Clonal analysis of *NRAS* activating mutations in *KIT-D816V* systemic mastocytosis

Todd M. Wilson,[1] Irina Maric,[2] Olga Simakova,[2] Yun Bai,[1] Eunice Ching Chan,[1] Nicolas Olivares,[2] Melody Carter,[1] Dragan Maric,[3] Jamie Robyn,[4] and Dean D. Metcalfe[1]

[1]Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases; [2]Department of Laboratory Medicine, Clinical Center; [3]Flow Cytometry Core Facility, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA; and [4]Department of Veterans Affairs, Springfield, Ohio, USA

ABSTRACT

Cooperating genetic events are likely to contribute to the phenotypic diversity of *KIT-D816V* systemic mastocytosis. In this study, 44 patients with *KIT-D816V* systemic mastocytosis were evaluated for coexisting *NRAS*, *KRAS*, *HRAS* or *MRAS* mutations. Activating *NRAS* mutations were identified in 2 of 8 patients with advanced disease. *NRAS* mutations were not found in patients with indolent systemic mastocytosis. To better understand the clonal evolution of mastocytosis, we evaluated the cell compartments impacted by the *NRAS* and *KIT* mutations. Clonal mast cells harbored both mutations. *KIT-D816V* was not detected in bone marrow CD34$^+$ progenitors, whereas the *NRAS* mutation was present. These findings suggest that *NRAS* mutations may have the potential to precede *KIT-D816V* in clonal development. Unlike other mature lineages, mast cell survival is dependent on *KIT* and the presence of these two activating mutations may have a greater impact on the expansion of this cell compartment and in resultant disease severity. *(Clinicaltrials.gov identifier: NCT00044122, NCT00001756)*

Key words: systemic mastocytosis, activating mutations, NRAS KIT.

Citation: Wilson TM, Maric I, Simakova O, Bai Y, Ching Chan E, Olivares N, Carter M, Maric D, Robyn J, Metcalfe DD. Clonal analysis of NRAS *activating mutations in* KIT-D816V *systemic mastocytosis. Haematologica 2011;96(03):459-463. doi:10.3324/haematol.2010.031690*

Introduction

Systemic mastocytosis (SM) is a heterogeneous disorder characterized by the pathological accumulation of mast cells within tissues. The majority of adult patients are classified with indolent systemic mastocytosis (ISM) which generally carries a low risk of transformation to an aggressive state and is not thought to affect lifespan. However, a subset of patients with aggressive systemic mastocytosis (ASM) have a poor prognosis and will require cytoreductive therapy. Somatic activating mutations in c-kit, most notably *KIT-D816V*, are detected in the majority of adult patients.[1] Although multilineage involvement by *KIT-D816V* clearly has an impact on disease severity and progression,[2] additional unidentified genetic abnormalities are likely to contribute to more advanced forms of the disease.

RAS proteins are small membrane associated GTPases that play a pivotal role in signal transduction events regulating cell proliferation, differentiation and survival. Somatic mutations which disrupt this intrinsic GTPase activity and lock RAS in an active GTP-bound state are frequent among myeloid malignancies, predominantly involving *KRAS* and *NRAS*. In murine models, oncogenic *NRAS* has not only produced chronic myelogenous leukemia and acute myelogenous leukemia-like diseases, but also increased mast cells in the blood, bone marrow, liver and spleen, a phenotype consistent with aggressive systemic mastocytosis.[3-4]

In this study, we demonstrate that *RAS* gene expression increases with mast cell maturation and that activating mutations, specifically in *NRAS*, are found exclusively in advanced forms of systemic mastocytosis and may precede the *KIT-D816V* mutation in clonal development.

Design and Methods

Patients

Forty-four patients with systemic mastocytosis were evaluated at the National Institutes of Health (NIH, Bethesda, MD, USA) between 2006 and 2009 as part of an Institutional Review Board-approved research protocol designed to study the pathogenesis and natural history of systemic mastocytosis (NCT00044122). This included 27 patients with indolent systemic mastocytosis (ISM), 9 patients with smoldering systemic mastocytosis (SSM), 4 patients with systemic mastocytosis with an associated clonal hematologic non-mast cell lineage disease (SM-AHNMD) and 4 patients with aggressive systemic mastocytosis (ASM). All patients were diagnosed according to the World Health Organization (WHO) criteria[5] and carried the *KIT-D816V* mutation.

The online version of this article has a Supplementary Appendix.

Funding: this study was funded by the Intramural Research Program of the NIAID, NIH.
Acknowledgments: we wish to thank the patients for their participation, as well as Alasdair Gilfillan and Kimberly Dyer and the clinical research staff for their scientific and medical assistance.
Manuscript received on August 9, 2010. Revised version arrived on November 3, 2010. Manuscript accepted on November 30, 2010.
Correspondence: Todd M. Wilson, Building 10, Room 12S235A National Institutes of Health, Bethesda, MD 20892-1881 USA. Phone: international +301.4968891. Fax: international +301.480-384. E-mail: twilson@niaid.nih.gov

Sample processing

RNA/cDNA was prepared from bone marrow mononuclear cells and cell lines as described.[6] Buccal gDNA was isolated using the Gentra Puregene DNA Purification Kit (Qiagen) followed by amplification using a Qiagen REPLI-g Mini kit. HMC1, LAD2 and CD34$^+$ derived human mast cells (NCT00001756) were cultured as described.[7]

Immunophenotypic analysis of mast cells and flow cytometry cell sorting

Bone marrow mast cells were analyzed as described[6] using CD45 PerCP, CD117 APC and CD25 FITC (BD Biosciences) antibodies and FACSCanto II flow cytometer (BD Biosciences).

To obtain mast cells, CD34$^+$ cells, monocytes, granulocytes, eosinophils, B- and T-cell fractions, a CD45$^+$ enriched population (Whole Blood CD45 MicroBeads; Miltenyi Biotec) were stained using CD45 Tri Color, CD3 PE-TR, CD19 PE-TR (Invitrogen), CD14 FITC, CD49d PE, CD34 FITC (BD Biosciences), CD117 PE (Dako), DAPI and sorted using a FACSVantage SE flow cytometer (BD Biosciences). Sort purity routinely exceeded 98%.

Mutational analysis

The *KIT-D816V* mutation was detected by RT-PCR/RFLP as described.[6] Two round PCR followed by RFLP was used for flow sorted cells. *NRAS*, *HRAS*, *KRAS* and *MRAS* open reading frames were amplified from cDNA either directly or by nested PCR (flow-sorted cells). PCR products were gel purified and directly sequenced in both sense and antisense directions using BigDye terminator v3.1 chemistry and an ABI-3100 genetic analyzer according to standard protocols. Sequencing data were analyzed by Sequencher (Version 4.5, Softgenetics). Primers and conditions used for all PCR reactions are found in the *Online Supplementary Table S1*.

RAS real-time PCR

Real-time PCR was performed using RT^2 SYBR® Green qPCR Master Mixes (SABiosciences) and the ABI7500 real-time PCR system (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of each gene to GAPDH.

Results and Discussion

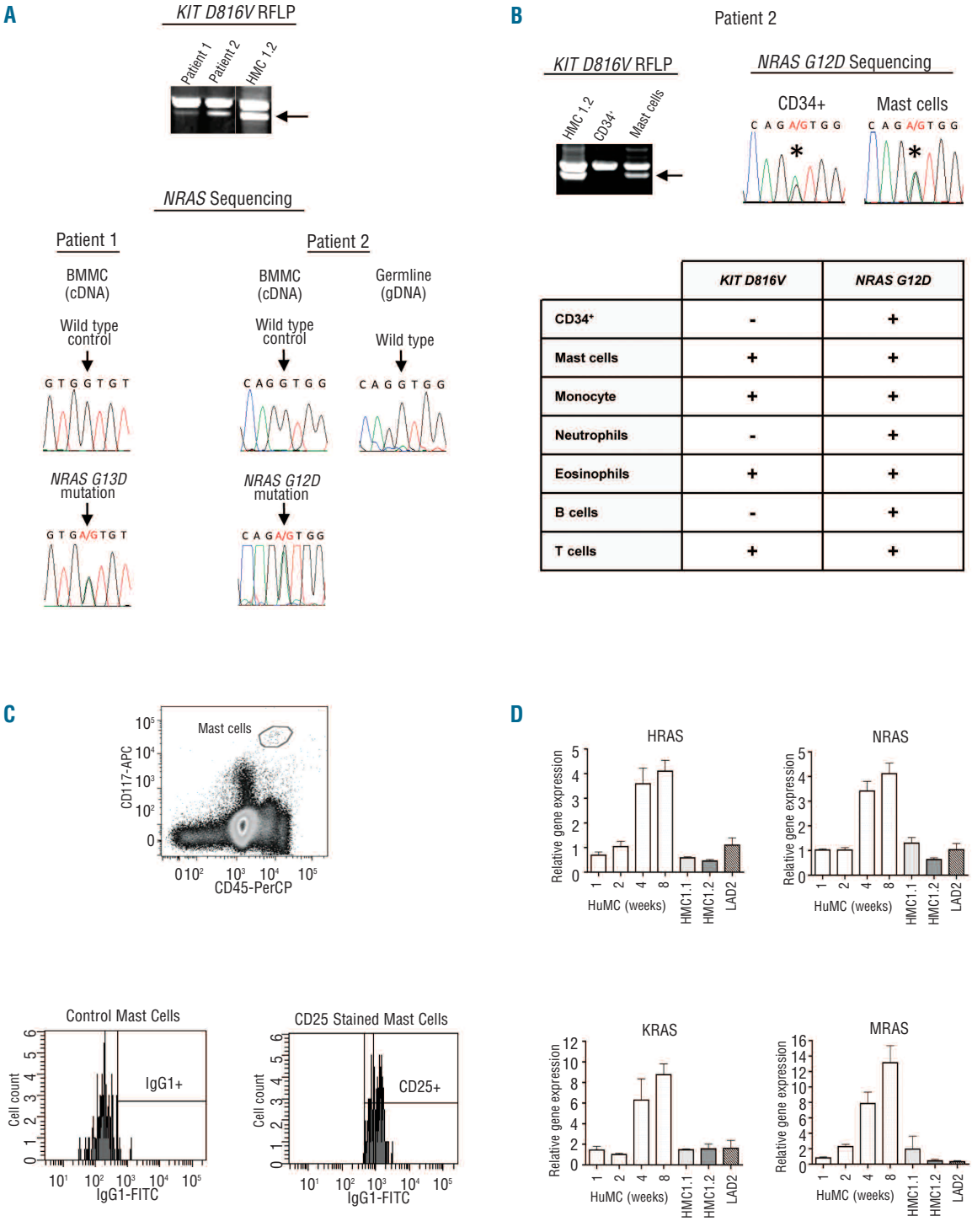
The phenotypic diversity displayed among myeloproliferative disorders is thought to be the result of multiple and complex molecular events. *KIT-D816V* SM shares this phenotypic heterogeneity and coexisting mutations are increasingly being identified. The *JAK2 V617F* mutation was detected in a rare subset of patients with *KIT D816V* systemic mastocytosis associated with chronic idiopathic myelofibrosis.[8] More recently, loss of function mutations in the putative tumor suppressor gene, *TET2*, were frequently found in patients with systemic mastocytosis although this did not appear to alter prognosis.[9] We now report coexisting *NRAS* activating mutations which potentially collaborate with *KIT-D816V* in disease pathogenesis.

Two of 44 patients (4.5%) harbored an *NRAS* activating mutation. *NRAS-G12D* and *NRAS-G13D* mutations were identified in one patient with SM-CMML and one patient with aggressive systemic mastocytosis, respectively (Figure 1A). Bone marrow histology supported these classifications and although a hypercellular marrow was observed in the patient with aggressive systemic mastocytosis, the overall findings did not meet 2008 WHO criteria for any myeloproliferative or myelodysplastic disorder (Figure 2). Together, 25% (2/8) of patients with advanced forms of systemic mastocytosis harbored activating *NRAS* mutations,

Table 1. Characteristics of the study population with advanced forms of *KIT-D816V* systemic mastocytosis. Shading indicates patients with additional *NRAS* mutations.

Patient	#1	#2	#3	#4	#5	#6	#7	#8
Age	68	54	54	53	54	74	67	59
Gender	M	F	F	F	M	F	M	M
WHO Classification	SM-CMML	ASM	ASM	ASM	ASM	SM-CMML	SM-MDS	SM-ET
Bone Marrow Biopsy Mast Cell %	<5	70	80	80	40	50	25	<5
Aspirate Mast Cell %	<1	<1	<1	5	NA	6	4	<1
Peripheral Blood Mast Cell %	0.03	0.01	0.02	0.01	0.04	0.14	0.01	0
Serum Tryptase (ng/mL)	7.6	528	229	194	196	762	18	76
Splenomegaly	Y	Y	Y	Y	Y	Y	Y	N
Hepatomegaly	Y	N	Y	N	Y	Y	N	N
Pathological Fractures	N	N	N	Y	N	N	N	N
Weight Loss	N	Y	Y	N	N	N	N	N
WBC (K/uL)	12.8	5.1	3.8	17.5	19	10.2	1.3	5.6
Hemoglobin (g/dL)	10.5	9.3	9.2	13.8	8.1	10.5	9.3	13.5
Platelets (K/uL)	40	127	25	439	133	283	21	584
Neutrophil (K/uL)	6.4	3.6	1.6	7.3	10.5	1.4	0.6	3.8
Lymphocyte (K/uL)	0.6	0.9	1.8	8.2	2.3	2.5	0.5	1.3
Monocyte (K/uL)	4.6	0.4	0.2	1.7	0.8	6.3	0.2	0.3
Eosinophil (K/uL)	0	0.2	0.1	0.3	5.5	0	0	0.2

SM-CMML: systemic mastocytosis with chronic myelomonocytic leukemia; SM-MDS: systemic mastocytosis with myelodysplastic syndrome; SM-ET: systemic mastocytosis with essential thrombocytosis; ASM: aggressive systemic mastocytosis; NA: not available.



	KIT D816V	NRAS G12D
CD34+	-	+
Mast cells	+	+
Monocyte	+	+
Neutrophils	-	+
Eosinophils	+	+
B cells	-	+
T cells	+	+

Figure 1. (A) Activating *NRAS* mutations in 2 patients with severe forms of *KIT-D816V* systemic mastocytosis. *KIT-D816V* RT-PCR/RFLP: Detection of *KIT-D816V* (arrow) in the bone marrow mononuclear cells (BMMC) of the 2 patients. HMC1.2 cells were used as positive control. *NRAS* sequencing: Patient 1: Compared to control, a heterozygous missense mutation GGT→GAT at codon 13 was identified resulting in a Glycine to Aspartic Acid substitution (*NRAS-G13D*). Patient 2: A similar heterozygous missense mutation GGT→GAT at codon 12 was identified resulting in a Glycine to Aspartic Acid substitution (*NRAS G12D*). A wild-type sequence in the buccal germline DNA confirmed a somatic event. (B) Patient 2: Segregation of *KIT-D816V* and *NRAS-G12D* in flow sorted bone marrow cell populations. *KIT-D816V* is present in mast cells (arrow), but absent in the CD34$^+$ cells. *NRAS-G12D* is present in both populations (asterisks). Results are representative of three separate flow sorting experiments and summarized in Table 1. (C) Mast Cell Immunophenotyping. Bone marrow mast cells were initially identified as CD117 bright positive, CD45 positive cells with characteristic forward and side scatter properties (circled). Compared to the isotype control, gated mast cells uniformly expressed the aberrant CD25 marker. (D) *RAS* gene expression in cultured human mast cells. *HRAS*, *NRAS*, *KRAS* and *MRAS* gene expression standardized to *GAPDH* is plotted on the Y axis. Mast cells on the X axis include CD34$^+$ derived human mast cells (HuMC) harvested at weeks one, 2, 4 and 8; HMC1.1, HMC1.2 and LAD2 mast cell lines. Error bars are the Standard Error Mean (SEM) of three separate experiments each performed in triplicate. Different CD34$^+$ donors were used for each HuMC experiment. *Note: *KIT-D816V* RT-PCR/RFLP gel lanes are cropped from the same gel without enhancement.

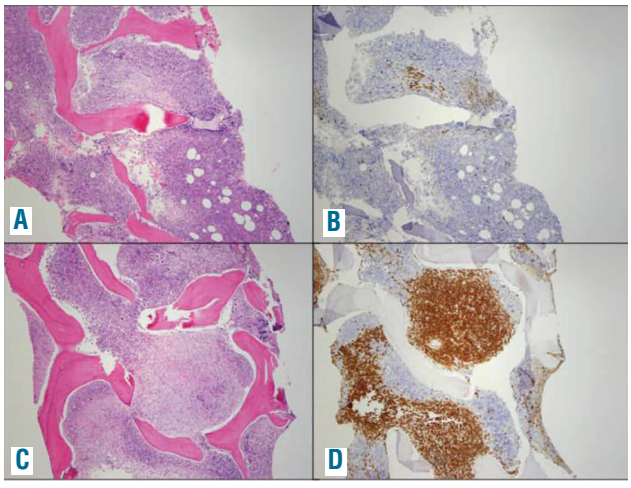


Figure 2. Histopathological changes in bone marrow biopsies from 2 *NRAS* positive patients. Both patients have hypercellular marrow biopsies, but with markedly different mast cell burden, as assessed by % mast cell involvement of the biopsy sections. Representative H&E (A) and CD117 (B) immunostained sections show that Patient 1 has minimal involvement by mast cell aggregates, while Patient 2 has extensive marrow replacement by mast cells [(C) H&E; (D) CD117]. In addition, Patient 1 has myeloid hyperplasia with increase in monocytic precursors and blasts, while Patient 2 has normal trilineage maturation. (Magnification 40x for all photographs)

although no associated phenotype was observed within this subset (Table 1). These findings parallel observations made in other myeloproliferative disorders such as acute myelogenous leukemia, where *RAS* mutation frequency does not vary with gender, age, leukocytosis, or WHO performance status.[10] Of similar importance is the absence of *NRAS* mutations in 36 patients with indolent systemic mastocytosis (n=27) or smoldering systemic mastocytosis (n=9). This observation supports the current premise that more benign forms of mastocytosis are mainly *KIT-D816V* driven and additional mutations may be required for more severe forms of the disease. Indeed, *NRAS* mutations associated with progression from myelodysplastic syndromes to acute myelogenous leukemia are described.[11-12]

Efforts to molecularly dissect the cell compartments impacted by these two mutations in aggressive systemic mastocytosis (Patient 2) revealed that bone marrow mast cells harbored both mutations and uniformly expressed the aberrant CD25 marker, indicating a clonal population (Figure 1B and C). *KIT-D816V* and *NRAS-G12D* were also detected in both myeloid and lymphoid lineages (Figure 1B). This is consistent with previous observations that aggressive forms of mastocytosis display multilineage involvement, likely the result of a common progenitor.[2, 13] However, detection of *KIT-D816V* in CD34$^+$ bone marrow progenitors has varied between studies. Akin *et al.* did not detect *KIT-D816V* in the CD34$^+$ cells of 3 of 3 patients with systemic mastocytosis displaying multilineage involvement.[13] In contrast, Garcia-Montero *et al*. observed that 3 of 4 of patients with aggressive systemic mastocytosis harbored *KIT-D816V* in CD34$^+$ progenitors.[2] In our study we detected only *NRAS-G12D* in the CD34$^+$ progenitors, despite the *KIT-D816V* RT-PCR/RFLP assay having greater sensitivity than cDNA sequencing. This observation suggests that *NRAS-G12D* may have preceded *KIT-D816V* in clonal development. According to the clonal expansion model, early mutations should be more prevalent in the clonal population than late mutations.[14] Consistent with this model, *NRAS-G12D* penetrated more cell populations than *KIT-D816V* (Figure 1B).

Cooperating Class I (enhanced proliferation and/or survival) and Class II (impaired differentiation) mutations are thought necessary for leukemogenesis.[15] *NRAS* and *KIT* mutations both represent Class I mutations. Although it is likely that an unidentified Class II mutation may exist, this "exception" has been observed in other studies.[16-17] A model utilizing information from the Cancer Genome Atlas recently predicted that *NRAS* activating mutations would coexist with *KIT* mutations in hematopoietic malignancies and were, therefore, strong candidates for cosequencing.[14] Mutations in other *RAS* genes were not predicted to coexist with *KIT* mutations. In support of this prediction, we did not detect *KRAS*, *HRAS* or *MRAS* mutations in our *KIT-D816V* population. Arguably, this may be a reflection of the sampling size or alternatively *NRAS* may have a significant role in mast cell homeostasis.

The *RAS* gene family appears to play a role in mast cell development, as their relative expression levels uniformly increased as mast cells matured *in vitro* (Figure 1D). Peak *RAS* expression was observed at eight weeks, at which time mature mast cells were the only cell type present. NRAS and KRAS, but not HRAS are reported to be the dominant isoforms in LAD2 and HMC1 cell lines.[18] We observed that the message for all isoforms was detectable, including *MRAS*, albeit expression was relatively low and comparable to that of an immature mast cell. This may reflect the maturity state of the cell lines and/or the cell division rate. Cell lines and cultured CD34$^+$ cells at 1-2 weeks are rapidly dividing and may not require significant *RAS* expression, whereas the relatively quiescent mature mast cell may be more dependent. *RAS* mutations were not observed in the cell lines.

Clinical trials targeting KIT-D816V have demonstrated only modest efficacy in systemic mastocytosis.[19-20] This study contributes to growing evidence that additional genetic alterations are present in *KIT-D816V* SM and effective treatment will likely require a multi-targeted approach.[21-22] Specifically identifying *RAS* mutations may influence the choice and dosing of cytoreductive therapy, as acute myelogenous leukemia patients carrying activated forms of RAS appear to benefit from higher cytosine arabinoside doses in response rate and overall survival.[23-24] As advanced systemic mastocytosis is relatively rare, large multicenter studies will be required to support not only our findings, but future studies investigating the molecular pathogenesis of systemic mastocytosis.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc Natl Acad Sci. USA. 1995;92(23):10560-4.
2. Garcia-Montero AC, Jara-Acevedo M, Teodosio C, Sanchez ML, Nunez R, Prados A, et al. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. Blood. 2006;108(7):2366-72.
3. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS rapidly and efficiently induces CMML- and AML-like diseases in mice. Blood. 2006;108(7):2349-57.
4. Wiesner SM, Jones JM, Hasz DE, Largaespada DA. Repressible transgenic model of NRAS oncogene-driven mast cell disease in the mouse. Blood. 2005;106(3): 1054-62.
5. Horny H-P, Metcalfe DD, Bennett JM, Bain BJ, Akin C, Escribano L, Valent P. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, et al. World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press, Lyon 2008.
6. Maric I, Robyn J, Metcalfe DD, Fay MP, Carter M, Wilson T, et al. KIT D816V-associated systemic mastocytosis with eosinophilia and FIP1L1/PDGFRA-associated chronic eosinophilic leukemia are distinct entities. J Allergy Clin Immunol. 2007;120(3):680-7.
7. Kulka M, Fukuishi N, Metcalfe DD. Human mast cells synthesize and release angiogenin, a member of the ribonuclease A (RNase A) superfamily. J Leukoc Biol. 2009;86(5):1217-26.
8. Sotlar K, Bache A, Stellmacher F, Bultmann B, Valent P, Horny HP. Systemic mastocytosis associated with chronic idiopathic myelofibrosis: a distinct subtype of systemic mastocytosis associated with a [corrected] clonal hematological non-mast [corrected] cell lineage disorder carrying the activating point mutations KITD816V and JAK2V617F. J Mol Diagn. 2008;10(1): 58-66.
9. Tefferi A, Levine RL, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia. 2009;23(5):900-4.
10. Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. Leukemia. 2008;22(5):915-31.
11. Shih LY, Huang CF, Wang PN, Wu JH, Lin TL, Dunn P, et al. Acquisition of FLT3 or N-ras mutations is frequently associated with progression of myelodysplastic syndrome to acute myeloid leukemia. Leukemia. 2004;18(3):466-75.
12. Christiansen DH, Andersen MK, Desta F, Pedersen-Bjergaard J. Mutations of genes in the receptor tyrosine kinase (RTK)/RAS-BRAF signal transduction pathway in therapy-related myelodysplasia and acute myeloid leukemia. Leukemia. 2005;19(12): 2232-40.
13. Akin C, Kirshenbaum AS, Semere T, Worobec AS, Scott LM, Metcalfe DD. Analysis of the surface expression of c-kit and occurrence of the c-kit Asp816Val activating mutation in T cells, B cells, and myelomonocytic cells in patients with mastocytosis. Exp Hematol. 2000;28(2):140-7.
14. Yeang CH, McCormick F, Levine A. Combinatorial patterns of somatic gene mutations in cancer. Faseb J. 2008;22(8): 2605-22.
15. Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. Nat Rev Cancer. 2002;2(7):502-13.
16. Shih LY, Liang DC, Huang CF, Chang YT, Lai CL, Lin TH, et al. Cooperating mutations of receptor tyrosine kinases and Ras genes in childhood core-binding factor acute myeloid leukemia and a comparative analysis on paired diagnosis and relapse samples. Leukemia. 2008;22(2):303-7.
17. Ishikawa Y, Kiyoi H, Tsujimura A, Miyawaki S, Miyazaki Y, Kuriyama K, et al. Comprehensive analysis of cooperative gene mutations between class I and class II in de novo acute myeloid leukemia. Eur J Haematol. 2009;83(2):90-8.
18. Shefler I, Mekori YA, Mor A. Stimulation of human mast cells by activated T cells leads to N-Ras activation through Ras guanine nucleotide releasing protein 1. J Allergy Clin Immunol. 2008;122(6):1222-5.
19. Verstovsek S, Tefferi A, Cortes J, O'Brien S, Garcia-Manero G, Pardanani A, et al. Phase II study of dasatinib in Philadelphia chromosome-negative acute and chronic myeloid diseases, including systemic mastocytosis. Clin Cancer Res. 2008;14(12): 3906-15.
20. Gotlib J, George TI, Corless C, Linder A, Ruddell A, Akin C, et al. The KIT tyrosine kinase inhibitor midostaurine (PKC412) exhibits a high response rate in aggressive systemic mastocytosis (ASM): interim results of a Phase II trial. Blood. 2007;110: 1035a.
21. Aichberger KJ, Sperr WR, Gleixner KV, Kretschmer A, Valent P. Treatment responses to cladribine and dasatinib in rapidly progressing aggressive mastocytosis. Eur J Clin Invest. 2008;38(11):869-73.
22. Ustun C, Corless CL, Savage N, Fiskus W, Manaloor E, Heinrich MC, et al. Chemotherapy and dasatinib induce long-term hematologic and molecular remission in systemic mastocytosis with acute myeloid leukemia with KIT D816V. Leuk Res. 2009;33(5):735-41.
23. Neubauer A, Maharry K, Mrozek K, Thiede C, Marcucci G, Paschka P, et al. Patients with acute myeloid leukemia and RAS mutations benefit most from postremission high-dose cytarabine: a Cancer and Leukemia Group B study. J Clin Oncol. 2008;26(28):4603-9.
24. Illmer T, Thiede C, Fredersdorf A, Stadler S, Neubauer A, Ehninger G, et al. Activation of the RAS pathway is predictive for a chemosensitive phenotype of acute myelogenous leukemia blasts. Clin Cancer Res. 2005;11(9):3217-24.