Prevalence of somatic mutations in patients with aplastic anemia using peripheral blood cfDNA as compared with BM

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Numerous studies in patients with cancer have demonstrated the presence of tumor-specific DNA, RNA and protein in the peripheral blood. Peripherally blood (PB) cell free DNA (cfDNA) can be used for the diagnosis and monitoring of cancer and is particularly useful in solid tumors in which tissue biopsy may be hazardous or not possible. The success of cfDNA for genomic screening depends on multiple factors, including disease stage and tumor size, vascularity and biology. In contrast, in hematologic neoplasms, neoplastic cells are immersed in blood and peripheral blood cfDNA has been reported to be as reliable as bone marrow (BM) DNA in detecting molecular abnormalities. The success of cfDNA for genomic screening is conduciive to emergence of abnormal hematopoietic clones, carrying mutations that are typically detected in patients with myelodysplastic syndrome (MDS). Most of these clones are detected at very low frequency, at low variant allele frequency (VAF). Therefore, patients with AA might be ideal to evaluate the sensitivity and reliability of cfDNA in the evaluation of mutations in the hematopoietic compartment. Furthermore, in general, patients with AA have a very low number of circulating neutrophils making results from testing peripheral blood cells questionable. In addition, we have previously reported that in patients with MDS, due to the increased bone marrow apoptosis and perhaps due to inability to differentiate and circulate, these subclones are not detected when peripheral blood cells are tested.

We tested the mutation profile in peripheral blood cfDNA in direct comparison to bone marrow aspiration samples. A panel of 54 gene (TruSight Myeloid Sequencing Panel, Illumina; QIAcube) extractions according to the manufacturer's instruction. Molecular abnormalities were called using the Illumina-developed Somatic Variant Caller. RefSeq (NCBI; Bethesda, MD, USA) annotations were applied and molecular abnormalities were called in Illumina Variant Studio then individually verified with the Integrated Genome Viewer (Broad Institute; Cambridge, MA, USA). NGS testing for mutations in CALR, FLT3-ITD and ASXL1 were complemented by using fragment length analysis to avoid misinterpretation of CNVs that could be missed by NGS. cfDNA from all samples was obtained and analyzed irrespective of the severity of the disease. The efficiency of sequencing of the cfDNA was similar to that of BM cellular DNA. As a quality control, with the exception of few exons of the CEBPA gene, all amplicons in the 54 genes must meet a depth of $>6000$, otherwise, the sequencing was repeated. Buccal mucosa samples were tested for any mutations with VAF around between 40 and 60%. As quality control, normal plasma cfDNA was tested with every run as well positive control.

Of the 96 patients, 33 (34%) had one or more mutation in either cfDNA or BM DNA. Of the 120 samples, 48 (40%) showed one or more mutations and the total number of mutations was 64. Of the 48 samples, 26 (54%) had one mutation, 15 (31%) had two mutations and 7 (15%) had three mutations. Overall, 45 unique mutations (Table 1) were detected in the following genes: ASXL1, BCOR, BCORL1, CBLC, CSF3R, DNMT3A, EZH2, IDH1, JAK2, NPM1, NRAS, PTEN, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TET2, U2AF1 and ZRSR2.

Table 1. List of detected mutations

| Gene   | Nucleotide | Amino acid |
|--------|------------|------------|
| DNMT3A | NM_022552.4:c.2470delA | NP_072046.2:p.Ile824Ter |
| ASXL1  | NM_015338.5:c.2287delC | NP_056153.2:p.Leu764TerfsTer8 |
| ASXL1  | NM_015338.5:c.1926_1927insG | NP_056153.2:p.Gly646ValfsTer7 |
| ASXL1  | NM_015338.5:c.1771_1772insA | NP_056153.2:p.Tyr591Ter |
| ASXL1  | NM_015338.5:c.2287insG | NP_056153.2:p.Asp741Val |
| ASXL1  | NM_015338.5:c.2222A > T | NP_001120680.1:p.Gln383Ter |
| TET2   | NM_001127208.2:c.1147C > T | NP_004963.1:p.Val617Phe |
| JAK2   | NM_004972.3:c.1849G > T | NP_056153.2:p.Gly646ValfsTer12 |
| ASXL1  | NM_015338.5:c.2222A > T | NP_0043477.2:p.Glu211ArgfsTer11 |
| U2AF1  | NM_001020374.1:p.Glu383Ter |
| DNMT3A | NM_022552.4:c.1913C > A | NP_056153.2:p.Gln733Ter |
| ASXL1  | NM_015338.5:c.1926_1927insG | NP_056153.2:p.Gly646ValfsTer7 |
| TET2   | NM_001127208.2:c.3763_3764insA | NP_0043477.2:p.Glu211ArgfsTer11 |
More mutations were detected in cfDNA (N = 64) (Figure 1a) than in cellular BM DNA (N = 57) (Figure 1b), and 6 of 33 patients with somatic mutations (18%) showed mutations in plasma cfDNA but not in BM; two patients (6%) had mutations in BM cells that were not present in cfDNA (P = 0.002) (Figure 1c). Mutations detected in cfDNA and not in BM DNA were in the following genes: BCOR, NPM1, PTEN, RUNX1, STAG2, and ZRSR2. Mutations in ASXL1 were detected in the two patients who had mutations in BM but not in plasma. One of these two patients was tested twice, few months apart, and at both time points showed an ASXL1 (Tyr591Ter) mutation in bone marrow and not in cfDNA, but in the later sample, a second mutation in ASXL1 (Gly646TrpfsTer12) was detected in both BM DNA and in cfDNA. The variant allele frequency (VAF) for the Tyr591 was at 21% and 13%, respectively. The second patient with a mutation in BM DNA and not in cfDNA had an ASXL1 Gln733Ter mutation detected at VAF of 6%. The seven mutations detected in the cfDNA and not in the BM DNA had VAF of 6, 7, 10, 6, 13, 6 and 5% in STAG2, PTEN, RUNX1, BCOR, NPM1, ZRSR2 and BCOR, respectively. The most common mutation was ASXL1 (22% of cfDNA and 27% of BM cells), followed by TET2 (19% of cfDNA and 21% of BM cells), DNMT3A (7% in both cfDNA and BM cells), then BCOR (7% of cfDNA and 4% of BM cells).

Upon comparing the VAF of various mutations as detected by BM DNA and cfDNA, there was significant correlation (r = 0.77; P-value < 0.0001) and overall no significant difference in VAF between the two sample types (P = 0.071, Sign test). The median VAF in cfDNA was 12.6 and 10.9 in BM DNA.

These data confirm that cfDNA is as reliable as BM cells for detecting mutations, even when these mutations are present at very low frequency in hypocellular bone marrows. If cfDNA testing proved more reliable, it might be preferred for multiple reasons to BM sampling for the purpose of serial monitoring of neoplastic processes in bone marrow, especially at early, premalignant stages. Minimal residual disease in patients with leukemia can be monitored using cfDNA, sparing the patient the need for bone marrow aspiration and biopsy. Furthermore cfDNA can be used for early diagnosis, especially in elderly patients and when a patient refuses biopsy. cfDNA may be an especially valuable source of mutation detection in marrow failure, in which marrow aspirates may not contain sufficient cells for accurate mutation analysis.

| Gene    | Nucleotide     | Amino acid                      |
|---------|----------------|---------------------------------|
| RUNX1   | NM_001754.4:4c.965C>G | G > C                           |
| STAG2   | NM_001042749.1c.1027G>T | T > G                           |
| PTEN    | NM_000314.4:c.674A>G   | A > G                           |
| ASXL1   | NM_015338.5:c.31100G>A | A > G                           |
| ASXL1   | NM_015338.5:c.2276_2280delGCCCAG | 6G > 7G, 4A > 5A |
| BCORL1  | NM_021946.4:c.3323C>T | T > C                           |
| ASXL1   | NM_015338.5:c.2513A>G | A > G                           |
| ZRSR2   | NM_005089.3:c.1314_1315insAGCGGG | 2A > 3A |
| TET2    | NM_001127208.2c.3662G>T | T > C                           |
| TET2    | NM_001127208.2c.3332T>A | T > C                           |
| RUNX1   | NM_001754.4:c.1440C>G | C > G                           |
| BCOR    | NM_001123385.1c.4988_4989delGG | 2GG > 2GA, 2C > 1C |
| SF3B1   | NM_012433.2:c.1973G>C | C > G                           |
| TET2    | NM_001127208.2c.5636A>T | T > C                           |
| CALR    | NM_004343.3:c.1192_1194delGAG | 3G > 3C, 2A > 2C |
| IDH1    | NM_005896.2:c.394C>G   | G > C                           |
| CBLC    | NM_0012116.3:c.1303C>T | T > C                           |
| TET2    | NM_001127208.2c.575_576insAAT | 2AAT > 2AAA |
| PTEN    | NM_002834.3:c.1178G>C  | C > G                           |
| PTPN1   | NM_002834.3:c.1178G>C  | C > G                           |
| RUNX1   | NM_001754.4:c.276dupC | C > C                           |
| NPM1    | NM_002520.3c.863_864insCCGC | CC > CC, 3G > 3C |
| ZRSR2   | NM_005089.3:c.1314_1315insAGCGGG | 2A > 3A |
| DNMT3A  | NM_022552.4:c.2109delA | A > A                           |
| DNMT3A  | NM_022552.4:c.976C>T   | T > C                           |
| BCORL1  | NM_021946.4:c.1942_1943insCCGC | CC > CC, 3G > 3C |
| TET2    | NM_001127208.2c.2715_2716insA | 2A > 3A |
| CSF3R   | NM_156039.3:c.2326C>T  | T > C                           |
| TET2    | NM_001127208.2c.2771A>G | G > C                           |
| BCOR    | NM_001123385.1c.4973_4974delAG | 2G > 1G, 2T > 1C |
| TET2    | NM_001127208.2c.1648C>T | T > C                           |
| ATRX    | NM_000489.3:c.5579A>G  | G > A                           |
| BCOR    | NM_001123385.1c.3809G>A | A > G                           |
| DNMT3A  | NM_022552.4:c.2578T>C  | C > C                           |
CONFLICT OF INTEREST

AA, WM, IDD, VF and MA are employed by a diagnostic company offering cfDNA testing. All other authors declare no competing interest.

AUTHOR CONTRIBUTIONS

Concept and design: AA, MA, DT and NSY. Conduct of Laboratory work: AA, WM, IDD, VF and MA. Acquisition of data and samples: AA, DT, WM, IDD, VF, NSY and MA. Analysis and interpretation of data: AA, WM, VF, NSY and MA. Writing, review and/or revision of manuscript: all authors. Study supervision: MA.

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