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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.[1–4] Peripheral blood (PB) cell free DNA (cfDNA) can be used for the diagnosis and monitoring of cancer,[2–4] and is particularly useful in solid tumors in which tissue biopsy may be hazardous or not possible.[1–4] The success of cfDNA for genomic screening depends on multiple factors, including disease stage and tumor size, vascularity and biology.[4] 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.[4–12] In theory, peripheral blood cfDNA might be more representative of the entire bone marrow and less influenced to sampling error than is BM DNA.[4]

The disease aplastic anemia (AA) is, in most cases, the result of immune-mediated destruction of hematopoietic stem cells.[13,14] However, recent studies have suggested that this inflammatory environment is conducive to emergence of abnormal hematopoietic clones, carrying mutations that are typically detected in patients with myelodysplastic syndrome (MDS).[15] Most of these clones are detected at very low frequency, at low variant allele frequency (VAF).[15] 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 obtained 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.[8]

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; San Diego, CA, USA) and next generation sequencing (NGS) were utilized to assess 120 paired bone marrow and peripheral blood cfDNA samples collected from 96 patients who had been diagnosed with AA. Paired samples were collected at the same time. All patients in the studied group had a very low neutrophils count (median 275, range 0–1380 neutrophils/ul). Twenty six of these patients had absolute neutrophils count $< 200/\mu L$.

Total nucleic acid was extracted from PB plasma via the NucliSenS EasyMAG automated platform (BioMerieux; Marcy-l'Étoile, France). DNA from whole bone marrow cells was extracted QIAamp DNA Mini Kit (Qiagen; Venlo, The Netherlands) in both manual and automated (QIAcube) extractions according to 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 missing large indels that can 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.Leu764TyrfsTer8
ASXL1	NM_015338.5:c.1926_1927insG	NP_056153.2:p.Gly646TrpfsTer12
ASXL1	NM_015338.5:c.1771_1772insA	NP_056153.2:p.Tyr591Ter
TET2	NM_001127208.2:c.1147C>T	NP_001120680.1:p.Gln383Ter
JAK2	NM_004972.3:c.1849G>T	NP_004963.1:p.Val617Phe
ASXL1	NM_015338.5:c.2222A>T	NP_056153.2:p.Asp741Val
U2AF1	NM_001025203.1:c.101C>T	NP_001020374.1:p.Ser34Phe
DNMT3A	NM_022552.4:c.1913C>A	NP_072046.2:p.Ser638Tyr
ASXL1	NM_015338.5:c.2197C>T	NP_056153.2:p.Gln733Ter
TET2	NM_001127208.2:c.3763_3764insA	NP_001120680.1:p.Tyr1255Ter
EZH2	NM_004456.4:c.630dupA	NP_004447.2:p.Glu211ArgfsTer11

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Table 1. (Continued)

Gene	*Nucleotide*	*Amino acid*
RUNX1	NM_001754.4:c.965C>G	NP_001745.2:p.Ser322Ter
STAG2	NM_001042749.1:c.1027G>T	NP_001036214.1:p.Val343Leu
PTEN	NM_000314.4:c.674A>G	NP_000305.3:p.Tyr225Cys
ASXL1	NM_015338.5:c.3110G>A	NP_056153.2:p.Trp1037Ter
ASXL1	NM_015338.5:c.2276_2280delGCCAG	NP_056153.2:p.Gln760LeufsTer12
BCORL1	NM_021946.4:c.3332C>T	NP_068765.3:p.Thr1111Met
ASXL1	NM_015338.5:c.2513A>G	NP_056153.2:p.Lys838Arg
ZRSR2	NM_005089.3:c.1314_1315insAGCCGG	NP_005080.1:p.Gly438_Ser439insSerArg
TET2	NM_001127208.2:c.3662G>T	NP_001120680.1:p.Cys1221Phe
TET2	NM_001127208.2:c.3332T>A	NP_001120680.1:p.Leu1111Ter
RUNX1	NM_001754.4:c.1440C>A	NP_001745.2:p.Tyr480Ter
BCOR	NM_001123385.1:c.4988_4989delGG	NP_001116857.1:p.Trp1663SerfsTer8
SF3B1	NM_012433.2:c.1973G>C	NP_036565.2:p.Trp658Ser
TET2	NM_001127208.2:c.5636A>T	NP_001120680.1:p.Glu1879Val
CALR	NM_004343.3:c.1192_1194delGAG	NP_004334.1:p.Glu398del
IDH1	NM_005896.2:c.394C>T	NP_005887.2:p.Arg132Cys
CBLC	NM_012116.3:c.1303C>T	NP_036248.3:p.Pro435Ser
TET2	NM_001127208.2:c.575_576insAAT	NP_001120680.1:p.Tyr192delinsTer
TET2	NM_001127208.2:c.1118_1122delAAAAT	NP_001120680.1:p.Gln373ArgfsTer15
EZH2	NM_004456.4:c.2109delA	NP_004447.2:p.Val704LeufsTer2
TET2	NM_001127208.2:c.5167C>T	NP_001120680.1:p.Pro1723Ser
BCOR	NM_001123385.1:c.756C>A	NP_001116857.1:p.Tyr252Ter
SRSF2	NM_001195427.1:c.284C>G	NP_001182356.1:p.Pro95Arg
NRAS	NM_002524.4:c.35G>C	NP_002515.1:p.Gly12Ala
NRAS	NM_002524.4:c.37G>C	NP_002515.1:p.Gly13Arg
SETBP1	NM_015559.2:c.2602G>A	NP_056374.2:p.Asp868Asn
PTPN11	NM_002834.3:c.178G>C	NP_002825.3:p.Gly60Arg
PTPN11	NM_002834.3:c.226G>C	NP_002825.3:p.Glu76Gln
RUNX1	NM_001754.4:c.276dupC	NP_001745.2:p.Asp93ArgfsTer45
NPM1	NM_002520.6:c.863_864insCCGC	NP_002511.1:p.Trp288CysfsTer12
ZRSR2	NM_005089.3:c.1346_1360delGGAGCCGCCGCAGCC	NP_005080.1:p.Ser450_Arg454del
SF3B1	NM_012433.2:c.1998G>T	NP_036565.2:p.Lys666Asn
DNMT3A	NM_022552.4:c.1634delA	NP_072046.2:p.Glu545GlyfsTer106
DNMT3A	NM_022552.4:c.976C>T	NP_072046.2:p.Arg326Cys
BCORL1	NM_021946.4:c.1942_1943insC	NP_068765.3:p.Val650ArgfsTer15
TET2	NM_001127208.2:c.2715_2716insA	NP_001120680.1:p.Met906AsnfsTer18
CSF3R	NM_156039.3:c.2326C>T	NP_724781.1:p.Gln776Ter
TET2	NM_001127208.2:c.2771A>G	NP_001120680.1:p.His924Arg
BCOR	NM_001123385.1:c.4973_4974delAG	NP_001116857.1:p.Gln1658ArgfsTer13
TET2	NM_001127208.2:c.1648C>T	NP_001120680.1:p.Arg550Ter
ATRX	NM_000489.3:c.5579A>G	NP_000480.2:p.Asn1860Ser
BCOR	NM_001123385.1:c.3809G>A	NP_001116857.1:p.Trp1270Ter
DNMT3A	NM_022552.4:c.2578T>C	NP_072046.2:p.Trp860Arg

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 4%. 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 cfDAN, 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 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.

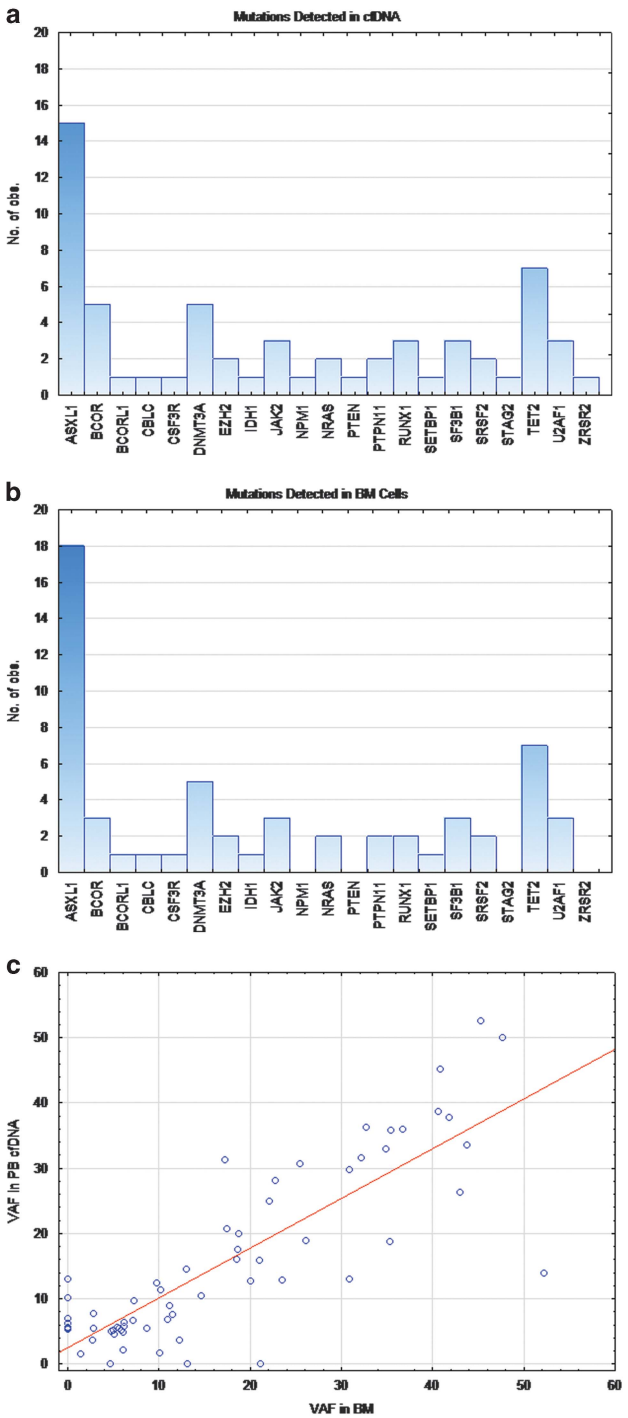

Figure 1. Comparison of cfDNA with bone marrow DNA: Frequency of mutations detected in each gene as detected in cfDNA are shown in (**a**) and as detected in BM cells are shown in (**b**). (**c**) shows variant allele frequency (VAF) in cfDNA as correlated with the VAF detected in the bone marrow cellular DNA ($r = 0.77$; P-value < 0.0001).

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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