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To cite this version:

HAL Id: hal-01325368
https://hal.sorbonne-universite.fr/hal-01325368
Submitted on 2 Jun 2016

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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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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.

Methods

Patients’ data

Seventy-two patients (35 men; 37 women) with mastocytosis diagnosis as defined by the WHO criteria 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 AHNM (SM-AHNM), aggressive SM (ASM), mast cell leukemia (MCL) and mast cell sarcoma (Online Supplementary Table S1). 13 AHNM 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, eg. 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.1 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 (AFIRM). 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.15,18 Other methods have been previously described as follows: DNA Sanger-sequencing of exon-coding sequences of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2 and EZH2, SRSF2, U2AF1, ZRSR2 and SF3B1.16

**FACS sorting**

Fresh or frozen whole bone marrow biopsy material from patients was stained with the following antibody cocktail: anti-humanCD3-EC, anti-humanCD14-alexa647, anti-humanCD25-PE and anti-humanFcpsilonRIa-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.

**Results**

**SRSF2-P95 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 SRSF2-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 mastocytosis1 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,3 3 patients who were negative for KIT mutation were positive for SRSF2-P95 mutation. SRSF2 mutation also coincides significantly with the presence of AHNM (15 of 17 patients with SRSF2-P95 mutation present with AHNM), but not with any particular form of AHNM (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

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(F) Fisher’s exact test.
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. 1518 originally diagnosed with ISM progressed to SM-AHNMD, and Patient n. D60 originally diagnosed with ASM developed MCL (Figure 1, 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, ASXL1, 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, 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).

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 S4) 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      |
| Tet2 mutation            |                          |                          |            |
| N.                       | 55                       | 17                       | 72         |
| WT                       | 48 (87%)                 | 9 (53%)                  | 57         |
| MUT                      | 7 (13%)                  | 8 (47%)                  | 15         |
| Epi-regulators*          |                          |                          |            |
| N.                       | 55                       | 17                       | 72         |
| at least 1              | 8 (16%)                  | 13 (77%)                 | 22         |
| 0                        | 46 (84%)                 | 4 (23%)                  | 50         |

(F) Fisher’s exact test, *TET2, IDH1/2, ASXL1.
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.19,21 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: EZH2.21 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.2 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 muta-

Funding

This work was financed by INSERM, the Ligue Nationale Contre le Cancer (équipe labellisée PD), INCA (PD), La Fondation de France (ES) and La Fondation de Recherche Médicale (FB).

Authorship and Disclosures

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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