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SRSF2-P95 Hotspot Mutation is Highly Associated with Advanced Forms of Mastocytosis and Mutations in Epigenetic Regulator Genes

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Abstract

Mastocytosis is a rare and chronic disease with phenotypes ranging from indolent to severe. Prognosis for this disease is variable and very few biomarkers to predict disease evolution or outcome are currently known. We have performed comprehensive screening in our large cohort of mastocytosis patients for mutations previously found in other myeloid diseases and that could serve as prognostic indicators. *KIT*, SRSF2-P95 and *TET2* mutations were by far the most frequent, detected in 81, 24 and 21 percent of patients, respectively. Where *TET2* and SRSF2-P95 mutation both correlated with advanced disease phenotypes, SRSF2-P95 hotspot mutation was found almost exclusively in patients diagnosed with associated clonal hematologic non-mast cell-disease. Statistically, *TET2* and SRSF2-P95 mutations were highly associated, suggesting a mechanistic link between these two factors. Finally, analysis of both clonal and sorted cell populations from patients confirms the presence of these mutations in the mast cell component of the disease, suggest an ontological mutation hierarchy and provide evidence for the expansion of multiple clones, highlighting the prognostic potential of such approaches, if applied systematically, for delineating the roles of specific mutations in predisposing and/or driving distinct disease phenotypes.

Keywords

Mastocytosis, AHNMD, SRSF2, *TET2*, mutation

Introduction

Mastocytosis is a rare and clonal hematopoietic disorder described by the accumulation of abnormal mast cells in the bone marrow[1,2]. In adults, mastocytosis most often presents as a persistent systemic disorder of variable course and prognosis[3,4]. Disease phenotypes range from indolent to aggressive and are defined by WHO criteria: mainly B- and C-findings that describe the extent of organ and tissue damage resulting from systemic mast cell infiltration. In about 40 percent of cases, systemic mastocytosis is diagnosed in conjunction with associated clonal haematological non-mast cell lineage diseases, AHNMD, which include myelodysplastic syndromes (MDS), myeloproliferative neoplasm (MPN) as well as both acute and chronic forms of myeloid leukemia (AML, CML, CMML)[4, 5].

It remains unclear why in some cases mastocytosis evolves aggressively while in other cases the disease remains indolent. Efforts to discriminate and to predict the clinical course of mastocytosis have uncovered genetic mutations that figure prominently in this disease. KITD816V mutation is the most common (>80% of mastocytosis cases) and is thought to drive the expansion of affected clones towards the mast cell lineage[6], but does not segregate with advanced disease. By contrast, TET2 mutation, found in about 20% of patients, is associated with aggressive forms of mastocytosis[7]. Mutations in other epigenetic modifiers have been described, but so far they have not been clearly associated to any particular form of disease and, overall, their prognostic relevance is not clear[8].

More recently, a hotspot mutation in SRSF2, a component of the RNA splicing machinery, has been identified and associated with leukemic transformation[9,10]. Among myelodysplastic syndromes and other haematological disorders, SRSF2 mutation is

most frequent in CMML, with reports ranging from 28.4 to 47.2%[11]. Like TET2, SRSF2 mutation occurs early in disease ontogeny and has been dubbed a founder mutation[12]. As such, SRSF2 mutation is thought to pre-dispose early progenitor cells to malignant selection, perhaps via its role in the acetylation/phosphorylation network and as an important regulator of DNA stability and mRNA splicing[13]. We have now sequenced for SRSF2 mutation in our cohort of mastocytosis patients, previously characterized for both KIT and TET2 mutations[7], and reveal a striking association between SRSF2 mutation and advanced disease types.

Methods

Patient Data

72 patients (35 men/37 women) with mastocytosis diagnosis as defined by the WHO criteria[14] were enrolled in a prospective national multicentre study between 2005 and 2013. The cohort consists of patients diagnosed with cutaneous mastocytosis (CM), CM (type TMEP), MCAS clonal, indolent SM (ISM), systemic mastocytosis with AHNMD (SM-AHNMD), Aggressive SM (ASM), mast cell leukemia (MCL) and mast cell sarcoma (Supplementary Table 1) [1-3]. AHNMD diagnosis for each patient has been indicated (Fig.1), and detailed in Supplementary Table 1. Patients were further grouped using the operational terms “advanced” and “non-advanced” to account for the number of patients in certain classifications, MCL and mast cell sarcoma for example that were not large enough for statistical analysis. Statistical analysis of predictive factors (anemia, blast count, thrombocytopenia, hypoalbuminemia etc.), *KIT* and *TET2* mutation for this cohort have been presented elsewhere[7]. All patients were included in a mastocytosis pathophysiological study, which started in 2003 and is sponsored by the Association-

For-Initiative-and-Research-on-Mast cell-and-Mastocytosis (AFIRMM). The study was approved by Necker Hospital ethical committee, and carried out according to the Helsinki convention. Each patient provided informed consent.

Mutation Screening

Mutation analysis for *KIT* and *NRAS*, *KRAS* and *TET2* has been described[15,16]. DNA Sanger-sequencing of exon-coding sequences of *ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2* and *EZH2* was performed as described [17], *SRSF2, U2AF1*, *ZSRSR2* as described [11], and *SF3B1* as described [18].

Clonal Analysis

Leukocytes were purified using Ficoll^{R} (Sigma) from fresh bone marrow from patients and plated at low density in methocult medium (H4035 without Epo, StemCell Technologies). Individual colonies were isolated at day 10-12 of culture and DNA was isolated for mutation screening.

FACS Sorting

Fresh or frozen whole bone marrow biopsy material from patients was stained with the following antibody cocktail: anti-humanCD3-ECD, anti-humanCD14-alexa647, anti-humanCD25-PE and anti-humanFcepsilonGR1a-FITC (all from BD Biosciences). Cells were sorted on an LSRII and using DIVA^{TM} (Becton-Dickinson) software. Sorted cell populations were directly lysed and DNA was isolated for mutation screening.

Statistical analysis

Statistical comparisons for predictive factors and mutations were based on Fisher's exact test. All reported p values were two tailed with confidence intervals of 95%. Survival data were analyzed using GraphPad Prism software version 5.01 (GraphPad

Software Inc., San Diego, CA) and both the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were applied to survival curves to determine significant differences.

Results

SRSF2P95 hotspot mutation is highly correlated with advanced forms of mastocytosis

Total bone marrow or peripheral blood was collected from a cohort of 72 patients with mastocytosis, categorized according to the WHO classification and for advanced or non-advanced disease (SupTable 1). Biopsied material from these patients was screened for mutation in genes commonly mutated in mastocytosis or other myeloid diseases (Fig 1). After *KIT* (81%), the SRSF2-P95 hotspot mutation was the most frequent mutation found in these patients: 17/72 (23.6%) patients. We detect four different mutations of the SRSF2-P95 codon in patients: SRSF2-P95H, SRSF2-P95L, SRSF2-P95T and SRSF2-P95R (SupTable 1).

We have previously reported an association between TET2 and advanced systemic mastocytosis[7], and, like *TET2*, we also see a significant correlation between SRSF2-P95 mutation and advanced disease ($p<0.0001$) (Table 1). Unlike *TET2* mutation that was found only in patients positive for KIT mutation[7], 3 patients that were negative for *KIT* mutation were positive for SRSF2-P95 mutation. *SRSF2* mutation also coincides significantly with the presence of AHNMD (15/17 patients with SRSF2-P95 mutation present with AHNMD), but not with any particular form of AHNMD (Table 2).

Advanced forms of mastocytosis are associated with short overall survival

Survival curves were generated to compare the survival of mastocytosis patients with advanced disease versus non-advanced disease (Fig.2A). We find a significant

reduction in the survival time at diagnosis for patients diagnosed with advanced forms of mastocytosis ($p>0.001$). Since the vast majority of patients with SRSF2-P95 mutation have advanced disease types, to eliminate this bias, we next generated survival curves for patients diagnosed with advanced disease types only, to compare survival time of patients with or without SRSF2-P95 mutation (Fig.2B). We find no significant difference between the overall survival of patients with advanced disease and SRSF2-P95 MUT compared to patients with advanced disease and SRSF2-P95 WT ($p>0.5$, Fig. 2B). Together, advanced disease diagnosis and not SRSF2-P95 mutation appears to be the dominant factor for predicting shorter overall survival times in patients.

Interestingly, 3 patients positive for SRSF2-P95 mutation at the original time of diagnosis developed an AHNMD during the course of this study (within 2 years): Patient M40 originally diagnosed with SM developed AHNMD (MDS), Patient 1318 originally diagnosed with ISM progressed to SM-AHNMD, and patient D60 originally diagnosed with ASM developed MCL (SupTable 1 and Fig.1, dotted boxes).

SRSF2-P95 mutation is significantly associated with mutations in epi-regulators

In addition to KIT and SRSF2-P95, amplicon screening was also performed for other known mutations: *SF3B1, U2AF1* and *ZRSR2* (splicing factors), *TET2, IDH1*, *IDH2, AXSL1, DNMT3A, EZH2* (epigenetic regulators), as well as *JAK2, CBL, NRAS* and *KRAS* (SupTable 1 and Fig.1).

Generally, more mutations were found in patients with advanced disease than patients with non-advanced disease. After *KIT*, SRSF2-P95 and *TET2* mutation were by far the most frequent mutations found in patients: 24 and 21 percent, respectively. Interestingly, statistical analysis shows that TET2 mutation is significantly correlated to SRSF2-P95 mutation in these patients ($p<0.01$) (Table 3), and an even more significant

correlation is found when mutations in epigenetic factors are considered as a whole (mutation of SRSF2 in combination with at least one epigenetic factor, $p<0.0001$).

Consistent with a previous report showing that SF3B1 mutations are infrequent in mast cell diseases[19], *SF3B1* mutations were detected in only 4 (5.6%) of our patients: 2 patients had mutations in exon 14 (codon 666 and the other at codon 663), and 2 patients had mutations in exon 15. We also identified 2 patients with mutation in *U2AF1* (both at codon 101 in exon 2). Interestingly, mutations in splicing factors *SRSF2, SF3B1* and *U2AF1* are exclusive in patients (Fig.1 and SupTable 1).

SRSF2-P95 mutation is present in mast cells

Given the strong association between *SRSF2* mutation and AHNMD, to ensure that the *SRSF2* mutation was present in the mast cell component of the disease, we sorted cell populations from total bone marrow of 7 patients with advanced disease phenotypes for genotype analysis (Fig.3A). Genomic DNA from the three collected populations, T-cells, monocytes and mast cells, was isolated and genotyped for *KIT, TET2* and *SRSF2* mutations (Fig.3B). In all 7 patients for which sorted populations were analysed, *KIT, SRSF2* and *TET2* mutations, when originally detected in whole bone marrow biopsies (SupTable 1) were also detected in the mast cell and monocyte compartments. In all patients *KIT, TET2* and *SRSF2* mutations were variably present in T-cells, depending on when, during hematopoietic differentiation, the individual mutations were acquired. For patient M40, in which *KIT* mutation was not detected, we detected JAK2 V617F mutation in mast cells and monocytes, but not in T-cells. And finally, in patients presenting with two different *TET2* mutations, both mutations were present in positive cell populations. Interestingly, in one patient, 1445, we detected

SRSF2 mutation in T-cells where *TET2* and *KIT* mutations were present only in mast cells and monocytes.

SRSF2-P95 mutation can occur early or late during clonal evolution

To determine the relative timing of *SRSF2* mutation during clonal evolution, we examined individual colonies derived from total bone marrow of 2 patients with advanced disease and KITD816V, *TET2* and SRSF2-P95 mutations. Mutational analysis of single colonies distinguished three different patterns of mutation accumulation (Fig.3C, arrows). In both patients, we identified clones harbouring *TET2* mutation alone, *TET2* and *SRSF2, TET2* and *KIT* or all three mutations. These data are consistent with *TET2* mutation preceding both *SRSF2* and *KIT* mutation in both patients, and either *SRSF2* or *KIT* mutation occurring next. Interestingly, where mutation screening of whole bone marrow for patient F50 identified KITD816V, TET2 Q1389* and SRSF2-P95L mutations (SupTable 1), clonal analysis revealed 5/14 colonies harbouring a distinct SRSF2-P95H mutation. Both SRSF2-P95L and SRSF2-P95H colonies harboured TET2 Q1389* mutation, but only SRSF2-P95L colonies were positive for KITD816V mutation.

Discussion

We now report that the SRSF2-P95 hotspot mutation is highly correlated with advanced forms of mastocytosis, and in contrast to *TET2*, is associated almost exclusively with, and might predict the onset of AHNMD.

We also find a small number of patients negative for the SRSF2-P95 mutation but harbouring a mutation in another splicing factor, either *SF3B1* or *U2AF1.* Combined, spliceosome mutations were found in as many as 32% of patients, pointing to a

pathogenic role for abnormal RNA splicing in SM. In addition to its role as an SR protein in promoting alternative exon inclusion and integrating other steps in RNA metabolism[22], a unique role for SRSF2 has been described in regulating PolII pausing and elongation at promoters[23, 24]. SF3B1 is part of a much larger complex associated with the catalytic activity of the spliceosome, and U2AF1 is an auxiliary U2-factor involved in splice-site recognition. So far, among these factors, only U2AF1 has been directly associated with the deregulated splicing of a specific, cancer relevant target: EZH2[25]. Future studies to address the global or specific role of gene splicing in haematological diseases should be informative in dissecting the molecular contribution of SRSF2 and other splicing mutations in these contexts.

Extensive mutation analysis of this cohort has also revealed an association between *SRSF2* mutation and mutation in genes whose products function in modifying the epigenome (epi-regulators), including *TET2*. This is consistent with a previous report showing an association between spliceosome mutations and mutations in epigenetic modifiers[26]. Mechanistically, splicing is often tightly coupled with transcription and recent work suggests that alternative splicing might be affected by chromatin structure and histone modification[27]. Together with their role in regulating DNA stability, it is interesting to speculate that *SRSF2* and epigenetic modifier mutations may act synergistically to promote advanced disease. At present, however, it is unclear why these mutations coincide with such high frequency.

Interestingly, from a clinical perspective, 3 patients with SRSF2-P95 mutation were re-diagnosed with more severe disease and AHNMD during the course of this study. Pursuant to reports of an association between *SRSF2* mutation, poor prognosis and leukemic progression of MPNs, these cases may provide support for a prognostic

relevance of SRSF2-P95 mutation in mast cells and in predicting advanced disease progression. A longer follow-up for the 2 patients with SRSF2-P95 mutation and no AHNMD as well as screening in all new patients for mutation in splicing factor genes will be necessary to validate this hypothesis. Indeed, here we have validated our sequencing results from whole bone marrow using sorted primary mast cells for a subset of patients. However, the advent of deep sequencing methods will in future be important and allow for more sensitive screening for mutations in mastocytosis patients using whole bone marrow, even when the mast cell burden in the bone marrow is low. By this approach, we may also reveal a larger number of mutations in patients with non-advanced disease to better address issues of specific mutations and their prognostic relevance.

Importantly, where *SRSF2* mutation has previously been associated with diseases relevant to AHNMD[26], mutation analysis of sorted cell populations from the bone marrow of mastocytosis patients shows that SRSF2-P95 mutation is present in both mast cells and monocytes from the bone marrow, supporting a role for SRSF2-P95 mutation in mast cell transformation and possibly a clonal relationship between the ASM and myeloid AHNMD components of this disease.

Finally, our previous analyses using clonal and sorted cell populations isolated from patient bone marrow, suggested that *TET2* mutation occurs prior to *KIT* mutation during clonal evolution of advanced forms of mastocytosis[7]. By this same approach, we now find that *SRSF2* mutation can occur relatively late during the ontogeny, while in other cases can precede both *TET2* and *KIT* mutation. We find evidence to support both: 1) a clone harbouring two mutations *TET2*, KITD816V but wild type for *SRSF2* was isolated from total bone marrow of a patient with advanced disease and positive for all

three mutations in the mast cell compartment (Fig.3B and C patient F50), and 2) SRSF2-P95 mutation was detected in all cell types from a patient where *TET2* and *KIT* mutation were detected in only mast cells and monocytes (Fig.3B, patient 1445). In this case, for SRSF2-P95 mutation to be present in T-cells, the mutation must have occurred either independently or else in a common progenitor, upstream of both *TET2* and *KIT* mutation. Since all three mutations are detected in both the mast cell and monocyte (AHNMD) components of the disease, it is unclear from these results whether clonal evolution favours one pattern over the other in different cell types. Finally, not only do these results reveal different patterns of mutation hierarchy and potentially multiple clones in a single patient, but they also suggest the possibility of parallel development of different diseases (mastocytosis and AHNMD).

Overall, we have performed a comprehensive screen for mutations previously associated with myeloproliferative disorders within our large cohort of mastocytosis patients. We report that in addition to TET2 mutation, mutation of the spliceosome factor SRSF2, is also frequent and correlates to advanced disease. In contrast to previous studies, our patient cohort contains a significant number of both advanced and non-advanced cases, and as such this study has a high clinical importance. Moreover, statistically, these two mutations are highly associated, suggesting that in addition to their known functions during differentiation, a mechanistic link between spliceosome and epigenetic regulators could promote transformation *in vivo*.

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Authorship and Disclosures

KH and JA processed/sequenced patient samples. SG-L, GD, MC, LC and OH contributed patient data and statistical analysis and with PdS and FB advised on manuscript. ES wrote the paper, designed/performed experiments. PD supervised all aspects.

The authors declare no conflict of interest and nothing to disclose.

References

1. Sánchez-Muñoz L, Alvarez-Twose I, García-Montero AC, Teodosio C, Jara-Acevedo M, Pedreira CE, et al. Evaluation of the WHO criteria for the classification of patients with mastocytosis. Mod Pathol. 2011; 24(9):1157-68.
2. Pardanani A. Systemic mastocytosis in adults: 2012 update on diagnosis, risk stratification, and management. Am J Hematol. 2012; 87(4):401-11.
3. Sperr WR, Valent P. Diagnosis, progression patterns and prognostication in mastocytosis. Expert Rev Hematol. 2012; 5(3):261-74.
4. Lim KH, Tefferi A, Lasho TL, Finke C, Patnaik M, Butterfield JH, et al. Systemic mastocytosis in 342 consecutive adults: Survival studies and prognostic factors. Blood. 2009; 113(23):5727-36.
5. Wang SA, Hutchinson L, Tang G, Chen SS, Miron PM, Huh YO, et al. Systemic mastocytosis with associated clonal hematological non-mast cell lineage disease:

Clinical significance and comparison of chomosomal abnormalities in SM and AHNMD components. Am J Hematol. 2013; 88(3):219-24.

6. Wilson TM, Maric I, Simakova O, Bai Y, Chan EC, Olivares N, et al. Clonal analysis of NRAS activating mutations in KIT-D816V systemic mastocytosis. Haematologica. 2011; 96(3):459-63.
7. Soucie E, Hanssens K, Mercher T, Georgin-Lavialle S, Damaj G, Livideanu C, et al. In aggressive forms of mastocytosis, TET2 loss cooperates with c-kitd816v to transform mast cells. Blood. 2012; 120(24):4846-9.
8. Vainchenker W, Delhommeau F, Constantinescu SN, Bernard OA. New mutations and pathogenesis of myeloproliferative neoplasms. Blood. 2011; 118(7):1723-35.
9. Kar SA, Jankowska A, Makishima H, Visconte V, Jerez A, Sugimoto Y, et al. Spliceosomal gene mutations are frequent events in the diverse mutational spectrum of chronic myelomonocytic leukemia but largely absent in juvenile myelomonocytic leukemia. Haematologica. 2013; 98(1):107-13.
10. Makishima H, Visconte V, Sakaguchi H, Jankowska AM, Abu Kar S, Jerez A, et al. Mutations in the spliceosome machinery, a novel and ubiquitous pathway in leukemogenesis. Blood. 2012; 119(14):3203-10.
11. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature. 2011; 478(7367):64-9.
12. Mian SA, Smith AE, Kulasekararaj AG, Kizilors A, Mohamedali AM, Lea NC, et al. Spliceosome mutations exhibit specific associations with epigenetic modifiers and proto-oncogenes mutated in myelodysplastic syndrome. Haematologica. 2013; 98(7):1058-66.

13. Edmond V, Moysan E, Khochbin S, Matthias P, Brambilla C, Brambilla E, et al. Acetylation and phosphorylation of SRSF2 control cell fate decision in response to cisplatin. EMBO J. 2011; 30(3):510-23.
14. Valent P, Horny HP, Escribano L, Longley BJ, Li CY, Schwartz LB, et al. Diagnostic criteria and classification of mastocytosis: A consensus proposal. Leuk Res. 2001; 25(7):603-25.
15. Bodemer C, Hermine O, Palmérini F, Yang Y, Grandpeix-Guyodo C, Leventhal PS, et al. Pediatric mastocytosis is a clonal disease associated with D816V and other activating c-kit mutations. J Invest Dermatol. 2010; 130(3):804-15.
16. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Massé A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009; 360(22):2289-301.
17. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, et al. Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. Genes, Chromosomes and Cancer. 2012; 51(8):743-55.
18. Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N Engl J Med. 2011; 365(15):1384-95.
19. Visconte V, Tabarroki A, Rogers HJ, Hasrouni E, Traina F, Makishima H, et al. SF3B1 mutations are infrequently found in non-myelodysplastic bone marrow failure syndromes and mast cell diseases but, if present, are associated with the ring sideroblast phenotype. Haematologica. 2013; 98(9):e105-7.
20. St John AL, Abraham SN. Innate immunity and its regulation by mast cells. J Immunol. 2013; 190(9):4458-63.

21. Donker ML, van Doormaal JJ, van Doormaal FF, Kluin PM, van der Veer E, de Monchy JG, et al. Biochemical markers predictive for bone marrow involvement in systemic mastocytosis. Haematologica. 2008; 93(1):120-3.

22. Zhong XY, Wang P, Han J, Rosenfeld MG, Fu XD. SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. Mol Cell. 2009; 35(1):1-10.

23. Lin S, Coutinho-Mansfield G, Wang D, Pandit S, Fu XD. The splicing factor SC35 has an active role in transcriptional elongation. Nat Struct Mol Biol. 2008; 15(8):819-26.

24. Ji X, Zhou Y, Pandit S, Huang J, Li H, Lin CY, et al. SR proteins collaborate with 7SK and promoter-associated nascent RNA to release paused polymerase. Cell. 2013; 153(4):855-68.

25. Khan SN, Jankowska AM, Mahfouz R, Dunbar AJ, Sugimoto Y, Hosono N, et al. Multiple mechanisms deregulate EZH2 and histone H3 lysine 27 epigenetic changes in myeloid malignancies. Leukemia. 2013; 27(6):1301-9.

26. Visconte V, Makishima H, Maciejewski JP, Tiu RV. Emerging roles of the spliceosomal machinery in myelodysplastic syndromes and other hematological disorders. Leukemia. 2012; 26(12):2447-54.

27. Brown SJ, Stoilov P, Xing Y. Chromatin and epigenetic regulation of pre-mrna processing. Hum Mol Genet. 2012; 21(R1):R90-6.

Table 1. SRSF2-P95 mutation is correlated with advanced mastocytosis

	Non-advanced	Advanced	Total	p-value
***SRSF2* mutation**				**<0.0001 (F)**
n	41	31	72	
WT	39 (95%)	16 (52%)	55 (76%)	
P95H/L/R/T	2 (5%)	15 (48%)	17 (24%)	

(F) Fisher's exact test

Table 2. Contingency analysis for SRSF2-P95 mutation and AHNMD

	SRSF2 WT	SRSF2-P95	Total	p-value
Associated disease				**<0.0001 (F)**
n	55	17	72	
no AHNMD	46 (84%)	2 (12%)	48	
AHNMD	9 (16%)	15 (88%)	24	
Type of AHNMD				**0.4731 (C)**
n	9	15	24	
CML	1	0	1	
AML	1	2	3	
CMML	0	4	4	
MDS	2	3	5	
MSD (AREB)	3	2	5	
MPN (SMG myeloid)	1	2	3	
MPN/MDS	1	1	2	
Waldenstrom	0	1	1	

(F) Fisher's exact test
(C) Chi-squared

Table 3. Contingency analysis for SRSF2-P95 mutation and Epi-regulator mutations

	SRSF2 WT	SRSF2-P95	Total	p-value
Tet2 mutation				**0.0049 (F)**
n	55	17	72	
WT	48 (87%)	9 (53%)	57	
MUT	7 (13%)	8 (47%)	15	
Epi-regulators*				**<0.0001 (F)**
n	55	17	72	
at least 1	9 (16%)	13 (77%)	22	
0	46 (84%)	4 (23%)	50	

(F) Fisher's exact test
**TET2, IDH1/2, ASXL1*

Figure Legends

Figure 1. The frequencies and distribution of 10 gene mutations in 72 patients with mastocytosis. Each column represents one individual patient with mutated gene(s) shown by different coloured bars. The last two rows describe the disease classification for each patient. Half boxes reveal evolved diagnosis during the course of this study and dotted lines highlight those patients with SRSF2-P95 mutation and disease evolution. No mutations were found for *ZRSR2, KRAS, IDH1, DNMT3A* or *EZH2*.

Figure 2. Survival proportions of patients with advanced forms of mastocytosis. (A) Percent survival of patients with advanced disease type and non-advanced disease type in years post-disease diagnosis. **(B)** Percent survival of patients with advanced disease phenotype, with or without SRSF2-P95 mutation (Adv SRSF2-P95 MUT or Adv SRSF2-P95 WT), in years post-disease diagnosis. P-values were calculated using the Log-rank (Mantel-Cox) test.

Figure 3. SRSF2, TET2 and KIT status of sorted cell populations for 7 patients with ASM. (A) Total bone marrow of 7 patients with advanced disease phenotypes were FACS sorted using lineage-specific antibodies. Figure shows a representative FACS profile from one patient. **(B)** DNA was isolated from the three collected populations, T-cell, monocytes and mast cells and genotyped for *KIT, TET2* and *SRSF2* mutation. **(B) Analysis of single colonies for mutations in *SRSF2, TET2* and *KIT*.** Single colony-forming units derived from mononuclear cells of whole bone marrow, were isolated and analyzed individually for the presence of *SRSF2, TET2* and KITD816V mutations. Each colony is represented by a dot that is placed in boxes according to genotype. The

unique patient numbers and the diagnoses are shown above the corresponding boxes. Light grey arrows indicate the suggested order of mutation events. A black dot indicates that SRSF2-P95H mutation was detected rather than SRSF2-P95L.

Figure 1

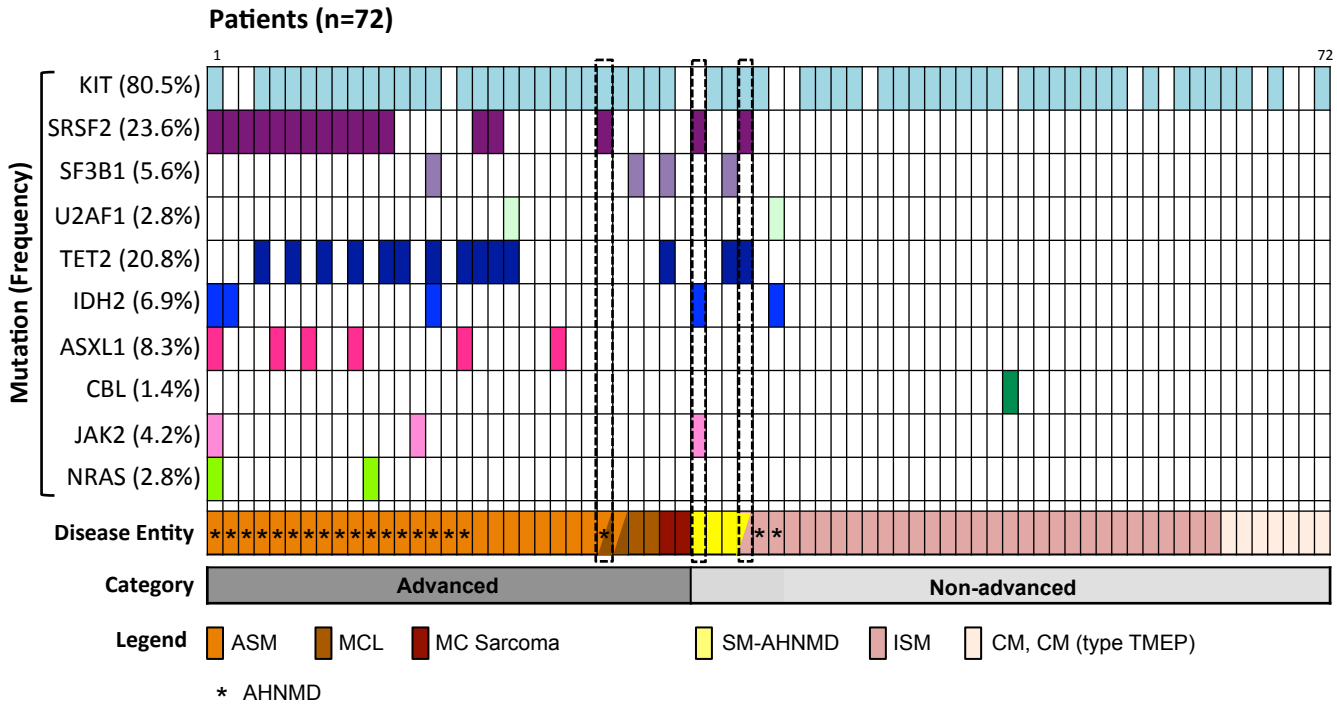

Patients (n=72)
1
72
Mutation (Frequency)
KIT (80.5%)
SRSF2 (23.6%)
SF3B1 (5.6%)
U2AF1 (2.8%)
TET2 (20.8%)
IDH2 (6.9%)
ASXL1 (8.3%)
CBL (1.4%)
JAK2 (4.2%)
NRAS (2.8%)
Disease Entity
Category
Advanced
Non-advanced
Legend
ASM
MCL
MC Sarcoma
SM-AHNMD
ISM
CM, CM (type TMEP)
* AHNMD

Figure 2

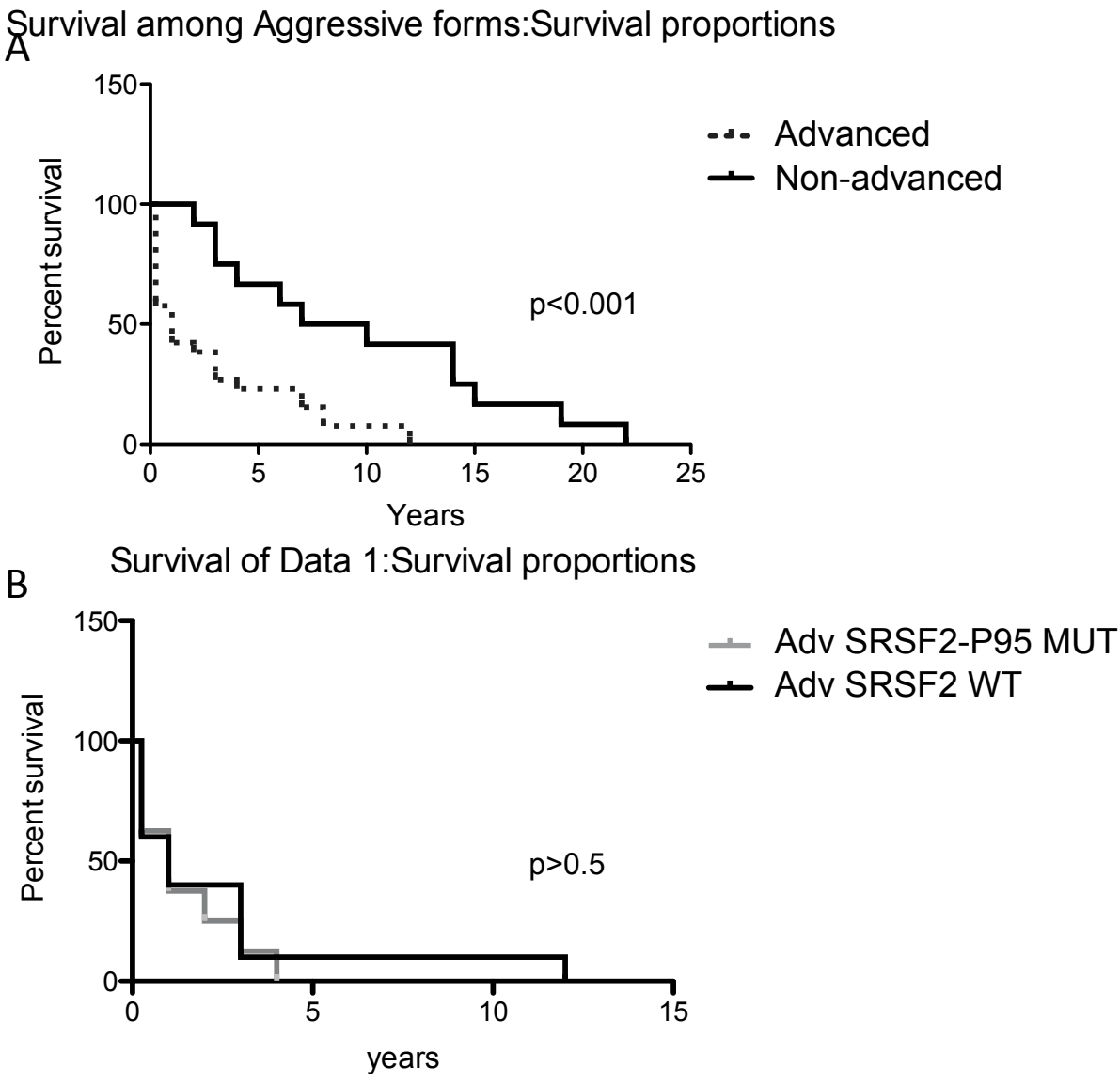
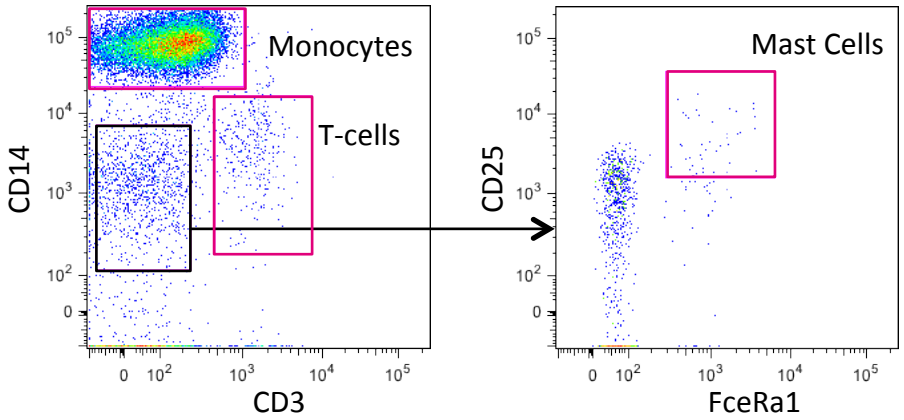

Survival among Aggressive forms:Survival proportions
A
Percent survival
150
100
50
0
0
5
10
15
20
25
Years
Advanced
Non-advanced
p<0.001
Survival of Data 1:Survival proportions
B
Percent survival
150
100
50
0
0
5
10
15
years
Adv SRSF2-P95 MUT
Adv SRSF2 WT
p>0.5

Figure 3

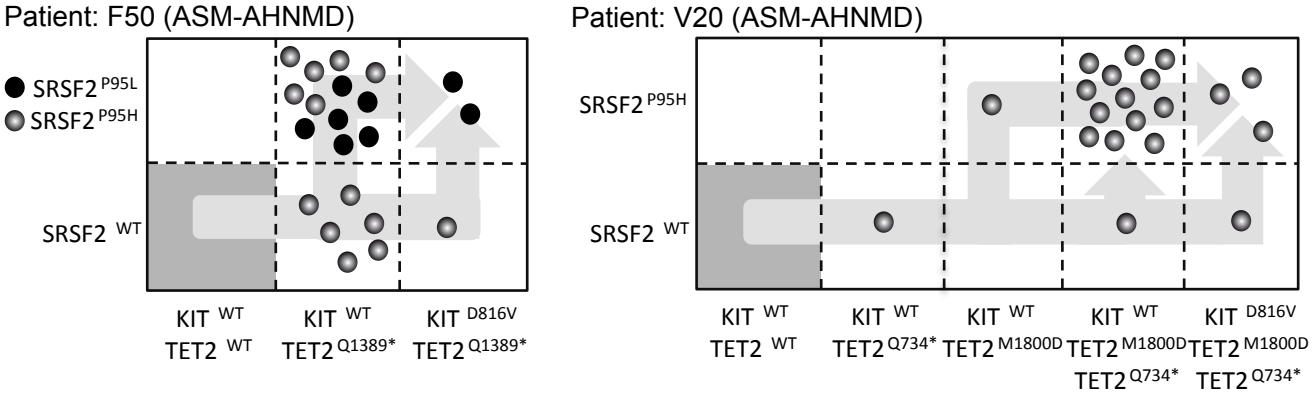
A



B

Patient	Mast Cells			Monocytes			T-cells		
	KITD816V	TET2	SRSF2P95	KITD816V	TET2	SRSF2P95	KITD816V	TET2	SRSF2P95
1639	+	+	+	+	+	+	+	+	+
1445	+	+	+	+	+	+	-	-	+
D60	+	-	+	+	-	+	+	-	+
F50	+	+	+	+	+	+	-	+	+
M40	-	-	+	-	-	+	-	-	+
289	+	+	-	+	+	-	+	+	-
1202	+	+	-	+	+	-	+	+	-

C



Patients	Sex	Age	WHO Classification	AHNMD	Type of AHNMD	CD2 masto	Tryptase ng/ml	B Findings	C findings	HMG	SMG	Ascite	Hb (<10g/dl)	Plq (<100)	PNN (<1000)	Alb	amaig >10%	osteoporosis	MCAS	Status Dead / Alive	Advanced (A)	KIT mutation	SRSF2	SF3B1 (exons13-16)	TET2	IDH2 (exon4)	ASXL1 (exon12)	CBL (exons8-9)	JAK2 (exon 14)	NRAS	U2AF1 (exon2,7)	ZRSR2 (exon8,9,11)
1534	M	81	ASM	yes	MPN (SMG myeloid)	neg	169	na	4	yes	yes	yes	9.2	59.000	14.700	37	yes	no	no	D	A	D816V	P95T			1	1		1	1		
D60	M	75	ASM>MCL	yes	MPN/MDS	neg	458		4	yes	yes	yes	9,2	31 000	12.000	24,3	yes	yes	no	A	A	D816V	P96R									
1386	M	90	ASM	yes	Waldenstrom		19,4	na	1	yes	yes	no	8.9	103.000		NK	yes	NK	no	D	A	WT	P95R									
1263	M	64	ASM	yes	MPN (SMG myeloid)		105	na	3	yes	yes	no	10;6	64.000	3200	NK	yes	NK	itching	A	A	WT	P95R			1						
F50	F	68	ASM	yes	CMML	neg	133	na	4	yes	yes	yes	10,6	240.000	2700	24,8	yes	no	no	A	A	D816V	P95L		1							
M174	M	72	ASM	no	na		330	na	4	Yes	Yes	Yes	10.3	116.000	4500	42	Yes	NK	no	D	A	D816V	P95L		1							
1349	M	90	ASM	no	na	neg	800	na	3	yes	yes	no	7.7	28.000	11.200	33	yes	NK	no	D	A	D816V	P95H		2							
1639	M	64	ASM	yes	AML	pos	1240	na	4	yes	yes	yes	8.4	144.000	6400	30,4	yes	no	no	D	A	D816V	P95H		2		1					
1445	M	78	ASM	yes	CMML	neg	209	na	4	yes	yes	yes	11.4	26.000	4200	30	yes	no	no	D	A	D816V	P95H		2							
1672	M	54	ASM	yes	MDS (AREB)	neg	312	na	4	yes	yes	yes	9,1	99.000	2300	26,8	yes	no	no	A	A	D816V	P95H							1		
1466	M	82	ASM	yes	CMML		235	na	3	yes	yes	yes	11	14000	58.000	40	no	no	flush, itching	D	A	D816V	P95H									
1218	M	77	ASM	yes	MDS (AREB)		775	na	3	yes	yes	no	9	53.000	1900	NK	yes	no	no	D	A	D816V	P95H				1					
169	M	62	ASM	yes	AML		NK	na	4	yes	yes	yes	8	10.000	8600	27	yes	NK	no	D	A	D816V	P95H				1					
V20	F	66	ASM	yes	MDS	neg	367	na	4	yes	yes	yes	7.3	44.000	5000	31	yes	no	no	A	A	D816V	P95H		2							
1154	M	77	ASM	yes	CMML		200	na	3	yes	yes	no	10,4	35.000	21576	38	yes	no	no	D	A	p.(F504_N505delinsLKFLT)	P95H		2							
289	F	76	ASM	no	na		NK	na	1	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	D	A	D816V			1					1		
394	F	58	ASM	no	na		27,5	na	1	no	no	no	13	60.000	8000	43	no	no	itching	A	A	D816V					1					
328	M	76	ASM	no	na		> 200	na	4	yes	yes	yes	8.4	94.000	6700	23.8	yes	yes	no	D	A	D816V										
397	M	47	ASM	no	na	pos	100	na	1	yes	yes	no	15;4	41.000	5.000	43	no	yes	no	A	A	D816V										
327	M	39	ASM	no	na	pos	615	na	4	yes	yes	yes	9.6	378.000	7400	28	yes	NK	flush	A	A	D816V										
1182	F	51	ASM	no	na		149	na	1	no	no	no	15,4	235.000	1.118	35,8	yes	yes	flush, itching, diarrhea, pollakiuria	A	A	D816V										
1070	M	83	ASM>MCL	no	na		1077	na	4	yes	yes	yes	8.4	31.000	3.800	27.4	yes	no	no	D	A	D816V										
984	F	56	ASM	yes	MPN (SMG myeloid)		212	na	3	yes	yes	no	7.8	129.000	2900	NK	no	no	flush, itching, diarrhea	D	A	D816V							1			
486	M	80	Mast cell Sarcoma	no	na		426	na	3	no	yes	yes	8,8	69.000	741	30	yes	yes	itching, diarrhea, flush, abdominal pain	D	A	D816V		1	2							
S10	F	80	ASM	yes	MDS (AREB)		822	na	2	no	yes	no	7.7	19.000	1680	NK	no	NK	no	D	A	D816V			1		1					
233	F	75	ASM	yes	MDS		389	na	4	yes	yes	yes	7.7	52.000	4180	NK	yes	no	itching	A	A	D816V			1							
1202	M	63	ASM	yes	AML		635	na	4	yes	yes	yes	9	87.000	3600	30	yes	no	no	D	A	D816V		1	1	1						
1725	F	29	MCL	no	na	neg	840	na	4	yes	yes	yes	7.5	68.000	10.500	26	yes	no	flush, diarrhea, musculo-squelettic pain	A	A	p.A502_Y503dup										
1186	F	70	MCL	no	na		410	na	2	no	yes	no	8.5	78.000	16000	NK	yes	NK	flush, malaise	D	A	p.S501_A502dup		1								
1427	M	70	ASM	yes	MPN (Vaquez) / MDS		73,4	na	2	no	yes	no	6,8	71.000	1590	NK	no	NK	no	A	A	WT										
1329	M	28	Mast cell Sarcoma	no	na	pos	900	na	1	no	yes	no	11.7	445.000	5200	NK	yes	NK	flush	D	A	WT										
M40	M	80	SM-AHNMD	yes	MDS		71	0	0	yes	yes	no	10.8	294.000	10.000	47	no	no	no	D	NA	WT	P95L			1			1			
1318	M	76	ISM>SM-AHNMD	yes	na		47,5	0	0	no	no	no	N	N	N	N	no	NK	diarrhea	NK	NA	D816V	P95H		2							
1571	F	51	ISM	no	na	pos	19,2	0	0	no	no	no	N	N	N	N	no	no	anaphylactic shock, diarrhea, itching, pollakiuria	A	NA	D816V										
1668	F	36	ISM	no	na		52,6	0	0	no	no	no	N	N	N	N	no	no	flush, diarrhea, psy trauma, dl os	A	NA	D816V										
1718	M	32	ISM	no	na		220	0	0	no	no	no	N	N	N	N	no	NK	psy trauma, itching, dl os, astenia	A	NA	D816V										
1705	F	51	ISM	yes	CML		3,7	0	0	no	no	no	N	N	N	N	no	yes	eurologic disorder, asthenia, anaphylactic shock, d	A	NA	D816V										
1739	M	82	ISM	yes	MDS (AREB)		9,5	0	0	no	no	no	7.6	36.000	800	N	no	NK	diarrhea, flush	A	NA	WT				1					1	
1231	F	77	ISM	no	na		131	2	0	yes	yes	no	12	167.000	3900	NK	no	no	itching, diarrhea, flush, abdominal pain	A	NA	D816V			1							
1598	M	55	SM-AHNMD	yes	MDS (AREB)	neg	12	SMG	0	no	yes	no	10,4	183.000	1975	46,4	no	no	itching, flush, pains, RGO, neuro-psy disorder	A	NA	D816V		1	1							
1010	F	75	CM	no	na		22	0	0	no	no	no	N	N	N	N	no	no	itching, diarrhea, anaphylactic shock	A	NA	D816V										
1159	F	57	CM	no	na		13,6	0	0	no	no	no	N	N	N	N	no	NK	gastric disorder, pollakiuria, itching	A	NA	D816V										
105	F	43	CM	no	na		11,3	0	0	no	no	no	N	N	N	N	no	NK	flush, shocks, itching, diarrhea, pollakiuria	A	NA	D816V										
435	F	52	ISM	no	na		40	0	0	no	no	no	N	N	N	N	no	yes	no	A	NA	D816V										
492	F	68	ISM	no	na	pos	24,5	HMG	0	yes	no	no	N	N	N	N	no	NK	not described	A	NA	D816V										
1005	F	65	ISM	no	na		125	0	0	no	no	no	N	N	N	N	no	yes	flush, diarrhea	A	NA	D816V										
356	F	57	ISM	no	na		38,6	0	0	no	no	no	N	N	N	N	no	A	flush, depression	A	NA	D816V										
1025	F	57	ISM	no	na		18,6	0	0	no	no	no	N	N	N	N	no	yes	not described	A	NA	D816V										
425	F	56	ISM	no	na		36,4	0	0	no	no	no	N	N	N	N	no	no	flush, itching, pollakiuria, fatigue, depression	A	NA	D816V										
427	F	56	ISM	no	na		63	0	0	no	no	no	N	N	N	N	no	yes	flush, itching, fatigue, anxiety	A	NA	D816V										
406	F	54	ISM	no	na		20,5	0	0	no	no	no	N	N	N	N	no	NK	itching,	A	NA	D816V										
1002	F	51	ISM	no	na		115	0	0	no	no	no	N	N	N	N	no	no	flush, itching	A	NA	D816V										
1016	F	45	ISM	no	na		79,6	0	0	yes	no	no	N	N	N	N	no	no	not described	A	NA	D816V										
245	F	43	ISM	no	na		23	0	0	no	no	no	N	N	N	N	no	yes	flush, itching	A	NA	D816V										
246	F	42	ISM	no	na		38,7	0	0	no	no	no	N	N	N	N	no	NK	flush, itching, diarrhea	A	NA	D816V										
1323	M	63	ISM	no	na		75	0	0	no	no	no	N	N	N	NK	no	NK	itching, diarrhea	A	NA	D816V										
421	M	60	ISM	no	na		63	0	0	no	no	no	N	N	N	N	no	yes	itching, flush, diarrhea, gastric disorder, pollakiuria	A	NA	D816V										
1096	M	57	ISM	no	na		25	0	0	no	no	no	N	N	N	NK	no	no	flush, itching, diarrhea	A	NA	D816V										
424	M	46	ISM	no	na		8,3	0	0	no	no	no	N	N	N	N	no	yes	feeding intolerances	A	NA	D816V										
372	M	45	ISM	no	na		42,2	0	0	no	no	no	N	N	N	N	no	yes	not described	A	NA	D816V										
409	M	28	ISM	no	na		31,5	0	0	no	no	no	N	N	N	N	no	yes	diarrhea	A	NA	D816V										
1083	M	53	ISM	no	na		26,2	0	0	no	no	no	N	N	N	N	no	no	itching, malaises	A	NA	D816V										
1006	M	33	MC (type TMEP)	no	na		8	0	0	no	no	no	N	N	N	N	no	NK	itching	A	NA	D816V										
468	F	63	SM-AHNMD	yes	MDS		191	0	0	no	no	no	11,8	186.000	1190	NK	no	yes	no	NK	NA	D816V										
1594	F	70	ISM	no	na		24,7	0	0	no	no	no	N	N	N	N	no	no	gastric disorder, flush, itching, sleep disorder	A	NA	WT										
1265	F	54	ISM	no	na		5,4	0	0	no	no	no	N	N	N	N	no	no	itching, flush	A	NA	WT										
1215	F	45	CM	no	na		24,3	0	0	no	no	no	N	N	N	N	no	no	no	A	NA	WT										
1122	F	54	CM	no	na		5,3	0	0	no	no	no	N	N	N	N	no	no	no	A	NA	WT										
1241	M	39	CM	no	na		7,3	0	0	no	no	no	N	N	N	N	no	NK	itching	A	NA	WT										
1001	F	79	ISM	no	na		6,7	HMG	0	yes	no	no	N	N	N	N	36	yes	NK	itching, flush, diarrhea, headache	A	NA	WT					1					
1376	F	46	ISM	no	na		14,8	0	0	no	no	no	N	N	N	N	no	NK	flush, malaises, itching	A	NA	WT										
398	F	33	ISM	no	na		2,6	0	0	no	no	no	N	N	N	N	no	yes	hing, pollakiuria, depression, diarrhea, anaphylact	A	NA	WT										

Abbreviations: WHO, World Health Organization; AHNMD, associated hematologic non-mast cell lineage disease; MCL, mast cell leukemia; ASM, aggressive SM; ISM, indolent SM; SM, systemic mastocytosis; CM, cutaneous mastocytosis;MC, bone marrow mastocytosis; MDS, Myelodysplastic Syndrome; RAEB, Refractory anemia with excess blasts;UP, Urticaria Pigmentosa; TMEP,Telangiectasia macularis eruptiva perstans; NK, Not Known; na, not applicable.