



Published in final edited form as:

*Leukemia*. 2018 September ; 32(9): 2049–2054. doi:10.1038/s41375-018-0050-z.

## Ongoing Clonal Evolution in Chronic Myelomonocytic Leukemia on Hypomethylating Agents: A Computational Perspective

Hein Than<sup>1,2</sup>, Yi Qiao<sup>3</sup>, Xiaomeng Huang<sup>3</sup>, Dongqing Yan<sup>1</sup>, Jamshid S. Khorashad<sup>1,4</sup>, Anthony D. Pomicter<sup>1</sup>, Tibor J. Kovacs<sup>4</sup>, Gabor T. Marth<sup>3</sup>, Thomas O'Hare<sup>1,5</sup>, and Michael W. Deininger<sup>1,5</sup>

<sup>1</sup>Huntsman Cancer Institute, The University of Utah, Salt Lake City, UT, USA

<sup>2</sup>Department of Hematology, Singapore General Hospital, Singapore

<sup>3</sup>Eccles Institute of Human Genetics, The University of Utah, Salt Lake City, UT, USA

<sup>4</sup>Centre for Hematology, Department of Medicine, Imperial College, London, UK

<sup>5</sup>Division of Hematology and Hematologic Malignancies, The University of Utah, Salt Lake City, UT, USA

### To the Editor

Chronic myelomonocytic leukemia (CMML) is a clinically heterogeneous myeloid neoplasm that combines myelodysplastic and myeloproliferative features, and carries a poor prognosis due to progression to acute myeloid leukemia or complications of cytopenias. *TET2*, *SRSF2* and *ASXL1* are the most commonly mutated genes in CMML, but somatic variants in additional genes have been identified<sup>1–3</sup>. Allogeneic stem cell transplantation is potentially curative, but most patients are ineligible due to advanced age and/or comorbidities. Hypomethylating agents (HMAs) such as 5-azacitidine (5-Aza) induce responses in ~40% of CMML patients, but their impact on survival remains debatable<sup>4</sup>. While *TET2* mutations have been reported to predict HMA response in myelodysplastic syndromes, data from patients treated with decitabine suggest that epigenetic profiles rather than somatic mutations govern response to HMAs in CMML<sup>5, 6</sup>. Previous work described unchanged mutant allele burden in CMML in patients responding to HMAs<sup>1</sup>, but detailed analyses of clonal evolution in relation to HMA response have not been reported. We applied SubcloneSeeker computational analysis algorithm to whole exome sequencing (WES) and single nucleotide polymorphism (SNP) array data to uncover clonal architecture and evolution in CMML patients treated with 5-Aza on a prospective trial<sup>7</sup>. Compared to

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: [http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

**Correspondence:** Michael W. Deininger, MD, PhD, The University of Utah, Huntsman Cancer Institute, 2000 Circle of Hope, Room 4280, Salt Lake City, Utah 84112-5550, 801/581-6363 phone, 801/585-0900 fax, michael.deininger@hci.utah.edu.

#### Authorship

Contribution: H.T., Y.Q. and M.W.D. conceived the project and analyzed data. H.T., T.O. and M.W.D. wrote the manuscript. Y.Q., X.H., and G.T.M. designed the software and analyzed data. H.T., D.Y. and J.S.K. prepared the samples and performed experiments. M.W.D. and T.J.K. provided clinical information and research materials. A.D.P. and T.O. critically reviewed the manuscript.

targeted sequencing, this approach allows reconstruction of clonal architecture using all somatic mutation calls, including driver and passenger mutations, in an unbiased fashion.

We studied a total of 46 samples obtained from twelve patients treated with single-agent 5-Aza for up to two years. All patients provided informed consent for the study. Ten patients were treated on a prospective clinical study (NCT01350947) and two in analogy to the study protocol. Median age at presentation was 70 years. Ten patients had CMML-1 and two had CMML-2. Three patients (25%) achieved complete remission (CR) and four (33%) partial remission (PR), one had stable disease (SD), and one had no response (International Working Group 2006 response criteria). Three patients (25%) progressed after a period of SD (Supplementary Table 1).

Mononuclear cells (MNCs) or monocytes were isolated from bone marrow or blood samples prior to treatment, at 3- and 6-month intervals on therapy, and at the end of study or disease progression. Cultured mesenchymal stromal cells (n=4), skin fibroblasts (n=6) or fluorescence-activated cell-sorted CD3<sup>+</sup> lymphocytes (n=2) were used as sources of constitutional DNA in CMML patients. WES was performed on paired tumor and control samples, with median of 3 longitudinal tumor samples per patient (n=46). Copy number variation (CNV) and loss of heterozygosity (LOH) were analyzed by whole-genome SNP arrays (Infinium Omni2.5-8 v1.3, Illumina). Variant allele frequencies (VAFs) were corrected for proportion of lymphocytes in MNC samples.

A median of 39 (range: 10 – 95) somatic mutations per patient exome was detected, with average read depth (DP) of >200X per sample. The most common variants were non-synonymous missense single-nucleotide variants (SNVs) (90%), followed by frameshift insertions/deletions and stop-gain mutations (Supplementary Figures 1a-c). The variants were predominantly transitions (65%) with transition-transversion ratio of 1.86, similar to other myeloid and lymphoid malignancies<sup>8, 9</sup>. Mutations in *TET2* and *SRSF2* were each found in 62% of the patients. Additional mutations detected in genes associated with CMML included *ASXL1* (38%), *RUNX1* (38%), *CBL* (31%), *KRAS* (23%), *DNMT3A* (15%) and *NRAS* (15%) (Figure 1a). *TET2*, *SRSF2* and *ASXL1* mutations persisted across pre- and post-treatment samples (Figure 1a). Two patients showed copy neutral (CN)-LOH. CNV and mutations with corresponding VAFs in longitudinal samples are summarized in Supplementary Table 2. Our data revealed higher median somatic mutations per exome than a previous report<sup>1</sup>, probably related to higher average read depth. However, somatic mutation burden was remarkably stable despite response to 5-Aza. Of 477 total somatic mutations identified prior to 5-Aza, 98% were still detectable in the last follow-up samples and only 13 new variants were acquired on therapy.

To map clonal architecture, we used the SubcloneSeeker computational algorithm to construct a set of clonal trees by clustering all somatic variants with similar VAFs and calculating their cellular prevalence values<sup>10</sup>. Compatible trees from multiple longitudinal samples at different time points were merged to establish a unified model of clonal evolution in each patient (Supplementary Methods). Clonal evolution patterns were studied in patients without LOH, based on changes in relative proportions of parental clones, pre- and post-treatment (Figure 1a, Supplementary Table 4). In patients P01 to P03, baseline clonal

architectures remained relatively stable with proportional changes between parental clones and progeny subclones on 5-Aza (Figure 1b, Supplementary Figure 2a). In patients P04 to P06, we saw increasing shifts from parental clones to progeny subclones on therapy (Supplementary Figures 2b-d). In patients P07 to P10, clonal architecture was characterized by expansion of maximally mutated progeny subclones on 5-Aza (Figure 1c, Supplementary Figures 2e-f).

We next delineated clonal evolution in patients with CN-LOH. Patient P11 harbored subclones bearing *SRSF2*<sup>P95H</sup>, *RUNX1*<sup>L144Q</sup> and two *CBL* variants (*CBL*<sup>C384Y</sup>, *CBL*<sup>C416Y</sup>) at presentation (Figure 2a). While in SD on 5-Aza treatment, the patient acquired chromosome 11q CN-LOH, with uniparental disomy of *CBL*<sup>C384Y</sup>. These subclones expanded after acquisition of additional *RUNX1* mutations and became dominant at disease progression. A similar pattern of clonal evolution was observed in patient P12 with CR. At 3 months on 5-Aza, CN-LOH of chromosome 12 led to elimination of *KRAS*<sup>A146V</sup>-containing subclones, with reversion to native *KRAS*; and focal CN-LOH in chromosome 17 led to reduction of *SRSF2*<sup>P95H</sup>-containing subclones. At 6 months, clonal architecture was largely simplified to subclones containing *TET2*<sup>V239fs</sup> and *SH2B3*<sup>V402M</sup> (Figure 2b).

Our data illustrate early clonal dominance and clonal heterogeneity with co-existence of parental and progeny populations at baseline. *TET2*, *SRSF2* and *ASXL1* mutations were detected as co-founding events in individual subclones across different CMML patient samples, and their VAFs were not altered despite clinical response, as previously described<sup>11</sup>. Distinct evolution patterns were observed, ranging from relative preservation of baseline clonal architecture to expansion and dominance of progeny subclones through successive acquisition of mutations or via LOH. As an example, in patient P11 with CN-LOH, disruption of the ring finger domain critical for E3 ligase activity via acquired biallelic *CBL*<sup>C384Y</sup> mutation correlated with myelomonocytic expansion<sup>12, 13</sup>. Subsequent acquisition of inactivating *RUNX1* mutations further enabled dominance of these *CBL*<sup>C384Y</sup>-bearing subclones. Overall, global suppression of myelomonocytic cells was achieved after 4 cycles of 5-Aza, with re-expansion of lymphocytes to a median of 24% in patients with CR or PR (n=6) (Supplementary Figure 3). However, clonal evolution patterns did not correlate with response to HMAs. Clonal evolution with expansion of maximally mutated progeny subclones occurred in 5 out of 8 patients with favorable clinical response, while progeny subclones evolved and expanded with successive acquisition of secondary mutations or LOH events in 2 out of 3 patients with disease progression. This suggests that subclones within the CMML compartment continue to evolve irrespective of clinical response, and that response is governed by complex genetic signals and epigenetic mechanisms. Consistent with a previous report<sup>11</sup>, our study highlights that current understanding of CMML biology is predominantly mechanistic, and accurate correlation of specific pathogenic clones with clinical response has yet to be determined.

Next generation sequencing (NGS) panels are increasingly used for molecular monitoring in CMML, but VAFs of somatic mutations alone do not adequately reflect clonal heterogeneity. Single cell sequencing provides the maximum resolution for delineation of clonal architecture, but is limited by costs and potential allelic bias<sup>14</sup>. Analysis of single cell colonies may be influenced by specific cytokines used in the cultures. While current clinical

practice is to monitor hematologic parameters and VAFs of mutations in CMML patients, we demonstrate that subclonal hierarchies and evolution can be delineated at high resolution from standard bulk NGS data using the SubcloneSeeker computational algorithm. Inclusion of all somatic mutations rather than only driver mutations is required to solve clonal architecture with high confidence. This strategy provides deeper insights into the hierarchy of acquisition and distribution of founding and secondary mutations within individual subclones in CMML, without resorting to single cell analysis. The predictive accuracy of the computational algorithm has recently been validated in a similar study for drug-resistant breast cancer subclones, using single-cell genotyping experiments<sup>15</sup>. Until single-cell sequencing technologies become widely available for routine testing, computational reconstruction of clonal evolution represents the most dynamic platform available for delineation of specific mutations and subclones in leukemia, and may become a useful tool for response monitoring and potentially therapeutic decision making.

Ongoing clonal evolution despite apparent clinical remission highlights gaps in the current mechanistic understanding of HMA therapy in CMML. Integration of epigenetic evolution and the influence of tumor microenvironment into clonality studies may establish strategies to unravel biological complexity and identify novel therapeutic targets in CMML.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by a Leukemia & Lymphoma Society (LLS) Translational Research Program Award (6086-12) (M.W.D.) and an LLS Specialized Center of Research Program Award (GCNCR0314A-UTAH) (M.W.D.). This work was also supported by the National Institutes of Health (NIH) National Cancer Institute grant R01CA178397 (M.W.D. and T.O.), and by the V Foundation for Cancer Research (M.W.D. and T.O.) and by U01HG006513 (G.T.M.). J.S.K. was a special fellow of the LLS and was supported by a Translational Research Training in Hematology Award from the American Society of Hematology (ASH). H.T. was a visiting scholar from Singapore, and was supported by Research Training Fellowship from National Medical Research Council of Singapore. We thank Brian K. Dalley, director of High-Throughput Genomics Core Facility, and James Marvin, director of Flow Cytometry Core Facility, at the University of Utah for their assistance with the experiments. The University of Utah Flow Cytometry Facility was supported by the National Cancer Institute through award 5P30CA042014-24 and the National Center for Research Resources of the National Institutes of Health under award 1S10RR026802-01. The University of Utah High-Throughput Genomics Core Facility was supported by Award Number P30CA042014 from the National Cancer Institute.

### Disclosure of Conflicts of Interest

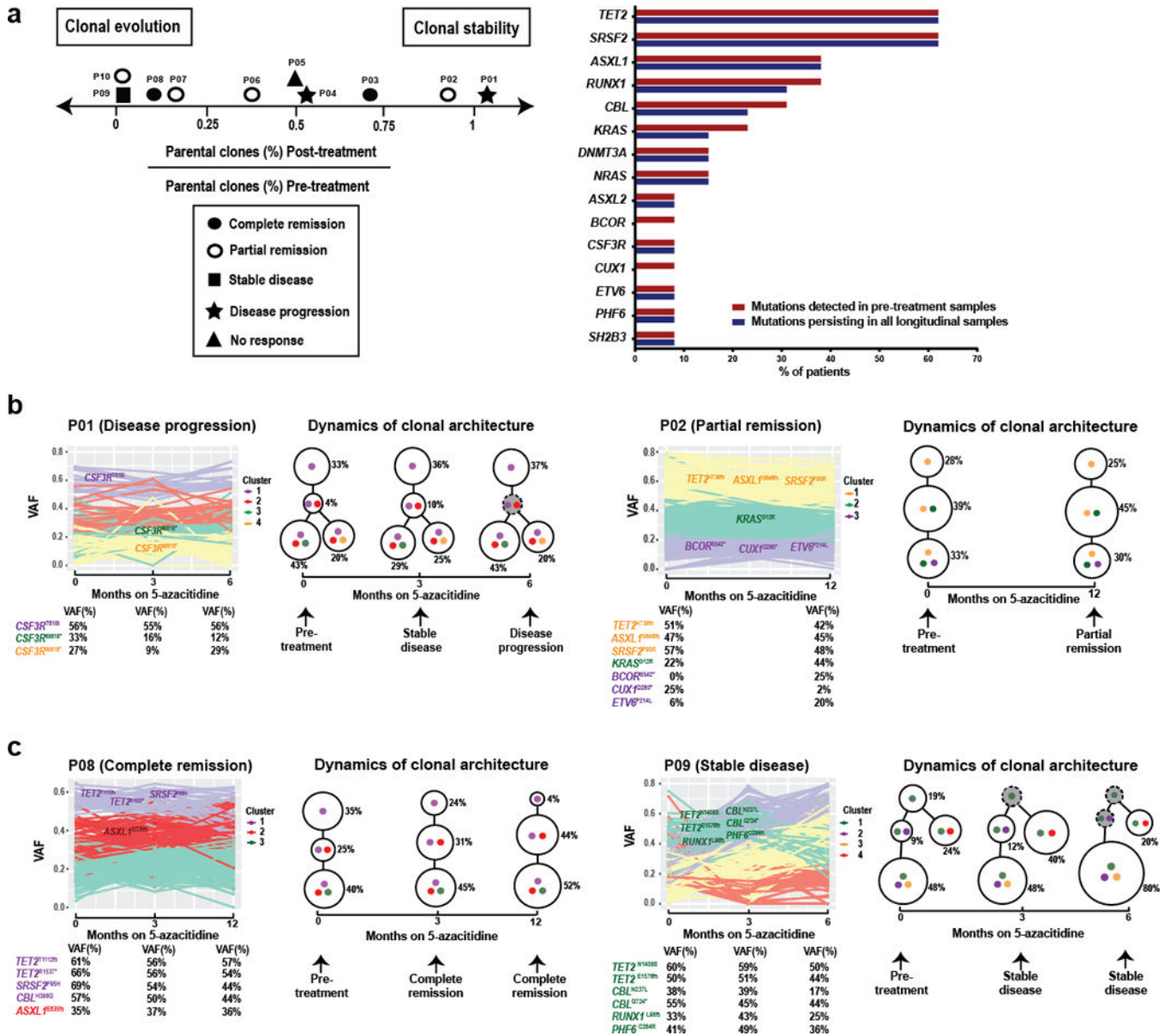
M.W.D. is on the advisory board and is a consultant for Incyte, Novartis and Pfizer, and serves on the advisory board for Ariad, Blueprint and Galena BioPharma. His laboratory receives research funding from Novartis and Pfizer.

## References

1. Merlevede J, Droin N, Qin T, Meldi K, Yoshida K, Morabito M, et al. Mutation allele burden remains unchanged in chronic myelomonocytic leukaemia responding to hypomethylating agents. *Nat Commun*. 2016 Feb 24;7:10767. [PubMed: 26908133]
2. Mason CC, Khorashad JS, Tantravahi SK, Kelley TW, Zabriskie MS, Yan D, et al. Age-related mutations and chronic myelomonocytic leukemia. *Leukemia*. 2016 Apr; 30(4):906–913. [PubMed: 26648538]

3. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature*. 2011 Sep 11; 478(7367):64–69. [PubMed: 21909114]
4. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *The Lancet Oncology*. 2009 Mar; 10(3):223–232. [PubMed: 19230772]
5. Meldi K, Qin T, Buchi F, Droin N, Sotzen J, Micol JB, et al. Specific molecular signatures predict decitabine response in chronic myelomonocytic leukemia. *J Clin Invest*. 2015 May; 125(5):1857–1872. [PubMed: 25822018]
6. Bejar R, Lord A, Stevenson K, Bar-Natan M, Perez-Ladaga A, Zaneveld J, et al. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood*. 2014 Oct 23; 124(17):2705–2712. [PubMed: 25224413]
7. Tantravahi SK, Szankasi P, Khorashad JS, Dao K-H, Kovacsovics T, Kelley TW, et al. A phase II study of the efficacy, safety, and determinants of response to 5-azacitidine (Vidaza®) in patients with chronic myelomonocytic leukemia. *Leukemia & Lymphoma*. 2016 Oct 02; 57(10):2441–2444. 2016. [PubMed: 26752680]
8. Kandath C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. *Nature*. 2013 Oct 17; 502(7471):333–339. [PubMed: 24132290]
9. Schuetz JM, Johnson NA, Morin RD, Scott DW, Tan K, Ben-Nierah S, et al. BCL2 mutations in diffuse large B-cell lymphoma. *Leukemia*. 2012 Jun; 26(6):1383–1390. [PubMed: 22189900]
10. Qiao Y, Quinlan AR, Jazaeri AA, Verhaak RG, Wheeler DA, Marth GT. SubcloneSeeker: a computational framework for reconstructing tumor clone structure for cancer variant interpretation and prioritization. *Genome biology*. 2014 Aug 26. 15(8):443. [PubMed: 25160522]
11. Patel BJ, Przychodzen B, Thota S, Radivoyevitch T, Visconte V, Kuzmanovic T, et al. Genomic determinants of chronic myelomonocytic leukemia. *Leukemia*. 2017 Dec; 31(12):2815–2823. [PubMed: 28555081]
12. Dunbar AJ, Gondek LP, O’Keefe CL, Makishima H, Rataul MS, Szpurka H, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res*. 2008 Dec 15; 68(24):10349–10357. [PubMed: 19074904]
13. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, et al. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood*. 2009 Jun 11; 113(24):6182–6192. [PubMed: 19387008]
14. Grun D, van Oudenaarden A. Design and Analysis of Single-Cell Sequencing Experiments. *Cell*. 2015 Nov 05; 163(4):799–810. [PubMed: 26544934]
15. Brady SW, McQuerry JA, Qiao Y, Piccolo SR, Shrestha G, Jenkins DF, et al. Combating subclonal evolution of resistant cancer phenotypes. *Nat Commun*. 2017 Nov 01. 8(1):1231. [PubMed: 29093439]





**Figure 1.** Clonal evolution via expansion of progeny subclones in CMML on 5-Aza (n=10). (a) Clonal evolution and persistence of mutations. Clonal evolution patterns in CMML patients on 5-Aza (left panel). Ratios of parental clones in the post- and pre-treatment samples in each patient were calculated. Ratios close to 1 reflect clonal stability, while ratios <1 indicate clonal evolution with expansion of progeny subclones. Frequency distribution of mutations detected pre- and post-5-Aza treatment (right panel). Red bars represent mutations detected pre-treatment and blue bars represent persistent mutations post-treatment. (b) Representative examples of clonal stability on 5-Aza. (c) Representative examples of clonal evolution with expansion of progeny subclones on 5-Aza. All somatic variants with similar VAFs are clustered (green, purple, yellow and red lines as shown). White circles represent viable clones/subclones and dashed circles in grey represent regressed clones/subclones superseded

by progeny subclones. P01 to P10 represent CMML patients; 5-Aza, 5-azacitidine; VAF, variant allele frequency.

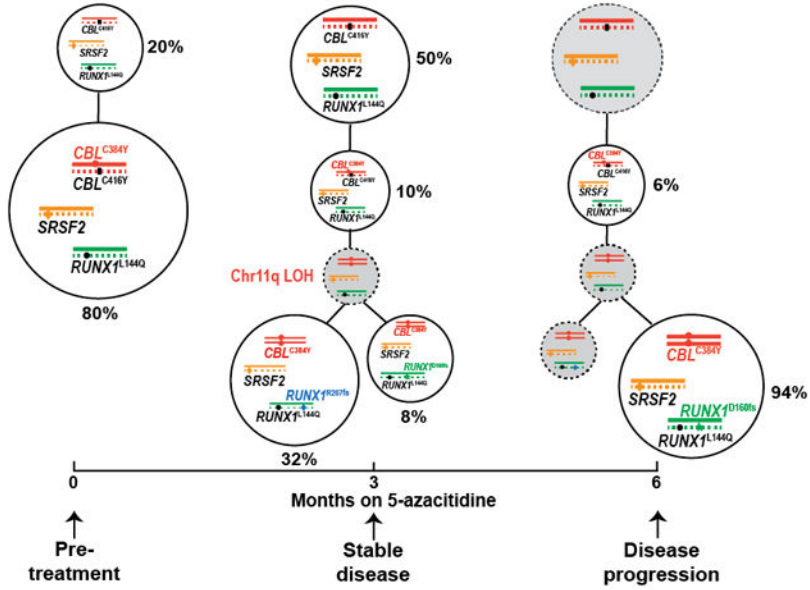
Author Manuscript

Author Manuscript

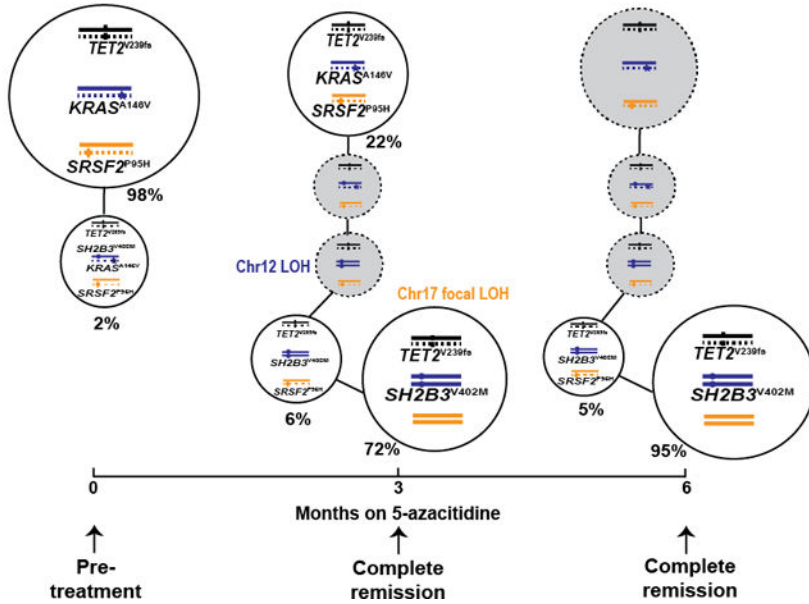
Author Manuscript

Author Manuscript

**a P11 (Disease progression)**



**b P12 (Complete remission)**



**Figure 2.** Clonal evolution via loss of heterozygosity in CMML on 5-Aza (n=2). (a) Clonal evolution with disease progression and (b) Clonal evolution with complete remission. Colored lines represent chromosomes; a solid line and a dashed line for an intact chromosome, and two solid lines for a chromosome with LOH. White circles represent viable clones/subclones and dashed circles in grey represent regressed clones/subclones superseded by progeny subclones. 5-Aza, 5-azacitidine; Chr, chromosome; LOH, loss of heterozygosity.