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TO THE EDITOR:

# Clonal evolution and heterogeneity in advanced systemic mastocytosis revealed by single-cell DNA sequencing

Ken-Hong Lim,[1-3] Jo-Ning Wu,[3] To-Yu Huang,[3] Jie-Yang Jhuang,[4] Yu-Cheng Chang,[1-3] Huan-Chau Lin,[1,3] Yi-Hao Chiang,[1,3] Ying-Wen Su,[1-3] Caleb Gon-Shen Chen,[1-3,5,6] Yi-Fang Chang,[1-3] and Johnson Lin[1]

[1]Division of Hematology and Oncology, Department of Internal Medicine, MacKay Memorial Hospital, Taipei, Taiwan; [2]Department of Medicine, MacKay Medical College, New Taipei City, Taiwan; [3]Good Clinical Research Center, Department of Medical Research, and [4]Department of Pathology, MacKay Memorial Hospital, Taipei, Taiwan; [5]Department of Nursing, MacKay Junior College of Medicine, Nursing, and Management, New Taipei City, Taiwan; and [6]Institute of Molecular Medicine, National Tsing-Hua University, Hsin-Chu, Taiwan



Advanced systemic mastocytosis (advSM) is a clonal stem cell neoplasm that includes aggressive SM (ASM), SM with an associated hematologic neoplasm (AHN), and mast cell leukemia.[1-3] The median survival is 41 months for ASM, 24 months for SM-AHN, and <6 to 18 months for mast cell leukemia.[4-6] *KIT* D816V mutation can be detected in >90% of SM patients.[7] Interestingly, multilineage involvement of *KIT* D816V and multimutations in other genes such as *SRSF2*, *ASXL1*, and *RUNX1* are frequently detected in advSM.[8] Some of the above-mentioned mutations were found to precede the *KIT* D816V mutation, indicating that the *KIT* mutation is a phenotypic mutation in SM.[9] The presence and number of several mutated genes such as the *SRSF2-ASXL1-RUNX1* panel are associated with worse prognosis in advSM.[10-12]

Recently, the multikinase inhibitor midostaurin was approved for the treatment of advSM on the basis of its clinical activity in 116 adults with advSM.[13] Currently, both primary and acquired resistance to midostaurin are clinical challenges in advSM. The complexity and dynamics of mutational profiles in midostaurin-treated advSM have been studied by using serial next-generation sequencing, and acquisition of additional mutations or increasing variant allele frequency (VAF) in *KRAS*/*NRAS*, *RUNX1*, *IDH2*, or *NPM1* were associated with progression.[14] However, the changes in clonal architecture under the selection pressure of midostaurin in advSM are still elusive. In contrast to traditional bulk DNA sequencing (DNA-seq), genomic analysis at single-cell resolution may provide a better opportunity to resolve clonal architecture in advSM. Here, we report the clonal evolution and heterogeneity in a case of ASM associated with chronic myelomonocytic leukemia (CMML) being treated with midostaurin and azacitidine at the single-cell level.

A 73-year-old woman presented with night sweats, weight loss of 13 kg, epigastric pain, and right chest wall pain, which she had experienced for 1 year. Her hemogram revealed leukocytosis, monocytosis, and leukoerythroblastosis. A bone marrow (BM) examination was performed, and it revealed a significant increase in abnormal mast cell infiltration and aggregation (~40%) in January 2018. She tested positive for the *KIT* D816V mutation, and plasma tryptase was >200 ng/mL. Her disease progressed to ASM with intermittent abdominal pain related to significant spleen involvement, ascites, sclerotic and osteolytic bone lesions, anemia, and thrombocytopenia in November 2018. She was treated with peginterferon alfa-2a plus methylprednisolone for 3 weeks, and she then discontinued treatment because of intolerance in January 2019. Starting in March 2019, she was given midostaurin up to 100 mg/day (maintained at 50 mg/day because of intolerance) to treat her advSM. Her symptoms improved gradually, and partial response was achieved after treatment. However, progressive monocytosis (absolute monocyte count of 31 372 cells per μL) was found in June 2019. Another BM examination revealed a slight decrease of abnormal mast cell aggregation to ~20% to 30% with increased monocytes, indicating a diagnosis of ASM associated with CMML-1. In addition to midostaurin, azacitidine at 75 mg/m$^2$ for 7 days once every 4 weeks was started in July 2019 to control her CMML-1. Complete hematologic remission was rapidly achieved after the first cycle of azacitidine,

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To request data, please contact Ken-Hong Lim at limkenhong@gmail.com.

The full-text version of this article contains a data supplement.

**Table 1. Mutations detected by bulk DNA next-generation sequencing in a patient with advSM**

| Variant | VAF, % | | |
|---|---|---|---|
| | PB1 | BM | PB2 |
| *RUNX1* R204Q | 34.82 | 46.15 | 20.94 |
| *SRSF2* P95H | 34.23 | 43.19 | 20.06 |
| *ASXL1* R693* | 35.71 | 45.01 | 20.93 |
| *KIT* D816V | 30.25 | 0.00 | 12.27 |
| *EZH2* V696E | 33.08 | 0.00 | 13.88 |
| *TET2* P851Lfs*22 | 0.00 | 44.03 | 6.55 |
| *NRAS* G12D | 0.00 | 40.87 | 0.00 |
| *RUNX1* R162S | 0.00 | 0.00 | 9.97 |

PB1, PBMC sample 7 months before treatment; BM, bulk BM sample 3 months after midostaurin treatment; PB2, PBMC sample at relapse after combination treatment with midostaurin and azacitidine for 8 months.

and her disease was well controlled with midostaurin plus azacitidine for 8 months. Nevertheless, she experienced rapid disease progression in March 2020, and then died as a result of severe hypercalcemia and sepsis. To investigate clonal evolution and to explore the molecular markers of drugs resistance, we analyzed peripheral blood mononuclear cells (PBMCs) and unsorted bulk BM cells at 3 distinct time points: before treatment (PB1), after midostaurin treatment (BM), and at relapse (PB2) using conventional bulk sequencing (bulk-seq) and single-cell DNA-seq.

Genomic testing was approved by the MacKay Memorial Hospital Institutional Review Board, and the patient had provided written informed consent. PBMCs and unsorted BM cells were used for testing. Single-cell targeted DNA-seq was performed using the Tapestri acute myeloid leukemia panel (Mission Bio, San Francisco, CA). The Archer VariantPlex Myeloid panel (ArcherDX, Boulder, CO) was used for bulk targeted DNA-seq. The detailed description of mutation analysis can be found in the supplemental Materials.

In the bulk-seq data, we identified heterozygous somatic *RUNX1* R204Q, *SRSF2* P95H, *ASXL1* R693*, *KIT* D816V, and *EZH2* V696E mutations in the patient before any treatment was given (Table 1). After treatment with midostaurin, *KIT* and *EZH2* mutations became undetectable, but secondary acquired *TET2* P851Lfs*22 and *NRAS* G12D mutations were detected. At the time of relapse after treatment with midostaurin plus azacitidine, significant decrease of the VAFs in *TET2* and *NRAS* mutations, re-emergence of the *KIT* and *EZH2* mutations, and a second *RUNX1* R162S mutation were detected. Notably, *SRSF2*, *ASXL1*, and *RUNX1* mutations were detected at all 3 time points with similar VAFs, indicating that they were likely co-mutations arising early in the hematopoietic stem and progenitor cells (Figure 1A).

In the single-cell DNA-seq data, we successfully detected all bulk-seq–verified mutations except the *SRSF2* P95H and *ASXL1* R693* mutations because there was no coverage by the DNA-seq amplicons at these 2 genomic loci (Table 2). All variants were annotated as acquired heterozygous mutations. In the BM sample, we identified a predominant and rapidly evolved *KIT* wild-type subclone with *RUNX1* R204Q/*TET2* P851Lfs*22/*NRAS* G12D co-mutations after treatment with midostaurin. This *KIT* wild-type subclone represented the CMML component in this SM patient that was dependent on NRAS signaling and was resistant to treatment with midostaurin. At the time of relapse, 2 major subclones were detected, each with *RUNX1* R204Q/*KIT* D816V/*EZH2* V696E/*RUNX1* R162S and *RUNX1* R204Q/*TET2* P851Lfs*22 co-mutations. It is likely that these 2 subclones related to acquired resistance to midostaurin and azacitidine, respectively. The subclone with *RUNX1* R204Q/*TET2* P851Lfs*22/*NRAS* G12D co-mutations diminished significantly after treatment with azacitidine, indicating that the subclone that depended on NRAS signaling could be effectively controlled by azacitidine in this patient. By using single-cell DNA-seq data, a fish plot showing the clonal phylogeny was constructed to demonstrate both linear and branching clonal evolution patterns in advSM (Figure 1B).

To our knowledge, this was the first study to successfully unravel the clonal evolution and heterogeneity in response to selection pressure of KIT inhibition in advSM at single-cell resolution. Our results clearly show that progression in advSM can be caused by expansion of subclones acquiring new mutations in other myeloid genes independent of the *KIT* D816V mutation. In accordance with previous reports, no secondary *KIT* mutation related to drug resistance was detected.[14] In addition, no copy number change in *KIT* or other myeloid genes was detected in the *KIT* D816V–mutated resistant subclone (supplemental Figure 1). Whether the activation of *KIT* D816V–independent signaling molecules such as Lyn and Btk or *KIT* D816V-dependent downstream pathways such as JAK/STAT and PI3K/AKT/mTOR is involved in the reemergence of a *KIT*-mutated subclone should be further explored in a future

**Table 2. Mutations detected by single-cell DNA sequencing in a patient with advSM**

| Subclone variants | Cell no. (subclone %) | | |
|---|---|---|---|
| | PB1 | BM (total N = 239)* | PB2 (total N = 4986)* |
| WT | NA | 2 (0.78) | 3626 (72.72) |
| *RUNX1* R204Q | NA | 1 (0.39) | 63 (1.26) |
| *RUNX1* R204Q/*TET2* P851Lfs*22 | NA | 5 (1.95) | 513 (10.29) |
| *RUNX1* R204Q/*TET2* P851Lfs*22/*NRAS* G12D | NA | 231 (96.88) | 4 (0.08) |
| *RUNX1* R204Q/*KIT* D816V | NA | 0 (0.00) | 33 (0.66) |
| *RUNX1* R204Q/*KIT* D816V/*EZH2* V696E | NA | 0 (0.00) | 181 (3.63) |
| *RUNX1* R204Q/*KIT* D816V/*EZH2* V696E/*RUNX1* R162S | NA | 0 (0.00) | 566 (11.35) |

NA, not available.
*The number of cells with allele dropout or missing genotype in BM or PB2 was 331 and 1000, respectively.

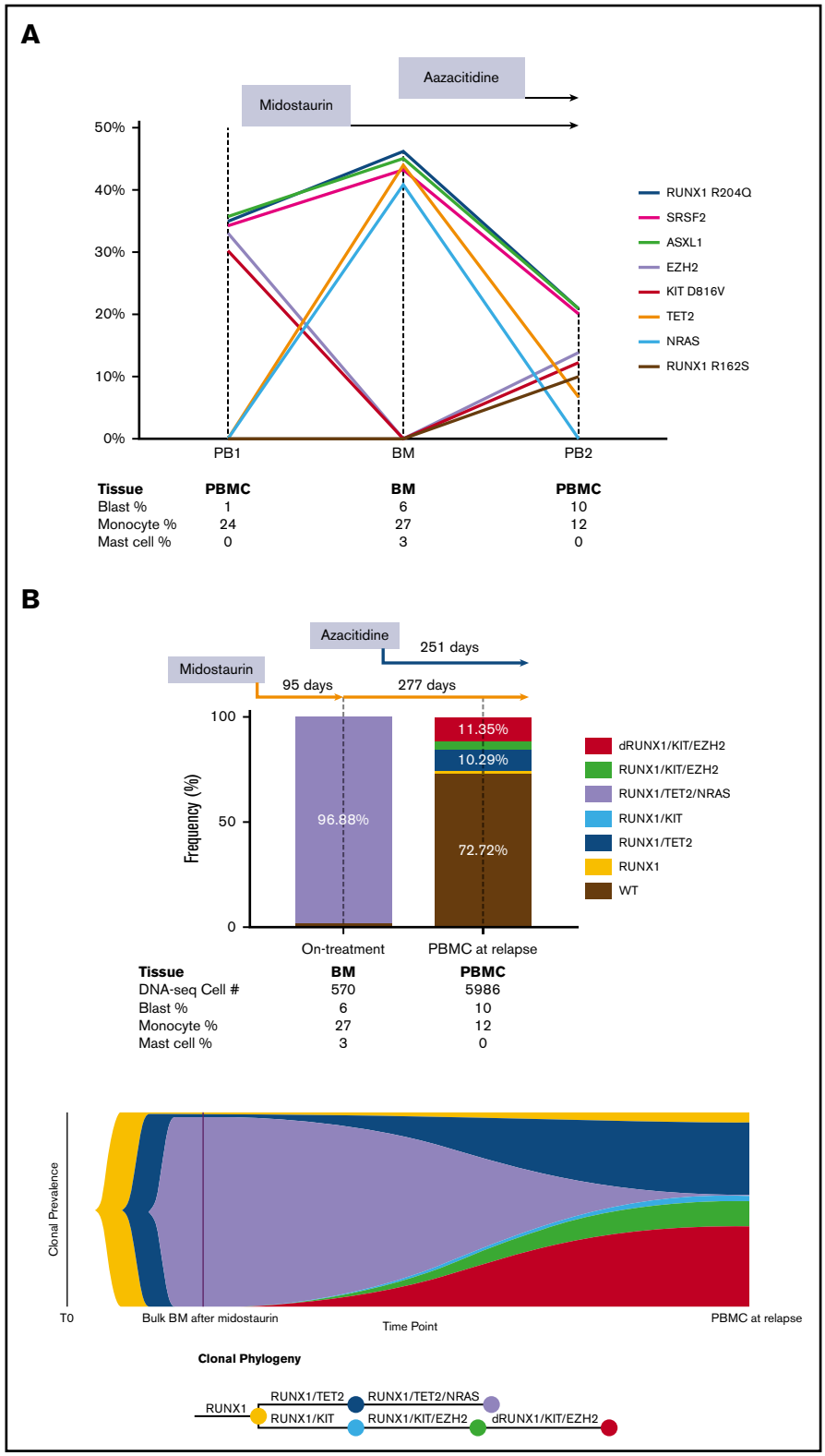


**Figure 1. Clonal evolution by serial bulk DNA next-generation sequencing and single-cell DNA-seq in a patient with** adv**SM.** (A) Clonal evolution in response to midostaurin and azacitidine by serial bulk DNA-seq. Founding *ASXL1*, *SRSF2*, and *RUNX1* mutations persisted during midostaurin and azacitidine treatment. *KIT* and *EZH2* mutation burden were initially high at the PB1 time point and were significantly diminished after midostaurin treatment. At the BM time point, allele frequency of *NRAS* and *TET2* mutations were initially high, but they later decreased as a result of azacitidine treatment. *KIT* and *EZH2* mutations, followed by disease relapse, recurred with an additional new mutation site at *RUNX1* R162S. The results were projected into a trend line with VAF shown on the Y-axis and collected samples listed below the graph. (B) Clonal evolution in response to treatment with midostaurin and azacitidine determined by single-cell DNA-seq. After treatment with midostaurin for 3 months, significant reduction of *KIT* D816V and *EZH2* mutations with significant expansion of *TET2* and *NRAS* mutations was detected. The addition of azacitidine resulted in significant reduction of *TET2* and *NRAS* mutations and progressive expansion of *KIT* D816V and *EZH2* mutations. Acquisition of a new mutation (*RUNX1* R162S) was followed by disease progression and rapid death after 8 months of combinatorial treatment. The results of single-cell DNA-seq are shown in a bar graph and fish plot with sequenced cell numbers listed below as combined blood and bone marrow cell count results. dRUNX1, double *RUNX1* mutations.

study.[15] The rapid emergence of the *KIT* wild-type subclone after treatment with midostaurin also provides a molecular basis for the explanation of relative insensitivity of monocytes to KIT inhibitors in those with SM-CMML.[13,16] Furthermore, the mutational history of driver genes as well as linear and branching clonal evolution were reconstructed in this patient with advSM. The detection of *KIT* wild-type and multimutated AHN subclones in advSM can be an important cause of acquired resistance to KIT inhibition and reinforces the

concept that AHN-directed therapy will be necessary in addition to the KIT inhibitor. Besides, persistence of subclones with *RUNX1*, *SRSF2*, and *ASXL1* mutations in early hematopoietic stem and progenitor cells probably linked to intrinsic resistant to midostaurin and azacitidine in advSM and importantly caused relapse.[10] The use of other molecular targeted therapy such as SRSF2 inhibitor may be necessary to overcome primary resistant to the KIT inhibitor.[17,18] In conclusion, our study has demonstrated the clonal evolution of malignant cells during the progression of ASM-CMML under treatment with midostaurin and azacitidine and provided a rationale for the combinatorial molecular targeted therapy in advSM.

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**ORCID profile:** K.-H.L., 0000-0003-2282-0416.

**Correspondence:** Ken-Hong Lim, Division of Hematology and Oncology, MacKay Memorial Hospital, No. 92, Sec. 2, Zhongshan N Rd, Taipei City 104217, Taiwan; e-mail: limkenhong@gmail.com.

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