Brief Report

Molecular disease monitoring using circulating tumour DNA in myelodysplastic syndromes.

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Key points: Circulating tumour DNA can monitor disease and predict treatment failure by tracking driver mutations and karyotypic abnormalities in MDS.

Running title: Circulating tumour DNA in myelodysplastic syndromes.
ABSTRACT

The diagnosis and monitoring of myelodysplastic syndromes (MDS) are highly reliant on bone marrow morphology, which is associated with substantial inter-observer variability. Azacitidine is the mainstay of treatment in MDS, however only half of all patients respond. Therefore, there is an urgent need for improved modalities for the diagnosis and monitoring of MDS. The majority of MDS patients have either clonal somatic karyotypic abnormalities and/or gene mutations that aid in the diagnosis and can be used to monitor treatment response. Circulating cell free DNA is primarily derived from haematopoietic cells, and we surmised that the malignant MDS genome would be a major contributor to cell free DNA levels in MDS patients as a result of ineffective haematopoiesis. Through analysis of serial bone marrow and matched plasma samples (n = 75) we demonstrate that cell free circulating tumour DNA (ctDNA) is directly comparable to bone marrow biopsy in representing the genomic heterogeneity of malignant clones in MDS. Remarkably, we demonstrate that serial monitoring of ctDNA allows concurrent tracking of both mutations and karyotypic abnormalities throughout therapy and is able to anticipate treatment failure. These data highlight the role of ctDNA as a minimally invasive molecular disease monitoring strategy in MDS.
INTRODUCTION

Current diagnostic and prognostic algorithms in myelodysplastic syndromes (MDS) rely heavily on peripheral blood (PB) counts and bone marrow (BM) morphology, which often has inter-observer variability. Recurrent chromosomal aberrations have helped refine current MDS prognostic models and more recently, the sequencing of MDS cancer genomes has also identified several recurrent mutations which give prognostic insights at diagnosis. However, there is little evidence of how the clonal and subclonal architecture is influenced by therapy.

Hypomethylating agents such as azacitidine and decitabine remain the mainstay of MDS treatment. However, response may take months to achieve and only half respond to therapy. Repeated BM biopsies to monitor response can be invasive, associated with procedure-related complications, and resource demanding. These limitations have, in part, compromised our ability to more regularly assess response to therapy and study clonal evolution in MDS. In this regard, we assessed the role of cell free circulating tumor DNA (ctDNA) as a novel and minimally invasive biomarker to monitor therapeutic response and clonal evolution in MDS.

METHODS

Serial BM (n=89) and plasma samples (n=83) were collected from 12 patients with MDS that received azacitidine and eltrombopag as part of a phase 1 clinical trial (Table 1). Targeted deep sequencing (TS) was performed on DNA derived from BM and plasma using a customised panel of 55 genes known to be recurrently mutated in MDS/AML (Supplemental Table 1). Sequencing of BM samples identified putative driver mutations in 10 of 12 patients (Figure 1A).

Digital PCR (dPCR) was performed to validate the TS results. Quantification of mutant allele fraction (MAF) showed excellent correlation (Supplemental Figure 1A) between TS and dPCR. Further methods are detailed in Supplemental Methods.

RESULTS AND DISCUSSION

cDNA accurately reflects the fractional abundance of somatic mutations detected in bone marrow.

The majority of cell free DNA is derived from haematopoietic cells. As MDS is a clonal disorder characterised by ineffective haematopoiesis, we postulated that the malignant MDS genome would be well represented in circulating cell free DNA. We first sought to understand how mutation detection in ctDNA compared to other haematopoietic compartments in a patient with MDS without significant cytopenia (Figure 1B). Here, the MAF of a TP53 and U2AF1 mutation from plasma ctDNA was comparable to DNA from
peripheral blood neutrophils, whole blood, and BM aspirate mononuclear (MNL) cells. The lower MAF in the PB MNL cells, most likely reflects the fact that many circulating lymphocytes, may not be derived from the malignant MDS clones. These data are representative of our findings with several pathogenic mutations in patients with MDS (data not shown).

PB cytopenias are common in MDS; as such molecular assessment of neutrophils and other peripheral blood cells is not always feasible. Therefore, the current ‘gold standard’ for molecular testing in MDS is from BM aspirate DNA. Indeed, much of our understanding of the MDS genomic landscape has been through sequencing this compartment. In all cases (n=10), the main driver mutations were detected in both BM and ctDNA (Table 1). Importantly, there was an excellent correlation ($r^2=0.84; \ p<0.0001$) between the MAF of these mutations in BM and ctDNA across multiple matched time-points (n=75) (Figure 1C). This is the highest correlation reported between a tumour compartment and ctDNA. Importantly, this correlation was preserved even when the patients were leucopenic (Supplemental Figure 1C and 1D) and there was no correlation between ctDNA MAF and peripheral blood white cell count (Supplemental Figure 1E). Together these data confirm the fact that ctDNA accurately reflects the genomic architecture of bone marrow blasts in MDS regardless of the peripheral blood white cell count. Not infrequently BM biopsies provide poor quality specimens due to technical difficulties such as BM fibrosis or hypoplasia. In situations where a sub-optimal, blood dilute BM aspirate was obtained, ctDNA analysis provided equal, and in some cases superior molecular information to BM sampling (Figure 1D and supplemental Figure 2).

cDNA dynamics reflect tumor burden during therapy for MDS.

Overall assessment of therapeutic response in MDS is measured using standard criteria. However, these criteria fail to appreciate the tumour heterogeneity in MDS and do not capture the clonal dynamics and evolutionary changes observed with therapeutic pressure. Therefore, to better understand this, serial TS and/or dPCR analysis was performed on BM and ctDNA in the 10 patients with detected mutations (Figure 1, Supplemental Figure 2). In each case, ctDNA dynamics closely followed that of BM DNA.

Studies have shown that TET2 mutations predict response to azacitidine, although benefit appears confined to patients with a MAF >10%. Furthermore, not all clones that harbor TET2 mutations show sensitivity to azacitidine. In case AZA004, there were two TET2 mutations present at baseline with a MAF>10%. Consistent with the findings of Bejar et al. a response to therapy was achieved, which was paralleled by a reduction in the MAF of both TET2 mutations in plasma ctDNA (Figure 1D). Importantly, we also found cases with distinct TET2 mutations where the TET2 mutant clones were not suppressed by azacitidine therapy (Supplemental Figure 2E).

Despite initial response to azacitidine-based therapies, progression invariably occurs. In case AZA009, despite initial clinical stability during treatment,
ctDNA demonstrated an expanding malignant sub-clone containing the \textit{NRAS} mutation, which ultimately resulted in the patient’s progression to AML (Figure 1E). Importantly, in several patients who progressed after an initial response to therapy, ctDNA reflected dynamic changes in tumor burden (Figure 1F, Figure 1H, Supplemental Figure 2B-F).

Together these findings highlight the ability of ctDNA analysis to mirror the genomic changes observed in the BM and track multiple driver mutations throughout therapy. Importantly, ctDNA shows the differential response of the malignant subclones during therapy and can be used to identify and pre-empt disease progression.

**Serial ctDNA analysis can monitor karyotypic abnormalities in plasma.**

Although used in MDS prognostic models\textsuperscript{2}, the difficulty in using karyotypic abnormalities as a monitoring tool is that they are present in less than 50\% of cases\textsuperscript{17,23}. This may be further compounded by lack of sensitivity of metaphase cytogenetics. To address this, we used low-coverage whole genome sequencing (LC-WGS) to monitor chromosomal aberrations in plasma from three MDS patients throughout therapy (Figure 1, Supplemental Figure 3).

Case AZA011 highlights a patient who had a del9q alteration detected in the BM by conventional cytogenetics (28/30 metaphase cells) (Table 1). Prior to treatment, this del9q was clearly identifiable in plasma ctDNA (Figure 1G). Notably, following clinical response to therapy, the copy number alteration was markedly reduced in plasma, suggesting that azacitidine was able to suppress the malignant clone and allow haematopoiesis to be restored from other haematopoietic stem and progenitor cells\textsuperscript{24}. The patient achieved a prolonged response (>4 years) but eventually progressed in the BM with an increasing myeloblast count (Day 1492). Interestingly, progression was associated with a re-emergence of the del9q clone, which ctDNA detected almost 3 months before confirmation by BM cytogenetic analysis (Figure 1G). Importantly, whilst no cytogenetic evolution was noted at progression, TS clearly showed clonal evolution with the emergence of a new \textit{ASXL1} mutation in ctDNA at this time (Figure 1H). \textit{ASXL1} mutations confer a poor outcome in the myeloid malignancies potentially negating the positive influence of \textit{TET2} mutations in mediating response to azacitidine therapy\textsuperscript{21,22}. It is currently unclear if azacitidine primarily acts by suppressing or differentiating the dominant malignant clone\textsuperscript{11,25,26}. Whilst, the data in this case would suggest the former, it remains entirely possible that in other cases azacitidine results in a restoration of blood counts by differentiating the malignant clone.

There is growing evidence supporting the importance of molecular assessment in MDS. Here we show that ctDNA mirrors the genomic information from BM, accurately reflects the dynamic clonal changes seen in response to therapy and can predict treatment failure. Together these data support the use of ctDNA analysis as a non-invasive biomarker to compliment existing monitoring strategies for MDS.
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AUTHORSHIP CONTRIBUTIONS

PY, SJD and MAD designed the project, interpreted data and wrote the manuscript. MD recruited study participants. MD, PY, DW and PB provided patient samples and clinicopathological data. SJD, MAD, PY, and RA developed the targeted gene panel with helpful input from PB and DW. PY, SF, DS, RV, SW and CF performed the experimental work. PY and TH analysed data with input from KD. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing financial interests.
REFERENCES


Table 1. Baseline clinical and molecular characteristics of patient cohort.

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SD, Stable Disease; CR-M, Complete response-Marrow; CR-P, Complete response-Peripheral blood; HI-P, Hematological improvement-Platelets; HI-N, Hematological improvement-Neutrophils; PD, Progressive Disease
F, Female; M, Male; ND, Not Determined
MAF Based on dPCR. MAF based on TS are shown as (#).
Figure 1. ctDNA as a disease monitoring strategy in MDS.

(A) Mutations and cytogenetic abnormalities present amongst all patients recruited to the azacitidine + eltrombopag study.

(B) Mutant allele fraction (MAF) measured by digital PCR (dPCR) of a TP53 P177R and U2AF1 Q157P gene mutation in bone marrow (BM) mononuclear layer (MNL), peripheral blood MNL, peripheral blood neutrophils, plasma ctDNA and whole blood DNA from a patient with high grade MDS. The patient had no circulating blasts in the peripheral blood detected by morphology. BM morphology revealed multi-lineage dysplasia with an excess of myeloblasts (11% of nucleated cells).

(C) Correlation between MAF measured by dPCR between BM and plasma ctDNA across 75 matched timepoints ($r^2=0.84$ p<0.0001).

(D) Case AZA004 – Serial comparison of the MAF of TET2 N1890I and TET2 D1376V mutation by dPCR between BM and plasma ctDNA. The patient had MDS with a classification of RAEB-2, which responded to azacitidine and eltrombopag therapy by a reduction in BM myeloblast percentage. At various time-points (day 193, day 318 and day 599), poor quality blood dilute aspirate samples were obtained (denoted by *). At these times, plasma ctDNA MAF was higher than BM MAF of the TET2 N1890I and TET2 D1376V mutation. There was also severe neutropenia at these time-points (neutrophils = 0.13, 0.15 and 0.16 x10⁹/L respectively).

(E) Case AZA009 – Serial MAF of a NRAS G13D mutation by dPCR of BM and plasma ctDNA. The patient had RAEB-1 and stable disease after azacitidine and eltrombopag therapy represented by a persistent but stable excess of bone marrow blasts. There was eventual progression at day 407 of therapy to acute myeloid leukemia.

(F) Case AZA007 – Serial MAF of a KRAS A59G and SRSF2 P95H mutation by TS of BM and plasma ctDNA respectively. The patient had RCUD with severe thrombocytopenia, which responded initially to azacitidine and eltrombopag therapy. The patient eventually progressed with an increase in BM myeloblast percentage. Of note, the SRSF2 mutation, despite being reduced, still remained detectable in ctDNA at all time-points sampled. At the time of disease progression, the MAF of the SRSF2 mutation in plasma had clearly increased, whilst the KRAS mutation remained undetectable.

(G) Depth of coverage (DOC) log2 ratio plots from LC-WGS of plasma in patient AZA011. At “baseline” (top panel), the plot shows the presence of a loss of copy number at chromosome 9 (yellow) prior to azacitidine therapy. At “response” on day 167 (middle panel) there is near resolution of the copy number alteration at chromosome 9. At day 1441, whilst still on therapy, at “pre-progression” (bottom panel) there is re-emergence of the loss of copy number at chromosome 9 (yellow).
(H) Serial MAF of a *CBL, U2AF1, TET2* and an *ASXL1* mutation of patient AZA011 throughout azacitidine and eltrombopag therapy. Response to therapy was achieved by an improvement in platelet count. The MAF of the *CBL, U2AF1* and *TET2* mutations reduced accordingly. At Day 1441 all three of these MAFs increased alongside emergence of a new *ASXL1* mutation. The patient subsequently progressed on day 1525 with thrombocytopenia and an increase in BM myeloblasts.
**A**

- **TET2**: 1 mutation
- **SRSF2**: 2 mutations
- **IDH2**: Detectable in plasma
- **ASXL1**: x
- **RUNX1**: x
- **TP53**: x
- **NRAS**: x
- **TP53**: x
- **WT1**: x
- **U2AF1**: x
- **KRAS**: x
- **CBL**: Normal
- **t11,17**: Normal
- **Normal**: Trisomy 8
- **Normal**: Del Y
- **Normal**: Trisomy 8, 21
- **Normal**: Del 9q

**B**

- **AZACOM6**:
  - TP53 P177R
  - U2AF1 Q157P
  - MAF (%)
  - Bone marrow MNL
  - Peripheral blood MNL
  - Peripheral blood neutrophils
  - Whole blood
  - Plasma

**C**

- Bone marrow MAF (%)
  - r² = 0.84
  - p < 0.0001

**D**

- **Azacitidine**
  - Marrow blasts (%)
  - Blood dilute aspirate
  - TET2 N1890I (Plasma)
  - TET2 D1376V (Plasma)
  - TET2 N1890I (Marrow)
  - TET2 D1376V (Marrow)

**E**

- **Azacitidine**
  - Marrow blasts (%)
  - NRAS G13D (Marrow)
  - NRAS G13D (Plasma)

**F**

- **Azacitidine**
  - Marrow blasts (%)
  - Platelet count (x10^9/L)
  - KRAS A59G (Plasma)
  - KRAS A59G (Marrow)
  - SRSF2 P95H (Plasma)
  - SRSF2 P95H (Marrow)
  - CBL H398Y (Plasma)
  - TET2 P761Lfs52 (Plasma)
  - U2AF1 S34F (Plasma)
  - ASXL1 c.2564_2567del (Plasma)
Molecular disease monitoring using circulating tumor DNA in myelodysplastic syndromes

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