Aims: In this study we aim to: i)Identify changes in size of immunophenotypic (IP) compartments in the progression from CP to BP-CML, ii)characterize which haemopoietic stem/progenitor cell (HSPC) subpopulations of BP-CML patients contain LSCs iii)understand the clonal structures associated with disease progression.
Methods: We have performed extensive IP analysis of 14 AP-CML, 4 CP-CML and 11 BP-CML patients and compared the various HSPCs compartments to normal bone marrow (BM) (N=3). Furthermore we purified HSPC-like populations from 5 BP-CML patient samples and performed *in vivo* xenograft studies using NSG mice with serial transplantation to identify populations with LSC potential. Finally multicolor FISH analysis was performed in 3 patient samples and in engrafted human cells from primary and secondary murine recipients.
Results: Our data conclusively demonstrate that functional LSCs are present in multiple stem/progenitors populations in myeloid BP-CML,. We have described the clonal structures in the samples of 3 patients. Sequential acquisition of 17p13 loss or of 17p13loss and isochromosome 17 was seen in patient samples. More complex clonal structures, with the presence of subclonal atypical BCR-ABL and 17p13loss were detected after transplantation in NSG mice. The monitoring of clonal structures in a patient with 2 sequential samples suggested that the clonal architecture is dynamic and can change with progression of the disease.
Summary/Conclusions: Our results demonstrate that myeloid BP-CML is a heterogeneous disorder with variable LSC populations. Further interrogation of these populations and of clonal hierarchy will identify novel therapeutic targets of LSC populations.
DK and HM are joint first authors, MC and PV are joint senior authors

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ROLE OF THE AURORA KINASE A AXIS IN IMATINIB RESISTANCE OF CHRONIC MYELOID LEUKEMIA CD34+ PROGENITORS

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Background: Aurora Kinase (AK) A has a pivotal role in chronic myeloid leukemia (CML) genomic instability. Its constitutive activation associated with the *BCR-ABL*1 TK activity promotes the progression of mitosis irrespective of the integrity of replicated DNA. Published studies have proven the therapeutic advantage of AK inhibitors in CML patients either responsive or resistant to imatinib (IM). Such AK inhibitors potential has been ascribed to their inhibitory activity on p210 TK activity.
Aims: In this study, we have focused on the specificity and mechanisms of action of AKs and Plk inhibitors in the putative LSC compartment (CD34+).
Methods: The CD34+ hematopoietic cell fraction was investigated for the phosphorylation levels of AKA and other pro-survival components of its signalling pathways: - FoxM1, a proliferation-associated transcription factor implicated in the advantage of clonal hematopoiesis over the normal counterpart, particularly in the leukemic stem cell (LSC) compartment, which is not dependent on BCR-ABL1 tyrosine kinase (TK) for proliferation and survival; - Polo-like kinase 1 (Plk1), a ser-thr kinase involved in M/G1 progression. CD34+ cells were isolated from bone marrow samples of 10 CML patients at clinical diagnosis by means of immuno-magnetic selection (miniMACS from Miltenyi Biotec). CD34+ cells from peripheral blood of healthy donors, pooled to avoid individual differences were used as normal controls. Informed consent was obtained from all the patients. RT-PCR (reverse transcriptase-polymerase chain reaction), WB (western blotting), IP/IB (immunoprecipitation and immunoblotting) were used to investigate gene expression and protein interactions.
Results: Our results proved a FoxM1 increment associated with IM resistance. An IM-resistant K562 cell line (LD50 0.37 microM *vs* 0.026 microM of parental cells) generated in our lab exhibited FoxM1 over-expression and hyper-phosphorylation contingent upon the upstream activation of AKA and Plk1. In fact, in IM-resistant K562 cells, both Plk1 inhibition by volasertib (1microM) and AKs inhibition by danusertib (1microM), activated a significant increment of apoptotic cell death compared to parental cell line. AKA, FoxM1 and Plk1 involvement in IM resistance was confirmed in mononuclear cell fraction from bone marrow samples of 3 CML patients who developed IM resistance independent from BCR-ABL1 point mutations. Interestingly, the putative BCR-ABL1+/CD34+ LSC compartment, which is neither dependent on BCR-ABL1 TK for proliferation and survival nor killed by IM and second generation inhibitors, showed a hyperphosphorylation of AKA and a consequent overexpression and hyper-activation of FoxM1 and Plk1. Moreover, clonogenic assays performed by using CD34+ progenitors from 3 CML patients at diagnosis showed that volasertib and danusertib are capable to reduce the clonogenic potential of the CD34+ compartment to a much greater extent compared to 1st and 2nd generation TKIs (see Table 1).

Table 1.

LD50	PT1	PT2	PT3
IMATINIB	0.255 M	0.374 M	0.472 M
NILOTINIB	0.197 M	0.277 M	0.324 M
DASATINIB	0.269 M	0.311 M	0.295 M
VOLASERTIB	0.112 M	0.093 M	0.162 M
DANUSERTIB	0.098 M	0.084 M	0.073 M

Summary/Conclusions: The BCR-ABL1+/CD34+ compartment provides a sanctuary for disease relapse upon drug withdrawal as well as a putative source of drug-resistance. We have identified a new signaling pathway involved both in drug resistance and in CD34+ cell survival. Our data open the route to novel therapeutic approaches worth exploring in order to overcome drug resistance. Danusertib and volasertib are in clinical trials in hematologic malignancies.

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PRESENCE OF SOMATIC AND GERMLINE MUTATIONS IN EPIGENETIC MODIFIERS IN CML-CP

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Background: Chronic myeloid leukaemia (CML) originates from a single genetic aberration (BCR-ABL1); however the clinical disease is remarkably heterogeneous and the genetic mechanisms of resistance to tyrosine kinase inhibitors (TKI) are still poorly understood. Recently, we have identified consistent differences in genome-wide DNA methylation patterns in chronic phase (CP) CML patients compared to healthy controls, whireas epigenetic modifying enzymes have been found frequently mutated in other haematological neoplasms.
Aims: The aim of this study is to analyse a panel of mutations in epigenetic modifiers in CML-CP using Ion Torrent PGM next-generation sequencing. The panel design was based on gene expression analysis we generated and literature search. Potential mutations found at diagnosis may be used as novel prognostic biomarkers for TKI response.
Methods: 52 samples from untreated patients with newly diagnosed CML-CP ($CD34^+$) who started on imatinib were included in the study, classified as responders (n=26) /non-responders (n=26) based on BCR-ABL1/ABL ratio at 3 months. As constitutional non-leukaemic DNA, for non-responders we used DNA from T cells, cultured *in vitro* for 7 days, and for responders DNA from the patients in deep molecular remission (whole blood). 14 samples from healthy donors ($CD34^+$) and 5 samples from CML-BC (blast crisis) were also used. A custom panel covering the coding region of 71 epigenetic enzymes was designed.
Results: A mean depth of 273/amplicon was obtained, detecting mutations as low as 4%. After excluding "bad" variants of low quality, common SNPs with minor allele frequency (maf) >1%, variants found in healthy controls and intronic variants, we kept the non-synonymous variants predicted disease causing, deleterious and damaging by Mutation Taster, PolyPhen-2 and SIFT respectively, and found 104 variants in 46/71 of the genes. However, when constitutional DNA was used as non leukaemic control, we found that only 35 were CML-related somatic mutations, including missense, nonsense, frameshift/non-frameshift insertions and splice site variants, present in 25 genes. 26 mutations were found in non-responders (NR), 13 in responders (R). Interestingly all nonsense variants (in *ASXL1, IKZF1, DNMT3A, EP300*) and most insertions (in *ASXL1, WT1),* were found only in non-responders. Similarly, nonsense mutations in *ASXL1 and IKZF1* were found in 2 CML-BC patients. The frequency of the mutated allele for most mutations was <10%. Mutations were detected in the R and NR group in equal proportion (11/26 patients), however, the presence of ≥2 mutations was more common in NR**.** In addition, we examined the correlation of presence of mutations with gene expression, detecting a correlation in some cases, and their influence on overall survival (OS), finding an influence, especially in the non-responders, more prominent when there were ≥2 mutations. Moreover, we found 69 missense variants that were also present in the constitutional DNA, with frequency of the mutated allele ~50%, considered as germline mutations. These variants were present in 32 genes with 39 and 34 variants in NR and R respectively**,** while there was no difference in the number of R, NR that carried at least one mutation (both groups 20/26 patients)**.** Specific variants in *RUNX1, TET1, TET2* were found in ≥3 patients.
Summary/Conclusions: The mutation analysis of epigenetic modifiers in CML-CP identified the presence of somatic mutations, some of which (found only in NR) can be considered as predictive biomarkers for IM failure, and germline mutations that may predispose to CML.