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LETTER TO THE EDITOR

ASXL1, *TP53* and *IKZF3* mutations are present in the chronic phase and blast crisis of chronic myeloid leukemia

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The mechanism of transformation from chronic phase (CP) to blast crisis (BC) chronic myeloid leukemia (CML) is heterogeneous and poorly understood.[1] The most frequently genetic aberration observed at this advanced stage includes a second Philadelphia (Ph) chromosome, trisomy 8, isochromosome 17 and trisomy 19, alone or in various combinations and complex aberrations.[1] Clonal cytogenetic evolution appears to be the most consistent predictor of blastic transformation, present in up to 80% of patients, until now.[2] At present, little is known about the molecular mechanisms underlying the mutational profilling at CP-CML disease progression and only limited changes occuring during clonal evolution at BC have been described.[3] We read with great interest a series of manuscripts reporting somatic mutations in patients with CML in both the CP and BC.[4–8] Mutations in genes such as *TP53*, *NPM1*, *IKZF1*, *RUNX1* and *ASXL1* have been described in CML progression. However, all these studies have analyzed a limited number of genes and mainly focused on BC phase. Interestingly, Soverini *et al.*[7] performed a massive parallel sequencing at three different stages (diagnosis, major molecular response and disease progression) of a patient who developed a lymphoid BC. In this study, *IDH2* R140Q was detected in a very low number of cases, and the mutation was only observed in BC.

To investigate the genetic changes associated with CML progression, we performed whole-exome sequencing (WES) of an individual patient at three different phases: CP, complete cytogenetic remission and BC. The patient was a 65-year-old man diagnosed with a Ph+ CML and presented with a hypercellular bone marrow (BM) containing 2% blast cells. Karyotype analysis showed the Ph chromosome in all analyzed metaphases at diagnosis. The patient was treated with Imatinib (400 mg/day) achieving, along the treatment, complete hematological response after 1 month and complete cytogenetic response (CCyR) after 12 months. Conversely, he never achieved major molecular response during this period. Unfortunately, after 14 months from diagnosis, the patient progressed to a myeloid BC that did not respond to a second-line treatment (Dasatinib + Idarubicin-AraC) and died of the disease 18 months after diagnosis. At that time, the G-banding analysis showed a complex karyotype (Supplementary Figure 1). To perform the WES technique, DNA samples were obtained from the normal tissue (oral mucosa) and BM blasts at diagnosis (CP), at CCyR and at the time of disease progression (BC) from the index CML patient. The preparation of shotgun libraries from the leukemic (CP, CCyR and BC) and non-leukemic genomic DNA followed by an in-solution exome capture was performed with the use of a commercial platform (Agilent) covering 50 Mb of coding exons (~1.60% of the genome). After massive parallel sequencing using the Genome Analyzer IIx (Illumina), candidate somatic mutations were identified using RUbioSeq software.[9] The bioinformatics analysis and the filtering steps to identify the coding variants are detailed in the Supplementary Material.

After discarding the variants present in the matched normal DNA and in the dbSNP132 database, we obtained a total of 719, 1839 and 869 single-nucleotide substitutions (SNSs) and small insertions and deletions (indels) for CP, CCyR and BC, respectively. Next, we selected only those variants within coding regions that, passing depth and quality controls, were frameshift, stop gain/loss and non-synonymous amino acid changes predicted to produce deleterious damage in the protein structure (Supplementary Table 1). Finally, this resulted in 13, 7 and 15 SNSs for CP, CCyR and BC, respectively (Figure 1a and Supplementary Table 2). The application of the WES approach allowed determining the

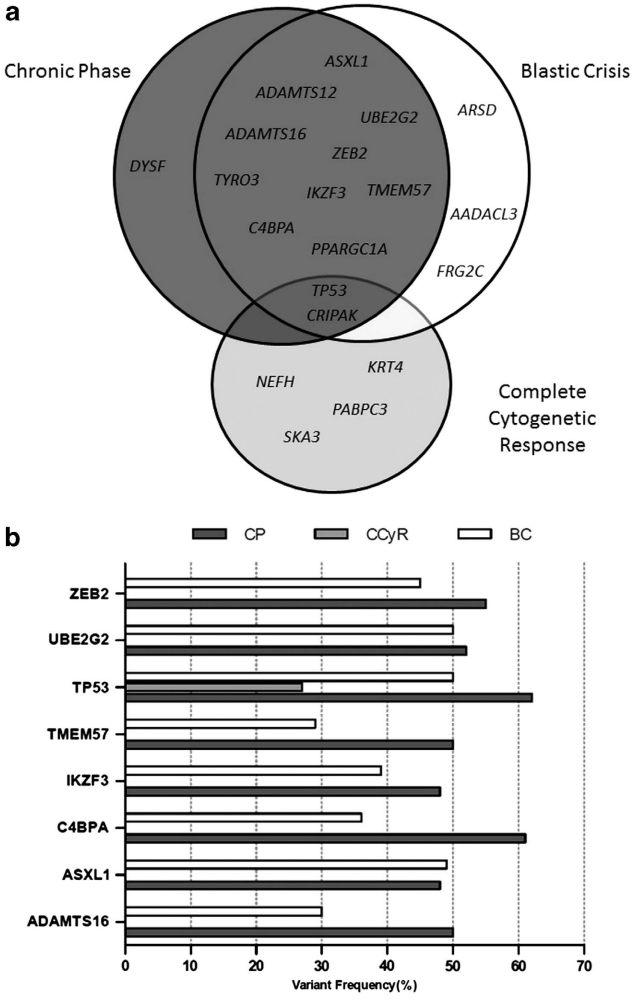

Figure 1. Mutational profile by whole-exome sequencing along the three phases of the index CML patient. (**a**) The Venn diagram representing the distribution of mutated genes in CML progression. (**b**) Frequencies of the mutant alleles of every affected gene at the three time points of CML progression. Sanger validation was conducted for those genes with frequencies above 20%: *ZEB2*, *UBE2G2*, *TP53*, *TMEM57*, *IKZF3*, *C4BPA*, *ASXL1* and *ADAMTS16*.

Table 1. Chromosomal and molecular features in 13 CP and BC/NCgR paired samples

Id	*Type*	*Age (years)*	*Follow-up (months)*	*Karyotype*	*Mutational status*		
					ASXL1	*IKZF3*	*TP53*
1[a]	CP	65/M	18	45,XY,t(9;22)(q34;q11.2),rob(13;14)(q10;q10)c[20]	c.2035G>T p.G679[a]	c.952G>A p.E318K	c.730G>A p.G244S
	BC			44,XY,t(9;22)(q34;q11.2),rob(13;14)(q10;q10)c,-17 [19]/ 46,XY,t(9;22)(q34;q11.2),+10,rob(13;14)(q10;q10)c,-17[1]	c.2035G>T p.G679[a]	c.952G>A p.E318K	c.730G>A p.G244S
2	CP	31/F	6	46,XX,t(9;22)(q34;q11.2)[20]	c.2498_2501del p.S833I	wt	wt
	NCgR			46,XX, t(9;22)(q34;q11.2)[20]	c.2498_2501del p.S833I	wt	wt
3	CP	70/M	3	46,XY,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	NCgR			46,XY,t(9;22)(q34;q11.2)[6]/46,XY[14]	wt	wt	wt
4	CP	41/F	5	46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	BC			47,XX,+der(1)t(1;?)(p12;?),+6,+8,t(9;22)(q34;q11.2),-14?,-17?,-18?,+21,+der(22)t(9;22)(q34;q11.2),+mar[cp2]/46,XX[6]	wt	wt	wt
5	CP	54/M	5	46,XY,t(2;13)(p36;?),t(9;22)(q34;q11)[20]	wt	wt	wt
	NCgR			46,XY,t(2;13)(p36;?),t(9;22)(q34;q11)[1]/46,XY[19]	wt	wt	wt
6	CP	85/M	7	46,XY,t(9;22)(q34;q11.2)[4]/46,XY,t(9;9;22)(p12;q34;q11.2)[10]/46,XY[6]	wt	wt	wt
	NCgR			46,XY[11]/46,XY,t(9;9;22)(p12;q34;q11.2)[5]	wt	wt	wt
7	CP	36//M	58	46,XY,t(9;22)(q34;q11.2)[7]/46,XY[8]	wt	wt	wt
	NCgR			46,XY,t(9;22)(q34;q11.2)[2]/46,XY[18]	wt	wt	wt
8	CP	34/M	12	46,XY,t(9;22)(q34;q11.2),der(3)t(3;?)(?;?)[7]/46,XY,t(9;22)(q34;q11.2)[8]	wt	c.71C>T p.A24V	wt
	BC			46,XY,t(9;22)(q34;q11.2)[2]/46,XY[26]	wt	c.71C>T p.A24V	wt
9	CP	8/M	4	46,XY,t(9;22)(q34;q11.2)[17]/46,XY[3]	c.2598A>G *(p.L866L)*	wt	wt
	BC			47,XY,+X,-7,ider(9)(p10)t(9;22)(q34;q11.2),+mar[18]/46,XY[2]	c.2598A>G *(p.L866L)*	wt	wt
10	CP	44/F	24	46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	NCgR			46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
11	CP	73/F	20	46,XX,t(9;22)(q34;q11.2)[20]	c.1937dup p.G649W	wt	wt
	NCgR			46,XX,t(9;22)(q34;q11.2)[20]	c.1937dup p.G649W	wt	wt
12	CP	60/F	14	46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	BC			46,XX,t(9;22)(q34;q11.2)[8]/47,XX,t(9;22)(q34;q11.2),+t(9;22)(q34;q11.2)[10]/46,XX[2]	wt	wt	wt
13	CP	69/F	3	46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	NCgR			46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
14	CP	NA	24	46,XX,t(9;22)(q34;q11.2)[20]	wt	wt	wt
	BC			45,XX,-7,t(9;22)(q34;q11.2)[8]/46,XX[12]	wt	wt	wt

Abbreviations: BC, blast crisis; CP, chronic phase; F, female; M, male; NCgR, no cytogenetic response; wt, wild type. [a]Whole-exome sequencing analyzed patient.

clonality and clonal evolution patterns during the CML progression. In this sense, 93% of the selected SNSs and indels that were present in the CP were also seen in BC. In fact, the percentages of reads of the mutant alleles identified for the most relevant genes were the same (around 50%) both at CP and at BC (Figure 1b). As expected, these data suggest that the same clone was present in CP and BC.

Interestingly, *TP53* mutation (p.G244S) is present in all the stages, pinpointing its potential role as a tumor-initiating event. In contrast, *ASXL1* (p.G679*), *UBE2G2* (p.D35V), *ZEB2* (p.L420R) and *IKZF3* (p.E318K) were present in CP and BC, also suggesting that these alterations are initiating events and co-occur with the *BCR/ABL1* fusion gene (Figure 1a). All theses SNSs were validated using PCR amplification and direct DNA sequencing of the same samples that were subjected to WES. Finally, SNSs unique to the BC sample (*ARSD, FRG2C* and *AADACL3* mutations) may contribute to leukemic transformation; however, we were not able to validate these two mutations by Sanger sequencing due to the limitations to detect variants with a frequency lower than 20%.

Among the genes previously reported in CML progression,[4–8] we detected *ASXL1* and *TP53* mutations. Interestingly, *TP53* mutation was found in the three phases of CML progression and is present in the Catalogue of Somatic Mutations In Cancer (COSMIC) database.[10] There are some controversial results regarding its role in blastic transformation. Whereas some authors have observed mutations in 25–30% of BC,[2] in a recent work by Grossman *et al.*,[6] in which the deep-sequencing approach was used, they found a *TP53* mutation only in 1 out of 39 (2.6%) BC patients analyzed. To fill in this gap, we analyzed the *TP53* gene in an independent set of 26 samples consisting of 13 paired CML and BC/NCgR (no cytogenetic response) samples, and no mutations were found (details in Supplementary Material). Although it seems that mutations affecting *TP53* gene can be considered as a rare event in the progression of CML, the presence of the mutation in all three phases confirms that it is clearly related to a non-standard response to the treatment and/or clinical progression. Regarding *ASXL1*, mutations in this gene during CML evolution were described for the first time by Boultwood *et al.*[5] They found mutation in 6 out of 41 patients analyzed (three CP and three BC non-paired samples; 14.5%); however, in the BC sample for which a CP paired sample was available, they did not detect the same mutation. Subsequently, other authors have observed *ASXL1* as a commonly affected gene in similar frequencies.[6,11,12] In accordance with these previous findings, we detected a missense mutation in the patient sequenced, and we also observed two different mutations in 4 out of 26 (15%) in BC/NCgR and also in the CP paired samples analyzed (Table 1). All these data together corroborate that *ASXL1* mutations constitute an early event and might cooperate with other alteration such *BCR/ABL1*.

Regarding mutations found in CP and BC, it is important to mention the mutation observed in *IKZF3*. *IKZF3* is a member of the Ikaros family of transcription factors, which are the important regulators of lymphoid differentiation. Among the five members of the family, *IKZF1* have been found deleted and mutated in acute lymphoblastic leukemia (ALL), CML blastic phase and *BCR/ABL1*-positive ALL, suggesting a pathogenic contribution to leukemic transformation.[13] In addition, this genetic alteration has been found also in 19% of patients with blast-phase myeloproliferative neoplasm, suggesting a potential pathogenic role in the myeloid lineage.[14] To date, mutations in other members apart from *IKZF1* have never been described in human leukemia. In order to explore this genetic feature, we screened for mutation in *IKZF3* gene in the 26 samples using conventional PCR and Sanger sequencing. *IKZF3* were also mutated in 8% of CP and BC/NCgR samples, suggesting a potential role of this gene in myeloid leukemia (Table 1).

In conclusion, WES allowed the identification of a large number of mutated genes, even at the CP of CML, which harbor prognostic and predictive significance, such as *ASXL1* and *TP53*, both found mutated at CP and BC. The study of the mutation profile through the course of the disease indicated that, at least in this patient, the number and the type of mutations were similar at CP and BC. In addition, we identified for the first time deleterious mutations in

IKZF3, *UBE2G2* and *ZEB2* in CML. Although current diagnostic procedures recommend the study of *ABL1* mutations in non-responder patients, our data suggest that sequencing a wider panel of genes could be also beneficial in the clinical management of these patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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J Menezes[1,6], RN Salgado[1,6], F Acquadro[1], G Gómez-López[2], MC Carralero[1], A Barroso[3], F Mercadillo[4], L Espinosa-Hevia[1], JG Talavera-Casañas[5], DG Pisano[2], S Álvarez[1] and JC Cigudosa[1]
[1]Molecular Cytogenetics Group, Human Cancer Genetics Program, Spanish National Cancer Research Center–CNIO, Madrid, Spain; [2]Bioinformatic Unit, Structural Biology and Biocomputing Program, Spanish National Cancer Research Center–CNIO, Madrid, Spain; [3]Human Genetics Group, Human Cancer Genetics Program, Spanish National Cancer Research Center–CNIO, Madrid, Spain; [4]Clinic Familial Cancer Unit, Human Cancer Genetics Program, Spanish National Cancer Research Center–CNIO, Madrid, Spain and [5]Hematology and Hemotherapy Service, Ntra. Sra. de Candelaria Hospital, Santa Cruz de Tenerife, Spain
E-mail: jccigudosa@cnio.es
[6]These authors contributed equally to this work.

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