# 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% 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, suggests an ontological mutation hierarchy and provides evidence for the expansion of multiple clones. This highlights the prognostic potential of such approaches, if applied systematically, for delineating the roles of specific mutations in predisposing and/or driving distinct disease phenotypes.

## Introduction

Mastocytosis is a rare and clonal hematopoietic disorder described as the accumulation of abnormal mast cells in the bone marrow (BM).[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 approximately 40% of cases, systemic mastocytosis is diagnosed in conjunction with associated clonal hematologic 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. In contrast, TET2 mutation, found in approximately 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 hematologic 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 have revealed a striking association between SRSF2 mutation and advanced disease types.

## Methods

### Patients' data

Seventy-two patients (35 men; 37 women) with mastocytosis diagnosis as defined by the WHO criteria[14] were enrolled in a prospective national multicenter study between 2005 and 2013. The cohort con-

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sists of patients diagnosed with cutaneous mastocytosis (CM), CM (type TMEP), indolent SM (ISM), systemic mastocytosis with AHNMD (SM-AHNMD), aggressive SM (ASM), mast cell leukemia (MCL) and mast cell sarcoma (*Online Supplementary Table S1*).[1-3] AHNMD diagnosis for each patient has been indicated (Figure 1), and this is detailed in the *Online Supplementary Table S1*. Patients were further grouped using the operational terms "advanced" and "non-advanced" to account for the number of patients in certain classifications, e.g. MCL and mast cell sarcoma 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 the Necker Hospital ethical committee, and carried out according to the Declaration of Helsinki. Each patient provided informed consent.

### Mutation screening

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

### Clonal analysis

Leukocytes were purified using Ficoll® (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 DIVA™ (Becton-Dickinson) software was used. 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, USA) and both the log rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were applied to survival curves to determine significant differences.

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

| | Non-advanced | Advanced | Total | *P* |
|---|---|---|---|---|
| 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.*

## 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 (*Online Supplementary Table S1*). Biopsied material from these patients was screened for mutation in genes commonly mutated in mastocytosis or other myeloid diseases (Figure 1). After *KIT* (81%), the SRSF2-P95 hotspot mutation was the most frequent mutation found in these patients (17 of 72; 23.6%). We detected four different mutations of the NSRSF2-P95 codon in patients: SRSF2-P95H, SRSF2-P95L, SRSF2-P95T and SRSF2-P95R (*Online Supplementary Table S1*). We have previously reported an association between TET2 and advanced systemic mastocytosis[7] and, like *TET2*, we also saw 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 who were negative for *KIT* mutation were positive for SRSF2-P95 mutation. *SRSF2* mutation also coincides significantly with the presence of AHNMD (15 of 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 (Figure 2A). We found 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 (Figure 2B). We found 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 ($P>0.5$) (Figure 2B). Taken together, advanced disease diagnosis and not

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

| | SRSF2 WT | SRSF2-P95 | Total | *P* |
|---|---|---|---|---|
| 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) $\chi^2$.*

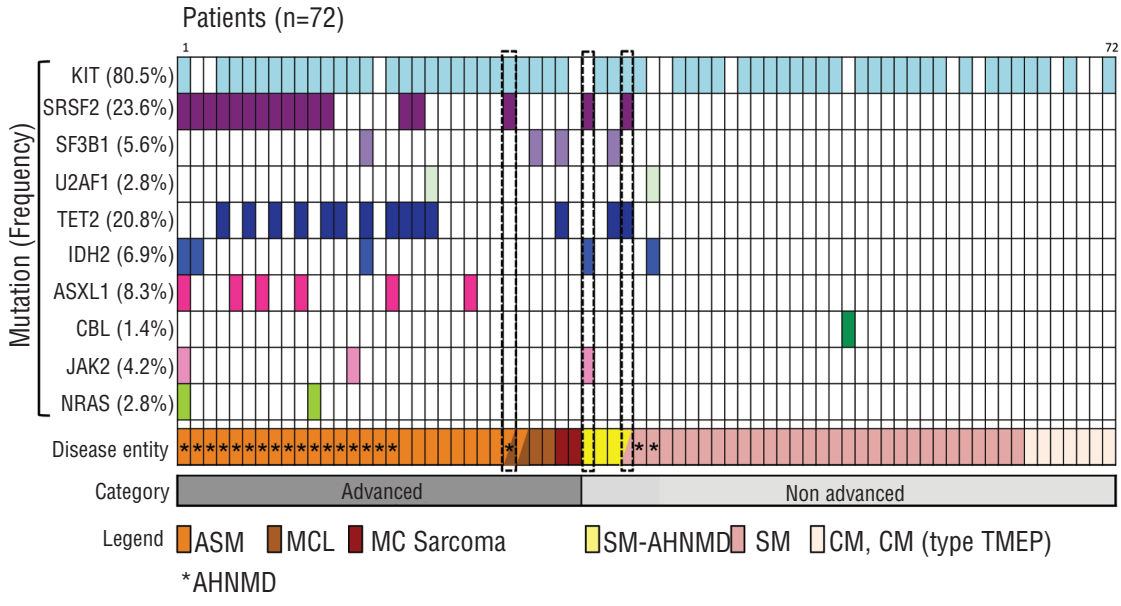



**Figure 1.** The frequencies and distribution of 10 gene mutations in 72 patients with mastocytosis. Each column represents one individual patient with mutated gene(s) shown by different colored bars. The last two rows describe the disease classification for each patient. Half boxes reveal evolved diagnosis 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.

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 n. M40 originally diagnosed with SM developed AHNMD (MDS), Patient n. 1318 originally diagnosed with ISM progressed to SM-AHNMD, and Patient n. D60 originally diagnosed with ASM developed MCL (Figure1, dotted boxes, and *Online Supplementary Table S1*).

### *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* (Figure 1 and *Online Supplementary Table S1*).

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%, 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 mutually exclusive in these patients (Figure 1 and *Online Supplementary Table S1*).

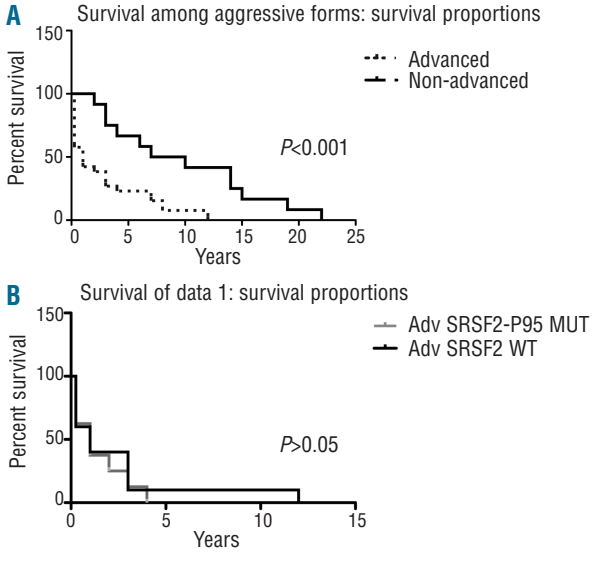



**Figure 2.** Survival proportions of patients with advanced forms of mastocytosis. (A) Percent survival of patients with advanced disease type and non-advanced disease type in years post-disease diagnosis. (B) Percent survival of patients with advanced disease phenotype, with or without SRSF2-P95 mutation (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.

### *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 sort-

ed cell populations from total bone marrow of 7 patients with advanced disease phenotypes for genotype analysis (Figure 3A). Genomic DNA from the three collected populations, T cells, monocytes and mast cells was isolated and genotyped for *KIT*, *TET2* and *SRSF2* mutations (Figure 3B). In all 7 patients for whom sorted populations, *KIT*, *SRSF2* and *TET2* mutations, were detected in whole bone marrow biopsies (*Online Supplementary Table S1*) as well as 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 whom *KIT* mutation was not detected, we detected JAK2 V617F mutation in mast cells and monocytes, but not in T cells. Finally, in patients presenting with two different *TET2* mutations, both mutations were present in positive cell populations. Interestingly, in one patient (Patient n. 1445), we detected *SRSF2* mutation in T cells where *TET2* and *KIT* mutations were present only in mast cells and monocytes.

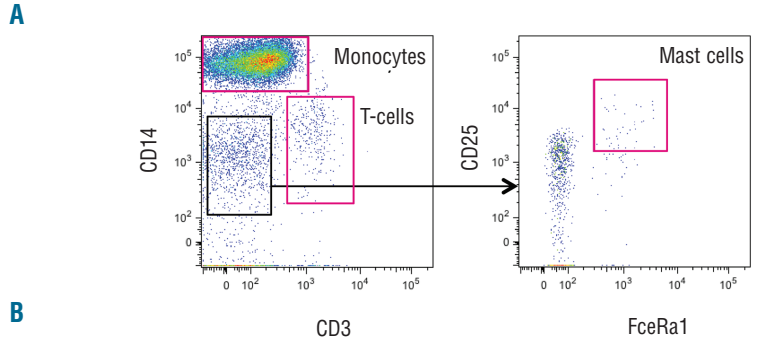
### *SRSF2-P95 mutation can occur early or late during clonal evolution*

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 (Figure 3C, arrows). In both patients, we identified clones harboring *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 n. F50 identified

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

| | SRSF2 WT | SRSF2-P95 | Total | P |
|---|---|---|---|---|
| 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.*




B

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

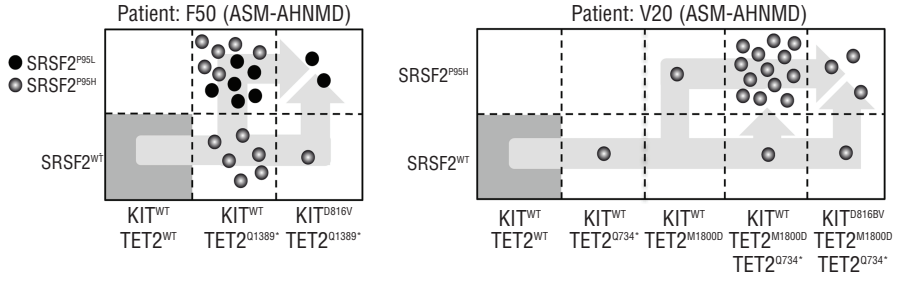



**Figure 3. SRSF2, TET2 and KIT status of sorted cell populations for 7 patients with ASM. (A) Total bone marrow of 7 patients with advanced disease phenotypes were FACS sorted using lineage-specific antibodies. Figure shows a representative FACS profile from one patient. (B) DNA was isolated from the three collected populations, T cells, monocytes and mast cells and genotyped for KIT, TET2 and SRSF2 mutation. (C) 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 gray arrows indicate the suggested order of mutation events. A black dot indicates that SRSF2-P95H mutation was detected rather than SRSF2-P95L.**

KITD816V, TET2 Q1389* and SRSF2-P95L mutations (*Online Supplementary Table S1*), clonal analysis revealed 5 of 14 colonies harboring a distinct SRSF2-P95H mutation. Both SRSF2-P95L and SRSF2-P95H colonies harbored 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 harboring 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,[20] a unique role for SRSF2 has been described in regulating PolII pausing and elongation at promoters.[21,22] 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.[23] Future studies to address the global or specific role of gene splicing in hematologic 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.[24] Mechanistically, splicing is often tightly coupled with transcription and recent work suggests that alternative splicing might be affected by chromatin structure and histone modification.[25] 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,[26,27] 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 the 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 this can precede both *TET2* and *KIT* mutation. We find evidence to support both: 1) a clone harboring 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 (Figure 3B and C; Patient n. F50); and 2) SRSF2-P95 mutation was detected in all cell types from a patient in whom *TET2* and *KIT* mutation were detected in only mast cells and monocytes (Figure 3B; Patient n. 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 favors 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 strongly 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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*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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