2918 An RNA-Based Next Generation Sequencing (NGS) Strategy Detects More Cancer Gene Mutations Than a DNA-Based Approach for the Prediction and Assessment of Resistance in CML

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**Background:** Mutation of genes linked to hematologic cancer have recently been reported in CML and are associated with early progression and resistance (Reviewed in Branford, Kim Leuk 2019). The mutations comprise single nucleotide variants (SNVs) and small insertions/deletions (indels), plus gene fusions and large focal gene deletions. In 39 patients (pts) in blast crisis (BC), all had at least 1 cancer gene mutation, including fusions in 33%: partner genes MLL, RUNX1, IKZF1, MECOM and CBFB. 50% of the fusions were novel and some were present at chronic phase diagnosis. BCR-ABL1 mutations rarely occurred as the sole mutant. NGS offers critical information for resistance assessment. For many clinical purposes, targeted DNA sequencing (seq) using panels of specific disease related genes is the most cost effective screening choice. However, this strategy could miss relevant fusions and deletions.

**Aim:** To determine whether an RNA based approach is more informative than DNA for detecting a broad range of mutations.

**Method:** A hybridization capture NGS gene panel was developed to target 126 genes relevant for myeloid/lymphoid leukemia. In a pilot study, DNA and RNA derived from 5 leukemia cell lines with well characterized mutations, including fusions and deletions, were panel sequenced. An additional 6 cell lines were sequenced using RNA, plus 49 pt samples with RNA stored for up to 14.6 years: 45 at diagnosis and 4 at BC/resistance. Six of these pt samples had prior whole exome and/or whole transcriptome seq. We used total RNA that detected intronic splice region variants from pre-spliced RNA. SNVs/indels were called from DNA/RNA with FreeBayes. Manta called focal deletions from DNA. Known and novel RNA fusions and novel splice junctions were detected using the STAR aligner. Gene expression used edgeR.
**Results:** For the 5 cell lines with DNA versus (v) RNA seq, SNVs/indels were reliably called in RNA, with a strong positive correlation of mutant allele frequency: DNA v RNA, r = 0.93. Two TP53 small deletions of 26 and 46 bp were not called in RNA, but were instead detected as novel RNA splice junctions. Read counts were 5.2 fold higher for RNA than DNA at sites of clinically relevant mutants, consistent with enrichment of seq read depth proportional to expression. Overall, RNA revealed a higher number of relevant mutants than DNA: RNA = 49 v DNA = 37, Fig A-C. Notably, the functional effect of splice region disrupting mutants and large focal deletions were evident by novel RNA splicing, Fig D-F.

In the total 11 cell lines tested with RNA, all 13 reported fusions were called, including BCR-ABL1 and RUNX1, MLL, ETV6 and CBFB fusions. For 7 cell lines with variants described in the COSMIC Cell Lines Project, 23/23 cancer gene SNVs/indels were called, plus 7 cancer gene SNVs/indels not reported. These were verified by DNA seq. 15 gene deletions were evident by atypical RNA splicing and verified by DNA seq: IKZF1, CDKN2A/B, PAX5, BTG1, RB1 and NCOR1. Five other cell lines had verified CDKN2A deletions that were evident by loss of gene expression, Fig G. Two BTG1 deletions were not detected.

For the 6 pt samples re-sequenced by the RNA panel, 8/8 verified fusion transcripts were detected with a 31 fold enrichment of read counts. 11/11 cancer gene SNVs/indels were called and 3/4 gene deletions. The exception was a CDKN2A deletion not detected by novel splicing but evident as loss of expression, Fig G. Seven other cancer gene SNVs were found at low allele frequency, including a resistant BCR-ABL1 mutation at 1.7% in the oldest sample.

Of the 43 diagnosis samples without prior NGS, BCR-ABL1 transcripts were detected in all. BCR-ABL1 genomic breakpoints were called at base pair resolution in 39, 91%. Two pts had mutated ASXL1 at diagnosis and both failed imatinib by 9 months with mutant BCR-ABL1. By gene expression analysis, all but 1 of the total 45 diagnosis samples clustered together. The exception was a pt who transformed to lymphoid BC at 6 months that clustered with the lymphoid cell lines and lymphoid BC pts, Fig H.

**Conclusion:** RNA gene panel seq demonstrated enhanced sensitivity and an increased yield of clinically relevant mutations compared with DNA panel seq. A single RNA assay has the capacity to detect SNV/indels, known and novel gene fusions, focal deletions and the likely functional effect of splice disrupting mutations. RNA panel seq is a valuable tool for the comprehensive assessment of mutations that drive CML treatment failure and drug resistance.
Background and rationale: In chronic myeloid leukemia (CML) about half of patients (pts) achieving a deep and stable molecular response (MR) with tyrosine kinase inhibitors (TKIs) may discontinue TKI treatment without disease recurrence. As such, treatment-free remission (TFR) has become an ambitious goal of treatment. Given the evidence that deepness and duration of molecular response are necessary but not sufficient requisites for a successful TFR, additional biological criteria to possibly identify more and better CML patients suitable for an efficacious discontinuation are today focus of research in CML. Leukemia stem cells (LSCs) are supposed to be the reservoir of disease. We first showed in a cross-sectional study including 112 pts in TFR for a median of 31 months (mos) that residual circulating CD34+/CD38-/CD26+ CML-specific LSCs were still detectable in the majority of CML pts despite stable and deep molecular response. This evidence suggested that the level of BCR-ABL transcript only may not reflect the actual residual CML LSCs burden and that there could be a “threshold” of LSCs predicting a successful TFR.

Aims: To further study the behavior of residual LSCs during TKI discontinuation we designed a prospective multicentered study (AIRC IG 20133 study) in which we monitored circulating CD26+ LSCs in CML pts from the time of TKI discontinuation until molecular relapse.

Methods: CML pts meeting the current molecular criteria for TKI withdrawal entered this multicenter study. At TKI stop (baseline) and at +1, +2, +3, +6, +12 mos after discontinuation and at any time if molecular relapse, CML pts were evaluated for peripheral blood number of CD34+/CD38-/CD26+ LSCs by centralized flow-cytometry analysis and for BCR-ABL transcript level by standard (IS) quantitative RT-PCR assay.

Results: 49 consecutive CML pts were enrolled in the study so far. Pts characteristics at diagnosis, type of TKI, disease response and treatment duration before discontinuation are shown in Tab. 1. After a median time of 7 mos since TKI stop (range 1-24), 13/49 (26.5%) pts lost their molecular response and restarted TKI treatment. Median time to relapse after discontinuation was 4 mos (range 2-7). 36/49 (73.4%) pts are still in TFR after a median time of 7.5 mos (range 1-24). If considering a cut-off of 6 mos from discontinuation as the period with higher risk of relapse, 14/36 pts actually in TFR have discontinued treatment for ≤ 6 mos (range 1-6) while 22/36 pts are in TFR for a median of 10 mos (range 7-24). Regarding residual CML LSCs evaluation, at baseline 23/49 (46%) pts had still measurable circulating CD26+LSCs with a median number of 0.0204µ/L (range 0.0077-0.1197), while 26/49 (54%) had no detectable CD26+ LCSs. Considering the small number of molecular relapses no statistical difference in number of
residual CD26+ LSCs at time of discontinuation was shown between pts losing vs maintaining TFR (13 pts median CD26+ LSC 0.0237/µ/L, range 0-0.1197 and 36 pts median CD26+ LSCs 0.0204/µ/L, range 0-0.1039, respectively). However, the number of pts with undetectable CD26+ LSCs at baseline was 6/13 (45%) and 20/36 (55%) in the two subgroups, respectively. Considering subsequent time points, the 13 relapsed pts showed a small yet progressive increase of residual CD26+ LSCs number until molecular relapse, while the 36 pts in TFR showed a fluctuation of CD26+ cells number. However, Kendall rank correlation coefficient, Mood test and bi-linear relation model of the whole cohort showed no correlation between BCR-ABL/ABLIS ratio and number of residual CD26+ LSCs either at baseline or at each time points after discontinuation, thus confirming our previous observations.

Conclusions: Yet very preliminary our results showed that CD26+ LSCs are detectable at time of TKI discontinuation and during TFR. Moreover, at least for the observation median time of the study (7.5 mos) the persistence of “fluctuating” values of residual CD26+ LSCs do not hamper the possibility to maintain a stable TFR. Due to the short follow up and the small number of molecular relapsed pts we could not find a threshold of CD26+ LSCs predictive of TFR loss. Our data may suggest other factors then LSCs “burden” to play an active role in controlling disease recurrence. Additional studies evaluating CD26+ LSCs ability to modulate the immune system through a variable expression of immune response inhibitory molecules and through their interactions with effectors cells are ongoing.
Marianna Romzova, PhD

BACKGROUND: Bcr-abl1 oncogene targeted treatment with tyrosine kinase inhibitors (TKI) showed an impressive efficacy against proliferating chronic myeloid leukemia (CML) cells. However, rapid relapses in more than half of CML patients after discontinuation of the treatment suggest a presence of quiescent leukemic stem cells inherently resistant to BCR-ABL1 inhibition. Understanding the heterogeneity of CML stem cell compartment is crucial for preventing the treatment failure. Specificity of already established leukemic stem cell (LSC) markers has been tested mainly in bulk CD34+CD38- populations at diagnosis. Phenotypes and molecular signatures of therapy resistant BCR ABL1 positive stem cells is however yet to be established.

AIMS: Identification of BCR-ABL1 dependent LSC markers at single cell level by direct comparison their surface and transcript expression with the levels and the presence of BCR-ABL1 transcript at diagnosis and after administration of TKI treatment.

METHODS: Total number of 375 cells were obtained from bone marrow and peripheral blood of 4 chronic phase CML patients. Cells were collected prior any treatment and three months after TKI treatment initiation. Normal bone marrow cells and BCR-ABL1 positive K562 cell line were used as controls. Indexed immuno-phenotyping and sorting of CD34+CD38- single cells was performed using a panel of 11 specific surface markers. Collected single cells were lysed and cDNA was enriched for 11 targets using 22 cycle pre-amplification. Expression profiling was carried on SmartChip real-time PCR system (Takara Bio) detecting following genes: BCR-ABL1, CD26, CD25, IL1-Rap, CD56, CD90, CD93, CD69, KI67, and control genes GUS and HPRT. Unsupervised clustering was performed using principal component analysis (PCA). Correlations were measured by Spearman rank method.

RESULTS: At diagnosis, majority of BCR-ABL1+ C34+CD38- stem cells co-express IL1-Rap, CD26, and CD69 on their surface (88%, 82%, 78% overlap). Only 56% of BCR-ABL1+ cells positive for aforementioned markers co-express CD25, 28% CD93 and 16% CD56. The expression of these markers could also be detected in 4-11% of BCR-ABL1- cell, although this could be technical inaccuracy caused by the single cell profiling. CD90 marker did not show any correlation with BCR-ABL1 expression. At transcript level the expression of IL-1Rap, CD26, CD25 and CD56 was observed in 62%, 52% 45% and 16% BCR-ABL1+ cells, and up to 7% of BCR-ABL1- cells. CD69 expression was observed in 90% of BCR-ABL1+ cells at transcript level, but also in 71% BCR-ABL- cells. BCR-ABL1 independent expression was observed for cKIT. (60% vs. 76 % in positive vs negative). Finally proliferation marker KI67 was expressed only in 6% of the BCR-ABL1+ cells. PCA analysis
divided cells into several distinct clusters with BCR-ABL1 as the main contributor, and cKIT, CD69 and CD26, IL-1RAP as other significant factors. Interestingly BCR-ABL1+ cells collected during TKI treatment showed persistent surface expression of IL-1Rap and CD26, while CD56, CD69 and CD93 were only on part of the BCR-ABL1+ cells. CD25 was significantly deregulated during TKI treatment.

**CONCLUSION:** At diagnosis up to 80% of LSC co-express 3 specific surface markers – IL-1RAP, CD26 and CD69. Variable portion of LSC co-express additional markers such are CD25, CD56 and CD93. During TKI treatment the surface expression of majority of markers is decreased, where the best correlated LSC marker is IL-1Rap, followed by CD26 and CD69. CD56 marker seems to persist in the same proportion of cells while CD25 disappears. cKIT is highly expressed in normal BM and HSC from CML patients, but also in some LSC. CD34+CD38- cells show non-proliferating phenotype.

2921 The Role of the Lipid Raft-Associated Protein Flotillin-2 during Development and Progression of Myeloid Leukaemia

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**Introduction:** The role of the bone marrow (BM) microenvironment (BMM) for the regulation of leukaemic stem cells (LSC) and their respective interplay is slowly being elucidated, but knowledge about how membrane structures or adhesion molecules on leukaemia cells may, specifically, interact with the BMM leading to regulation of leukemia cell maintenance and proliferation is limited. Flotillin (flot)-1 and -2 are highly conserved, ubiquitously expressed proteins localised in lipid microdomains in cellular membranes. These dynamic microdomains can serve as platforms for signal transduction, endocytosis and interactions with the actin cytoskeleton, as well as cell adhesion.

**Hypothesis:** As flotillins in the leukaemia cell membrane are associated with adhesion molecules known to be involved in the engraftment of leukaemia-initiating cells (LIC), we hypothesized that flotillins may play a role in LIC engraftment in the BMM.

**Results:** Using the murine retroviral transduction/transplantation model of BCR-ABL1-driven chronic myeloid leukaemia (CML) we observed a significant prolongation of survival of recipients of BCR-ABL1+, flot-2-deficient bone marrow compared to the respective control. The homing of BCR-ABL1+LIC to wild-type recipient bone marrow was reduced. Consistent with the known association of flotillins in the cell membrane with P-selectin glycoprotein ligand-1, a selectin ligand similar to CD44, both of which are known to be involved in the engraftment of CML LIC (Krause DS et al., Nat Med, 2006, and Krause DS et al., Blood, 2014), we demonstrated that flot-2 physically interacts and co-localizes with CD44. Flot-2-deficiency led to a significant reduction of the expression of CD44 and an
impairment of the cytoskeleton in BCR-ABL1+ cells, as well as impaired migration. Further, Cdc42, a member of the Rho-GTPase family, was differentially distributed in wildtype versus flotillin-2-deficient BCR-ABL1+ LSC and may have compromised activity. Consistently, intrafemoral transplantation of flot-2-deficient CML-initiating cells or coexpression of CD44 in flot-2-deficient bone marrow 'rescued' or restored the CML-like disease.

In contrast, in an MLL-AF9-driven model of acute myeloid leukemia (AML) a similar prolongation of survival or reduced homing of AML-initiating cells were not observed. However, expression of CD44, known to play a role for engraftment in this disease (Jin L et al., Nat Med, 2006), in AML-initiating cells was similarly reduced and migration, adhesion and the cytoskeleton was similarly compromised. We demonstrated that this might be due to compensatory upregulation of C-X-C chemokine receptor type 4 (CXCR-4), the receptor for stromal-derived factor (SDF)-1a.

Conclusions: In summary, these data suggest that lipid raft molecules, particularly flotillins, play a previously unknown and possibly leukaemia-specific role in CML progression via modification of the levels and function of CD44 and possible regulation of Cdc42.

2922 Multicolor Immunophenotyping of Candidate Leukemic Stem Cell Markers in CD34+CD38- Chronic Myeloid Leukemia Stem Cells

Martin Culen, PhD

Introduction: Detection of leukemic stem cells (LSCs) may represent a new potential prognostic parameter in chronic myeloid leukemia (CML) and a tool for minimal residual disease monitoring in combination with standard qPCR method. To date CD26 is studied as a most specific marker for CML LSC detection. Several other candidate LSC markers have been reported, such as IL1-RAP, CD25 and CD93, however a side-by-side testing of their specificity is lacking. Recently, we have identified CD69 molecule to be overexpressed in CD34+CD38-CD26+ cells, which makes this antigen another candidate marker for LSC.

Aim: To compare the surface expression of LSC markers CD69, CD26, CD25, CD56, IL1-RAP, CD56, CD93 in bulk CD34+CD38- population at diagnosis using a multicolor phenotypization assay.

Methods: In total, 44 patients were analyzed at diagnosis of chronic phase CML before administration of any treatment. Fresh (n=38) or cryopreserved (n=6) leukocytes obtained by erythrolysis were stained with CD45, CD34, CD38, CD25, CD26, CD56, CD69, CD93, IL1-RAP antibodies and 7-AAD for selection of live cells. Analysis was performed on FACSaria Fusion (BD Biosciences). Acquisition of live mononuclear cells ranged from 2×104 to 2.5×106.
**Results:** Expression of candidate LSC markers CD26, CD25, CD56, IL1-RAP, CD56, CD93 was analyzed in BM of 35 patients who carried at least 30 CD34+CD38- cells. Median percentage of marker positive cells was 34% for IL1-RAP, 31% for CD25, 23% for CD26, 16% for CD56 and 2% for CD93, from the parent CD34+CD38-population. Next we analyzed the overlap and combination for the three best markers – IL1-RAP, CD25 and CD26. The 3-combination (defined as IL1-RAP or CD25 or CD26 expression) identified 40% of CD34+CD38- positive cells, which was more than any of the markers alone. Expression of the three markers showed good overlap and ruled out mutually exclusive expression of the markers. This was demonstrated by median difference of 0.4% of CD34+CD38- cells (range: 0-18%) and a correlation coefficient $r^2=0.9914$, when comparing the 3-combination and the best performing marker in each patient. In contrast, in 12/35 (34%) of patients, one of the three markers failed to identify at least half of the cells positive for another marker.

In 21/35 patients, we also analyzed the expression of CD69 in the CD34+CD38-compartment. The CD69 showed similar performance as the 3-combination of CD26, CD25, and IL1-RAP, 59% vs 54% positive cells, respectively. We observed excellent overlap between CD69 and 3-combination expression in individual patients with median difference of 0% of CD34+CD38- cells (range 0-15%) and correlation coefficient $r^2=0.9890$.

Furthermore, we compared marker positivity in BM vs PB in 19 paired samples. Both, sample types showed similar frequency of CD34+CD38- cells ($5\times10^{-3}$ in BM, and $2\times10^{-3}$ for PB), but PB carried higher percentage of LSCs identified by the 3-combination – median 76 vs 52% cells.

**Conclusions:** We show an overlap in surface expression of three previously reported CML LSC markers – IL1-RAP, CD25 and CD26. Nevertheless, a combination of these markers can detect more positive cells than any of the markers alone. Moreover, we demonstrate that CD69 identifies the same cells within the CD34+CD38- compartment as the combination of three above mentioned markers, which makes CD69 the best candidate for routine CML LSC quantification.
2923 NF-κB-Dependent Activation of the Proteasome Components, PSMD1 and PSMD3, As a Mechanism of Resistance to Imatinib

Idaly Maria Olivas, BS

Tyrosine kinase inhibitors (TKIs) targeting BCR-ABL1 are remarkably effective therapies in chronic myeloid leukemia (CML). Despite clinical success, TKIs do not target the CML leukemic stem cell (LSC), and the majority of patients must be treated for life to maintain remission. Our previous work has shown that BCR-ABL1-independent resistance is driven by STAT3 in CML stem/progenitor cells (Eiring et al. Leukemia 2015). Unexpectedly, RNA-sequencing on TKI-resistant K562 cells (K562-R) versus parental controls (K562-S) revealed that resistance is not associated with STAT3-mediated transcription, but is rather reminiscent of TNFa signaling via NF-κB (p=0.024). Nucleocytoplasmic fractionation confirmed these findings, demonstrating higher levels of phospho-NF-κB in the nucleus of CD34+ cells from TKI-resistant patients (n=3) compared to newly diagnosed CML patients (n=2) or normal individuals (n=2). Surprisingly, ELISA results revealed that K562-R cells do not produce autonomous TNFa, but they do produce IL-6 (p<0.01). These data suggest that NF-κB may be driving the gene expression signature of BCR-ABL1-independent resistance, and implicate non-canonical functions for STAT3.

To better understand the mechanism by which NF-κB drives resistance, we correlated our RNA sequencing data with gene expression profiles of CML patients not responding to imatinib (McWeeney et al. Blood 2010), identifying 36 genes commonly dysregulated in both TKI-resistant cell lines and patient samples. Of the 30 upregulated genes, 21 had p65-NF-κB bound to their promoter regions via ChIP in hematopoietic cells (UCSC Genome Brower). Two of these genes are members of the ubiquitin proteasome system, including PSMD1 and PSMD3, both of which were implicated as hits in a previously published shRNA library screen for BCR-ABL1-independent resistance (Khorashad et al. Blood 2015). PSMD1 and PSMD3 are non-ATPase subunits of the 19S regulatory complex in the 26S proteasome, likely involved in proteasome substrate recognition and binding. In breast cancer, PSMD1 was shown to regulate cell growth by inducing p53 degradation (Okumura et al. 2018), whereas PSMD3 was shown to protect HER2 from degradation (Fararjeh et al. 2019). qRT-PCR confirmed upregulation of PSMD1 and PSMD3 by 3-fold and 6-fold, respectively, in K562-R cells versus parental controls in the presence of imatinib. Interestingly, according to data from The Cancer Genome Atlas (TCGA), higher levels of PSMD1 and PSMD3 mRNA correlates with a worse prognosis in acute myeloid leukemia (PSMD1, p=0.0138; PSMD3, p=0.0229). We hypothesized that PSMD1 and PSMD3 upregulation contributes to NF-κB activation and TKI resistance.
We used doxycycline-inducible shRNAs to assess the function of PSMD1 and PSMD3 in CML cell survival and TKI response. Induction of knockdown (100 ng/mL doxycycline, 72h) resulted in a reduction of PSMD1 and PSMD3 mRNA and protein by ~73% and ~77%, respectively, in K562-R cells. Importantly, immunoblot analysis revealed that knockdown of either PSMD1 or PSMD3 in TKI-resistant K562-R cells resulted in a significant reduction of phospho-NF-κB (p65), suggesting that upregulation of these proteins promotes NF-κB activation. Reduced phospho-NF-κB (p65) correlated with phenotypic effects, including reduced colony formation, increased response to TKIs as assessed in MTS assays, and increased apoptosis in both the presence and absence of imatinib. Our results suggest that NF-κB activation in TKI resistance depends on the proteasome components, PSMD1 and PSMD3, forming a positive feedback loop potentiating NF-κB signaling. Our data also suggest that specific targeting of the ubiquitin proteasome system through either PSMD1 or PSMD3 may be a novel strategy to restore TKI sensitivity in patients with BCR-ABL1-independent TKI resistance. Future studies will address the non-canonical functions of STAT3 in TKI resistance.