Mutated *ASXL1* and number of somatic mutations as possible indicators of progression to chronic myelomonocytic leukemia of myelodysplastic syndromes with single or multilineage dysplasia

Myelodysplastic syndromes (MDS) are heterogeneous clonal disorders characterized by ineffective hematopoiesis and transformation in acute myeloid leukemia (AML).[1] However, disease progression is not always to overt AML; MDS may develop myeloproliferative features and move to overlap-diseases [MDS/myeloproliferative neoplasms (MPN)], such as chronic myelomonocytic leukemia (CMML).

Some authors reported unexpected transformation of *de novo* MDS to CMML independently of the presence of relative monocytosis (>10%) at initial diagnosis of MDS.[2,3] In these reports, apart from an evident increase in total white blood cell count (WBC) and monocytosis, alterations of other clinical parameters and/or cytogenetic and molecular abnormalities were not associated directly with progression.[3] Deep sequencing demonstrated the high frequency of somatic mutations in MDS as well as the acquisition of additional mutations during disease history.[4,5] Some somatic mutations are often shared by MDS and MDS/MPN and contribute in different ways to the prognosis of these disorders.[6-8]

Padron *et al.*[4] identified a similar pattern of mutations at diagnosis and in the myeloproliferative stage of MDS patients that transformed to CMML, with maintained variant allele frequency (VAF). The acquisition of new mutations was nevertheless evident at progression (*ETV6, NRAS, CBL*, and *RUNX1*) and differently to this, but in the same line of multi-step clonal evolution, it was observed that MDS progressing to AML acquired mutations of *RUNX1, ETV6, PHF6, NRAS* and *KRAS*.[5]

Here, on a molecular level, we describe a cohort of lower risk MDS patients who evolved to CMML. All consecutive cases diagnosed at Azienda Ospedaliero Universitaria Careggi (Florence, Italy) as MDS in accordance with World Health Organization (WHO) 2008 criteria between April 2010 and September 2016 were revised. Out of 360 cases analyzed, we identified 9 patients reclassified with revised WHO 2016[9] presenting a diagnosis of MDS with single lineage dysplasia (MDS-SLD) or MDS with multilineage dysplasia (MDS-MLD) who progressed to overt CMML after variable follow-up periods (referred as Group 1) (Table 1). In all patients, absolute and relative monocytosis was absent during the entire duration of MDS and the median time to CMML transformation was 21 months (range 10-41) (Table 1).

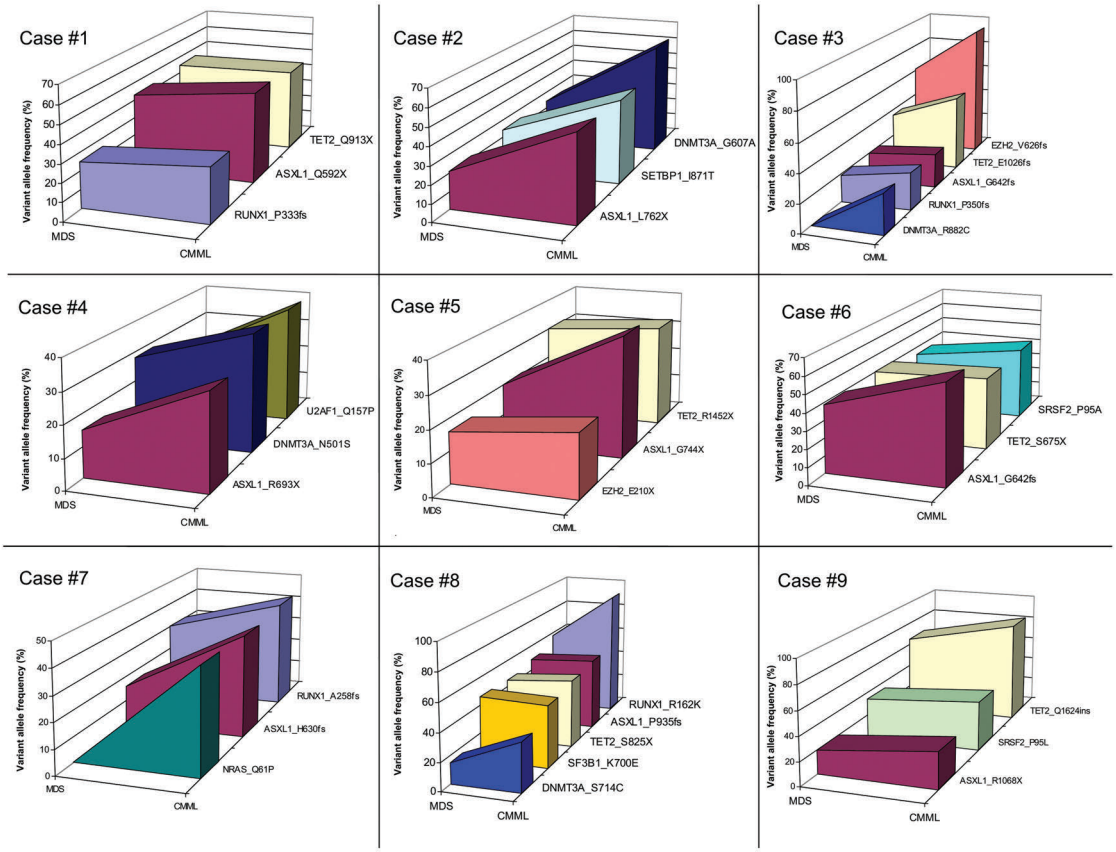

Figure 1. Representation of mutations and variant allele frequency (VAF) (%) in the paired DNA samples of the myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML) phases in the Group 1 of patients. Each graph represents cases #1 to #9 of Group 1. Genes are color coded and VAFs (y-axis) and protein change (x-axis) are represented for each variant. Our panel gene was as follows (only the first nine genes were mutated in Group 1 of patients at MDS diagnosis): *ASXL1, EZH2, RUNX1, SETBP1, SF3B1, U2AF1, SRSF2, TET2, DNMT3A, TP53, ETV6, NPM1, FLT3, CBL, CSF3R, CEBPA, IDH1, IDH2, JAK2, MPL, CARL*.

Paired DNA samples of the MDS and CMML phases were isolated in all 9 cases. Bone marrow (BM) mononuclear cells were collected and cryopreserved at diagnosis (MDS phase) and at transformation (CMML phase). Informed consent was provided in accordance with the Declaration of Helsinki and local ethics committee approval. Twenty-two candidate genes were selected for sequencing analysis (See *Online Supplementary Appendix* for methods), referred to in literature as those most frequently mutated in MDS and CMML:[10-12] *ASXL1, EZH2, TP53, SF3B1, U2AF1, SRSF2, TET2, DNMT3A, ETV6, RUNX1, NPM1, FLT3, CBL, SETBP1, CSF3R, CEBPA, IDH1, IDH2, JAK2, MPL, CARL, NRAS*. Patients' characteristics are presented in Table 1. In brief, 5 were males, and the median age at diagnosis was 67 years (range 56-76 years). At diagnosis, 4 patients had MDS-SLD and 5 patients had MDS-MLD. At transformation, 3 patients presented a CMML-0, 4 patients a CMML-1, and 2 patients a CMML-2 according to the WHO 2016 classification. WBC, absolute monocyte count (AMC) and blast count in CMML phase were statistically higher than in MDS phase (mean WBC 3.64×10^9/L *vs.* 13×10^9/L, P=0.001; AMC 0.21×10^9/L *vs.* 1.6×10^9/L, P=0.004; blast count 0% *vs.* 6%, P=0.0001). There was no significant statistical difference in hemoglobin level (10.85 g/dL *vs.* 9.7 g/dL, P=0.42) or platelet count (147×10^9/L *vs.* 86×10^9/L, P=0.11) between the MDS and CMML phases. After the initial diagnosis of MDS, all patients were treated exclusively with erythropoiesis-stimulating agents, and all patients with and without stable disease were followed every three months. In both phases, 5 patients presented a normal karyotype, one patient a monosomy 7, one patient a trisomy 8, and one patient a trisomy 14. Only case #1 presented a new trisomy 8 at progression (Table 1). After sequencing, coverage across targeted regions was 2500X (minimum 316 and maximum 9877). In the MDS phase, somatic mutations were identified in 9 genes, resulting in 29 variants. All mutations were heterozygous, and comprised 10 missense, 10 nonsense, and 9 frameshift mutations. Eight of the 9 CMML-progressing cases presented three mutations or more (median number of mutations: 3, range 2-5) (*Online Supplementary*

Table 1. Clinical and cytogenetic characteristics of myelodysplastic syndrome patients that progress to chronic myelomonocytic leukemia.

Case#		1	2	3**	4	5	6	7	8	9
Age		63	56	70	76	63	67	71	72	64
Sex		Female	Female	Male	Male	Male	Male	Male	Female	Female
Time to transformation CMML (months)		20,8	12,1	21,2	39,2	10,5	17,0	20,6	41,6	27,6
*WHO classification		MDS-MLD	MDS-MLD	MDS-SLD	MDS-MLD	MDS-MLD	MDS-SLD	MDS-SLD	MDS-SLD	MDS-MLD
IPSS/IPSS-R		Low / Very low	Int-1 / Low	Low / Very low	Int-1 / Int	Int-1 / Int	Low / Very low	Int-1 / Int	Low / Very low	Low / Low
WBC count ($x10^9$/L)	MDS	4.04	2.24	10.3	2.79	5.7	3.64	1.74	4.54	2.40
	CMML	13.2	12.1	59.1	24.4	13	4.9	33.3	12.1	4.5
ANC count ($x10^9$/L)	MDS	2.2	1.7	6.8	2.01	3.4	2.1	1.2	2.66	1.9
	CMML	9.4	8.2	56.2	10.5	8.7	2.7	26.4	6.3	2.7
AMC count ($x10^9$/L)	MDS	0.48	0.12	0.72	0.25	0.30	0.44	0.06	0.17	0.03
	CMML	2.1	1.6	1.48	8.6	3.60	1.60	5.3	1.4	1.5
Monocytes (%)	MDS	9.4	5.4	6.9	8.9	5.2	9.3	3.5	3.7	1.3
	CMML	15.9	13.2	2.5	35.2	27.7	32.6	16	11.5	33.3
BM blasts (%)	MDS	0	0	0	4	4	0	4	0	0
	CMML	12	4	6	9	19	5	9	4	0
Hemoglobin (g/dL)	MDS	11.2	12.4	8	5.6	14.7	10.8	10.4	10.5	13.3
	CMML	7.4	9.7	8.8	7.2	15	11.1	9.1	10.1	12.3
Platelets ($x10^9$/L)	MDS	145	78	173	208	86	145	37	354	149
	CMML	80	82	94	110	45	142	30	86	104
Cytogenetics	MDS	Normal	-7	Normal	+14	+8	Normal	Normal	Normal	Normal
	CMML	+8	-7	Normal	+14	+8	Normal	Normal	Normal	Normal
Organomegaly	MDS	No	No	No	No	No	No	No	No	No
	CMML	Yes	No	Yes	Yes	Yes	No	No	Yes	No
Time AML evolution (months)		8.8	NA	NA	NA	6.9	NA	NA	3.1	NA

*At initial diagnosis of myelodysplastic syndromes (MDS). **Criteria for diagnosis of chronic myelomonocytic leukemia (CMML) for case #3 are not completely fulfilled; the percentage of monocytes is less than 10% [level required in the World Health Organization (WHO) 2016 definition of CMML]. This patient cannot be diagnosed as atypical chronic myeloid leukemia (CML) because the first condition according to the WHO for such a diagnosis is not satisfied: "PB leukocytosis due to increased numbers of neutrophils and their precursors (promyelocytes, myelocytes, metamyelocytes) comprising ≥10% of leukocytes". Myelodysplastic syndromes/myeloproliferative neoplasms (MDS/MPN)-unclassifiable is also not applicable because absolute monocyte count (AMC) in the progression to MDS/MPN was more than 1000/uL. MDS-SLD: myelodysplastic syndrome with single lineage dysplasia; MDS-MLD: myelodysplastic syndrome with multilineage dysplasia; IPSS: International Prognostic Score System; IPSS-R: revised International Prognostic Score System; Int-1: intermediate-1; Int: intermediate; WBC: white blood cells; ANC: absolute neutrophil count; BM: bone marrow; AML: acute myeloid leukemia; NA: not applicable; PB: peripheral blood.

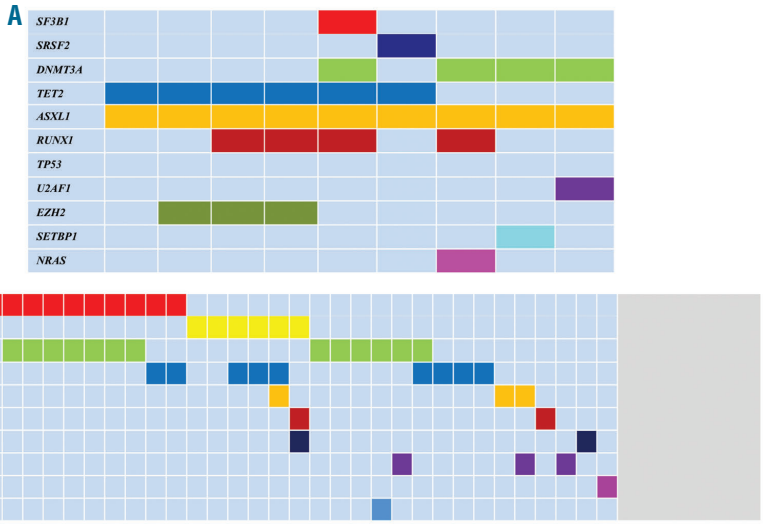

Figure 2. Mutated genes in patients included in Group 1 (A) and Group 2 (B). (A) All 9 patients of Group 1 presented at least two mutations. (B) In Group 2, 13 cases (28%) did not present any mutation and are all represented in the last gray column. Genes are color coded and every case is represented in a column.

Figure S1). The mutational analysis of the CMML phase confirmed the presence of all the 29 variants found at diagnosis (Figure 1). In general, mutation VAF did not change significantly, while some mutated clones were marginally expanded during progression. An acquisition of two missense variants was presented in the CMML samples: case #3 with a new variant of the *DNMT3A* gene and case #7 with a new variant of the gene NRAS (Figure 1). In our cohort of patients, the acquisition of new somatic mutations was not closely correlated with progression, as 7 of the 9 cases studied did not acquire new mutations. We then compared the mutational profile of Group 1 with a second cohort of lower risk MDS patients that did not present CMML transformation or progression to higher risk MDS or AML (referred as Group 2). Clinical data of Group 2 are summarized in *Online Supplementary Table S1*. Groups 1 and 2 were matched by age, sex and International Prognostic Scoring System (IPSS) risk; Group 2 had a longer OS than Group 1 (67 *vs.* 38 months) (*Online Supplementary Figure S2*). After sequencing of Group 2, coverage across the targeted regions of 3441X (minimum 410 and maximum 11401) was obtained. Thirteen cases (28%) did not present any mutation in the 22 genes analyzed, and 33 cases (72%) presented at least one mutation (median number of mutations: 1, range 1-3). The first important observation is that Group 1 cases presented with a higher number of mutated genes: 88% cases of Group 1 showed 3 or more mutations compared with 2 cases (4%) of Group 2 (Figure 2 and *Online Supplementary Figure S1*). The second observation is that the most frequently mutated genes in Group 1 were *ASXL1* and *TET2* that were mutated in all cases and in 6 of 9 cases, respectively (Figure 2A). Consistent with reports in literature, *TET2, SF3B1* and *DNMT3A* were the most frequently mutated genes in Group 2 cases (19%, 24% and 28%, respectively),[10,11] while *ASXL1* was present in only 3 cases (7%): in 2 cases as an isolated mutation and in one case in combination with *TET2* and *SRSF2* (Figure 2B). The latter case has been censored because the patient received an early hematopoietic stem cell transplant, and therefore it was not possible to evaluate progression. Group 1 cases shared the constant presence of *ASXL1* mutations. Based on average sequencing depth *versus* VAF, *ASXL1* mutations in Group 1 appear both as clonal and subclonal. Moreover, *VAF* of *ASXL1* in cases #3 and #8 did not vary significantly while other mutations presented in the same samples increased in size (Figure 1). It seems that, together with the presence of *ASXL1* mutations, the increase in size of other clones could be related to the disease progression. An *ASXL1* mutation is present in 45% of CMML cases and has an adverse prognostic impact on OS.[12,13] *TET2* mutations, the second most frequent in Group 1, are widely prevalent in CMML (46%) and their prognostic relevance remains unclear;[14] in our series *TET2* mutations were not present in any of our cases that progressed from MDS to CMML. Co-mutation of *ASXL1* and *TET2* is associated with very poor survival in CMML.[14] In order to evaluate the weight of *ASXL1* and *TET2* mutation positive clones in this peculiar MDS-to-CMML progression, we compared VAF data of the MDS and CMML phases using the paired sign test. VAF of *ASXL1* was significantly higher in CMML than in the corresponding MDS phase (42% *vs.* 29%, respectively, P=0.039) while VAF of *TET2* was not significantly varied in either phase (55% *vs.* 46%, respectively, P=0.38) (*Online Supplementary Figure S3A* and *B*), suggesting that early presence and increase in the burden of mutated *ASXL1* during progression of the disease to myeloproliferative features have a prominent and determining role. It should be stressed that the presence of *ASXL1* mutation was a rare event (7%) in matched lower risk MDS cases of Group 2 without any kind of progression. Although our study was limited by the number of cases evaluated, no significant changes were observed in the mutation profile during progression to CMML. Although the presence of acquired mutations in other genes not included in this study cannot be excluded, nevertheless, our observations strongly suggest that a "pre-CMML state" could possibly be identified. All "pre-CMML" cases were characterized by worsening anemia that required treatment, in line with the role of *ASXL1* in erythropoiesis.[15] These very particular "pre-CMML"-MDS cases seem to be well characterized by enhanced genetic instability, justifying multiple co-mutations, and by the constant feature of early *ASXL1* mutation. To note, none of

the patients with a "pre-CMML"-MDS presented *TP53* mutations, which is very rarely found in CMML. This suggests that the presence of *ASXL1* mutations and *TP53* wild type could be part of this definition of "pre-CMML" state. Although this kind of progression MDS-to-CMML is quite rare, further studies involving larger numbers of these patients should be carried out in order to confirm whether this kind of evolution is predictable and would, therefore, justify an earlier start to therapy in lower risk MDS with *ASXL1* mutations.

Ana Valencia-Martinez,[1] Alessandro Sanna,[1] Erico Masala,[1] Elisa Contini,[2] Alice Brogi,[1,3] Antonella Gozzini[1] and Valeria Santini[1]

[1]Department of Hematology, Università degli Studi di Firenze, AOU Careggi, Florence; [2]SOD Diagnostica Genetica and [3]Department of Biotechnologies, University of Siena, Italy

Funding: this work was supported by Associazione Italiana Ricerca sul Cancro (AIRC: IG 2015 Id 16861).

Correspondence: valeria.santini@unifi.it
doi:10.3324/haematol.2017.166124

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.

References

1. Steensma DP, Bennett JM. The myelodysplastic syndromes: diagnosis and treatment. Mayo Clin Proc. 2006;81(1):104-130.
2. Rigolin GM, Cuneo A, Roberti MG, et al. Myelodysplastic syndromes with monocytic component: hematologic and cytogenetic characterization. Haematologica. 1997;82(1):25-30.
3. Wang SA, Galili N, Cerny J, et al. Chronic myelomonocytic leukemia evolving from preexisting myelodysplasia shares many features with de novo disease. Am J Clin Pathol. 2006;126(5):789-797.
4. Padron E, Yoder S, Kunigal S, et al. ETV6 and signaling gene mutations are associated with secondary transformation of myelodysplastic syndromes to chronic myelomonocytic leukemia. Blood. 2014;123(23):3675-3677.
5. Pellagatti A, Roy S, Di Genua C, et al. Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression. Leukemia. 2016;30(1):247-250.
6. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R mutations in chronic neutrophilic eukemia and atypical CML. N Engl J Med. 2013;368(19):1781-1790.
7. Meggendorfer M, Roller A, Haferlach T, et al. SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). Blood. 2012;120(15):3080-3088.
8. Papaemmanuil E, Cazzola M, Boultwood J, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N Engl J Med. 2011;365(15):1384-1395.
9. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-2405.
10. Papaemmanuil E, Gerstung M, Malcovati L, et al. Clinical and biological implications of driver mutations in myelodysplastic syndromes. Blood. 2013;122(22):3616-3627.
11. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia. 2013;28(2):241-247.
12. Itzykson R, Kosmider O, Renneville A, et al. Prognostic score including gene mutations in chronic myelomonocytic leukemia. J Clin Oncol. 2013;31(19):2428-2436.
13. Patnaik MM, Itzykson R, Lasho TL, et al. ASXL1 and SETBP1 mutations and their prognostic contribution in chronic myelomonocytic leukemia: a two-center study of 466 patients. Leukemia. 2014;28(11):2206-2212.
14. Patnaik MM, Lasho TL, Vijayvargiya P, et al. Prognostic interaction between ASXL1 and TET2 mutations in chronic myelomonocytic leukemia. Blood Cancer J. 2016;6:e385.
15. Shi H, Yamamoto S, Sheng M, et al. ASXL1 plays an important role in erythropoiesis. Sci Rep. 2016;6:28789.