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TITLE: *KIT* D816V mutated bone marrow mesenchymal stem cells in indolent systemic mastocytosis are associated with disease progression.

RUNNING HEAD: KIT D816V MUTATED MESENCHYMAL STEM CELLS IN SM

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KEY POINTS:

- Acquisition of the *KIT* D816V mutation in an early pluripotent progenitor cell confers ISM cases a greater risk for disease progression.
- Despite the early acquisition of the *KIT* mutation, onset of clinical symptoms of ISM is often delayed to the middle-adulthood.

ABSTRACT

Multilineage involvement of bone marrow (BM) hematopoiesis by the somatic *KIT* D816V mutation is present in a subset of adult indolent systemic mastocytosis (ISM) patients in association with a poorer prognosis. Here we investigated the potential

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involvement of BM mesenchymal stem cells (MSC) from ISM patients by the *KIT* D816V mutation and its potential impact on disease progression and outcome. The *KIT* D816V mutation was investigated in highly-purified BM MSC and other BM cell populations from 83 ISM patients followed for a median of 116 months. *KIT* D816V-mutated MSC were detected in 22/83 (27%) cases. All MSC-mutated patients had multilineage *KIT* mutation (100% vs 30%, p=0.0001) and they more frequently showed involvement of lymphoid plus myeloid BM cells (59% vs 22%; *P*=.03) and a polyclonal pattern of inactivation of the X-chromosome of *KIT*-mutated BM mast cells (64% vs 0%; *P*=0.01) vs other multilineage ISM cases. Moreover, presence of *KIT*-mutated MSC was associated with more advanced disease features of ISM, a greater rate of disease progression (50% vs 17%; *P*=.04) and a shorter progression-free survival at 10, 20 and 30 years ($P \leq .003$). Overall, these results support the notion that ISM patients with mutated MSC may have acquired the *KIT* mutation in a common pluripotent progenitor cell, prior to differentiation into MSC and hematopoietic precursor cells, before the X-chromosome inactivation process occurs. From a clinical point of view, acquisition of the *KIT* mutation in an earlier BM precursor cell confers a significantly greater risk for disease progression and a poorer outcome.

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INTRODUCTION

The *KIT* D816V mutation[1] is the most common genetic alteration of systemic mastocytosis (SM)[2,3] being present in >95% of adults with indolent (ISM) and aggressive (ASM) SM[4]. Therefore, on its own this mutation cannot explain the different clinical outcomes[5] of indolent cases that show a normal life expectancy (e.g. most ISM)[6,7] versus the more severe forms of SM -i.e. ASM and mast cell leukemia (MCL)- in whom patient's outcome is significantly compromised[8]. Thus, secondary genetic lesions on top of the *KIT* mutation or upon cooperation with a particular genetic background are potentially needed for malignant transformation of ISM into severe disease[9-11].

In recent years, we have shown that around one-third of ISM patients carry multilineage myeloid and/or lymphoid involvement of hematopoiesis by the *KIT* D816V mutation in bone marrow (BM) cell compartments other than mast cells (MC)[4], this being associated with a higher risk for disease progression[7]. Altogether, these results suggest that occurrence of the *KIT* D816V mutation in an early precursor cell could be associated with higher numbers of mutated hematopoietic progenitors, a greater extent of involvement of hematopoiesis by the mutation and a higher probability of progression of ISM to advanced disease -e.g. ASM, MCL and SM associated to other hematological non-MC diseases (SM-AHNMD)-.

The potential occurrence of the *KIT* mutation in an uncommitted hematopoietic stem and precursor cell (HPC) was first evidenced by the demonstration of this mutation in multiple non-MC myeloid[12-15] and also lymphoid[12,15,16] cells, in addition to $CD34^+$HPC[4]. In parallel, involvement of a multipotent $CD34^+/CD38^-$HPC has also been demonstrated in other myeloproliferative neoplasms (MPN), myelodysplastic

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syndromes (MDS)[17-19], and acute myeloid leukemia[20,21]. Moreover, the AHNMD cells from SM-AHNMD patients often carry the *KIT* D816V mutation[22-24] supporting a common clonal origin for both disease components in an HPC with multilineage potential.

Despite the above, at present it still remains to be established whether in SM the somatic *KIT* D816V mutation occurs at the level of CD34+HPC or at an earlier precursor cell. Most likely, early (indolent) forms of SM are accumulative diseases with limited proliferation and expansion of clonal MC[25-27]; in aggressive cases, additional secondary genetic alterations[9] are likely to occur in *KIT* D816V$^+$ precursors. The earlier the *KIT* mutation would emerge, the greatest extent of involvement of hematopoiesis would occur with a potentially greater risk for additional secondary genetic alterations and progression of the disease. In this regard, mesenchymal stem cells (MSC) have been long described as precursor cells that have the ability for differentiation into various mesodermal lineages and tissues[28]. MSC are present in the BM stroma to provide microenvironmental support to HPC with whom they share an ontogenic link[29,30]. Despite MSC share cytogenetic alterations with HPC in MPN, MDS and other hematological conditions[31-34], no study has been reported so far in which the presence of the *KIT* D816V mutation is investigated in BM MSC from ISM patients.

Here we investigated the potential involvement of BM MSC by the *KIT* D816V mutation in a cohort of 83 ISM patients in order to determine its potential impact on disease progression and patient outcome.

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METHODS

Patients. Eighty-three patients (37 men and 46 women; median age at diagnosis of 42 years) diagnosed with ISM at the reference centres of the Spanish Network on Mastocytosis (REMA, Toledo, Spain) were selected from a series of 169 consecutive ISM patients (43 cases having MC-restricted and 40 multilineage *KIT* mutation according to previously defined criteria[7]) and prospectively included in this study. Patient selection (n=83) was based on the availability of sufficient ($\geq 3x10^3$) highly-purified BM MSC to perform further molecular analyses. Diagnosis and classification of SM was made according to the World Health Organization (WHO) 2008[35] and the more recent European Competence Network on Mastocytosis[36] criteria. Prior to entering the study, patients gave their written informed consent to participate according to the Declaration of Helsinki; the study was approved by the local institutional Ethics Committees.

Follow-up studies and disease progression. At diagnosis, and subsequently every 6-12 months, or whenever disease progression was suspected, a complete clinical and physical examination, including a BM study, a skin biopsy, evaluation of serum baseline tryptase levels and bone lesions, were performed, as previously described[7].

Disease progression was defined as transformation of ISM into a more aggressive subtype of mastocytosis[35,36] including smoldering SM (SSM), ASM and/or SM-AHNMD, after a median follow-up from disease onset of 116 months (range: 28 to 544 months). In detail this included emergence of ≥2 “B” findings (i.e. organomegaly without impaired organ function; BM infiltration with >30% of focal and dense aggregates of MC; serum tryptase >20ng/ml; dysplasia or myeloproliferation with non AHNMD and normal blood counts) in the absence (e.g. SSM) or in the presence (ASM)

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of ≥1 "C" findings (i.e. organomegaly with organ failure; BM dysfunction with cytopenia; large osteolytic lesions and/or pathological fractures; malabsorption with weight loss) according to the WHO criteria.[35]

Immunophenotypic characterization of BM cell populations. Fresh EDTA-anticoagulated BM aspirated samples collected from the iliac crest, were used for multiparameter flow cytometry immunophenotypic analysis of BM cell populations, after they had been stained with a large panel of monoclonal antibodies (MoAbs) (Table 1), following previously described protocols[37].

Purification of MC, MSC and other BM cell populations. Isolation of antibody-stained (Table 1) BM cell populations was performed using well established stain-and-then-lyse-and-wash procedures[38] and a 4-way fluorescence-activated cell sorter (FACSAria III, Becton/Dickinson Bioscences, San Jose, CA), as described elsewhere[4]. For BM cell isolation purposes, MC were identified as CD117^{high}/CD45^{+}/CD34^{-}/CD3^{-}/CD14^{-}/CD105^{-} cells, monocytes as CD45^{high}/CD14^{high}/CD34^{-}/CD117^{-}/CD3^{-} cells, maturing neutrophils were defined as CD45^{+}/CD34^{-}/CD117^{-}/CD3^{-}/CD14^{-} cells, eosinophils as CD45^{+}/CD13^{+}/CD34^{-}/CD117^{-}/CD3^{-}/CD105^{-}/CD14^{-} sideward scatter (SSC)^{high} with high green and orange autofluorescence cells, CD34^{+}HPC were identified as cells with a CD34^{+}/CD45^{low}/CD117$^{-/+}$/CD13$^{-/+}$/CD105$^{-/+}$/CD3^{-}/CD14^{-} phenotype and SSC$^{low/int}$, and T cells were defined as CD3^{+}/CD45^{high}/CD34^{-}/CD13^{-}/CD14^{-}/CD105^{-}/CD117^{-} cells. Identification and purification of MSC was based on a CD105^{+}/CD13^{high}/CD45^{-}/CD34^{-}/CD14^{-}/CD3^{-} immunophenotype[39]. The purity of each of the isolated BM cell populations was systematically >98% in the absence of cross-contamination by MC (<0.001%) or any other *KIT* D816V^{+} BM cell population.

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Validation of the CD105/CD13/CD45/CD34/CD14/CD3 antibody combination for the identification of MSCs was performed in each individual sample, by further evaluating their immunophenotypic profile using a broad panel of MoAbs (e.g. CD10, CD13, CD34, CD45, CD73, CD90, CD105, CD117, CD146, HLA-DR) including MoAbs for those proteins currently required for the definition of MSC[40,41] (Table 1). In a subgroup of 7 ISM patients, the identity of the isolated $CD105^{+}/CD13^{high}/CD45^{-}/CD34^{-}/CD14^{-}$ $/CD3^{-}$ MSCs population was confirmed using an expanded MoAb panel containing additional MSC-associated markers such as the homing cell adhesion glycoprotein (CD44), the platelet-derived growth factor receptor beta (CD140b), the nerve growth factor receptor (CD271), the mesenchymal stem cell antigen (MSCA-1), the stage-specific embryonic antigen-4 (SSEA-4), and the stromal cell precursor antigen (STRO-1). Furthermore, the functionality of the isolated $CD105^{+}/CD13^{high}/CD45^{-}$ MSC population, was validated in 4 ISM patients carrying both myeloid plus lymphoid multilineage involvement of hematopoiesis by the *KIT* D816V mutation and mutated MSCs, through *in vitro* culture and expansion of the isolated MSC and evaluation of their ability to undergo adipogenic and osteogenic differentiation, following previously described culture and staining conditions.[39]

DNA extraction and molecular studies. Genomic DNA (gDNA) was extracted from purified cell populations using previously described methods[4]. Positivity for the *KIT* D816V mutation was determined in gDNA of FACS-purified BM cell populations using a peptide-nucleic acid (PNA)-mediated PCR-clamping technique[4]. In order to validate those findings and to rule out any false positive result due to potential contamination of purified MSC by other *KIT* D816V mutated BM cells, the *KIT* mutational status of MSC was also investigated in parallel by a quantitative real-time allele-specific oligonucleotide qPCR (ASO-qPCR) method[42] that provided an accurate measurement of

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the mutated allele burden of the isolated MSC populations under study. Cases carrying *KIT* mutated MSC systematically showed a mutated allele burden greater than the contamination of the purified MSC by other BM cells. Multilineage (vs MC-restricted) *KIT* mutation was defined by the presence of the *KIT* mutation in maturing/matured BM cell compartments other than MC (e.g. neutrophils, monocytes, eosinophils, and/or lymphocytes, plus the $CD34^{+}$HPC) vs only the MC. MC clonality was further assayed on gDNA from 26/46 female patients by the human-androgen receptor (HUMARA) X-chromosome inactivation test[43].

Statistical analyses. To assess the statistical significance (set at *P*-values<.05) of differences observed between groups, either the Mann-Whitney U or the Pearson's Chi-square and the Fisher's exact tests were used for continuous and categorical variables, respectively (SPSS 20.0, SPSS, Chicago, IL, USA). Progression-free survival (PFS) curves were estimated according to the Kaplan-Meier method and compared with the Breslow (i.e. generalized Wilcoxon) test (SPSS 20.0).

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RESULTS

Immunophenotypic and functional characterization of BM MSCs from ISM patients. All purified $CD105^{+}/CD13^{high+}/CD45^{-}$ BM cells showed immunophenotypic features which were fully consistent with previously defined criteria for MSC[39-41], such as: absence of CD11b, CD14, CD19, CD34 and CD45 expression; heterogeneous reactivity for CD10 and HLA-DR, and; expression of the CD44, CD73, CD90, CD105, CD140b, CD146, CD271, MSCA-1, SSEA-4 and STRO-1 MSC-associated markers (Supplemental Figure 1).

Ex vivo culture and expansion of purified $CD105^{+}/CD13^{high}/CD45^{-}$ MSCs in 4 ISM cases carrying *KIT* $D816V^{+}$ MSC (median allele burden of 17%, range: 9%-34%) confirmed the presence of the *KIT* mutation in cultured MSC from all 4 cases in the first culture passage (median of 17 days of culture; range: 14-26 days) with a median *KIT* D816V mutated allele burden of 11% (range: 6-26%). In contrast, no *KIT* D816V mutated MSCs were detected after passage 3 (median of 39 days of culture; range: 27-47 days). After passage 4 (median of 45 days of culture; range: 32-55 days), in 3/4 cases enough cultured cells were obtained to evaluate the adipogenic and osteogenic differentiation of cultured MSC. Two cases showed normal adipogenic and osteogenic differentiation while the third case reached senescence prior to differentiating (Supplemental Figure 2).

Presence of the *KIT* D816V mutation in BM MSC and its association with ISM disease features at diagnosis. Overall, 22/83 ISM patients (27%) had the *KIT* D816V mutation in purified BM MSC (Table 2). All *KIT* D816V mutated-MSC patients showed a mutated allele burden >8% for the purified MSC population (median 18%; range: 8%-100%). Despite patients who had mutated MSC had a similar median follow-

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up and distribution by sex and age at onset, to cases having non-mutated MSC, both patient groups showed markedly different disease features at diagnosis (Table 2). Thus, patients that had *KIT* D816V^{+} MSC displayed higher levels of BM MC infiltration (median: 0.49% vs 0.08%; *P*=.008) and greater serum baseline tryptase (median: 170ng/ml vs 31.1ng/ml; *P*=.001), together with an increased frequency of bone lesions (39% vs 8%; *P*=.002), organomegalies (39% vs 7%; *P*=.006), multilineage *KIT* mutation (100% vs 30%; *P*<.0001) and, within multilineage cases, a greater number of cases with *KIT* mutated myeloid and lymphoid cells (59% vs 22%; *P*=.03) (Table 2). Interestingly, HUMARA analysis showed a polyclonal X-chromosome inactivation pattern (XCIP) in purified BM MC from 7/11 female patients who carried *KIT* D816V^{+} MSC; by contrast, all 15 female patients analyzed who showed *KIT* D816V^{-} MSC had BM MC with a clonal pattern of X-chromosome inactivation by HUMARA (Table 2).

Association between the presence of *KIT* D816V^{+} BM MSC and patient outcome. Overall, 14 ISM cases showed disease progression: 8 (57%) ISM evolved to ASM, 4 (29%) to SSM and 2 (14%) to SM-AHNMD (an MPN and a B-cell non-Hodgkin lymphoma) (Table 3). Median time from disease onset to progression was of 15 years. Except for a patient who progressed to ASM and displayed features associated with a moderate BM MC load (0.13% BM MC and 52.6ng/mL of serum baseline tryptase), all other patients displayed very high serum baseline tryptase levels (median: 275ng/mL; range: 144ng/mL to 2,036ng/mL) and/or of BM MC percentages (median: 3.1%; range: 0.06% to 18%) at progression. Of note, all ISM patients that progressed showed multilineage involvement of hematopoiesis by the *KIT* D816V mutation already at diagnosis.

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From those 14 ISM cases that showed progression, 11 belonged to the *KIT* D816V$^+$ MSC group (11/22, 50%), while only 3/61 ISM cases with non-mutated MSC showed disease progression (5%; *P*=.0005) (Table 2). Moreover, ISM patients with *KIT* D816V$^+$ MSC also showed a significantly (*P*<.001) shorter PFS vs patients who had non-mutated MSC (75% PFS of 13 years vs not reached; *P*<.001) (Figure 1A) with significantly lower PFS rates at 10- (82%±8% vs 98%±2%; *P*=.003), 20- (63%±12% vs 98%±2%; *P*<.001) and 30-years (54%±13% vs 78%±18%; *P*=.001) (Table 4).

In order to determine whether the presence of *KIT* D816V$^+$ MSC would be a better predictor for disease progression than the occurrence of multilineage involvement of BM hematopoiesis by the *KIT* mutation, we further restricted the comparison between patients with (n=22) vs without (n=18) *KIT* D816V$^+$ MSC to multilineage cases (n=40). Once again, ISM cases with *KIT* D816V$^+$ MSC more frequently had disease progression (50% vs 17%; *P*=.04) (Table 2). The adverse impact of having *KIT* D816V$^+$ MSC also translated into progressively shorter PFS rates (vs ISM cases having non-mutated MSC) at 10- (82%±8% vs 94%±5%), 20- (63%±12% vs 94%±5%) and 30-years (54%±13% vs 63%±26%), although differences only reached marginal statistical significance at 20- and 30-years (*P*≤.08) (Table 4). Despite the above differences in PFS rates, no statistically significant differences were found as regards overall survival between patients with vs without *KIT* D816V mutated MSC (overall survival rate at 10 years of 93.8% vs 100%, respectively). This is probably due to the fact that at the moment of closing this study, only 3/83 patients (3.6%) had died 7, 13 and 40 years after disease onset. Of note 2/3 cases had multilineage *KIT* mutation with *KIT* mutated MSC, the other patient having non-mutated MSC died from sepsis 40 years after disease onset.

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DISCUSSION

Presence of the somatic *KIT* D816V mutation[1,44] in BM MC is a molecular hallmark of adult SM[45] and a (minor) diagnostic criterion of the disease[22,35,36]. Additionally, detection of the *KIT* D816V mutation is thoroughly applied to establish the clonal nature of the disease[3,22,46], and it has also been used as a molecular marker to track the clonal origin of different hematological cell lineages within a patient (i.e. through the definition of MC-restricted vs multilineage involvement of hematopoiesis) and to establish the clonal relationship between SM MC and the AHNMD tumor cells in SM-AHNMD[12,13,15,16,23,47,48]. Around half of all 83 ISM cases here analyzed showed multilineage involvement of BM cells by the *KIT* D816V mutation and half of these multilineage cases also had *KIT* mutated MSC. Most interestingly, the presence of mutated MSC in the BM of ISM patients was associated with features of more advanced disease, a greater rate of disease progression and a shorter PFS. Of note, the greater frequency of multilineage *KIT* mutation among our cases vs previously published series from our group was due to the need for enough purified BM MSC for further molecular analyses, as discussed above in the methods section.

Interestingly, purified pathological *KIT* D816V$^+$ BM MC from a significant proportion of female ISM cases here analyzed showed a "polyclonal" XCIP, despite these cells most likely have a "clonally"-related origin. The cellular mosaicism resulting from the analysis of the XCIP in females has long been used as a marker to investigate clonal development and relationship among distinct cell compartments in different human hematopoietic disorders[19,49,50]; thus, a polyclonal XCIP in the absence of other genotypic markers (i.e. *KIT* D816V), could be interpreted as an accumulation of otherwise reactive, non-clonal MC. The apparent discrepancy observed between the genotypic clonality defined by the *KIT* D816V mutation and the polyclonal XCIP could

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be explained either by a hypothetical reactivation of the inactive X-chromosome[51] at any stage after the occurrence of the mutation in a committed HPC, or by the emergence of the *KIT* mutation in an uncommitted precursor/stem cell at relatively early stages of development (i.e. embryogenesis), prior to the inactivation of the X-chromosome during hematopoiesis. Since in human somatic cells XCIP appears to be rather stable[52], the second hypothesis seems most feasible. Thus, occurrence of the *KIT* D816V mutation during ontogeny would potentially target an early precursor/stem cell leading to multiple (instead of one) involved HPC; most likely, this would more frequently lead to multilineage involvement of hematopoiesis (and potentially also other tissues) by the *KIT* mutation. In line with this hypothesis, all ISM cases that displayed a polyclonal XCIP for their BM MC, also had multilineage involvement of hematopoiesis by the *KIT* mutation. These findings support previous observations in advanced SM about the origin of clonal MC in a pluripotent HPC with the ability to differentiate to other non-MC myeloid and even lymphoid lineages[12-16,45]. Most interestingly, this polyclonal XCIP found across multilineage ISM cases was restricted to two-thirds of the ISM female patients carrying *KIT* mutated MSC, while absent among ISM female patients with wild-type *KIT* MSC. MSC are multipotent mesodermal progenitor cells present in the BM stroma which diverged from the hematopoietic lineages early during embryogenesis[28]; thereby, these results would support the notion that in these patients, the *KIT* mutation could had been acquired in a common pluripotent progenitor cell early during ontogeny, prior to differentiation into MSC and HPC. This hypothesis is also consistent with a report of two monozygotic twins with adult-onset ISM carrying the somatic *KIT* D816V mutation[53] who both presented a mosaic pattern for the *KIT* mutation compatible with multilineage involvement of hematopoiesis. Noteworthy, despite the (potentially) very early acquisition of this mutation during ontogeny, onset

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of typical clinical symptoms of mastocytosis was delayed to their middle-adulthood, one of the twins progressing from ISM to an SM-AHNMD 30 years after skin lesions had appeared[53]; this is in line with our observations and those of other groups[7,54].

As a consequence of the occurrence of the *KIT* mutation in a common mesodermal ancestor of MSC and HPC, a mosaic of the mutated pluripotent precursor cell progeny would also carry the *KIT* genetic alteration, including both myeloid and lymphoid committed HPC, as found in our cases. The more frequent (simultaneous) involvement of myeloid and lymphoid cells observed among ISM cases with mutated vs wild-type BM MSC may be due to a greater number of involved HPC and the more extended involvement of hematopoiesis in the former vs the latter group. In turn, the existence of a subset of MSC-mutated ISM patients showing only involvement of myeloid BM cells, could be explained by preferential signaling of the mutated *KIT* in HPC to the myeloid rather than the lymphoid lineages, the significantly different production and renewal rates of mature lymphocytes vs monocytes and neutrophils (longer vs shorter turnover times, respectively) or both[55-58]. In addition, occurrence of a sporadic *KIT* D816V mutation that constitutively activates the SCF/KIT signaling pathway could lend those CD117^{+} precursor cells with a proliferative and/or survival advantage. Thereby, the ability of a *KIT* D816V^{+} myeloid-committed HPC to colonize the patient's BM will be faster than that of the mutated lymphoid-committed precursors, which would not have a proliferative advantage because of the activating *KIT* D816V mutation, due to loss of CD117 expression early during commitment to the lymphoid lineage[59]. In turn, the presence of the *KIT* D816V mutation in BM MSC should impair their function and potentially affect their role in supporting hematopoiesis and bone turnover. Even though we have not found any evidence of differences in bone lesions between patients with vs without mutated MSC, purified CD105^{+}/CD13^{high} /CD45^{-} BM

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MSC from SM patients showed slower growth *in vitro* vs MSC from healthy donors[39]. These results are in line with recent observations by Nemeth K et al. who described the presence of abnormal MSC with slow proliferation, signs of senescence and impaired osteogenic function (vs normal MSC colonies) in SM patients.[60] Of note, these authors do not find the *KIT* D816V mutation in cultured BM MSC from any of the 5 SM patients they analyzed. The apparent discrepancy between these findings and our observations could be due to the relatively low number of patient analyzed or to a preferential growth of normal vs mutated MSC after medium to long term in vitro culture. In this regard, our results also showed that while the *KIT* D816V mutation was initially detected in cultured (purified) $CD105^{+}/CD13^{high}$ $/CD45^{-}$ MSC, it became negative in vitro after 3 culture passages further supporting a survival/proliferative advantage in vitro for normal vs *KIT*-mutated MSC from ISM patients.

Overall, one might expect that the greater number of *KIT*-mutated HPC, the greatest level of BM involvement by the *KIT* mutation, which would most likely contribute to pave the way for secondary (driver) genetic lesions and more frequent disease progression. In line with this, all cases having *KIT*-mutated MSC also showed multilineage *KIT* mutation with a greater frequency of myeloid plus lymphoid (vs only myeloid) involvement of hematopoiesis. Moreover, such MSC-mutated ISM cases showed a greater frequency of progression to more advanced disease and shorter PFS rates, together with significantly greater levels of BM MC infiltration and serum baseline tryptase, and a greater frequency of organomegalies and bone lesions already at diagnosis. Of note, the rate of disease progression among multilineage cases, was also significantly higher for patients with *KIT*-mutated vs non-mutated MSC. Altogether, these results are consistent with recent observations in a mouse model with conditional expression of a constitutively active D814V mutated KIT which showed a greater MC

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disease severity when the mutation was expressed in undifferentiated HPC vs (only) more mature cells[61]. In addition, our findings might also contribute to explain why ISM patients with MC-restricted *KIT* mutation in the BM have a normal life expectancy and very rarely progress to more aggressive disease[7]. Of note, here we reported a higher rate of progression of ISM patients than that observed in previous (large) series of ISM patients[7,54,62]; this is probably due to the preferential selection of cases with multilineage involvement of hematopoiesis by the *KIT* D816V mutation vs MC-restricted *KIT* D816V mutation, since the latter cases typically showed very low rate of disease progression[7] and/or a longer follow-up in this vs other previously reported series.

Overall, the above findings suggest that the clinical impact of constitutive activation of the SCF/KIT signaling pathway critically depends on the stage of development at which the somatic *KIT* D816V mutation has been acquired, and the extent of involvement of the $CD34^+$HPC compartment (and therefore of the whole hematopoietic compartment). Thus, occurrence of the *KIT* mutation in an early progenitor cell during ontogeny will potentially lead to greater involvement of hematopoiesis, providing clonal cells an increased probability of acquiring secondary (driver) mutations/genetic alterations, particularly after long periods of time[9]; such genetic lesions might more frequently lead to progression and/or transformation of ISM to more severe disease and/or to the development of secondary myeloid (most frequently) and lymphoid neoplasias. Of note, acquisition and maintenance of these secondary mutations could be facilitated by the anti-apoptotic and survival pathways which are differentially activated within the pathological MC due to the *KIT* D816V mutation[25,27], in association with the more immature immunophenotype of BM MC from patients carrying multilineage vs MC-restricted *KIT* D816V mutation[37]. In line with this hypothesis, it has been recently shown in a murine model of SM, as well as

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among advanced SM patients[63], that coexistence of the *KIT* D816V mutation and loss of function of TET2 (or other mutations) in progenitor cells causes a more aggressive phenotype, typically mimicking advanced disease (i.e. ASM)[64].

In summary, the results here presented demonstrate the occurrence of the *KIT* D816V mutation in BM MSC from a substantial fraction of ISM patients that systematically showed multilineage involvement of hematopoiesis, in association with a greater risk for disease progression and shorter PFS. These findings suggest that among ISM cases, occurrence of the *KIT* D816V mutation in an earlier precursor cell is associated with a poorer outcome.

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AUTHORSHIP CONTRIBUTIONS:

AC. G-M. designed the research, analyzed the data, interpreted results, made the figures and wrote the paper; M. J-A performed experiments, analyzed the data, interpreted results, made the figures and wrote the paper; I. A-T collected the samples, performed the clinical follow-up of the patients and critically reviewed the paper; C. T performed experiments and critically reviewed the paper; C. M performed MSC experiments and critically reviewed the paper; L. S-M performed experiments and critically reviewed the paper; JI. M-G performed experiments and critically reviewed the paper; A. M performed experiments and critically reviewed the paper; A. M performed the clinical follow-up of the patients and critically reviewed the paper; C. C, contributed with technical support and critically reviewed the paper; JM. M. collected the samples and critically reviewed the paper; L.E. supervised the study, performed clinical follow-up of the patients and critically reviewed the paper; A. O. designed the research, supervised the study and wrote the paper.

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Table 1: Immunophenotypic markers used for the identification and characterization of bone marrow mesenchymal stem cells and other bone marrow cell populations.

MARKER	FLUOROCHROME	CLONE	SOURCE
CD3	PB	UCHT1	BD Bioscience[1]
CD10	APC-H7	HI10A	BD Bioscience[1]
CD11b	FITC	Bear1	BeckmanCoulter[6]
CD13	APC / PE	WM15 / L138	BD Bioscience[1]
CD14	APC-H7	M�P9	BD Bioscience[1]
CD19	APC-H7	SJ25C1	BD Bioscience[1]
CD34	PerCPCy5.5 / APC	8G12	BD Bioscience[1]
CD44	PerCP-Cy5.5	IM7	eBioscience[2]
CD45	PO / OC515	HI30 / GA90	Invitrogen[4] / Cytognos[3]
CD73	PE	AD2	BD Bioscience[1]
CD90	PE	5E10	BD Bioscience[1]
CD105	FITC / PE	166707 / 1G2	R&D Systems[5] / BeckmanCoulter[6]
CD117	PECy7	104D2D1	BeckmanCoulter[6]
CD140b	PE	28D4	BD Bioscience[1]
CD146	PE	P1H12	BD Bioscience[1]
CD271	PE Cy7	ME20.4	Biolegend[7]
HLA-DR	PB	L243	Biolegend[7]
MSCA-1	PE	W8B2	Miltenyi Biotec[8]
SSEA-4	PE	MC-813-70	Biolegend[7]
STRO-1	FITC	STRO-1	Biolegend[7]

APC, allophycocyanin; Cy7, cyanin7; FITC, fluorescein isothiocyanate; H7, Hilite7; OC515, Orange Cytognos 515; PB, pacific blue; PO, pacific orange; PE, phycoerythrin; PE Cy7, phycoerythrin cyanine 7; PerCPCy5.5, peridinin chlorophyll protein–cyanin5.5. [1]BD Biosciences, San Diego, CA, USA; [2] eBioscience, San Diego, CA, USA; [3]Cytognos SL, Salamanca, Spain; [4]Invitrogen, Carlsabad, CA, USA; [5]R&D Systems, Minneapolis, MN, USA; [6]Beckman Coulter, Brea, CA, USA; [7]Biolegend, San Diego, CA, USA. [8]Miltenyi Biotec (Bergisch Gladbach, Germany)

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Table 2.- ISM (n=83): disease features at diagnosis according to the presence vs absence of the *KIT* D816V mutation in bone marrow mesenchymal stem cells.

DISEASE FEATURES	*KIT* D816V+ Mesenchymal stem cells	*KIT* D816V-negative Mesenchymal stem cells			
		All cases		Cases with multilineage *KIT* mutation	
	(n=22)	(n=61)	*P*-value	(n=18)	*P*-value
Gender (M/F)	45%/55%	44%/56%	NS	40%/60%	NS
Age at onset (years)[+]	34 (0-76)	37 (9-63)	NS	34 (9-63)	NS
Follow-up (years)[+]	15.2 (2.4-45)	7.4 (3.6-37)	NS	15.5 (5.5-37)	NS
% of BM MC[+]	0.49% (0.03%-4.3%)	0.08% (0.004%-0.4%)	.008	0.16% (0.01%-0.4%)	NS
Serum tryptase(ng/mL)[+]	170 (12.1-404)	31.1 (8.6-182)	.001	55.5 (8.6-136)	NS
Bone lesions*	39%	8%	.002	22%	NS
Cytopenias	0%	2%	NS	6%	NS
Organomegalies[#]	39%	7%	.006	19%	NS
% of Multilineage *KIT* D816V+ mutation	100%	30%	.0001	100%	NS
% Myeloid plus lymphoid multilineage KIT D816V+ mutation	59%	7%	.0001	22%	.03
MC with a clonal X-chromosome inactivation pattern[a]	36%	100%	.0005	100%	.01
Disease progression	50%	5%	.0001	17%	.04

Results expressed as percentage of cases or as [+]median (range).*Bone lesions include osteoporosis and/or diffuse bone sclerosis due to SM. [#]Organomegaly includes palpable hepatomegaly and/or splenomegaly without organ failure. [a]Percentage of female cases with clonal MC as assessed by the human androgen receptor (HUMARA) *X* chromosome inactivation test. BM MC, bone marrow mast cells. NS, statistically not-significantly different.

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Table 3.- Relevant clinical and biological features at progression of ISM patients that progressed to more advanced disease.

	Patient ID.													
	#1	**#7**	**#8**	**#10**	**#15**	**#22**	**#30**	**#34**	**#38**	**#41**	**#47**	**#102**	**#104**	**#108**
Age at onset/ at progression (years)	35/60	76/78	24/64	0/31	63/76	73/76	33/61	25/37	64/69	30/64	28/46	50/51	48/49	31/62
Sex	M	M	F	M	M	F	F	F	M	M	M	M	F	M
Disease subtype at progression	SM-AHNMD (NHL)	SM-AHNMD (MPN)	ASM	ASM	SSM	ASM	SSM	SSM	ASM	ASM	SSM	ASM	ASM	ASM
Organomegalies	Yes*	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes*	Yes*	Yes	No	Yes*	Yes*
Cytopenias	No	No	No	Yes	No	Yes	No	No	Yes	No	No	Yes	Yes	Yes
Bone lesions	O+F	O	O+F	O, DBS	No	DBS	DBS	DBS	O	No	DBS	O	DBS	DBS
Serum tryptase (ng/mL)	182	144	239	430	170	357	173	329	52.6	151	310	238	312	2036
% of BM MC	0.4	0.6	9	11	3.2	18	0.06	0.35	0.13	0.5	3.1	0.47	3.7	4.8
% of *KIT* D816V$^+$ MSC	100%	100%	17%	49%	11%	21%	-	9%	-	-	31%	10%	24%	41%
***KIT* D816V$^+$ BM hematopoietic cell compartments**	MC+ M+L	MC+ M+L	MC+ M+L	MC+ M+L	MC+ M+L	MC+M	MC+M	MC+M	MC+M	MC+M	MC+M	MC+M	MC+ M+L	MC+M

ASM, aggressive systemic mastocytosis; BM MC, bone marrow mast cells; DBS, diffuse bone sclerosis; MC+M, MC plus other myeloid lineage cells; MC+M+L, MC plus other myeloid and lymphoid lineage cells; MPN, myeloproliferative neoplasm; NHL, non-Hodgkin lymphoma; O, osteoporosis; O+F, small size osteolysis and/or osteoporosis with pathologic fractures; SM-AHNMD, systemic mastocytosis associated with a clonal hematological non-mast cell lineage disease; SSM, smoldering systemic mastocytosis.
*with organ failure.

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Table 4.- ISM (n=83): cumulative probability of disease progression from disease onset according to the presence vs absence of the D816V *KIT* mutation in bone marrow mesenchymal stem cells.

Progression free survival rate	*KIT* D816V-positive mesenchymal stem cells	*KIT* D816V-negative mesenchymal stem cells			
		All cases		Cases with multilineage *KIT* mutation	
	(n=22)	(n=61)	*P*-value	(n=18)	*P*-value
10-year PFS	82% ± 8%	98% ± 2%	.003	94% ± 5%	.19
20-year PFS	63% ± 12%	98% ± 2%	<.001	94% ± 5%	.07
30-year PFS	54% ± 13%	78% ± 18%	.001	63% ± 26%	.08

Results expressed as percentage of cases ± one standard deviation. PFS: progression free survival.

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FIGURE LEGENDS

Figure 1.- Progression-free survival (PFS) of ISM patients classified according to the presence (dotted line) vs absence (full line) of the D816V *KIT* mutation in bone marrow mesenchymal stem cells (MSC). Panel **A** shows PFS from disease onset for all (n=83) patients analyzed, while in panel **B**, analysis of PFS is restricted to patients (n=40) with multilineage involvement of hematopoiesis by the *KIT* D816V mutation with (n=22) or without (n=18) D816V *KIT* mutated MSC.

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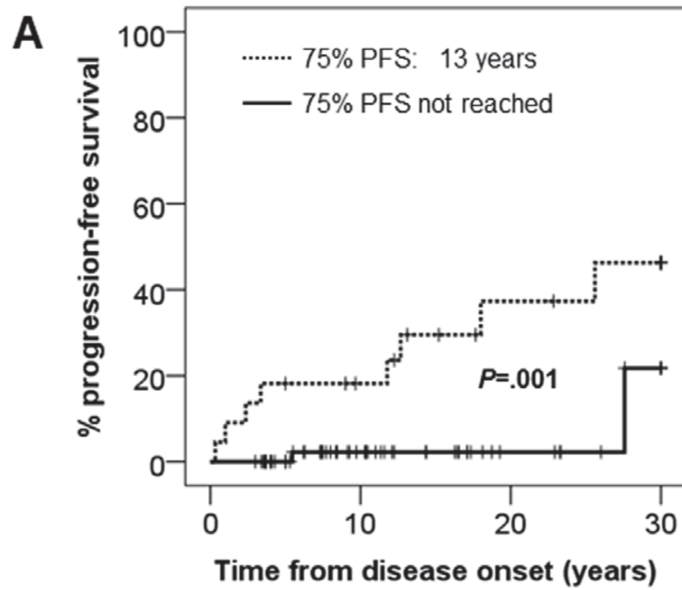
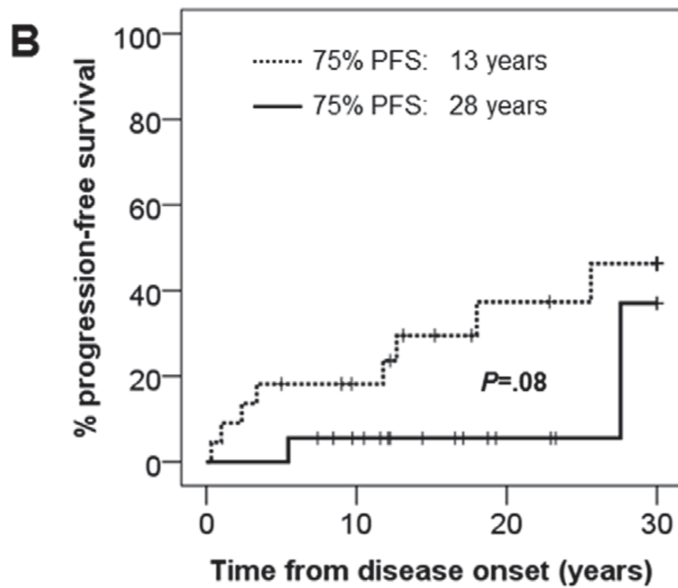


	0	10	20	30
N. of MSC+ cases	22	15	8	6
N. of MSC- cases	61	29	9	3



	0	10	20	30
N. of MSC+ cases	22	15	8	6
N. of MSC- cases	18	14	5	2

Figure 1.-

FIGURE LEGENDS

Figure S1.- Immunophenotypic features of bone marrow mesenchymal stem cells (MSC) identified as $CD105^{+}/CD13^{high}/CD45^{-}$ cells. Panels **A**-**D** depict the gating strategy used for the identification of MSC vs mast cells (MC) (Panel **D**). Panels **E**-**T** show the immunophenotypic features of MSC from a representative SM patient. Baseline autofluorescence and expression levels for each protein are indicated in the histogram plot in *gray* and *black*, respectively, using the overlay histogram function of the Infinicyt software (Cytognos SL, Salamanca, Spain). Panel **U** shows the pattern of expression of MSC-associated markers in purified BM $CD105^{+}/CD13^{high}/CD45^{-}$ MSC from 7 SM patients. Normalized median fluorescence intensity values are displayed after subtracting the background autofluorescence levels observed for each individual marker-associated fluorochrome channel. Open circles represent individual samples, short horizontal and vertical lines display median values and 95% CI of the levels of antigen expression (MFI values), respectively. *SSC,* sideward light scatter; *MFI,* median fluorescence intensity (arbitrary units scaled from 0 to 262 000).

Figure S2.- Functional features of bone marrow mesenchymal stem cells identified as $CD105^{+}/CD13^{high}/CD45^{-}$ cells. Panels **A**-**F** display the functional features of cultured FACS-sorted $CD105^{+}/CD13^{high}/CD45^{-}$ BM MSC which have been induced to differentiate *in vitro* towards the adipose tissue (panels **A**-**B**) as assessed by the Oil red O staining (Sigma-Aldrich, St. Louis, MO, USA), and the bone tissue as assessed by the alkaline phosphatase (Panels **C**-**D**) and the alizarin red S (Sigma-Aldrich) (Panels **E**-**F**) stainings. For each staining technique, undifferentiated control FACS-sorted BM MSC counterstained with hematoxylin are also displayed (Panels **A**, **C** and **E**). Images were acquired in an Eclipse Ti-s inverted microscope (Nikon, Tokyo, Japan) equipped with a ProgRes® C3 camera (Jenoptik Optical Systems, Jena, Germany). Magnification x200 (Panels **A** and **B**), x100 (Panels **C**-**D**) and x40 (Panels **E**-**F**).

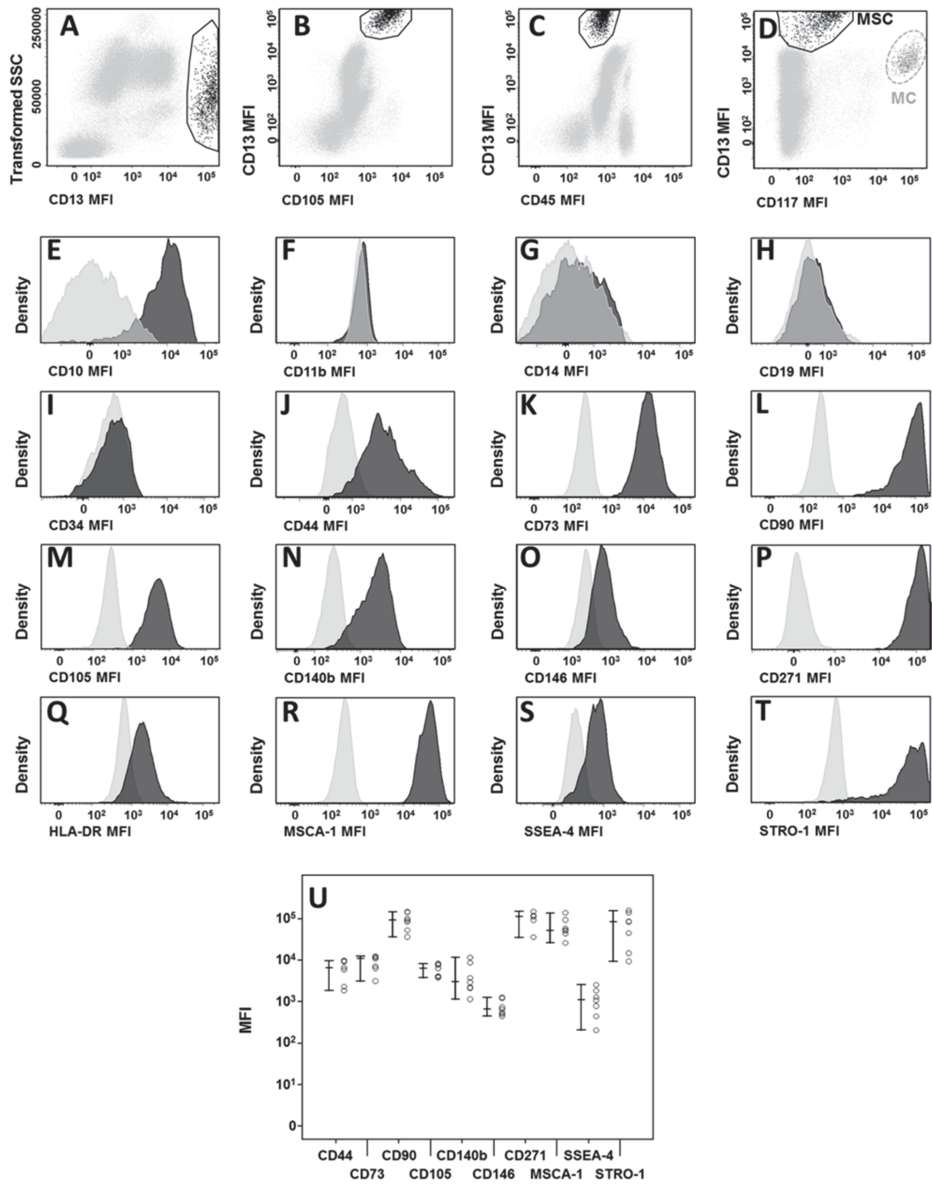


Figure S1.-

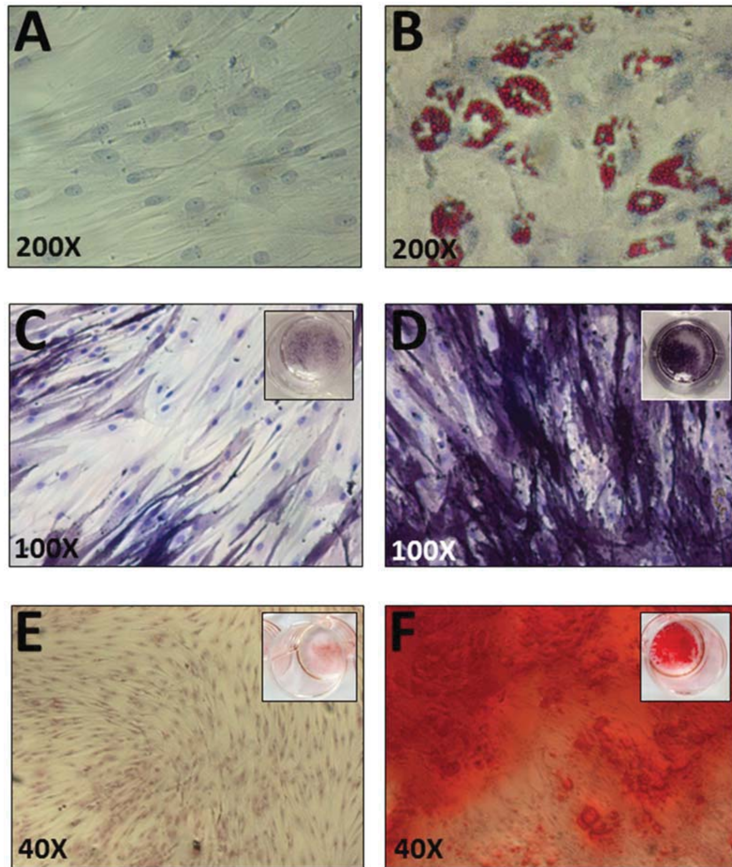


Figure S2.-