Determining Tumor Load and Biallelic Mutation in Patients with CALR Mutation Using Peripheral Blood Plasma

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ABSTRACT

Background: CALR gene is frequently mutated in patients with myeloproliferative neoplasms (MPN). Almost all mutations are indel - some with large (>50 bp) deletion. Detecting these mutations with acceptable sensitivity is difficult by sequencing. Fragment length analysis (FLA) is a reliable technique in detecting this type of mutations. Furthermore, FLA allows quantifying the mutant DNA and better evaluation of tumor load. Determining tumor load can be confusing and difficult when the mutation is biallelic. Distinguishing patients with single allele mutation from those with biallelic mutations might add another dimension in predicting clinical behavior and determining the tumor load. We explored using cell free DNA in peripheral blood plasma to test for CALR mutations and determining tumor load.

Methods: Using direct bidirectional sequencing and FLA, we detected CALR indel mutations in 71 of 522 (14%) patients suspected of having MPN and referred for testing for CALR mutation. No sample showed point mutation. DNA from cells and cell-free DNA in plasma was available from 31 of the 71 cases. The mutant DNA peak was quantified and the relative percentage of mutant DNA was calculated in both cellular DNA and cell free plasma DNA. FLA and Sanger sequencing data were compared between cellular DNA and cell-free plasma DNA. Any ratio >55% was considered as evidence of biallelic mutation.

Results: As expected all positive samples by cellular DNA testing also showed the indel mutation in cell-free plasma by FLA. However, four of the 31 (13%) positive samples by FLA failed to show the mutation on Sanger sequencing. The most likely cause for failing to detect the mutation by Sanger is low level mutation load and the lower sensitivity of Sanger sequencing. When we compared ratios of mutant peak (tumor load) between cellular DNA and cell-free DNA in plasma , mutant CALR DNA was significantly higher (P=0.0002, Wilcoxon matched pairs test) in cell free DNA in plasma than in cellular DNA. More importantly, we were able to determine the presence of homozygous mutation (>55% mutant DNA) in 5 of 31 (16%) patients when cell-free DNA in plasma is used. In contrast, only 1 of 31 (3%) patients showed evidence of biallelic mutation when cellular DNA is used. Most of the mutations (25 of 31, 81%) were deletions. As deletions results in smaller size amplicon on the FLA and better amplification efficiency, we set 55% as a cut-off for biallelic mutation to account for the more efficient amplification of the deleted peak.

Conclusions: Cell-free DNA in plasma is more reliable than cellular DNA for the detection of CALR mutations and for determining tumor load. Testing for CALR must include fragment length analysis. More importantly, biallelic CALR mutation is frequent and the clinical relevance of biallelic CALR mutation needs to be investigated.

OBJECTIVE

(a) Compare analysis of cell-free DNA in plasma with cellular DNA in peripheral blood or bone marrow for the detection of CALR mutations and

(b) Compare fragment length analysis (FLA) with Sanger sequencing for the quantification of the CALR mutant allele burden

INTRODUCTION

Chronic myeloproliferative neoplasms (MPNs) are diagnosed and confirmed by morphologic and characteristic molecular abnormalities. Almost all cases with polycythemia vera are characterized by a mutation in JAK2. Primary myelofibrosis and essential thrombocythemia show JAK2 mutation in 40% of cases and MPL gene mutation in 2% of cases. Mutations in calreticulin (CALR) mutation is reported in 67% cases with essential thrombocythemia and 88% of cases with primary myelofibrosis that lack JAK2 or MPL gene mutations. Detection of CALR mutation is not only necessary for confirming diagnosis, but also for determining prognosis. Patients with MPN and CALR mutation have significantly lower risk of thrombosis and longer overall survival than patients with mutations in JAK2.

Mutations in CALR are insertions or deletions in exon 9 and highly unlikely to be missense mutation. The two most common mutations in CALR are 52-bp deletions (type 1 mutation) and 5-bp deletions (type 2 mutation), accounting for approximately 53% and 31.7% of detected CALR mutations respectively.

Allele burden of mutants has also been shown to play a role in determining the clinical phenotype and disease evolution, most likely because it reflects homozygous or hemizygous state of the mutation.

Bone marrow aspirates and peripheral blood specimens have been the specimens of choice for monitoring patients with MPNs. The invasive nature of obtaining bone marrow aspirates and difficulty in obtaining adequate specimen from bone marrow aspirates are limitations in using aspirates in monitoring. Analysis of tumor-derived cell-free DNA in plasma has been used in disease monitoring. In patients with MPNs, utilizing plasma rather than peripheral blood or bone marrow may be superior, as plasma may be less diluted with DNA from normal cells and more concentrated with tumor-specific DNA, increasing the sensitivity of molecular diagnostics. Sequencing of cell free DNA from plasma has been shown to be more reliable than cellular DNA in distinguishing between heterozygosity and homozygosity/hemizygosity for the JAK2 V617F mutant allele. Fragment length analysis (FLA) may be a more efficient approach for the quantification of allele burden compared to traditional Sanger Sequencing.

METHODS

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 extracted from bone marrow or peripheral blood specimens using QIACube as recommended by the manufacturer.

Fragment Length Analysis:

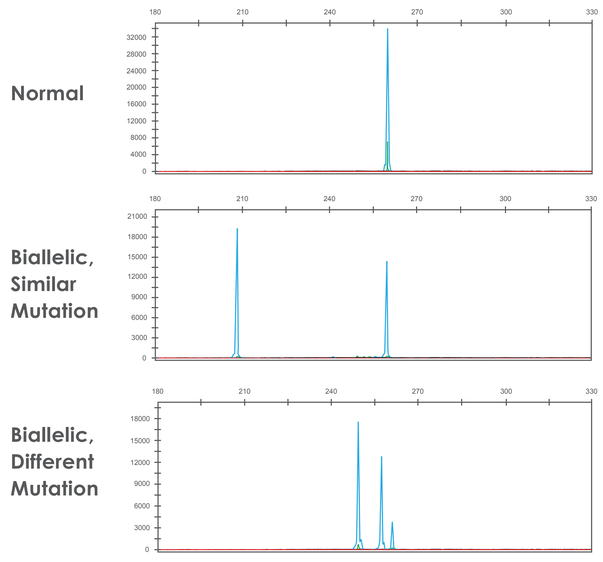
PCR/Fragment reaction covering CALR gene mutation hot spot exon 9 was performed using 6-Fam labeled primer. The CALR exon 9 mutated or wildtype products were verified by determining the size of PCR products using ABI Genetic Analyzer. Wildtype specimens display a 260 bp peak, while those with mutated CALR (with insertion/deletion) display various size peaks in addition to the CALR 260bp wildtype peak. The intensity of the mutant and wildtype peaks was used to calculate the mutant rate (semi-quantitation).

RESULTS

1. Fragment length analysis is highly sensitive

Low level (<10%) mutant CALR DNA was not detectable by sequencing, while the FLA was very sensitive in detecting this low level of mutation. This is true when the plasma or cellular DNA was used. Four of the 31 (13%) samples positive by FLA failed to show the mutation by Sanger sequencing. Furthermore, when biallelic mutation is present, detecting biallelic mutations was missed by Sanger, but easily detected by FLA. This was very obvious when the two mutations are different in the length of deletion or insertion (Figure 1).

Figure 1. Detection of biallelic CALR mutation by fragment length analysis

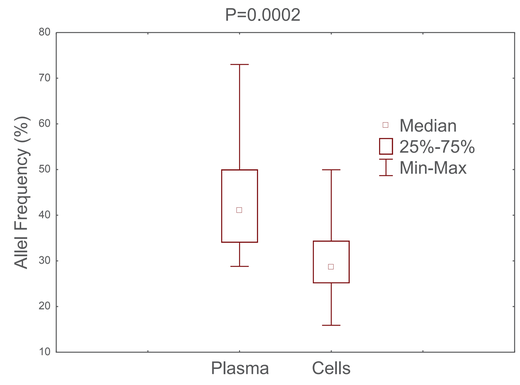


2. Cell-free DNA in plasma is more sensitive than DNA from cells

By FLA, the mutant peak was measured as a ratio of the wild-type peak to determine tumor load. The cellular DNA tumor load was compared with that of the cell-free plasma DNA. Mutant CALR DNA was significantly higher (P=0.0002, Wilcoxon matched pairs test) (Fig. 2) in cell-free DNA in plasma than in cellular DNA reflecting enrichment of the plasma by the tumor-specific DNA.

More importantly, analysis of cell-free DNA enabled detection of biallelic mutation, which most likely represents more aggressive disease. Any mutant peak that is >55% [Mutant: Mutant+normal) X 100] was considered to represent biallelic mutation. Based on this, 5 of 31 (16%) patients showed biallelic mutation when cell-free DNA in plasma was analyzed compared with only 1 of 31 (3%) patients when cellular DNA is analyzed. Most mutations (25 of 31, 81%) were deletions. As deletions results in smaller size amplicon on the FLA and better amplification efficiency, we set 55% as a cut-off for biallelic mutation to account for the more efficient amplification of the deleted peak.

Figure 2. Significantly higher relative level of mutant CALR in cell-free DNA in plasma compared to DNA from cells



CONCLUSIONS

1. Testing for CALR mutations using cell-free plasma DNA is highly reliable and can quantify the tumor load and determine biallelic (homozygous) mutations more accurately than cellular DNA.

2. Fragment length analysis is more sensitive than direct sequencing in detecting CALR mutation and tumor load.

3. Analysis of cell-free DNA in plasma by fragment length analysis is the best method for detecting CALR mutations

4. Fragment length analysis allows detection of the presence of two different types of mutation that may be present as biallelic or subclonal variation. Presence of two mutations would have been missed if sequencing alone is used.