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***KIT* GNNK splice variants: Expression in systemic mastocytosis and influence on the activating potential of the D816V mutation in mast cells**

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Abstract

Stem cell factor-dependent *KIT* activation is an essential process for mast cell homeostasis. The two major splice variants of *KIT* differ by the presence or absence of four amino acids (GNNK) at the juxta-membrane region of the extracellular domain. We hypothesized that the expression pattern of these variants differs in systemic mastocytosis and that transcripts containing the *KIT* D816V mutation segregate preferentially to one GNNK variant. A quantitative real-time PCR assay to assess GNNK⁻ and GNNK⁺ transcripts from bone marrow mononuclear cells was developed. The GNNK⁻/GNNK⁺ copy number ratio showed a trend toward a positive correlation with the percentage of neoplastic mast cell involvement, and *KIT*D816V containing transcripts displayed a significantly elevated GNNK⁻/GNNK⁺ copy number ratio. Relative expression of only the GNNK⁻ variant correlated with increasing percentage of neoplastic mast cell involvement. A mast cell transfection system revealed that the GNNK⁻ isoform of wild type *KIT* was associated with increased granule formation, histamine content, and growth. When accompanying the *KIT* D816V mutation, the GNNK⁻ isoform enhanced cytokine-free metabolism and moderately reduced sensitivity to the tyrosine kinase inhibitor, PKC412. These data suggest that neoplastic mast cells favor a GNNK⁻ variant predominance, which in turn enhances the activating potential of the *KIT* D816V mutation and thus could influence therapeutic sensitivity in systemic mastocytosis.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

KIT, the receptor for stem cell factor (SCF), is expressed on the surface of various hematopoietic progenitor cells and on mature mast cells [1]. Binding of SCF induces KIT dimerization, inherent tyrosine kinase activation, and resulting activation of downstream signaling pathways, including the PI3-kinase, MAPK, and Ras/ERK pathways [2]. These KIT-mediated signaling cascades are critical for proper mast cell proliferation, activation, and differentiation [3].

As a result of alternative messenger RNA splicing, two major isoforms of KIT are expressed, characterized by the presence or absence of four amino acids (GNNK) in the juxta-membrane region of the extracellular domain [4–6]. These isoforms are generally coexpressed, often with the GNNK[−] variant as the predominant transcript. Biological differences between the two GNNK isoforms have been described. The GNNK[−] isoform generally exhibits stronger signal transduction [7,8] and potential tumorigenicity [9]. Expression differences of the two variants in malignant cell lines, solid tumors, and hematologic malignancies have suggested a possible prognostic utility [5,10–12].

Systemic mastocytosis is a myeloproliferative neoplasm characterized by the clonal expansion of neoplastic mast cells [13]. The KIT D816V activating mutation, located in the intracellular tyrosine kinase domain, is observed in more than 90% of adult patients with systemic mastocytosis [14], and early acquisition of this mutation during hematopoiesis contributes to disease severity [15]. Alterations in KIT messenger RNA processing can also have a critical role in disease pathogenesis, as novel *KIT* transcripts have been detected in aggressive mast cell malignancies [16,17]. We hypothesized that alterations in the expression pattern of the GNNK variants exist in systemic mastocytosis; therefore, we developed a novel real-time PCR assay to examine the GNNK transcripts and their relationship to the *KIT*D816V mutation.

In this study, we report that *KIT*D816V containing transcripts in mastocytosis displayed an elevated GNNK[−]/GNNK⁺ copy number ratio. Furthermore, the GNNK[−] isoform, in association with the KIT D816V mutation, enhanced cytokine-free metabolism and reduced sensitivity to the tyrosine kinase inhibitor, PKC412. This study suggests that normal mast cell homeostasis is dependent on the relative levels of the KIT GNNK isoforms expressed and, furthermore, preferential expression might influence the molecular pathogenesis and therapeutic responses in KIT D816V systemic mastocytosis.

Methods

Study subjects

Following informed consent, 25 patients with systemic mastocytosis (11 men, 14 women, ages 24–74 years) and 16 healthy subjects (10 men, 6 women, ages 29–62 years) underwent bone marrow biopsies as part of research protocols approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board (NCT00044122, NCT00806364, NCT00090662). Systemic mastocytosis was diagnosed and classified according to the World Health Organization criteria [18] (Table 1). All bone marrow microscopic examinations were performed in a blinded manner by a hematopathologist. cDNA was prepared from isolated bone marrow mononuclear cells as described previously [19]. As a clinical diagnostic, the presence of the *KIT*D816V mutation was determined by PCR/RFLP as described previously [20].

Generation of control plasmids to validate real-time quantitative PCR assay

To validate the KIT GNNK quantitative (qPCR) assay, plasmids containing either the GNNK⁺ or GNNK[−] sequences were generated. The GNNK variants were amplified from

cultured human mast cell (HuMC) cDNA using the following primer sets: the GNNK⁺ primer set, GNNK⁺ F1 5'-GCA TTT AAA GGT AAC AAC AAA GAG C-3' and GNNK⁺ R1 5'-ATC TGC ATC CCA GCA AGT CT-3'; the GNNK⁻ primer set, GNNK⁻ F1 5'-CCT ATT TTA ACT TTG CAT TTA AAG AGC-3' and GNNK⁻ R1 5'-CCC AGC AAG TCT TCA TTA TGT C-3'. The PCR products were cloned into the pCR 2.1-TOPO plasmid using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. These plasmids were used as positive, negative, and interfering template controls to validate the qPCR assay. In addition, the molecular weight of the plasmids was calculated, and a serial dilution of plasmid DNA was prepared to produce standard curves from which transcript copy numbers could be derived in the samples.

Real-time qPCR assay

Quantitative PCR was performed using RT² SYBR Green Master Mixes (SABiosciences, Valencia, CA, USA) according to the manufacturer's instructions with modified PCR cycles on the ABI PRISM 7500 SDS system (Applied Biosystems, Carlsbad, CA, USA). To distinguish GNNK⁺ and GNNK⁻ splice variants in the samples, it was critical to develop and validate highly specific primers selective for the alternatively spliced variants. This process is described in detail in Supplementary Figure 1A (online only, available at www.expchem.org). The primer sets used for each specific transcript are as follows: GNNK⁺ F1 5'-GCA TTT AAA GGT AAC AAC AAA GAG C-3' and GNNK⁺ R2 5'-CTG TAC TTC ATA CAT GGG TTT CTG-3'; GNNK⁻ F1 5'-CCT ATT TTA ACT TTG CAT TTA AAG AGC-3' and GNNK⁻ R2 5'-ATA ATG CAC ATC ATG CCA GCT A-3'. The amplification cycles were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min 30 sec. All reactions were performed in triplicate, and the specificity of the reactions was confirmed by dissociation curve analysis. The GNNK variant transcript copy numbers were determined using standard curves generated with the control plasmids, and the GNNK⁻/GNNK⁺ copy number ratio was calculated for each sample. In addition, the 2^{-Ct} method was used to calculate the expression level of each GNNK variant relative to that of a healthy control. GAPDH C_t values were consistent between samples, and GAPDH was used as the housekeeping/reference transcript to normalize for technical variations.

Detection of the KIT D816V mutation in the GNNK isoforms

GNNK⁺ F1/R1 and GNNK⁻ F1/R1 primer sets were used to amplify the GNNK variant-specific transcripts, including the D816V region. The amplification cycles were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 63°C for 1 min and 72°C for 90 sec, and the final extension of 72°C for 7 min. The PCR products were run on 2% agarose gels. DNA was recovered from the agarose gels using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions and eluted in 15 µL of water. The presence of the KITD816V mutation was determined by PCR/RFLP [20] using *HinfI* restriction enzyme digestion, which generates a confirmatory 188-bp band.

Amplification of KIT D816V transcripts

KITD816V containing transcripts were amplified using the following allele-specific primers: D816V F 5'-AGT GCA TTC AAG CAC AAT GG-3' and D816V R 5'-TTA GAA TCA TTC TTG ATG A-3'. As part of the primer validation, the KITD816V mutation was readily detected in the cDNA of the neoplastic HMC1.2 (KITD816V positive) cell line. In contrast, no amplification product was detected in normal human mast cells and the HMC1.1 (KITD816V negative) cell line under various annealing temperatures (Supplementary Figure 1B, online only, available at www.expchem.org). The optimized amplification cycles were as follows: 94°C for 5 min, followed by 36 cycles of 94°C for 45 sec, 55°C for 90 sec and 72°C for 90 sec, and the final extension of 72°C for 7 min. The PCR products were run on 2% agarose gels. DNA was recovered from the agarose gels using the QIAEX II Gel

Extraction Kit (Qiagen) according to the manufacturer's instructions and eluted in 15 μ L of water. The eluted PCR products were diluted, and the GNNK variant transcript copy number quantified by qPCR.

Expression constructs and mutagenesis

Human wild type *KIT*^{GNNK⁺} (WT⁺) or GNNK⁻ (WT⁻) open reading frames were cloned into the pcDNA 3.1 expression vector (Invitrogen) using standard molecular biology techniques. The D816V mutation associated with either the GNNK⁺ (D816V⁺) or the GNNK⁻ (D816V⁻) variant was generated using the Quick-Change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The *KIT* variants (designated as WT⁻, WT⁺, D816V⁻, and D816V⁺; Fig. 1) were confirmed by Sanger sequencing and subsequently subcloned into the pMX-puro retroviral expression vector (Cell BioLabs, San Diego, CA, USA) using standard molecular biology techniques.

Mast cell culture and stable expression of KIT GNNK isoforms

The immature murine mast cell line, IC2, was a gift from Tatsuki Kataoka (Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan). IC2 cells express Fc RI, lack surface KIT expression, and exhibit minimal granule formation [21]. The IC2 cells were cultured in Alpha MEM (Mediatech, Manassas, VA, USA) medium containing 10% FBS (Gibco, Carlsbad, CA), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 20 ng/mL mouse interleukin (IL) 3 (PeproTech, Rocky Hill, NJ, USA). Transfection of the pMX-puro/*KIT* variants was performed using the Platinum Retroviral Expression System (Cell BioLabs) according to the manufacturer's instructions. Transduction of the virus into IC2 cells was performed using the ViraDuctin Retrovirus Transduction Kit (Cell BioLabs) according to the manufacturer's instructions and followed by positive selection with 1.8 μ g/mL puromycin for 1 week. Expression was confirmed with flow cytometry using PE-conjugated anti-human CD117 (BioLegend, San Diego, CA, USA) and PE-conjugated isotype control (BD Biosciences, San Jose, CA, USA) on a LSRII flow cytometer (BD Biosciences) and using FlowJo software (Tree Star, Ashland, OR). Aliquots of the stable transduced cell lines were frozen and revived as needed.

Mast cell growth and metabolism assays

Following puromycin selection, the *KIT*-transduced IC2 cells were washed free of IL-3 and cultured in the presence or absence of human SCF (hSCF; 100 ng/mL; PeproTech) for 1 week. To assess cell proliferation differences, 1×10^5 cells/mL were subsequently seeded in a six-well plate, and viable cells were counted daily using the Cellometer Auto T4 cell counter (Nexcelom Bioscience, Lawrence, MA, USA). An MTT assay was performed to assess cell metabolism by measuring the activity of living cells via mitochondrial dehydrogenase activity (Sigma-Aldrich, St. Louis, MO, USA). Cells (2×10^4) in 100 μ L medium were incubated in a 96-well plate for 24 hours. Medium was used as the blank control. After 21 hours, 10 μ L of MTT solution was added into each well and incubated for 3 hours. The cells were centrifuged at 3000 rpm for 10 min; 75 μ L supernatant was removed from the samples and controls. MTT solvent (100 μ L) was added into each well and resuspended thoroughly to dissolve the crystals. Absorbance was measured at 570 nm within 1 hour.

Mast cell maturation studies

The *KIT*-transduced IC2 cells were cultured in the presence or absence of hSCF 100 ng/mL (PeproTech) for 4 weeks. To assess granule formation, cytospin preparations were prepared and toluidine blue staining was performed [22]. In addition, 1×10^5 cells were lysed in 100

μL of H_2O , and the histamine content was measured by ELISA (Cell-cult/Urine kit, Immuno-Biological Laboratories, Minneapolis, MN, USA) according to the manufacturer's instructions.

Immunoblotting

Cell lysates were prepared using 1×10^6 cells and 25 μL aliquots were loaded on to 4–12% NuPAGE Bis-Tris gels (Invitrogen) for electrophoretic separation and immunoblotting as described previously [23]. The following antibodies were used for immunoblotting: anti-KIT mAb (Cell Signaling, Danvers, MA, USA), anti-phospho-KIT pAb (pY936; Life Technologies), anti-Akt pAb, anti-phospho-Akt pAb (Ser473), anti-Erk pAb, anti-phospho-Erk pAb (Thr202/Tyr204) pAb (Cell Signaling), and anti- β -actin mAb (Sigma). Immunoreactive proteins were visualized with enhanced chemiluminescence ECL (Thermo Scientific, Rockford, IL, USA).

Mast cell drug inhibition assays

The *KIT*D816V transduced IC2 cells were plated at 4×10^5 cells/mL with PKC412 (1–1000 nmol/L) in a final concentration of 0.1% DMSO [24]. After 72 hours, an equal volume of $2 \times$ CyQuant direct detection reagent (Invitrogen) was added to the cells in culture. Following a 1-hour incubation at 37°C with detection reagent, sample fluorescence was detected by using 508/527-nm wavelength filter sets.

Statistical analysis

The analysis was conducted primarily with a nonparametric approach. Thus, comparisons between groups were made with the Wilcoxon rank sum test, correlations were estimated with the Spearman ρ , and the paired comparison was made with Wilcoxon signed rank test. IC_{50} values were estimated from a sigmoidal dose-response model, in which differences were assessed with an F test; $p < 0.05$ was considered statistically significant. Analysis was performed using PRISM software, version 5 (GraphPad).

Results

GNNK[−]/GNNK⁺ copy number ratio and GNNK[−] relative expression increase with percent neoplastic mast cell involvement in systemic mastocytosis

We first examined the cDNA of bone marrow mononuclear cells obtained from normal volunteers and patients with systemic mastocytosis for the absolute expression of the GNNK variants. The absolute copy number of each GNNK variant was derived using standard curves, and the GNNK[−]/GNNK⁺ copy number ratio was calculated for each sample. No statistical difference between copy number ratios in normal and mastocytosis patients was observed ($p = 0.779$; Fig. 2A). However, the copy number ratios increased with percent mast cell involvement, and the correlation approached significance (Spearman $\rho = 0.39$; $p = 0.053$; Fig 2B).

To determine whether preferential expression of one or both variants contributes to this observation, the relative expression of each variant was examined as compared to a healthy control. No correlation between the relative expression of the GNNK⁺ variant and percent mast cells was observed (Spearman $\rho = 0.15$; $p = 0.477$; Fig 2C), whereas GNNK[−] relative expression directly correlated with the percent mast cells in the bone marrow mononuclear cell fraction (Spearman $\rho = 0.51$; $p = 0.009$; Fig 2D). Too few mast cells were present in the healthy control bone marrow mononuclear cells to determine whether this trend observed in patients with mastocytosis was also present in the general population.

***KIT* D816V transcripts have higher GNNK⁻/GNNK⁺ copy number ratios in systemic mastocytosis**

Given the association of the *KIT*D816V mutation with mast cell neoplasia, we next investigated whether *KIT*D816V was preferentially present in either GNNK variant. The *KIT*D816V PCR/RFLP assay was performed on purified GNNK⁺ or GNNK⁻ PCR amplification products from patients with mastocytosis and healthy control subjects. Representative gels are shown in Figure 3A. As expected, *KIT*D816V was not detected in the healthy control subjects or an SM-MDS patient with wild type *KIT* (Patient 19). A *KIT* D816V-specific RFLP product (188 bp), however, was detectable in both GNNK⁺ and GNNK⁻ transcripts from systemic mastocytosis patients harboring the *KIT*D816V mutation (Patients 15, 16, 17, and 20).

With the *KIT*D816V confirmed in both GNNK variants, we next determined the GNNK⁻/GNNK⁺ copy number ratio of *KIT*D816V-specific transcripts. A *KIT*D816V allele-specific primer was used to amplify *KIT*D816V containing transcripts from patients with *KIT*D816V-positive systemic mastocytosis (n = 21). These *KIT*D816V-enriched transcripts displayed a significantly higher GNNK⁻/GNNK⁺ copy number ratio compared with the total *KIT* transcript pool of the same sample ($p < 0.0001$, Wilcoxon signed rank test; Fig 3B). These data thus support our earlier observation that GNNK⁻ predominance is associated with increasing mast cell involvement (Fig 2D).

GNNK⁻/GNNK⁺ copy number ratio in bone marrow mononuclear cells was unable to distinguish disease severity in systemic mastocytosis

We next attempted to determine a potential association of the GNNK variants with disease severity. The subjects with systemic mastocytosis were divided into three groups: (1) indolent systemic mastocytosis (ISM), (2) indolent with one B finding (ISM-B) or smoldering systemic mastocytosis (SSM), and (3) advanced (ADV) disease as indicated in Table 1 [18]. No statistical difference in GNNK⁻/GNNK⁺ copy number ratio (or *KIT* D816V enriched copy number ratio) was detected between the three disease groups (data not shown). It should be noted, however, that there was no significant difference in the median percentage of mast cells in the bone marrow mononuclear cell fraction between the groups (ISM, 0.07%; ISM-B/SSM, 0.14%; ADV, 0.07%). Other groups have also reported that mast cell involvement within the bone marrow mononuclear cell fraction can be similar in indolent and other forms of systemic mastocytosis [25].

Enhanced IC2 mast cell growth and maturation is associated with the GNNK⁻ *KIT* isoform

To explore the functional effect of these clinical observations, we stably transduced the immature murine mast cell line, IC2, with human WT *KIT* and *KIT* D816V in both the GNNK⁺ and GNNK⁻ isoforms. Comparable surface and total *KIT* expression was observed between the GNNK isoforms in cells transduced with either WT *KIT* or *KIT* D816V (Supplementary Fig 2, online only, available at www.exphem.org). The GNNK⁻ isoform significantly enhanced mast cell proliferation (WT⁻ in Fig 4A; $p = 0.004$, Wilcoxon rank sum test) and metabolism (WT⁻ in Fig 4C; $p < 0.0001$, Wilcoxon rank sum test) of WT *KIT* transduced cells cultured in hSCF at day 3. No difference in the growth rate was observed in cells transduced with *KIT* D816V in either isoform (D816V⁺ and D816V⁻ in Fig 4A). These observations were supported by metabolic studies (MTT assay) displaying similar results (Fig 4C). In the absence of hSCF, only *KIT*D816V transduced cells survived and, although cell proliferation was similar between GNNK variants (Fig 4B), a significant difference ($p = 0.003$, Wilcoxon rank sum test) in SCF-independent metabolism was observed (Fig 4C).

Upon culturing the *KIT*-transduced IC2 cells in the presence or absence of hSCF for 4 weeks, cell morphology was examined using toluidine blue staining. As predicted, the

control cells (IC2 cells transduced with empty vector, cultured in mouse IL-3; Fig 5A) were hypogranular in appearance. In contrast, notable granule formation in all KIT-transduced cells was observed (Fig 5B). This difference in the number of granules between the empty vector-transduced cells and the KIT-transduced cells was reflected by the histamine content in the cells. In the presence of hSCF, the GNNK⁻ isoform significantly increased the histamine content in WT KIT (WT⁻ in Fig 5C; $p = 0.005$, Wilcoxon rank sum test) and KIT D816V (D816V⁻ in Fig 5C; $p = 0.03$, Wilcoxon rank sum test) transduced cells. The KIT D816V cytokine-independent histamine content was also significantly increased by the GNNK⁻ isoform (D816V⁻ in Fig 5C; $p = 0.007$, Wilcoxon rank sum test). Of note, despite increased granule formation and Fc RI expression, immunoglobulin E-mediated degranulation of the transformed IC2 cells remained deficient.

GNNK⁻ isoform increases KIT D816V activation of AKT and decreases KIT D816V sensitivity to the tyrosine kinase inhibitor, PKC412, in the absence of SCF

Immunoblotting was performed to investigate the effect of the GNNK isoforms on KIT D816V signaling (Fig 6A). With comparable levels of KIT D816V expression, KIT phosphorylation was similar between isoforms independent of hSCF. However, AKT activation appeared enhanced by the GNNK⁻ isoform with the GNNK⁺ isoform displaying a moderate dependence on hSCF.

We next investigated the potential therapeutic consequences of the determination that the GNNK variants might influence KIT D816V-driven, cytokine-independent mast cell activity. Inhibition assays were performed in the KIT D816V transduced mast cells with the tyrosine kinase inhibitor, PKC412. In the presence of hSCF, no statistical differences in the IC₅₀ values between GNNK variants were observed (Fig 6B). However, a significant difference in IC₅₀ values ($p = 0.003$, F test) was observed in the absence of hSCF. KIT D816V associated with the GNNK⁺ isoform was more sensitive to PKC412 (IC₅₀ = 32.2 nmol/L) when compared with KIT D816V associated with the GNNK⁻ isoform (IC₅₀ = 95.7 nmol/L; Fig 6C), whereas KIT D816V associated with the GNNK⁻ isoform displayed virtually identical IC₅₀ values regardless of the presence of hSCF (IC₅₀ = 98.4 nmol/L with SCF; IC₅₀ = 95.7 nmol/L without SCF).

Together, these studies suggest that the GNNK⁻ isoform is more supportive of KIT D816V cytokine-independent metabolism, maturation, and therapeutic resistance to PKC412.

Discussion

In this study, we present evidence to support the conclusion that the *KIT*GNNK⁻ variant is predominantly expressed in the mast cells of patients with systemic mastocytosis and specifically in the *KIT*D816V containing transcripts. The GNNK⁻/GNNK⁺ copy number ratio in the bone marrow mononuclear cells of systemic mastocytosis appeared similar to that in healthy control subjects (Fig 2A), which agrees with the reported conclusion that no differences are present [14,26]. However, further analysis suggested that the GNNK⁻/GNNK⁺ copy number ratio increases because mast cells comprise a larger percent of the of the bone marrow mononuclear cells (Fig 2B). This observation was maintained when specific amplification of the *KIT*D816V transcripts resulted in an elevated GNNK⁻/GNNK⁺ copy number ratio (Fig 3B). This novel observation in systemic mastocytosis is supported by a similar preferential expression of the GNNK⁻ variant in gastrointestinally pronounced systemic mast cell activation disorders [27] and an increased GNNK⁻/GNNK⁺ ratio in mucosal biopsy specimens infiltrated by mast cells [11].

In an effort to determine whether altered expression of one or both GNNK variants contributes to the increased GNNK⁻/GNNK⁺ ratio, the relative expression of each was

examined. As mast cell numbers increase, a positive correlation with both variants would be expected; however, a significant correlation was observed only with the GNNK⁻ variant. We surmise that GNNK⁺ expression in the mast cell compartment is low compared with the other KIT-expressing cells in the sample; therefore, a significant correlation was not established. Additional studies using enriched mast cell populations could confirm this hypothesis and determine whether the GNNK variants influence disease prognosis. Variable mast cell recovery in the bone marrow mononuclear cell fraction, particularly in advanced disease, clearly limited our assessment of disease severity. It is likely that bone marrow fibrosis, frequently encountered in advanced disease, might have contributed to a diminished yield not reflective of the disease state. As it is generally accepted that disease severity increases with increasing total mast cell burden, analyzing the heavily mast cell infiltrated core biopsy might offer an improved approach to correlate increased GNNK⁻/GNNK⁺ copy number ratios with advanced disease.

We selected the IL-3-dependent immature murine mast cell line, IC2, to investigate potential proliferation and developmental differences between the KIT GNNK isoforms [21]. This system has been used to examine the effect of WT and mutated mouse KIT on mast cell growth factor independence, tumorigenicity, differentiation, and signal transduction [28–30]. In this system, transfection of IC2 cells with the GNNK⁻ isoform led to an apparent increase in granule formulation, along with an increase in histamine content, reflecting enhanced maturation. This increase was likely a consequence of the enhanced signaling potential associated with the GNNK⁻ isoform [7,8,31,32].

As predicted, only with the expression of the *KIT*D816V mutation were cytokine-independent cell growth and development achieved [33]. Although the GNNK variants had no significant effect on *KIT*D816V cell proliferation, differences in metabolism, mast cell development, and drug sensitivity suggested that the KIT extracellular GNNK region could retain an influence on intracellular signaling in the *KIT*D816V-driven mast cells. Indeed, enhanced signaling through AKT was observed in *KIT*D816V associated with the GNNK⁻ isoform—a pathway known to be critical to the maturation of mast cells [34]. In line with our findings, differences in AKT activation between GNNK isoforms with similar levels of KIT phosphorylation have been reported [31]. Of significance, a study performed in Ba/F3 cells [33] reported that the D816V mutation eliminated differences in biological response between the two GNNK isoforms. Although mast cell differentiation antigens have been induced in this KIT-independent pro-B cell lineage [35], our study supports that KIT-driven biological differences exist between cell lines [31] and a mast cell background might offer a more reflective disease model [28,29].

In general, hSCF had minimal influence on the activity of KIT D816V with the GNNK⁻ isoform (increased oncogenic potential), whereas the GNNK⁺ isoform retained a modest reliance on hSCF. This finding was exemplified in the PKC412 inhibition studies in which the GNNK⁺ variant was more sensitive in the absence of hSCF. In support of this observation, increased resistance to therapy has recently been described in multiple myeloma cell lines transduced with the WT GNNK⁻ variant compared with the WT GNNK⁺ variant, with AKT activation proposed as a possible mechanism of resistance [7]. Constitutive AKT activation is reported to protect partially against PKC412 cytotoxicity [36] and might account for the survival advantage we observed in *KIT*D816V with the GNNK⁻ isoform. Preclinical studies seldom define the GNNK variant used for KIT signaling experiments, and this study suggests that this would be of value.

Our combined observation that a GNNK⁻ predominance exists in *KIT*D816V systemic mastocytosis and that the GNNK⁻ isoform might enhance KIT D816V-driven mast cell activity is provocative. It can be speculated that *KIT* GNNK splicing can influence or

contribute to the molecular pathogenesis of the disease. Significant questions remain to be explored relating to the controls by which *KIT* is spliced, especially whether the D816V mutation influences GNNK⁻ splicing preference in human mast cells. These questions are beyond the scope of this current study, but may lie at the heart of understanding the oncogenic transformation occurring in systemic mastocytosis as well other KIT driven neoplastic disorders.

In summary, our study contributes to the growing evidence that RNA splicing abnormalities are frequent in hematologic disorders, including systemic mastocytosis. More importantly, such alternative splicing might influence the biological activity and response of the transformed cells to therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic diagram of KIT protein. The two major isoforms of KIT are characterized by the presence or absence of four amino acids (GNNK⁻ or GNNK⁺) in the juxta-membrane region of the extracellular domain. The KIT D816V activating mutation is located in the intracellular tyrosine kinase domain. Adapted from Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev.* 2009;228:149–169.

Figure 2.

GNNK⁻/GNNK⁺ copy number ratio and GNNK⁻ variant relative expression increase with neoplastic mast cell involvement. A real-time qPCR assay was used to analyze the expression of GNNK⁺ and GNNK⁻ variants in the bone marrow mononuclear cells of healthy control subjects (n = 16) and patients with systemic mastocytosis (n = 25). **(A)** GNNK⁻/GNNK⁺ copy number ratio shown for individuals in each study group. For the patients with mastocytosis, **(B)** GNNK⁻/GNNK⁺ copy number ratio **(C)** GNNK⁺ relative expression, and **(D)** GNNK⁻ relative expression were plotted against the percentage of mast cells in the bone marrow mononuclear cell fraction. All reactions were performed in triplicate, and specificity of the reactions was confirmed by dissociation curve analysis.

Figure 3.

*KIT*D816V is present in both GNNK variants, and *KIT*D816V transcripts have higher GNNK⁻/GNNK⁺ copy number ratios in systemic mastocytosis. **(A)** The *KIT*D816V PCR/RFLP assay was performed on purified GNNK⁻ or GNNK⁺ PCR amplification products from normal controls (n = 16) and patients with systemic mastocytosis (n = 25). The presence of the *KIT*D816V was confirmed by the generation of a 188-bp band in the patients known to be positive for the D816V mutation (N = 21; in this representative blot, patients 15, 16, 17, and 20). **(B)** For individual patients with mastocytosis with the *KIT* D816V mutation (n = 21), the GNNK⁻/GNNK⁺ ratio of *KIT*D816V-specific transcripts was determined, and a paired analysis with the GNNK⁻/GNNK⁺ ratio of total *KIT* transcripts was performed.

Figure 4.

GNNK⁻ isoform enhances hSCF-induced WT KIT proliferation and KIT D816V factor-independent IC2 mast cell proliferation. The immature murine mast cell line, IC2, was stably transduced with human WT KIT and KIT D816V in either the GNNK⁺ or GNNK⁻ variant. After culturing for 1 week in (A) the presence of hSCF or (B) no cytokine, 2×10^5 cells were seeded in 2 mL of media and counted daily for 3 days. The data are presented as the mean $\pm$ SEM of three separate experiments each performed in duplicate. (C) Metabolism of the cells cultured in the presence of hSCF or no cytokine for 1 week was examined using an MTT assay. The data are presented as the mean $\pm$ SEM of three separate experiments each performed in triplicate. *Control* = IC2 cell transduced with empty vector; *D816V⁺* = D816V with GNNK⁺ isoform; *D816V⁻* = D816V with GNNK⁻ isoform; *WT⁺* = WT with GNNK⁺ isoform; *WT⁻* = WT with GNNK⁻ isoform.

Figure 5.

GNNK⁻ isoform enhances KIT IC2 mast cell granulation and histamine content. IC2 mast cells stably transduced human WT KIT and KIT D816V in either the GNNK⁺ or GNNK⁻ variant were cultured in the presence of hSCF or no cytokine for 4 weeks. (A) Toluidine blue staining of the empty vector control compared with (B) KIT transduced cells. (C) Histamine content of the KIT transduced cells cultured in hSCF or cytokine free medium. The data are presented as the mean $\pm$ SEM of six separate experiments each performed in duplicate. *Control* = IC2 cell transduced with empty vector cultured in IL-3; *WT⁺* = WT with GNNK⁺ isoform; *WT⁻* = WT with GNNK⁻ isoform; *D816V⁺* = D816V with GNNK⁺ isoform; *D816V⁻* = D816V with GNNK⁻ isoform. Scale bars = 10 μ m.

Figure 6.

GNNK⁻ isoform enhances KIT D816V signaling and factor-independent resistance to PKC412. IC2 mast cells, stably transduced with KIT D816V in either the GNNK⁺ or GNNK⁻ variant, were cultured in the presence of hSCF or no cytokine for 1 week. **(A)** Activation of the KIT D816V transduced cells was investigated by immunoblotting using antibodies against p-KIT, pAKT, and pERK. To assess potential expression differences, immunoblotting of KIT, AKT, ERK, and Actin was performed in tandem. The blots are representative of three separate experiments. The KIT D816V transduced cells cultured in **(B)** the presence of hSCF or **(C)** no cytokine were subsequently cultured with PKC412 (1–

1000 nmol/L, 0.1% dimethyl sulfoxide) for 72 hours, and the CyQuant cell proliferation assay was performed to assess growth inhibition. Values represent the percent of viable cells compared with untreated control cells. The data are presented as the mean $\pm$ SEM of three separate experiments each performed in triplicate.

Table 1

Characteristics of study subjects with systemic mastocytosis

Patient	WHO classification	Age (y)	Gender	% mast cells of bone marrow mononuclear cells	% mast cells of bone marrow core biopsy	Serum tryptase (ng/mL)	CD2/CD25 expression on mast cells	KIT D816V
1	Indolent	44	F	0.01	3	5	+	+
2	Indolent	71	F	0.07	15	55	+	+
3	Indolent	65	M	0.05	20	78	+	+
4	Indolent	49	M	0.06	7	61	+	+
5	Indolent	51	F	0.14	20	56	+	+
6	Indolent	73	M	0.16	15	75	+	+
7	Indolent	54	M	0.09	20	37	+	+
8	Indolent	28	F	0.01	3	16	+	+
9	Indolent	54	M	0.01	10	66	+	+
10	Indolent	60	F	0.20	10	136	+	+
11	Indolent (B)	34	F	0.22	40	88	+	+
12	Indolent (B)	24	F	0.19	30	55	+	-
13	Indolent (B)	68	F	0.05	40	186	+	+
14	Smoldering	40	F	0.3	60	115	+	+
15	Smoldering	43	F	0.16	30	369	-	+
16	Smoldering	64	M	0.08	10	96	+	+
17	Smoldering	59	F	0.04	20	365	+	+
18	Smoldering	52	M	0.12	30	236	+	+
19	SM with MDS	67	M	0.07	30	18	+	-
20	SM with ET	59	M	0.08	10	84	+	+
21	SM with CMML	74	F	0.56	60	762	+	-
22	Aggressive	52	F	0.13	50	171	+	-
23	Aggressive	55	F	0.06	40	234	+	+
24	Aggressive	50	M	0.03	15	58	+	+
25	Aggressive	47	M	0.04	10	22	+	+

CMML = chronic myelomonocytic leukemia; ET = essential thrombocythosis; F = female; Indolent (B) = indolent with one B finding; M = male; MDS = myelodysplastic syndrome; SM = systemic mastocytosis; WHO = World Health Organization.

Light gray shading indicates the indolent disease group; medium gray indicates the indolent (B)/smoldering disease group; dark gray indicates the advanced disease group.

Normal serum tryptase <11.5 ng/mL.