Mutational analysis in serial marrow samples during azacitidine treatment in patients with post-transplant relapse of acute myeloid leukemia or myelodysplastic syndromes

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Supplemental Data

Supplemental Methods

Study design

"Treatment of Post-Transplant Relapse and Persistent Disease in Patients with MDS and AML with Azacitidine" was a prospective, open-label, single center phase II study performed at Fred Hutchinson Cancer Research Center and University of Washington. The study was conducted in accordance with the Declaration of Helsinki and received Institutional Review Board approval by the Fred Hutchinson Cancer Research Center. Written informed consent was given by all patients. Primary endpoint was 6-month overall survival. Secondary endpoints included the rate of response by IWG criteria.

Patients

MDS or AML patients (as diagnosed by WHO criteria) with evidence of relapse or persistent disease at ≥ day 28 and < day 100 post-transplant as evidenced by recurrent or persistent cytogenetic abnormalities using standard karyotyping or FISH (the cytogenetic abnormalities had to be documented before stem cell transplantation), morphologic recurrence or persistence of abnormal myeloblasts in peripheral blood or marrow, flow cytometric aberrancy or extramedullary relapse (local radiotherapy was allowed). Major exclusions included refractory disease at the time of stem cell transplantation (i.e. patients who received chemotherapy prior to transplant with no evidence of response by IWG criteria); ≥ 10% bone marrow myeloblasts; evidence of CNS disease at time of relapse; serum creatinine >2 × ULN (upper limit of normal); AST/ALT >2x ULN; performance status >2 (ECOG scale); patients with severe disease other than MDS or AML that would be expected to prevent compliance with treatment; and patients with severe infections (pneumonia, sepsis, etc.) within the 2 weeks prior to the anticipated start of protocol treatment.

Treatment

Azacitidine was started within two weeks of relapse and was given at doses of 75 mg/m^2/day × 7 days every 28 (± 3) days until loss of response/disease progression. Patients with evidence of progressive relapse during therapy with azacitidine could be withdrawn from treatment and offered alternative therapy. Patients continued to receive azacitidine for a total of at least 6 cycles unless they were offered alternative therapy before that time point.

Evaluation

Complete blood counts with differential were obtained at least weekly for the first two months, and thereafter at least monthly while still receiving treatment. Marrow aspirations were performed at 3, 6, 9 and 12 months (±14 days). The clinical testing performed on these marrows included flow cytometry, morphologic analysis and cytogenetics, and research samples were obtained at the same time. Interval history and physical exam was performed on a monthly basis.

Molecular analysis

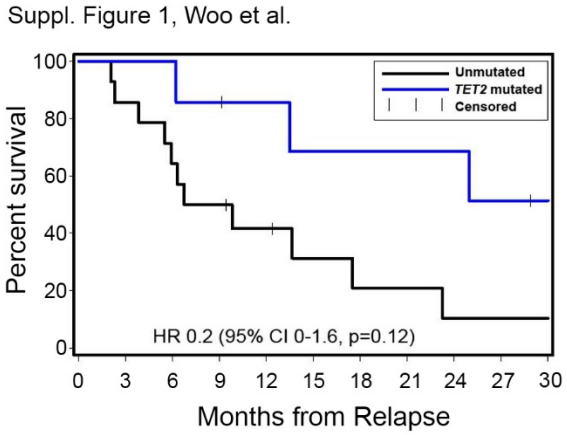
Mononuclear cells from bone marrows were isolated and frozen. Frozen cells were thawed in batches, cultured overnight in H3000 media supplemented with CC110, a combination of recombinant human cytokines, including Flt3 ligand, SCF, and TPO (StemCell, Vancouver, BC, Canada), and dead cells and debris were removed subsequently by Dead-cell removal kit. DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Subsequent assays and analysis have been developed and performed in conjunction with the Genomics & Bioinformatics Shared Resources at the Fred Hutchinson Cancer Research Center. NGS libraries were prepared from 50 ng genomic DNA using the TruSight Myeloid Sequencing Panel (Illumina, Inc. San Diego, CA, USA), following the manufacturer's instructions. Library size distributions were validated using an Agilent 2200

TapeStation (Agilent Technologies, Santa Clara, CA, USA). Additional library QC, blending of pooled indexed libraries, and cluster optimization was performed using Life Technologies' Invitrogen Qubit 2.0 Fluorometer (Life Technologies-Invitrogen, Carlsbad, CA, USA). The TruSight libraries were pooled (96-plex) and sequenced on an Illumina HiSeq 2500 in rapid-mode employing a paired-end, 150 base read length (PE150) sequencing strategy. Paired-end short reads were first aligned to the human genome reference assembly (GRCh37/hg19) using Burrows-Wheeler Aligner (BWA, v0.7.12) (1). The resulting alignment data were further processed based on the best practice of Genome Analysis Toolkit (GATK, v3.5) (Broad Institute, Cambridge, Massachusetts, USA, https://www.broadinstitute.org/gatk/). The overview of sequence alignment statistics was computed for each sample using Samtools (v 1.0, http://samtools.sourceforge.net/). The sequence coverage was computed for each sample using GATK DepthOfCoverage, and the average of depth of coverage was 10461.5X. Variants were called using the standard variant caller GATK HaplotypeCaller, and annotated using Annovar (version 2015Mar22) (2) for further evaluation. Variants that were reported in dbSNP (www.ncbi.nlm.nih.gov/SNP), but not reported in the Catalogue of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and categorized tolerated or benign variants by SIFT (http://sift.jcvi.org/) or Polyphen (http://genetics.bwh.harvard.edu/pph2/), were excluded for mutation calls. In addition, variants that are present in ExAC (http://exac.broadinstitute.org) (3) at a population frequency > 0.1% were excluded unless they were a known somatic variant hotspot. All variants were visually inspected in Integrative Genomics Viewer (IGV, http://www.broadinstitute.org/igv/). In-house perl script was used to generate the final desired output. To identify the mutations derived from recipient clones (relapsed disease), we compared the bone marrows at relapse to bone marrows obtained prior to HCT. We hypothesized that single nucleotide variants (SNVs), which are associated with high frequency of single nucleotide polymorphisms (SNPs) and which were specific for the recipient, could determine clonal frequency in recipient cells at the time of relapse. We first normalized

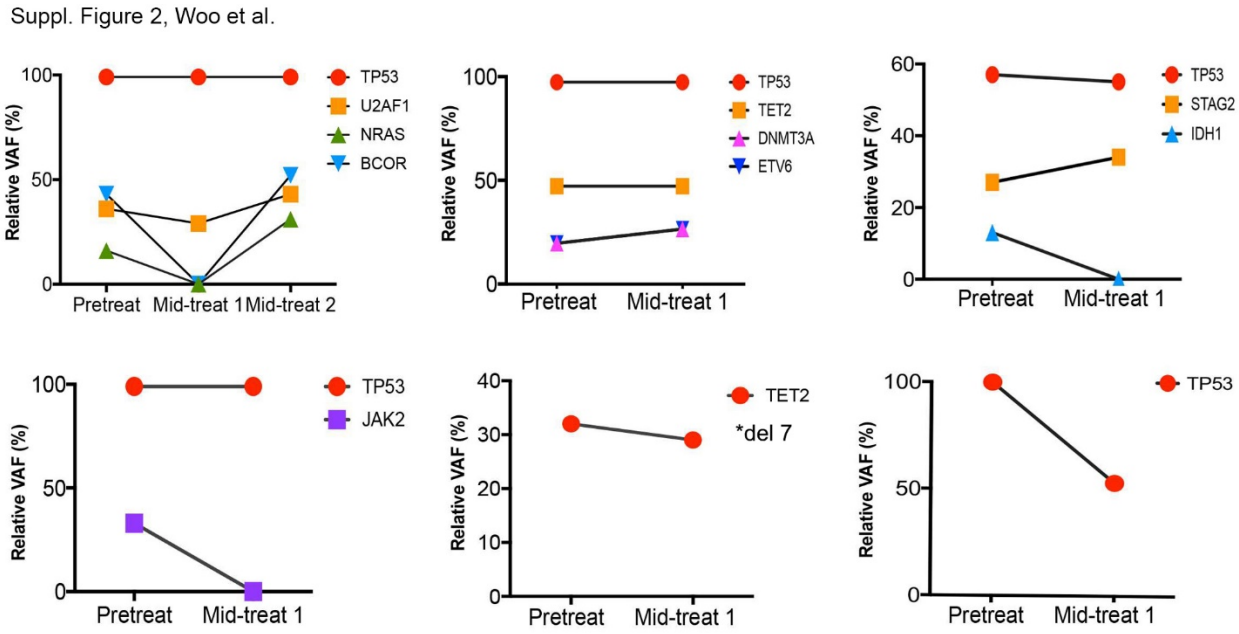
variant allele frequencies (VAFs) by fraction of at least two SNPs consistently specific to the recipient, and identified the mutations that were present in both the bone marrow samples prior to HCT and the bone marrow samples at relapse. Mutational composites in **Supplemental Figure 1** and *TP53* structural domains and associated mutations in **Supplemental Figure 2** were generated by cBioPortal (http://www.cbioportal.org/tools.jsp) (4)

References to Supplemental Methods

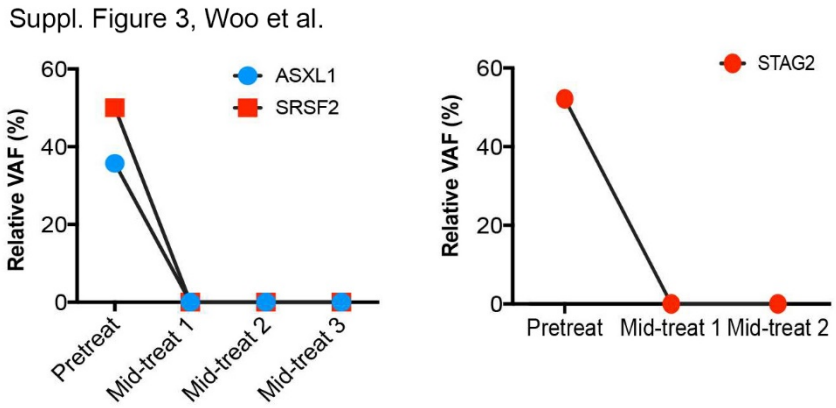
1. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-60.
2. Chang X, Wang K. wANNOVAR: annotating genetic variants for personal genomes via the web. J Med Genet. 2012 Jul;49(7):433-6.
3. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016 Aug 18;536(7616):285-91.
4. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013 Apr 2;6(269):pl1.



Supplemental Figure 1. Kaplan–Meier estimates of overall survival (OS) for patients with and without the *TET2* mutation. Survival outcomes were favorable in patients with *TET2* mutation who received azacitidine after relapse.



Supplemental Figure 2. Serial mutation profiles and clonal evolution during azacitidine treatment from paired bone marrows in 6 patients who failed to respond. * del (7q) was persistent throughout the course in the patient with *TET 2* mutation.



Supplemental Figure 3. Serial mutation profiles and clonal evolution during azacitidine treatment from paired bone marrows in 2 patients who responded (in addition to one patient shown in Figure 2D). An additional patient who responded did not have detectable mutations, but had del (11q), which ultimately disappeared after treatment