Identification of novel potential predisposing variants in familial acute myeloid leukemia

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Abstract

Myeloid neoplasms, including acute myeloid leukemia, have been traditionally among the less investigated cancer types concerning germline predisposition. Indeed, myeloid neoplasms with germline predisposition are challenging to identify because often display similar clinical and morphological characteristics of sporadic cases and have similar age at diagnosis. However, a misidentification of familiarity in myeloid neoplasms have a critical impact on clinical management both for the carriers and their relatives. We performed a thorough genomic analysis using a large custom gene panel, the Myelo-Panel, targeted on cancer predisposing genes. In particular, we assessed both germline and somatic variants in 4 families, each with two siblings, who developed hematological neoplasms: 7 acute myeloid leukemia and 1 Philadelphia-positive chronic myeloid leukemia. In each family, we identified at least one novel potentially predisposing variant, affecting also genes not included in the current European LeukemiaNet guidelines for AML management. Moreover, we suggest reclassification of 2 germline variants as pathogenic: likely pathogenic p.S21Tfs*139 in *CEPBA* and VUS p.K392Afs*66 in *DDX41*. Our data underline how familiar predisposition to hematological neoplasms is currently underestimated and call for revision of clinical practices that should include thorough reconstruction of family history and genetic testing with gene panels targeted for cancer predisposing genes.

INTRODUCTION

Advent of next-generation sequencing and large-scale analysis of mutations in cancer have shown that germline mutations in cancer-predisposing genes are more common than previously thought and found in a variable fraction from 5 to 20% of cancer patients, with different prevalence among cancer types. Germline variants are often scored also in sporadic cases of cancer with no familial history. To date, approximately 100 cancer-predisposing genes have been associated to hereditary syndromes. The PanCancer analysis scored 8% frequency of pathogenic and likely pathogenic germline mutations in a large cohort of 10,389 patients with 33 different cancer types. Some cancer types show a significant association with mutations in well-known cancer predisposing genes, i.e. ovarian cancer in *BRCA1/2* , while new associations were revealed, such as loss of function mutations in *BUB1B* in lung cancer and in *SDHA* in melanoma. These data suggest that a relevant number of cancer-predisposing genes and their phenotypic consequences are yet to be identified.

Investigation of an increased risk for cancer development has several important clinical implications, both for carriers of the pathogenic variants and their relatives. For cancer patients, identification of a germline pathogenic variant can yield critical information about prognosis and direct therapeutic choices, both in terms

of efficiency and toxicity of chemiotherapeutic/radiotherapeutic regimens and of surgical intervention strategies. Concerning other family members, identification of pathogenic germline variants in cancer-predisposing genes may enhance targeted cancer surveillance and improve cancer prevention.

One of the biggest challenges in this field is the classification of the identified germline variants, for two main reasons: i) most are classified as variants of unknown significance (VUS) and, therefore, have not been assigned a defined biological role nor have they been attributed to particular disease phenotypes. Consequently, such variants have no relevance for clinical applications. ii) each cancer-predisposing gene may confer different cancer risks, ranging from high to low penetrance (probability) of cancer development, depending both on cancer type and type of variants affecting the genes.

Acute myeloid leukemia (AML) is among the malignancies with the lowest frequency of germline variants (4.2% in PanCancer) and traditionally it has been among the less investigated cancer types from this point of view. However, germline predisposition of hematological neoplasms is increasingly assessed and, in recent years, advent of molecular testing has led to identification of specific hereditary hematological syndromes . The current guidelines from the European LeukemiaNet (ELN) for diagnosis and management of AML in adult patients recommend investigation of germline predisposition. This is particularly critical for clinical management of AML patients that often receive allogeneic stem-cell transplantation to consolidate disease remission. Considering that usually relatives are the best donors, it is critical to exclude the presence of hematopoietic stem cells harboring pathogenic variants from the donor before transplantation. The list of cancer predisposing genes in hematological neoplasms is likely to be largely incomplete and we are in need for further investigations in order to uncover other players of hematological hereditability and define the penetrance of the identified variants.

We designed a comprehensive gene panel for the assessment of germline pathogenic variants in hematological neoplasms, covering the entire coding sequence of 256 genes. The employment of large gene panels enlarges the testing potential compared to genetic screens restricted to high penetrance genes, allowing discovery of more potential predisposing variants; however, it also poses the challenge of assigning a specific functional/phenotypic consequence to newly identified VUS. Variants can also be reclassified when more information on gene function and/or data from family history become available over time, based on new studies. We studied 4 families with two siblings each affected by myeloid neoplasms in order to exploit the potential of family segregation studies in this context.

METHODS

Patients . We enrolled 4 families with history of hematological disorders. Each family comprised two siblings, of which at least one was diagnosed and treated in our Institute. For routine clinical management, patients underwent bone marrow (BM) evaluation including morphological, immunophenotypic and cytogenetic analysis. The main patient characteristics are reported in Table 1. All patients gave written informed consent to their participation to the diagnostic and treatment program, according to the IEO ethical committee approval.

Molecular analyses . We extracted DNA from BM aspirates or peripheral blood draws for the analysis of somatic variants, and from buccal swabs for the analysis of germline variants, using the QIAamp DNA mini kit following the manufacturer's instructions (Qiagen). As previously described, we performed two genomic characterizations: one for routine clinical practice and one for research purposes. For clinical assessment, we used a commercially available diagnostic NGS assay, the Oncomine Myeloid Research Assay (40 key DNA target genes and 29 driver genes in a broad fusion panel). Data analysis was performed with IonReporter software applying the last release of Myeloid workflow (ThermoFisher Scientific). For research purposes, we used the Myelo-panel, a custom gene panel for analysis of 256 cancer-predisposing genes, purposely designed to stratify patients for possible targeted therapies and to identify germline variants associated with predisposition to develop myeloid neoplasms. The 256 cancer-predisposing genes include: 79 susceptibility genes, the most frequently associated with risk of development of hematological tumors; 38 AML drivers, with a key role in leukemogenesis; 113 actionable genes, with a concrete clinical manageability; 26 genes

belonging to more than 1 of these categories and 126 pharmacogenomics single nucleotide polymorphisms (SNPs), allelic variants associated with susceptibility to certain drug (see Supplementary Table S1 for gene list).

Library preparation was performed using Ion Torrent Ion AmpliSeq Library Kit 2.0 and libraries were sequenced with Ion S5 system according to the manufacturer's recommendations (ThermoFisher Scientific). Variant analysis was performed with Ion Reporter software and filtering as previously described. Variants passing our filters were annotated in terms of pathogenicity using different computational tools, including ClinVar, CancerVar, Intervar, Varsome and RENOVO.

RESULTS

Clinical history

We identified 4 families, each with two siblings, who developed hematological neoplasms. Of these 8 patients, 7 (87.5%) developed acute myeloid leukemias (AML; see Table 1 for clinical details). Patient ID4 of Family2 developed a Philadelphia-positive chronic myeloid leukemia (Ph+ CML). The detailed clinical history of each family is described below.

Family1. Sister ID1 was diagnosed with hyperleukocytotic AML with normal karyotype (NK) at 17 years of age. She received a regimen (LAME 89 pilot study) including an intensive induction phase (mitoxantrone plus cytarabine) and obtained complete remission. She then received two consolidation courses: the first containing timed-sequential high-dose cytarabine, asparaginase and amsacrine; the second consisting of mercaptopurine plus cytarabine for 18 months. She is now 40 years old and still in complete remission (CR).

Sister ID2 also received a diagnosis of AML with NK when she was 32 years old. She obtained CR following induction therapy with the 3+7 schedule (cytarabine 100 mg/mq G1, daunorubicin 60 mg/mq G1-3-5). She then received 4 consolidation therapy courses with high doses of cytarabine. After 2 years from end of treatment, she is persistently in CR. Both sisters are currently under close clinical monitoring.

Family2. Brother ID3 developed AML with karyotype 46,XY,del(16)(q22qter)[7]/46,XY[13] at age 62. He received ICE (idarubicine, cytarabine and etoposide) as induction chemotherapy and achieved CR. He then received a consolidation cycle with high dose cytarabine, and peripheral-blood stem cells (PBSC) were harvested during recovery. He underwent autologous PBSC transplantation as final consolidation therapy, but relapsed a year later. Molecular evaluation identified a known *JAK2 V617F* mutation (Table 1) and additional mutations in the *ASXL1* (p.G1185Cfs*4, Varian Allele Frequency (VAF)=3.09%) and *RUNX1* (p.L405Cfs*, VAF=4.71%) genes (not shown). The patient, therefore, received reinduction chemotherapy with FLAI (fludarabine, idarubicine and cytarabine) and a consolidation cycle with high-dose cytarabine, achieving CR. Four months later he underwent myeloablative allogeneic haploidentical stem-cell transplantation from a family donor (conditioning: fludarabine, cyclophosphamide, total body irradiation and cyclophosphamide +3 and +4 post reinfusion), complicated by occurrence of veno-occlusive disease, which was treated and resolved with defibrotide. Approximately 1 year and 9 months after transplantation, he is in CR in fair general condition.

Brother ID4 was diagnosed with Ph+ CML, Sokal score low risk, in absence of additional cytogenetic alterations at age 53. He started therapy with imatinib but immediately developed intolerance and, therefore, changed to dasatinib (tyrosine kinase inhibitor - TKI- 2ndgeneration), achieving complete molecular response. Three years after starting therapy, he developed laterocervical lymphadenopathy and persistent fever; he was deemed intolerant to dasatinib, which was discontinued. He then started therapy with ponatinib (3rd generation TKI), with persistence of molecular remission to date.

Family3. Brother ID5 was diagnosed with AML characterized by myelodysplastic changes, NK and mutations in*TET2, ASXL1* and *U2AF1* genes (Table 1) at age 74. He was treated with ICE to which the patient was refractory. Therefore, reinduction chemotherapy according to FLAI plus venetoclax was performed, resulting in CR. Four months later, he underwent myeloablative haploidentical allogeneic transplantation (conditioning as for brother ID3), maintaining CR and achieving complete full chimera. Seven months after

transplantation, he showed reduction in chimerism with worsening of blood counts and progressive need for transfusion support. Subsequent BM evaluation showed no increase in blasts, a progressive increase in VAF of *TET2* , *ASXL1* and*U2AF1* mutations and acquisition of a *TP53* mutation (Figure 1A). These clinical and molecular parameters were considered indicative of disease relapse. The patient was then treated with salvage therapy (azacitidine + venetoclax) followed by worsening of his general condition and progressive leukocytosis; BM evaluation showed refractoriness of the disease with transformation into megakaryoblastic AML. The patient died of disease progression approximately one year after transplantation.

Brother ID6 was diagnosed with AML bearing trisomy of chromosome 8 at age 61. He received induction chemotherapy with ICE achieving CR. He received further cycles of IC (idarubicin and cytarabine) as consolidation and cycle A8 (cytarabine) followed by reinfusion of autologous PBSCs. When in chronic remission, he underwent allogeneic myeloablative transplantation from a volunteer donor (conditioning: busulfan, fludarabine, ATG). Post-transplantation recovery, he was undermined by a state of immunosuppression, showing Cytomegalovirus (CMV) reactivation resistant to antiviral treatments, which resulted in graft failure (requiring a boost of PBSCs from the same donor), Gram-positive encephalitis and finally Geotrichum sepsis, which led to patient's death approximately 10 years after transplantation.

Family4. Sister ID7 received diagnosis of AML with myelodysplastic changes and NK when she was 58 years old. NGS mutational analysis of blasts for clinical assessment showed presence of mutations in *SF3B1* , *TET2* and *ETV6* (Table 1). She received induction therapy with 3+7 scheme plus gentuzumab ozogamicin (as part of the GIMEMA 1819 protocol). However, she did not obtain remission and underwent a cycle of reinduction according to FLAI plus venetoclax, obtaining only partial response with persistence of the *SF3B1* ,*TET2* and *ETV6* mutations (Figure 1B). Within one month, the disease relapsed and she underwent a further cycle of therapy with azacitidine plus venetoclax, obtaining complete response. She then underwent haploidentical allogeneic transplantation (conditioning with treosulfan + fludarabine). Nine days after, she had an acute episode of cerebral hemorrhage, which caused her death.

Brother ID8 was diagnosed at age 73 with AML displaying myelodysplastic changes, NK and presence of the hotspot *IDH2* mutation (Table 1). He achieved CR after the first course of azacitidine plus venetoclax therapy. To date, he underwent 8 chemotherapeutic cycles, maintaining CR.

Germline and somatic mutational analysis

To define if the patients described above had germline predisposition to developing myeloid neoplasms and, when possible, define the somatic landscapes of their malignancies, we performed mutational analyses using the Ion Torrent technology and a custom gene panel designed in our laboratory, the Myelo-panel (see Materials and Methods for details).

We performed germline analysis on buccal DNA and, whenever available, somatic analysis on the pathological sample at diagnosis, as previously described. We report all germline variants (VAF>20%) identified in common between the sibling pairs (Table 2) and, for somatic mutations, all pathogenic and likely pathogenic variants plus VUS identified with VAF>5% (Table 3). All germline variants were validated by Sanger sequencing (Supplementary Figure 1).

Families with mutations in genes reported in hereditary hematologic malignancy (HHM) syndromes

In Family1 and Family2 of our cohort, we identified germline mutations in two genes known to confer inherited risk to development of MDS/AML:*CEBPA* and *DDX41* . In particular, both sisters in Family1 harbored a likely pathogenic indel p.S21Tfs*139 in *CEBPA* . The mutation is heterozygous in both sisters with VAF=51.9% and 50.8%, respectively (Table 2). As expected for CEBPA-associated familial AML, both sisters developed their disease at very young age (Table 1). We performed somatic mutational analysis only for sister ID1 and detected no somatic mutations (Table 3). However, this result may not be significant since immunophenotypic analysis of BM aspirate used for NGS showed less than 1% blast infiltration.

In Family2 both brothers had the germline heterozygous frameshift indel p.K392Afs*66 in *DDX41* , with VAF=51.7% and 48.6%, respectively. This variant is currently annotated as VUS (Table 2). As for Family1,

we had tumor DNA available only for one brother. As often reported for *DDX41* -associated familial AML, patient ID3 harbored also the hotspot p.R525H somatic mutation for *DDX41* (VAF=7.95%), together with two other somatic mutations: the most common pathogenic variant in *JAK2* p.V617F (VAF=3.56%) and the likely pathogenic mutation p.P777H in *DNMT3A* (VAF=10.58%; Table 3).

Family with a germline mutation in the LYST gene

In Family3 both brothers displayed a germline missense single-nucleotide mutation in the *LYST* gene, translating in p.R3398Q (Table 2). This variant is currently annotated as VUS and the functional consequences are unknown. Both siblings developed AML and somatic mutational analysis of their leukemic samples identified pathogenic variants. Brother ID5 harbored truncating pathogenic indels in *ASXL1* (p.R417*) and in *RAD50* (p.N934Ifs*6), a likely pathogenic truncating indel in *TET2* (p.L1721Ffs*24) and a VUS in *IGFN1* (p.D2249G). Brother ID6 harbored the second most common somatic pathogenic variant in *IDH2* , p.R172K, with VAF=36.9% (Table 3).

In order to reconstruct the genomic evolution of the disease in patient ID5, we exploited NGS analysis performed in our Institute on BM aspirates for clinical monitoring using the commercially available Oncomine Myeloid Research Assay. As shown in Figure 1A, there was partial response to treatment post-induction with disappearance of cells bearing *TET2* p.L1721Ffs*24 and *U2AF1* p.S34F variants, but there were no effects on the blasts harboring *ASXL1* p.R417* and *TET2* p.D77Tfs*18 mutations. The patient underwent CR post-allo TMB, however, unfortunately, he relapsed very rapidly with re-expansion of the same AML clone detected pre-transplant and appearance of a new mutation in *TP53* , p.C141Y, that reached a VAF around 90%. The patient succumbed of disease less than 8 months after relapse.

Family with a complex germline mutational landscape

In Family4, both siblings carried three heterozygous germline mutations annotated as VUS: p.S858R in *FANCA* , p.G571S in *JAK2* and p.E1280Q in *ERBB4* (Table 2). These patients also shared the presence in their AML samples of the most common somatic pathogenic variant in *SF3B1* : p.K700E, with a VAF of 34.2% and 31.7% in patient ID7 and ID8, respectively (Table 3). Using Sanger sequencing, we confirmed this is an acquired somatic mutation in a hematopoietic clone with high VAF; indeed, the variant was not detected in buccal DNA collected from either siblings (Supplementary Figure 2). Patient ID7 harbored also two likely pathogenic somatic variants (*TET2* p.E566* and *ETV6* p.V158Pfs*10) and 3 VUS with VAF>5% (*GATA2* p.H323P, *FGFR3* p.A634T and *BRCA2* p.L1545P; Table 3). Patient ID8 harbored two subclonal pathogenic mutations in both *IDH2* (p.R140Q; VAF=11.64%) and *IDH1* (p.R132H; VAF=2.38%).

As for patient ID5, we were able to reconstruct the genomic evolution of AML in patient ID7, based on NGS analysis performed for clinical monitoring. Following induction chemotherapy, the patient showed persistence of the hematopoietic clone harboring the *SF3B1* p.K700E mutation, which maintained almost constant VAF (30-40%), showing complete resistance along the entire course of the disease. After induction, the blasts acquired two new variants: *TET2* p.T1093Kfs*12 and *GATA2* p.N317S. Therefore, the patient underwent re-induction chemotherapy, that affected the primary responsive clone, but again showed no efficacy on the clone with *SF3B1* p.K700E variant or clones with the two mutations acquired post-induction. This patient succumbed to the disease within three months post-relapse, with reappearance of all mutations identified both pre- and post-induction therapy (Figure 1B).

DISCUSSION

We report the results of detailed genomics studies on four families in which pairs of siblings developed myeloid neoplasms and, in particular, AML in 7 out of 8 cases. According to the current World Health Organization (WHO) classification of hematological neoplasms with germline predisposition and the last published recommendations from ELN for diagnosis and management of AML, we identified germline mutations both in genes with a well-established role in predisposing to the development of MDS/AML and genes with no defined role in this context. Indeed, *CEBPA* and *DDX41* are included in myeloid neoplasms with germline predisposition without a pre-existing platelet disorder; *FANCA* is listed in the category of genes mutated in

myeloid neoplasms associated with bone marrow failure syndromes; *JAK2* is included in emerging disorders with germline predisposition; and, finally, *LYST* and *ERBB4* have not been reported yet in such classifications. None of the identified mutations is currently annotated as pathogenic in cancer databases; however, they could all play an important role in hematological diseases development.

In particular, the variant identified in *CEBPA* is a truncating mutation and, importantly, it is located at the 5'-end of the gene (c.62, p.S21). N-terminal mutations in *CEBPA* are known to have a penetrance close to 100% of leukemia development; however, they correlate with a favorable prognosis. Due to the unavailability of AML cells, we could not assess if in our patients there was acquisition of a somatic *CEPBA* mutation; nonetheless, both siblings are currently in CR and under clinical monitoring. The frameshift indel identified in*DDX41* tumor suppressor gene is currently annotated as VUS and it is not listed either in COSMIC or cBioportal. This mutation is expected to truncate the protein before its functional helicase domain and likely causes a loss of function. Truncating mutations in *DDX41* have been shown to increase the risk of developing myeloid neoplasms and are associated with faster progression to leukaemia. Both our patients are males who, in presence of *DDX41* mutations, are expected to develop myeloid malignancies more frequently than females. Moreover, patient ID3 harbored the hotspot mutation p.R525H in *DDX41* , frequently acquired as somatic mutation in carriers of germline*DDX41* variants. No pathological DNA was available for the other sibling, who however developed a Ph+ CML.

The *LYST* gene is mutated in autosomal recessive mode in Inborn Errors of Immunity syndromes and, in particular, in Familial Hemophagocytic Lymphohistiocytosis (FHL) syndromes with Hypopigmentation: Chediak-Higashi syndrome and Hemophagocytic Lymphohistiocytosis (HLH). LYST is a lysosomal trafficking regulator and a key effector of cytotoxic granules' biogenesis. It is involved in the modulation of cytotoxic T-lymphocytes (CTL) and natural killer (NK)-cell functions by regulating degranulation. LYST-deficient CTLs and NK-cells display impaired ability to kill target cells and accumulate giant cytotoxic granules. Patients with Chediak-Higashi syndrome display oculocutaneous albinism, easy bruising, recurrent pyogenic infections and exhibit abnormal functions of NK-cells and alterations in neutrophils, leading to neutropenia. We have no evidence to infer a direct role for the identified *LYST* mutation in predisposing to development of myeloid neoplasms; however, we can envision a possible role for a deregulated immune system in controlling the homeostasis of hematopoietic differentiation.

In the fourth family we scored a more complex landscape with three germline VUS shared by both siblings. The most noteworthy is mutation p.S858R in *FANCA* gene. It is annotated as VUS in cancer databases; however, notably, it is annotated as pathogenic in FAMutdb, a database of variants identified in Fanconi Anemia (FA) (http://www2.rockefeller.edu/fanconi/) and it is reported in several FA patients of different origins (Italian, German, Indian-Jewish). FA is an autosomal recessive disease usually associated to mutations of other *FANC* members for its manifestation. The penetrance and phenotypic manifestations of the syndrome are highly variable. Our patients had no signs of FA and we did not identify pathogenic mutations in other sequenced members of *FANC* gene family. However, carriers of heterozygous FA mutations present increased risk for development of MDS and AML. Therefore, we can envision an incompletely penetrant phenotype imposed by p.S858R *FANCA*mutation, which requires cooperation with other germline lesions.

Germline predisposition to myeloid neoplasms due to pathogenic or likely pathogenic variants of *JAK2* gene are emerging as new disorders; however, in both siblings of Family4, we identified a germline variant currently annotated as VUS: p.G571S. This mutation is located in exon 13 and, as the most common oncogenic variant in *JAK2* , p.V617F, within the region encoding for the autoinhibitory JH2 pseudokinase domain of the protein. Although the biological significance of this variant is not well established yet, in vitro assays in Ba/F3 cells suggest no significant impact on the JAK2 protein functions. This variant has been reported associated to MPN with a frequency of around 0.01% and as germline both in sporadic and in familial cases of Essential Thrombocythemia (ET)[·]. Moreover, it is listed in COSMIC (COSM29107, COSM142855: 10 mutations, 7 of which in the Haematopoietic and lymphoid category).

Finally, the missense variant in the receptor tyrosine kinase*ERBB4* has an unknown biological significance and it has not been reported in COSMIC. We currently have no clues on its possible role in our clinical

context.

Although functional characterization of these mutations is still required, we speculate that in Family4 the full MDS/AML phenotype may result from the cooperation between the *FANCA* and *JAK2*missense mutations. Interestingly, in a WES analysis of mutations in a cohort of patients with BM failure syndromes of suspected inherited origin, 11.6% of patients carried 2 co-occuring potential alterations.

In Family4, in siblings sharing this same complex germline landscape, the clinical history of the disease followed independent pathways, with acquisition of independent somatic mutations except for a common prevalent clone (VAF>30% in both siblings) with p.K700E mutation in *SF3B1,* suggesting a selective pressure imposed by the germline variants on acquisition of this somatic mutation. In a cohort of 16 FA patients, this same mutation in *SF3B1* was identified in a patient with a germline *FANCA* mutation, who developed refractory anemia with ring sideroblasts and mutations in *SF3B1*and *JAK2* seem to co-occur in myelodysplastic/myeloproliferative neoplasms with ring sideroblasts and thrombocytosis [9]. Notably, clonal evolution reconstruction by single cell sequencing in a FA patient harboring a mutation in *FANCA* , showed appearance, in the early stage of MDS, of a clone with the *SF3B1* p.K700E mutation, together with a mutation in *RUNX1* , which expanded to become dominant with progression of the disease to AML.

Currently, a field of intense investigation is the study of clonal hematopoiesis (CH), a premalignant state in which hematopoietic stem and progenitor cells clonally expand due to acquisition of somatic mutations in genes that confer selective growth advantages. CH has been associated to increase risk of development of a number of diseases that include both hematological tumors and non-malignant conditions such as ischemic cardiovascular, inflammatory and autoimmune diseases. The analysis of CH is performed on peripheral blood in several clinical contexts, independent from hematological disease, by NGS that include both WES and targeted gene panels. The search for CH in an ever-growing number of non-hematological patients will generate a critical mass of genomic data on the hematopoietic system that could prove extremely helpful in the field of germline predisposition to myeloid neoplasms, significantly increasing the wealth of information available for this traditionally under investigated cancer type.

In conclusion, we show that thorough genomic analysis using large gene panels appositively targeted on cancer predisposing genes allow identification of novel germline variants. Indeed, in each family of our cohort, we identified at least one novel variant, affecting also genes not included in the current ENL guidelines for AML management. According to The American College of Medical Genetics and Genomics (ACMG) guidelines for interpretation of sequencing variants, a critical component for understanding of significance of a VUS is the observed clinical phenotype. On these basis, we suggest reclassification as pathogenic of the likely pathogenic p.S21Tfs*139 in *CEPBA* and the VUS p.K392Afs*66 in *DDX41* . Finally, considering that myeloid neoplasms with germline predisposition often display clinical and morphological characteristics similar to sporadic cases and that age at diagnosis in the two groups often overlap, it is really challenging to suspect familiarity in the context of hematological tumours. Indeed, based exclusively on their clinical parameters, none of the cases of our cohort would have been suspected of familiarity. However, studying affected siblings, we identified potential germline predisposition in each family. Our data underline how current clinical practice underestimate familial cases within hematological neoplasms and calls for implementation of novel clinical practices that should include thorough reconstruction of personal and family history and genetic testing with large gene panels targeted for predisposing genes.

AUTHOR CONTRIBUTIONS

CR, FG, VA, MF, RF participated in data collection; MF, RF prepared libraries; CR, MF, RF, GG, FB performed data analysis and interpreted the results; CR, FG wrote the manuscript with input and critical revision from all co-authors; FG, VA, FG, MR, FL, AB carried out clinical work; ED, CT, PGP, critically revised the study; MA, ET conceived and supervised the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Main clinical features of our families' cohort

Family	Patient ID	Age at diagnosis	Sex	Disease	Karyotype	Molecular Biology at diagnosis	Chemiotherapeutic Treatment	Follow-u
Family1	ID1	17	F	AML	46,XX [20]	negative	*Induction:* mitox-antrone plus cytarabine *Consolida-tion:* - HD-ARA-C, ASP, AMSA - 6-MP, ARA-C	CR
	ID2	32	F	AML	46,XX	NA	*Induction:* scheme 3+7 *Consoli-dation:* 4 cycles HD-ARA-C	CR

Family	Patient ID	Age at diagnosis	Sex	Disease	Karyotype	Molecular Biology at diagnosis	Chemiotherapeutic Treatment	Follow-u
Family2	ID3	62	M	AML	46,XY,del(16)(q22q24)[7]/46,XY[13]	JAK2 p.V617F (VAF=3.55%)	*Induction:* scheme ICE *Consolidation:* - 2 cycles HD-ARA-C - autologous PBSC transplant *Reinduction:* scheme FLAI *Consolidation:* 1 cycle HD-ARA-C Allogeneic haploidentical PBSC transplant	CR pos Allo-TM
	ID4	53	M	Ph+ CML	46,XY,t(9;22)	NA	Imatinib; dasatinib; ponatinib	Molecul remissio
Family3	ID5	74	M	AML	46,XY [20]	ASXL1 p.R417* (VAF=10.75%)	*Induction:* ICE scheme *Reinduction:* FLAI plus Ven scheme. Allogeneic haploidentical PBSC transplant *Salvage therapy:* azacitidine plus Ven	Dead

Family	Patient ID	Age at diagnosis	Sex	Disease	Karyotype	Molecular Biology at diagnosis	Chemiotherapeutic Treatment	Follow-
						TET2 p.L1721Ffs*24 (VAF=10.83%) TET2 p.D77Tfs*18 (VAF=21.12%) U2AF1 p.S34F (VAF=11.5%)		
	ID6	61	M	AML	46,XY,+8	NA	*Induction:* ICE scheme *Consolidation:* - IC scheme - 1 cycle ARA-C plus re-infusion of autologous PBSC Allogeneic PBSC transplant from a VUD	Dead
Family4	ID7	58	F	AML with myelodisplastic features	46,XX [15]	SF3B1 p.K700E (VAF=33.7%) TET2 p.T229Nfs*25 (VAF=32.93%)	*Induction:* 3+7 scheme plus GO *Reinduction:* - FLAI plus Ven scheme - Azacitidine plus Ven 1 cycle Allogeneic haploidentical PBSC transplant	Dead

Family	Patient ID	Age at diagnosis	Sex	Disease	Karyotype	Molecular Biology at diagnosis	Chemiotherapeutic Treatment	Follow-
						TET2 p.E566* (VAF=31.32%) ETV6 p.V158Pfs*10 (VAF=17.87%)		
	ID8	73	M	AML with myelodis-plastic features	46,XY [20]	IDH2 p.R140Q	Azacitidine plus Ven 8 cycles	CR

AML, acute myeloid leukaemia; Ph+ CML, Philadelphia-positive (Ph+) chronic myeloid leukaemia; NA, not available; VAF, variant allele frequency; CR, complete remission;

MRD, minimal residual disease; Allo-HSCT, allogeneic haematopoietic stem cell transplantation; HD, high dose; ARA-C, cytarabine; ASP, asparaginase; AMSA, amsacrine; 6-MP, mercaptopurine; 3+7: Cytarabine+Daunorubicin; ICE: Idarubicine, Cytarabine and etoposide; PBSC, peripheral blood stem cells; Ven, venetoclax; IC: Idarubicine, Cytarabine; VUD, volunteer unrelated donor; GO, gentuzumab ozogamicin.

*Next generation sequencing analysis of PB DNA using the Oncomine Myeloid Research Assay and the Ion Torrent S5 technology (ThermoFisher).

Table 2. Germline variants identified in our cohort

Family	Patient ID	GENE	Transcript	Nucleotide change	AA change	Variant Classification#	VAF (%)
Family1	ID1	*CEBPA*	NM_-004364.4	c.62delG	p.S21Tfs*139	Likely Pathogenic	51.97
	ID2	*CEBPA*	NM_-004364.4	c.62delG	p.S21Tfs*139	Likely Pathogenic	50.84
Family2	ID3	*DDX41*	NM_-016222.4	c.1174_-1175delAA	p.K392Afs*66	VUS	51.66
	ID4	*DDX41*	NM_-016222.4	c.1174_-1175delAA	p.K392Afs*66	VUS	48.64
Family3	ID5	*LYST*	NM_-001301365.1	c.10193G>A	p.R3398Q	VUS	51.83
	ID6	*LYST*	NM_-001301365.1	c.10193G>A	p.R3398Q	VUS	51.96
Family4	ID7	*FANCA*	NM_-000135.4	c.2574C>G	p.S858R	VUS (Pathogenic in FAMutdb)	42.39
		JAK2	NM_-004972.4	c.1711G>A	p.G571S	VUS	47.95
		ERBB4	NM_-005235.3	c.3838G>C	p.E1280Q	VUS	49.79

Family	Patient ID	GENE	Transcript	Nucleotide change	AA change	Variant Classification#	VAF (%)
	ID8	*FANCA*	NM_-000135.4	c.2574C>G	p.S858R	VUS (Pathogenic in FAMutdb)	45.15
		JAK2	NM_-004972.4	c.1711G>A	p.G571S	VUS	47.95
		ERBB4	NM_-005235.3	c.3838G>C	p.E1280Q	VUS	48.41

AA change, aminoacid change; VAF, variant allele frequency; VUS, variant of unknown significance; FA-Mutdb, Fanconi Anemia Mutation Database.

#Laboratory classification based on annotation in ClinVar, Varsome and Intervar.

Table 3. Somatic variants identified in our cohort

Family	Patient ID	GENE	Transcript	Nucleotide change	AA change	Variant Classification#	VA
Family1	ID1	None	/	/	/	/	/
	ID2	NA	/	/	/	/	/
Family2	ID3	*DDX41*	NM_016222.4	c.1574G>A	p.R525H	Pathogenic	7.9
		JAK2	NM_004972.3	c.1849G>T	&p.V617F	Pathogenic	3.5
		DNMT3A	NM_175629.2	c.2330C>A	p.P777H	Likely pathogenic	10
	ID4	*NA*	/	/	/	/	/
Family3	ID5	*ASXL1*	NM_015338.6	c.1249C>T	&p.R417*	Pathogenic	13
		RAD50	NM_005732.4	c.2801delA	p.N934Ifs*6	Pathogenic	13
		TET2	NM_001127208.2	c.5163delG	&p.L1721Ffs*24	Likely pathogenic	17
		IGFN1	NM_001164586.2	c.6746A>G	p.D2249G	VUS	12
	ID6	*IDH2*	NM_002168.4	c.515G>A	p.R172K	Pathogenic	36
Family4	ID7	*SF3B1*	NM_012433.3	c.2098A>G	&p.K700E	Pathogenic	34
		TET2	NM_001127208.2	c.1696G>T	&p.E566*	Likely pathogenic	29
		ETV6	NM_001987.4	c.472_473delGT	&p.V158Pfs*10	Likely pathogenic	16
		GATA2	NM_032638.4	c.968A>C	p.H323P	VUS	31
		FGFR3	NM_000142.4	c.1900G>A	p.A634T	VUS	5.3
		BRCA2	NM_000059.3	c.4634T>C	p.L1545P	VUS	5.0
	ID8	*SF3B1*	NM_012433.3	c.2098A>G	p.K700E	Pathogenic	31
		IDH2	NM_002168.3	c.419G>A	&p.R140Q	Pathogenic	11
		IDH1	NM_005896.3	c.395G>A	p.R132H	Pathogenic	2.3

AA change, aminoacid change; VAF, variant allele frequency; VUS, variant of unknown clinical significance; NA, not available.

&Variants found in common with clinical assessment of mutational analysis (see Table 1).

#Laboratory classification based on annotation in ClinVar and CancerVar.

FIGURE LEGENDS

Figure 1. Evolution of the genomic landscape in two patients of our cohort. For each patient, we show the variant allele frequency (VAF, left Y axis) of each somatic mutation identified by clinical monitoring

with the NGS Oncomine Myeloid Research Assay. Each mutation is labelled according to the left legend shown above the graphs. On the X axis we report the date of testing. Response to treatment was monitored in the BM of the patient by immunophenotype of the myeloid blasts (% blasts, right Y axis). **A.** Patient ID5. In panel A, we also show the percentage of donor cells in the peripheral blood (% donor PB cells, right Y axis), following allogeneic BM transplantation (post Allo-TMB). The small black arrow indicates the timing of induction chemotherapy. **B.** Patient ID7. The first black arrow shows the timing of induction therapy and the second of re-induction therapy. Beg., beginning.

FIGURE 1

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`image1.emf` available at https://authorea.com/users/771255/articles/853023-identification-of-novel-potential-predisposing-variants-in-familial-acute-myeloid-leukemia