TITLE:

Clinical impact of oncogenetic profiles in Systemic mastocytosis with an associated hematological non-mast cell disease.

RUNNING TITLE: *KIT* mutation and AHNMD-associated genetic alterations in SM-AHNMD patients

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ABSTRACT

In a substantial fraction of systemic mastocytosis (SM) patients, SM coexists with an associated clonal hematological non-mast cell (MC) lineage disease (AHNMD). Most SM-AHNMD patients carry *KIT* mutations and AHNMD-associated genetic alterations; however, there is limited information about the frequency and clinical impact of the coexistence of both types of genetic/molecular alterations in distinct bone marrow (BM) cell compartments. Here we studied 65 SM-AHNMD patients grouped into SM-AHNMD cases with: i) unrelated genetic alterations; ii) shared *KIT* mutation in BM MC and AHNMD tumor cells, in the absence of AHNMD-associated genetic alterations in BM MC, and; iii) shared AHNMD-associated genetic alterations. Overall, patients with shared AHNMD-associated genetic alterations showed a significantly poorer progression-free survival (PFS) and overall survival (OS) vs. the other two groups ($p < 0.01$). In addition, the pattern of involvement of BM cell compartments other than MC by the *KIT* mutation and the subtypes of SM and AHNMD, were also relevant prognostic factors in the univariate analysis ($p < 0.01$). Multivariate analysis confirmed that the best combination of independent prognostic factors for OS and PFS were the pattern of involvement of BM cells by the *KIT* mutation ($p < 0.001$ and $p < 0.01$, respectively) and the oncogenetic subgroup of AHNMD ($p = 0.02$ and $p < 0.01$, respectively) together or not with the type of AHNMD (HR, 27.9; $p < 0.001$), respectively. Thus, in SM-AHNMD patients, the pattern of involvement of SM and AHNMD tumor cell compartments by the *KIT* mutation and AHNMD-associated genetic alterations adds prognostic information to that of specific diagnostic subtypes of SM and AHNMD.

KEY POINTS

1. SM-AHNMD patients show different oncogenetic profiles with an impact on disease outcome.

2. Coexistence of the *KIT* mutation and AHNMD-associated genetic markers in BM MC and AHNMD cells is an adverse prognostic factor in SM-ANMD.

INTRODUCTION

Systemic mastocytosis (SM) comprises a heterogeneous group of diseases characterized by an abnormal accumulation of clonal mast cells (MC) in one or more tissues.[1-3] According to their clinical, histopathological and biological features, seven different diagnostic subtypes of SM are currently recognized by the World Health Organization (WHO), including mild and poor-prognosis variants of the disease [1,2]. Among the later cases, SM with an associated clonal hematological non-MC lineage disease (SM-AHNMD) remains a particularly heterogeneous subgroup which most frequently consists of indolent (ISM) or aggressive SM (ASM) associated with a (coexisting) malignant myeloid disease –e.g. acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML) [2,4,5]-. However, in a significant proportion of SM-AHNMD cases, SM also coexists with a lymphoid and/or plasma cell neoplasm [6-11]. The specific subtypes of SM and AHNMD coexisting in individual patients are currently considered as the most relevant prognostic factors.[12,13]

Similarly to other diagnostic subtypes of SM, most (>90%) SM-AHNMD patients carry the D816V activating *KIT* mutation[14-16]. Despite this, previous observations indicate that [17-19] while in some SM-AHNMD patients the *KIT* mutation and/or other coexisting genetic alterations are present in both the SM and AHNMD cell compartments due to the potential involvement of a common stem cell, in other cases the *KIT* mutation is restricted to the MC; these findings point out the potential existence of distinct underlying molecular pathways for the two coexisting diseases [19,20] which might also potentially contribute to explain the heterogeneous clinical behavior of SM-AHNMD, even when considering cases presenting with the same subtypes of SM and AHNMD[17,21-23]. The clonal relationship between the SM and AHNMD cell compartments has been previously investigated in a limited number of studies including single case reports and a few SM-AHNMD patients series [18,24-28]; however, the clinical impact of the distinct ontogenetic pathways leading to the development of both diseases, in addition to their specific subtypes, still remains to be investigated [14,28-30].

Here, we report on the clinic-biological, immunophenotypic, genetic and prognostic features of 65 SM-AHNMD patients classified into three different ontogenetic groups based on the pattern of

involvement of BM MC, AHNMD tumor cells and other residual BM cells, by both the *KIT* mutation and AHNMD-associated cytogenetic/molecular alterations. To our knowledge, this is the largest series in which the clinical, histopathological and prognostic features of the disease are described for SM-AHNMD patients carrying distinct oncogenetic profiles.

MATERIALS AND METHODS

Patients and samples. Overall, 65 patients (40 males and 25 females; median age of 63 years, ranging from 10 to 87 years) with newly-diagnosed SM-AHNMD, were studied. SM and AHNMD were diagnosed and classified according to the WHO2008 criteria [31] (Table 1). For each patient, EDTA-anticoagulated BM samples were collected at diagnosis, after informed consent was given by the patient; the study was approved by the local Ethics Committee of the two REMA (Spanish Network on Mastocytosis) centers: Instituto de Estudios de Mastocitosis de Castilla-La Mancha (Toledo, Spain) and Cancer Research Center (University of Salamanca, Salamanca, Spain). Disease progression was defined as progression of SM and/or AHNMD to more advanced forms of either one or the two diseases, based on well-established criteria[32-40]. At the moment of closing this study, median follow-up was of 25 months (range:1-217 months) and 54% of patients remained alive.

Cell Purification. Purification of specific BM cell populations was performed using a FACSAria flow cytometer (BD). Prior to sorting, cells were stained with ≥4-color combinations of fluorochrome-conjugated MAb aimed at specific and simultaneous identification of the different cell populations of interest in the sample -e.g. blast/tumor (i.e. AHNMD) cells, maturing neutrophils, monocytic cells, nucleated red blood cells (NRBC) and mature lymphocytes-, as previously described in detail [41,42]. The purity achieved for the different FACS-sorted cell populations was systematically >97%.

Interphase fluorescence *in situ* hybridization (iFISH) and human androgen receptor assay (HUMARA) studies. iFISH studies aimed at detection of t(9;22), t(8;21), inv(16), 11q abnormalities, -5/del(5q), -7/del(7q), del(20q), trisomy 8, nulisomy Y, trisomy 12, del(17p13.1), del(13q14), t(14q32), t(18q21), t(11;14), t(3q27) and del(6q21) were performed on interphase nuclei from different FACS-purified and methanol/acetic fixed 3/1 (v/v) cell populations. The following panel of Spectrum Orange^{TM} and Spectrum Green^{TM} DNA probes (Vysis Inc, Downers Grove, IL) was used: LSI BCR/ABL, LSI AML1/ETO, LSI CBFB dual color breakapart,(DCBA) LSI MLL DCBA, LSI EGR1/D5S23, D5S721, LSI D7S486/CEP7, LSI D20S108, CEP8 (alpha satellite), CEPY (satellite III), CEP12 (alpha satellite), LSI ATM, LSI MLL, LSI P53, LSI 13/RB1, LSI D13S25, LSI IgH DCBA, LSI MALT1 DCBA, LSI

IgH/CCND1, and LSI BCL6 DCBA DNA probes. Additional DNA probes (Kreatech Diagnostics, Amsterdam, The Netherlands) used included the ON 6q21/SE Probe cocktail. Hybridization with fluorochrome-labeled FISH probes was performed as recommended by the manufacturer with slight modifications [43]. Fluorescence signals were evaluated using an Eclipse Ci fluorescence microscope (Nikon, Tokyo, Japan) and a 100x oil objective, as previously described [43].

In order to determine the pattern of inactivation of chromosome X, FACS-purified BM maturing neutrophils, monocytic cells, NRBC, CD34+ hematopoietic precursors (HPC) and mature lymphocytes were analyzed using HUMARA, as previously reported [44].

***KIT* mutational analysis.** The *KIT* D816V mutation was assessed in genomic DNA from FACS-purified cell populations, using a previously described polymerase chain reaction and peptide nucleic acid-clamping technique [14,45].

Statistical methods. For all continuous variables, median values and range were calculated using the Statistical Package for the Social Sciences Software (SPSS 23.0, IBM, Chicago, IL); for categorical variables, frequencies were used. For categorical and continuous variables, comparisons between two or more groups were made using the chi-square test, and either the Student T (for parametric data) or the Mann-Whitney U and the Kruskal-Wallis tests (for non-parametric data), respectively. Survival curves were plotted according to the method of Kaplan and Meier [46] and compared by the (one-side) log-rank test. Based on those variables which had a significant effect on overall survival (OS) and progression-free survival (PFS) in the univariate analysis, multivariate Cox proportional-hazard models were built using those parameters which showed independent predictive value for OS and PFS, and a forward stepwise approach. P-values <0.05 were considered to be associated with statistical significance.

RESULTS

Diagnostic subgroups of SM-AHNMD. The majority of SM-AHNMD cases (58/65; 89%) had SM associated with one AHNMD; in the other 7 patients (11%) SM was associated with two AHNMD (Table 1). Most cases in the former group (48/65; 74%) had SM plus a myeloid AHNMD (Table 1) consisting of: i) MDS in 25 cases, (38%); ii) AML in 12 (18%), iii) MDS/myeloproliferative neoplasm (MDS/MPN) in 7, (11%) including CMML in 5 (8%), MDS/MPN with eosinophilia (MPNEo) in 1 (2%) and MDS/MPN-unclassificable (MPN-UC) in the remaining case (2%), and; iv) MPN in 4 cases (6%) -two (3%) polycythaemia vera (PV) patients, one (1%) essential thrombocythemia (ET) and one (2%) hypereosinophilic syndrome (HES)- (Table 1). In turn, lymphoid AHNMD were less frequently observed (17/65 patients; 26%) (Table 1) and included monoclonal gammopathy of undetermined significance (MGUS) in 7 (11%) patients, multiple myeloma (MM) in 2 (3%), chronic lymphocytic leukemia (CLL) in another 2 (3%) and marginal zone lymphoma (MZL) in 1 patient (2%) (Table 1). In those 7 patients (11%) who presented with two AHNMD neoplasms, these consisted of a myeloid plus a lymphoid AHNMD in 4 cases (6%) -i.e. ET together with MZL, MDS plus MM, CMML with an MGUS and CMML plus MM in one patient each- and of two coexisting lymphoid neoplasms in the other 3 patients (5%)- i.e. CLL plus MZL in 2 cases (3%) and diffuse large B-cell lymphoma (DLBCL) plus large granular T-cell leukemia (T-LGL) in one (2%)- (Table 1). According to the WHO2008 classification, 34 patients (52%) had ISM-AHNMD, 26 (40%) had ASM-AHNMD and 2 (4%) were diagnosed with mast cell leukemia (MCL)-AHNMD. No significant association was found between the subtypes of SM and AHNMD, except for a greater frequency (p=0.05) of CMML in ASM vs ISM cases -6/26 (23%) vs 2/34 (6%)- and a higher proportion (p=0.03) of lymphoid AHNMD in ISM vs ASM patients -12/34 (35%) vs 3/26 (12%)-.

Frequency of *KIT* mutation and AHNMD-associated genetic alterations in different BM cell compartments of SM-AHNMD patients. The *KIT* D816V mutation was present in BM MC from all SM-AHNMD patients; in 28/65 cases (43%) it was restricted to MC, while in 37/65 patients (57%) it was shared by both MC and AHNMD cells (Table 1 and Supplementary Table 1). In addition, AHNMD-associated genetic alterations other than the *KIT* mutation were also detected in 33/65 (51%) SM-AHNMD cases. In a subset of these patients (12/33; 36%), the *KIT* mutation was restricted to BM MC in the absence

(in these cells) of AHNMD-associated genetic alterations; in contrast, in another 12/33 cases (36%) BM MC and AHNMD cells carried one or more AHNMD-associated cytogenetic alterations in common (Supplementary Table 1) together (9/33; 27%) or not (3/33; 9%) with the *KIT* mutation in both cell compartments (Table 2); in the remaining 9 patients (27%) who had AHNMD-associated genetic alterations, both BM MC and non-MC compartments, including AHNMD cells, shared the *KIT* mutation but not the AHNMD-associated alterations (Supplementary Table 1).

In all cases that shared the *KIT* mutation in BM MC and AHNMD tumor cells, this mutation was detected in both BM MC and other myeloid BM cells; additionally, a significant fraction of them - 10/28 cases (36%)- showed also *KIT*-mutated BM lymphoid cells (Tables 1 and 2). Interestingly, purified BM mesenchymal stem cells (MSC) from 5/6 (83%) SM-AHNMD cases investigated, who carried myeloid (n=1) or myeloid plus lymphoid (n=5) involvement by the *KIT* mutation, showed *KIT*-mutated MSC. In turn, all 12 cases that shared AHNMD-associated genetic alterations in BM MC and AHNMD cells (together or not with the *KIT* mutation) showed involvement of myeloid but not lymphoid residual BM cells by such genetic alterations. (Tables 1 and 2 and Supplementary Table 1). Of note, in the majority of these patients (7/12; 58%) the *KIT* mutation was detected in all myeloid and lymphoid BM cell lineages analyzed, while AHNMD-associated genetic alterations were restricted to BM AHNMD and myeloid cells, but absent in lymphoid cells (Table 2), suggesting the *KIT* mutation was an earlier event.

Based on the above patterns of involvement of BM MC, AHNMD tumor cells and other BM myeloid and lymphoid cell compartments by the *KIT* mutation and/or AHNMD-associated genetic alterations, an hypothetical model of clonal evolution was built for each individual patient (Figure 1). Altogether, individual patient models showed that, while there are cases where the *KIT* mutation is restricted to BM MC (28/65; 43%) and patients with AHNMD-associated genetic alterations restricted to BM AHNMD tumor cells (25/65; 39%), in most SM-AHNMD patients either one or the two alterations coexisted in BM MC, AHNMD tumor cells and/or other BM cells (37/65; 57%). In the later cases, the *KIT* mutation almost systematically appears to be the first hit, being positive across most myeloid and lymphoid cell populations analyzed, while AHNMD-associated genetic changes displayed a more restricted pattern of involvement of the different BM myeloid and/or lymphoid compartments (Figure 1 and Table 2).

Oncogenetic subgroups of SM-AHNMD. Depending on whether the *KIT* mutation and/or the AHNMD-associated genetic alterations were shared or not by BM MC and AHNMD tumor cells, SM-AHNMD patients were subclassified into three distinct groups (Table 1): i) SM-AHNMD with unrelated genetic alterations in whom the *KIT* mutation and AHNMD-associated genetic alterations were present in different tumor cell compartments (n=25; 39%); ii) SM-AHNMD which shared the *KIT* mutation (but not AHNMD-associated genetic alterations) in both BM MC and AHNMD cells (n=28; 43%), and; iii): cases who shared AHNMD-associated genetic alterations in BM MC and AHNMD tumor cells, the *KIT* mutation being present (n=7) or absent (n=5) in the later cell population (n=12; 18%).

Overall, SM-AHNMD cases whose BM MC and AHNMD tumor cells shared the *KIT* mutation and/or AHNMD-associated genetic alterations, more frequently showed myeloid AHNMD (31% vs 69%, p= 0.04). Interestingly, within these cases the frequency of MDS plus MDS/MPN was also significantly higher (25% vs 75%, p=0.03). In turn, AML was more frequently observed among patients who did not have the *KIT* mutation in AHNMD cells -2/12 (17%) vs 10/12 (83%), (p=0.02)-. By contrast, cases who have no shared genetic alterations between BM MC and AHNMD cells showed predominance of lymphoid AHNMD, in association or not with another myeloid AHNMD (p= 0.03): 10/25 (40%) vs 7/40 (18%) cases, respectively (Table 1).

Clinical and biological features of SM-AHNMD cases grouped according to the pattern of involvement of BM cells by the *KIT* mutation and AHNMD-associated genetic alterations. Overall, SM-AHNMD patients who showed AHNMD-associated genetic alterations in both BM AHNMD cells and MC at diagnosis, had lower age (p=0.03), lower frequency of skin lesions (p=0.03), lower peripheral blood (PB) platelet counts (p=0.05) and higher creatinine serum levels (p<0.01) (Table 3). In contrast, cases who had no genetic alterations in common in BM MC and AHNMD tumor cells had a lower frequency of splenomegaly (p<0.01), a higher percentage of skin lesions (p=0.03) and anaphylaxis (p<0.01), together with lower creatinine (p<0.01) and tryptase serum levels (p=0.03) (Table 3). Cases presenting with the *KIT* mutation in both BM MC and AHNMD cells had intermediate features between the other two groups, more closely resembling those of patients who shared AHNMD-associated genetic alterations (Table 3).

Most interestingly, SM-AHNMD cases who showed no shared genetic alterations between BM MC and AHNMD tumor cells, more frequently had ISM, while ASM was more commonly observed in the other two groups of patients (p<0.01) and the two SM-MCL cases had AHNMD-associated genetic alterations also in BM MC (Table 3).

Clinical outcome of SM-AHNMD cases. Overall, PFS and OS progressively decreased from SM-AHNMD patients who did not share any genetic alterations - median PFS and OS: not reached (NR) - to cases that shared the *KIT* mutation (median PFS and OS: 30 and 48 months, respectively) and patients with AHNMD-associated genetic alterations in both BM MC and AHNMD cells (median PFS and OS: 6 and 11 months, respectively) (p<0.01) (Table 4 and Figure 2). Of note, differences in PFS and OS rates were maintained when the analysis was restricted to patients who had SM associated with a myeloid malignancy (p=0.04 and p=0.02 respectively), SM associated with MDS and/or MDS/MPN (p=0.06 and p=0.04, respectively) or just to SM-MDS patients (p=0.04 and p=0.02, respectively) (Figure 2). Other prognostic factors in the univariate analysis included: the subtype of SM –median PFS and OS of ISM vs ASM/MCL: NR vs 13 months (p=0.001) and NR vs 18 months (p=0.005), respectively-, the pattern of involvement of BM cells by the *KIT* mutation -median PFS and OS of MC-restricted vs myeloid vs myeloid plus lymphoid involvement of NR vs NR vs 7 months (p=0.007 and 0.001, respectively)- and the type of AHNMD - median PFS and OS of AML vs MDS plus MDS/MPN vs other AHNMD of 9 vs 21 vs 167 months (p<0.001) and 4 months vs 43 months vs NR (p<0.001), respectively)-.

Multivariate analysis of prognostic factors for the whole patient series showed that the best combination of independent variables to predict for OS included the type of AHNMD (AML vs other; HR of 27.9, p<0.001), the pattern of involvement of BM cells by the *KIT* mutation -myeloid plus lymphoid or myeloid vs MC-restricted; HR of 23.9 (p≤0.001) and 6.2 (p=0.03), respectively- and the oncogenetic subgroup of SM-AHNMD (shared genetic alterations by BM MC and AHNMD cells vs the other groups; HR of 5.0, p=0.02). Similarly, the best combination of prognostic factors to predict for PFS included the oncogenetic group -shared AHNMD-associated genetic alterations or shared *KIT* mutation vs the other group; HR of 12.1 (p<0.001) and 3.1 (p=0.03), respectively- and the type of AHNMD -AML or CMML vs other AHNMD; HR of 5.2 (p<0.001) and 3.2 (p=0.03), respectively-.

When multivariate analysis was restricted to SM-MDS plus SM-MDS/MPN patients, the pattern of involvement of BM cells by the *KIT* mutation (myeloid plus lymphoid vs both myeloid and MC-restricted; HR of 4.3, p=0.03) emerged as the most powerful independent (adverse) prognostic factor for OS. For this specific subgroup of SM-AHNMD patients, the type of SM (ASM vs ISM; HR of 2.8, p=0.04) was the only independent predictor for PFS.

DISCUSSION

The association of SM with another non-MC hematopoietic neoplasm has been long recognized as a common finding among mastocytosis patients, occurring in between 5% to 40% of all SM patients,[1,3,17] such cases being considered by the WHO as a specific poor-prognosis subgroup of SM[1]. Despite this, SM-AHNMD still remains a highly heterogeneous diagnostic entity from the clinical, genetic and prognostic point of view. In fact, both the SM (e.g.ISM vs AML and MCL) and AHNMD diagnostic components of SM-AHNMD patients vary substantially, leading to many different combinations of malignant (e.g. ASM or MCL and AML or MDS, respectively) and/or indolent (e.g.ISM and MGUS) disorders[2,47]. Despite few studies have been reported so far in which relatively small series of SM-AHNMD patients are characterized in detail, the diagnostic subtypes of both SM and AHNMD are generally considered as the major variables that influence disease behavior and patient outcome [12,48] and thereby, the most relevant prognostic factors for SM-AHNMD cases[5]. In turn, at present it is also well-established that SM-AHNMD patients might display distinct oncogenetic profiles, even within well-defined categories of SM-AHNMD such as ASM-MDS [12,19,20,48-52]. However, the potential clinical impact and prognostic relevance of the underlying oncogenetic pathways, defined by the pattern of involvement of BM MC and AHNMD cells (and other non-MC hematopoietic cells) by the *KIT* mutation and other AHNMD-associated genetic alterations, has not been thoroughly investigated.

Here, we confirmed the presence of distinct oncogenetic profiles in SM-AHNMD patients, based on the pattern of involvement of the coexisting hematopoietic tumor cell compartments by the *KIT* mutation and AHNMD-associated genetic alterations. Thus, SM-AHNMD patients could be classified into three major oncogenetic groups depending on whether such alterations were shared or not by BM MC and AHNMD tumor cells. Of note, the former patterns were more frequently associated with ASM and myeloid AHNMD, particularly MDS, MDS/MPN and AML, while the later predominate in patients with ISM and lymphoid neoplasms.

Previous studies indicate that coexistence of SM with AHNMD is not random [13,18,53-56], occurrence of an AHNMD being a relatively common finding among SM patients, either at diagnosis or during disease progression[12,14,48]. In addition, recent studies also found a direct relationship between a greater degree of

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involvement of distinct BM hematopoietic cells compartments [9,13] and MSC [57] by the *KIT* mutation, and an increased risk of progression of ISM to poor-prognosis categories of the disease, including ASM and SM-AHNMD. Altogether, these findings suggest that the occurrence of the *KIT* mutation in an early hematopoietic stem cell could favor the development of another (typically myeloid) hematological malignancy. In line with these findings, here we observed a substantial degree of involvement of BM hematopoietic cell compartments other than MC by the *KIT* mutation, including AHNMD tumor cells, particularly among SM-AHNMD patients who had ASM and myeloid malignancies[12,18,47,48]. Most importantly, in a substantial fraction of our cases, BM MC also shared AHNMD-associated genetic alterations, frequently together with *KIT*-mutated AHNMD cells; in these cases, the later genetic alteration was associated with a more extended pattern of involvement of BM hematopoiesis, frequently including both myeloid and lymphoid cells. Altogether, these findings suggest that in most SM-AHNMD patients who show shared genetic alterations, the *KIT* mutation could occur at an earlier stage of hematopoietic development, AHNMD-associated genetic changes emerging later in an already *KIT*-mutated hematopoietic stem cell which is more prone to undergo myeloid than lymphoid differentiation; this could contribute to explain the higher frequency of myeloid malignancies in these patients. These findings are in contrast with previous *in vitro* culture observations that suggested SM-AHNMD is a multi-mutated neoplasm where the *KIT* mutation appears to be a late event[19]. However, these investigations were developed in single cell-derived granulocyte-macrophage colonies (CFU-GM) (and not primary single tumor cells) from a relatively limited number of SM-AHNMD patients who mostly had SM-MDS/MPN[19]. In line with this, a small fraction of our patients also had *KIT* mutation restricted to BM MC with AHNMD-associated genetic alterations positive in AHNMD cells and *KIT*-mutated MC. Of note, this later pattern was also more frequently observed among AML and high risk MDS cases, and it may lead to an earlier development of AHNMD, as reflected by the younger age found here for this patient subgroup.

Apart from the above two patient groups, a significant proportion of our SM-AHNMD patients, showed no genetic markers in common between tumoral BM MC and AHNMD cells, suggesting they had two unrelated diseases, in line with previous observations[18,20,52]. It should be noted however, that we did not specifically search for all molecular changes that have been reported to be associated with myeloid

malignancies (e.g. MDS and AML) such as *NMPM1*, *ASXL1*, *TET2, DNMT3A* gene mutations[19,49-53]. So far, these later AHNMD-associated molecular markers have not been separately investigated in BM MC and AHNMD cells from individual SM-AHNMD patients, further investigations being necessary to determine whether in this subset of patients both diseases are in fact ontogenetically independent. Despite this, in a significant fraction of those SM-AHNMD patients who shared no genetic alterations, the AHNMD corresponded to (some of) the most prevalent (benign/indolent) hematological disorders in the elderly such as MGUS, CLL and low-grade lymphomas, in the absence of involvement of the hematopoiesis by the *KIT* mutation. These findings suggest that at least in a substantial fraction of cases, both tumor cell types could be genetically unrelated.

From the clinical point of view, cases who had no genetic/molecular markers in common between BM MC and AHNMD cells, more frequently displayed symptoms/signs of disease associated with early forms of mastocytosis such as anaphylaxis and skin lesions; in contrast, other SM-AHNMD cases had more advanced forms of SM (e.g. ASM and MCL) together with disease features associated with advanced disease (e.g. greater serum tryptase levels and creatinine and/or higher frequency of splenomegaly), although some of these features could also be related or influenced by the coexisting AHNMD. These results are in line with previous observations from our and other groups [9,13,14,44,56,58,59], which demonstrated that most ASM and MCL patients display multilineage involvement of BM hematopoiesis by the *KIT* mutation, whereas ISM patients most frequently show a MC-restricted *KIT* mutational profile, independently of the AHNMD[9,14,20,44]. From the prognostic point of view, our results extend on these observations and show that SM-AHNMD cases who share AHNMD-associated genetic alterations and/or the *KIT* mutation by both BM MC and AHNMD tumor cells display a significant poorer outcome than SM-AHNMD patients in which such genetic alterations are restricted to the AHNMD tumor cell and BM MC compartments, respectively. Interestingly, the pattern of involvement of BM MC and AHNMD cells by both molecular/genetic alterations emerged as a powerful prognostic factor for both PFS and OS among SM-AHNMD patients, independently of the specific subtypes of SM and AHNMD, even when the analysis was restricted to the most frequent subtypes of AHNMD (e.g. MDS plus MDS/MPN).

In summary, our results confirm the clinical, genetic and prognostic heterogeneity of SM-AHNMD and indicate that both disease behavior and patient outcome depend on the underlying oncogenetic profile of tumor MC and AHNMD cells, in addition to the specific diagnostic subtypes of SM and AHNMD.

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FIGURE LEGENDS:

FIGURE 1: Clonal evolution patterns observed in SM-AHNMD patients according to the distribution of the *KIT* mutation as well as AHNMD-associated cytogenetic/molecular alterations in different BM cell compartments. The sequence of acquisition of the *KIT* D816V mutation and other AHNMD-associated cytogenetic/molecular alterations by MC and other non-MC BM cell compartments (from CD34+ HSC to the common myeloid and lymphoid precursors, maturing myeloid cells, mast cells and lymphoid cells) is shown for the 65 SM-AHNMD patients analyzed. Numbers the small circles represent cases who acquired the *KIT* mutation and AHNMD-associated genetic/molecular alterations (gray and black areas in the upper and lower part of the small circle) at the indicated level. The large pie charts represent the accumulated frequency (%) of cases carrying the *KIT* mutation and AHNMD-associated genetic/molecular alterations at each cell differentiation stage.*In 5/6 cases, involvement of bone marrow mesenchymal stem cell (BM MSC) was also confirmed, suggesting that in such cases the genetic abnormalities have most probably targeted an earlier precursor than the HSC. **C-Ly Progenitor**, Common lymphoid progenitor; **C-My Progenitor,** common myeloid progenitor; **GM cells**; maturing neutrophils and monocytic cells; **HSC**, hematopoietic stem cell.

FIGURE 2: Progression-free survival (left panels) and overall survival (left panels) of SM-AHNMD patients grouped according to the pattern of involvement of BM MC and other non-MC hematopoietic cell compartments by the *KIT* mutation and other AHNMD-associated cytogenetic/molecular alterations. Numbers indicate median survival in months (95% confidence interval). ━ No genetic/molecular alteration shared. ⋯ Shared *KIT* mutation. ▬ Shared AHNMD associated cytogenetic/molecular alteration w/o *KIT* mutation.

Table 1. Distribution of SM-AHNMD cases according to the pattern of involvement of BM MC and other BM non-MC hematopoietic cell compartments by the *KIT* mutation and other AHNMD-associated cytogenetic/molecular alterations.

Subtype of AHNMD	TOTAL (n=65)	Pattern of involvement of BM MC and other non-MC hematopoietic cell compartments by the *KIT* mutation and AHNMD-associated genetic alterations			*P*
		No genetic alterations shared (n=25)	Shared *KIT* mutation (n=28)	Shared AHNMD-associated genetic alterations (n=12)[a]	
AHNMD of myeloid origin	**48/65 (73%)**	**15/25 (60%)**	**22/28 (78%)**	**11/12 (92%)**	*0.04
AML	12/65 (18%)	5/25 (20%)	2/28 (7%)	5/12 (42%)	0.05 ; *0.02
MDS	25/65 (38%)	7/25 (28%)	13/28 (46%)	5[a]/12 (42%)	NS
MDS/MPN[1]	7/65 (11%)	1/25 (4%)	5/28 (18%)	1/12 (8%)	*0.05
MPN[2]	4/65 (6%)	2/25 (8%)	2/28 (7%)	0/12 (0%)	NS
AHNMD of lymphoid origin	**10/65 (16%)**	**7/25 (28%)**	**3/28(11%)**	**0/12 (0%)**	0.05 ; ***0.03
B-CLPD or T-CLPD	3/65 (5%)	2/25 (8%)	1/28(4%)	0/12 (0%)	NS
MGUS	7/65 (11%)	5/25 (20%)	2/28(7%)	0/12	**0.05
Multiple myeloid and/or lymphoid AHNMD	**7/65 (11%)**	**3/25 (12%)**	**3/28 (11%)**	**1/12 (8%)**	NS
B-CLPD + B-CLPD[3]	2/65 (3%)	2/25 (8%)	0/28	0/12 (0%)	NE
B-CLPD + T-CLPD[4]	1/65 (1.5%)	0/25	1/28(4%)	0/12 (0%)	NE
B-CLPD + MPN[5]	1/65 (1.5%)	1/25 (4%)	0/28	0/1 (0%)	NE
MDS/MPN + MGUS	1/65 (1.5%)	0/25	1/28(4%)	0/1 (0%)	NE
MDS + MM[6]	1/65 (1.5%)	0/25	1/28(4%)	0/1 (0%)	NE
MDS/MPN + MM	1/65 (1.5%)	0/25	0/28	1/12 (8%)	NE

AML, acute myeloid leukemia; **ANHMD**, associated clonal hematological non-mast cell lineage disease; **B-CLPD**, B-cell chronic lymphoproliferative disorder; **MDS,** myelodysplastic syndrome; **MDS/MPN** myelodysplastic syndrome/myeloproliferative neoplasm; [1]Chronic myelomonocytic leukemia (CMML), 5 cases; myeloproliferative neoplasm with eosinophilia (MPNEo), 1 case; unclassified myeloproliferative neoplasm (MPN-UC), 1 patient; **MGUS**, monoclonal gammopathy of undetermined significance 8 cases; **MM**, multiple myeloma 2 cases; [2]polycythemia vera (PV), 2 cases; essential thrombocythemia (ET), 1 case; hypereosinophilic syndrome (HES) 1 patient; **T-CLPD,** T-cell chronic lymphoproliferative disorder; **SM**, systemic mastocytosis; [3] Chronic lymphocytic leukaemia (CLL) + marginal zone B cell lymphoma (MZL) 2 cases; [4]Diffuse large B-cell lymphoma (DLBCL) + large granular T cell leukemia/lymphoma (LGL), 1 case; [5]ET + MZL,1 case; [a]3 cases showed *KIT* mutation restricted to MC but MC showed MDS genetic markers of clonality. * Shared *KIT* mutation group vs the other two groups. ** Shared AHNMD-associated genetic alterations group vs the other two groups. ** No shared genetic alterations group vs the other two groups. **NE:** not evaluable due to the low number of cases/group. **NS,** no statistically significant differences found for all group comparisons (p>0.05).

Table 2. Distribution of the *KIT* mutation and AHNMD-genetic alterations detected in BM MC, AHNMD tumor cells and different non-MC BM cell compartments in SM-AHNMD patients (n=65).

Patient code	SM-AHNMD subgroups	Diagnosis	MC		ANHMD tumor cells		Myeloid cells								Lymphoid cells		MSC
							CD34+		Neutrophils		Monocytes		NRBC				
			KIT	AHNMD*	*KIT*	AHNMD	*KIT*	AHNMD	*KIT*	AHNMD	*KIT*	AHNMD	*KIT*	AHNMD	*KIT*	AHNMD	*KIT*
1		SM-AML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
2		SM-AML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
3		SM-AML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
4		SM-AML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
5		SM-AML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
6		SM-MDS	+	-	-	+	-	+	-	+	-	-	-	-	-	-	NA
7		SM-MDS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
8		SM-MDS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
9		SM-MDS	+	-	-	+	-	+	-	+	-	+	-	+	-	-	NA
10		SM-MDS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
11		SM-MDS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
12		SM-MDS	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
13	No genetic alteration shared	SM-CMML	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
14		SM-TE	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
15		SM-PV	+	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
16		SM-MGUS	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
17		SM-MGUS	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
18		SM-MGUS	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
19		SM-MGUS	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
20		SM-MGUS	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
21		SM-CLPD	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
22		SM-CLPD	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
23		SM+ET+MZL	+	-	-	+	-	+	-	+	-	-	-	+	-	-	NA
24		SM+CLL+MZL	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
25		SM+CLL+MZL	+	-	-	+	-	-	-	-	-	-	-	-	-	-	NA
26		SM-AML	+	-	+	-	+	-	+	-	-	-	-	-	-	-	NA
27		SM-MDS	+	-	+	+	+	+	+	+	+	+	+	+	-	-	NA
28		SM-MDS	+	-	+	+	+	+	+	+	+	+	+	+	-	-	NA
29		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
30		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
31		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
32		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
33		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
34		SM-CMML	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
35		SM-CMML	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
36		SM-CMML	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
37		SM-CMML	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
38	Shared *KIT* mutation	SM-PV	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
39		SM-HES	+	-	+	-	+	-	+	-	+	-	+	-	-	-	NA
40		SM-MGUS	+	-	+	+	+	-	+	-	+	-	+	-	-	-	NA
41		SM-MGUS	+	-	+	+	+	-	+	-	+	-	+	-	-	-	NA
42		SM-CLPD	+	-	+	+	+	-	+	-	+	-	+	-	-	-	NA
43		SM+MGUS+CMML	+	-	+	+	+	-	+	-	+	-	+	-	-	-	NA
44		SM-AML	+	-	+	+	+	+	+	+	+	-	+	-	+	-	+
45		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	+	-	NA
46		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	+	-	NA
47		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	+	-	NA
48		SM-MDS	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+
49		SM-MDS	+	-	+	+	+	-	+	+	+	-	+	+	+	-	NA
50		SM-MDS	+	-	+	-	+	-	+	-	+	-	+	-	+	-	NA
51		SM-CMML	+	-	+	-	+	-	+	-	+	-	+	-	+	-	NA
52		SM+DLBCL+LGL	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
53		SM+MM+ SMD	+	-	+	-	+	-	+	-	+	-	+	-	+	-	-
54		SM-AML	+	+	-	+	-	+	-	+	-	+	-	+	-	-	NA
55		SM-MDS	+	+	-	+	-	+	-	+	-	+	-	NA	-	-	NA
56		SM-AML	+	+	+	+	+	+	+	+	+	+	+	+	-	-	NA
57		SM-AML	+	+	+	+	+	+	+	+	+	+	+	+	-	-	NA
58	Shared *AHNMD*-associated genetic alteration	SM-MDS	+	+	-	+	-	+	-	+	-	+	-	+	-	-	NA
59		SM-AML	+	+	+	+	+	+	+	+	+	NA	+	-	+	-	NA
60		SM-AML	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
61		SM-MDS	+	+	+	+	+	+	+	+	+	+	+	-	+	-	NA
62		SM-MDS	+	+	+	+	+	+	+	+	+	+	+	-	+	-	NA
63		SM-MDS	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
64		SM-CMML	+	+	+	+	+	+	+	+	+	+	+	-	+	-	NA
65		SM+MM+CMML	+	+	+	+	+	+	+	+	+	+	+	-	+	-	NA
TOTAL			65/65 (100%)	12/65 (18%)	38/65 (59%)	33/65 (51%)	38/65 (59%)	19/65 (29%)	38/65 (59%)	19/65 (29%)	38/65 (59%)	12/64 (19%)	37/65 (57%)	12/64 (19%)	18/65 (28%)	0	5/6 (83%)

AML, acute myeloid leukemia; **ANHMD**, associated clonal hematological non-mast cell lineage disease; **CLL,** Chronic lymphocytic leukaemia**; CLPD,** chronic lymphoproliferative disorder; **CMML,** chronic myelomonocytic leukemia**; DLBCL,** diffuse large B-cell lymphoma; **ET,** essential thrombocythemia; **HES**, hypereosinophilic syndrome; **LGL,** large granular T cell leukemia/lymphoma; **MDS,** myelodysplastic syndrome**; MGUS**, monoclonal gammopathy of undetermined significance**; MM**, multiple mieloma; **MSC,** mesenchymal stem cell; **MZL,** marginal zone B cell lymphoma; **PV,** polycythemia vera; **SM**, systemic mastocytosis; **NA,** not analyzed. *AHNMD-associated genetic alteration.

Table 3. SM-AHNMD: clinical and biological disease features of the disease at diagnosis according to the pattern of involvement of BM MC and other non-BM MC hematopoietic cell compartments by the *KIT* mutation and other AHNMD-associated cytogenetic/molecular alterations

Clinical and laboratory parameters	Pattern of involvement of BM MC and other non-MC hematopoietic cell compartments by the *KIT* mutation and AHNMD-associated genetic alterations			
	No genetic alterations shared (n=25)	**Shared *KIT* mutation (n=28)**	**Shared AHNMD-associated genetic alterations (n=12)**	***P***
Demographics and clinical findings				
Age	62 ±15 (25-82)	63 ± 12 (40-87)	53 ± 17 (10-72)	0.05/ ** 0.03
Sex (male/female)	13/12 (52%/48%)	20/8 (71%/29%)	7/5 (58%/42%)	NS
Constitutional symptoms	8/24 (33%)	6/28 (21%)	5/11 (46%)	NS
Fever	2/21 (9%)	7/24 (29%)	5/11 (46%)	NS
Lymphadenopathies	0/19 (0%)	5/21 (24%)	1/11 (9%)	NS
Splenomegaly	6/24 (25%)	17/25 (68%)	5/11 (46%)	<0.01
Hepatomegaly	1/19 (8%)	8/24 (33%)	3/11 (27%)	NS
Skin lesions	12/21 (57%)	9/23 (39%)	1/11 (10%)	0.03
Anaphylaxis	6/14 (43%)	0/23 (0%)	0/11 (0%)	<0.01
Anemia (<100g/L)	4/21 (19%)	14/28 (50%)	6/12 (50%)	NS
Thrombocytopenia (<100 x 10^9/L)	7/19 (37%)	12/26 (46%)	7/12 (58%)	NS
Laboratory parameters[1]				
Hb (g/L)	123 ± 19 (88-154)	115 ± 29 (54-188)	111 ± 20 (82-150)	NS
WBC (x10^9/L)	91 ± 68 (16-225)	134 ± 19 (19-963)	189 ± 23 (21-791)	NS
N. of platelets (x10^9/L)	205 ± 173 (24-725)	143 ± 99 (12-356)	95 ± 60 (14-197)	0.05
Serum LDH (IU/mL)	326 ± 316 (85-1450)	324 ± 208 (114-1130)	363 ± 206 (116-790)	NS
Serum ß-2microglobulin (µg/mL)	2.3 ± 0.8 (0.9-3.8)	4.1 ±2.9 (0.6-12.8)	5.5 ± 5.5 (1.9-18.7)	NS
Serum CRP (mg/L)	5.4 ± 3.7 (0.5-11.1)	18.1 ± 19.3 (3.0-66.1)	17.6 ± 34.2 (0.1-87)	NS
Serum creatinine (mg/dL)	0.86 ± 0.17 (0.6-1.2)	0.98 ± 0.38 (0.6-2.3)	7.6 ± 20.4 (0.5-62)	** <0.01
Serum tryptase (ng/mL)	71 ± 73 (8.1-232)	226 ± 278 (12.6-1328)	242 ± 190 (19-598)	* 0.03
Subtype of SM #				
ISM (n=34)	19/34 (56%)	11/34 (32%)	4/34 (12%)	
ASM (n=26)	3/26 (12%)	17/26 (65%)	6/26 (23%)	<0.01
MCL(n=2)	0/2 (0%)	0/2 (0%)	2/2 (100%)	
Patient outcome				
% Deaths	6/21 (29%)	13/23 (57%)	7/9 † (78%)	0.03
Median PFS[2] (95% confidence interval)	NR (0-167)	30 (0-138)	6 (0-21)	<0.01
Median OS[2] (95% confidence interval)	NR (0-217)	48 (0-138)	11 (1-24)	<0.01

Results expressed as number of cases from all cases in the corresponding patient group and percentage between brackets, or [1] as mean ± one standard deviation (SD) unless otherwise specified. **ANHMD**, associated clonal hematological non-mast cell lineage disease; **SM**, systemic mastocytosis; **WBC,** white blood cell count; **ASM**, aggressive systemic mastocytosis; **CRP**, C-reactive protein; **Hb**, hemoglobin; **ISM**, indolent systemic mastocytosis; **LDH,** lactate dehydrogenase ; **MCL**, mast cell leukemia; **NR**, not reached; [2]**OS**, overall survival expressed in months; [1]**PFS**, progression-free survival expressed in months. †Excluding from the comparison three cases that showed the *KIT* mutation restricted to MC while MC shared the same cytogenetic abnormality than myeloid BM cells involved in the AHNMD, all three cases, remaining alive at the moment of closing this study. # 3 SM cases could not be classified as ISM, ASM or MCL. *No genetic alterations shared vs the other two groups. ** Shared AHNMD-associated genetic alterations vs the other two groups. **NS,** no statistically significant differences found for all group comparisons (p>0.05).
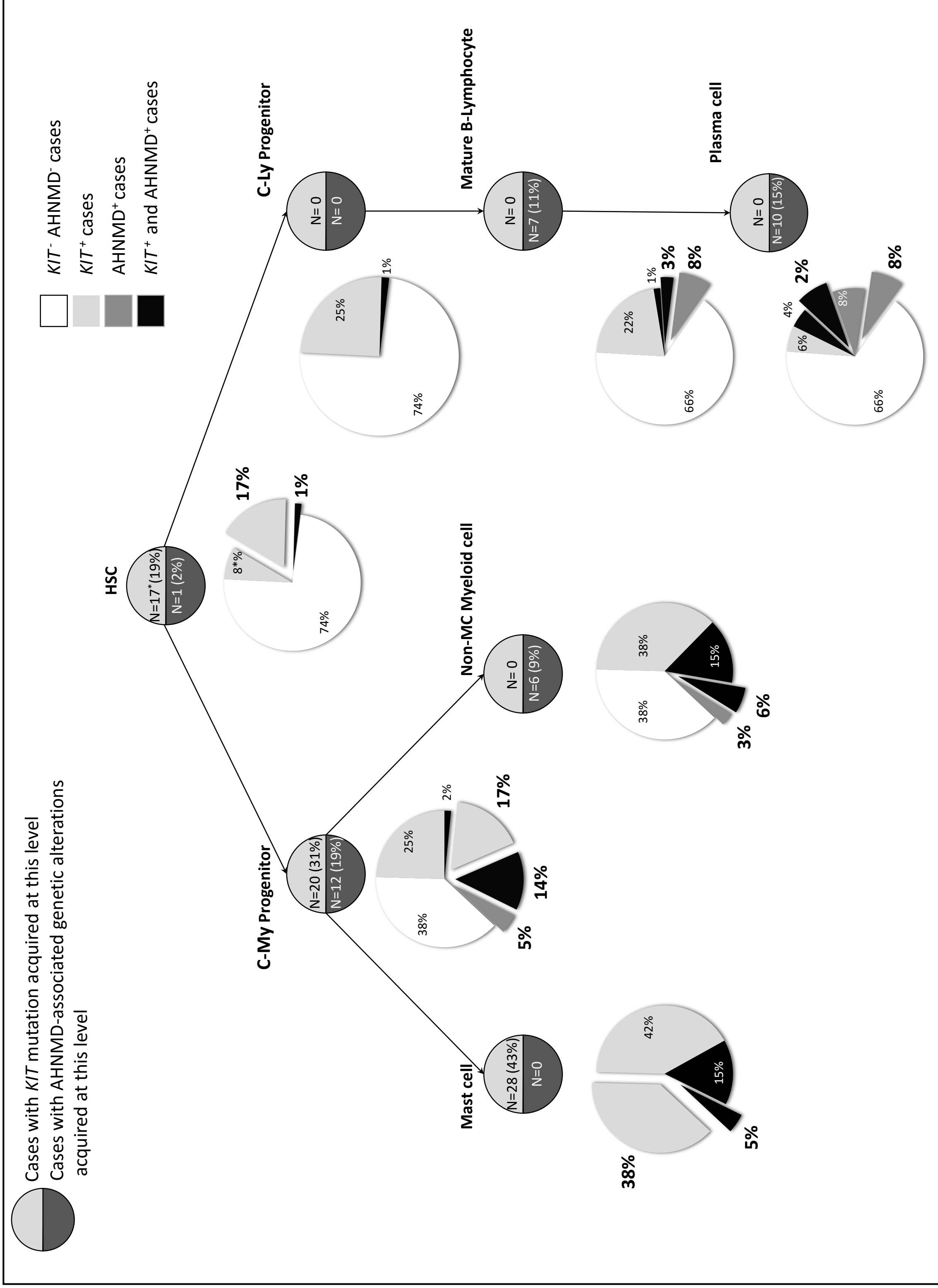
FIGURE 1

FIGURE 2

A SM-AHNMD (all cases)

p<0.01

No shared alteration
NR (0-167)

Shared *KIT* mutation
30 (0-138)

Shared AHNMD alteration
6 (0-21)

SM-AHNMD (all cases)

p<0.01

No shared alteration
NR (0-217)

Shared *KIT* mutation
48 (0-138)

Shared AHNMD alteration
11 (1-24)

B SM+ myeloid malignancy

p=0.04

No shared alteration
57 (0-167)

Shared *KIT* mutation
18 (0-81)

Shared AHNMD alteration
6 (0-21)

SM+ myeloid malignancy

p=0.02

No shared alteration
67 (0-217)

Shared *KIT* mutation
43 (0-85)

Shared AHNMD alteration
11 (1-24)

C SM+ MDS or MDS/MPN

NS

No shared alteration
57 (1-109)

Shared *KIT* mutation
30 (0-76)

Shared AHNMD alteration
7 (6-15)

SM+ MDS or MDS/MPN

p=0.04

No shared alteration
67 (11-119)

Shared *KIT* mutation
43 (3-85)

Shared AHNMD alteration
11 (11-24)

D SM+ MDS

p=0.04

No shared alteration
57 (1-109)

Shared *KIT* mutation
30 (0-76)

Shared AHNMD alteration
7 (6-15)

SM+ MDS

p=0.02

No shared alteration
67 (32-119)

Shared *KIT* mutation
48 (3-84)

Shared AHNMD alteration
11 (11-16)

% of progression free survival

% of overall survival

Time (months) from diagnosis