

Case Report

Next generation sequencing guided treatment modulation and prognosis in Acute myeloid leukemia: Case vignettes

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Abstract: Objective: The genomic mutational landscape of Acute Myeloid Leukemia has contributed to better treatment options, risk stratification and prognostication of this genetically heterogeneous disease. With several approved new drugs targeting specific mutations with better outcomes, we describe here two cases of AML in which, NPM1 was detected at diagnosis. The impact of age, type of treatment, stability of NPM1 mutation, and co-occurring mutations on survival are the essential parameters for investigation. Method: Both the cases of AML were females, >60 years of age with normal 46XX karyotype. Allele specific RT-PCR and fragment analysis was performed for the detection of NPM1-A mutation at diagnosis. Both the patients were unfit for intensive chemotherapy therefore reduced intensity induction chemotherapy regimen was initially administered. Next-generation sequencing was performed for comprehensive mutational profiling, which guided targeted treatment, prognostic stratification, and response assessment. Result: We report that the older AML patients with NPM1 mutation may not have a good outcome with intensive chemotherapy, especially patients with concurrent DNMT3A/IDH-1/2 mutations. In the second case with mutated NPM1, concurrent FLT3-ITD mutation served as a therapeutic target. The FLT3 inhibitor used in combination with standard therapy showed promising results in this case. Conclusion: Here, we emphasize on the utility of next generation sequencing in guiding the treatment initiation or modulation during the disease course and risk stratification in AML. In conclusion, conventional chemotherapy in AML gives very poor overall survival rates and targeted chemotherapy against specific mutations may drastically improve patient survival and treatment outcomes.

Keywords: Acute myeloid leukemia, NPM1, FLT3-ITD, allelic ratio, DNMT3A, IDH1/2, next-generation sequencing

Introduction

Acute myeloid leukaemia (AML) is a rapidly advancing aggressive haematological malignancy characterised by genetic alterations in normal myeloid precursors leading to uncontrolled proliferation, causing impaired haematopoiesis and bone marrow failure [1]. The incidence of AML is 4.3 per 100,000 (age-adjusted) annually with a median age at diagnosis of 68 years and a poor 5-Year period survival of 29.3% in the United States alone [2]. Despite the advancements in AML research, increased risk of relapse/refractory disease is common and high mortality rate is reported as no single superior approach is promising for its treat-

ment [3]. The recent advent of novel therapies has changed the outlook of AML with eight new drugs approved in the last decade for both fit and unfit patients of AML (Table 1). Five of these new drug discoveries are attributable to the better understanding of the molecular architecture of AML.

There has been an incredible surge in our understanding of the mutational landscape of AML [4-6], which has introduced modifications in the existing AML classification and transformed the prognostic stratification, treatment and response assessment of AML [7, 8]. "Passenger lesions" (TET2, DNMT3A, GNAS, ASXL1, SF3B1, PPM1D) have been identified, which

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Table 1. Novel therapies approved in AML

S.No.	Name	Function	Use	FDA Approval	
A) Drugs not Targeting any specific Mutations					
1.	CPX-351	Liposomal formulation of AraC and Daunorubicin	Secondary AML as front-line treatment	March 2017	
2.	Gemtuzumab Ozogamicin		CD33+ AML alone or in combination with CT for newly diagnosed or R/R AML	September 2017	
3.	Glasdegib	Inhibitor of hedgehog signaling pathway	Combination with LDAC for patients with newly diagnosed AML >75 yrs or with comorbidities	November 2018	
A) Drugs Targeting Molecular Target Points					
1.	Midostaurin	Mutated FLT3	Newly diagnosed AML in combination with standard chemotherapy	April 2017	TKI-inhibitors
2.	Gilteritinib	Inhibitor of FLT3 and AXL	Approved for R/R FLT3 mutated AML	November 2018	TKI-inhibitors
3.	Enasidenib	Inhibitor of IDH2	Approved for R/R AML with an IDH2 mutation	August 2017	IDH2 inhibitors
4.	Ivosidenib	Inhibitor of IDH1	R/R AML with an IDH1 mutation	July 2018	IDH1 inhibitors
5.	Venetoclax	Inhibitor of Bcl-2, independent of TP53 mutations	Newly diagnosed AML unfit for intensive chemotherapy in combination with HMAs/LDAC	November 2018	Binds to Bcl-2, translocation of proapoptotic proteins (BIM, BAX) to the mitochondria

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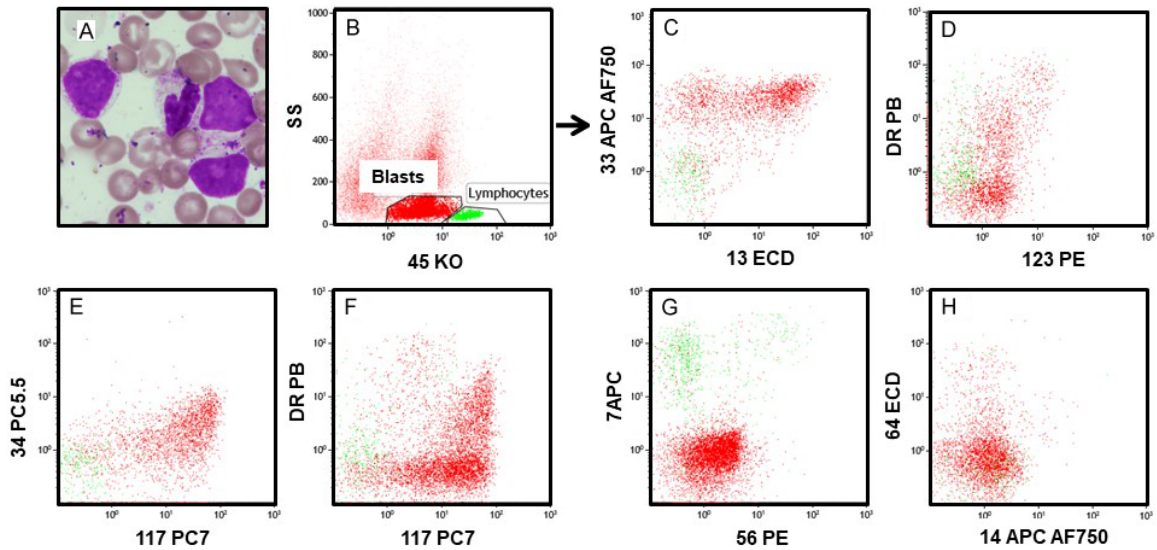


Figure 1. Morphological and flow cytometric characterization of blasts from case 1. (A) Peripheral blood smear showing myeloblasts (Jenner Giemsa; 1000X), (B-H) Blasts are CD45dim+, CD34dim+, CD117+, CD13+, CD33+, HLA-DR+, CD123-, CD7-, CD56-, CD14-, CD64-.

are initiator mutations present at a low allelic frequency during the beginning of the disease and are insufficient to lead to AML. The incidence of these mutations also known as ‘CHIP’ mutations (clonal hematopoiesis of indeterminate potential) [9-12] progressively increase with age to an incidence of ~10% in normal population older than 70 years with a risk of evolution to myeloid neoplasms at 1% per year. Few disease defining mutations with higher risk, also known as “driver mutations” like *BCR-ABL*, *JAK2*, *RUNX*, *FLT3*, *KRAS*, *HRAS*, called as clonal hematopoiesis of oncogenic potential (CHOP) have also been identified. An average of 13 genes per patient of AML were found to be mutated (23 genes being recurrently mutated). Moreover, half of the patients carried at least one sub-clone along with the founder clone [6]. There is partial adoption of these mutations into the clinical practice by European leukemia network (ELN) and National comprehensive Cancer network (NCCN). Both ELN and NCCN recommend *NPM1*, *FLT3*, *CEBPA* (biallelic), *RUNX1*, *ASXL1* and *TP53* testing for all patients with newly diagnosed AML. The NCCN also recommends testing for *IDH1/2* and *c-KIT* (CBF-AML). Based on these mutations, these guidelines risk stratify the patients and personalise treatment [8].

Here, we discuss two cases of AML and report their treatment outcome and disease course

wherein either the treatment initiation or modulation was guided by Next Generation Sequencing.

Case 1

A 68-year-old obese woman with type II Diabetes, had history of fever and repeated blood transfusions over the past two months. On evaluation, she was found to have anaemia, thrombocytopenia, and a white blood cell count (WBC) of 51,700 cells/mm³. Her bone marrow aspirate showed 60% CD45 dim positive myeloblasts which were immunoreactive for CD13, CD33, CD117 and were CD34dim. The blasts did not express cMPO, CD79a, cCD3, CD19, CD14, and HLA-DR. The patient was classified as M1 under the FAB classification (Figure 1). Cytogenetics studies showed normal 46XX karyotype. Molecular studies revealed *NPM1-A* mutation by allele-specific oligo PCR. Other molecular markers including *RUNX1-RUNX1T1*, *CBFB-MYH11* fusion transcripts and *FLT3-ITD* and *FLT3-TKD* mutations were not detected. Considering the age and poor performance status, patient was started on single agent Decitabine therapy (20 mg/m²/day IV for 5 days). After four cycles, the bone marrow was documented to be in complete morphological remission. However, the qualitative PCR for *NPM1-A* mutation remained positive. After eight cycles of decitabine, blasts reappeared in the peripheral

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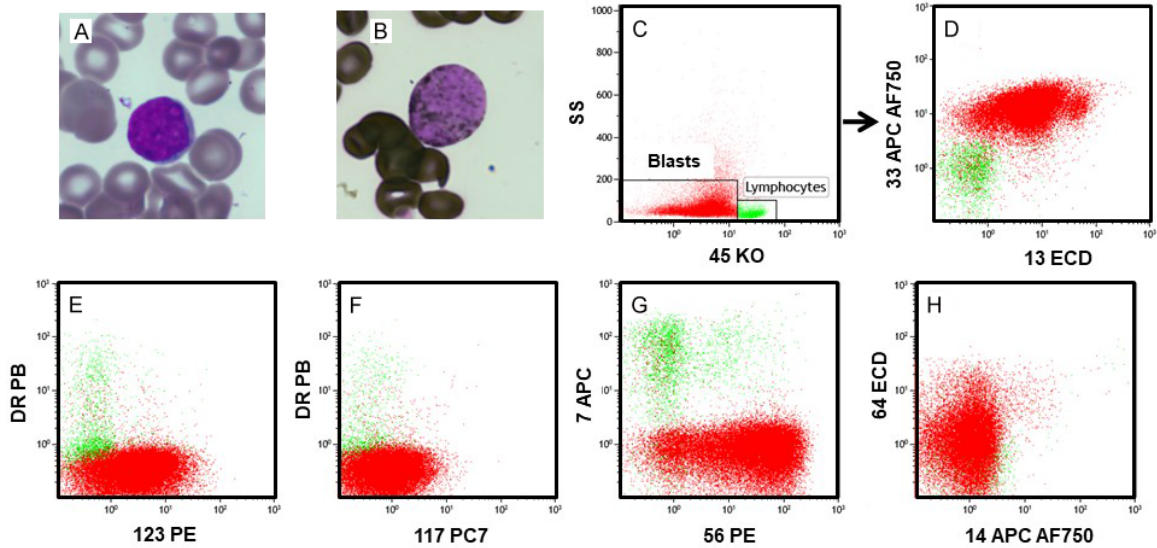


Figure 2. Morphological and flow cytometric characterization of blasts from case 2. (A) Peripheral blood smear showing myeloblasts (Jenner Giemsa; 1000X) (B) myeloblasts with cytochemical reactivity for Myeloperoxidase (1000X); (C-H) Blasts are CD45dim+, CD13+, CD33+, CD123+, CD117dim+, CD56+, HLA-DR-, CD7-, CD14-, CD64-.

blood (10%) and the bone marrow (22%). The patient was given a reduced intensity 3 + 5 induction chemotherapy regimen with Daunorubicin (45 mg/m²/day IV on days 1 to 3) and Cytarabine (100 mg/m²/day continuous IV infusion on days 1 to 5), following which morphological, immunophenotypic (<0.1%) and molecular remission (negative *NPM1-A* on ASO-PCR) was documented. Consolidation therapy with two cycles of high-dose cytarabine (HiDAc, 2 g/m² IV BD on days 1, 3, and 5, Total = 12 g/m²) was given.

A second relapse was seen after seven months with 84% blasts and the immunophenotypic profile similar to diagnostic time point. The patient also had chest infection at this point. The *NPM1-A* mutation was detected at second relapse at mutant to wild-type allelic ratio of 0.8 by fragment analysis. Targeted NGS revealed *DNMT3A* (p.R882H; COSM52944), and *IDH1* (p.R132H; COSM28746) missense mutations in addition to *NPM1-A* (p.W288Cfs*12; c.860_863dupTCTG). The *NPM1* (VAF: 0.42), *DNMT3A* (VAF: 0.3), and *IDH1* (VAF: 0.38) genes had a median VAF ≤0.5, suggesting these to be heterozygous mutations carried by most cells in the specimen rather than a subpopulation of the sequenced cells.

The patient was started on broad-spectrum antibiotics initially with prophylactic voricon-

azole and had responded. The fever and chest symptoms decreased. A combination regimen of Azacitidine (75 mg/m²/day SC for 7 days) and Venetoclax (10 mg PO OD on day 1, ramped up to 100 mg PO OD by day 4 due to co-administration with voriconazole) was initiated. Venetoclax was however stopped on day 9. The leucocytosis subsided quickly with complete clearance of peripheral blasts and appearance of severe pancytopenia. The patient again developed a recurrence of febrile neutropenia and succumbed to sepsis subsequently on day 19 of therapy.

Case 2

A 64-year-old lady with obesity and Type II Diabetes mellitus presented with complaints of increasing fatigue and worsening of anaemia over a period of four months. On evaluation, the peripheral blood smear showed 75% myeloblasts which were CD45dim+ with immunoreactivity for CD33, CD13, CD123, CD56, CD38, CD64, cMPO. The blasts were negative for cCD79a, CD19, CD7, cCD3, sCD3, CD34, CD16, CD11b, HLA-DR, CD36, CD4, CD14, cCD61, CD41, CD10, CD4, CD8, and CD57 (**Figure 2**). The haemoglobin, TLC and platelet count of the patient over the course of treatment have been summarised in **Table 2**. Molecular workup revealed a normal 46XX karyotype with mutations in *NPM1-A* (mutant to wild type allelic ratio

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Table 2. Periodic changes in CBC parameters over the duration of treatment

Time Point	Hb (g/dL)	TLC (/mm ³)	Platelets (/mm ³)	Blast (%)
Initial	6.7	4200	186000	-
Time of Presentation	8.2	9200	36000	75
D9 of 1# AZA	8.2	5520	5200	35
Post1# AZA	7.4	4900	4000	1
Post2#AZA+Sorafenib	9.2	4400	17000	0
Post3#AZA+Sorafenib	7.1	4500	57000	0
Post4#AZA+Sorafenib	6.8	5380	79000	0
Post5#AZA+Sorafenib	8.9	3570	118000	0
Post6#AZA+Sorafenib	9.7	3400	153000	0
Post7#AZA+Sorafenib	11.7	6430	187000	0
Post8#AZA+Sorafenib	11.6	8170	115000	0
Post9#AZA+Sorafenib	11.5	7060	154000	0, Flow-CR
Last Follow up	11.8	5970	179000	0

= 0.75) and *FLT3-ITD* (mutant 1 = 32 bp insertion; allelic ratio = 0.12 and mutant 2 = 41 bp insertion; allelic ratio = 0.4). Other common molecular markers including *RUNX1-RUNX1T1*, *CBFB-MYH11* fusion transcripts and *FLT3-TKD* mutation were not detected. Mutations in *NPM1* (p.W288Cfs*12; c.863_864insCCTG; COSM28937), and *TP53* (p.I195T; COSM116924 and p.V274D; COSM44448 and p.F270I; COSM437484) genes were detected by NGS. On account of poor performance status (ECOGII) and relatively advanced age, she was deemed 'unfit' for intensive chemotherapy, and was instead started on azacitidine (75 mg/m²/day SC/IV for seven days) and low-dose cytarabine (LD-AraC, 20 mg SC BD) therapy. In view of *FLT3-ITD* positive status, sorafenib (200 mg PO BD) was added from second chemotherapy cycle onwards. Post four cycles of therapy, patient attained both complete morphological and immunophenotypic remission. A year after the initial diagnosis, having completed 12 cycles with no significant toxicities, she continues to be in remission.

Discussion

The *NPM1*-mutated AML is the largest genetic group accounting for ~30% of all AML across both young and older patients and up to 50-60% of cytogenetically normal AML where it is slightly more common in females (56.2% vs. 43.8%) [7, 13-15]. The outcome is dependent upon the age of presentation [16-18], karyotype [15], co-occurring mutations especially

FLT3-ITD, *IDH-1/2*, and *DNMT3A* [16, 19, 20], probably allelic dominance [21-23], the type of treatment (intensive chemotherapy, HMA+Venetoclax) [18], and *NPM1*-negativity following treatment [24].

The present case 1 to summarize is a case of *NPM1*-mutated AML in an older lady, normal karyotype without any *FLT3-ITD* at the baseline who achieved CR without MRD-negativity and achieved second MRD-negative CR with intensive chemotherapy, which was

short lasting. The NGS performed at relapse revealed additional mutations including *DNMT3A*^{R882H} and *IDH1*^{R132}. Whether these mutations were present at baseline could not be established due to inadequate sample. Moreover, at baseline the *NPM1-A* mutant to wild-type allelic ratio could not be determined because of unavailability of the assay at that time. The impact of age, type of treatment, stability of *NPM1* mutation, co-occurring mutations on the survival are the essential points of discussion in the present case.

The favourable impact of *NPM1* mutation is diminished markedly in the older patients treated with intensive chemotherapy especially in the patients of >65 years with worse CR-rate (53% vs. 88%) and 2-year OS in the *NPM1*⁺*FLT3-ITD*⁻ cohort (27% vs. 70%; p<0.001) as compared with 55-65 year age group [20]. The outcome of HMA appears inferior in context of CR (28% vs. 56%) and survival (6 vs. 10.8 months; P = 0.076) as compared to intensive chemotherapy in >65 years although it is confounded by the fitness/performance status [18]. However, when HMA is combined with venetoclax, it appears to be more effective than intensive chemotherapy despite older age or poor fitness [18].

The *NPM1* mutations are usually secondary events following *DNMT3A*, *IDH1*, or *NRAS* mutations, but remains stable during the disease as in the present case [7, 24]. There are several

mutations which are gained (*WT1*, *FLT3-ITD/TKD*, *IDH-1/2*, *TP53*, *RAS*) and lost (*FLT3-TKD*, *RAS*, *PTPN11*, *CEBPA*) during the relapse. The presence of *IDH1*^{R132} in addition to *NPM1/DNMT3A* like in the present case [19], portends poor prognosis which is irrespective of the age when treated with intensive chemotherapy and an important finding in contrast to *FLT3-ITD* co-mutation which has an impact only in the younger subset and not in the older cases [15]. The *IDH1/2* co-mutations achieve better CR rates than *IDH*-wild type patients when treated with HMA+venetoclax combination and remains to be seen how durable they are in long-term follow up [18].

Venetoclax, an inhibitor of Bcl-2 has been approved by FDA to be given in newly diagnosed AML patients who seem to be unfit for intensive chemotherapy in combination with HMA for its remarkable activity and tolerability [25]. The maximum sensitivity was demonstrated in patients with *NPM1* mutation and *IDH-1/2* mutations. Venetoclax in combination with HMA at a low-intensity regimen has shown promising efficacy and a tolerable safety profile in elderly patients with AML. The HMA+Venetoclax combination appears to be promising in this subset of patients as its associated with low early mortality rates, a high CR + CRi rate of 73%, and OS >17 months [26]. Therefore, a comprehensive NGS panel is crucial in characterising these patients and would also be useful in treatment planning.

The learning from case 1 is that, *NPM1* mutated AML is a heterogenous category with diverse prognosis with age and co-occurring molecular mutations. The interactions between the mutations require more understanding in the future studies. At the same time, therapeutic decisions should take into account the molecular information in addition to fitness for intensive chemotherapy. Older AML patients with *NPM1* mutation may not have a good outcome [27] as compared to the younger patients with intensive chemotherapy especially patients with concurrent *DNMT3A/IDH-1/2* mutations.

In the absence of *FLT3-ITD* mutations, *NPM1* mutations are prognostically favourable [28]. It is well documented that the non-favourable impact of *FLT3-ITD* surpasses the prognostic benefit of *NPM1* mutation [29]. Stratification using both *NPM1* and *FLT3-ITD* markers identi-

fied three prognostic groups: *NPM1⁺FLT3-ITD⁻* (good), *NPM1⁻FLT3-ITD⁻* or *NPM1⁺FLT3-ITD⁺* (intermediate) and *NPM1⁻FLT3-ITD⁺* (poor) [29] assigning an intermediate risk category to our case 2. In cytogenetically normal AML, the frequency of *FLT3-ITD* ranges from 28-34%, whereas 39% in AML with *NPM1* mutation [7]. *FLT3* (FMS-like tyrosine kinase) is a transmembrane tyrosine kinase that is assigned to receptor tyrosine kinases (RTK) class III. These receptors are triggered by ligand binding that initiates a pro-proliferative signaling cascade. Approximately 30% of the AML patients have activating mutations in *FLT3* and these are not restricted to any specific AML subgroups. *FLT3*-internal tandem duplication (ITD) mutation within the cytoplasmic juxtamembrane (JM) region occurs at higher frequency (~25%) as compared to point mutations in the activation loop of the tyrosine kinase domain (*FLT3-TKD* mutation), such as the D835Y mutation (~7%) [13]. The *FLT3-ITD* mutations are associated with short duration of remission, high relapse rates after conventional induction therapy and poor prognosis [30]. When treated with conventional chemotherapy, patients with *FLT3-ITD* mutation and normal karyotype have reduced overall survival (OS) [31]. The levels of the cytokine *FLT3* ligand rise two- to three-log-fold in response to chemotherapy, and blasts with mutated *FLT3-ITD* are exquisitely sensitive to the rise in *FLT3* ligand levels. This suggests that post chemotherapy, this patient's residual blasts would be exposed to an environment rich in *FLT3* ligand and, hence, conducive to their continued growth; a phenomenon which explains the increased relapse rate and reduced overall survival reported in *FLT3-ITD* AML patients [32].

The length of in frame duplicated DNA in *FLT3-ITD* is variable and can range from three to greater than 400 base pairs (bp). Although the exact site of insertion varies from case to case [33], but it is primarily in frame and therefore generates a protein with functional kinase domain. It is reported that the frequency of *FLT3-ITDs* in an individual patient may vary from one to five different mutants of varying sizes and relative levels [34]. In our case 2, we detected two *FLT3-ITD* insertions of variable lengths. Discrepancies exist with regard to the *FLT3-ITD* mutation, although longer ITD length is associated with higher risk of relapse but this assessment is not included in the routine risk

stratification criteria [35, 36]. The size and insertion site of the inserted DNA may be of prognostic significance [37]. These insertions lead to constitutive activation of the receptor [38] and are associated with leucocytosis, a high percentage of bone marrow blasts, higher relapse incidence, and reduced disease-free survival [35, 39, 40].

One of the key factors that may influence the prognostic impact of FLT3-ITDs is the mutant/wild-type allelic ratio (AR) [33, 37]. Patients with AML harbouring *FLT3-ITD* with a high (≥ 0.5) allelic ratio have poor outcomes [41-43]. Recently, the 2017 ELN recommendations categorised FLT3-ITD genotypes based on the ITD allelic ratio and the *NPM1* mutational status into four distinct molecular subgroups [44]. However, it is proposed that *NPM1* and *FLT3-ITD* mutations alone are insufficient factors in AML prognosis, and the occurrence of *DNMT3A* mutation, may influence the decision-making for treatment options in *NPM1*-mutated AML [7, 45]. More recently, the prognostic impact of *NPM1*⁺*FLT3-ITD*⁺ mutation in adult AML was shown to be age-dependent [46]. Of note, *FLT3-ITD* indicated poor survival in younger patients (<60 years) but had no effect in older patients (60-74 years), whereas *NPM1* mutation indicated better survival in older patients as compared to younger patients. In patients with *NPM1* and *FLT3-ITD* dual mutations, the survival was less dependent on age than in the other molecular subgroups that is applicable in our case.

The TP53 mutations in *de-novo* AML are reported at a frequency of 5-10%. Patients with wild-type TP53 had significantly higher incidence of *NPM1*, *FLT3-ITD* and *DNMT3A* mutations as compared to the AML cases with TP53 mutations [47]. The presence of TP53 mutations is significantly associated with chemo-resistance, poor overall and disease-free survival. These mutations primarily occur in the TP53 region encoding DNA-binding domain covering exons 5-8. Of note, six mutational hot-spot codons were identified R175, G245, R248, R249, R273 and R282, of which R273 and R248 are recurrently mutated in AML [48]. The impact of these mutations on TP53 protein is either by direct disruption of the DBD or induction of conformational changes, thus resulting in impaired TP53 function. In our case 2, although the mutations

in TP53 were identified in the DBD region (I195T, V274D and F270I) but none of them were hot-spot residues hence implicating their relevance as most likely neutral in nature.

The FLT3 has emerged as a therapeutic target in AML and FLT3 inhibitors have shown promising results in combination with standard therapy. Both FLT3 and *NPM1* status have influence on risk stratification and the presence of FLT3-mutation, guides the inclusion of the multikinase inhibitor midostaurin [49] into primary treatment as was given in our patient. Moreover, these inhibitors are included as front-line drug and maintenance therapy after consolidation chemotherapy, in relapsed/refractory disease, or allogeneic stem cell transplantation. Midostaurin is a multi-targeted kinase inhibitor and when combined with standard chemotherapy in patients with *FLT3*-mutated AML, its known to prolong the overall survival (OS) and event-free survival (EFS) [50]. However, its use is limited by its cost, especially in resource limited countries including India. Sorafenib is a type II FLT3 inhibitor as it acts on both the active and kinetic state of the receptor. Tao et al [20] showed that mono-chemotherapy + Sorafenib significantly improved the overall response rate (ORR) but not the overall survival and relapse free survival. In patients ≥ 60 years of age, sorafenib combined with 7 + 3 showed two times better 1-year OS for the FLT3-ITD-mutated patients than the control group.

Conclusion

In the ever-evolving era, where molecular characteristics of almost every disease play a role in disease classification, prognostication and targeted therapy, AML is no exception. ELN and NCCN have recommended genetic testing for all new cases of AML for *NPM1*, *CEBPA*, *RUNX1*, *FLT3*, *TP53*, *ASXL1*. Molecular testing is useful in not only predicting outcome but also helps in detecting actionable mutations such as *FLT-3*, *IDH-1*, *IDH-2*, *NPM1* and *CBF*. Small sub-clones harbouring an unfavourable genetic mutation might later relapse as dominant clone and thus may require treatment as unfavourable or high-risk in the beginning of treatment itself. In the new NGS era, the current costs of this technique in a high output, resource limited health-care system will progressively become more affordable and hence NGS will be accessible ubiquitously. Conventional chemotherapy in

AML gives very poor overall survival rates and targeted chemo-immunotherapy against specific mutations may drastically improve patient survival and outcomes.

Disclosure of conflict of interest

None.

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References

- [1] De Kouchkovsky I and Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. *Blood Cancer J* 2016; 6: e441.
- [2] Howlader N, Noone A, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis D, Chen H, Feuer E and Cronin Ke. SEER Cancer statistics review, 1975-2017. Bethesda, MD: National Cancer Institute. <https://seer.cancer.gov/csr/1975-2017/> based on November 2019 SEER data submission, posted to the SEER web site, April 2020. Accessed 25/05/2020.
- [3] Ramos NR, Mo CC, Karp JE and Hourigan CS. Current approaches in the treatment of relapsed and refractory acute myeloid leukemia. *J Clin Med* 2015; 4: 665-695.
- [4] Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, Wartman LD, Lamprecht TL, Liu F, Xia J, Kandoth C, Fulton RS, McLellan MD, Dooling DJ, Wallis JW, Chen K, Harris CC, Schmidt HK, Kalicki-Veizer JM, Lu C, Zhang Q, Lin L, O'Laughlin MD, McMichael JF, Delehaunty KD, Fulton LA, Magrini VJ, McGrath SD, Demeter RT, Vickery TL, Hundal J, Cook LL, Swift GW, Reed JP, Alldredge PA, Wylie TN, Walker JR, Watson MA, Heath SE, Shannon WD, Varghese N, Nagarajan R, Payton JE, Baty JD, Kulkarni S, Klco JM, Tomasson MH, Westervelt P, Walter MJ, Graubert TA, DiPersio JF, Ding L, Mardis ER and Wilson RK. The origin and evolution of mutations in acute myeloid leukemia. *Cell* 2012; 150: 264-278.
- [5] Martelli MP, Sportoletti P, Tiacci E, Martelli MF and Falini B. Mutational landscape of AML with normal cytogenetics: biological and clinical implications. *Blood Rev* 2013; 27: 13-22.
- [6] Cancer Genome Atlas Research Network, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, Hoadley K, Triche TJ Jr, Laird PW, Baty JD, Fulton LL, Fulton R, Heath SE, Kalicki-Veizer J, Kandoth C, Klco JM, Koboldt DC, Kanchi KL, Kulkarni S, Lamprecht TL, Larson DE, Lin L, Lu C, McLellan MD, McMichael JF, Payton J, Schmidt H, Spencer DH, Tomasson MH, Wallis JW, Wartman LD, Watson MA, Welch J, Wendl MC, Ally A, Balasundaram M, Birol I, Butterfield Y, Chiu R, Chu A, Chuah E, Chun HJ, Corbett R, Dhalla N, Guin R, He A, Hirst C, Hirst M, Holt RA, Jones S, Karsan A, Lee D, Li HI, Marra MA, Mayo M, Moore RA, Mungall K, Parker J, Pleasance E, Plettner P, Schein J, Stoll D, Swanson L, Tam A, Thiessen N, Varhol R, Wye N, Zhao Y, Gabriel S, Getz G, Sougnez C, Zou L, Leiserson MD, Vandin F, Wu HT, Applebaum F, Baylin SB, Akbani R, Broom BM, Chen K, Motter TC, Nguyen K, Weinstein JN, Zhang N, Ferguson ML, Adams C, Black A, Bowen J, Gastier-Foster J, Grossman T, Lichtenberg T, Wise L, Davidsen T, Demchok JA, Shaw KR, Sheth M, Sofia HJ, Yang L, Downing JR and Eley G. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 2013; 368: 2059-2074.
- [7] Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, Potter NE, Heuser M, Thol F, Bolli N, Gundem G, Van Loo P, Martincorena I, Ganly P, Mudie L, McLaren S, O'Meara S, Raine K, Jones DR, Teague JW, Butler AP, Greaves MF, Ganser A, Döhner K, Schlenk RF, Döhner H and Campbell PJ. Genomic classification and prognosis in acute myeloid leukemia. *N Engl J Med* 2016; 374: 2209-2221.
- [8] Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T, Dombret H, Ebert BL, Fenaux P, Larson RA, Levine RL, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz M, Sierra J, Tallman MS, Tien HF, Wei AH, Lowenberg B and Bloomfield CD. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 2017; 129: 424-447.
- [9] Busque L, Patel JP, Figueroa ME, Vasanthakumar A, Provost S, Hamilou Z, Mollica L, Li J, Viale A, Heguy A, Hassimi M, Socci N, Bhatt PK, Gonen M, Mason CE, Melnick A, Godley LA, Brennan CW, Abdel-Wahab O and Levine RL. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet* 2012; 44: 1179-1181.
- [10] Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burt N, Chavez A, Higgins JM, Moltchanov V, Kuo FC, Kluk MJ, Henderson B, Kinnunen L, Koistinen HA, Ladenvall C, Getz G, Correa A, Banahan BF, Gabriel S, Kathiresan S, Stringham HM, McCarthy MI, Boehnke M, Tuomilehto J, Haiman C, Groop L, Atzmon G, Wilson JG, Neuberg D, Altshuler D and Ebert

- BL. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 2014; 371: 2488-2498.
- [11] Genovesi G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K, Mick E, Neale BM, Fromer M, Purcell SM, Svantesson O, Landén M, Höglund M, Lehmann S, Gabriel SB, Moran JL, Lander ES, Sullivan PF, Sklar P, Grönberg H, Hultman CM and McCarroll SA. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 2014; 371: 2477-2487.
- [12] McKerrell T, Park N, Moreno T, Grove CS, Ponstingl H, Stephens J, Crawley C, Craig J, Scott MA, Hodgkinson C, Baxter J, Rad R, Forsyth DR, Quail MA, Zeggini E, Ouwehand W, Varela I and Vassiliou GS. Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis. *Cell Rep* 2015; 10: 1239-1245.
- [13] Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, Van Vlierberghe P, Dolgalev I, Thomas S, Aminova O, Huberman K, Cheng J, Viale A, Socci ND, Heguy A, Cherry A, Vance G, Higgins RR, Ketterling RP, Gallagher RE, Litzow M, van den Brink MR, Lazarus HM, Rowe JM, Luger S, Ferrando A, Paietta E, Tallman MS, Melnick A, Abdel-Wahab O and Levine RL. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. *N Engl J Med* 2012; 366: 1079-1089.
- [14] Nagel G, Weber D, Fromm E, Erhardt S, Lübbert M, Fiedler W, Kindler T, Krauter J, Brossart P, Kündgen A, Salih HR, Westermann J, Wulf G, Hertenstein B, Wattad M, Götze K, Kraemer D, Heinicke T, Girschikofsky M, Derigs HG, Horst HA, Rudolph C, Heuser M, Göhring G, Teleanu V, Bullinger L, Thol F, Gaidzik VI, Paschka P, Döhner K, Ganser A, Döhner H and Schlenk RF. Epidemiological, genetic, and clinical characterization by age of newly diagnosed acute myeloid leukemia based on an academic population-based registry study (AMLSG BIO). *Ann Hematol* 2017; 96: 1993-2003.
- [15] Angenendt L, Röhlig C, Montesinos P, Martínez-Cuadrón D, Barragan E, García R, Botella C, Martínez P, Ravandi F, Kadia T, Kantarjian HM, Cortes J, Juliusson G, Lazarevic V, Höglund M, Lehmann S, Recher C, Pigneux A, Bertoli S, Dumas PY, Dombret H, Preudhomme C, Micol JB, Terré C, Ráčil Z, Novák J, Žák P, Wei AH, Tiong IS, Wall M, Estey E, Shaw C, Exeler R, Wagenführ L, Stölzel F, Thiede C, Stelljes M, Lenz G, Mikesch JH, Serve H, Ehninger G, Berdel WE, Kramer M, Krug U and Schliemann C. Chromosomal abnormalities and prognosis in NPM1-mutated acute myeloid leukemia: a pooled analysis of individual patient data from nine international cohorts. *J Clin Oncol* 2019; 37: 2632-2642.
- [16] Mrózek K, Marcucci G, Nicolet D, Maharry KS, Becker H, Whitman SP, Metzeler KH, Schwind S, Wu YZ, Kohlschmidt J, Pettenati MJ, Heerema NA, Block AW, Patil SR, Baer MR, Kolitz JE, Moore JO, Carroll AJ, Stone RM, Larson RA and Bloomfield CD. Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia. *J Clin Oncol* 2012; 30: 4515-4523.
- [17] Ostronoff F, Othus M, Lazenby M, Estey E, Appelbaum FR, Evans A, Godwin J, Gilkes A, Kopecky KJ, Burnett A, List AF, Fang M, Oehler VG, Petersdorf SH, Pogossova-Agadjanyan EL, Radich JP, Willman CL, Meshinchi S and Stirewalt DL. Prognostic significance of NPM1 mutations in the absence of FLT3-internal tandem duplication in older patients with acute myeloid leukemia: a SWOG and UK National Cancer Research Institute/Medical Research Council report. *J Clin Oncol* 2015; 33: 1157-1164.
- [18] Lachowiec CA, Loghavi S, Kadia TM, Daver N, Borthakur G, Pemmaraju N, Naqvi K, Alvarado Y, Yilmaz M, Short N, Ohanian M, Pierce SR, Patel KP, Qiao W, Ning J, Sasaki K, Takahashi K, Jabbour E, Andreeff M, Ravandi F, Kantarjian HM, Konopleva M and Di Nardo CD. Outcomes of older patients with NPM1-mutated AML: current treatments and the promise of venetoclax-based regimens. *Blood Adv* 2020; 4: 1311-1320.
- [19] Dunlap JB, Leonard J, Rosenberg M, Cook R, Press R, Fan G, Raess PW, Druker BJ and Traer E. The combination of NPM1, DNMT3A, and IDH1/2 mutations leads to inferior overall survival in AML. *Am J Hematol* 2019; 94: 913-920.
- [20] Tao S, Wang C, Chen Y, Deng Y, Song L, Shi Y, Ling L, Ding B, He Z and Yu L. Prognosis and outcome of patients with acute myeloid leukemia based on FLT3-ITD mutation with or without additional abnormal cytogenetics. *Oncol Lett* 2019; 18: 6766-6774.
- [21] Patel SS, Kuo FC, Gibson CJ, Steensma DP, Soiffer RJ, Alyea EP 3rd, Chen YA, Fathi AT, Graubert TA, Brunner AM, Wadleigh M, Stone RM, DeAngelo DJ, Nardi V, Hasserjian RP and Weinberg OK. High NPM1-mutant allele burden at diagnosis predicts unfavorable outcomes in de novo AML. *Blood* 2018; 131: 2816-2825.
- [22] Abbas HA, Ravandi F, Loghavi S, Patel KP, Borthakur G, Kadia TM, Jabbour E, Takahashi K, Cortes J, Issa GC, Konopleva M, Kantarjian HM and Short NJ. NPM1 mutant variant allele frequency correlates with leukemia burden but does not provide prognostic information in

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- NPM1-mutated acute myeloid leukemia. *Am J Hematol* 2019; 94: E158-E160.
- [23] Linch DC, Hills RK, Burnett AK, Russell N and Gale RE. Analysis of the clinical impact of NPM1 mutant allele burden in a large cohort of younger adult patients with acute myeloid leukaemia. *Br J Haematol* 2020; 188: 852-859.
- [24] Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, Patel Y, Bhudia N, Farah H, Mason J, Wall K, Akiki S, Griffiths M, Solomon E, McCaughan F, Linch DC, Gale RE, Vyas P, Freeman SD, Russell N, Burnett AK and Grimwade D. Assessment of minimal residual disease in standard-risk AML. *N Engl J Med* 2016; 374: 422-433.
- [25] DiNardo CD, Kantarjian H, Konopleva M, Pratz KW, Letai A, Jonas BA, Wei AH, Thirman M, Arellano M, Frattini MG, Popovic R, Chyla B, Xu T, Dunbar M, Agarwal SK, Humerickhouse R, Mabry M, Potluri J and Pollyea DA. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol* 2018; 19: 216-228.
- [26] DiNardo CD, Pratz K, Pullarkat V, Jonas BA, Arellano M, Becker PS, Frankfurt O, Konopleva M, Wei AH, Kantarjian HM, Xu T, Hong WJ, Chyla B, Potluri J, Pollyea DA and Letai A. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood* 2019; 133: 7-17.
- [27] Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI and Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. *Leukemia* 2017; 31: 798-807.
- [28] Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, Bullinger L, Fröhling S and Döhner H. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood* 2005; 106: 3740-3746.
- [29] Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK and Linch DC. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood* 2008; 111: 2776-2784.
- [30] Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, Walker H, Wheatley K, Bowen DT, Burnett AK, Goldstone AH and Linch DC. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* 2001; 98: 1752-1759.
- [31] Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, Habdank M, Späth D, Morgan M, Benner A, Schlegelberger B, Heil G, Ganser A and Döhner H; German-Auid Leukemia Study Group. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* 2008; 358: 1909-1918.
- [32] Zheng R, Bailey E, Nguyen B, Yang X, Piloto O, Levis M and Small D. Further activation of FLT3 mutants by FLT3 ligand. *Oncogene* 2011; 30: 4004-4014.
- [33] Breitenbuecher F, Schnittger S, Grundler R, Markova B, Carius B, Brecht A, Duyster J, Haferlach T, Huber C and Fischer T. Identification of a novel type of ITD mutations located in non-juxtamembrane domains of the FLT3 tyrosine kinase receptor. *Blood* 2009; 113: 4074-4077.
- [34] Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, Löffler H, Sauerland CM, Serve H, Büchner T, Haferlach T and Hidde-mann W. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 2002; 100: 59-66.
- [35] Stirewalt DL, Kopecky KJ, Meshinchi S, Engel JH, Pogossova-Agadjanyan EL, Linsley J, Slovak ML, Willman CL and Radich JP. Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. *Blood* 2006; 107: 3724-3726.
- [36] Ponziani V, Gianfaldoni G, Mannelli F, Leoni F, Ciolli S, Guglielmelli P, Antonioli E, Longo G, Bosi A and Vannucchi AM. The size of duplication does not add to the prognostic significance of FLT3 internal tandem duplication in acute myeloid leukemia patients. *Leukemia* 2006; 20: 2074-2076.
- [37] Schlenk RF, Kayser S, Bullinger L, Kobbe G, Casper J, Ringhoffer M, Held G, Brossart P, Lübbert M, Salih HR, Kindler T, Horst HA, Wulf G, Nachbaur D, Götze K, Lamparter A, Paschka P, Gaidzik VI, Teleanu V, Späth D, Benner A, Krauter J, Ganser A, Döhner H and Döhner K. Differential impact of allelic ratio and insertion site in FLT3-ITD-positive AML with respect to allogeneic transplantation. *Blood* 2014; 124: 3441-3449.
- [38] Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Muller C, Gruning W, Kratz-Albers K, Serve S, Steur C, Buchner T, Kienast J, Kanakura Y, Berdel WE and Serve H. FLT3 mutations from patients with acute myeloid leukemia

- mia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood* 2000; 96: 3907-3914.
- [39] Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, Wermke M, Bornhäuser M, Ritter M, Neubauer A, Ehninger G and Illmer T. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002; 99: 4326-4335.
- [40] Brunet S, Labopin M, Esteve J, Cornelissen J, Socié G, Iori AP, Verdonck LF, Volin L, Gratwohl A, Sierra J, Mohty M and Rocha V. Impact of FLT3 internal tandem duplication on the outcome of related and unrelated hematopoietic transplantation for adult acute myeloid leukemia in first remission: a retrospective analysis. *J Clin Oncol* 2012; 30: 735-741.
- [41] Pratcorona M, Brunet S, Nomdedéu J, Ribera JM, Tormo M, Duarte R, Escoda L, Guàrdia R, Queipo de Llano MP, Salamero O, Bargay J, Pedro C, Martí JM, Torreadell M, Díaz-Beyá M, Camós M, Colomer D, Hoyos M, Sierra J and Esteve J; Grupo Cooperativo Para el Estudio y Tratamiento de las Leucemias Agudas Mieloblásticas. Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1 mutation: relevance to post-remission therapy. *Blood* 2013; 121: 2734-2738.
- [42] Blau O, Berenstein R, Sindram A and Blau IW. Molecular analysis of different FLT3-ITD mutations in acute myeloid leukemia. *Leuk Lymphoma* 2013; 54: 145-152.
- [43] Linch DC, Hills RK, Burnett AK, Khwaja A and Gale RE. Impact of FLT3ITD mutant allele level on relapse risk in intermediate-risk acute myeloid leukemia. *Blood* 2014; 124: 273-276.
- [44] Dohner K, Thiede C, Jahn N, Panina E, Gambi-etz A, Larson RA, Prior TW, Marcucci G, Jones D, Krauter J, Heuser M, Voso MT, Ottone T, Nomdedeu JF, Mandrekar SJ, Klisovic RB, Wei AH, Sierra J, Sanz MA, Brandwein JM, de Witte T, Jansen JH, Niederwieser D, Appelbaum FR, Medeiros BC, Tallman MS, Schlenk RF, Ganser A, Serve H, Ehninger G, Amadori S, Gathmann I, Benner A, Pallaud C, Stone RM, Dohner H and Bloomfield CD. Impact of NPM1/FLT3-ITD genotypes defined by the 2017 European LeukemiaNet in patients with acute myeloid leukemia. *Blood* 2020; 135: 371-380.
- [45] Ma J, Dunlap J, Paliga A, Traer E, Press R, Shen L and Fan G. DNMT3A co-mutation is required for FLT3-ITD as an adverse prognostic indicator in intermediate-risk cytogenetic group AML. *Leuk Lymphoma* 2018; 59: 1938-1948.
- [46] Juliusson G, Jädersten M, Deneberg S, Lehmann S, Mollgard L, Wennstrom L, Antunovic P, Cammenga J, Lorenz F, Olander E, Lj Lazarevic V and Hoglund M. The prognostic impact of FLT3-ITD and NPM1 mutation in adult AML is age-dependent in the population-based setting. *Blood Adv* 2020; 4: 1094-1101.
- [47] Bowen D, Groves MJ, Burnett AK, Patel Y, Allen C, Green C, Gale RE, Hills R and Linch DC. TP53 gene mutation is frequent in patients with acute myeloid leukemia and complex karyotype, and is associated with very poor prognosis. *Leukemia* 2009; 23: 203-206.
- [48] Kadia TM, Jain P, Ravandi F, Garcia-Manero G, Andreef M, Takahashi K, Borthakur G, Jabbour E, Konopleva M, Daver NG, Dinardo C, Pierce S, Kanagal-Shamanna R, Patel K, Estrov Z, Cortes J and Kantarjian HM. TP53 mutations in newly diagnosed acute myeloid leukemia: Clinicomolecular characteristics, response to therapy, and outcomes. *Cancer* 2016; 122: 3484-3491.
- [49] Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, Thiede C, Prior TW, Dohner K, Marcucci G, Lo-Coco F, Klisovic RB, Wei A, Sierra J, Sanz MA, Brandwein JM, De Witte T, Niederwieser D, Appelbaum FR, Medeiros BC, Tallman MS, Krauter J, Schlenk RF, Ganser A, Serve H, Ehninger G, Amadori S, Larson RA and Dohner H. Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *N Engl J Med* 2017; 377: 454-464.
- [50] Short NJ, Kantarjian H, Ravandi F and Daver N. Emerging treatment paradigms with FLT3 inhibitors in acute myeloid leukemia. *Ther Adv Hematol* 2019; 10: 1-18.