

Clinical Characteristics and Whole Exome/Transcriptome Sequencing of Coexisting Chronic Myeloid Leukemia and Myelofibrosis

Malathi Kandarpa¹, Yi-Mi Wu², Dan Robinson², Patrick William Burke¹, Arul M. Chinnaiyan² and Moshe Talpaz¹

¹Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI 48109, USA.

²Michigan Center for Translational Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

Corresponding Author:

Moshe Talpaz, MD

Department of Internal Medicine, Division of Hematology Oncology

1500 East Medical Center Drive

4302 CCC-SPC 5936

Ann Arbor, MI 48109-5936

Phone: 734-764-8195

Fax: 734-647-9654

Email: mtalpaz@med.umich.edu

Abstract Word Count: 248

Text Word Count: 2440

Number of Tables: 2

Number of Figures: 2

Supplemental Tables: 2

Short Running Title: Molecular characteristics of dual-MPN

Keywords: myelofibrosis, CML, myeloproliferative neoplasm

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version record](#). Please cite this article as [doi:10.1002/ajh.24728](https://doi.org/10.1002/ajh.24728).

ABSTRACT

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell (HSC) disorders that can be classified on the basis of genetic, clinical, phenotypic features. Genetic lesions such as JAK2 mutations and BCR-ABL translocation are often mutually exclusive in MPN patients and lead to essential thrombocythemia, polycythemia vera or myelofibrosis (ET/PV/MF) or chronic myeloid leukemia, respectively. Nevertheless, coexistence of these genetic aberrations in the same patient has been reported. Whether these aberrations occur in the same stem cell or a different cell is unclear, but an unstable genome in the HSCs seems to be the common antecedent. In an effort to characterize the underlying genetic events that might contribute to the appearance of more than one MPN in a patient, we studied neoplastic cells from patients with dual MPNs by next-generation sequencing. We observed that most patients with two MPNs harbored mutations in genes known to contribute to clonal hematopoiesis through altered epigenetic regulation such as TET2, ASXL1/2, SRSF2, and IDH2 at varying frequencies (1-47%). In addition, we found that some patients also harbored oncogenic mutations in N/KRAS, TP53, BRAF, EZH2, GNAS at low frequencies, which probably represent clonal evolution. These findings support the hypothesis that hematopoietic cells from MPN patients harbor multiple genetic aberrations, some of which can contribute to clonal dominance. Acquiring mutations in JAK2/CALR/MPL or the BCR-ABL translocation probably drive the oncogenic phenotype towards a specific MPN. Further, we propose that the acquisition of BCR-ABL in these patients is frequently a secondary event resulting from an unstable genome.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are characterized by an expansion of one or more lineages of myeloid stem cells. According to the WHO classification, there are 8 different phenotypes of MPNs [1]. Several lines of evidence suggest that genomic instability in the HSC leads to development of molecular lesions that generate the myeloproliferative phenotype [2]. However, the underlying cause of this genomic instability is not well understood. The presence of the BCR-ABL1 fusion gene leads to a chronic myeloid leukemia (CML) phenotype, while mutations in the JAK2 gene are linked to essential thrombocythemia (ET), polycythemia vera (PV) and myelofibrosis (MF). In addition exclusive of JAK2, ET and MF patients can harbor CALR or MPL mutations [3]. These genetic aberrations result in dysregulated tyrosine kinases that generate proliferative signals in the disease-initiating cells. Since these molecular aberrations are usually perceived as mutually exclusive [4], once one MPN is diagnosed, tests for the other mutation/fusion are rarely performed.

Recently, several case studies have described either concomitant CML and ET/PV/MF or emergence of one of these diseases in patients previously diagnosed with another MPN [5-9]. In two cases where CML emerged after PV, the BCR-ABL translocation was suggested to be a secondary event in the JAK2-mutated clone [7, 8]. However, others have suggested the mutations may arise in two independent clones [10]. Nevertheless, both these scenarios presuppose an unstable genome that induces multiple changes in a stem cell or favors emergence of other competing clones. In this study, we evaluated the molecular landscape of hematopoietic stem and progenitor cells from patients with coexistent CML and MF using next-generation sequencing (NGS) methods.

METHODS

Patients and samples

Patients were diagnosed and treated at the University of Michigan Health system. Criteria for diagnosis of CML and post-ET and post-PV MF were based on the WHO classification [1]. Samples from consenting patients were obtained with approval from the institutional review board of the University of Michigan. Bone marrow and peripheral blood mononuclear cells were prepared by Ficoll density gradient centrifugation. For samples that were sequenced, bone marrow mononuclear cells were CD34 enriched using CD34 magnetic microbeads (Miltenyi Biotec, San Diego CA), or total bone marrow or peripheral blood mononuclear cells were used

for isolation of nucleic acids. Matched buccal swabs were used as the source of normal control DNA for each patient. Patients were identified throughout the study using their study ID assigned during enrollment.

Clinical data

Clinical characteristics of each consenting patient were extracted from patient charts. Laboratory results (e.g., diagnostic molecular testing), treatment timelines and drug regimens were compiled from patient charts. Most cases of CML were diagnosed based on classical karyotype and/or FISH. Quantitative molecular diagnosis of BCR-ABL-positive CML to determine response was based on PCR-based testing and was available from some patients for a few time points and the data is presented in International Scale (IS) or as otherwise indicated. Spleen size measurements were based on palpation unless otherwise stated.

Integrative high-throughput sequencing and mutation calls

Nucleic acid preparation, sequencing library construction and high-throughput sequencing were performed using standard protocols in our sequencing laboratory, which adheres to the Clinical Laboratory Improvement Amendments. Paired-end whole-exome libraries from tumor and matched normal DNA were prepared using the Agilent SureSelect human all exon v4 probes (Agilent Technologies, Santa Clara CA). Transcriptome libraries were prepared from total RNA and captured by the Agilent SureSelect human all exon v4 probes [11]. All the libraries were sequenced using the Illumina HiSeq2500 (Illumina Inc., San Diego CA). Aligned exome and transcriptome sequences were analyzed to detect putative somatic mutations, insertions and deletions (indels), copy-number alterations, gene fusions, and gene expression as described previously [12, 13].

COSMIC v 79 was interrogated using the Cancer Browser tool on the COSMIC web application, <http://cancer.sanger.ac.uk/cosmic/browse/tissue>.

RESULTS

Concomitant vs sequential diagnosis of dual MPN phenotype

We identified eight patients with diagnosis of two different MPNs (one being CML) during the course of their treatment or at initial diagnosis. Clinical characteristics of the patients and their diagnosis criteria for each disease are summarized in Table I. In two of the patients, both

diseases were diagnosed concomitantly; in the other patients, the second condition was diagnosed during treatment due to ongoing and complex physical findings (splenomegaly), cytogenetic findings of the Philadelphia chromosome (Ph), and pathological findings in the bone marrow, such as CML patient with dysmegakaryopoiesis indicating more than one myeloproliferative disease. The time between the establishments of the two diagnoses varied from 0-15 years. In addition to the patients presented, we identified three other MF patients who had minimal levels of BCR-ABL by PCR without hematological disease (data not shown). The predominant diagnosis at presentation varied among the eight patients. Patient (Pt) 1471 was under observation for ET, which progressed to MF and exhibited a BCR-ABL translocation along with a JAK2V617F mutation. Similarly, Pt2105 with post-ET MF was found to have BCR-ABL translocation during testing prior to enrollment onto a clinical trial. On the other hand, Pt1191 was Ph^{+ve} and did not have a JAK2V617F mutation at the time of CML diagnosis; however, after achieving a major molecular response, the patient's bone marrow showed a fibrotic myeloid neoplasm with prominent large megakaryocytes in clusters with sinusoidal dilation containing hematopoietic elements. The bone marrow findings, positive reticulin staining and persistent splenic enlargement implicated an overlap diagnosis of MF, which was eventually confirmed by the presence of the CALR mutation. Overall, CML was the first MPN diagnosis in two patients, second diagnosis in four patients, and concurrent diagnosis in five patients (data not shown for three) following low level BCR-ABL detection.

Treatment paradigms for the dual disease patients

Treatment was tailored to the clinically dominant disease in cases of concomitant diagnosis; in the other cases treatment was adjusted to address the second condition. The patients' treatment timelines are summarized in Figure 1A. Each patient's response to the treatment, as measured by spleen size, WBC count and BCR-ABL levels, is summarized in Figure 1B. An addition or change in therapy was warranted when a patient did not respond to a treatment as indicated by an increase in BCR-ABL transcripts, increase in WBCs, or persistence or increase in splenomegaly. A few of the patients were switched to TKI therapy for CML combined with a JAK inhibitor or another therapy for MF, either given together (Pt2105, dosed with ruxolitinib and imatinib/dasatinib every day) or in an alternating schedule (Pt1137, alternating schedule of nilotinib 4 days on/1 day off, then ruxolitinib 8 days on/1 day off). Most of these patients demonstrated improved response and safe tolerability with this regimen, potentially endorsing a

new treatment paradigm for dual MPN patients which can be tested in a carefully designed clinical study.

Integrative high throughput sequencing of patient samples

We obtained bone marrow and/or peripheral blood samples from 7 of the 8 patients described in this cohort and assessed all genomic alterations in the stem and progenitor cells wherever possible (Table II). A sufficient number of HSPCs were enriched by CD34-positive selection from 3 patients, and for the other 4 patients, total bone marrow or peripheral blood mononuclear cells were sequenced. Samples were subjected to integrative sequencing, which includes whole-exome sequencing of the tumor and matched normal sample, transcriptome sequencing and whole-genome sequencing. The samples for sequencing were obtained at different time points during treatment and stage of disease; therefore, some genetic aberrations at diagnosis were undetectable (Table II). Pt1191 had a BCR-ABL translocation at CML diagnosis, but achieved a major molecular response to dasatinib treatment, which was supported by negative PCR results for BCR-ABL in the sequencing sample (Figure 2B). CALR mutations in patients with post-ET MF have only recently been reported [3], and routine testing was unavailable when patients in this study were first diagnosed with an MPN. Although CALR mutations were not initially detected in Pt2105 during active treatment and stable disease, a length-affecting mutation in exon 9 of the CALR gene was later discovered once the patient's MF symptoms worsened. In most patients, the sequencing results confirmed the molecular diagnostics in the patient charts. For Pt1505, clinical diagnostics did not identify the MPLY591N mutation detected by NGS (10% frequency). The MPLY591N is an atypical weak gain-of-function mutation that increases MPL signaling [14].

In addition to validating clinical findings, NGS detected several additional genetic aberrations in the patients with concomitant MPNs. We found varying frequencies (1-47%) of mutations in genes involved in epigenetic regulation, including TET2, ASXL1/2, IDH2, SRSF2, and EZH2 (Table II), which have known incidences in MF [15, 16]. The 4 (out of 7) patients who harbored at least one of these gene mutations probably have a higher incidence of epigenetic modifications than the <14% incidence in CML (COSMIC data, Supplemental Table 2).

Some patients also harbored oncogenic mutations in N/KRAS, TP53 and BRAF at frequencies varying from 0.5-39% (Table II). The sub-clonal frequencies of these mutations might indicate clonal evolution of the disease. BRAF mutations found included V600E, a well-established gain-

of-function mutation, as well as G469V and D594E, which are two atypical mutations that like V600E, occur within the kinase domain. BRAF D594E has been previously reported in CMML cases [17].

We interrogated the COSMIC database to determine the genes that have been previously reported in CML (including blastic phase CML), ET, PV and MF. The top 20 genes in CML and blastic phase CML were compared to each of the top 20 genes from ET/PV/MF (Figure 2). The common genes found to be mutated in these diseases included the same epigenetic regulators we found mutated in our patient set, namely TET2, ASXL1, IDH1/2, SRSF2 and EZH2. Outside of ABL1 (47-28%), genetic variations in CML occur infrequently. Frequency of TP53 mutations increased from 4% in chronic-phase CML to 26% in blast-phase CML. In Ph^{+ve} MPNs, TP53 variant frequency was only 2-6%, suggesting that TP53 variants are associated with advanced disease and are not driver mutations in these diseases.

DISCUSSION

This report of eight patients with coexistent ET/PV/MF and CML, along with other reports in the literature underscore that the frequency of this phenomenon is significant. Most often one MPN is diagnosed, precluding further testing for other diseases. Lack of response to therapy is often the reason for alternate/additional diagnoses. Here we describe the diagnosis and management of patients with coexistent MPNs. The treatment was tailored to the patient's clinical presentation and tolerance to therapy, and guided by our experience.

We used a whole-genome sequencing approach to understand the pathophysiology of the dual disease phenotype. Our observations suggest that patients with concurrent MPNs acquire either simultaneous or sequential mutations in the HSPC. We therefore propose two models to explain the development of concurrent MPNs at the cellular level: 1) two independent clones arise from genetically unstable HSPCs and compete with each other, and 2) an already mutated HSPC acquires a "second hit". Previous reports from patients with two MPNs suggest that BCR-ABL1 and JAK2^{V617F} can be present in the same clone of cells or in distinct clones [6, 7, 9, 10, 18-21]. In some patients (n=4) from our cohort, the NGS analysis did not detect a previously identified BCR-ABL1 translocation because patients were in molecular remission at the time of sampling. JAK2 or MPL mutations were detected in these samples after the CML sub-clone was suppressed. The other interpretation is that the JAK2 mutation precedes BCR-ABL1 translocation and therefore JAK2 persisted after BCR-ABL1 eradication. Therefore, our data

suggest that genetic events that lead to ET/PV/MF arise in the HSPC and CML is a result of a “second hit”.

The literature supports that genetic instability in HSPCs is a precondition for MPN initiation. HSPCs from “healthy” individuals, who have no overt disease, can acquire mutations in several genes that might contribute to a pre-leukemic state, which over time could further destabilize the genome and result in the disease phenotype [22, 23]. These pre-leukemic genes in healthy individuals could provide a competitive advantage to the HSPCs, thus resulting in clonal hematopoiesis. Mutations in epigenetic regulators such as DNMT3A, TET2 and ASXL1 genes occur frequently in these individuals and in patients with MPNs including MF/ET/PV [16, 24, 25]. These epigenetic regulators might contribute to the instability of the HSPC genome [26, 27]. In our studies, mutations in TET2 (n=2) and ASXL1 (n=3) were found in some patients who were stable or in remission at the time of sampling. We did not detect any DNMT3A mutations, which is consistent with the low frequencies previously reported in MPNs [15]. TET2 loss-of-function mutations in MPNs can worsen the course of JAK2V617F-induced disease and increase the proliferative state of HSCs [27]. ASXL1 was found to be mutated frequently in MF (36%) and rarely in ET and PV [25]. The pathophysiology of loss-of-function ASXL1 mutations is not clear, though some reports have suggested that mutant ASXL1 can collaborate with NRASG12D in promoting myeloid leukemogenesis in mice [26]. Interestingly, ASXL1 mutations along with KRAS or NRAS mutation were found in 2 of the 7 patients with concomitant MPNs. Based on COSMIC data, TET2 loss-of-function (4%) and ASXL1 loss-of-function (10%) mutations are rarely found in CML, and the biological significance of these mutants in a Ph⁺ setting is not yet understood [24]. The relative high frequency of these mutations in our series suggests either dominance of MF with later development of CML, or may explain the presence of two diseases in the same patient.

Novel findings from this small data set include GNAS and BRAF mutations. Although BRAF mutations are frequent in Langerhans cell histiocytosis and hairy cell leukemia (HCL), they are not considered driver mutations in any myeloid neoplasms [28]. Expression of BRAFV600E in murine HSPCs resulted in features consistent with HCL in mice [29]. In our study cohort, 2 of 7 patients had BRAF mutations albeit at low frequency and probably due to clonal evolution. One patient harbored a GNAS hotspot mutation at a relatively high frequency (21%), and this mutation is reported in several gastrointestinal tumors and endocrine tumors [30]. The small

sample size of this study does not allow us to conclude whether these mutations are of significance to the dual-disease phenotype.

In conclusion, this is the first report of whole-genome sequencing to determine genomic changes that might contribute to the manifestation of more than one myeloid neoplasm in the same patient. Our data affirms that HSCs accumulate multiple genetic variants, which is a hallmark of patients with hematological malignancies. Identification of one genetic variant did not preclude the presence of another that could drive a phenotypically distinct disease. The data also suggest that the CML in these patients might be a secondary disease arising from underlying genetic instability. Therefore, treatment paradigms for these unusual cases should be tailored to target more than one signaling pathway to sustain remission and improve outcomes of patients.

ACKNOWLEDGEMENTS

We thank the patients and their families for their participation in this study. We thank Jessica Mercer for her editorial assistance. This study was supported by the Dan and Betty Kahn Foundation Grant to MT.

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Figure Legends

Figure 1: Clinical timeline of dual MPN diagnosis, treatment, response and NGS sampling. (A) Each line depicts the clinical course of one patient. The bars below show the timeframe of treatment directed towards MF or CML. The diagnosis of MF or CML is shown by a vertical arrow on the top of the timeline. The time point of NGS sample acquisition is also shown as a vertical arrow. (B) Each line represents one patient as in A, with the same time intervals. The treatment changes are shown as vertical arrows. Spleen size measurements as based on palpation in cm below the right costal margin or CT scans where indicated by a CT superscript. WBC counts are represented as 1000s of WBC/microL of blood. BCR-ABL was measured by PCR and is presented in IS (superscript % indicates % BCR-ABL1/ABL1, where IS units were unavailable).

Figure 2: COSMIC query of the top 20 genes in CML and ET/PV/MF. (A) Venn diagram showing overlap of genes in both disease subtypes (B) List of genes represented in the circles in A.

Table I: Clinical characteristics of dual MPN patients

Table II: Driver gene mutations in dual disease patients identified by integrative high-throughput sequencing

Table 1: Clinical characteristics of dual MPN patients

Patient ID	Sex	Race	Age at 1st Dx	Time to 2nd Dx	Diagnosis*	Splenomegaly	Size of Spleen	WBC	PLT	Hgb	% PB Blasts	Bcr-Abl quantitation	Jak2, Mpl, CRT mutation	Bone marrow reticulin
1471	Male	Caucasian	63	2 yrs	Post-ET MF	Yes	3 cm	5.4	496	13.3	0	negative	JAK2 V617F	Moderate
					CML	Yes	18 cm	24	575	13.3	0.5	50% by FISH & 4.4 by PCR	JAK2 V617F	MF-3
1191	Male	Caucasian	54	2 yrs	CML	Yes	7cm	55.2	255	13.9	2	17.44 % p210(b2a2/b3a2)	negative for JAK2V617F	ND (Mild MF)
					MF	Yes	10 cm	5.6	270	12.5	0	0.062 % (IS)	CALR Positive	Diffuse MF-2
2105	Male	Caucasian	70	4 yrs	Post-ET MF	Yes	nd	3.8	589	14.3	nd	nd	nd	Moderate
					CML	Yes	10 cm	15.2	287	10.2	1	93.3 % (IS)	CALR Positive	MF-3
1505	Female	Caucasian	59	13 yrs	ET	No	None	48.2	380	11.7	0	Negative	JAK2 V617F	Mild to none
					CML	No	None	9.7	383	8	0	3.291 % (IS)	JAK2 V617F	MF-3
1137	Male	Caucasian	61	3 yrs	CML	Yes	Not palpable (18.4 cm by CT)	15.7	327	12.2	3	99.25% by FISH	negative	Mild to moderate
					PMF	Yes	19 cm	46.8	275	10.5	2	20 m Post-Dx 0.167% (IS)	MPLW515L Pos	Mild to moderate
2158	Female	Caucasian	68	15 yrs	Post-PV MF	Yes	18.1 by Ultrasound	9.3	334	10.9	Rare	Negative	JAK2 V617F	Moderate
					CML	Yes	16 cm	4	41	9.5	1	11% by FISH	JAK2 V617F	High
1565	Female	Unknown	56	concomitant	Post-PV MF	Yes	14.5 cm	10.5	161	11.2			JAK2 exon 12	MF 1-2
					CML	Yes						0.02% by PCR		
6281	Male	Caucasian	70	concomitant	CML	Yes	17 cm	98.7	179	15.3	0		JAK2 V617F	Moderate
					Post-PV MF							p210(b3a2) +ve		

* The disease diagnosed 1st or is more prominent is listed first

Table II: Driver gene mutations in dual disease patients identified by integrative high throughput sequencing

Patient	Time point of sample	Sample type	Driver Gene Mutations				
			BCR-ABL1 Gene fusion	JAK2 (% Allele frequency)	CALR (% Allele frequency)	MPL (% Allele frequency)	Other mutations (variant, % Allele frequency)
1471	5.5 yrs post 2nd Dx	BM (CD33/34+)	Positive	V617F (87%)	Negative	Negative	BRAF (V600E, 0.5%) BRAF (G469V, 3%) KRAS (A146V, 1.5%) TP53 (C238Y, 1%) NF1 (R2258*, 4%) PIK3R3 (R105W, 6%) ASXL1 (G645V, 4%) KMT2C (N729D, 7%)
1191	1 yr post 2nd Dx	BMMCs	Negative*	Negative	Negative	Negative	KMT2D (A1740T, 37%) FLG (S2366T, 20%)
2105	2.5 yrs post 2nd Dx	BMMCs and PBMCs	Positive	Negative	E364fs (32%)	Negative	-
1505	2 yrs post 2nd Dx	BM (CD33/34+)	Negative*	V617F (94%)	Negative	Y591N (10%)	TET2 (Splice donor, E1268, 34%) TET2 (S217fs, 23%) SH2B3 (Y572fs, 3%)
1137	At 2nd Dx	PBMCs	Negative*	Negative	Negative	W515L (95%)	NRAS (G12V, 39%) SRSF2 (P95H, 47%) IDH2 (R140W, 43%) EZH2 (S695L, 22%) ASXL1 (D457fs, 44%)
2158	At 2nd Dx	PBMCs	Positive (low level)	V617F (4%)	Negative	Negative	BRAF (D594E, 1%) ASXL1 (G658*, 1%) ASXL2 (R614*, 2%)
1565	5mos post 2nd Dx	BM (CD33/34+)	Negative*	I540_E543 delinsMK	Negative	Negative	GNAS (R202H, 21%)

* denotes negative finding for BCR-ABL1 fusion gene due to sample timing was when patient was in major molecular response.

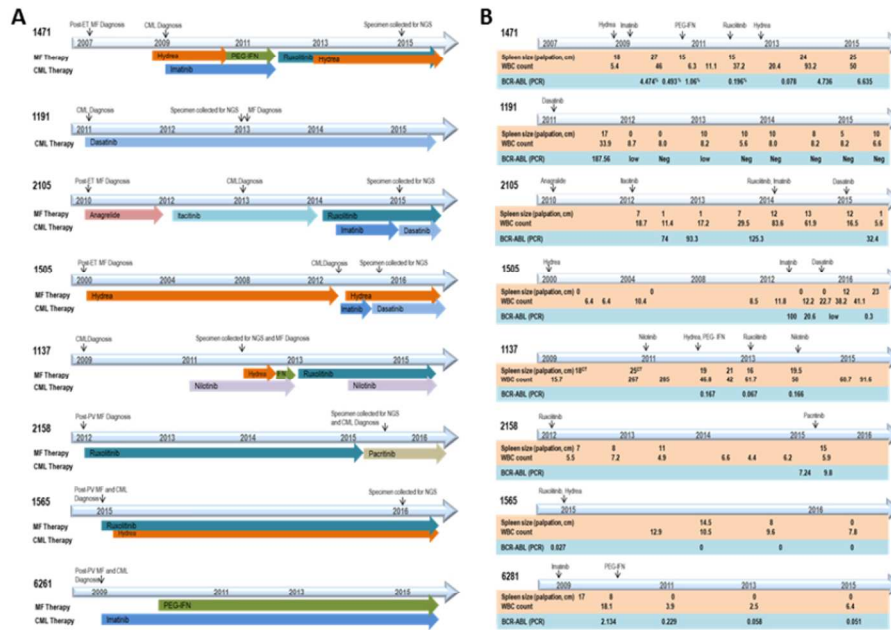


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Figure 1
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Figure 2

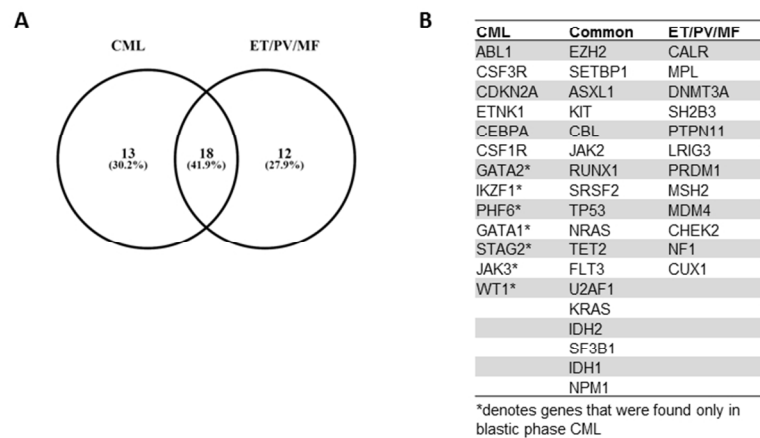


Figure 2: COSMIC query of the top 20 genes in CML and ET/PV/MF. (A) Venn diagram showing overlap of genes in both disease subtypes (B) List of genes represented in the circles in A.

Figure 2

254x190mm (96 x 96 DPI)

Accep