Correlation of Next-Generation Sequencing Based Genotypic Profiles with MICM Characteristics in *NPM1* Mutated Acute Myeloid Leukemia

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Abstract

The purpose of this study was to analyze association between next-generation sequencing (NGS) genomic profile and conventional MICM characteristics in patients with acute myeloid leukemia (AML) with *NPM1* mutation (*NPM1*^{mut}). We selected 238 *NPM1*^{mut} patients with available NGS information on 112 genes related to blood diseases, using χ^2 and Mann-Whitney U test to analyze the distribution correlation between genomic alterations and MICM parameters. Compared with *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾, the *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group presented a slightly common M5 [78/143 (54.5%) vs. 64/95 (67.4%); $P=0.048$], more higher CD34 and CD7 expression rates (CD34: 20.6% vs. 47.9%, $P<0.001$; CD7: 29.9% vs. 61.5%, $P<0.001$), and lack of favorable- and adverse-risk karyotypes (6.4% vs. 0%; $P=0.031$). In entire *NPM1*^{mut} cohort, totaling 240 *NPM1* mutation events were identified, of whom 10 (10/240, 4.2%) were missense types. When confining analysis to the 205 cases with *NPM1*^{mut} insertions/deletions type and normal karyotype, multivariable logistic analysis showed that *FLT3*-ITD was positively correlated with CD34 and CD7 expression ($HR=5.29$ [95% CI 2.64-10.60], $P<0.001$; $HR=3.47$ [95% CI 1.79-6.73], $P<0.001$; respectively). Ras-pathway mutation was positively correlated with HLA-DR expression ($HR=4.05$ [95% CI 1.70-9.63], $P=0.002$), and *KRAS* mutation negatively with MPO expression ($HR=0.18$ [95% CI 0.05-0.62], $P=0.007$). *DNMT3A*-R882 was positively correlated with CD7 and HLA-DR expression ($HR=3.59$ [95% CI 1.80-7.16], $P<0.001$; $HR=13.41$ [95% CI 4.56-39.45], $P<0.001$; respectively). *DNMT3A* mutation was negatively correlated with MPO expression ($HR=0.35$ [95% CI 1.48-8.38], $P=0.004$). *TET2*/*IDH1* mutations were negatively correlated with CD34 and CD7 expression ($HR=0.26$ [95% CI 0.11-0.62], $P=0.002$; $HR=0.30$ [95% CI 0.14-0.62], $P=0.001$; respectively), and positively with MPO expression ($HR=3.52$ [95% CI 1.48-8.38], $P=0.004$). *NPM1*^{mut} co-existing mutations in signaling pathways (*FLT3*-ITD and Ras-pathway) and methylation modifiers (*DNMT3A* and *TET2*/*IDH1*) are linked with the expression of CD34, CD7, HLA-DR and MPO, thereby providing a mechanism explanation for the immunophenotypic heterogeneity of this AML entity.

Background

The human *NPM1* gene, located on chromosome 5q35.1 and containing 12 exons, encodes for a nucleolar phosphoprotein which possesses multifunctions including chromatin remodeling, ribosome biogenesis, genomic stability, regulation of tumor suppressors and transcription factors.(1–3) Given its biological diversity and important role, the functional category of *NPM1* belongs to a separate one according to TCGA data.(4)

NPM1 gene abnormalities are involved in fusion(5), deletion(2) and mutation, therein, the mutation is most largely studied. The incidence of *NPM1* mutation (*NPM1*^{mut}) occupies approximately one-third of cases with *de novo* acute myeloid leukemia (AML), and up to ~ 60% in normal karyotype (NK) AML.(6, 7) The clinicobiological presentation of *NPM1*^{mut} AML are characterized by multiple aspects of MICM typing. For instance, French-American-British (FAB) morphologies commonly have monocytic differentiation (M4 or M5)(8, 9), and are likely to have cup-like nuclei.(10) Immunophenotypically, most *NPM1*^{mut} cases showed CD34 negativity.(11) According to analysis of the myeloid blast population, nearly half of *NPM1*^{mut} patients show an acute promyelocytic leukemia (APL)-like antigen expression feature represented by CD34⁽⁻⁾/HLA-DR⁽⁻⁾/MPO^(str+).(12) *NPM1*^{mut} AML mainly arises in a NK situation, mutually exclusive with recurrent cytogenetic abnormalities.(7, 13) *NPM1*^{mut} AML has unique gene expression profiles, especially the overexpression of HOX family members.(14)

Immunophenotype is not only used in the differential diagnosis of AML, but also has prognostic value. The CD34⁽⁺⁾(11, 15), leukemic stem cells (LSCs) phenotype CD34⁽⁺⁾/CD38⁽⁻⁾/CD123⁽⁺⁾(16), APL-like phenotype CD34⁽⁻⁾/HLA-DR⁽⁻⁾/MPO^(str+)(12) and clustered type-Ⅱ phenotype CD34⁽⁺⁾/HLA-DR⁽⁺⁾/CD7⁽⁺⁾(17) have been reported to convey prognostic effects on *NPM1*^{mut} AML. However, data on genetic information were less integrated into analysis in those relatively earlier studies. In recent years, studies regarding prognostic heterogeneity in *NPM1*^{mut} AML mainly focused on cytogenetic and gene mutations. Comutations in *DNMT3A*(18), *TET2*(19, 20) or *IDH1/2*(21, 22) have been shown as an adverse predictor, and *NRAS*(23), *FLT3*-TKD(24) or *moCEBPA*(25) as a favorable predictor on clinical outcome in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾/low AML.

Because *NPM1*^{mut} AML is mainly seen in an intermediate-risk cytogenetics, especially in NK background, we hypothesize that the diversity of leukemic phenotypes depends to a certain extent on the heterogeneity of coexisting gene mutations in this subtype of AML. Whole genome or exome sequencing revealed an average of 13 mutations in AML(7), indicating the interplay across mutations as an important pathomechanism of leukemic development and overt onset.

In addition, the *NPM1*^{mut} in association with prognostication is generally described as insertions and/or deletions (indels), which is predominantly characterized by 4 base pair (bp) insertion in the C-terminus within exon 12 and results in a frameshift consequence. However, data involving other mutational types of *NPM1*^{mut} were scarcely reported. Moreover, the types of *NPM1*^{mut} were not specifically designated in AML classification and treatment guidelines.(26, 27) The development of large-scale parallel sequencing technology, with its enlargement of higher throughput and wider coverage, is bound to detect more diversified mutational loci and types within the *NPM1* gene, as well as more concurrent mutations.

In this study, we selected newly diagnosed patients with *de novo* *NPM1*^{mut} AML and evaluate the correlations of MICM features with next-generation sequencing (NGS) determining genetic alterations in 112 genes related to blood diseases, aiming to profoundly understand the clinicopathological heterogeneity of this AML subtype.

Patients And Methods

Patient selection and MICM workup

We performed a retrospective review of newly diagnosed *de novo* AML patients in our institute and Shengjing Hospital of China Medical University from October 2014 to September 2019. AML diagnosis fulfilled the World Health Organization (WHO) criteria(28), according to which the MICM workup included cytomorphology, immunotyping, chromosome karyotyping and fluorescence in situ hybridization (FISH), molecular biology and gene mutation analysis (see below). The cytomorphological subtype was based on the FAB classification. Immunophenotyping was performed on freshly EDTA-anticoagulated or heparinized bone marrow (BM) or peripheral blood (PB) samples obtained at the time of initial diagnosis. The four-color analysis was conducted on FACSCalibur (Becton-Dickinson, USA) using the following sets of FITC (fluorescein-isothiocyanate), PE (phycoerythrin), PerCP (peridinin-chlorophyll-protein) and APC (allophycocyanin) labeled mouse antihuman fluorescent monoclonal antibodies: 1) CD34/CD10/CD45/CD19; 2) CD7/CD117/CD45/CD33; 3) CD9/CD2/CD45/CD56; 4) CD15/CD38/CD45/HLA-DR; 5) CD16/CD13/CD45/CD11b; 6) CD4/CD64/CD45/CD14; 7) cMPO/cCD79a/CD45/cCD3; and 8) TdT/CD123/CD45/HLA-DR. G-band karyotyping analysis was conducted using BM aspirate samples. When obtaining BM samples was difficult, PB was used instead. A total of 20 metaphase cells were analyzed for each patient, chromosomal abnormalities described according to the International System for Human Cytogenetic Nomenclature.(29) Additionally, *KMT2A* (*MLL*) rearrangements (11q23 abnormality) were verified by fluorescence in situ hybridization (FISH) using Dual-Color, Break-Apart Rearrangement Probe (Vysis, USA), and *TP53* deletions (17p-) by locus-specific Probe (Vysis, USA), respectively. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board (IRB) of all the participating institutions. All patients provided written informed consent for using their records.

Detection of mutations by NGS and conventional methods

Genomic DNA extraction (Qiagen, Hilden, Germany), quality control and quantification measurement (Nanodrop Technologies, USA), ultrasonic fragmentation (Covaris, USA), library construction and target enrichment (SureSelect, Agilent Technologies, USA; Illumina, San Diego, USA) were conducted according to the manufacturers' protocols. High-throughput targeted measurement of gene mutations were performed on an Ion torrent MiSeq/HiSeq (Illumina) sequencer or PGM™ (Life Technologies) platform with an average sequencing depth of 800×. The custom-designed panel consisted of 112 potentially mutated genes involved in hematological disorders which are related to the following functional categories: signaling pathways, epigenetic regulators, **transcription factors**, spliceosomes, cohesin complex, tumor suppressors, *NPM1* and others. Single nucleotide variants (SNVs) and short fragment indels in protein coding sequence (CDS) were analyzed by using Ion

Reporter™ and Variant Reporter pipelines and annotated referencing to the dbSNP, 1000 Genomes, Polyphen-2 and COSMIC databases. *NPM1* (exon12), *FLT3*-ITD, and potential complex indels in *CEBPA* (TAD and bZIP domains) were additionally examined by the PCR followed by direct sequencing as previously reported.(30-32)

Statistical analysis

Descriptive statistics were presented as "median (range)" for non-normally distributed variables and "frequency (incidence)" for categorical variables. The χ^2 test and Mann-Whitney U test were used to calculate statistical significance for associations between coexisting mutations and MICM features. To extract independent factors, those with a *P*-value <0.15 were included as covariates in multivariate Logistic model using the forward stepwise selection procedure. All calculations were performed applying IBM SPSS v25.0 for Windows. In all analyses, *P*-values <0.05 were considered statistically significant. The Microsoft Excel 2013, Graphpad Prism 7.03, and Circos-0.69-9 software were also used for figure plotting.

Results

FAB subtypes of *NPM1*^{mut} AML

In this study, we picked out 238 patients with *NPM1*^{mut} AML for our purpose analysis. The study cohort consisted of 105 males and 133 females, with a median age of 49 (range 15–81) years. The most common FAB subtypes of *NPM1*^{mut} AML were AML-M5 (59.7%), followed by M2 (17.6%) and M4 (15.5%), similar to other findings.(8, 9) According to *FLT3*-ITD, M2 is more common in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group [34/143 (23.8%) vs. 8/95 (8.4%); *P* = 0.002], while M5 is slightly more common in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group [64/95 (67.4%) vs. 78/143 (54.5%); *P* = 0.048], as shown in Fig. 1a.

The expression incidence of CD34 and CD7 in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group were higher than in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group.

As per literature(33), The leukemic blasts at the initial diagnosis could be divided into leukemic myeloid blasts and leukemic immature monocyte populations, with the latter detected in about 50% cases and mostly in M4 or M5 morphologic subtypes. The leukemic myeloid blasts recurred when AML relapsed, while leukemic immature monocyte populations often disappeared, indicating that the leukemic myeloid blasts may enrich more LSCs which serve as source of disease relapse. Consequently, in the description of baseline FCM characteristic, we only analyzed the antigen expression aspects of leukemic myeloid cells. In entire *NPM1*^{mut} cohort, the antigens positively expressed at an incidence of 80% or more were CD117 (211/235, 89.8%), CD13 (207/233, 88.8%), CD33 (233/233, 100%), CD123 (230/232, 99.1%) and CD38 (209/233, 89.7%). The positive incidence of CD34 and TDT were 31.5% (74/235) and 6.9% (16/231), respectively. The positive incidence of HLA-DR was 67.4% (157/233) and MPO was 74.9% (57/227). CD7 was positively expressed in 43.1% (94/218), CD19 in 3.5% (8/228) and CD79a in 0.4% (1/227) cases, as shown in Fig. 1b. According to *FLT3*-ITD, the positive incidence of CD34 and CD7 in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ were significantly higher than in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group (CD34: 47.9% vs. 20.6%, *P* < 0.001; CD7: 61.5% vs. 29.9%, *P* < 0.001), while the positive incidence of other antigens were not different between the two genotypic groups.

Chromosomal karyotypes in *NPM1*^{mut} AML

Of all 238 patients with *NPM1*^{mut}, 234 patients had evaluable metaphases, in whom 208 (88.9%) were normal karyotypes (NK) and 26 (11.1%) were abnormal karyotypes. Among 143 cases with *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾, 140 had evaluable metaphases, with 131 cases in the intermediate-risk group (including 121 cases NK; 10 cases intermediate-risk abnormal karyotype, Fig. 1c), 5 cases in the favorable-risk group [including 4 cases t(8;21)(q22;q22); 1 case inv (16) (p13q22)] and 4 cases in the adverse-risk group (including 1 case complex karyotype, monosomy karyotype, t(6;9)(p23;q34), t(8;9;22)(q24;q34;q11.2) for each]. Among 95 cases with *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾, 94 had evaluable metaphases, with all of them in the intermediate-risk group (including 87 cases NK; 7 cases intermediate-risk abnormal karyotype, Fig. 1c) and none in the favorable- or adverse-risk group. There was no difference in the distribution of both NK and intermediate-risk abnormal karyotype between *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ and *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group (*P* = 0.144 and 0.930, respectively), while the

favorable- and adverse-risk karyotypes were only enriched in the *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group and not in the *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group (6.4% vs. 0%; *P* = 0.031). No correlation was found between other coexisting gene mutations and abnormal karyotypes (all *P* > 0.05, data not shown). No *KMT2A* (*MLL*) or *TP53* rearrangement was identified in 206 *NPM1*^{mut} patients with available FISH data.

NPM1^{mut} loci, types and co-mutation patterns

In entire *NPM1*^{mut} cohort, totaling 240 *NPM1* mutant events were identified, among whom 230 (230/240, 95.8%) were out-of-frame indels. Ten cases (10/240, 4.2%) were detected to harbour *NPM1*^{mut} missense events, i.e., 3 cases with c.578A > G→p.K193R, 2 with c.676G > A→p.E226K and 5 with c.733G > C→p.E245Q. All these missense codons did not disrupt any of the tryptophan residues W288 and W290 which are indispensable for the nucleolar localization signal (NoLS). Furthermore, all but one of these missense mutations (9/10, 90.0%) were accompanied by an AML subtype-defining recurrent cytogenetic or molecular abnormality, with 7 cases in the favorable risk and 2 in adverse risk (Table 1).

Table 1
AML subtype-defining cytogenetic or molecular abnormalities accompanied by *NPM1*^{mut} missense mutations

<i>NPM1</i> ^{mut} missense	AML subtype-defining cytogenetic or molecular abnormalities
c.578A > G→p.K193R	t(8;21)(q22;q22),-Y
c.578A > G→p.K193R	inv(16)(p13q22)
c.578A > G→p.K193R	t(6;9)(p23;q34)
c.676G > A→p.E226K	<i>NPM1</i> ^{mut} [p.W288Cfs*12]
c.676G > A→p.E226K	<i>CEBPA</i> ^{dm} [p.E309_D322indelsGQTQQKVLELTS; p.D80Gfs]
c.733G > C→p.E245Q	t(8;21)(q22;q22)
c.733G > C→p.E245Q	Complex: +8,-14,i(17)(q10),+2 ~ 3mar
c.733G > C→p.E245Q	<i>NPM1</i> ^{mut} [p.W288Cfs*12]
c.733G > C→p.E245Q	<i>CEBPA</i> ^{dm} [p.K313dupK; p.Q87X]
c.733G > C→p.E245Q	—
Abbreviations: <i>NPM1</i> ^{mut} , <i>NPM1</i> mutation; <i>CEBPA</i> ^{dm} , double allele mutation of <i>CEBPA</i>	

At least one co-mutation was detected in all 238 *NPM1*^{mut} cases. Including *NPM1*^{mut}, the median number of mutated genes per individual was 4.5 (2–14), with 4.0 (2–14) in the *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾, insignificantly different from 5.0 (2–10) in the *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group (*P* = 0.378, Fig. 2a). According to gene function categories, the order of incidence is as follows: signaling pathways (72.7%), epigenetic regulators (71.4%), tumor suppressors (31.9%) and myeloid transcription factors (8.8%, Fig. 2b). *DNMT3A* (104, 43.7%), *FLT3*-ITD (95, 39.9%) and *FAT1* (57, 23.9%) represented the top three most frequently mutated genes (more details on relatively common genes with a mutation frequency > 5% are listed in Fig. 3). Analysis of gene-gene relationship across *NPM1*^{mut} coexisting mutations showed that *FLT3*-ITD and *DNMT3A* had significant accompaniment (*P* = 0.005), while *FLT3*-ITD was mutually exclusive of *FLT3*-nonITD (*P* < 0.001), *NRAS* (*P* < 0.001), *PTPN11* (*P* = 0.017) and *IDH1* (*P* = 0.005, Fig. 4).

Association between *NPM1*^{mut} coexisting mutations and immunophenotypic markers

Our results have shown that the expression of CD34 and CD7 is significantly associated with *FLT3*-ITD. Because *NPM1*^{mut} AML mostly occurs in the context of NK, we hypothesize that the diversity of antigen expression in leukemia cells to a certain extent is determined by heterogeneity of coexisting mutations. In order to rule out the influence of abnormal karyotype on immunophenotype, as well in view of the deductively insufficient pathogenicity of *NPM1*^{mut} missense mutations, only patients with NK and *NPM1*^{mut} indels type were included for subsequent analysis. A total of 205 *NPM1*^{mut} patients meeting the above conditions were submitted to the distributional crosstabulation between immunophenotypic markers and coexisting mutations. The significant results from χ^2 test and multivariate analysis are shown in Table 2. Logistic analysis showed that, in entire *NPM1*^{mut} cohort, *FLT3*-ITD was positively correlated with CD34 and CD7 (*HR* = 5.29 [95% CI 2.64–10.60], *P* < 0.001; *HR* = 3.47 [95% CI 1.79–6.73], *P* < 0.001). Ras-pathway mutation was positively correlated with HLA-DR expression (*HR* = 4.05 [95% CI 1.70–9.63], *P* = 0.002) and negatively with MPO expression (*HR* = 0.18 [95% CI 0.05–0.62], *P* = 0.007) in entire *NPM1*^{mut} cohort. Stratified analysis indicated that this effect was only seen in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group (*HR* and *P* values are detailed in Table 2), but not in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group.

Table 2

Correlation between immunophenotypic markers with mutations of coexisting genes in *NPM1*^{mut} AML

Association		<i>NPM1</i> ^{mut} / <i>FLT3</i> TD ⁽⁻⁾			<i>NPM1</i> ^{mut} / <i>FLT3</i> TD ⁽⁺⁾			Entire cohort		
		χ ² P	HR (95%CI)	P	χ ² P	HR (95%CI)	P	χ ² P	HR (95%CI)	P
CD34 (N = 202)	× <i>FLT3</i> TD	NA	NA	NA	NA	NA	NA	< 0.001	5.29 (2.64–10.60)	< 0.001
	× <i>DNMT3A</i>	NS	NA	NA	0.028	2.60 (1.00–6.79)	0.051	0.026	NA	NA
	× <i>TET2</i> / <i>IDH1</i>	NS	NA	NA	0.005	0.21 (0.06–0.71)	0.012	0.001	0.26 (0.11–0.62)	0.002
CD7 (N = 186)	× <i>FLT3</i> TD	NA	NA	NA	NA	NA	NA	< 0.001	3.47 (1.79–6.73)	< 0.001
	× <i>DNMT3A</i>	0.008	NA	NA	0.007	3.30 (1.15–9.46)	0.026	< 0.001	NA	NA
	× <i>DNMT3A</i> -R882	0.002	3.93 (1.61–9.59)	0.003	0.009	NA	NA	< 0.001	3.59 (1.80–7.16)	< 0.001
	× <i>TET2</i> / <i>IDH1</i>	0.048	NA	NA	0.001	0.18 (0.05–0.60)	0.005	NS	0.30 (0.14–0.62)	0.001
HLA-DR (N = 200)	×Ras pathways	0.002	3.83 (1.40–10.46)	0.009	0.055	NA	NA	< 0.001	4.05 (1.70–9.63)	0.002
	× <i>DNMT3A</i> -R882	< 0.001	26.77 (3.44–208.46)	0.002	< 0.001	8.65 (2.28–32.89)	0.002	< 0.001	13.41 (4.56–39.45)	< 0.001
	× <i>TET2</i> / <i>IDH1</i>	NS	NA	NA	0.002	0.26 (0.09–0.78)	0.016	0.046	NA	NA
MPO (N = 196)	× <i>KRAS</i>	0.002	0.13 (0.03–0.56)	0.006	NS	NA	NA	0.003	0.18 (0.05–0.62)	0.007
	× <i>DNMT3A</i>	0.003	NA	NA	0.071	NA	NA	< 0.001	0.35 (0.17–0.70)	0.003
	× <i>DNMT3A</i> -R882	0.002	0.27 (0.10–0.74)	0.011	NS	NA	NA	0.001	NA	NA
	× <i>TET2</i> / <i>IDH1</i>	0.040	NA	NA	0.021	4.32 (1.16–16.15)	0.029	0.001	3.52 (1.48–8.38)	0.004
APL-like (N = 198)	×Ras pathways	0.008	0.32 (0.11–0.96)	0.041	0.025	NA	NA	< 0.001	0.22 (0.08–0.57)	0.002
Abbreviations: NS, no significance; NA, not applicable										

× <i>DNMT3A</i> -R882	< 0.001	NA	NA	< 0.001	0.04 (0.01–0.36)	0.004	< 0.001	0.02 (0.00–0.18)	< 0.001
× <i>TET2/IDH1</i>	NS	NA	NA	< 0.001	6.73 (1.83–24.78)	0.004	0.008	2.26 (1.07–4.78)	0.033

Abbreviations: NS, no significance; NA, not applicable

DNMT3A-R882 was positively correlated with CD7 and HLA-DR expression ($HR = 3.59$ [95% CI 1.80–7.16], $P < 0.001$; $HR = 13.41$ [95% CI 4.56–39.45], $P < 0.001$), and *DNMT3A* mutation negatively with MPO expression ($HR = 0.35$ [95% CI 1.48–8.38], $P = 0.004$). Stratified analysis according to *FLT3*-ITD status indicated that the independent correlation of *DNMT3A* mutation (especially *DNMT3A*-R882) with CD7 and with HLA-DR expression was significant both in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ and in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ groups (HR and P values are detailed in Table 2). *TET2/IDH1* mutations were negatively correlated with CD34 and CD7 expression ($HR = 0.26$ [95% CI 0.11–0.62], $P = 0.002$; $HR = 0.30$ [95% CI 0.14–0.62], $P = 0.001$), and positively with MPO expression ($HR = 3.52$ [95% CI 1.48–8.38], $P = 0.004$). Stratified analysis indicated the above effects to be prominent only in the *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group (HR and P values are detailed in Table 2), but not in the *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group. There were no significant correlations between *NPM1*^{mut} coexisting mutations with other antigen expressions.

We finally analyzed association of *NPM1*^{mut} coexisting mutations with APL-like phenotype CD34⁽⁻⁾/HLA-DR⁽⁻⁾/MPO^(str+), which has been reportedly to predict a presence of *TET2/IDH1* mutation.(12) In entire cohort, mutations of Ras-pathway, *DNMT3A*-R882 and *TET2/IDH1* were each significantly linked with APL-like phenotype. When layered by *FLT3*-ITD, in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, only Ras-pathway mutation presented an association with APL-like phenotype ($HR = 0.32$ [95% CI 0.11–0.96], $P = 0.041$). Comparatively, negative correlation of *DNMT3A*-R882 ($HR = 0.04$ [95% CI 0.01–0.36], $P = 0.004$) and positive correlation of *TET2/IDH1* mutation ($HR = 6.73$ [95% CI 1.83–24.78], $P = 0.004$) with this phenotype were seen only in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, but not in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group (Table 2).

Discussion

Previous studies concerning prognosis of *NPM1*^{mut} AML often depicted its mutational type as indels, and there was little information about other types of *NPM1*^{mut}. In our cohort of 238 *NPM1*^{mut} patients, 240 *NPM1* mutant events were identified, therein the vast majority (232, 99.1%) were indels type, all of them deranging the tryptophan residues W288 and W290 which are indispensably responsible for NoLS.(2) Ten *NPM1*^{mut} missense mutations were clustered in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, and none of them disrupt the NoLS loci, nor were they involved in *NPM1* post-translational modification sites(3). Moreover, all except one (9, 90.0%) missense mutations were accompanied with an AML subtype-defining favorable- and adverse-risk cytogenetic or molecular abnormality, indicating that *NPM1*^{mut} missense may be insufficient to drive leukemogenesis and necessitate other well-characterized pathomechanism. When we mention theme of *NPM1*^{mut} prognostication, we will be referring to mutations exclusively in the context of its indels type, rather than including missense mutations with relatively rarity and inadequate pathogenicity.

In this study, NK reached ~ 90% in entire *NPM1*^{mut} cohort with analyzable metaphases and accounted for 84.6% in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, similar with the finding of 82.4% in a large sample survey.(13) Moreover, the recurrent cytogenetic translocations were uncommon and FISH did not detect any of *KMT2A* (*MLL*) translocations or *TP53* deletions, implying the leukemogenesis of frameshift *NPM1*^{mut} not relying on chromosomal abnormality. Nonetheless, all *NPM1*^{mut} indels arised together with coexisting mutations, especially those affecting epigenetic regulators and signaling pathways, which points to the necessity of interactivity of *NPM1*^{mut} with other genetic lesions to promote leukemic overt occurrence. The favorable- and adverse-risk abnormal karyotypes were only aggregated in the *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ but not in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, suggestive of a mutual exclusivity or pathogenic independence between *FLT3*-ITD and these karyotypes in *NPM1*^{mut} AML.

Compared with *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ had higher incidences of CD34 and CD7 expression, similar to other reports.(34) FCM immunophenotyping is not only used in the differential diagnosis of AML, but also has prognostic value. In terms of an individual immunomarker, CD34⁽⁺⁾ in *NPM1*^{mut} AML was associated with a poor prognosis.(11, 15) CD123 was only expressed in leukemia and other neoplastic cells, but hardly in normal hematopoietic cells.(35) The percentage of CD123⁽⁺⁾ cells in *NPM1*^{mut} patients divided by cutoff 52% was also reported to predict prognosis.(36) Going forward, combination of multiple aspects of antigen expression can more potently predict survival. Especially, the CD34⁽⁺⁾/CD38⁽⁻⁾/CD123⁽⁺⁾ which represents a LSC phenotype has prognostic relevance.(16) In addition, most LSC phenotypes also present cross-lineage, antigen overexpression or asynchronous expression phenomena.(16) In this study, the positive incidences of stem cell antigen CD34 and cross-lineage antigen CD7 were higher in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, which may be implied to encompass more LSCs at initial presentation. LSCs are in the relatively silent cell cycle G0 phase and highly express the drug-resistant efflux transporter P-glycoprotein (PGP) or multidrug-resistant protein (MDR1).(11, 37) Chen CY et al. (17) clustered immunophenotyping in 94 *NPM1*^{mut} patients and divided them into two categories according to CD34, CD7 and HLA-DR, showing that the prognosis of type-I class characterized by CD34⁽⁺⁾/HLA-DR⁽⁺⁾/CD7⁽⁺⁾ was significantly poorer versus the type-II class CD34⁽⁻⁾/CD7⁽⁻⁾. However, their results may be affected by the biased distribution of concurrent *FLT3*-ITD, which has a positive correlation with CD34 and CD7. Due to limited number of cases, it was not clear whether the differential effect of class I and II features on prognosis is independent of *FLT3*-ITD although stratified analysis has been carried out.

We investigated the relationship between *NPM1*^{mut} coexisting mutations and immunophenotypic markers. In general, there is a distributional association of signaling and methylating mutations with CD34, CD7, HLA-DR and MPO expression. The regulatory effect of Ras-pathway mutation on expression of these antigens was only found in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, but not in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, partly owing to the reciprocal exclusivity of *FLT3*-ITD with Ras-pathway mutation. Although both involved in methylation regulation, *DNMT3A* mutation was positively correlated with the expression of CD34, CD7 and HLA-DR, while *TET2*/*IDH1* mutation negatively with these antigens. By contrast, *DNMT3A* mutation was negatively correlated with MPO expression, while *TET2*/*IDH1* mutation positively with MPO expression. As per *FLT3*-ITD status, in *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, specific *DNMT3A*-R882 was independently correlated with CD7, HLA-DR and MPO expression, while *TET2*/*IDH1* mutation showed no correlation with the expression of these antigens. In *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, *DNMT3A* and *TET2*/*IDH1* mutations were each independently related to, and resultingly may jointly determine the trend of expression of CD34, CD7 and HLA-DR.

In *NPM1*^{mut}/*FLT3*-ITD⁽⁻⁾ group, Ras-pathway mutation and *DNMT3A*-R882 were positively correlated with the expression of monocyte marker HLA-DR, and negatively with myeloid marker MPO, which is linked to the morphological association of FAB typing in monocytic differentiation (M4/M5) or granulocytic differentiation (M2). Comparatively, in *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ group, although *TET2*/*IDH1* mutation was negatively correlated with HLA-DR expression, the more commonly coexisting *DNMT3A*-R882 which was positively correlated with HLA-DR expression was in concordance with a more frequent M4/M5 morphology in this genotypic subset.

Mason EF et al.(12) had analyzed the myeloid blast populations excluding monocytic differentiation in *NPM1*^{mut} patients. Nearly half of cases (48%) had an APL-like phenotype represented by CD34⁽⁻⁾/HLA-DR⁽⁻⁾/MPO^(str+), which could predict a presence of *TET2* or *IDH1/2* mutations, a result in line with our findings. Moreover, they demonstrated the APL-like phenotype to beneficially impact RFS and OS, and its combination with *TET2* or *IDH1/2* coexisting mutations to be more explicit to refine prognostic subgroups. The present study extends those findings. In addition to *TET2* or *IDH1/2*, we also showed an independent association of both Ras-pathway mutations and *DNMT3A*-R882 with APL-like phenotype, and this relationship was only found in the *NPM1*^{mut}/*FLT3*-ITD⁽⁺⁾ genotypic background. These results suggest that interplay of *NPM1*^{mut} coexisting genetic lesions may jointly determine the trend of antigen expression, partly explaining the immunophenotypic heterogeneity in *NPM1*^{mut} AML.

To sum up, *NPM1*^{mut} missense mutations may be of leukemogenic insufficiency, and largely rely on other well-defined pathomechanism to the development of leukemia. Correlation of coexisting mutations with antigen expressions in *NPM1*^{mut} AML partly explains the immunophenotypic diversity. Comprehensively evaluating FCM immunophenotype and NGS landscape of genetic lesions allows us to gain insight into the clinicopathological heterogeneity for this distinct AML entity.

Declarations

Author Contributions:

B.W. collected clinical-laboratory data and wrote the article; B.Y. and W.W. performed statistical analysis; X.L. performed sequencing and interpreted mutational data; Hq.L. was responsible for study conception and design. All co-authors have read and approved the final manuscript.

Conflict of interest statement:

The authors declare that they have no conflict of interests.

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Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

(a) Composition ratio of morphologic FAB subtypes in 238 patients with NPM1mut AML (by FLT3-ITD). (b) Positive expression rate of antigens on FCM (by FLT3-ITD); *, $P < 0.05$. (c) Conventional banding karyotype (by FLT3-ITD and cytogenetic risk classification).

Figure 2

Number of mutated genes per patient (a) and incidence of gene functional categories (b) in NPM1mut AML (by FLT3-ITD).

Figure 3

Mutational landscape for the relatively common mutated genes in NPM1mut AML; Row represents each different gene, and column represents each patient subject; Black cell indicates a presence of mutation, and blank cell wild type; The 17 individual genes were grouped into seven functional categories as labeled on the left, and mutational incidence of each gene was listed on the right.

Figure 4

Circos plot illustrating pairwise relationships across the relatively common mutated genes in NPM1mut AML; Red ribbon indicates a significant coexistence, and black ribbon a mutual exclusivity; White ribbon indicates a non-significant association; Width of ribbon corresponds to number of patients who have simultaneous presence of a first and a second gene in parallel.