Early Detection of Myelodysplastic Syndrome/Leukemia-associated Mutations Using NGS Is Critical in Treating Aplastic Anemia*

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Summary: Distinguishing between aplastic anemia (AA) and hypoblastic myelodysplastic syndrome (hMDS) with a low percentage of bone marrow (BM) blasts (<5%) can be difficult due to the overlap in clonality and a spectrum of genetic alternations between the two subtypes of diseases. However, due to recent advances in DNA sequencing technology, both spectrum and frequency of mutations can be accurately determined and monitored by next-generation sequencing (NGS) at initial diagnosis and during immunosuppressive therapy (IST) in patients with AA or hMDS. This improvement in acquiring a patient's genetic status and clonal evolution can provide more proper, precise, and on-time information to guide disease management, which is especially helpful in the absence of traditional morphologic/cytogenetic evidence.
Key words: aplastic anemia; hypoblastic myelodysplastic syndrome; immunosuppressive therapy; next-generation sequencing

Aplastic anemia (AA) is an acquired marrow failure syndrome, characterized by peripheral pancytopenia and hypoplastic bone marrow (BM), sharing clinical manifestations and BM morphological characteristics with other acquired or congenital BM failure syndromes[1]. Hypocellular myelodysplastic syndrome (hMDS) accounts for 10%–15% of all MDS patients[2]. It is characterized by low bone marrow cellularity, which is age-dependent, being lower in aged patients (<30% cellularity if ≤60 years or <20% cellularity if >60 years). Interestingly, both AA and hMDS have similar histological characteristics of a hypocellular bone marrow, thus the distinction between them mostly relies on other morphological or immunophenotypic characteristics such as megakaryocytic or CD34+ blast counts. However, such hyperplasia may not be observed because of the hypocellular characteristics. In some cases, it is difficult to obtain enough BM cells for immunophenotyping, genetic screening, and cytogenetics. Moreover, the high clonality in AA[3, 4] may result in an overlap in the spectrum of genetic alterations between AA and MDS[5, 6], which may further complicate the diagnosis. Next-generation sequencing (NGS) has revolutionized the determination of DNA sequences. The accumulated knowledge of the major driver mutations in MDS/acute myelocytic leukemia (AML) has promoted the investigation of somatic mutations in AA associated with its clonal evolution to MDS/AML. Thus, NGS can be a powerful tool for detecting mutations that are usually associated with MDS/AML transformation in AA.

1 Mutation Frequency and the Common Somatic Mutation Genes in AA

It has been reported that healthy individuals are also known to accumulate structural genomic rearrangements and somatic mutations as a part of normal ageing (≤1% in those <50 years old *vs.* ≤10% in those >65 years old)[7–9]. Thus, pre-existing age-related somatic mutations in AA could serve as a substrate that is then shaped by autoimmune selection and hematopoietic stress to create the unique clonal signature of AA. It was reported that through 5–11 years of observation, 1.7%–15% of patients with AA progressed to MDS[10, 11], especially in those not attaining complete response (CR) following immunosuppressive therapy (IST). Therefore, it is critical to monitor clonal hematopoiesis combined with clinical evaluation to estimate prognosis and guide therapy in AA[7, 12].

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*This work was supported by the National Natural Science Foundation of China (No. 81470009).

Several NGS-based mutation studies have reported various AA mutation frequencies in the range of 5.3%–72%[4, 6, 13, 14]. These studies found that 19% of AA patients carried somatic mutations that were frequently found in MDS, including *ASXL1*, *DNMT3A* and *BCOR*. Other MDS/AML-related mutations, such as *TP53*, *RUNX1*, and *CSMD1*, were also detected but at lower frequencies[15]. It has been reported that mutations in *DNMT3A*, *ASXL1*, *TP53*, *RUNX1*, and *CSMD1* ("unfavorable" mutations) are significantly associated with faster progression to MDS/AML, shorter overall survival, and poor response to IST than in *PIGA* and *BCOR*/*BCORL1* ("favorable" mutations)[16]. It is likely that mutations in *BCOR*/*BCORL1* and *PIGA* are associated with a decreasing or stable clone size during the clonal evolution of AA[17]. Interestingly, high-risk cytogenetics were detected more frequently in MDS transformed from AA (AA-MDS) than in *de novo* MDS, suggesting that IST may contribute to the production of a high-risk karyotype and malignant transformation[18].

2 Difference of Somatic Mutation Spectrum in AA and MDS

Although both AA and MDS have a risk of progression to acute leukemia, the risk associated with the latter is much higher. Therefore, it is important to distinguish MDS, especially hMDS, from AA to avoid misdiagnosis and inappropriate treatment. Recent application of high-density genotyping with single nucleotide polymorphism arrays (SNP-A) and NGS have uncovered a plethora of clonal somatic changes in the bone marrow of AA patients. *DNMT3A* and *ASXL1* are among the common mutational targets in both AA and MDS/AML, while *PIGA/BCOR/BCORL1* mutations are correlated with a better response to IST[16, 19]. *TET2*, *TP53*, *RUNX1*, and genes encoding cohesion and splicing factors were underrepresented in AA compared with MDS/AML[20], which likely reflects the difference in the mechanism of clonal selection between the diseases. It was also reported that these "MDS/AML-like" mutations expressed at low levels in AA may predict a substantial risk for progression to MDS/AML[6, 14].

ZRSR2 is involved in RNA-splicing and *TET2* regulates DNA methylation[21]. It was reported that mutations in genes regulating RNA-splicing and DNA methylation appeared to occur early as 'founder' mutations in over 50% of MDS patients[16]. *TET2* mutation was found to have an independent prognostic response to hypomethylating agents[22, 23], while some studies found no association between TET2 mutation and hypomethylating agents treatment response[24]; mutational status of *ZRSR2* did not affect the response rate or survival in MDS patients who had received first-line decitabine treatment[25]. So, detection of MDS-associated mutations by NGS can be a life-and-death matter in AA disease management.

3 Case Presentation

This case was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. A 72-year-old woman presented with cytopenia accompanied by fatigue since 2006. In June 2013, she visited the local hospital for gonarthromeningitis. The blood-routine test showed severe anemia and leucopenia (the exact values were unclear). In July 2013, she visited our hospital for treatment. At this time, her blood routine test displayed pancytopenia with a white blood cell count of 3.12×10^9/L, 65 g/L of hemoglobin, 2.2×10^9/L of neutrophils and 91×10^9/L of platelets (table 1). She also received the following conventional examinations: BM aspiration showed severe hypocellularity (fig. 1A); flow cytometry detected only 0.92% of CD34+ cells without any abnormal immunophenotyping, a phenotype between AA and hMDS; fluorescence *in situ* hybridization (FISH) and chromosome banding showed no increase in GPI-AP-deficient blood cells or abnormal signals by either technique; clinical markers related to autoimmune diseases were also negative and blood chemistry parameters were almost normal; the expression level of HBV-DNA, folate and vitamin B12 were all within normal limits (table 1). Therefore, this patient was diagnosed with AA, while hMDS could not be completely excluded. Treatment with oral cyclosporine, androgen and stimulating factors were initiated. She received a blood routine test and blood

Table 1 Clinical features at initial diagnosis and post-treatment

Clinical features	Pre-treatment	Post-treatment
WBC ($\times10^9$/L)	3.12	0.25
Platelet count ($\times10^9$/L)	91	11
Hemoglobin (g/L)	65	71
Neutrophils ($\times10^9$/L)	2.2	0.06
Blast cells (%)	0.92	3
Bone marrow hematopoiesis	Hypoproliferative	Severe hypoproliferative
Fluorescence *in situ* hybridization	Normal	Normal
Chromosome karyotype	46, XX	46, XX

chemistry examination once a week and bone marrow aspiration once a year. A blood component transfusion was given when her hemoglobin was lower than 60 g/L and the platelet count lower than 20×10^9/L. However, the blood routine test showed no improvement and cytopenia became more aggravated during the course of treatment between July 2013 and Jan. 2017 (fig.1B and 1C, fig. 2).

Meanwhile, nearly all bone marrow morphological examinations showed severe hypocellularity with hyperplasia of neutrophils and erythroid cells; only about 1%–3.27% of CD34+ blasts were detected by immunophenotyping. In addition, this patient showed a normal karyotype as determined by both FISH and chromosome banding and a normal level of WT1 expression.

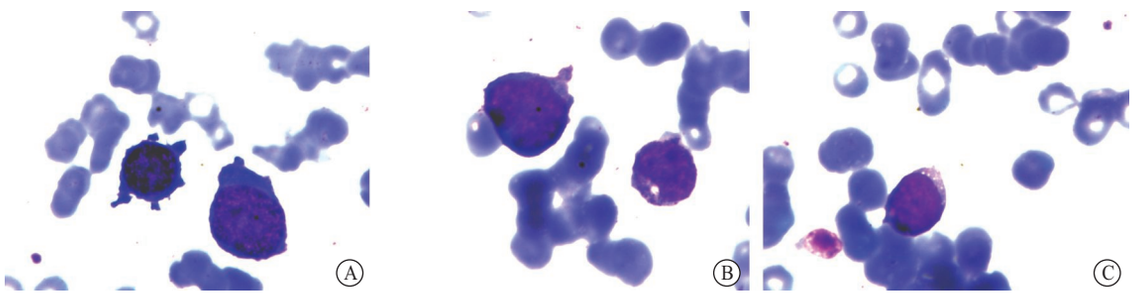

Fig. 1 Bone marrow morphology of this case displaying hypoplasia and few hematopoietic cells on the slides (×100)
Bone marrow results were from three examinations. A: July 2013 (before therapy); B: August 2014 (during therapy); C: January 2017 (during therapy)

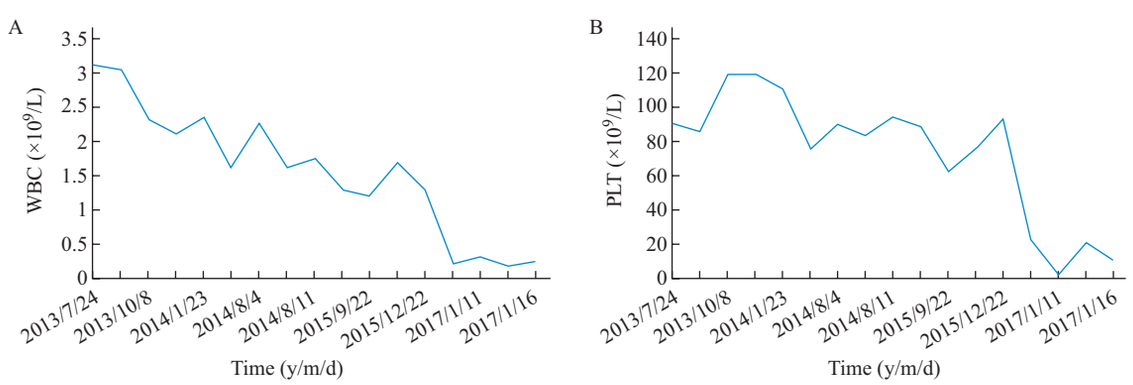

Fig. 2 Blood cell count profiles during treatment from July 2013 to January 2017
A: WBC count; B: platelet count

Although the percentage of CD34+ cells in this patient once reached the threshold considered typical of hMDS, she was not diagnosed with MDS due to the lack of other morphological or cytogenetic supporting evidence. Based on that diagnosis, she continued with oral cyclosporine accompanied by supportive care. In Dec. 2016, she was hospitalized for severe pneumonia, complicating tuberculosis. She was immediately prescribed an anti-infective regimen that soon relieved these symptoms. When conditions permitted, she received a bone marrow routine inspection to obtain a better evaluation of her disease condition. This time, we added NGS to the diagnostics to detect possible tumorigenic mutations. The results of the routine tests were the same as before, while NGS detected 3 mutated genes, *TET2*, *ZRSR2* and *BCOR* with mutation burdens of 53.2%, 49.2%, and 47.5%, respectively (fig. 3). As

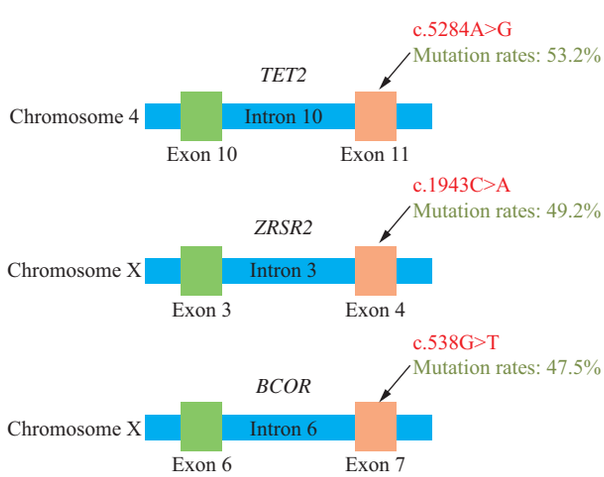

Fig. 3 Diagram showing mutations in *TET2*, *ZRSR2*, and *BCOR* identified by NGS

reported, mutations in the *BCOR* gene are frequently detected in AA, correlated with a better response to IST and better overall and progression-free survival[16–18]. However, *ZRSR2* and *TET2* mutations are commonly found in MDS, not in AA[4]. So this NGS data revealed very important information: AA-related and MDS-related mutations were both detected in the same case after 3 years of IST. Although this patient had a *BCOR* mutation suggesting a better response to IST, her blood routine test result was not improved at all after 3 years of IST. Therefore, it is possible that other factors are present, such as mutations in *ZRSR2* and *TET2*, affecting the efficacy of IST. In the end, this patient unfortunately died from severe infection and related complications before suitable therapy against MDS could be provided.

In addition, mutations associated with MDS can be divided into 2 groups: group 1, including *FLT3*, *PTPN11*, *WT1*, *IDH1*, *NPM1*, *IDH2* and *NRAS*, has been correlated with faster sAML progression and a shorter overall survival time; group 2, including *TP53*, *GATA2*, *KRAS*, *RUNX1*, *STAG2*, *ASXL1*, *ZRSR2* and *TET2*, has less influence on sAML progression and overall survival[16]. So based on the NGS result of mutations in the *ZRSR2* and *TET2* genes, it is likely that a better outcome could have been achieved if this patient had been treated from the beginning as MDS rather than AA. Therefore, for patients with these mutations, it would make a big difference in outcome if NGS was used at the time of initial diagnosis and decitabine was included as part of the ongoing treatment. Such an adjustment corresponding to the mutation status in *ZRSR2* and *TET2* might significantly improve the outcome measured as clinical response and quality of life.

4 Summary and Prospects

Distinguishing pathologically between AA and hMDS can sometimes be very challenging, due to subtle differences and the availability of bone marrow tissue. This difference is especially more important since IST therapy, which is commonly used to treat AA patients, has limited success in a variable proportion of MDS cases[26], such as younger patients with low-risk MDS, which have been demonstrated by studies conducted on a large number of IST-treated patients from more than 10 centers worldwide[27–30]. Our case further demonstrates that morphological criteria may not be enough to distinguish between AA and MDS, which differ by a subtler molecular mechanism. She responded poorly with a gradual decrease in white blood cells and platelets and died from severe infection. If NGS was available at the initial diagnosis and during the course of treatment, a more proper treatment targeting MDS-like genetic mutations, namely *TET2* and *ZRSR2*, could have been prescribed and perhaps would have achieved a better outcome.

It has been reported that some somatic mutations (like *DNMT3A*, *TET2*, and *ASXL1*) in leukemia-associated driver genes exist in older healthy individuals[7–9], thus detection of common aging-associated mutations in a cytopenic patient may lead to diagnostic uncertainty. Despite this potential confused factor, the high likelihood of disease progression exists when some somatic mutation patterns are observed in cytopenic patients and a provisional diagnosis of MDS may be suggested when morphologic dysplasia and other diagnostic criteria are absent. Recent European series has stressed that in patients with unexplained persistent cytopenias, mutations in leukemia-associated driver genes confer a substantial risk of progression to WHO-defined MDS or AML, with a hazard ratio >13.9-fold higher than the risk of MDS/AML progression in patients with idiopathic cytopenia of uncertain significance (ICUS) without a clonal mutation[31]. The risk of progression for the patients with "clonal cytopenias of indeterminate significance" (CCUS) was greatest for those with a higher mutation burden (VAF >20%), >1 mutation, or a mutation in a splicing factor[32].

Recently, the development of parallel sequencing technologies demonstrated unquestionable power to discover genetic changes across the entire genomes or protein-coding sequences in human cancers. The interpretation of these results yet needs caution. These advances can help us achieve a better understanding of disease pathogenesis and provide therapeutic targets for ongoing clinical trials[17, 33]. This is especially important when there is no morphologic evidence of MDS. The detection of MDS/AML-like mutations in AA may represent the early stage of disease or an intermediary state that may require acquisition of further cooperating mutations or biological events. Therefore, it is possible that we can distinguish the initial stage of MDS or atypical MDS from other hypocellular diseases by detecting the clonal dynamics of mutations in AA patients with the help of NGS, even in the absence of overt morphological or cytogenetic evidence.

Conflict of Interest Statement

All authors have no conflict of interest to report.

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(Received Dec. 1, 2018; revised Dec. 24, 2018)