

Mastocytosis associated with a rare germline *KIT* K509I mutation displays a well-differentiated mast cell phenotype

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Background: Mastocytosis associated with germline *KIT* activating mutations is exceedingly rare. We report the unique clinicopathologic features of a patient with systemic mastocytosis caused by a *de novo* germline *KIT* K509I mutation. **Objectives:** We sought to investigate the effect of the germline *KIT* K509I mutation on human mast cell development and function.

Methods: Primary human mast cells derived from CD34⁺ peripheral blood progenitors were examined for growth, development, survival, and IgE-mediated activation. In addition, a mast cell transduction system that stably expressed the *KIT* K509I mutation was established.

Results: *KIT* K509I biopsied mast cells were round, CD25⁺, and well differentiated. *KIT* K509I progenitors cultured in stem cell factor (SCF) demonstrated a 10-fold expansion compared with progenitors from healthy subjects and developed into mature hypergranular mast cells with enhanced antigen-mediated degranulation. *KIT* K509I progenitors cultured in the absence of SCF survived but lacked expansion and developed into hypogranular mast cells. A *KIT* K509I mast cell transduction system revealed SCF-independent survival to be reliant on the preferential splicing of *KIT* at the adjacent exonic junction.

Conclusion: Germline *KIT* mutations associated with mastocytosis drive a well-differentiated mast cell phenotype distinct to that of somatic *KIT* D816V disease, the oncogenic potential of which might be influenced by SCF and selective *KIT* splicing. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *KIT*, *K509I*, mastocytosis, germline, mast cells, well differentiated

Abbreviations used

EM: Electron microscopy
FITC: Fluorescein isothiocyanate
HuMC: CD34⁺ derived human mast cell
PE: Phycoerythrin
PGD₂: prostaglandin D₂
SCF: Stem cell factor
WDSM: Well-differentiated systemic mastocytosis
WT: Wild-type

Systemic mastocytosis is a myeloproliferative neoplasm characterized by the pathologic expansion and infiltration of mast cells within tissues.¹⁻³ Affecting mainly adults, the onset is sporadic and often associated with acquired gain-of-function mutations in the receptor tyrosine kinase *KIT*. *KIT* signaling through its ligand, stem cell factor (SCF), influences mast cell proliferation, activation, and differentiation. The most common somatic mutation, *KIT* D816V, is located in the second intracellular tyrosine kinase domain, induces SCF-independent activation, and is observed in greater than 90% of adult patients with systemic mastocytosis.⁴

Rarely, mastocytosis may be associated with germline *KIT* mutations, as underscored by 7 reports in the literature.⁵⁻¹¹ The inheritance pattern is generally autosomal dominant and a consequence of nonsynonymous point mutations involving either the extracellular, transmembrane, or juxtamembrane regions of *KIT*. These mutations are thought to enhance *KIT* dimerization, impair kinase regulation, or both, while generally maintaining sensitivity to the tyrosine kinase inhibitor imatinib. An exception is the recent report of a family with cutaneous mastocytosis accompanied by a germline *KIT* N822I mutation.¹⁰ This mutation is located within the kinase domain and was found to be resistant to imatinib. To date, a germline *KIT* D816V mutation has not been reported.

Cell-culture systems to effectively study the primary mast cells of patients with mastocytosis are lacking, mainly because of the limited recovery of neoplastic mast cells from tissues and a lack of significant clonal expansion *ex vivo*. Therefore studies to understand the effects of *KIT* activating mutations *in vitro* have relied primarily on mast cell lines or transduction experiments, often in non-mast cell lineages. Although much has been learned by using these alternative approaches, the capacity to expand and study primary mast cells from patients with mastocytosis is favored.

In this study we report the unique clinicopathologic features of well-differentiated systemic mastocytosis (WDSM) driven by a *de novo* germline *KIT* K509I mutation. WDSM is a rare variant of systemic mastocytosis characterized by compact aggregates of mature, round, fully granulated mast cells in the bone marrow that lack the *KIT* D816V mutation, as well as the aberrant expression of CD25/CD2 markers.¹²⁻¹⁵ The germline nature of

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this presentation permitted the growth of *KIT* K509I CD34⁺ derived human mast cells (HuMCs) from the patient. The HuMCs displayed a mature phenotype with enhanced proliferation, granulation, and activation. Moreover, SCF-independent growth and development were determined to be dependent on the preferential splicing of *KIT*. We propose that the activating potential of germline *KIT* mutations might retain significant ligand and molecular regulation, thus resulting in a well-differentiated HuMC phenotype.

METHODS

The patient

The patient is a white woman who, at the age of 6 weeks, was reportedly given a diagnosis of cutaneous mastocytosis after having “blisters” on her skin. Throughout childhood, she reported sporadic flushing, pruritus, and urticaria (Fig 1, A). In addition, she reported recurrent episodes of abdominal discomfort, requiring hospitalization on 3 occasions. By the age of 19 years, her gastrointestinal symptoms regressed and skin symptoms stabilized to the point of requiring no antihistamines. At 22 years old, she had significant morning stiffness and arthralgia involving her hands, shoulders, and knees; she was subsequently given a diagnosis of seronegative rheumatoid arthritis.

At the age of 24 years, the patient’s mastocytosis-related symptoms flared after moving to Arizona. Symptoms included diarrhea, abdominal pain, musculoskeletal pain, flushing, and headaches. Her skin displayed a diffuse pattern of involvement that was erythrodermic in nature and accompanied by scattered nodules (subcutaneous benign lipoma) and significant pruritus. This diffuse cutaneous presentation is in contrast to the urticaria pigmentosa classically observed in patients with adult-onset *KIT* D816V systemic mastocytosis (Fig 1, B). A bone marrow examination revealed 90% cellularity (almost entirely mast cells), and the aspirate was 75% mast cells, with round nuclei and variable granularity. The bone marrow mast cells displayed no evidence of “spindling” or CD25 expression (Fig 1, C). The serum total tryptase level was 189 ng/mL. A diagnosis of indolent systemic mastocytosis was established according to World Health Organization criteria,^{2,3} and the disease was further defined as WDSM. Sanger sequencing of *KIT* was performed after initial *KIT* D816V mutation test results were negative. A heterozygous *KIT* K509I mutation was identified in the cDNA of the bone marrow mononuclear cells (Fig 1, D), as well as the genomic DNA of PBMCs, buccal mucosa, and hair samples (see Fig E1 in this article’s Online Repository at www.jacionline.org). This mutation was not detected in either parent, and this suggests the *KIT* K509I mutation was a *de novo* germline event.

The patient’s significant symptoms and bone marrow mast cell involvement and the known sensitivity of the *KIT* K509I mutation to imatinib⁹ prompted a trial of 300 mg/d imatinib. Although an encouraging decrease in her serum tryptase level was noted (57 ng/mL), she temporarily discontinued imatinib after an exacerbation of her headaches. Imatinib was restarted at 100 mg/d, and on increasing to 200 mg/d, her headaches and skin erythema worsened (Fig 2, A), requiring imatinib to be held. Prednisone, 40 mg/d, was started 1 week before reinitiation of 100 mg of imatinib every other day to control the reactions. Prednisone was tapered weekly by 10-mg increments until reaching a stable regimen of 10 mg/d prednisone and 100 mg of imatinib every other day. At this time, mast cells involved approximately 25% of the bone marrow and 10% of the aspirate, and her serum total tryptase level was 37.2 ng/mL. Despite these measures, imatinib was ultimately discontinued by the patient because of intolerance and a desire to possibly conceive.

The patient received symptoms-based treatment under guarded observation for approximately 3 years. After an initial increase, her tryptase level reached a plateau at approximately 100 ng/mL (Fig 2, B). However, her bone marrow mast cell involvement progressively increased to 50%, accompanied by increased daily flushing, pruritus, and severe bone pain, limiting her daily activity. Aspirin, cromolyn sodium (Gastrocrom; Celltech Pharmaceuticals, Rochester, NY), and UVA/UVB phototherapy were added to her antihistamine regimen, with minimal relief. At 28 years old, imatinib was restarted at

50 mg/d and gradually increased over 4 months. She ultimately achieved a dosage of 100 mg/d, which resulted in a normal tryptase level (Fig 2, B), clearance of the bone marrow mast cells (Fig 2, C), and a modest reduction in symptoms.

All patients and healthy donors provided informed consent on National Institutes of Health Institutional Review Board–approved protocols (NCT00044122, NCT00050193 and NCT00001756).

Mutational analysis

Total RNA, cDNA, and genomic DNA was prepared, as previously described.¹⁶ Overlapping *KIT* PCR amplification products were purified and directly sequenced by Macrogen USA (Rockville, Md). Sequencing data were analyzed with Sequencher (Version 4.5; SoftGenetics, State College, Pa). Detection of the *KIT* D816V mutation was assessed by using PCR/RFLP, as previously described.¹⁷

HuMC cultures

Leukapheresis and enrichment of peripheral CD34⁺ cells was performed, as previously described,¹⁸ with the exception that a mobilizing agent (granulocyte colony-stimulating factor) was not administered to the patient. The percentage of enriched CD34⁺ cells obtained by means of leukapheresis was determined with a fluorescein isothiocyanate (FITC)–conjugated anti-CD34⁺ antibody (BD Biosciences, San Jose, Calif) on a FACSCalibur (BD Biosciences) with CellQuest 3.3 software (BD Biosciences). HuMCs were cultured in StemPro complete media including L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 mg/mL), IL-3 (30 ng/mL for the first week only), and IL-6 (100 ng/mL) in the presence or absence of SCF (100 ng/mL; PeproTech, Rocky Hill, NJ) by using an equal starting number of CD34⁺ progenitor cells.¹⁹ For all HuMC studies, different healthy donors were used as control subjects for each set of experiments. HMC 1.1, HMC1.2, and LAD2 mast cell lines were cultured, as previously described.^{20,21}

Light and electron microscopy

Cytospin preparations followed by toluidine blue staining were performed by using standard protocols.¹⁸ For electron microscopy (EM), HuMCs grown on 13-mm Thermanox Coverslips (Nunc, Naperville, Ill) were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7. Samples were postfixed for 30 minutes with 0.5% osmium tetroxide/0.8% potassium ferricyanide in 0.1 mol/L sodium cacodylate, for 1 hour with 1% tannic acid, and overnight with 1% uranyl acetate at 4°C. Samples were dehydrated with a graded ethanol series and embedded in Spurr resin. Thin sections were cut with a Leica EM UC6 ultramicrotome (Leica, Vienna, Austria) and stained with 1% uranyl acetate and Reynold lead citrate before viewing at 120 kV on a Tecnai BT Spirit transmission electron microscope (FEI, Hillsboro, Ore). Digital images were acquired with a Hamamatsu XR-100 side mount digital camera system (Advanced Microscopy Techniques, Danvers, Mass) and processed with Adobe Photoshop (Adobe Systems, San Jose, Calif).

Multiparameter flow cytometry

Bone marrow mast cells and HuMCs were analyzed, as previously described,¹⁷ with a FACSCanto II flow cytometer (BD Biosciences) and the following antibodies: CD45-PerCP, CD2–phycoerythrin (PE), CD25-FITC, CD117-allophycocyanin, CD69-FITC, CD63-FITC, CD203-PE (BD Biosciences) and/or FcεRI-PE (eBioscience, San Diego, Calif).

MTT and apoptosis assays

HuMCs were plated at 2×10^5 cells/mL (MTT assay) or 1×10^5 cells/mL (apoptosis assay) with different concentrations of SCF for 72 hours. The MTT assay (Cell Growth Determination Kit; Sigma, St Louis, Mo) was performed at the final 3 hours of incubation, according to the manufacturer’s instructions. Apoptosis was evaluated by using the cellular Annexin V–FITC

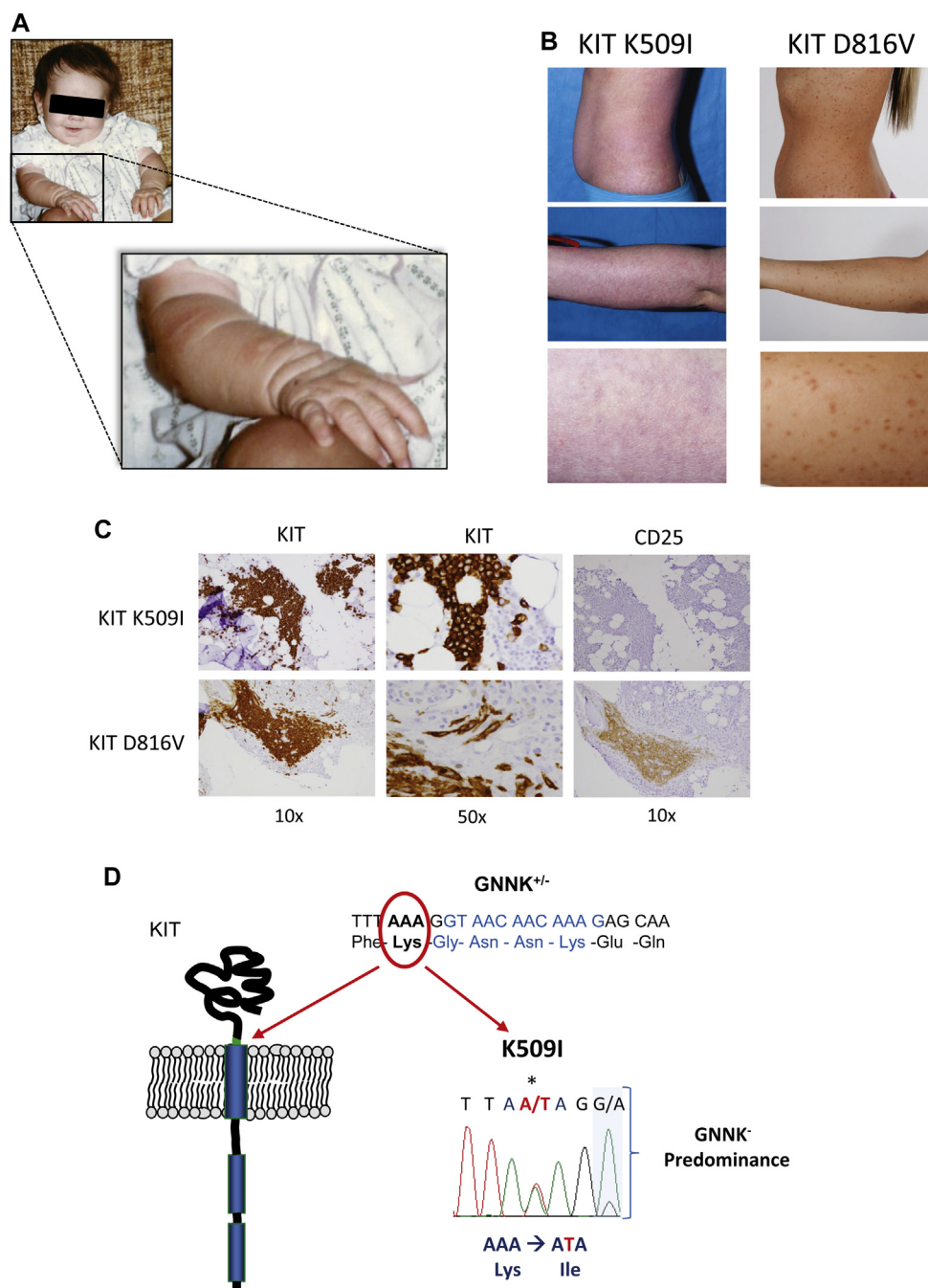


FIG 1. Clinicopathologic features of germline *KIT* K509I WDSM. **A**, Cutaneous presentation as an infant. **B**, Diffuse cutaneous presentation as an adult with comparison with typical *KIT* D816V urticarial pigmentosa. **C**, KIT and CD25 staining of bone marrow mast cells with comparison with typical *KIT* D816V morphology. **D**, Diagram of KIT and location of the heterozygous K509I mutation in relationship to GNNK splice site.

Apoptosis Kit (Biovision, Mountain View, Calif), according to the manufacturer's instructions.

HuMC antigen-mediated activation assays

HuMCs were sensitized overnight with biotinylated human IgE (100 ng/mL) in the absence of SCF and subsequently stimulated with various concentrations of streptavidin in the presence or absence of SCF (100 ng/mL) at 37°C for 30 minutes.²² β -Hexosaminidase release was determined, as previously described.²³ Prostaglandin D₂ (PGD₂) release was determined by

using the PGD₂-MOX ELISA kit (Cayman Chemical Company, Ann Arbor, Mich), according to the manufacturer's instructions. Ca²⁺ flux was measured in sensitized and activated Fura 2-AM (2 μ mol/L; Molecular Probes, Eugene, Ore)-loaded HuMCs, as previously described.²⁴

IC2 mast cell transduction system

The IL-3-dependent immature murine mast cell line IC2 expresses Fc ϵ RI, but lacks surface KIT expression and exhibits minimal granule formation.²⁵ *KIT* wild-type (WT) and D816V associated with GNNK^{+/-} variants were

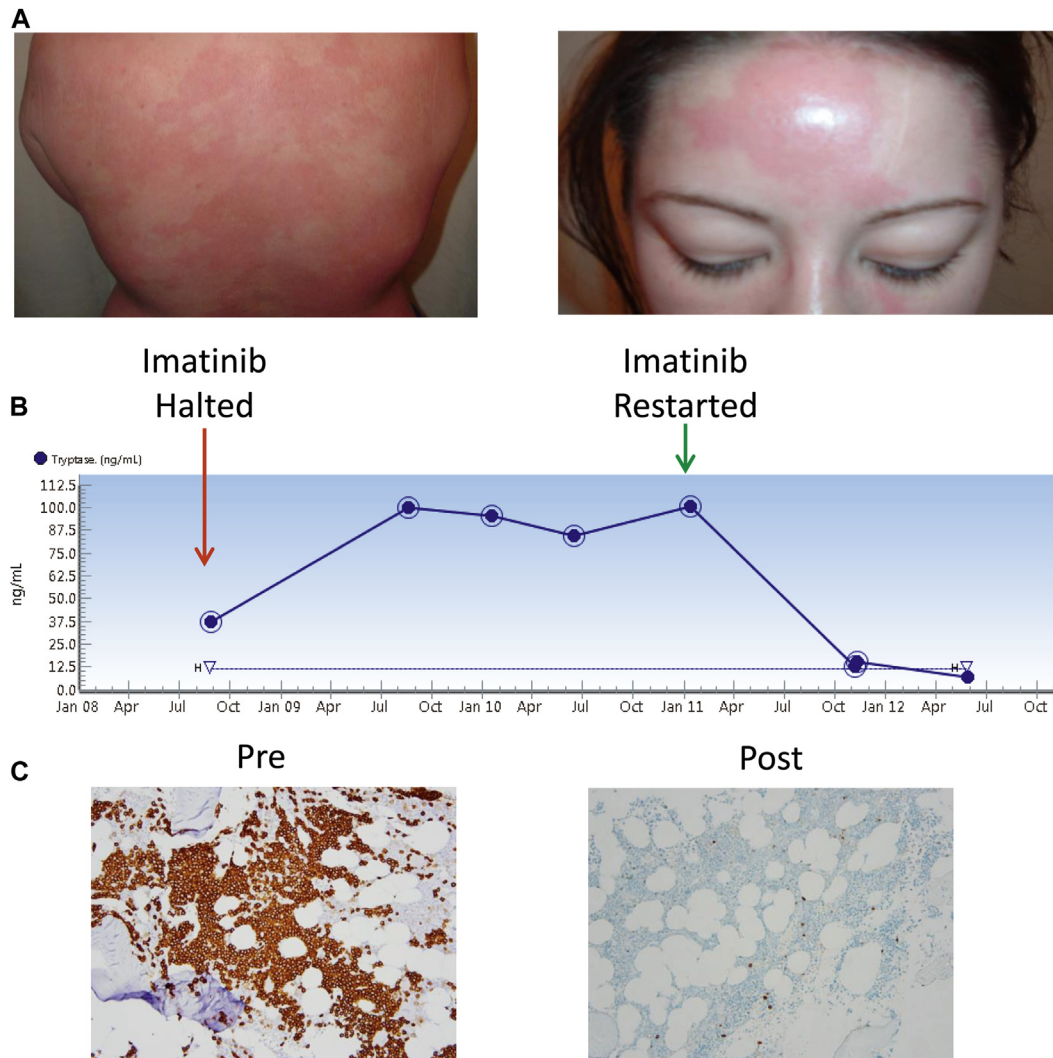


FIG 2. Clinical reaction and response to imatinib. **A**, Erythematous rash exaggerated by the initiation of imatinib. **B**, Serum total tryptase levels on discontinuation and reinitiation of imatinib. **C**, Tryptase staining of a bone marrow biopsy specimen demonstrating mast cell involvement before and after imatinib.

cloned into the pMX-puro retroviral expression vector (Cell BioLabs, San Diego, Calif), as previously described.²⁶ *KIT* K509I was subsequently generated by using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, Calif), according to the manufacturer's instructions. Transduction of IC2 cells (including empty vector control) was performed, as previously described.²⁶ For metabolism experiments, transduced IC2 cells were washed free of IL-3 and cultured in the presence or absence of SCF (100 ng/mL) for 1 week. Cells (2×10^5 /mL) were subsequently incubated in a 96-well plate for 24 hours, and the MTT assay was performed as above. The transduced IC2 cells maintained in SCF were subsequently seeded at 2×10^5 cells/mL in a 96-well plate in the presence or absence of SCF to assess proliferation. Viable cells were counted on day 3 with the Cellometer Auto T4 cell counter (Nexcelom Bioscience, Lawrence, Mass).

Immunoblotting

For KIT activation experiments, HuMCs sensitized in the absence of SCF overnight were stimulated with SCF (10 ng/mL) at 37°C for 2 minutes. Cell lysates were prepared and loaded onto 4% to 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, Calif) for electrophoretic separation and immunoblotting, as previously described.²⁷ Anti-human c-Kit mAb (Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-human phospho-c-Kit

(Tyr703) mAb (Cell Signaling Technology, Danvers, Mass), anti-human CD226 mAb (Santa Cruz Biotechnology), and anti- β -actin mAb (Sigma) were used for immunoblotting. Immunoreactive proteins were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences, San Jose, Calif).

Statistical analysis

Data are presented as means $\pm$ SEMs. Comparisons between 2 groups were analyzed by using the unpaired Student *t* test. *P* values of less than .05 were considered statistically significant. Analysis was performed with PRISM software, version 5 (GraphPad Software, San Diego, Calif).

RESULTS

KIT K509I CD34⁺ progenitors display enhanced proliferation and develop into hypergranular HuMCs

CD34⁺ progenitors were cultured *ex vivo* to investigate the effect of the *KIT* K509I mutation on primary mast cell development. At 8 weeks, the *KIT* K509I progenitors cultured in SCF demonstrated a 10-fold expansion compared with progenitors from healthy donors (*P* < .05; Fig 3, A). *KIT* K509I HuMC

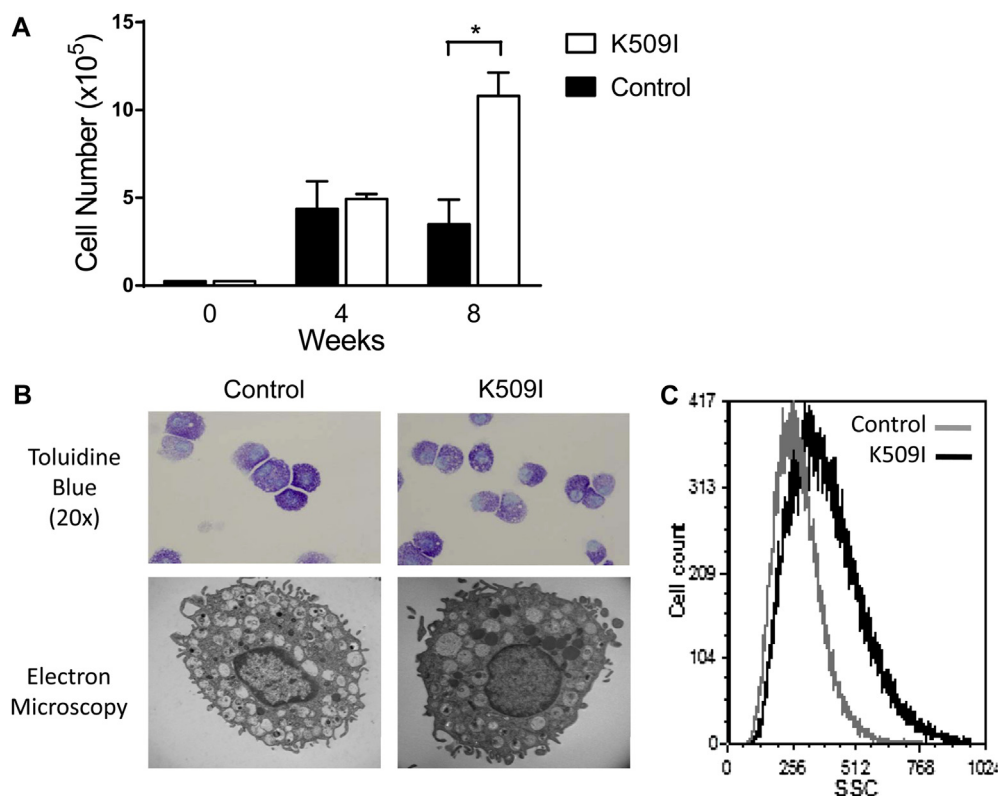


FIG 3. *KIT* K509I CD34⁺ progenitors display enhanced proliferation and develop into hypergranular HuMCs in the presence of SCF. **A**, Growth of K509I HuMCs in the presence of SCF. Data are means $\pm$ SEMs of 3 independent experiments performed in duplicate. **B**, Light microscopy with toluidine blue staining ($\times 20$ magnification) and EM. **C**, Side scatter (SSC) histogram. Results are representative of 3 experiments. * $P < .05$.

expansion continued for up to 20 weeks, exceeding normal survival (10-12 weeks). Preservation of the *KIT* K509I mutation at 8 weeks was confirmed by using Sanger sequencing (see Fig E1). The morphology of the *KIT* K509I HuMCs was examined by using light microscopy and EM. Similar to our bone marrow observations, the *KIT* K509I HuMCs grown in SCF appeared round and mature, as indicated by the well-condensed nuclei, with an apparent increase in granulation determined by using EM (Fig 3, B). This hypergranular phenotype was supported by an increase in side scatter when the cells were analyzed by means of flow cytometry (Fig 3, C).

SCF depletion affects *KIT* K509I HuMC proliferation, development, and survival

As HuMCs are dependent on SCF for survival, proliferation, activation, and differentiation, we next investigated the oncogenic potential of the *KIT* K509I HuMCs in the absence of SCF. After 3 days of SCF depletion, a significant decrease in the survival of both control HuMCs ($P < .0001$) and *KIT* K509I HuMCs ($P < .0001$) was observed by using the MTT assay (Fig 4, A). However, the *KIT* K509I HuMCs were less sensitive to SCF depletion and demonstrated a survival advantage over the control HuMCs (85% vs 26% survival, $P < .0001$). An apoptosis assay performed in parallel revealed that SCF withdrawal resulted in minimal apoptosis in the *KIT* K509I HuMCs ($P = .055$) and a significant induction of apoptosis ($P < .05$) in the control HuMCs (Fig 4, B). This observation is further illustrated when the degree of

apoptosis in the *KIT* K509I HuMCs is directly compared with that in control cells (15% vs 67% apoptosis, $P < .01$). Consistent with these observations, the *KIT* K509I HuMCs displayed minimal autophosphorylation of KIT in the absence of SCF (Fig 4, C).

With the observation that the *KIT* K509I HuMCs retained a marginal survival dependence on SCF, we sought to determine whether the *KIT* K509I mutation might support SCF-independent HuMC growth and development. *KIT* K509I and control CD34⁺ progenitors were cultured in the absence of SCF for 8 weeks. Only the *KIT* K509I CD34⁺ progenitors survived and developed into HuMCs; however, these cells displayed minimal expansion by week 8 (Fig 4, D). HuMCs accounted for nearly 100% of the culture and appeared mature, although hypogranular (Fig 4, E).

KIT K509I-transduced IC2 cells are dependent on the *KIT* GNNK⁻ isoform for SCF-independent survival

As a result of alternative mRNA splicing, 2 major isoforms of KIT are coexpressed in mast cells characterized by the presence or absence of 4 amino acids (GNNK) in the juxtamembrane region of the extracellular domain.^{26,28-30} The GNNK⁻ variant is the predominant transcript, as observed in the patient's bone marrow mononuclear cells (Fig 1, D). The *KIT* K509I mutation is located adjacent to this GNNK splice region, and therefore we

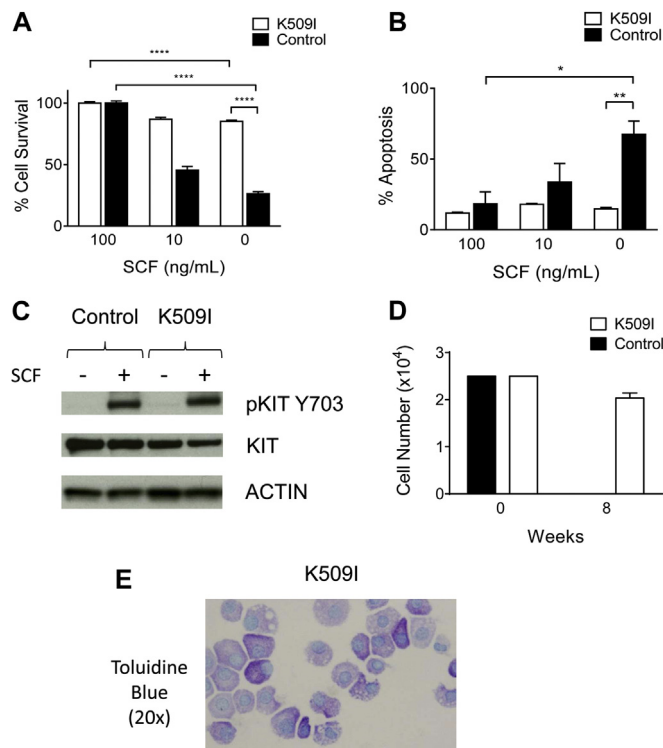


FIG 4. SCF depletion affects *KIT* K509I HuMC proliferation, development, and survival. **A** and **B**, *KIT* K509I HuMC survival (Fig 4, **A**) and apoptosis (Fig 4, **B**) with various SCF concentrations. Data are means $\pm$ SEMs of 3 independent experiments performed in triplicate. **C**, Immunoblot of KIT phosphorylation before and after SCF stimulation. Results are representative of 3 experiments. **D**, SCF-independent *KIT* K509I HuMC growth. Data are means $\pm$ SEMs of 3 independent experiments performed in duplicate. **E**, Light microscopy with toluidine blue staining ($\times 20$ magnification). * $P < .05$, ** $P < .01$, and **** $P < .0001$.

hypothesized that the activating potential of the mutation might be influenced by GNNK splicing.

We expressed the *KIT* K509I GNNK⁻ or GNNK⁺ isoforms in the IL-3-dependent immature mast cell line IC2 to assess the potential effect on SCF independent growth and survival. As reported, in the presence of SCF, WT *KIT* metabolism (Fig 5, **A**) and growth (Fig 5, **C**) were enhanced when associated with the GNNK⁻ isoform compared with the GNNK⁺ isoform ($P < .0005$). However, this difference was negated by the presence of either *KIT* K509I or *KIT* D816V (Fig 5, **A** and **C**). With SCF absent, WT *KIT*-transduced IC2 cells succumbed, whereas *KIT* D816V supported ligand-independent metabolism (Fig 5, **B**) and growth (Fig 5, **D**). SCF-independent survival of *KIT* K509I-transduced IC2 cells was only observed when associated with the GNNK⁻ isoform (Fig 5, **B** and **D**) and displayed a far weaker oncogenic potential compared with the *KIT* D816V mutation. Of note, the GNNK⁻ isoform appears to influence *KIT* D816V ligand-independent metabolism (Fig 5, **B**) but not cell proliferation (Fig 5, **D**), as previously reported.²⁶

***KIT* K509I HuMCs display enhanced antigen-mediated degranulation, PGD₂ release, and intracellular calcium flux**

Enhanced HuMC degranulation through *KIT* activation is well recognized.^{24,31} In the presence (Fig 6, **A**) or absence (Fig 6, **B**) of

SCF, the *KIT* K509I HuMCs displayed enhanced Fc ϵ RI-mediated degranulation with increasing antigen concentrations compared with those seen in control cells. No increase in baseline degranulation was observed in the *KIT* K509I HuMCs in the absence of antigen, irrespective of the presence of SCF. In contrast, SCF alone produced a baseline PGD₂ release in both the *KIT* K509I and control HuMCs (Fig 6, **C**), a previously reported effect.³² However, in the absence of SCF, the *KIT* K509I HuMCs displayed enhanced PGD₂ release with increasing antigen concentrations (Fig 6, **D**). Antigen-mediated intracellular calcium flux was also examined in the presence (Fig 6, **E**) or absence (Fig 6, **F**) of SCF, with an enhanced signal observed in the *KIT* K509I HuMCs.

***KIT* K509I HuMCs exhibit increased expression of Fc ϵ RI and CD226**

With enhanced antigen-mediated activation confirmed in the *KIT* K509I HuMCs, we sought to identify potential contributing features. Fluorescence-activated cell sorting analysis revealed no difference in CD117, CD25, CD2, CD203c, CD63, or CD69 expression. However, surface expression of the IgE receptor Fc ϵ RI was markedly increased (Fig 6, **G**). A limited gene expression array was performed with the *KIT* K509I HuMCs to identify alterations in gene regulation (not shown). Compared with a control, the gene with the most profound increase in expression (10-fold) was the immunomodulating transmembrane glycoprotein CD226. CD226 immunoblotting with *KIT* K509I HuMC lysates confirmed this preliminary finding, and minimal CD226 expression was observed in the control HuMCs (Fig 6, **H**). We expanded this analysis and observed moderate CD226 expression in HMC1.1 cells (*KIT* D816V negative) and a lack of CD226 expression in HMC1.2 cells (*KIT* D816V positive). LAD2 cells (*KIT* D816V negative) were found to overexpress CD226 in excess of the positive control Jurkat cells.

DISCUSSION

In this study we demonstrate that a germline juxtamembrane *KIT* K509I activating mutation enhances proliferation and development of mature, round, hypergranular HuMCs, which lack the CD2/CD25 activation markers characteristically observed in *KIT* D816V-driven disease. This well-differentiated HuMC phenotype^{12,14,15} appears common to mastocytosis associated with germline *KIT* mutations, as summarized in Table I.⁵⁻¹¹ Interestingly, this shared morphology does not appear entirely dependent on the location of the activating mutation within *KIT*. Although the mutations tend to cluster in the *KIT* juxtamembrane and transmembrane regions, mutations in the extracellular and tyrosine kinase domains are also reported and suggest other factors might be involved in the tolerance of these oncogenic events.

We have herein demonstrated that *KIT* K509I HuMCs retain a modest dependency on SCF for growth, development, and survival. Perhaps germline *KIT* mutations share a common reliance on SCF, and as a result, their oncogenic potential is limited and compatible with a mature HuMC phenotype and long-term survival. Consistent with this hypothesis, the *KIT* K509I HuMCs displayed negligible ligand-independent autophosphorylation of *KIT* and an enhanced response to SCF, as evidenced by increased expansion and activation. It is plausible this hyperresponsiveness

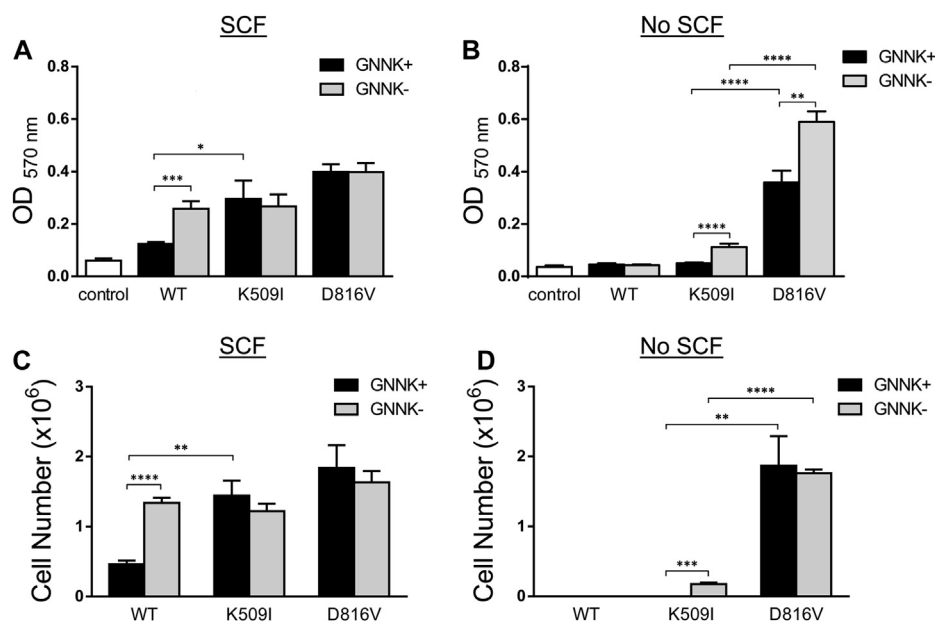


FIG 5. Activating potential of KIT K509I is dependent on the GNNK⁻ isoform. **A** and **B**, MTT assay of transduced IC2 cells cultured in the presence (Fig 5, **A**) or absence (Fig 5, **B**) of SCF. Data are means $\pm$ SEMs of 3 independent experiments performed in triplicate. **C** and **D**, Growth and survival of transduced IC2 cells cultured in the presence (Fig 5, **C**) or absence (Fig 5, **D**) of SCF. Data are means $\pm$ SEMs of 2 independent experiments performed in duplicate. Control, Empty vector. * $P < .05$, ** $P < .01$, *** $P < .0005$, and **** $P < .0001$.

coupled with the potential for autocrine SCF production by the *KIT* K509I HuMCs³³ might account for the survival and protection from apoptosis observed in the “SCF-free” culture conditions. Future studies with the *KIT* K509I HuMCs, as well as other germline *KIT* mutations, might define the critical signaling pathways, contributing to the enhanced responsiveness to SCF and their association with a well-differentiated phenotype.

Alternative splicing has a recognized role in oncogenesis,^{34,35} and the proximity of the K509I mutation to the *KIT* GNNK splicing region has prompted interest. A normal GNNK⁻ predominance in the patient’s bone marrow mononuclear cells was in agreement with Zhang et al,⁹ who observed that the K509I mutation did not alter the GNNK splicing profile in a family with mastocytosis. Nonetheless, selective expression of the GNNK isoforms had a significant effect on KIT K509I oncogenic potential, with SCF-independent survival only achieved when the KIT K509I mutation was associated with the GNNK⁻ isoform in the transduced IC2 mast cells. In contrast, the GNNK isoforms have relatively little influence on the robust oncogenic potential of the KIT D816V mutation.^{26,36} Our observation suggests that the survival, growth, and development of the *KIT* K509I HuMCs in the absence of SCF were supported solely by the GNNK⁻ isoform. It is provocative to consider that HuMCs *in vivo* could regulate the activating potential of the KIT K509I mutation through selective GNNK splicing; however, no evidence presently exists to support this conclusion.

In our attempt to identify additional factors contributing to the enhanced degranulation of the *KIT* K509I HuMCs, we observed increased expression of FcεRI and CD226. Aggregation of surface FcεRI, the high-affinity IgE receptor, is central to antigen-mediated HuMC degranulation, and it is plausible that upregulation of FcεRI might enhance this activation. CD226 is

a transmembrane glycoprotein that mediates cell adhesion, cytokine secretion, and cytotoxicity.³⁷ Moreover, coengagement of CD226 with FcεRI on mast cells synergistically augments degranulation.³⁸ Interestingly, CD226 appeared overexpressed in neoplastic mast cell lines lacking the *KIT* D816V mutation, most profoundly the LAD2 cell line.²³ Taken together, these observations suggest the existence of interplay between CD226, FcεRI, and KIT signaling, which enhances mast cell activation.

To our knowledge, this study is the first to evaluate IgE-mediated degranulation and activation of primary mast cells from a patient with mastocytosis. The *KIT* K509I HuMCs displayed heightened antigen-dependent activation. These data support the clinical observation that IgE-mediated systemic reactions (anaphylaxis) are more prevalent in adult patients with systemic mastocytosis.^{39,40} Indeed, enhanced degranulation was observed at lower antigen concentrations in the *KIT* K509I HuMCs (most notably in the presence of SCF); however, IgE sensitization alone (no antigen) was insufficient to induce basal degranulation in our assays.

In summary, our study contributes to a larger body of evidence suggesting that germline *KIT* mutations associated with mastocytosis drive a well-differentiated mast cell phenotype markedly different to that of somatic *KIT* D816V disease, the activating potential of which might be influenced by SCF and selective *KIT* splicing.

We thank the patient and her family, as well as the LAD and Mayo clinical research staff for their contributions.

Clinical implications: Mastocytosis associated with reported germline *KIT* activating mutations, in this case *KIT* K509I, display a mature, well-differentiated mast cell phenotype distinct to that of somatic *KIT* D816V disease.

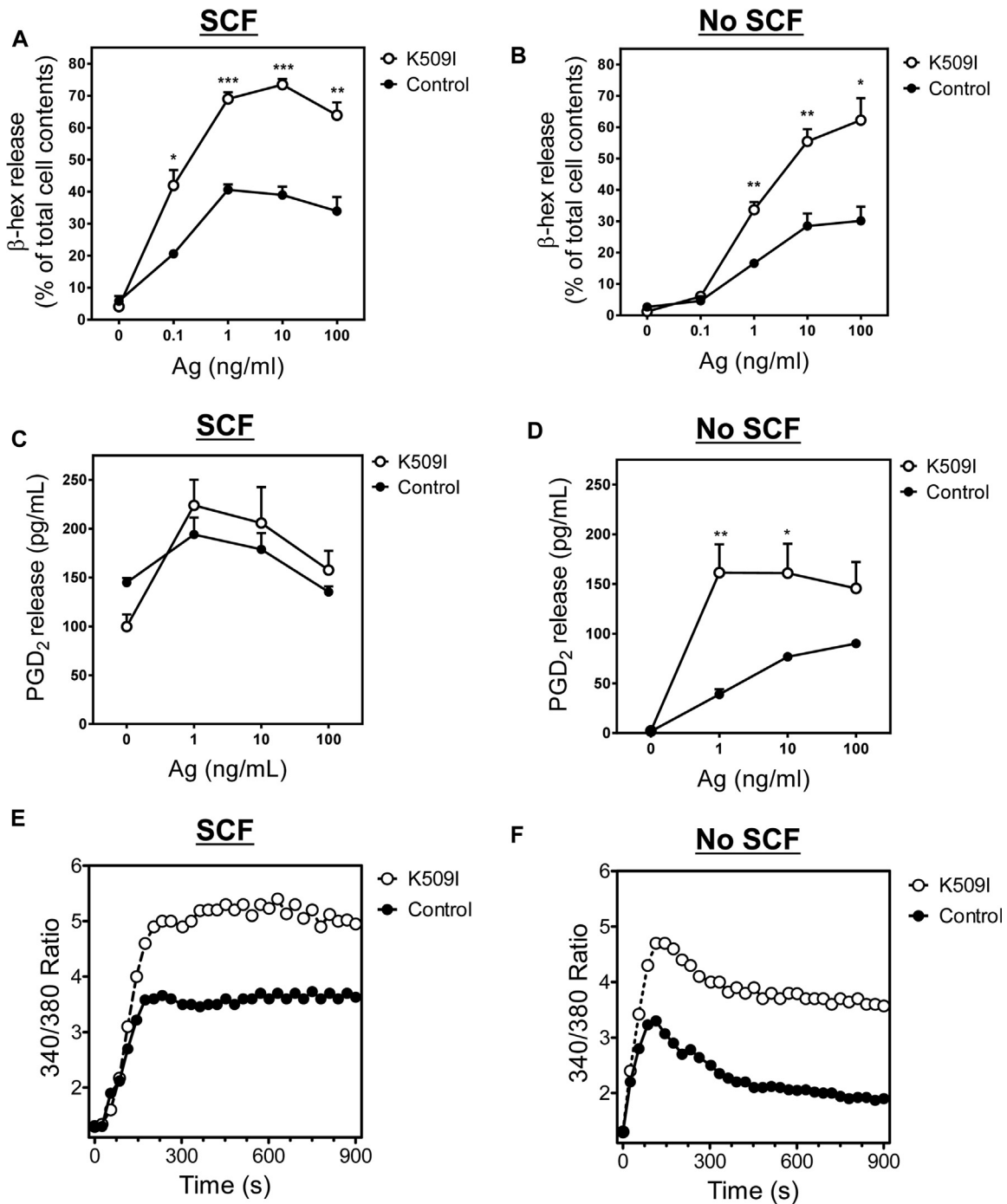


FIG 6. *K/TK509I* HuMCs display enhanced antigen-mediated activation. **A-F**, β -Hexosaminidase release (Fig 6, **A** and **B**), PGD_2 release (Fig 6, **C** and **D**), and intracellular Ca^{2+} flux (Fig 6, **E** and **F**) in the presence or absence of SCF. Data are means $\pm$ SEMs of 3 independent experiments performed in triplicate. Ca^{2+} flux represents an experiment performed in triplicate. **G**, $\text{Fc}\epsilon\text{RI}$ surface expression determined by means of flow cytometry. Results are representative of 3 experiments. **H**, CD226 immunoblotting of whole-cell lysates. Results are representative of 2 experiments. * $P < .05$, ** $P < .01$, and *** $P < .0005$.

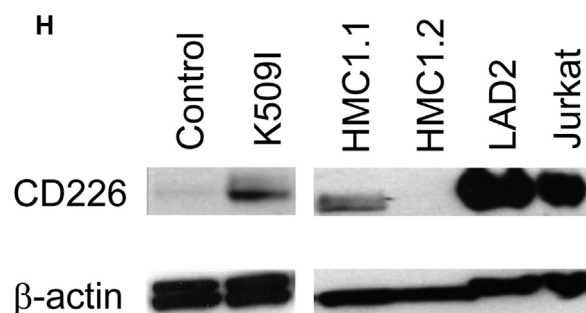
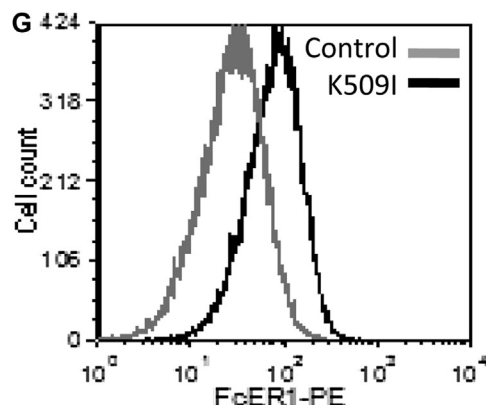


FIG 6. (continued).

TABLE I. Mastocytosis associated with germline *KIT* mutations

Reference	<i>KIT</i> mutation	Exon	Location	Genotype	Inheritance pattern	Mast cell morphology	CD25 expression	Cutaneous involvement	Systemic progression	Imatinib sensitive
Beghini et al ⁵	A559V	10	JMI	Heterozygous	AD	Round, mature	*	Urticaria pigmentosa	*	*
Tang et al ⁶	A533D	10	TM	Heterozygous	AD	Round, mature	*	Diffuse cutaneous	Yes	*
Akin et al ⁷	F522C	10	TM	Heterozygous	<i>De novo</i>	Round, mature, hypergranular	Negative	Urticaria pigmentosa	Yes	Yes
Hartmann et al ⁸	Del 419 Asp	8	EX	Heterozygous	AD	Round, mature†	*	Diffuse cutaneous	Yes	*
Zhang et al ⁹	K509I	9	JME	Heterozygous	AD	Round, granular	*	Diffuse cutaneous	Yes	Yes
Wasag et al ¹⁰	N822I	17	TK	Heterozygous	AD	Round, mature	Negative	Urticaria pigmentosa	No	No
Speight et al ¹¹	K509I	9	JME	Heterozygous	<i>De novo</i>	Round, mature	*	Diffuse cutaneous	Yes	No

AD, Autosomal dominant; EX, extracellular; JME, juxtamembrane extracellular; JMI, juxtamembrane intracellular; TK, tyrosine kinase domain; TM, transmembrane.

*Not reported.

†Personal communication.

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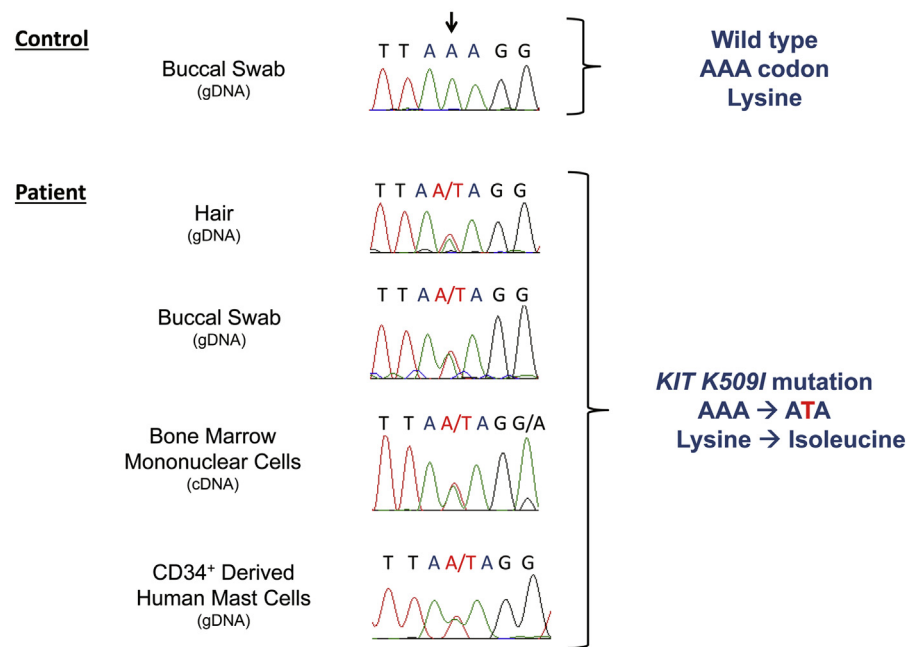


FIG E1. Confirmation of the heterozygous germline *KIT* K509I mutation in various tissues and cell populations.

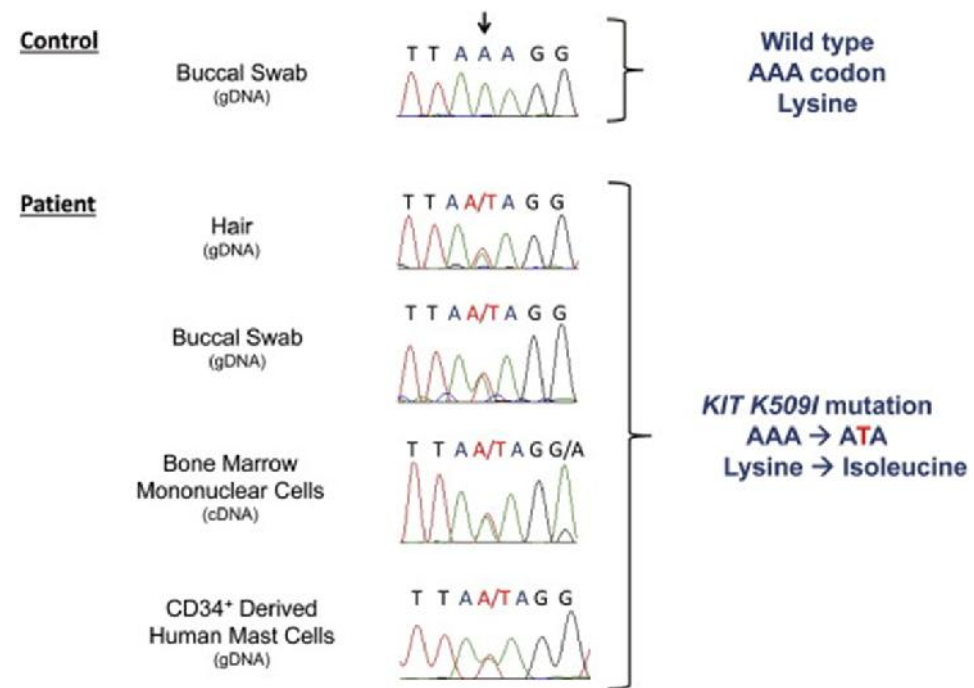


Fig E1 Confirmation of the heterozygous germline KIT K509I mutation in various tissues and cell populations.

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