Review

Molecular Diagnosis of Mast Cell Disorders

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Mastocytosis is a disease characterized by pathological mast cell accumulation and activation in tissues. Most patients with mastocytosis exhibit the D816V point mutation in the tyrosine kinase domain of the transmembrane receptor protein Kit, leading to its constitutive activation in bone marrow or lesional skin tissue. Detection of a codon 816 *c-kit* mutation is included as a minor diagnostic criterion in the World Health Organization's diagnostic criteria for systemic mastocytosis. Determining mutational status of the *c-kit* gene also has pharmacogenomic implications in patients considered for investigational mast cell cytoreductive therapies. This article reviews diagnostic and therapeutic implications of *c-kit* mutations as well as other less common molecular abnormalities observed in mast cell disease. (*J Mol Diagn* 2006, 8:412–419; DOI: 10.2353/jmoldx.2006.060022)

Mastocytosis is a disorder resulting from pathological accumulation and activation of mast cells in tissues such as skin, bone marrow, liver, spleen, and lymph nodes.¹ The clinical spectrum of mastocytosis is variable and may range from a self-limited disease confined to skin in children to progressive and life-threatening variants associated with poor prognosis (Table 1). The symptoms observed in mastocytosis are attributable to the spontaneous or triggered release of mast cell mediators or the consequences of the clonal expansion of the hematopoietic clone giving rise to mast cells.²

Mastocytosis: When to Suspect

The most common clinical scenarios leading to diagnosis of mastocytosis are as follows. 1) A child or an adult

patient presents with a cutaneous rash diagnosed as urticaria pigmentosa, the characteristic skin finding of mastocytosis. 2) A patient exhibits an unexplained abnormality in complete blood counts, hepatosplenomegaly, or lymphadenopathy leading to bone marrow biopsy. 3) A patient presents with recurrent mast cell degranulation symptoms such as flushing and anaphylaxis. 4) A patient is discovered to have diffuse bone remodeling or pathological fractures, incidentally or after diagnostic workup of bone pain.

The major physical examination finding suggesting the diagnosis of mastocytosis is the pathognomonic skin lesion of urticaria pigmentosa, a rash consisting of individual hyperpigmented and telangiectatic papules measuring up to a few centimeters in diameter, diffusely involving trunk and extremities with a tendency to spare sun-exposed areas such as face and hands.³ These lesions may urticate after physical stimulation such as rubbing (Darier's sign) or exposure to heat. Almost all adult patients with urticaria pigmentosa and some children particularly with late onset of skin lesions (after 2 years of age) have pathological mast cell collections in bone marrow and internal organs. These patients are termed to have systemic mastocytosis.⁴ Lesions of urticaria pigmentosa, however, may be absent in up to 20% of patients with systemic mastocytosis and especially in those with more advanced and aggressive categories of disease. In these patients, when the index of suspicion for systemic disease is high, bone marrow biopsy and aspirate are recommended to check for presence of The

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Table 1. WHO Classification of Mastocytosis, Prognosis, and the Age Group Most Commonly Affected⁸

Category	Prognosis	Age group
Cutaneous mastocytosis	Good	Children
Indolent systemic mastocytosis	Good	Adults
Systemic mastocytosis with associated clonal, hematological nonmast cell lineage disease	Poor	Adults
Aggressive systemic mastocytosis	Poor	Adults
Mast cell leukemia	Poor	Adults
Mast cell sarcoma	Poor	Adults
Extracutaneous mastocytoma	Good	All

World Health Organization's diagnostic criteria for systemic mastocytosis.

Approximately 20% of patients with systemic mastocytosis are diagnosed after a bone marrow biopsy prompted by an abnormal blood count such as an elevated or decreased white blood cell or platelet count, abnormal differential count, or unexplained anemia. In these patients, the bone marrow biopsy reveals diagnostic mast cell collections associated with another hematological disorder causing the abnormal blood count. Such disorders generally arise from the myeloid compartment and commonly include myeloproliferative disorders and myelodysplastic syndromes, but acute leukemias, myelomas, lymphomas, and other lymphoproliferative disorders may also be diagnosed.⁵

Bone mineralization abnormality is the initial presenting symptom leading to diagnosis in a small percentage of patients, although pathological changes in bones can be found in all categories of mastocytosis.⁶ Examples of this clinical presentation range from incidental findings of sclerosis and osteoporosis in computed tomography scans obtained for other medical reasons to persistent bone pain associated with imaging abnormalities or pathological fractures.

Mastocytosis is considered in the differential diagnosis of patients with unexplained symptoms attributable to mast cell mediator release.⁷ These symptoms may variably include recurrent episodic flushing, anaphylaxis, lightheadedness, syncope, near syncope, tachycardia, gastrointestinal cramping, nausea, vomiting, and diarrhea. On the other hand, recurrent hives, angioedema, and wheezing are curiously not very common in mastocytosis. Confirming the diagnosis in these patients may be challenging, especially in the absence of skin lesions, because none of these symptoms is specific for mast cell disease. An important clue in patient history is the occurrence of one or more of these symptoms in self-limited episodes lasting from a few minutes up to several hours.

Diagnostic Criteria for Systemic Mastocytosis

Diagnosis of mastocytosis should be confirmed by a tissue biopsy.⁸ In children with skin involvement, the tissue of choice for biopsy is the skin. A bone marrow biopsy is recommended to exclude or confirm systemic mastocytosis in adult patients with skin involvement, in patients without skin involvement if the suspicion for systemic disease is high based on clinical presentation as discussed above, and in children with urticaria pigmentosa only if they have an unexplained abnormality in peripheral blood counts, hepatosplenomegaly, or lymphadenopathy. The age of onset of skin lesions is an important determinant of systemic disease. Most children with urticaria pigmentosa who experience the onset of lesions before age 2 have the skin-limited disease, which resolves or improves as the child reaches adolescence.^{9,10} In contrast, most patients with adult-onset disease have bone marrow involvement at the time of diagnosis and follow a persistent or progressive course.¹¹

The World Health Organization's diagnostic criteria for systemic mastocytosis are shown in Table 2.⁸ These include one major and four minor criteria. Demonstration of the major and one minor or three minor criteria is needed before a diagnosis of systemic mastocytosis is confirmed. Bone marrow is the preferred tissue source to evaluate the presence of World Health Organization criteria. The major criterion, ie, multifocal mast cell collections of 15 or more cells, is present in more than half of patients with systemic mastocytosis (Figure 1). These patients usually display various mast cell morphological aberrations in biopsy sections stained for tryptase and aspirate smears (eg, spindling and hypogranulation) (Figure 2)¹² and have elevated serum tryptase levels¹³ and therefore readily satisfy the World Health Organization diagnostic criteria.

The major criterion, however, may be absent or not detectable if the biopsy sample is small or of suboptimal quality or in patients with limited systemic disease who

Table 2. WHO Diagnostic Criteria for Systemic Mastocytosis

Major
Multifocal dense infiltrates of 15 or more mast cells in bone marrow and/or other extracutaneous organ sections
Minor
25% of mast cells in tissue sections or bone marrow aspirate smear are spindle shaped or have atypical morphology
<i>C-kit</i> point mutation at codon 816
Expression of CD2 and/or CD25 by mast cells
Baseline serum tryptase persistently >20 ng/ml (not valid in presence of another nonmast cell clonal disorder)

Major plus one minor or three minor criteria are needed to diagnose systemic mastocytosis.⁸

Figure 1. Multifocal clusters of mast cells in a bone marrow biopsy visualized by immunohistochemical staining for tryptase satisfy the World Health Organization major diagnostic criterion in this patient with indolent systemic mastocytosis.

Figure 2. Mast cells in bone marrow aspirate smears show aberrant morphological features such as spindling and hypogranulation (**A**), as compared to normal mast cells with a round and centrally located nucleus and dense cytoplasmic granulation (**B**).

usually do not display marked elevations in their serum tryptase levels. In these patients, it becomes critical to assess the presence of minor criteria. Immunohistochemical staining of the biopsy section for tryptase and Wright-Giemsa staining of the aspirate smear are routinely performed in diagnostic hematology laboratories and provide information about the presence of the first minor criterion.^{12,14} Likewise, total baseline tryptase level measurements are available through many commercial laboratories in the United States.

Aberrant expression of CD25 and CD2 on mast cells is a sensitive marker of systemic mast cell disease.^{15,16} CD25 expression is generally stronger than that of CD2 and can be detected by flow cytometry or immunohistochemistry. Flow cytometric analysis of the mast cells requires special gating strategies because mast cells are generally present in small numbers in bone marrow (less than 0.1% of nucleated cells in many cases). Excellent methodological guidelines for bone marrow mast cell flow cytometry have been published.¹⁷

C-kit Mutations in Mastocytosis

C-kit is a proto-oncogene that encodes a transmembrane receptor (Kit) for the cytokine stem cell factor.¹⁸ Binding of stem cell factor homodimers to the extracellular domains of Kit induces crosslinking of the receptor molecules, activating the intrinsic tyrosine kinase activity of its intracellular portion. Cross-phosphorylation of tyrosine residues on Kit by adjacent receptor enzymatic domains creates docking sites for SH2 domain-containing adaptor proteins and signal transduction molecules that mediate the biological effects of Kit.¹⁹ Activation of Kit is critical for a number of mast cell functions such as induction of differentiation from hematopoietic progenitors, survival, chemotaxis, and potentiation of IgE-mediated functional activation.²⁰ Therefore, structural alterations in functionally important domains of the molecule are likely to have profound effects in mast cell survival and function.

The most common molecular abnormality consistently detected in systemic mastocytosis is a somatic point mutation, A>T in nucleotide 2468 of *c-kit* cDNA, affecting codon 816 with resulting replacement of an aspartic acid by valine (D816V)²¹ (Figure 3). Codon 816 is a critical residue contributing to the structure of the activation loop of the tyrosine kinase enzymatic domain of Kit by forming a hydrogen bond with N819. Disruption of this bond by replacement of aspartic acid at codon 816 destabilizes the inactive conformation of the kinase domain and results in ligand-independent constitutive activation and autophosphorylation of Kit.^{22–24} The D816V *c-kit* mutation accounts for more than 90% of all mutations described in mastocytosis and has been reported in all categories of disease (Table 1). Replacement of D816 by another residue or mutations in extracellular, transmembrane, or juxtamembrane domains of the molecule have also been described (Figure 3). Some of these unusual *c-kit* mutations have been detected in a germline pattern, although

Figure 3. *C-kit* mutations in mastocytosis. EC, extracellular (ligand binding); TM, transmembrane; JM, juxtamembrane; TK, tyrosine kinase domain. The most common mutation, D816V, is shown boxed.

familial transmission of mastocytosis is rare and has been shown to occur in less than 5% of pediatric-onset disease in several cohorts.^{25–27}

Role of c-kit Mutational Analysis in Diagnosis of Mast Cell Disease

Detection of a codon 816 *c-kit* mutation is a minor diagnostic criterion of systemic mastocytosis.⁸ In our experience, D816V *c-kit* mutation is detectable in ~70% of patients with systemic mastocytosis in bone marrow mononuclear cells, although enrichment of lesional mast cells by cell sorting or microdissection significantly increases its detectability. According to the World Health Organization guidelines, demonstration of only one of four minor criteria is required for the diagnosis of systemic mast cell disease in the presence of the major criterion. In a patient with multifocal mast cell clusters consisting of 15 or more cells in the bone marrow biopsy (major criterion), morphological examination of the biopsy or aspirate smear for atypical spindle-shaped mast cells or a serum tryptase level greater than 20 ng/ml usually satisfies the World Health Organization diagnostic criteria. Therefore, in many patients, diagnosis of mastocytosis can be established by histopathology alone.

On the other hand, mutational analysis of the *c-kit* gene is generally required to establish the diagnosis in patients with low mast cell burden, particularly in those who have normal or mildly elevated serum tryptase levels and lack multifocal mast cell clusters in bone marrow biopsy. For example, most children and some adults with urticaria pigmentosa have tryptase levels less than 20 ng/ml. Although workup for systemic disease is rarely indicated for children, patients with adult-onset urticaria pigmentosa are routinely recommended to undergo evaluation for systemic disease. In addition, some patients are suspected to have systemic mastocytosis based on recurrent symptoms of episodic mast cell degranulation such as anaphylaxis, hypotension, flushing, and gastrointestinal cramping. These patients may have serum tryptase levels less than 20 ng/ml and may lack urticaria pigmentosa. Morphological examination of the bone marrow in

these patients with low tryptase levels may reveal small clusters of mast cells or spindle-shaped mast cells displaying tropism for blood vessels or bony trabeculae but not sufficiently large enough to meet the major criterion. Mutational analysis of the *c-kit* gene along with mast cell flow cytometry thus becomes essential in the diagnostic workup of such patients in demonstrating the presence of a pathological mast cell clone.

Sample Source and Methodological Considerations

As an acquired somatic mutation, detectability of D816V *c-kit* in mastocytosis is enhanced proportionally to the amount of lesional cells carrying the mutation in the sample to be analyzed. Because mast cells reside in tissues and there are no mast cells in peripheral circulation under normal circumstances, analysis of the peripheral blood for *c-kit* mutations has a low yield of positive results even when the mutation is detectable in lesional tissues such as bone marrow and skin.

Evidence from single-cell polymerase chain reaction (PCR) and cell-sorting experiments suggests codon 816 *c-kit* mutation affects mast cell hematopoietic progenitors at different levels of commitment. Patients with involvement of a multipotential hematopoietic progenitor carry the mutation not only in their mast cells but also in other peripheral blood lineages such as monocytes, neutrophils, basophils, and B cells.²⁸ These patients generally have higher serum tryptase levels and more extensive bone marrow involvement with mast cell disease (systemic smoldering mastocytosis),^{29,30} and some may have evidence of other nonmast cell hematological disorders such as myeloproliferative disorders or myelodysplastic syndromes. In contrast, in many patients with indolent systemic mastocytosis with mild to moderate bone marrow involvement, the mutation appears to be restricted to a mast cell-committed progenitor and is not detectable in other cell lineages or in peripheral blood.^{31,32} Therefore, analysis of the bone marrow aspirate cells or lesional skin biopsy tissue has a higher likelihood of yielding positive results than peripheral blood, especially in patients with limited indolent disease.

The probability of detecting the *c-kit* mutation increases even further if mast cells are enriched from lesional tissues by such techniques as microdissection or magnetic bead selection. In our laboratory, we were able to demonstrate the D816V *c-kit* mutation by reverse transcriptase (RT)-PCR followed by restriction fragment length polymorphism (RFLP) analysis in all patients with systemic mastocytosis when the bone marrow aspirate mast cells expressed CD25 and they were enriched based on expression of this marker (C. Akin, unpublished observation). In this case, RT-PCR of the *c-kit* gene in CD25-sorted cells allow preferential detection of transcripts from mast cells because other CD25⁺ populations, such as activated lymphocytes, do not express *c-kit* in significant amounts.

Figure 4. Demonstration of D816V *c-kit* mutation by RFLP (A) and capillary sequencing (B). **A:** *HinfI* digestion of the RT-PCR product from HMC1.2 cell line (lane 1, positive control) and patient samples in lanes 4 and 6 show the presence of an additional band (arrow) as a result of creating a new restriction site by A>T nucleotide change in *c-kit* codon 816. Lanes 2, 3, and 5 show wild-type pattern codon 816. **B:** Demonstration of the heterozygous A>T nucleotide change in cDNA position 2468 (corresponding to amino acid change D816V) by direct capillary sequencing (right), and its comparison to the wild-type sequence (left).

The sensitivity of mutation detection also depends on the technique used. D816V *c-kit* mutation has been detected by using several techniques such as PCR amplification of the mutated region of the gene followed by direct sequencing³³ or RFLP analysis,²¹ allele-specific PCR,³⁴ and peptide-nucleic acid-mediated PCR (PNA-mediated PCR) clamping with hybridization probes and single-cell PCR (Figure 4).³⁵ Among these techniques, direct sequencing is the least sensitive and therefore is not recommended as a routine screening method. RFLP analysis is widely used owing to its simplicity and low cost. The *HinfI* RFLP assay commonly reported in the literature is based on detection of the creation of a new restriction site by A>T change at cDNA nucleotide position 2468. Although this assay is reasonably sensitive, it is important to realize that it will miss rare mutations caused by other nucleotide changes not affecting the restriction enzyme recognition pattern of the amplified PCR fragment. Likewise, allele-specific PCR will fail to detect mutations other than what the PCR primer is designed to complement. Different techniques can be used sequentially when one fails to demonstrate the mutation. For example, a negative RT-PCR *HinfI* RFLP result in a bone marrow aspirate sample can be followed by direct se-

quencing to exclude the presence of other atypical *c-kit* mutations.

Analysis by RT-PCR of RNA extracted from lesional tissue (such as bone marrow mononuclear cells) is more sensitive than analysis of genomic DNA in downstream applications mentioned above. This is because mast cells express *c-kit* message at much higher quantities than other cells in the sample, resulting in enhanced representation of mutated nucleic acid in the sample to be analyzed. This advantage is lost when analyzing the DNA because each lesional and nonlesional cell contributes equal amounts of DNA. A consensus conference was recently held in Vienna to standardize diagnostic techniques including *c-kit* mutational analysis, and its recommendations are currently being prepared for publication (P. Valent, C. Akin, L. Escribano, M. Föding, K. Hartmann, K. Brockow, M. Castells, W.R. Sperr, H.C. Kluin-Nelemans, N. Hamdy, O. Lortholary, J. Robyn, J. van Doormaal, K. Sotlar, A.W. Hauswirth, M. Arock, O. Hermine, A. Hellmann, M. Triggiani, M. Niekisz, L.B. Schwartz, A. Orfao, H.-P. Horny, and D.D. Metcalfe, submitted).

Mutations and Chromosomal Rearrangements Involving Genes Other than c-kit in Mastocytosis

Mutations or rearrangements of genes involving genes other than *c-kit* are rare in systemic mastocytosis and have been generally reported in the context of an associated clonal hematological nonmast cell disorder, as discussed below. Chronic eosinophilic leukemia is an atypical myeloproliferative disorder caused by an interstitial deletion in chromosome 4q12 leading to the fusion gene FIP1L1-PDGFR α , which results in constitutive activation of the tyrosine kinase activity of the platelet-derived growth factor receptor α (PDGFR α).^{36,37} Similar to systemic smoldering mastocytosis, chronic eosinophilic leukemia is a multilineage disorder in which FIP1L1-PDGFR α fusion gene has been demonstrated in multiple lineages including mast cells.^{38,39} In addition to the prominent peripheral blood eosinophilia, patients with FIP1L1-PDGFR α -associated disease have a myeloproliferative bone marrow with eosinophilia, increased numbers of bone marrow mast cells with atypical spindle shapes, and elevated serum tryptase levels.⁴⁰ Some of these patients may also have multifocal mast cell clusters in bone marrow biopsies, meeting the diagnostic criteria for systemic mastocytosis.⁴¹ Such patients are diagnosed as having systemic mastocytosis with associated chronic eosinophilic leukemia. FIP1L1-PDGFR α fusion can be detected by RT-PCR using primers specific for FIP1L1 and PDGFR α genes⁴⁰ or by demonstrating the deletion of the CHIC2 locus between these genes by fluorescence *in situ* hybridization.⁴¹ Mutational analyses of the *c-kit* gene revealed a wild-type sequence in patients with FIP1L1-PDGFR α fusion.⁴⁰ It is recommended to check for FIP1L1-PDGFR α fusion gene in mastocytosis

patients who have persistent eosinophilia, especially because of the therapeutic implications of this molecular defect, which is highly sensitive to the tyrosine kinase inhibitor imatinib (see below).

BCR-ABL-positive chronic myeloid leukemia is rarely seen together with mastocytosis. Nevertheless, it is prudent to exclude the presence of BCR-ABL in patients with mastocytosis who have myeloproliferative bone marrow findings because of the diagnostic and therapeutic implications. Case reports describing detection of both BCR-ABL translocation and codon 816 *c-kit* mutation in patients with SM-CML have been published.^{42,43} The D816V *c-kit* mutation was detectable in mast cells microdissected from bone marrow but not in CD15⁺ myeloid cells, suggesting the presence of two clones in one patient.⁴³ JAK2 V617F mutation associated with other classic myeloproliferative diseases is not commonly observed in mastocytosis.

C-kit mutations have been reported in up to 46% of adult and 70% of pediatric patients with core-binding factor leukemias, associated with cytogenetic abnormalities t(8;21)(q22;q22) and inv(16)(p13q22) (AML M2 and M4).^{44,45} These mutations involve exon 8 (generally deletion of codon 419 corresponding to extracellular domain), juxtamembrane as well as codon 816 of *c-kit*. Presence of *c-kit* mutations in patients with t(8;21)(q22;q22) have been shown to be associated with poorer prognosis in adults.⁴⁴ Similar prognostic studies in children have reported conflicting results.^{45,46} A systematic evaluation of presence of co-existing mastocytosis according to World Health Organization criteria in patients with core-binding factor leukemias and codon 816 *c-kit* mutations have not been published; however, in one study, 2 of 33 patients with AML and codon 816 *c-kit* mutations were reported to have mastocytosis.⁴⁷

Pharmacogenomic Implications of c-kit Mutational Analysis in Treatment of Mastocytosis

Mutational analysis of the *c-kit* gene not only has diagnostic value but also has implications for mast cell cytoreductive therapy.¹⁸ Mast cell cytoreductive therapy is indicated for aggressive variants of disease associated with poor prognosis; however, such options are currently limited to interferon- α and 2-CDA, which fail to produce complete remission in most patients.⁴⁸ Tyrosine kinase inhibitor imatinib, now used as a first line therapy of CML, inhibits not only BCR-ABL but also Kit and PDGFR by binding to the inactive conformation of these kinases and stabilizing the molecules in their inactive state.⁴⁹ However, codon 816 *c-kit* mutation causes a structural change in the kinase domain of Kit by impairing maintenance of this inactive conformation and thus prevents the binding of imatinib to Kit.²³ Consistent with this notion, mast cells carrying codon 816 *c-kit* mutations have been shown to be resistant to killing by imatinib.^{50,51} Therefore, imatinib is not predicted to be a useful therapy for the majority of patients with systemic mastocytosis carrying

the D816V *c-kit* mutation. On the other hand, wild-type *c-kit* and *c-kit* with mutations in extracellular, transmembrane, and juxtamembrane domains remain sensitive to imatinib.^{33,51,52} Likewise, FIP1L1-PDGFR α -associated variants of systemic mastocytosis respond to imatinib as long as they do not acquire secondary mutations in the kinase domain of the PDGFR α interfering with imatinib binding.^{41,53}

Newer conformation-tolerant tyrosine kinase inhibitors such as PKC412 and dasatinib have been shown to inhibit D816V *c-kit* autophosphorylation as well as survival of the mast cells carrying this mutation.^{54–56} These investigational agents are currently being evaluated for their safety and efficacy in mastocytosis in clinical trials. Mutational analysis of the *c-kit* gene is therefore expected to find widespread use in the future to select mast cell cytoreductive therapy of mastocytosis.

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