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The clinical and prognostic significance of *FIS1, SPI1, PDCD7* and *Ang2* expression levels in acute myeloid leukemia

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Abstract

Objectives: The marked heterogeneity of acute myeloid leukemia (AML) renders precisely predicting patient prognosis extremely difficult. Genetic alterations, fusions and mutations, may result in misexpression of key genes in AML. We aimed to investigate the expression patterns of 4 novel genes; *FIS1, SPI1, PDCD7* and *Ang2* to determine their potential prognostic role in AML patients.

Methods: Bone marrow mononuclear cells were analyzed for of *FIS1, SPI1, PDCD7* and *Ang2* expression levels by real-time quantitative PCR as well as of *FLT3/ITD* and *NPM1* mutations in 100 newly diagnosed cytogenetically normal (CN-AML) patients, and 100 non-malignant controls.

Results: FIS1, SPI1, PDCD7 and *Ang2* were significantly overexpressed in CN-AML patients ($p < 0.001$). Their high expression levels were significantly associated with lower complete remission (CR) rate, shorter relapse-free survival (RFS) and overall survival (OS). On multivariate analysis, high *FIS1* expression showed a significant impact on CR response after induction therapy (OR = 88.777, 95% C.I: 2.85–2765.78, $p = 0.011$) while high *PDCD7* appeared to be an independent risk factor for RFS (HR = 5.107, 95% C.I: 1.731–15.066, $p = 0.003$) and OS (HR = 7.353, 95% C.I: 1.859–29.079, $p = 0.004$) in CN-AML patients.

Conclusions: FIS1 and *PDCD7* expression are considered independent risk factors and should be integrated into the current AML stratification system.

Keywords AML, Gene expression, *FIS1*, *PDCD7*, Prognosis.

Introduction

Acute myeloid leukemia (AML) is a group of malignant clonal hematopoietic stem cell disorders characterized by aggressive clonal proliferation of myeloid progenitor cells in the bone marrow [1,2].

The marked heterogeneity of AML has rendered precisely predicting patient prognosis extremely difficult. Although the conventional cytogenetic analysis was assumed to provide the best marker for risk stratification and prognosis in AML, it was estimated that 50% of AML cases have normal karyotyping and variable outcomes [3–5]. For these patients, some molecular alterations including gene mutations and expression patterns are used as prognostic indicators [6]. Some of the gene mutations are already well-established risk factors and are now carried out as a part of the routine work-up for AML management, such as Nucleophosmin (*NPM1*), FMS-like tyrosine kinase 3/ internal tandem duplication (*FLT3/ITD*) and CCAAT enhancer binding protein alpha (*CEBPA*) [7,8].

Genetic alterations, including fusions and mutations, may result in misexpression of key genes and disrupt the transcription factors dependent genetic network in AML [9]. The dysregulated expression of several genes such as brain and acute leukemia cytoplasmic *(BAALC)*, Ets-related gene *(ERG)* and Wilms tumor 1 (*WT1),* has been studied in AML and proved to be associated with the treatment outcome [10–12]. Although intensive research has been conducted in recent decades, systematic investigation of gene expression

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patterns and their cytogenetic and gene mutation background still need to be fully addressed in AML. In addition, better and more precise biomarkers are still required for prediction of disease outcome and designing tailored treatment for AML patients [13,14].

From this context, this study was done to investigate the expression patterns of 4 novel genes; Fission 1 mitochondrial outer membrane (*FIS1*), spleen focus forming virus proviral integration site 1 (*SPI1*), programmed cell death 7 (*PDCD7*) and Angiopoietin-2 (*Ang2*), in order to determine their potential prognostic role in patients with AML.

Materials and methods

The current study was conducted on one hundred newly diagnosed non-M3 cytogenetically normal AML (CN-AML) patients admitted to Alexandria Main University Hospital (Alexandria, Egypt) from 2014 till 2018. Bone marrow (BM) samples from 100 subjects of matched age and sex with hypersplenism, iron deficiency anemia and primary immune thrombocytopenia were recruited as a control group. The sample size was calculated using Epi info 7 software programs with a confidence level of 95% and power of 80%. Peripheral blood and BM samples were obtained from both AML patients and controls with a written informed consent after explaining the nature, steps and aim of the study. The study was conducted after approval of the Medical Ethics Committee of Alexandria Faculty of Medicine.

All AML patients were subjected to a complete blood count (CBC), BM aspiration and/or biopsy, standard morphological examination, immunophenotyping and cytogenetic studies using BM samples at initial diagnosis. The diagnosis of AML was based on the French-American-British classification (FAB) classification and the World Health Organization (WHO) criteria [15]. The blast count cutoff point for the diagnosis of AML was 20%. In order to avoid the interference of different cytogenetic abnormalities with the results of target genes expression levels, only CN-AML patients were enrolled in the study.

Treatment protocols

All AML patients were treated according to the adopted protocol of the Clinical Hematology Unit, Alexandria University Hospital. AML patients aged $\leq$60 years received standard first-line treatment of DA like regimen, which consisted of daunorubicin 45–60 mg/m^2, from day 1 to 3; and Ara-C 100 mg/m^2, from day 1 through 7. If the patient did not attain complete remission (NCR), this protocol was repeated. If no or minimal response, patients were shifted to high dose chemotherapy. Patients who attained complete remission (CR) were treated with high-dose cytarabine-based chemotherapy for 4 cycles as consolidation therapy. For older patients who aged $>$60 years, the treatment was mainly decided by the physician according to the fitness of the patient: fit patients underwent a regimen similar to younger patients, but with a reduced consolidation cycles of high dose Ara-C to 2 cycles; unfit patients, underwent either low dose treatment, demethylation treatment or palliative care [16]. Bone marrow examination to assess treatment response was done on day 28 after completion of induction chemotherapy. For post-remission therapy, all patients were assessed for risk of relapse by bone marrow examination at 3 months interval.

Assessment of the response to induction chemotherapy

By the end of induction therapy, CR response was defined by normalization of the neutrophil (at least $\geq 1.5 \times 10^9$/L) and platelet ($> 100 \times 10^9$/L) counts, as well as marrow examination that demonstrated at least 20% cellularity, less than 5% blasts and no Auer rods, in addition to the absence of extramedullary infiltration. Resistance to treatment was defined as more than 25% blasts in the BM, lack of regeneration of normal hematopoiesis, persistence of peripheral blood blasts and/ or extramedullary leukemia after induction. Death during induction was defined as death during or after the first course of therapy with aplastic or hypocellular marrow.

FLT3/ITD and *NPM1* mutations detection

DNA and RNA were extracted from BM mononuclear cells (BMNCs) according to the manufacturer's protocol using the QIAamp DNA and RNA blood mini kits (Qiagen, USA), respectively. The concentration and purity of the isolated nucleic acids were measured by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). The reverse transcription by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and the PCR amplification were carried out on the SimpliAmp Thermal Cycler (Applied Biosystems, USA).

Exons 11 and 12 and the intervening intron of the *FLT3* gene were amplified using MyTaq Red Mix (Bioline, London, United Kingdom), 11F and 12R primers as previously described [17]. An *NPM1* 4 bp insertion mutation (type A mutation) in exon 12 was analyzed using allele-specific oligonucleotide reverse transcriptase PCR (ASO-RT-PCR). Forward (NPM-A) and reverse (NPM-REV-6) primers were used as previously described [18]. The Abelson (*ABL*) gene was included in this assay as an internal control using the same ASO-RT-PCR. Amplified products were electrophoresed through 2% agarose gels in 0.5X TBE buffer and visualized under UV light with ethidium bromide staining. For *FLT3/ITD* mutation, a fragment of 328 bp was produced from the wild allele and an additional band appeared for *FLT3/ITD* mutation. Regarding *NPM1* mutation, successful amplification of a 258 bp fragment for the *ABL* gene was used as an internal control in all samples regardless the presence of the mutation. A 320-bp fragment was visualized in *NPM1* mutated samples while unmutated wild samples lacked this band.

Target genes expression analysis

Quantitative determination of the expression levels of *FSI1, SPI1, PDCD7,* and *Ang2* genes was carried out on Rotor-Gene Q Real-time PCR system (Qiagen, USA) using the pre-synthesized cDNA, Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) and sequence-specific primers for each gene (table S1). *GADPH* and β*-actin* were used as endogenous references; their expression was stable in all the samples and independent of the analyzed variables. Relative

expression levels of target genes were determined using the $2^{-\Delta\Delta CT}$ method [19].

Statistical analysis of the data

After a median duration of follow up for 40 months, the overall survival (OS) was calculated from the first day of therapy to death or the last follow-up visit. Relapse-free survival (RFS) for patients who attained CR was defined from the date of CR to relapse, death, or the last follow up visit. Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov–Smirnov, Shapiro and D'agstino tests were used to verify the normality of distribution of variables, Comparisons between groups for categorical variables were assessed using the Chi-square test (Fisher or Monte Carlo). Student *t*-test was used to compare two groups for normally distributed quantitative variables. The Kruskal Wallis test was used to compare different groups for abnormally distributed quantitative variables and the Mann Whitney test was used for comparing every two groups. Spearman coefficient was used to correlate between quantitative variables. A multivariate logistic regression model was applied to analyze the factors related to the probability of CR response. Associations between genetic mutations and expression levels with RFS, or OS were initially explored using the Kaplan–Meier method and log-rank test. Multivariable models were fit using Cox proportional hazards models for survival studies. The significance of the obtained results was judged at the 5% level.

Results

The mutational status of *FLT3/ITD* and *NPM1* as well as the expression data of *FIS1, SPI1, PDCD7, Ang2* in addition to the clinicopathological characteristics of the study subjects are summarized in Table 1.

Target genes expression levels and clinical characteristics at diagnosis

The bone marrow of CN-AML patients had significantly higher *FIS1, SPI1, PDCD7* and *Ang2* expression levels compared to non-malignant controls with a median of 6.8, 4.9, 7.7 and 5.7 versus 0.95, 0.9, 0.97 and 0.93 respectively ($p < 0.001$) (Table 1 and Fig. 1).

For each gene, AML Patients were divided into two groups with high and low expression by the median value of the target gene expression level. No statistical significance was observed between the different expression levels of *FIS1, SPI1, PDCD7* or *Ang2* and other variables, including age, sex, FAB subtypes, WBCs count, platelets count and peripheral blood blast count. Low *PDCD7* levels were significantly associated with secondary AML (Table 2). On the other hand, the *SPI1, PDCD7* and *Ang2* expression levels positively correlate with the initial BM blast count at diagnosis ($r = 0.238$, $p = 0.017$ for *SPI1,* $r = 0.523$, $p < 0.001$ for *PDCD7* and $r = 0.239$, $p < 0.001$ for *Ang2*). *FIS1* transcripts were positively correlated with Hb concentration ($r = 0.198$, $p = 0.049$) (Table S2).

Target genes expression levels and mutational status

In the total group of 100 patients, 24 patients (24%) had *FLT3/ITD* mutations and 31 patients (31%) had *NPM1* mutations. Details of the presenting features of the *FLT3/ITD* and *NPM1* mutated AML patients are given in Table 3.

The presence of the *FLT3/ITD* and *NPM1* mutations was not related to gender or age. Mutations were found in all FAB subtypes except AML-M7 (which was a single case). *FLT3/ITD* mutated patients altogether had significantly higher WBC counts and bone marrow blast percent at diagnosis with a median count of 53.5×10^9/L and 89.0% versus 29.5×10^9/L and 64.5% in unmutated patients ($p = 0.026$, $p = 0.012$, respectively). *NPM1* mutated patients had significantly lower BM blasts compared to patients with a wild gene (54% versus 82%, $p = 0.012$) (Table 3).

The *FLT3/ITD* mutations were significantly associated with high expression of both *SPI1* and *PDCD7* genes. Regarding *SPI1* expression, *FLT3* was mutated in 17 patients (33.3%) with high *SPI1* versus 7 patients (14.3%) with low *SPI1* ($p = 0.026$). Also, 21 patients (42%) with high *PDCD7* were *FLT3/ITD* mutated versus 3 patients (6%) with low *PDCD7* ($p < 0.001$). No significant associations could be observed between *FLT3/ITD* mutation and different *FIS1* or *Ang2* expression levels. Furthermore, significant associations were found between *NPM1* mutation and low expression levels of *SPI1, PDCD7* and *Ang2* genes. *NPM1* was mutated in 20 patients (40.8%) with low *SPI1* versus 11 patients (21.6%) with high *SPI1* ($p = 0.037$), 23 patients (46%) with low *PDCD7* versus 8 patients (16%) with high *PDCD7* ($p = 0.001$) and 24 patients (48%) with low *Ang2* versus 7 patients (14%) with high *Ang2* expression ($p < 0.001$). On comparing *FIS1* expression levels according to *NPM1* mutational status, significant associations were not observed (Table 2).

Target genes expression levels and clinical outcomes

Response to induction therapy

Firstly, we investigated the prognostic significance of gene mutations in CN-AML patients who received uniformed therapy. *FLT3/ITD* mutated AML patients showed significantly lower rates for attaining CR responses after induction chemotherapy compared to unmutated patients (47.8% versus 72.2%, $p = 0.031$, OR = 2.836, 95% C.I: 1.078–7.461, $p = 0.035$). On the contrary, CR rates were significantly higher in *NPM1* mutated than unmutated AML patients (83.9% versus 57.8%, $p = 0.012$, OR = 0.264 95% C.I: 0.090–0.774, $p = 0.015$) (Tables 3 and S3).

After that, we studied the impact of different expression levels of target genes on the CR response. High *FSI1* (OR = 108.5, 95% C.I: 13.58–866.77, $p < 0.001$), high *SPI1* (OR = 26.157, 95% C.I: 7.041–97.176, $p < 0.001$), high *PDCD7* (OR = 3.826, 95% C.I: 1.545–9.472, $p = 0.004$) and high *Ang2* OR = 28.396, 95% C.I: 7.607–105.99, $p < 0.001$) were significantly associated with failure of attaining CR response after induction therapy (Table S3).

On Multivariate logistic analysis, only *FIS1*expression levels showed a significant impact on complete remission rate

Table 1 Clinicopathological characteristics of study subjects.

	AML patients ($n=100$)	Controls ($n=100$)	p
Age	48.56 ± 12.77	48.1 ± 13.3	0.807
≤60	87(87.0%)	77(77.0%)	0.066
>60	13(13.0%)	23(23.0%)	
Sex			
Male	61(61%)	56(56%)	0.473
Female	39(39%)	44(44%)	
CBC			
PLT x10^9/L	54(8–540)	278(159–422)	<0.001*
WBC x10^9/L	32(1.9–222)	7.2(4–10.9)	<0.001*
Hb (g/dl)	8.4 ± 2.9	13.3 ± 0.9	<0.001*
Initial PB blast count %	20.5(0–90)	–	–
Initial BM blast count %	69(20–99)	–	–
FAB subtypes			
M0	13(13.0%)	–	–
M1	18(18.0%)	–	–
M2	25(25.0%)	–	–
M3	0(0.0%)	–	–
M4	16(16.0%)	–	–
M5	24(24.0%)	–	–
M6	3(3.0%)	–	–
M7	1(1.0%)	–	–
Type of AML			
Denovo	89(89%)	–	–
Secondary	11(11%)	–	–
NPM1			
Unmutated	69(69.0%)	–	–
Mutated	31(31.0%)	–	–
FLT3/ITD			
Unmutated	76(76.0%)	–	–
Mutated	24(24.0%)	–	–
FIS1	6.8(0.4–31.8)	0.95(0.23–4.79)	<0.001*
SPI1	4.9(1.0–17.1)	0.9(0.4–5.0)	<0.001*
PDCD7	7.7(0.2–31.8)	0.97(0.3–2.77)	<0.001*
Ang2	5.7(1.3 – 15.4)	0.93(0.26–3.29)	<0.001*
Complete Remission ($n=95$)	63(66.3%)	–	–
Intensity of consolidation regimen ($n=95$)			
Low	54(56.8%)	–	–
High	41(43.2&)	–	–

PLT: Platelet, WBC: white blood cell, Hb: Hemoglobin.
Qualitative data were described using number and percent, while normally quantitative data was expressed in mean ± SD, abnormally distributed data was expressed in median (Min.–Max.).
* Statistically significant at $p \leq 0.05$.

after induction therapy (OR = 88.777, 95% C.I: 2.85–2765.78, $p=0.011$) while other factors were not associated with induction outcome in CN-AML patients (Table 4).

Survival analysis

The overall actuarial survival at 4 years from diagnosis was 74% for all CN-AML patients enrolled in this study. The relapse rate was significantly higher in *FLT3/ITD* mutated patients (50% versus 19.7%, $p=0.004$) while *NPM1* mutated patients experienced significantly lower relapse rates compared to unmutated patients (3.2% versus 37.7%, $p<0.001$) (Table 3). High *FIS1, PDCD7* and *Ang2* expression levels were significantly associated with higher relapse rates compared to low expression levels (35% versus 18%, $p=0.043$ for *FIS1*, 46% versus 8%, $p<0.001$ for *PDCD7* and 42% versus 12%, $p=0.001$ for *Ang2*) (Table 2).

Survival analysis was performed using the Kaplan-Meier method. Firstly, according to gene mutations, *FLT3/ITD* mutations were associated with poor RFS and OS (HR = 3.288, 95% C.I: 1.717–6.299, $p<0.001$ for RFS and HR = 3.284, 95% C.I: 1.515–7.119, $p=0.003$) while *NPM1* mutations were associated with favorable RFS and OS (HR = 0.139, 95% C.I: 0.043–0.454, $p=0.001$ for RFS and HR = 0.153, 95% C.I: 0.036–0.646, $p=0.011$) in CN-AML patients. Secondly, according to target genes expression levels, high *SPI1* (HR = 2.766, 95% C.I: 1.370–5.587, $p=0.005$ for RFS and HR = 2.357, 95% C.I: 1.024–5.422, $p=0.044$ for OS), high *PDCD7* (HR = 8.879, 95% C.I: 3.690–21.367, $p<0.001$ for RFS and HR = 10.166, 95% C.I: 3.046–33.928, $p<0.001$

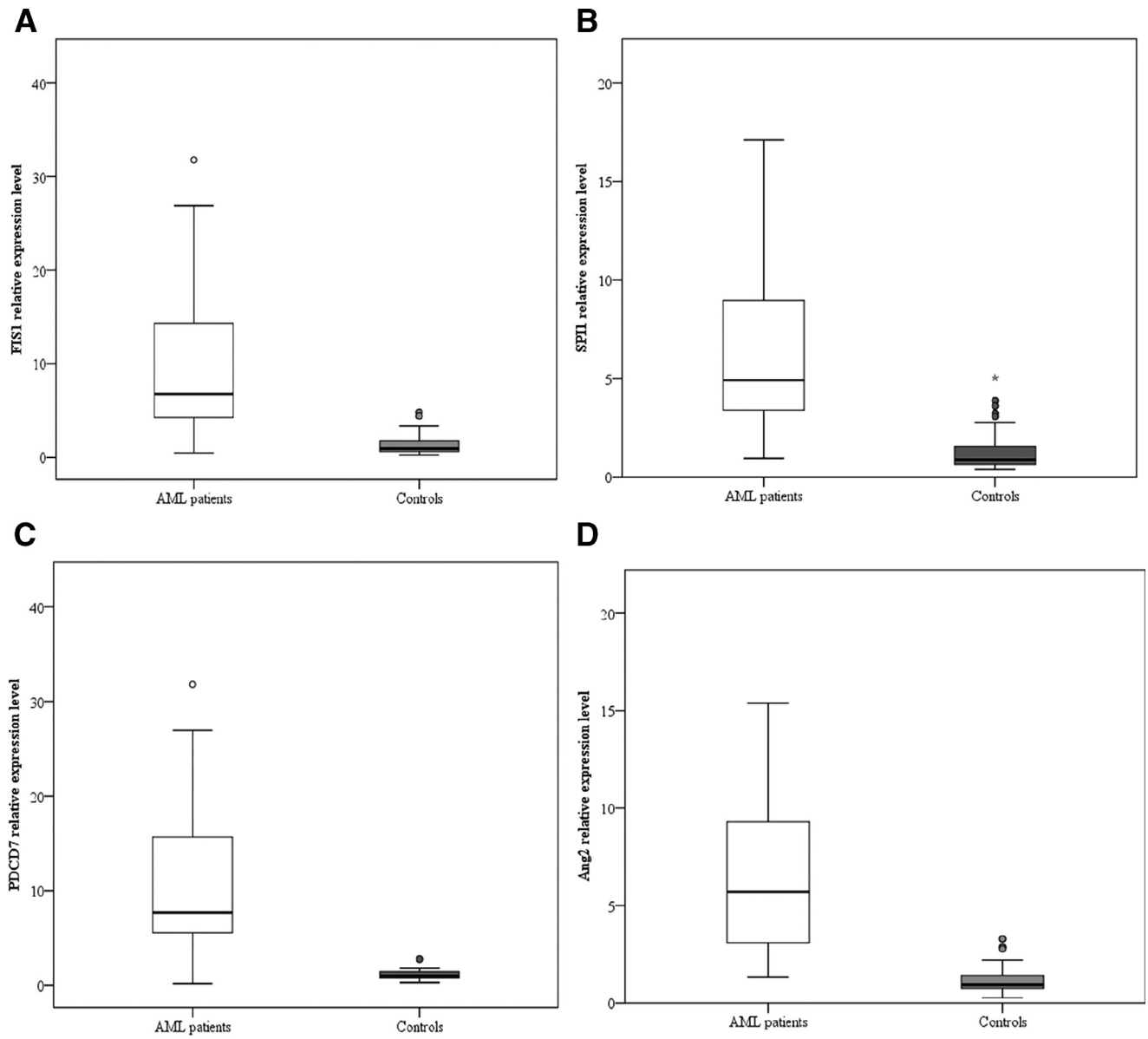

Fig. 1 Relative expression levels of (A) *FIS1* (B) *SPI1* (C) *PDCD7* (D) *Ang2* in CN-AML and non- malignant controls. o: Outlier and *: Extreme outlier.

for OS) and high *Ang2* (HR = 3.612, 95% C.I: 1.751–7.450, $p = 0.001$ for RFS and HR = 2.630, 95% C.I: 1.143–6.052, $p = 0.023$ for OS) were associated with poor RFS and OS. Regarding *FIS1* expression levels, high *FIS1* levels were associated with shorter RFS (HR = 2.919, 95% C.I: 1.445–5.897, $p = 0.003$) but it was not associated with OS (OR = 2.053, 95% C.I: 0.915–4.608, $p = 0.081$) (Fig. 2).

A Cox multivariate analysis was performed for RFS and OS, the variables considered were age (above or below 60 years), type of AML (denovo or secondary), presentation WBCs count, initial peripheral and bone marrow blast percent at diagnosis, the response after induction therapy (CR versus NCR), the intensity of consolidation regimen (high versus low intensity), the presence of genetic mutations and gene expression levels. High expression of *PDCD7* was the only significant risk factor adversely affecting RFS (HR = 5.107, 95% C.I: 1.731–15.066, $p = 0.003$) and OS (HR = 7.353, 95% C.I: 1.859–29.079, $p = 0.004$) (Table 4).

Discussion

Acute myeloid leukemia (AML) represents a highly heterogeneous clonal stem cell disorder in both the clinical and the biological levels. It is characterized by uncontrolled clonal expansion of myeloid hematopoietic precursors in the bone marrow. A large proportion of AML patients shows cytogenetic abnormalities, genetic mutations and abnormal gene expression patterns of oncogenes and tumor suppressor genes that

Table 2 Clinical characteristics and gene expression levels in CN-AML patients.

	FIS1		*SPI1*		*PDCD7*		*Ang2*	
	High	Low	High	Low	High	Low	High	Low
Age								
≤60	46(92.0%)	41(82%)	45(88.2%)	42(85.7%)	42(84.0%)	45(90%)	46(92.0%)	41(82%)
>60	4(8.0%)	9(18%)	6(11.8%)	7(14.3%)	8(16.0%)	5(10%)	4(8.0%)	9(18%)
p	0.137		0.708		0.372		0.137	
Sex								
Male	33(66%)	28(56%)	33(64.7%)	28(57.1%)	32(64%)	29(58%)	31(62%)	30(60%)
Female	17(34%)	22(44%)	18(35.3%)	21(42.9%)	18(36%)	21(42%)	19(38%)	20(40%)
p	0.305		0.438		0.539		0.838	
FAB subtypes								
M0	5(10%)	8(16%)	5(9.8%)	8(16.3%)	6(12%)	7(14%)	4(8%)	9(18%)
M1	10(20%)	8(16%)	10(19.6%)	8(16.3%)	9(18%)	9(18%)	10(20%)	8(16%)
M2	13(26%)	12(24%)	14(27.5%)	11(22.4%)	14(28%)	11(22%)	11(22%)	14(28%)
M3	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
M4	10(20%)	6(12%)	9(17.6%)	7(14.3%)	8(16%)	8(16%)	11(22%)	5(10%)
M5	11(22%)	13(26%)	12(23.5%)	12(24.5%)	10(20%)	14(28%)	12(24%)	12(24%)
M6	1(2%)	2(4%)	1(2%)	2(4.1%)	2(4%)	1(2%)	1(2%)	2(4%)
M7	0(0%)	1(2%)	0(0%)	1(2%)	1(2%)	0(0%)	1(2%)	0(0%)
p	0.795		0.875		0.927		0.415	
Type of AML								
Denovo	45(90%)	44(88%)	46(90.2%)	43(87.8%)	48(96%)	41(82%)	46(92%)	43(86%)
Secondary	5(10%)	6(12%)	5(9.8%)	6(12.2%)	2(4%)	9(18%)	4(8%)	7(14%)
p	0.749		0.697		0.025*		0.338	
WBC	31.5(1.9–222)	32(2–215)	32(1.9–222)	32(2–132)	28.5(1.9–222)	32(2–215)	32.5(1.9–222)	27(2–215)
p	0.893		1.000		0.287		0.572	
NPM1								
Unmutated	38(76%)	31(62%)	40(78.4%)	29(59.2%)	42(84%)	27(54)%	43(86%)	26(52%)
Mutated	12(24%)	19(38%)	11(21.6%)	20(40.8%)	8(16%)	23(46%)	7(14%)	24(48%)
p	0.130		0.037*		0.001*		<0.001*	
FLT3/ITD								
Unmutated	35(70%)	41(82%)	34(66.7%)	42(85.7%)	29(58%)	47(94%)	34(68%)	42(84%)
Mutated	15(30%)	9(18%)	17(33.3%)	7(14.3%)	21(42%)	3(6%)	16(32%)	8(16%)
p	0.160		0.026*		<0.001*		0.061	
Response to induction therapy								
CR	14(31.1%)	49(98%)	17(37%)	46(93.9%)	23(51.1%)	40(80%)	16(35.6%)	47(94%)
NCR	31(68.9%)	1(2%)	29(63%)	3(6.1%)	22(48.9%)	10(20%)	29(64.4%)	3(6%)
p	<0.001*		<0.001*		0.003*,*		<0.001*	
Intensity of consolidation regimen								
Low	19(38%)	40(80%)	20(39.2%)	39(79.6%)	23(46%)	36(72%)	19(38%)	40(80%)
High	31(62%)	10(20%)	31(60.8%)	10(20.4%)	27(54%)	14(28%)	31(62%)	10(20%)
p	<0.001*		<0.001*		0.008*		<0.001*	
Relapse								
No relapse	32(64%)	41(82%)	34(66.7%)	39(79.6%)	27(54%)	46(92%)	29(58%)	44(88%)
Relapse	18(35%)	9(18%)	17(33.3%)	10(20.4%)	23(46%)	4(8%)	21(42%)	6(12%)
p	0.043*		0.146		<0.001*		0.001*	
Survival								
Survived	33(66.0%)	41(82.0%)	33(64.7%)	41(83.7%)	27(54.0%)	47(94.0%)	32(64.0%)	42(84.0%)
Died	17(34.0%)	9(18.0%)	18(35.3%)	8(16.3%)	23(46.0%)	3(6.0%)	18(36.0%)	8(16.0%)
p	0.068		0.031*		<0.001*		0.023*	

CR: complete remission NCR: no complete remission.

Abnormally distributed data was expressed in median (Min.–Max.)

* Statistically significant at $p \leq 0.05$.

Table 3 Clinical characteristics and mutational status of *FLT3/ITD* and NMP1.

	FLT3/ITD		*NPM1*	
	Unmutated	Mutated	Unmutated	Mutated
Age	48.2 ± 13.1	49.8 ± 12.0	47.0 ± 12.5	52.1 ± 12.8
p	0.590		0.063	
≤60	66(86.8%)	21(87.5%)	61(88.4%)	26(83.9%)
>60	10(13.2%)	3(12.5%)	8(11.6%)	5(16.1%)
p	1.000		0.534	
Sex				
Male	46(60.5%)	15(62.5%)	42(60.9%)	19(61.3%)
Female	30(39.5%)	9(37.5%)	27(39.1%)	12(38.7%)
p	0.863		0.968	
FAB subtypes				
M0	11(14.5%)	2(8.3%)	7(10.1%)	6(19.4%)
M1	16(21.1%)	2(8.3%)	14(20.3%)	4(12.9%)
M2	21(27.6%)	4(16.7%)	16(23.2%)	9(29%)
M3	0(0%)	0(0%)	0(0%)	0(0%)
M4	11(14.5%)	5(20.8%)	12(17.4%)	4(12.9%)
M5	14(18.4%)	10(41.7%)	17(24.6%)	7(22.6%)
M6	2(2.6%)	1(4.2%)	2(2.9%)	1(3.2%)
M7	1(1.3%)	0(0%)	1(1.4%)	0(0%)
p	0.212		0.817	
Type of AML				
Denovo	65(85.5%)	24(100%)	62(89.9%)	27(87.1%)
Secondary	11(14.5%)	0(0%)	7(10.1%)	4(12.9%)
p	0.062		0.735	
PLT x10^9/L	48.0(8.0–403.0)	72.5(11.0–540.0)	55(8–540)	46(10– 403)
p	0.394		0.884	
WBC x10^9/L	29.5(1.9–132.0)	53.5(11.0–222.0)	32.0(1.9–222.0)	25.0(2.0–87.0)
p	0.026*		0.106	
Hb g/dl	8.5 ± 3.0	8.2 ± 2.4	8.3 ± 2.9	8.6 ± 2.8
p	<0.001*		0.598	
Initial PB blast count%	20.5(0.0–90.0)	21.0(0.0–90.0)	22(0–90)	19(1–90)
p	0.923		0.704	
Initial BM blast count%	64.5(23.0–98.0)	89.0(20.0–99.0)	82(20–99)	54(26–96)
p	0.012*		0.012*	
CR	52(72.2%)	11(47.8%)	37(57.8%)	26(83.9%)
NCR	20(27.8%)	12(52.2%)	27(42.2%)	5(16.1%)
p	0.031*		0.012*	
Relapse				
No relapse	61(80.3%)	12(50%)	43(62.3%)	30(96.8%)
Relapse	15(19.7%)	12(50%)	26(37.7%)	1(3.2%)
p	0.004*		<0.001*	
Survival				
Died	14(18.4%)	12(50%)	24(34.8%)	2(6.5%)
Survived	62(81.6%)	12(50%)	45(65.2%)	29(93.5%)
p	0.002*		0.003*	

PLT: Platelet, WBC: white blood cell, Hb: Hemoglobin, CR: complete remission, NCR: no complete remission.
Qualitative data were described using number and percent, while normally quantitative data was expressed in mean ± SD, abnormally distributed data was expressed in median (Min.–Max.).
* Statistically significant at $p \leq 0.05$.

affect AML prognosis [20]. However, accurate risk stratification of AML patients at diagnosis stayed difficult.

Recently, the development of sequencing techniques has allowed the discovery of new molecular markers and led to a better understanding of the molecular pathogenesis of AML. This technical progress permitted the diagnosis of AML to stride from simple morphological 7 FAB subtypes to a much-sophisticated classification involving cytogenetic abnormalities, genetic mutations, and recently gene expression levels. However, there is controversy about the prognostic significance of other novel molecular markers based on previous research [21].

Since accurate risk stratification and prognostic evaluation for AML patients upon diagnosis can greatly prolong survival and improve patients' quality of life, this study aimed to investigate the expression patterns and clinical relevance

Table 4 Multivariate analysis of prognostic value of AML patients.

	CR		RFS		OS	
	p	OR(95%C.I)	p	HR(95%C.I)	p	HR(95%C.I)
Age (>60)	NS		NS		NS	
Type of AML (Secondary)	NS		NS		NS	
WBC count	NS		NS		NS	
Initial PB blast count	NS		NS		NS	
Initial BM blast count	NS		0.791	1.002(0.985–1.020)	0.998	1.000(0.982–1.018)
CR versus NCR	NS		0.813	1.185(0.291–4.821)	NS	
intensity consolidation (High versus low)	0.098	4.494(0.757–26.682)	0.698	1.227(0.437–3.444)	NS	
NPM1	0.646	0.611(0.075–4.995)	0.037*	0.253(0.070–0.922)	0.080	0.260(0.058–1.172)
FLT3/ITD	0.443	2.104(0.315–14.070)	0.192	1.660(0.776–3.551)	0.444	1.370(0.612–3.070)
FISI	0.011*	88.777(2.85–2765.78)	0.742	1.321(0.252–6.919)	NS	
SPI1	0.526	0.378(0.019–7.650)	0.505	0.657(0.0191–2.261)	0.770	1.171(0.406–3.380)
PDCD7	0.778	0.775(0.131–4.573)	0.003*	5.107(1.731–15.066)	0.004*	7.353(1.859–29.079)
Ang2	0.056	7.200(0.953–54.400)	0.786	0.848(0.259–2.784)	0.696	0.804(0.268–2.406)

NS: no significance OR: Odd's ratio, C.I: Confidence interval.
* Statistically significant at $p \leq 0.05$.

of 4 novel molecular biomarkers in CN-AML patients, in order to improve the precision of AML prognostic evaluation. Target genes, *FIS1, SPI1, PDCD7* and *Ang2*, were selected based on previous studies that highlighted their probable significance in AML prognosis [9,22].

Our first target was the fission 1 mitochondrial outer membrane (*FIS1*) gene which is located on chromosome 7 and encodes the FIS1 protein, a major component of the ARCosome that promotes mitochondrial fission. Mitochondria are complex organelles that influence tumorigenesis. Mitochondrial fission and fusion dynamics maintain functional mitochondria in dysregulated cancer cells [23,24]. *FIS1* is well known to be implicated in cell cycle and apoptosis regulation [25–27]. In addition, it has been established to be involved in many neurodegenerative diseases and cancer cell signaling pathways [28,29]. Our results revealed significant upregulation of *FIS1* in BM samples of AML patients. The *FIS1* expression levels were positively correlated with the BM blast counts and leukemic burden at initial diagnosis. High *FIS1* was significantly associated with unfavorable therapeutic outcomes and in multivariate analysis, *FIS1* was an independent risk factor for attaining CR response after induction therapy. Our results were consistent with those reported by Tian et al. [22] and the previously published findings that higher levels of *FIS1* were found in leukemic stem cells (LSCs) derived from primary human AML specimens relative to non-LSCs [30]. Importantly, knocking-down of *FIS1* in LSCs was found to simultaneously activate AMPK and inhibit GSK3 resulting in global up-regulation of a hematopoietic lineage differentiation gene signature, and profound loss of LSCs self-renewal potential which elucidates a potential role for *FIS1* gene as a novel therapeutic target in AML [30].

Our second target was the spleen focus forming virus proviral integration site 1 (*SPI1*) proto-oncogene which encodes the PU.1 member of E-twenty six (ETS) transcription factor family. PU.1 is a hematopoietic transcription factor that stimulates gene expression during the myeloid and B- lymphoid lineage evolution [31]. PU.1 binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. PU.1 overexpression was associated with longer survival in follicular lymphoma [32] while its underexpression was reported in AML and indolent types of lymphomas [33,34]. However, *SPI1* expression patterns in AML were not fully addressed; hence, we investigated it in the current study and revealed significant overexpression of *SPI1* in BM of AML patients compared to controls. Our results are supported by the findings of other studies which revealed that overexpression of the oncogenic *SPI1* blocks erythroid progenitor differentiation and apoptosis by modulating the epigenetic control of the expression of the pro-apoptotic factor Bim [35,36]. Moreover, *SPI1* overexpression was reported to shorten S phase duration and raise genetic instability by increasing mutability in the absence of DNA breaks which favor the accumulation of mutations and the progression from the pre-leukemic to the leukemic stage [37].

In the same direction, our results revealed a significant association between high *SPI1* and *FLT3/ITD* mutation, failure of attaining CR response and higher mortality rates. In multivariate analysis, *SPI1* expression was not significantly associated with CR, RFS or OS. These results were consistent with those reported by Zhu et al. in AML [9].

Another new player in the transcriptional network of AML is the programmed cell death 7 (*PDCD7*) gene which is located on 15q22.31 and encodes a 59 kDa protein. PDCD7 protein associates with the U11 small nuclear ribonucleoprotein (snRNP), which is a component of the minor U12-type spliceosome responsible for catalyzing pre-mRNA splicing of U12-type introns [38,39]. In humans, the *PDCD7* gene was not associated with a specific disease or cancer and has not been studied adequately [40]. Only one previous study revealed *PDCD7* overexpression in human malignancy and it was on AML [22]. In order to validate these findings, we investigated the *PDCD7* expression and revealed its significant overexpression in the bone marrows of CN-AML patients. Moreover, *PDCD7* was positively correlated with bone marrow blast count at diagnosis emphasizing a link between the leukemic burden and *PDCD7* expression levels. High *PDCD7*

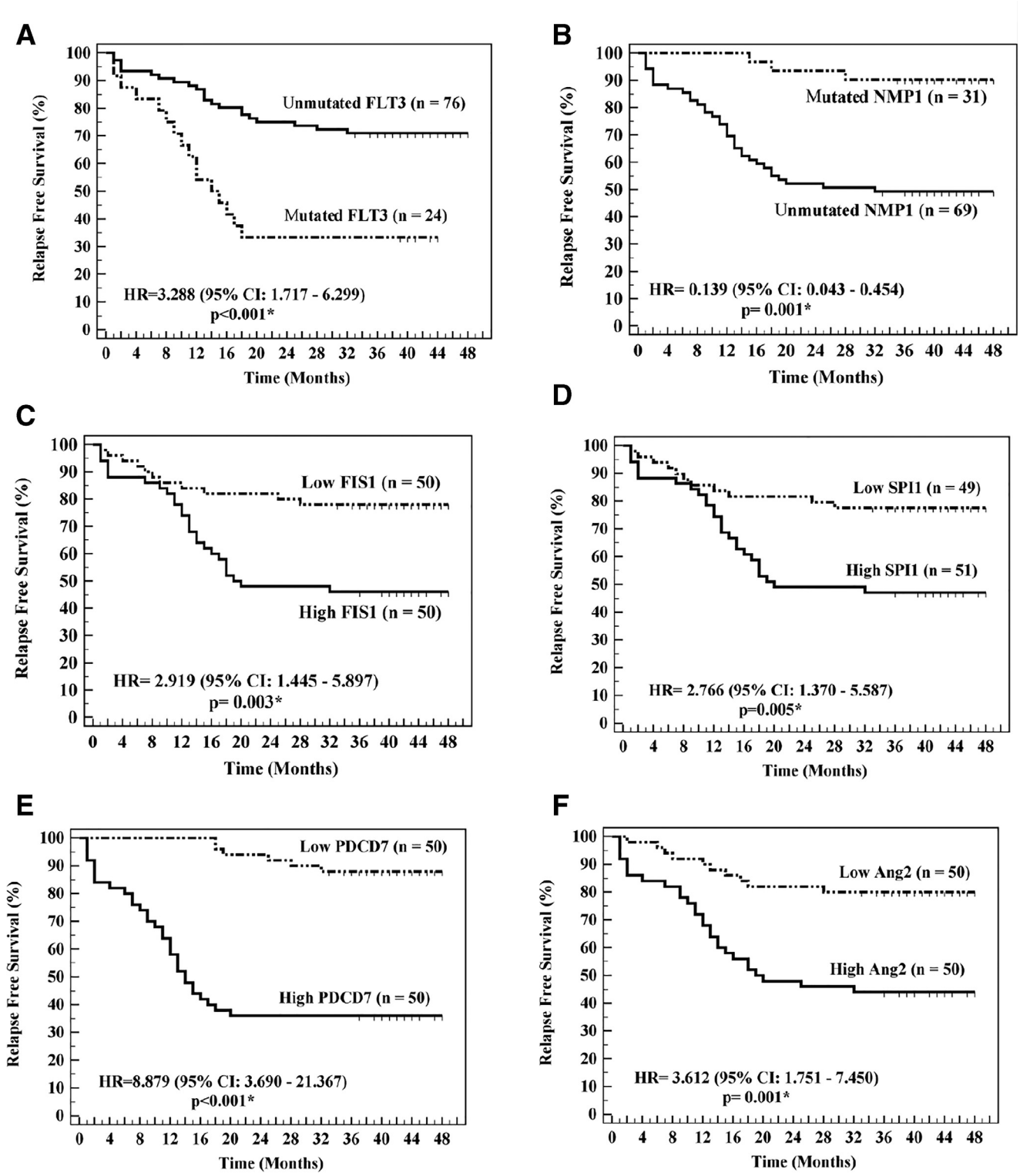

Fig. 2 The Kaplan–Meier survival curves for the Comparison of RFS between CN-AML patients according to the mutational status of (A) *FLT/ITD*, (B) *NPM*1, and different expression levels of (C) *FIS*1, (D) *SPI1*, (E) *PDCD7* and (F) *Ang2*. Also, OS was analyzed in CN-AML patients according to the mutational status of (G) *FLT/ITD*, (H) *NPM*1, and different expression levels of (I) *FIS1*, (J) *SPI1*, (K) *PDCD7* and (L) *Ang2*.

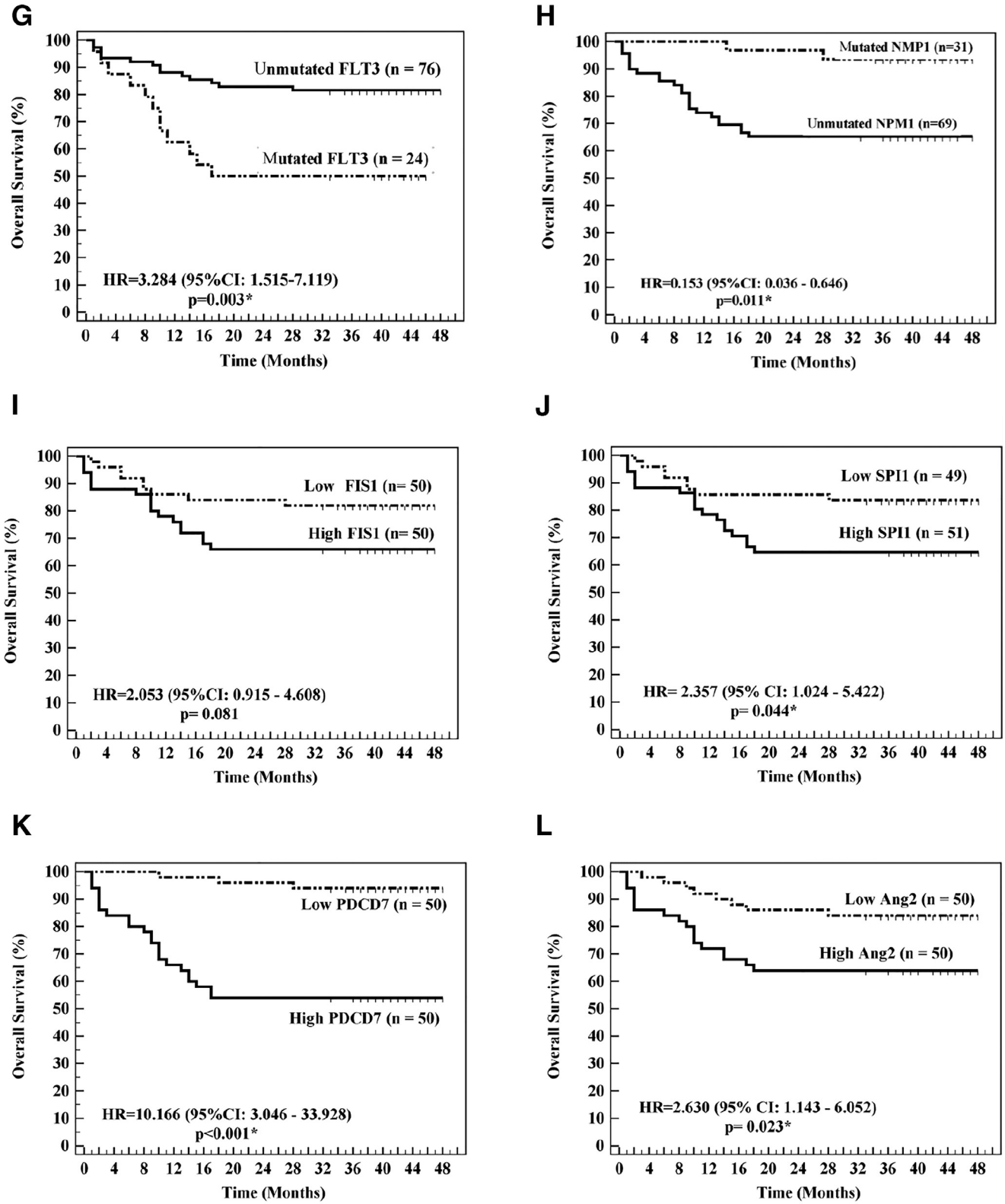

Fig. 2 Continued

was significantly associated with lower CR response rate, higher relapse rate, shorter RFS and OS compared to low expression levels. In multivariate analysis, only *PDCD7* was found to be an independent risk factor for RFS and OS. These results go hand in hand with those reported by Tian et al. [22] suggesting an effective role for *PDCD7* as a risk factor and potential therapeutic target in AML.

After that, we focused on Angiopoietin-2 (*Ang2*) which is a key regulator of tumor angiogenesis and metastasis. Ang2 plays a pivotal role in the progression of myeloid malignancies by acting as an antagonist for Ang1 suppressing Ang1-promoted Tie2 signaling, which is critical for blood vessel maturation and stabilization. It is encoded by *Ang2* gene on chromosome 8. The *Ang2* expression has been linked to tumor progression, growth, invasion and metastasis. Moreover, *Ang2* has been found to have a great potential as a therapeutic target, a prognostic marker, and an inhibitor of human cancers. However, there is a great debate about the role of *Ang2* in AML.

Our study revealed overexpression of *Ang2* in CN-AML. High *Ang2* was significantly associated with failure to attain CR response after induction therapy, poor RFS and OS. In multivariate analysis, RFS and OS seemed to be not influenced by *Ang2* expression levels alone. Similarly, Schliemann et al. [41] and Tian et al. [22] reported *Ang2* to be associated with a lower CR rate in AML. Furthermore, Hou et al. [42] reported that high pre-treatment level of Ang2 in the BM is an independent poor prognostic factor for OS in AML patients. On the contrary, Loges et al. [43] found that high *Ang2* expression in AML was significantly associated with a good response after initiation of induction chemotherapy. Also, Schliemann et al. [44] reported that AML patients with high levels of *Ang2* had significantly longer OS than those with low *Ang2* levels. The controversial results regarding the role of *Ang2* in AML prognosis may be attributed to its interaction with the pro-angiogenic vascular endothelial growth factor (*VEGF*) as they play a complementary role in the process of vascular remodeling. *VEGF* upregulation coincident with *Ang2* expression induces angiogenesis while late expression of tumor-derived *VEGF* may serve to repress the signal for vessel regression by *Ang2* [41–44].

In conclusion, the *FIS1* expression has a significant impact on CR response after induction therapy while high *PDCD7* expression is an independent risk factor for RFS and OS in CN-AML patients. Therefore, *FIS1* and *PDCD7* expression patterns provide important biological and clinical information and should be integrated into the current AML stratification system as their high expression levels demarcate a more aggressive disease form in AML patients who should be candidates for alternative therapeutic strategies.

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Declarations of interest

Authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2018.12.001.

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