis in pancreatic adenocarcinoma and glioblastoma patients (23, 24). In addition, functional analysis showed that expression of miR-196a in esophageal, breast, and endometrial cancer cells promotes proliferation and suppresses apoptosis through downregulation of *ANXA1* (18). These results suggest that miR-196a contributes to oncogenesis in cancer. On the other hand, miR-196a is significantly downregulated in melanoma, and its reexpression inhibited the invasive behavior of melanoma cells by targeting HOXC8 (25). Similarly, miR-196a suppressed HOXC8 and inhibited invasion and metastasis by breast cancer cells (26). Thus miR-196a seems to exert opposite effects in tumors of different origins.

The *HOX* genes are a highly conserved subgroup of the homeobox superfamily, and they play essential roles in a variety of biologic processes, including development, differentiation, apoptosis, and angiogenesis (27). In humans, 4 *HOX* clusters containing 39 *HOX* genes have been identified, and dysregulation of their expression is observed in various malignancies. Although the role of HOXs in cancer is not fully understood, its aberrant expression is thought to affect pathways that promote tumorigenesis and metastasis (27). For instance, *HOXC8* mRNA is overexpressed in prostate cancer cells and is associated with tumor cell proliferation and metastasis (28–30). In addition, *HOXC5* and *HOXC8* mRNAs are upregulated in cervical cancer cells (31), and one recent study suggested HOXC10 plays a key role of in the progression and invasion in cervical cancer (32).

An association between miR-196a and *HOX* expression in cancer has also been reported. Reduced expression of miR-196a in malignant melanoma cells leads to upregulation of HOXB7 and, in turn, activation of BMP4, a major modulator of migration (33). As mentioned above, miR-196a also inhibits invasion and metastasis by downregulating HOXC8 in melanoma and breast cancer cells (26, 34). Taken together, these results suggest that miR-196a acts as a tumor suppressor by targeting *HOX* genes in these tumor types. By contrast, we show in this study that both the miR-196a and *HOXC* genes are concurrently upregulated in malignant GISTs. Our findings are reminiscent of an earlier report showing that the expression patterns of miRNAs embedded in *HOX* clusters are very similar to those of *HOX* genes during mammalian embryogenesis (35). Global gene expression analysis revealed that expression of multiple putative miR-196a targets, including *ANXA1*, is diminished in GISTs overexpressing miR-196a, whereas their expression is enhanced upon inhibition of miR-196a in cultured GIST-T1 cells. In addition, inhibition of miR-196a in GIST cells overexpressing that miRNA moderately suppressed cell invasion. Taken together, our results indicate that upregulation of *HOXC* genes along with miR-196a may contribute to the malignant potential of GIST.

HOTAIR is located within the *HOXC* cluster and encodes a lincRNA known to repress its target genes by directly interacting with histone modification complexes. Epigenetic gene

regulation is closely associated with histone modifications in which di- or trimethylation of histone H3 lysine 4 (H3K4me2 or H3K4me3) is enriched within active gene promoters. In addition, trimethylation of histone H3 lysine 27 (H3K27me3) is a marker of gene silencing. In normal adult fibroblasts, *HOTAIR* suppresses the *HOXD* locus by recruiting the PRC2 complex, which consists of the histone H3K27 methylase EZH2, SUZ12, and EED (20). It was also recently shown that *HOTAIR* serves as a scaffold for multiple repressor complexes, including PRC2 and LSD1/CoREST/REST (36). LSD1 is a demethylase that specifically mediates demethylation of H3K4, leading to repression of the target genes. *HOTAIR* is also strongly implicated in cancer metastasis. In breast cancer cells, *HOTAIR* induces retargeting of the PRC2 complex throughout the genome, which leads to the silencing of multiple tumor suppressor and metastasis suppressor genes (17). Overexpression of *HOTAIR* is also predictive of recurrence in hepatocellular carcinoma patients after liver transplantation (37). We observed that upregulation of *HOTAIR* is closely associated with GIST aggressiveness and metastasis. In addition, functional analysis using GIST-T1 cells showed that RNAi-mediated knockdown of *HOTAIR* suppressed cell invasion. These results strongly suggest that upregulation of *HOTAIR* is one of the mechanisms that promote aggressiveness in GISTs. Interestingly, depletion of *HOTAIR* induced a significant change in the gene expression profile in GIST cells, suggesting that *HOTAIR* may regulate a spectrum of genes other than the previously reported target genes. Further studies, including genome-wide histone modification analysis, may reveal as yet unidentified roles played by *HOTAIR* in the malignant progression of GISTs.

The mechanism underlying upregulation of *HOX* cluster genes and noncoding RNAs in GISTs is intriguing. Our array CGH analysis did not detect chromosomal aberrations in any *HOX* loci, making it unlikely that gene amplification is the cause of their overexpression. However, we found that the transcription start sites of multiple genes in the *HOXC* cluster are marked by an active histone mark, H3K4me3, in GIST-T1 cells. Moreover, high-resolution ChIP-seq analysis revealed that, in cancer cells, the entire region is significantly enriched with H3K4me3, leading to overexpression of the affected genes. Our results are reminiscent of the recent finding that rearrangement of *MLL* in leukemia induces active histone modifications at the promoters of *HOXA* genes and miR-196b, resulting to their overexpression (38–40). Although such rearrangements are not known in GISTs, further study to clarify the involvement of epigenetic modifiers in malignant GISTs may lead to identification of new therapeutic targets.

Overall, our study has shown that noncoding RNAs encoded in the *HOXC* cluster could be useful predictive markers as well as novel therapeutic targets in malignant GISTs. As miRNAs are well preserved in FFPE specimens (41), miR-196a could be a reliable biomarker for risk assessment. We also provide evidence that *HOTAIR* is significantly upregulated in high-risk GISTs, indicating that this lincRNA also could be a useful biomarker, as well as a novel therapeutic target. Further study of the causes and functions of *HOXC* locus activation in GISTs may provide new strategies for the treatment of GIST patients.

Disclosure of Potential Conflicts of Interest

T. Nishida has received a research grant from Novartis Pharma K.K. The remaining authors disclose no conflicts of interest.

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References

1. Shinomura Y, Kinoshita K, Tsutsui S, Hirota S. Pathophysiology, diagnosis, and treatment of gastrointestinal stromal tumors. J Gastroenterol 2005;40:775–80.
2. Rubin BP, Heinrich MC, Corless CL. Gastrointestinal stromal tumour. Lancet 2007;369:1731–41.
3. Corless CL, Fletcher JA, Heinrich MC. Biology of gastrointestinal stromal tumors. J Clin Oncol 2004;22:3813–25.
4. Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, et al. Diagnosis of gastrointestinal stromal tumors: A consensus approach. Hum Pathol 2002;33:459–65.
5. Yamaguchi U, Nakayama R, Honda K, Ichikawa H, Hasegawa T, Shitashige M, et al. Distinct gene expression-defined classes of gastrointestinal stromal tumor. J Clin Oncol 2008;26:4100–8.
6. Igarashi S, Suzuki H, Niinuma T, Shimizu H, Nojima M, Iwaki H, et al. A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. Clin Cancer Res 2010;16:5114–23.
7. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
8. Esquela-Kerscher A, Slack FJ. Oncomirs–microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69.
9. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704–14.
10. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
11. Choi HJ, Lee H, Kim H, Kwon JE, Kang HJ, You KT, et al. MicroRNA expression profile of gastrointestinal stromal tumors is distinguished by 14q loss and anatomic site. Int J Cancer 2010;126:1640–50.
12. Haller F, von Heydebreck A, Zhang JD, Gunawan B, Langer C, Ramadori G, et al. Localization- and mutation-dependent microRNA (miRNA) expression signatures in gastrointestinal stromal tumours (GISTs), with a cluster of co-expressed miRNAs located at 14q32.31. J Pathol 2010;220:71–86.
13. Maruyama R, Choudhury S, Kowalczyk A, Bessarabova M, Beresford-Smith B, Conway T, et al. Epigenetic regulation of cell type-specific expression patterns in the human mammary epithelium. PLoS Genet 2011;7:e1001369.
14. Suzuki H, Takatsuka S, Akashi H, Yamamoto E, Nojima M, Maruyama R, et al. Genome-wide profiling of chromatin signatures reveals epigenetic regulation of microRNA genes in colorectal cancer. Cancer Res 2011;71:5646–58.

15. Taguchi T, Sonobe H, Toyonaga S, Yamasaki I, Shuin T, Takano A, et al. Conventional and molecular cytogenetic characterization of a new human cell line, GIST-T1, established from gastrointestinal stromal tumor. Lab Invest 2002;82:663–5.
16. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004;101:2999–3004.
17. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010;464:1071–6.
18. Luthra R, Singh RR, Luthra MG, Li YX, Hannah C, Romans AM, et al. MicroRNA-196a targets annexin A1: a microRNA-mediated mechanism of annexin A1 downregulation in cancers. Oncogene 2008;27: 6667–78.
19. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science 2004;304:594–6.
20. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 2007;129:1311–23.
21. Rink L, Skorobogatko Y, Kossenkov AV, Belinsky MG, Pajak T, Heinrich MC, et al. Gene expression signatures and response to imatinib mesylate in gastrointestinal stromal tumor. Mol Cancer Ther 2009;8: 2172–82.
22. Maru DM, Singh RR, Hannah C, Albarracin CT, Li YX, Abraham R, et al. MicroRNA-196a is a potential marker of progression during Barrett's metaplasia-dysplasia-invasive adenocarcinoma sequence in esophagus. Am J Pathol 2009;174:1940–8.
23. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007;297:1901–8.
24. Guan Y, Mizoguchi M, Yoshimoto K, Hata N, Shono T, Suzuki SO, et al. MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance. Clin Cancer Res 2010;16: 4289–97.
25. Mueller DW, Bosserhoff AK. MicroRNA miR-196a controls melanoma-associated genes by regulating HOX-C8 expression. Int J Cancer 2011;129:1064–74.
26. Li Y, Zhang M, Chen H, Dong Z, Ganapathy V, Thangaraju M, et al. Ratio of miR-196s to HOXC8 messenger RNA correlates with breast cancer cell migration and metastasis. Cancer Res 2010;70:7894–904.
27. Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. Nat Rev Cancer 2010;10:361–71.
28. Waltregny D, Alami Y, Clausse N, de Leval J, Castronovo V. Overexpression of the homeobox gene HOXC8 in human prostate cancer correlates with loss of tumor differentiation. Prostate 2002; 50:162–9.
29. Miller GJ, Miller HL, van Bokhoven A, Lambert JR, Werahera PN, Schirripa O, et al. Aberrant HOXC expression accompanies the malignant phenotype in human prostate. Cancer Res 2003;63: 5879–88.
30. Kikugawa T, Kinugasa Y, Shiraishi K, Nanba D, Nakashiro K, Tanji N, et al. PLZF regulates Pbx1 transcription and Pbx1-HoxC8 complex leads to androgen-independent prostate cancer proliferation. Prostate 2006;66:1092–9.
31. Alami Y, Castronovo V, Belotti D, Flagiello D, Clausse N. HOXC5 and HOXC8 expression are selectively turned on in human cervical cancer cells compared to normal kèratinocytes. Biochem Biophys Res Commun 1999;257:738–45.
32. Zhai Y, Kuick R, Nan B, Ota I, Weiss SJ, Trimble CL, et al. Gene expression analysis of preinvasive and invasive cervical squamous cell carcinomas identifies HOXC10 as a key mediator of invasion. Cancer Res 2007;67:10163–72.
33. Braig S, Mueller DW, Rothhammer T, Bosserhoff AK. MicroRNA miR-196a is a central regulator of HOX-B7 and BMP4 expression in malignant melanoma. Cell Mol Life Sci 2010;67:3535–48.
34. Mueller DW, Bosserhoff AK. MicroRNA miR-196a controls melanoma-associated genes by regulating HOX-C8 expression. Int J Cancer 2011;129:1064–74.
35. Mansfield JH, Harfe BD, Nissen R, Obenauer J, Srineel J, Chaudhuri A, et al. MicroRNA-responsive `sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. Nat Genet 2004;36:1079–83.
36. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, et al. Long noncoding RNA as modular scaffold of histone modification complexes. Science 2010;329:689–93.
37. Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F, et al. Overexpression of long non-coding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann Surg Oncol 2011;18:1243–50.
38. Okada Y, Feng Q, Lin Y, Jiang Q, Li Y, Coffield VM, et al. hDOT1L links histone methylation to leukemogenesis. Cell 2005;121:167–78.
39. Krivtsov AV, Feng Z, Lemieux ME, Faber J, Vempati S, Sinha AU, et al. H3K79 methylation profiles define murine and human MLL-AF4 leukemias. Cancer Cell 2008;14:355–68.
40. Popovic R, Riesbeck LE, Velu CS, Chaubey A, Zhang J, Achille NJ, et al. Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization. Blood 2009;113:3314–22.
41. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T, et al. Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Invest 2009;89:597–606.

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原　著

炎症性皮膚疾患と鑑別を要する皮膚T細胞リンパ腫の免疫組織化学および分子生物学的解析

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要約：原発性皮膚リンパ腫は，全体の約85%をT/NK細胞が占め，そのうち半数以上が菌状息肉症（mycosis fungoides; MF）とSezary症候群である．近年では，臨床像および細胞の形態に加え，細胞表面形質によって機能的に分類し，さらにクロナリティー解析を行うことにより以前では診断に至らなかったリンパ腫も診断することが可能になっている．しかし多彩な臨床像を呈し長期的な経過をとる症例が多く，初回生検時には炎症性変化であっても10数年の経過を経てリンパ腫へと移行するものもあり，現在のところそれらを事前に予測する手段は確立されていない．今回われわれは，皮膚T細胞リンパ腫と臨床的に鑑別を要する炎症性皮膚疾患として，局面状類乾癬（parapsoriasis en plaques; PP）を中心に免疫組織化学的にリンパ腫への移行を予測しえるか比較検討した．1993～2011年に昭和大学病院で臨床・病理学的にPPと診断された18例，MFと診断された8例（28検体），炎症性皮膚疾患として扁平苔癬（lichen planus; LP）14例を対象とし免疫組織化学的染色を施行，PPとMFについてはさらにクロナリティー解析を行い検討した．CD4/CD8間の解離はPP，MFではそれぞれ33%，88%に認めたが，LPでは全例で認められなかった．またCD7の減弱についても，PP，MFでその傾向が強かった．CCR3は，PP，MF，LPの全例で陰性だったが，CCR4，CXCR3はLPに比してPP，MFで陽性例が多かった．T細胞性クロナリティー解析では，PPは全例陰性であったが，MFは50%でモノクローナルな増殖を認めた．以上より，PPはLPに比してよりMFに近い表面形質の発現が認められ，炎症性皮膚疾患に加え腫瘍性変化の側面も持つことが示唆された．また経時的に生検されたMF症例の病勢については，クロナリティー解析は補助診断として有効なことが確認された．

キーワード：原発性皮膚リンパ腫，局面状類乾癬，扁平苔癬，免疫組織化学的染色，ケモカイン

原発性皮膚リンパ腫とは，確定診断時に皮膚以外の臓器に腫瘍細胞を認めないものと定義され，節外性リンパ腫の中では消化管，鼻咽腔について多いとされる[1]．悪性リンパ腫全体ではB細胞腫瘍が多いが，原発性皮膚リンパ腫では，全体の約85%をT/NK細胞が占め，そのうち半数以上が菌状息肉症（mycosis fungoides; MF）とSezary症候群である[2]．MFは紅斑期として発症し数年～数十年以上の経過の後に扁平浸潤期となり，さらに腫瘤期へと進行する．紅斑期の期間が長く緩徐な進行のため一般的には低悪性度な疾患とされるが，末期には皮膚外浸潤や形質転換を生じ悪性度の高いリンパ腫となるため，より早期での確実な診断が求められる．近年では，臨床像・細胞の形態に加え表面形質によって機能的に分類し，クロナリティー解析を行うことにより以前では診断に至らなかったものも診断することが可能になっているが，早期のMFに関しては診断がつかない症例も少なくない．

一方，類乾癬（parapsoriasis）とは乾癬に類似した角化性紅斑が慢性に経過する疾患の総称であり，