Deep Sequencing of Peripheral Blood Plasma DNA as a Reliable Test for Confirming the Diagnosis of Myelodysplastic Syndrome

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ABSTRACT

Background: In patients presenting with cytopenia, myelodysplastic syndrome (MDS) should be considered, but confirmation of diagnosis requires bone marrow biopsy and morphologic and cytogenetic evaluation. It is extremely difficult to rely on subjective morphologic features to confirm the diagnosis of MDS, when the karyotype is normal and blasts are not increased. Objective criteria for the diagnosis of MDS are needed in these cases. With recent advances in the characterization of molecular abnormalities in MDS, diagnosis of early MDS is becoming more objective by documenting the presence of MDS-specific molecular abnormalities in cases with appropriate clinical presentation. Since MDS is a disease of excessive apoptosis in bone marrow, DNA resulting from the apoptosis is abundant in circulation. We explored the potential of using cell-free DNA in peripheral blood plasma and next-generation sequencing (NGS) to confirm the diagnosis of early MDS without the need for marrow biopsy.

Methods: Total nucleic acid was extracted from the plasma of 16 patients presenting with cytopenia and the diagnosis of early MDS with blasts <5% was confirmed by the presence of mutations in one or more MDS-specific genes in DNA from cells in bone marrow. Plasma samples from 4 age-matched normal controls were used as negative controls. We performed targeted sequencing of 14 genes (581 amplicons) using the Illumina MiSeq platform. This panel included the following genes: ASXL1, ETV6, EZH2, IDH1, IDH2, NRAS, CBL, RUNX1, SF3B1, SRSF2, TET2, TP53, U2AF1 and ZRSR2. NGS and Sanger sequencing were used for testing. Results of cell-free DNA in plasma were compared to results from cells or whole peripheral blood.

Results: Deep sequencing of cell-free DNA in plasma from the 16 patients with early MDS showed at least one or more mutated gene, confirming the diagnosis of MDS. Eight patients (50%) showed mutation in one gene and the remaining 8 patients (50%) showed mutations in two or more genes. Cell-free DNA in plasma from normal controls showed no evidence of mutations. When NGS data of cell-free DNA from plasma were compared with Sanger sequencing data of DNA from whole blood, 8 of the 16 patients (50%) showed additional mutations in cell-free DNA in plasma that were not detected in whole blood DNA by Sanger sequencing. Of these, 5 (31%) showed abnormalities only in plasma and none in whole blood. Mutations detected by NGS in cell-free DNA in plasma were below the detection level of the Sanger technique using whole blood DNA. NGS was performed on whole blood DNA on 5 samples; four showed the same findings in plasma DNA and whole blood DNA, and one sample showed a mutation in plasma in the RUNX1 gene at 7% frequency, but not in whole blood. NGS allowed the measurement of relative tumor load (allele frequency) in plasma. Allele frequency in plasma as detected by NGS was significantly (P=0.008) higher than that detected in cellular DNA, suggesting higher sensitivity of the former in detecting minimal residual disease and a better tool for monitoring therapy. Without exception, all detected mutations showed higher tumor load in plasma as compared with DNA from cells or whole blood, supporting the concept that plasma is enriched in tumor-specific DNA.

Conclusions: NGS of cell-free DNA in plasma using a limited number of MDS-specific genes, when used in patients with cytopenia, presents an objective test for the confirmation of the diagnosis of MDS. Plasma is enriched in tumor-specific DNA in patients with MDS. Furthermore, mutation analysis of cell-free DNA in plasma can detect subclones with mutations and can predict the emergence of new clones. Analysis of cell-free DNA in plasma using NGS provides important data on tumor load, which can be used to monitor therapy and predict progression, and also reduces the need for performing bone marrow biopsies.

INTRODUCTION

Myelodysplastic syndromes (MDSs) are a group of heterogeneous clonal bone marrow disorders characterized by ineffective hematopoiesis manifesting as peripheral blood cytopenias. Cytopenia results from a high rate of apoptosis within the bone marrow environment and consequent lack of release of cells into the peripheral blood circulation. The diagnosis of MDS can be very difficult, especially in the early stage when the patient presents with cytopenia. There are numerous reactive processes that cause cytopenia including drug reaction, nutritional deficiency, or hormonal deficiencies, autoimmune diseases, or chronic infection. The major criteria for the diagnosis of MDS are the presence of peripheral cytopenia and dysplasia. However, evaluating dysplasia is subjective and can be more difficult without bone marrow biopsy.

Molecular studies can be very help in providing objective means for the demonstration of abnormal mutant clones to confirm the diagnosis of MDS. However, because of the cytopenia in peripheral blood, molecular studies on DNA extracted from cells in peripheral blood may not be able to identify abnormal clones; hence bone marrow is required for reliable results. Since MDS is a disease of excessive apoptosis in bone marrow, DNA resulting from apoptosis is abundant in circulation.

OBJECTIVE

To determine if:
Cell-free DNA from peripheral blood plasma can be used for mutation analysis to provide a reliable means for detecting molecular abnormalities in bone marrow

A diagnosis of MDS can be confirmed without bone marrow biopsy

A 14 gene panel is adequate to make a diagnosis of MDS

METHODS

Samples: Cell-free DNA from plasma and DNA from cells in peripheral blood from 16 patients presenting with cytopenia and confirmed diagnosis of early MDS with <5% blasts.

DNA isolation: Total nucleic acid was isolated from plasma using NucliSens extraction kit (bioMerieux Inc., Durham, NC) according to the manufacturer's instructions.

DNA was isolated from whole blood cells using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Genes sequenced: ASXL1, ETV6, EZH2, IDH1, IDH2, NRAS, CBL, RUNX1, SF3B1, SRSF2, TET2, TP53, U2AF1 and ZRSR2

Next-generation sequencing: Performed using Illumina MiSeq (San Diego, CA).

The NGS sequencing was complemented by Sanger sequencing for NRAS exon 3, EZH2 exons 10 and 11, TET2 exons 4 and 13-16, and TP53 exon 4. NGS, amplification, and indexing were performed as recommended by the manufacturer. Amplicons were confirmed for each sample by running on agarose gel. Samples were pooled and the experiment sheet was generated using Illumina Experiment Manager. MiSeq Reporter was used for analysis and Variant Studio was used for calling. For confirmation of variant calling, NextGene software (SoftGenetics, State College, PA) was used. Average sequencing coverage (X) across the entire coding regions was 4000 in 94% of the sequenced amplicons. This reliably allowed detection of mutations if present in at least 3% of DNA.

RESULTS

A. Detection of mutations in cell-free DNA in plasma

Deep sequencing of cell-free DNA in plasma from the 16 patients with early MDS showed at least one mutated gene in all patients, confirming presence of cell free DNA from neoplastic cells in plasma that could aid in the diagnosis of MDS.

Eight patients (50%) showed mutation in one gene and the remaining 8 patients (50%) showed mutations in two or more genes (Figure 1).

Cell-free DNA in plasma from normal controls showed no evidence of mutations in the genes included in the panel.

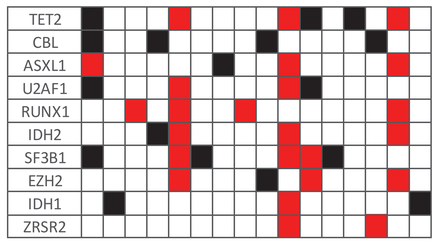
B. Comparison of NGS data of cell-free DNA from plasma with Sanger sequencing data of DNA from cells in whole blood

8 of the 16 patients (50%) showed additional mutations in cell-free DNA in plasma by NGS that was not detected in whole blood DNA by Sanger sequencing.

5 (31% of all patients) showed abnormalities only in plasma and none in whole blood. These patients would likely be wrongly presumed normal if Sanger sequencing alone was performed on whole blood.

Mutations detected in cell free DNA in plasma by NGS, but not by Sanger in DNA from cells, were at frequency <15%, which is in general below the detection level of Sanger sequencing even if they are present at the same frequency in DNA from cells. It is likely that the level or frequency of the mutated allele in the whole blood DNA is significantly lower as confirmed in the samples that were tested by NGS (see Results section C).

Figure 1. Mutation analysis in 16 patients.



Black: Mutations detected by Sanger sequencing in whole blood DNA and NGS in cell-free plasma DNA.

Red: Additional mutations detected by NGS in cell-free plasma DNA only.

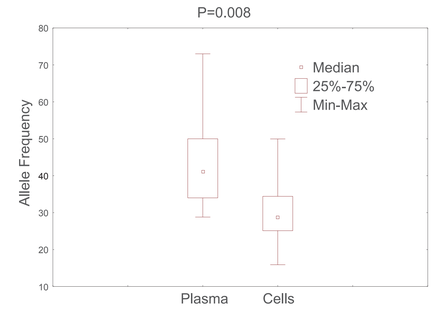
C. Comparison of NGS results in cell-free DNA in plasma and DNA from cells in whole blood (5 samples)

Four of the 5 pairs showed identical results.

One sample pair showed a mutation in RUNX1 at 7% in cell-free plasma DNA while the mutation was not detected in DNA from whole blood. Therefore, NGS allowed the measurement of relative tumor load (allele frequency) in plasma alone in this case.

More importantly, when comparing the frequency of the detected mutations, allele frequency in plasma as detected by NGS was significantly (P=0.008) higher, suggesting higher sensitivity and enrichment of neoplastic DNA in plasma (Figure 2).

Figure 2. Frequency of mutant allele in cell-free DNA in plasma vs. DNA in cells in peripheral blood by NGS.



DISCUSSION

Even when NGS is used, it is possible to miss the detection of mutations in cells in peripheral blood. It may be possible to detect mutations in cell-free DNA from plasma in these cases.

Use of peripheral blood plasma, which is easily accessible may be useful in confirming the diagnosis of MDS and can significantly reduce the needs for invasive bone marrow sampling.

Plasma is enriched with DNA from neoplastic cells, most likely due to the relatively high apoptosis of the neoplastic cells in MDS. While more studies and more data are needed, ruling out MDS is potentially possible using cell free DNA from plasma.

CONCLUSIONS

Peripheral blood cell-free DNA is enriched in neoplasm-specific DNA.

Cell-free plasma DNA is more reliable than DNA from cells in peripheral blood in detecting molecular abnormalities in patients with MDS even when the blast count is less than 5%.

Next-generation sequencing is more accurate than Sanger sequencing and should be considered as the method of choice for analyzing cell-free DNA in plasma.

NGS of cell-free DNA in plasma using a limited number of MDS-specific genes (ASXL1, ETV6, EZH2, IDH1, IDH2, NRAS, CBL, RUNX1, SF3B1, SRSF2, TET2, TP53, U2AF1 and ZRSR2), in patients with cytopenia, presents an objective test for the confirmation of the diagnosis of MDS.

Mutation analysis of cell-free DNA in plasma can detect subclones with mutations and can predict the emergence of new clones.

Analysis of cell-free DNA in plasma using NGS provides important data on tumor load, which can be used to monitor therapy and predict progression, and also reduces the need for performing invasive bone marrow biopsies.