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FLT3 and *NPM1* mRNA expression-based risk stratification of *de novo* acute Myeloid Leukemia

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ABSTRACT

Prognostication of acute myeloid leukemia (AML) at initial diagnosis relies on identification of pre-determined underlying genetic abnormalities. Nevertheless, the disease course of AML remains highly unpredictable and robust reliable prognostic biomarkers for newly diagnosed AML are lacking. We retrospectively explored two publicly available AML RNA-Seq datasets and found that inferior overall survival was associated with high-*FLT3* and low-*NPM1* transcript levels ("*FLT3*^{high}/*NPM1*^{low}") compared to low-*FLT3* and high-*NPM1* transcript levels ("*FLT3*^{low}/*NPM1*^{high}") in adult *de novo* AML patients, with a hazard ratio for death of at least 2. Transcript level-dependent differential overall survival was independent from the underlying *FLT3* or *NPM1* genotypes. Our two-gene RNA expression-based *de novo* AML risk stratification may supplement and fine-tune traditional genetic aberration-based prognostication methods.

1. Introduction

Acute myeloid leukemia (AML) is a cancer of myeloid-lineage cells. The age-adjusted annual incidence of AML in the United States is reported to be approximately 4.3 per 100,000 [1]. Cytotoxic intensive chemotherapy (e.g., 7 + 3) with or without mutation-targeted agent(s) is the standard of care (SOC) induction therapy, followed by consolidation therapy with chemotherapy or stem cell transplantation. The specific choice of therapeutic modality is ultimately made upon evaluating the estimated risk of relapse based on the presence of underlying genetic abnormalities as proposed by *Cancer and Leukemia Group B (CALGB)* or *European LeukemiaNet (ELN)*, as well as baseline performance status of the individual. Frequently encountered genetic abnormalities in AML include mutations in the FMS-like tyrosine kinase 3 (*FLT3*) and nucleophosmin 1 (*NPM1*) genes which arise in approximately 30 % of cases. *FLT3* gene alteration frequently occurs in the form of activating internal tandem duplication (*FLT3*-ITD) or tyrosine kinase domain mutation (*FLT3*-TKD). *NPM1* mutation frequently occurs in exon 12, resulting in loss of posttranslational modification of C-terminus (*NPM1*c) and aberrant cytoplasmic delocalization of the nucleolar protein *NPM1*. According to current ELN risk stratification guidelines, *NPM1* mutation without *FLT3*-ITD is associated with "favorable" risk, whereas the presence of *FLT3*-ITD is associated with "intermediate" risk regardless of *NPM1* genotype in the absence of other mutations associated with "adverse" risk [2]. Survival analysis of *de novo* AML patients enrolled in CALGB 8641 demonstrated median overall survival (mOS) of CALGB "favorable," "intermediate," and "adverse" risk groups as 7.6 years, 1.3 years, and 0.5 years, respectively [3]. A recent German study showed mOS of ELN2022 "favorable," "intermediate" and "adverse" risk groups in *de novo* AML was 9.5 years, 1.7 years, and 0.8 years, respectively [4].

However, AML is highly heterogenous at the molecular level and the prognosis of individual AML cases vary widely even within each CALGB or ELN risk category [5], making standard guidelines often not straightforwardly applicable. Only a handful of reliable gene expression-based prognostication biomarkers have been validated in hematologic malignancies, such as MYC and BCL2 in double-expressor lymphoma. Therefore, we explored novel gene expression-based risk stratification biomarkers using the two publicly available adult *de novo* AML bone marrow RNA-Seq databases and surprisingly found that *FLT3* and *NPM1* transcript levels alone allow reliable estimation of the overall survival (OS) in *de novo* AML.

2. Materials and methods

Normalized annotated bone marrow whole genome RNA-sequencing (RNA-Seq) transcript count (RPKM) of adult (age ≥18) *de novo* AML

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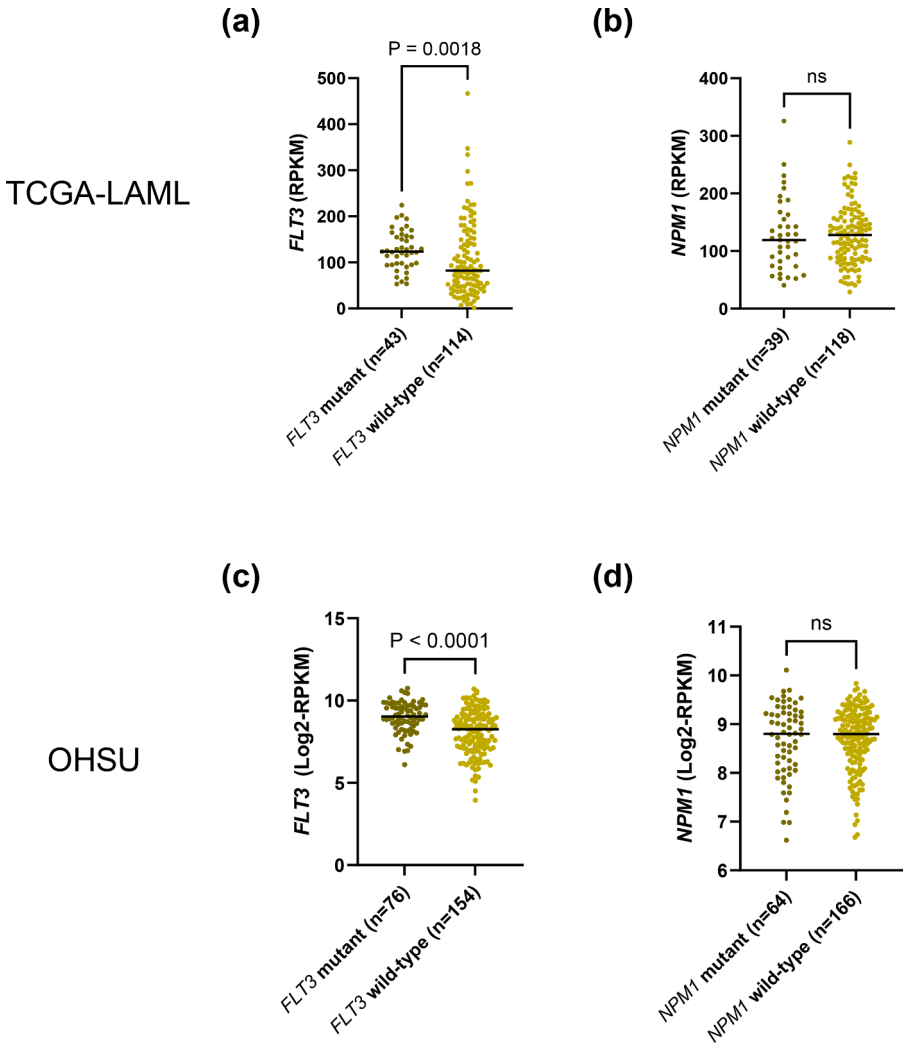

Fig. 1. Transcript levels of *FLT3* and *NPM1* in their respective mutant and wild-type genotypic backgrounds. (a) *FLT3* transcript levels in *FLT3* mutant genotypes (left) and wild-types (right), and (b) *NPM1* transcript levels in *NPM1* mutant genotypes (left) and wild-types (right) in TCGA-LAML cohort. (c) *FLT3* transcript levels in *FLT3* mutant genotypes (left) and wild-types (right), and (d) *NPM1* transcript levels in *NPM1* mutant genotypes (left) and wild-types (right) in the OHSU cohort. Horizontal lines in black indicate the median. All p-values are derived from two-tailed Mann-Whitney test. ns: not significant.

from The Cancer Genome Atlas AML project (TCGA-LAML; $n = 157$) [6] was downloaded from the National Cancer Institute Genomics Data Commons portal) and the log2-transformed RPKM from the Oregon Health Science University Beat AML project (OHSU; $n = 230$) [7] was downloaded from the cBioPortal [8]. Downloaded data was compiled with Microsoft Excel software. RNA-Seq transcript level of genes in individual patients were classified as "high" if higher than or equal to the cohort median RPKM, and "low" if lower than the cohort median RPKM. Kaplan-Meier survival analysis was performed using GraphPad Prism software and log-rank p-value and Mantel-Haenszel hazard ratio (HR) are reported unless specified otherwise.

3. Results

3.1. TCGA-LAML cohort

We first dichotomized patients with *FLT3* transcript levels. The median overall survival (mOS) was 15.9 months (484 days) in the $FLT3^{high}$ group versus 24.0 months (731 days) in the $FLT3^{low}$ group with HR for death of 1.63 (95 % CI 1.07–2.47, $p = 0.02$, **Figure S1a**). When patients were dichotomized with *NPM1* transcript level, the mOS was 26.0 months (792 days) in the $NPM1^{high}$ group versus 12.0 months (366 days) in the $NPM1^{low}$ group with an HR for death of 0.63 (95 % CI 0.41–0.95, $p = 0.027$, **Figure S1b**). With this observation, patients were classified into four groups based on *FLT3* and *NPM1* transcript levels – $FLT3^{high}/NPM1^{low}$, $FLT3^{low}/NPM1^{high}$, $FLT3^{high}/NPM1^{high}$, and $FLT3^{low}/NPM1^{low}$. The mOS was 10.0 months (305 days) in the $FLT3^{high}/NPM1^{low}$ group versus 28.0 months (854 days) in the $FLT3^{low}/NPM1^{high}$ group with an HR for death of 3.33 (95 % CI 1.67–6.66, $p = 0.0007$, **Figure S3a**). The mOS of $FLT3^{high}/NPM1^{high}$ and $FLT3^{low}/NPM1^{low}$ groups were intermediate, 18.9 months (576 days) and 12 months (366 days), respectively (**Figure S3a**).

FLT3 transcript levels of *FLT3* mutant genotypes were significantly different with higher median compared to that of *FLT3* wild-types (median RPKM 123.4 versus 82.15, two-tailed Mann Whitney $p = 0.0018$, Fig. 1**a**). However, *NPM1* transcript levels of *NPM1* mutant genotypes were not significantly different from that of *NPM1* wild-types (median RPKM 119.0 versus 127.9, two-tailed Mann Whitney $p = 0.68$, Fig. 1**b**). To eliminate such confounding factors that may influence survival outcomes, we carried out further analyses excluding any cases with *FLT3* or *NPM1* mutant genotypes. Acute promyelocytic leukemia (APL) is a subtype of AML with unique superior survival outcomes

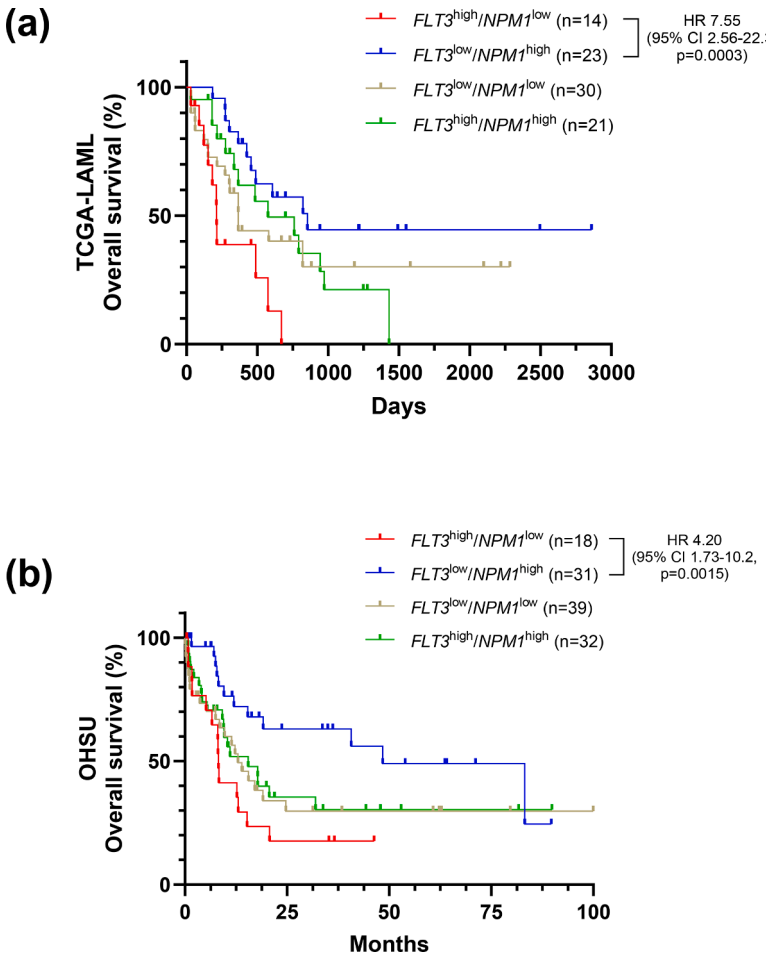

Fig. 2. Overall survival of *FLT3*^{high}/*NPM1*^{low}, *FLT3*^{low}/*NPM1*^{high}, *FLT3*^{high}/*NPM1*^{high}, and *FLT3*^{low}/*NPM1*^{low} groups in adult *de novo* AML with APL cases and mutant *FLT3* and *NPM1* genotypes excluded, in (a) TCGA-LAML and (b) OHSU cohorts.

compared to all other AML subtypes due to excellent response to all-trans retinoic acid (ATRA), which was FDA approved for APL induction therapy in 2004 [9]. Therefore, we also excluded APL cases which comprised 10 % of the TCGA cohort cases. With this new exclusion criteria, the mOS was 7 months (212 days) in the *FLT3*^{high}/*NPM1*^{low} group versus 28.1 months (854 days) in the *FLT3*^{low}/*NPM1*^{high} group with an HR for death of 7.55 (95 % CI 2.56–22.3, $p = 0.0003$, Fig. 2**a**). The mOS of *FLT3*^{high}/*NPM1*^{high} and *FLT3*^{low}/*NPM1*^{low} groups were intermediate, 18.9 months (576 days) and 12 months (366 days), respectively (Fig. 2**a**). The differential OS associated with differential expression of *FLT3* and *NPM1* was observed within each CALGB risk class, with the highest significance in the intermediate risk class with HR for death of 4.61 (95 % CI 1.39–12.27, $p = 0.01$, Fig. 3**a-d**). *TP53* mutation was not significantly prevalent in the *FLT3*^{high}/*NPM1*^{low} group (9 %, Fisher's exact $p = 0.71$, **Table S1**). As an internal control, we also dichotomized patients with wild-type *TP53* using *TP53* transcript levels and found no significant difference in the OS of *TP53*^{high} group compared to that of *TP53*^{low} group, with HR for death of 0.73 (95 % CI 0.47–1.13, $p = 0.16$, **Figure S4a**). Lastly, we performed Cox proportional hazard regression on adult *de novo* AML patients excluding APL and cases with *FLT3, NPM1* or *TP53* mutant genotypes to investigate any clinicopathological characteristics (e.g., age, sex, CALGB risk class) that may affect HR for death, which revealed *FLT3*^{high}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high} group (HR 3.5; 95 % CI 1.140–10.88, $p = 0.028$) but otherwise no additional independent prognostic factor (Table 1).

3.2. OHSU cohort

In the OHSU cohort, the mOS of *FLT3*^{high} group compared to *FLT3*^{low} group was not significantly different. The mOS was 17.8 months in the *FLT3*^{high} group versus 48.4 months in the *FLT3*^{low} group with an HR for death of 1.35 (95 % CI 0.93–1.97, $p = 0.12$, **Figure S2a**). Similarly, the difference in mOS between *NPM1*^{high} and *NPM1*^{low} groups was also not significant. The mOS was 40.1 months in the *NPM1*^{high} group versus 15.5 months in the *NPM1*^{low} group with an HR for death of 0.80 (95 % CI 0.55–1.17, $p = 0.25$, **Figure S2b**). However, the mOS was 8.7 months in the *FLT3*^{high}/*NPM1*^{low} group versus 48.4 months in the *FLT3*^{low}/*NPM1*^{high} group with an HR for death of 2.01 (95 % CI 1.08–3.74, $p = 0.03$, **Figure S3b**), consistent with our findings with TCGA-LAML cohort. The mOS of *FLT3*^{high}/*NPM1*^{high} and *FLT3*^{low}/*NPM1*^{low} groups were 28.4 months and 24.7 months, respectively (**Figure S3b**).

Again, the *FLT3* transcript levels of *FLT3* mutant genotypes were significantly different with higher median compared to that of *FLT3* wild-types (median log2-RPKM 9.03 versus 8.26, two-tailed Mann-Whitney $p < 0.0001$, Fig. 1**c**), and the *NPM1* transcript levels of *NPM1* mutant genotypes were not significantly different from that of *NPM1* wild-types (median log2-RPKM 8.8 versus 8.8, two-tailed Mann-Whitney $p = 0.97$, Fig. 1**d**). As in TCGA-LAML survival analysis above, we then excluded any *FLT3* or *NPM1* mutant genotypes, as well as APL cases which comprised 6 % of the OHSU cohort cases. The mOS was 8.3 months in the *FLT3*^{high}/*NPM1*^{low} group versus 48.4 months in the *FLT3*^{low}/*NPM1*^{high} group with an HR for death of 4.20 (95 % CI 1.73–10.2, $p = 0.0015$, Fig. 2**b**). The mOS of *FLT3*^{high}/*NPM1*^{high} and

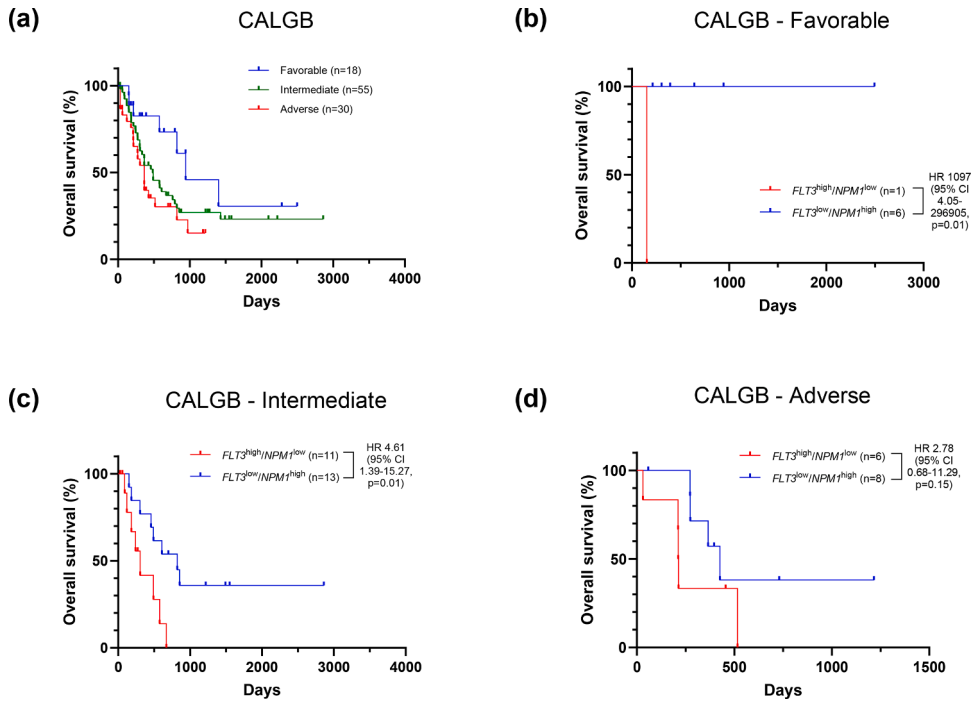

Fig. 3. mRNA expression-dependent OS within each CALGB risk class in TCGA-LAML cohort. (a) OS of CALGB risk classes, OS of *FLT3*^{high}/*NPM1*^{low} and *FLT3*^{low}/*NPM1*^{high} groups in CALGB (b) favorable risk class, (c) intermediate risk class, and (d) adverse risk class. *FLT3* and *NPM1* mutant genotypes and APL cases are excluded.

FLT3^{low}/*NPM1*^{low} groups were intermediate, 15.5 months and 13 months, respectively (Fig. 2**b**). The differential OS associated with differential expression of *FLT3* and *NPM1* was observed within each ELN2017 risk class, with the highest significance in the intermediate risk class with HR for death of 6.99 (95 % CI 0.90–54.3, $p = 0.06$, Fig. 4**a-d**). Again, *TP53* mutation was not significantly prevalent in the *FLT3*^{high}/*NPM1*^{low} group (16 %, Fisher's exact $p = 0.14$, **Table S2**), and there was no significant difference in the OS of *TP53*^{high} group compared to that of the *TP53*^{low} group, with HR for death of 0.88 (95 % CI 0.54–1.43, $p = 0.61$, **Figure S4b**). Finally, we performed Cox proportional hazard regression on adult *de novo* AML patients excluding APL and cases with *FLT3, NPM1* or *TP53* mutant genotypes to investigate any clinicopathological characteristics (e.g., age, gender, ELN2017 risk class) that may affect HR for death, which revealed *FLT3*^{high}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high} group (HR 2.943; 95 % CI 1.076–7.858, $p = 0.031$) but also male vs. female gender (HR 2.809; 95 % CI 1.380–6.337, $p = 0.007$) and ELN2017 "favorable" vs. "intermediate" risk class (HR 0.3018; 95 % CI 0.1095–0.7965, $p = 0.017$) as independent prognostic factors (Table 1).

4. Discussion

In this study, we demonstrate a promising prognostic potential of *FLT3* and *NPM1* RNA-Seq transcript levels in adult *de novo* AML by retrospectively analyzing two independent AML bone marrow RNA-Seq datasets. *FLT3*^{high}/*NPM1*^{low} portends poor prognosis compared to *FLT3*^{low}/*NPM1*^{high}, whereas *FLT3*^{high}/*NPM1*^{high} and *FLT3*^{low}/*NPM1*^{low} portend intermediate prognosis consistently in both TCGA-LAML and OHSU cohorts. Reassuringly, such differential OS was not present between *TP53*^{high} and *TP53*^{low} groups, which underscores the significance of differential OS observed between *FLT3*^{high}/*NPM1*^{low} and *FLT3*^{low}/*NPM1*^{high} groups. Cox proportional hazard regression analyses revealed male sex as an independent prognostic factor, although this was seen only in the OHSU cohort. The genotype of *FLT3* and *NPM1* has prognostic values in AML [2] and mutant genotypes may influence their transcript levels. We did observe higher levels of *FLT3* transcripts in patients with *FLT3* mutant genotypes compared to those with *FLT3* wild-type, although *NPM1* transcript levels in patients with *NPM1* mutant genotypes were not significantly different from that of *NPM1* wild-types. Regardless, the *FLT3* and *NPM1* mRNA expression-dependent differential OS was present even in the absence of underlying *FLT3* and *NPM1* mutations, suggesting that *FLT3* and *NPM1* transcript level profiles may represent a global oncogenic state regardless of the underlying *FLT3* and *NPM1* genotype. This is supported by the previously reported "*FLT3* mutation-like transcriptomic profiles" observed in *FLT3* wild-type AML cases [10,11]. The differential OS associated with differential expression of *FLT3* and *NPM1* observed within each CALGB and ELN2017 risk class suggest that differential *FLT3* and *NPM1* mRNA expressions are prognostic biomarkers that are likely independent from the underlying cytogenetics and genotypes. This is also supported by our observation that *TP53* mutation, which is associated with very poor prognosis in AML [12], is not significantly prevalent in the *FLT3*^{high}/*NPM1*^{low} group in both TCGA-LAML and OHSU cohorts.

Limitations underlie this study. First, we adopted the simple sample median-based approach to classify transcript levels into either "high" or "low." Next-generation RNA-Seq is a highly quantitative and sensitive method due to its large dynamic range, which has greatly advanced the field of transcriptomic gene expression studies. Even though a variety of approaches have been proposed ranging from a simple sample median-based dichotomization [13] to data-adaptive regression algorithms [14], there is no consensus on how to differentiate transcript levels into "high" or "low" levels to date. Data-adaptive computational methods might have improved the power of this study, but our findings were reproducible across two independent AML RNA-Seq datasets using sample median-based dichotomization. Second, while the two cohorts used in this study, TCGA-LAML and OHSU, are by far the largest publicly available AML bone marrow RNA-Seq datasets, their patient enrollment periods were more than a decade apart in time. In addition to advances in sequencing technology, considerable numbers of new drug approvals for newly diagnosed AML patients and advances in supportive care strategies occurred during this time span, any of which might have affected the landscape of transcriptomic gene expression as well as OS of

Table 1
Cox regression analyses on overall survival of adult *de novo* AML, excluding APL and cases with *FLT3, NPM1, TP53* mutant genotypes.

TCGA-LAML Cohort			
Variable	HR	95 % CI	*p* value
Age (≥65 vs. <65)	1.876	0.9297 to 3.719	0.074
Gender (Male vs. Female)	0.8063	0.4290 to 1.522	0.503
CALGB risk			
Favorable vs. Intermediate	0.5556	0.2176 to 1.247	0.180
Adverse vs. Intermediate	1.177	0.5544 to 2.335	0.654
Gene mRNA expression			
FLT3^{high}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high}	3.5	1.140 to 10.88	0.028 (*)
FLT3^{high}/*NPM1*^{high} and *FLT3*^{low}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high}	1.655	0.7896 to 3.719	0.198
TP53^{high} vs. *TP53*^{low}	1.051	0.5560 to 2.021	0.879
OHSU Cohort			
Variable	HR	95 % CI	*p* value
Age (≥65 vs. <65)	1.372	0.6875 to 2.753	0.368
Gender (Male vs. Female)	2.809	1.380 to 6.337	0.007 (*)
ELN 2017 risk			
Favorable vs. Intermediate	0.3018	0.1095 to 0.7965	0.017 (*)
Adverse vs. Intermediate	0.9757	0.4913 to 2.009	0.945
Gene mRNA expression			
FLT3^{high}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high}	2.943	1.076 to 7.858	0.031 (*)
FLT3^{high}/*NPM1*^{high} and *FLT3*^{low}/*NPM1*^{low} vs. *FLT3*^{low}/*NPM1*^{high}	1.737	0.8362 to 3.900	0.156
TP53^{high} vs. *TP53*^{low}	0.7604	0.3980 to 1.532	0.422

* indicate p value < 0.05

the patient groups analyzed in this study.

Our findings suggest that the bone marrow *FLT3* and *NPM1* transcript level obtained at the initial diagnosis of AML in adults may allow significant enhancement of risk stratification power when combined with the traditional ELN or CALGB prognostication guidelines in this population. For example, *de novo* AML patients at ELN "intermediate" with *FLT3*^{low}/*NPM1*^{high} (blue curve, Fig. 4**c**) who are clinically suboptimal for consolidative allogeneic stem cell transplantation may defer this option without significantly compromising their survival. On the other hand, those at ELN "favorable" with *FLT3*^{high}/*NPM1*^{low} (red curve, Fig. 4b) who prefer aggressive treatment may opt for upfront consolidation with allogeneic stem cell transplantation. The poor prognosis observed in *FLT3*^{high}/*NPM1*^{low} individuals with wild-type *FLT3* genotype in this study may imply potential benefit of *FLT3* wild-type targeted therapy such as quizartinib, a second-generation class III tyrosine kinase inhibitor with reported therapeutic activity in *FLT3* wild-type *de novo* AML [15], in this unique population. Prospective validation studies are warranted.

Disclosures

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CRediT authorship contribution statement

Donghyun Kim: Conceptualization, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Grerk Sutamtewagul:** Formal analysis, Investigation, Writing – review & editing, Resources. **Yeonhwa Yu:** Formal analysis, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

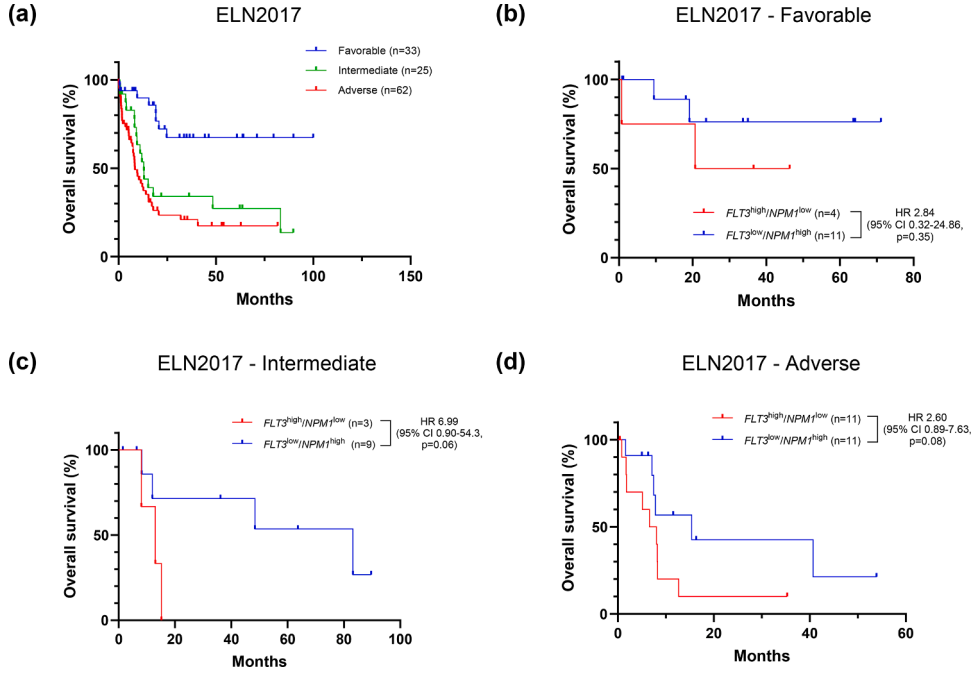

Fig. 4. mRNA expression-dependent OS within each ELN2017 risk class in the OHSU cohort. (a) OS of ELN2017 risk classes, OS of *FLT3*^{high}/*NPM1*^{low} and *FLT3*^{low}/*NPM1*^{high} groups in ELN2017 (b) favorable risk class, (c) intermediate risk class, and (d) adverse risk class. *FLT3* and *NPM1* mutant genotypes and APL cases are excluded.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.lrr.2024.100494.

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