

# *CD97* expression is associated with poor overall survival in acute myeloid leukemia

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**CD97, a member of the adhesion G-protein coupled receptor family, is normally expressed on leukocytes and smooth muscles. CD97 is also expressed in a variety of solid cancers, particularly those with aggressive metastatic phenotypes. Here we characterize the clinical significance of *CD97* in acute myeloid leukemia (AML). We analyzed 173 patients from the TCGA AML data set and found that *CD97* was higher in cytogenetically normal patients compared with cytogenetically abnormal patients ( $p = 0.023$ ). High *CD97* was also associated with *NPM1* mutations ( $p = 0.0033$ ). Patients with high *CD97* expression had shorter overall (median: 7.35 months vs. 24.1 months,  $p = 0.0015$ ) and disease-free (median DFS: 8.2 months vs. 18.2 months,  $p = 0.017$ ) survival. Importantly, we identified pathways involved in the leukemia stem cell interaction with the bone marrow niche, such as integrin, CXCR4, and interleukin-8, among the most upregulated signaling pathways in patients with high *CD97* expression. Our results suggest that high *CD97* expression is associated with poor clinical outcome and indicate a need for future functional and mechanistic studies to investigate the role of *CD97* in AML. © 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

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Acute myeloid leukemia (AML) is a heterogeneous, hematologic malignancy characterized by clonal proliferation of myeloid precursors [1]. It is the most common acute leukemia in adults. Overall survival of patients with AML remains dismal (<50% for younger patients and <10% for older patients) because of the high rate of relapse [2]. Cytogenetic and molecular genetic alterations provide significant prognostic information for determining the response to chemotherapy and survival outcome.

Adhesion G protein-coupled receptors (aGPCRs) were recently reported to be widely deregulated in AML [3,4]. Among aGPCRs, CD97 is particularly interesting; it is expressed predominantly in hematopoietic cells [5] and has been recently identified as a marker for leukemia stem cells [6]. Several ligands have been reported to bind

CD97: CD55, which is a negative regulator of the complement cascade [7]; chondroitin sulfate, a component of the extracellular matrix (ECM) [8]; and the integrin  $\alpha5\beta1$  [8]. The association with integrins is relevant to AML, as integrin and ILK signaling pathways play crucial roles in leukemia stem cell (LSC) survival and interaction with the bone marrow (BM) niche [9,10].

In solid cancers, CD97 association with integrin was found to regulate invasion, migration, and angiogenesis [11]. In undifferentiated anaplastic thyroid carcinoma, CD97 overexpression was associated with metastatic lesions [12,13]. Additionally, high CD97 expression was also reported to be associated with increased lymph vessel invasion and poor clinical outcome in colorectal carcinomas [14]. In pancreatic cancer, CD97 expression significantly correlated with tumor aggressiveness [15]. More recently, CD97 was also shown to be the first member of the EGF-TM7 family to be directly involved in cell signaling. Ward et al. [16] demonstrated

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functional synergy and heterodimerization between CD97 and lysophosphatidic acid receptor 1 (LPAR1), resulting in increased RHO-GTP levels in a  $G\alpha_{12/13}$ -dependent signaling pathway in thyroid and prostate cancers, as well as correlating with increased tumor invasion.

The role of CD97 in invasion and signaling suggests that it is an important therapeutic target. In this study, we characterized *CD97* expression in patients with AML using publicly available data sets to determine its association with patient's clinical and molecular characteristics and clinical outcome. We also identified signaling pathways that are deregulated in patients with high *CD97*.

## Methods

### Patient data sets

Available data on 173 of 200 patients with previously untreated AML with complete clinical and RNA expression data were studied from the TCGA data set [17]. This data set includes patients with previously untreated AML, all of whom had been diagnosed and received treatment according to the National Comprehensive Cancer Network (NCCN) guidelines between November 2001 and March 2010. The risk group stratification of the patients was done according to NCCN guidelines. The patients were assigned subtype classifications according to the French–American–British (FAB) classifications. Patients included in the study were assessed for the most frequently found somatic mutations in AML, such as *FLT3*, *NPM1*, *IDH1/2*, and *TET2*. Patient's clinical, gene expression (*Z* score), mutation, gene methylation, and survival data were downloaded from the TCGA database available at cBioPortal on September 9, 2018. Metzeler-163 [18], Metzeler-79 [18], and Bullinger [19] data sets were downloaded from OncoPrint.

Ethics approval and consent to participate were not necessary, as we studied public de-identified data.

### Gene expression analyses

We downloaded publicly available and analyzed RNA sequencing data of 173 patients with acute myeloid leukemia and complete clinical and molecular data [20,21]. We dichotomized patients into two groups based on *Z* scores  $\geq 1$  (high) and  $< 1$  (low). We also validated the associations using a *Z* score of 2 as the cutoff as well as the median of the mRNA  $\log_2$  transformed data.

### Statistical analyses

Overall survival (OS) was defined as the time between diagnosis and death from any reason, and disease-free survival (DFS) was defined as the time between diagnosis and removal from study because of relapse or death (two of the patients with missing information regarding their DFS were not included in the DFS analysis). We generated Kaplan–Meier survival curves for comparison of OS and DFS between patients with  $Z \geq 1$  and  $Z < 1$  *CD97* expression. Additionally, Kaplan–Meier survival curves were generated for patients with  $Z \geq 1$  and  $Z < 1$  *CD97* expression after stratification by age, cytogenetic status, transplant status, and *NPM1* mutation status. To determine the association between *CD97* expression and patients' clinical and molecular

characteristics, we employed T test and Fisher's exact test for continuous and categorical variables, respectively, using the GraphPad Prism software package (Version 5.0, GraphPad Software Inc., La Jolla, CA). We used Stata SE 12.0 to perform a Cox proportional hazards model to conduct survival analysis of the effect of *CD97* expression on OS and DFS after adjusting for other clinical/molecular factors. A statistical cutoff of  $p < 0.05$  was used for inclusion of variables into the multivariate survival analysis from univariate analysis.

### Ingenuity pathway analysis (IPA)

Patients were grouped as *CD97* high ( $Z > 2$ ) and *CD97* low ( $Z < -1$ ). Pearson correlation scores of *CD97* versus all the available gene expression data in TCGA were calculated, and upregulated genes with a score  $> 0.5$  and downregulated genes with a score  $< -0.5$  were exported into IPA. Signaling pathways were evaluated for *CD97* high and *CD97* low patients.

## Results

### *CD97* expression in AML

*CD97* mRNA (RNA Seq V2 RSEM) expression data were downloaded from the TCGA data set on cBioPortal [20,21]. Histograms representing the distribution of *CD97* mRNA  $\log_2$ -transformed data and *CD97* *Z*-scores are provided in [Supplementary Figure E1A and B](#) (online only, available at [www.exphem.org](http://www.exphem.org)). The scatterplot of *CD97*  $\log_2$ -transformed mRNA expression against *CD97* *Z* score is shown in [Figure E1C](#) (Pearson's  $r = 0.944$ ,  $p \leq 0.001$ ). *CD97* mRNA levels were compared among patients classified according to the FAB. Although patients in the M5 subgroup had relatively higher *CD97* expression, there was no significant difference in *CD97* expression among the subgroups ([Supplementary Figure E1D](#)). When patients were classified based on cytogenetic status into cytogenetically normal AML (CN-AML) ( $N = 80$ ) and cytogenetically abnormal AML (CA-AML) ( $N = 90$ ), we found that patients with CN-AML had significantly higher *CD97* expression compared with patients with CA-AML (1.31-fold,  $p = 0.023$ ) ([Supplementary Figure E1E](#)).

We also analyzed *CD97* expression according to the NCCN AML classification in which patients are grouped based on their molecular and cytogenetic risk status into favorable, intermediate, and poor risk groups and found no significant difference between these groups ([Supplementary Figure E1F](#)).

Additionally, we used the Vizome data analysis tool, which contains data from the BEAT AML cohort [22], and examined the level of *CD97* expression in relapsed ( $N = 46$ ) versus de novo AML ( $N = 288$ ) samples and found no significant difference between the two groups ([Supplementary Figure E1G](#)).

We dichotomized the patients in the TCGA data set based on their *CD97* mRNA expression *Z* score (RNA Seq V2 RSEM) into *CD97* high (*Z* score  $\geq 1$ ,  $N = 26$ ) and

CD97 low ( $Z$  score  $<1$ ,  $N=147$ ). Patients with high CD97 expression had significantly higher white blood cell counts (WBCs) (median: 56.1 vs. 13.1 [ $\times 10^9/L$ ],  $p=0.004$ ) and higher percentages of bone marrow blasts (median: 83% vs. 71%,  $p=0.019$ ) (Table 1).

#### High CD97 expression is associated with NPM1 mutations

To understand potential molecular genetic aberrations that may lead to or be associated with high CD97 in AML, we analyzed its expression with respect to the mutational status of patients with AML. CD97 was significantly higher in patients with NPM1 mutation ( $n=48$ ) than in patients with NPM1 wild type ( $n=125$ , 1.56-fold,  $p < 0.0001$ ; Figure 1A). CD97 was also significantly higher in patients with FLT3 mutations (ITD and point mutations) ( $n=49$ ) than in patients carrying FLT3 wild type ( $n=124$ , 1.4-fold,  $p=0.0008$ ; Figure 1B). Additionally, CD97 was significantly lower in the patients with RUNX1 mutations ( $n=17$ ) than in patients with the wild-type RUNX1 ( $n=156$ ) (42.1% lower,  $p=0.0002$ ; Figure 1C). No significant association was observed between CD97 expression and mutations in DNMT3A, IDH1, IDH2, TET2, TP53, CEBPA, WT1, and KIT.

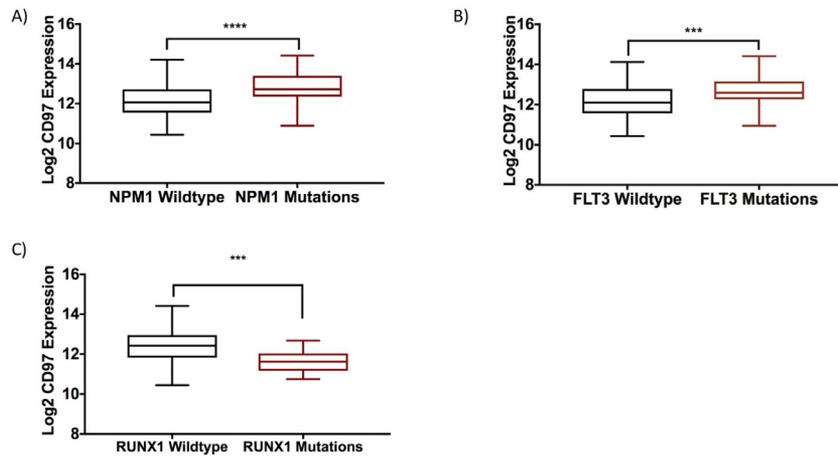
When we dichotomized patients according to CD97  $Z$  scores, we found a higher frequency of NPM1 mutations in high CD97 ( $Z \geq 1$ ) patients than in low CD97 ( $Z < 1$ ) patients (52% vs. 30.6%, Fisher exact  $p=0.0065$ ). No association was found between CD97 upregulation and mutations in other genes in the dichotomized analysis (Table 2).

#### High CD97 expression is associated with poor clinical outcome

The overall survival of patients in the CD97 high group ( $Z \geq 1$ ) was significantly shorter than that of patients in the CD97 low group (median OS: 7.35 vs. 24.1 months,  $p=0.0015$ ; Figure 2A). Patients with high CD97 expression had significantly shorter DFS than patients with low CD97 (median DFS: 8.2 months vs. 18.2 months,  $p=0.017$ ; Figure 2B). To further validate the association between high CD97 and poor clinical outcome, we stratified patients into  $Z \geq 2$  and  $Z < 2$  for survival analysis. Patients with high CD97 expression had significantly shorter OS and DFS than patients with low CD97 (OS—median: 7.5 months vs. 20.5 months,  $p=0.04$ ; DFS—median: 7.3 months vs. 17 months,  $p=0.03$ ; Supplementary Figure E2A and B, online only, available at [www.elsevier.com/locate/ymh](http://www.elsevier.com/locate/ymh)).

**Table 1.** Clinical characteristics of the AML cohort in the TCGA data set with respect to CD97 expression

	Total	CD97 low ( $Z < 1$ )	CD97 high ( $Z \geq 1$ )	$p$ Value	
				T test	Fisher exact test
Sex, No. (%)					1
Female	81 (46.8)	69 (85.2)	12 (14.8)		
Male	92 (53.1)	78 (84.8)	14 (15.2)		
Age, y (range)				0.385	
Median	58	57	62.5		
Mean	55.2 $\pm$ 1.22	54.7 $\pm$ 1.32	57.7 $\pm$ 3.38		
WBC count $\times 10^9/L$				0.004	
Median	17	13.1	56.1		
Mean	36.63 $\pm$ 3.50	32.44 $\pm$ 3.81	60.30 $\pm$ 7.48		
PB blasts, %				0.25	
Median	39	34.5	49		
Mean	39.59 $\pm$ 2.44	38.38 $\pm$ 2.68	46.2 $\pm$ 5.85		
BM blasts, %				0.019	
Median	72	71	83		
Mean	69.08 $\pm$ 1.45	67.65 $\pm$ 1.59	77.15 $\pm$ 3.17		
NCCN subtype, No.					vs. favorable
Favorable	33	29	4		
Intermediate	92	76	16		0.5
Poor	45	40	5		0.6
FAB subtype, No.					vs. M5
M0	16	15	1		0.042
M1	44	39	5		0.028
M2	38	34	4		0.026
M3	16	12	4		0.476
M4	34	29	5		0.081
M5	18	11	7		
M6	2	2			
M7	3	3			



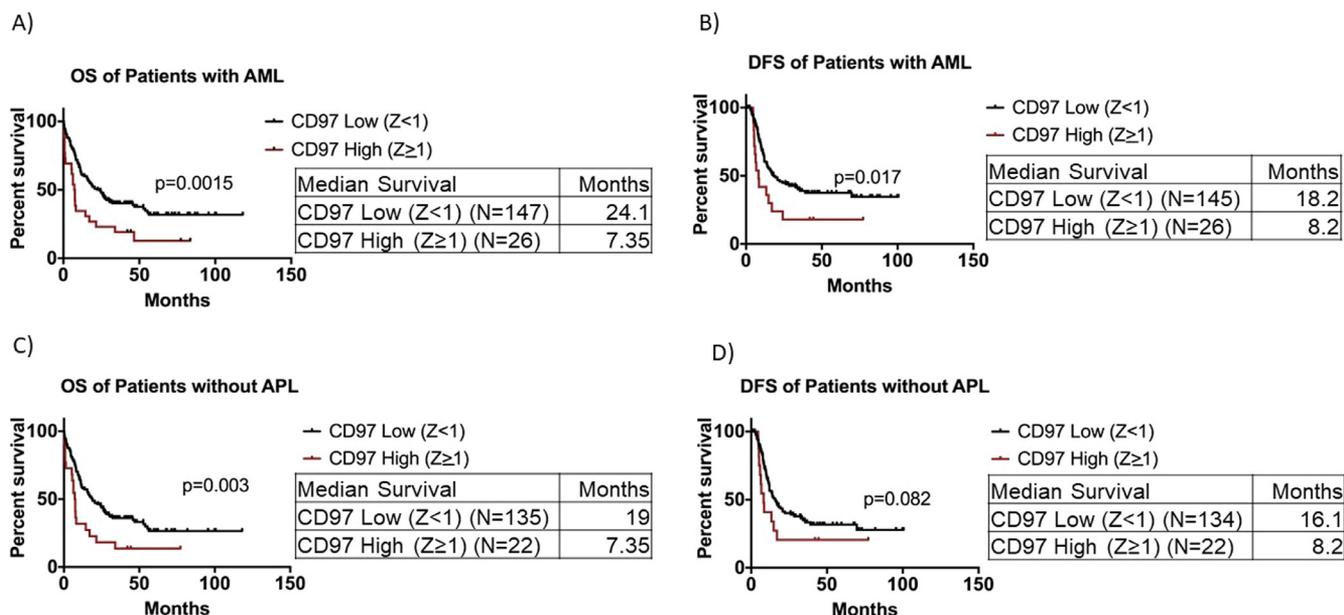
**Figure 1.** Association of *CD97* mRNA expression with patient mutational status. Relative *CD97* log<sub>2</sub> mRNA expression in (A) patients with *NPM1* mutations versus wild type; (B) patients with *FLT3* mutations versus wild type; and (C) patients with *RUNX1* mutations versus wild type. \*\*\**p* < 0.001. \*\*\*\**p* < 0.0001.

expchem.org). Because patients with APL or t(15, 17) are treated differently (with ATRA) and have better clinical outcome than the rest of patients with AML, we

excluded these patients from the survival analysis. Yet, the OS of the *CD97* high group ( $Z \geq 1$ ) remained significantly shorter than that of the *CD97* low patients

**Table 2.** Association of *CD97* expression with patient mutational status in the TCGA data set

	Total	<i>CD97</i> low ( $Z < 1$ )	<i>CD97</i> high ( $Z \geq 1$ )	<i>p</i> Value	
				T test	Fischer exact test
<b>FLT3, No. (%)</b>					
Present	49 (28.3)	38 (77.5)	11 (22.4)	0.0008	0.1005
Absent	124 (71.67)	109(87.9)	15 (12.1)		
<b>IDH1, No. (%)</b>				0.09	0.4718
Mutated	16 (9.2)	15 (93.7)	1 (6.2)		
Wild type	157 (92.3)	132 (84.0)	25 (15.9)		
<b>IDH2, No. (%)</b>				0.77	0.7238
Mutated	17 (9.8)	14 (82.3)	3 (17.6)		
Wild type	156 (90.1)	133 (85.2)	23 (14.7)		
<b>RUNX1, No. (%)</b>				0.0002	0.070
Mutated	17 (9.8)	17 (100)	0 (0)		
Wild type	156 (90.1)	130 (83.3)	26 (16.6)		
<b>TET2, No. (%)</b>				0.10	0.1311
Mutated	15 (8.6)	15 (100)	0 (0)		
Wild type	158 (91.3)	132 (83.5)	26 (16.4)		
<b>NRAS, No. (%)</b>				0.57	1
Mutated	12 (6.9)	10 (83.3)	2 (16.6)		
Wild type	161 (93.0)	137 (85.0)	24 (14.9)		
<b>CEBPA, No. (%)</b>				0.54	0.6944
Mutated	13 (7.5)	12 (92.3)	1 (7.6)		
Wild type	160 (92.5)	135 (84.3)	25 (15.6)		
<b>WT1, No. (%)</b>				0.42	0.3624
Mutated	10 (5.7)	10 (100)	0 (0)		
Wild type	163 (94.2)	137 (84.0)	26 (15.9)		
<b>DNMT3A, No. (%)</b>				0.122	0.1453
Mutated	45 (26.0)	35 (77.7)	10 (22.2)		
Wild type	128 (73.9)	112 (87.5)	16 (12.5)		
<b>NPM1, No. (%)</b>				<0.0001	0.0033
Mutated	48 (27.7)	34 (70.8)	14 (29.1)		
Wild type	125 (72.3)	113 (90.4)	12 (9.6)		
<b>TP53</b>				0.34	0.6970
Mutated	14 (8.09)	13 (92.8)	1 (7.1)		
Wild type	159 (91.9)	134 (84.2)	25 (15.7)		



**Figure 2.** Survival analysis of AML patients with respect to *CD97* expression. (A) Overall survival of 173 AML patients with *CD97* Z score  $\geq 1$  and *CD97* Z score  $< 1$ . (B) Disease-free survival of 171 AML patients with *CD97* Z score  $\geq 1$  and *CD97* Z score  $< 1$ . (C) Overall survival of patients without APL with *CD97* Z score  $\geq 1$  and *CD97* Z score  $< 1$ . (D) Disease-free survival of patients without APL with *CD97* Z score  $\geq 1$  and *CD97* Z score  $< 1$ .

(median: 7.35 months vs. 19 months,  $p = 0.003$ ; Figure 2C). A similar trend was observed in DFS (median: 8.2 months vs. 16.1 months,  $p = 0.08$ ; Figure 2D). Among patients with APL, the OS of patients in the *CD97* high group ( $Z \geq 1$ ) was significantly shorter than that of the *CD97* low patients ( $p = 0.03$ ; Supplementary Figure E3A, online only, available at [www.expchem.org](http://www.expchem.org)). APL patients with high *CD97* expression had significantly shorter DFS than patients with low *CD97* ( $p = 0.003$ ; Supplementary Figure E3B).

When patients were stratified according to their cytogenetic status, we found that among CA-AML patients, those with *CD97* high ( $Z \geq 1$ ) had shorter overall survival than those with *CD97* low; however, this was not statistically significant (median OS: 7.2 months vs. 19.2 months,  $p = 0.159$ ; Figure 3A). Yet the difference in OS between the two groups was statistically significant among the CN-AML patients (median OS: 7.5 months vs. 24.6 months,  $p = 0.0045$ ; Figure 3B). Also, there was a significant decrease in the DFS of *CD97* high patients ( $Z \geq 1$ ) compared with *CD97* low patients (median: 10.8 months vs. 39 months,  $p = 0.043$ ; Figure 3C) in CA-AML but not in CN-AML patients (median: 8.2 months vs. 12.1 months,  $p = 0.25$ ; Figure 3D).

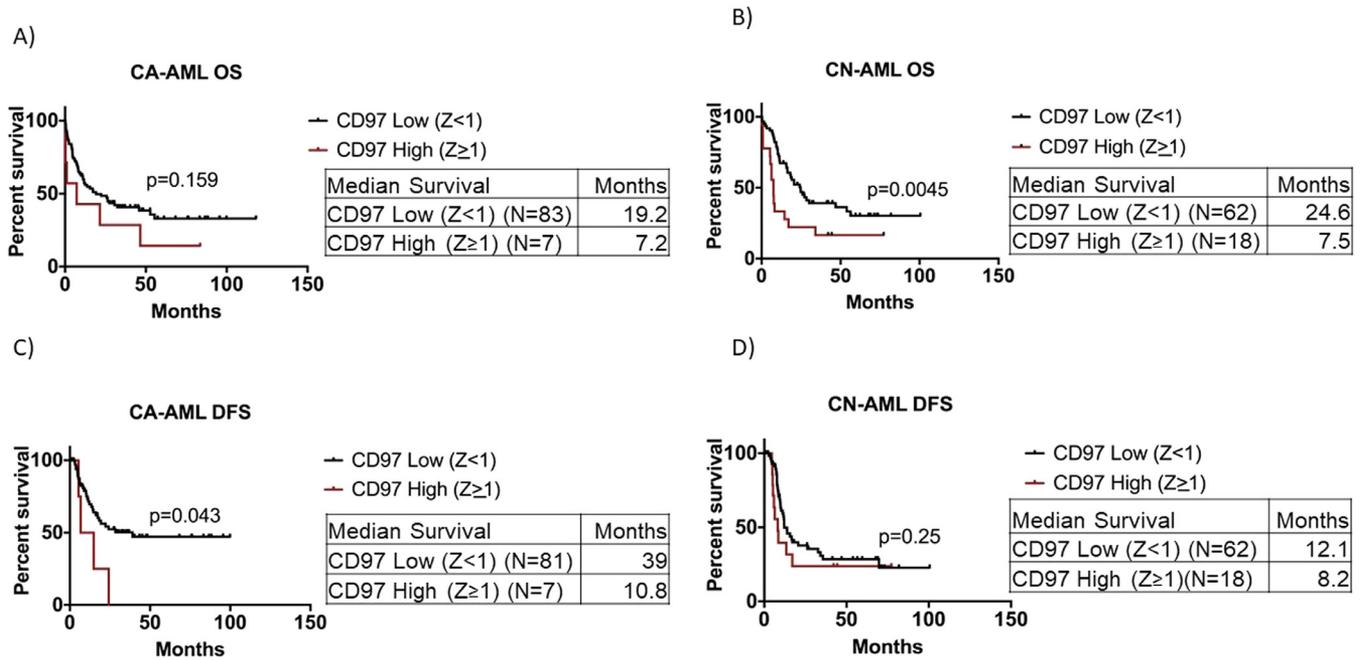
In multivariate survival analysis, *CD97* high expression ( $Z \geq 1$ ) was associated with shorter overall survival when adjusted for age, cytogenetic risk, and transplant status (HR = 1.96, 95% CI: 1.19–3.24,  $p = 0.009$ ; Table 3). A similar trend was observed when patients with t(15,17) were excluded from the analysis: *CD97* expression ( $Z \geq 1$ )

was associated with shorter overall survival (HR = 1.56, 95% CI: 0.97–2.83,  $p = 0.066$ ).

When patients were stratified according to whether they received a transplant or not, we found that only in patients who did not receive a transplant was *CD97* high expression ( $Z \geq 1$ ) associated with significantly shorter OS and DFS (median OS: 5.5 months vs. 11.4 months,  $p = 0.0078$ ; median DFS: 5.9 months vs. 32.7 months,  $p = 0.007$ ) (Figure 4A, C).

No significant difference was observed in OS (Figure 4B) and DFS (Figure 4D) between the *CD97* high and low in patients who received a transplant.

We also analyzed the association of *CD97* expression with clinical outcome in additional data sets: Metzeler-163 [18] (N = 163 CN-AML patients), Metzeler-79 [18] (N = 79 CN-AML patients) and Bullinger (N = 105 CN-AML and CA-AML patients) [19] data sets (histograms representing the distribution of *CD97* mRNA expression are illustrated in Supplementary Figure E4A–C [online only, available at [www.expchem.org](http://www.expchem.org)]). Patients were dichotomized based on median *CD97* expression into *CD97* high (above median) and *CD97* low (below median). In the Metzeler-79 data set (which includes CN-AML only), patients with *CD97* high expression had shorter overall survival (median OS: 8.9 months vs. 42.01 months,  $p = 0.0027$ ; Figure 5A). Similarly, in the Metzeler-163 data set (which includes CN-AML only), patients with high *CD97* expression survived for shorter times than patients with low *CD97*



**Figure 3.** Survival analysis of AML patients with respect to *CD97* expression after stratification based on cytogenetic status. (A) Overall survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in cytogenetically abnormal (CA-AML) patients. (B) Overall survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in cytogenetically normal (CN-AML) patients. (C) Disease-free survival of AML patients with *CD97* high ( $Z$  score  $> 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in CA-AML patients. (D) Disease-free survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in CN-AML patients.

expression; however, the difference in survival was not statistically significant (median OS: 8.6 months vs. 14.2 months,  $p = 0.23$ ; Figure 5B). On the contrary, in the Bullinger data set, which includes both CN and CA-AML, there was no significant association between *CD97* expression and clinical outcome (median survival: 13.15 months vs. 18.74 months,  $p = 0.6$ ; Figure 5C). This discrepancy between the Bullinger data set survival analysis and those of other data sets is possibly due to the difference in the microarray gene probe used to measure *CD97*. We also analyzed the TCGA data set using *CD97* median expression to dichotomize patients into high and low *CD97* expression groups. We found that patients with high *CD97* expression had shorter overall survival (median OS: months 11 vs. 27.4 months,  $p = 0.008$ ; Figure 5D).

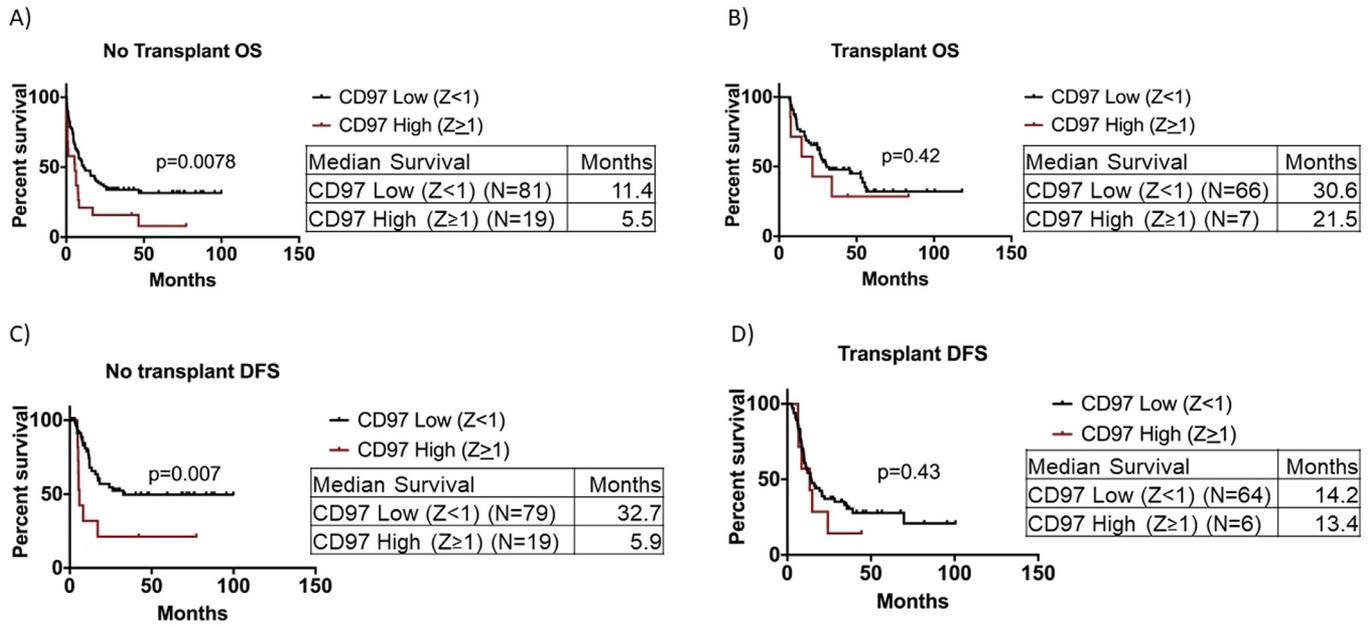
**Table 3.** Cox proportional hazards model for overall survival in patients with AML comparing patients with high ( $Z \geq 1$ ) *CD97* expression and patients with low ( $Z < 1$ ) *CD97* expression.

Variable	Hazard ratio	95% Confidence interval	$p$ Value	
Age	1.03	1.01	1.04	0.002
Molecular risk				0.002
Intermediate	3.03	1.51	6.07	
Poor	6.57	3.10	13.9	<0.001
Transplant status (Y/N)	0.47	0.29	0.74	0.001
<i>CD97</i> ( $Z \geq 1$ )	1.96	1.19	3.24	0.009

#### *CD97 expression is associated with survival status of patients with NPM1 mutation*

Because *CD97* expression was associated with *NPM1* mutation, which is a favorable prognostic marker in AML, we examined the association between *CD97* expression and clinical outcome in patients with *NPM1* mutation. We stratified patients according to *NPM1* mutational status and performed survival analysis in each group. We found that in patients with wild-type *NPM1*, high *CD97* expression was associated with a significantly shorter OS (median survival: 6.35 months vs. 22.3 months,  $p = 0.0081$ ; Figure 6A) and DFS (median survival: 6.7 months vs. 22.2 months,  $p = 0.0015$ ; Supplementary Figure E5A, online only, available at [www.exphem.org](http://www.exphem.org)). In patients with *NPM1* mutation, a similar trend but not statistically significant association was observed between high *CD97* expression and shorter OS (median survival: 7.5 months vs. 24.1 months,  $p = 0.11$ ; Figure 6B) but not DFS (median survival: 10.8 months vs. 12.1 months,  $p = 0.75$ ; Supplementary Figure E5B).

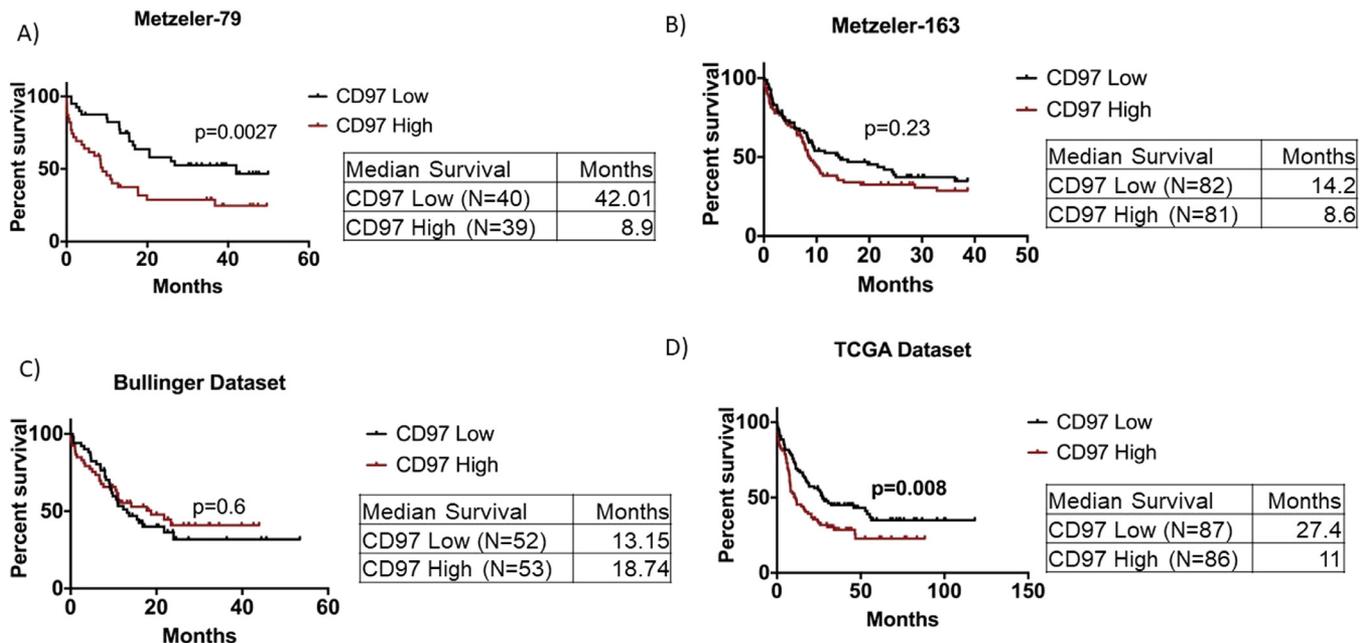
As patients with *NPM1* mutation have a better prognosis and patients with *FLT3* mutation have poor prognosis, we further analyzed the association between *CD97* expression and patients harboring *FLT3* and *NPM1* mutations to gain better insight. We identified 8 of 173 patients in the TCGA data that have both *FLT3*



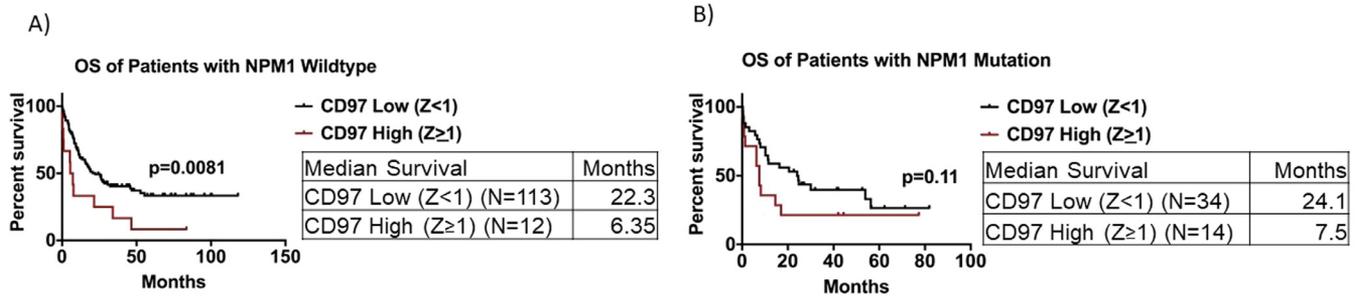
**Figure 4.** Survival analysis of AML patients with respect to *CD97* expression after stratification based on patient transplant status. (A) Overall survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in patients who did not receive a transplant. (B) Overall survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in patients who received a transplant. (C) Disease-free survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in patients who did not receive a transplant. (D) Disease-free survival of AML patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) in patients who received a transplant.

mutation, *NPM1* mutation, and *CD97*  $Z \geq 1$ . When we compared overall survival between patients with *CD97*  $Z > 1$  (N=8) and those with *CD97*  $Z < 1$  (N=18) among those with *FLT3* and *NPM1* mutations, we found that

patients with *CD97*  $Z \geq 1$  had significantly worse survival compared with the  $Z \leq 1$  group ( $p=0.04$ , median survival: 7.5 months vs. 24.6 months; [Supplementary Figure E5C](#)).



**Figure 5.** Survival analysis of AML patients with respect to *CD97* expression in different data sets. Overall survival of patients with AML in four data sets, in which patients were dichotomized based on *CD97* median mRNA expression into *CD97* high and *CD97* low according to the  $\log_2$  median-centered expression. (A) Metzeler-79 data set. (B) Metzeler-163 data set. (C) Bullinger data set. (D) TCGA data set.



**Figure 6.** Survival analysis of AML patients with respect to *CD97* expression after stratification based on *NPM1* mutation status. (A) Overall survival of patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) among patients with *NPM1* wild-type gene. (B) Overall survival of patients with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ) among patients with *NPM1* mutated gene.

### High *CD97* expression is associated with poor survival in older patients

Because older patients with AML have significantly worse overall survival, we performed survival analysis in patients with AML stratified by their age into younger patients ( $< 60$ ) and older patients ( $\geq 60$ ). In patients  $\geq 60$ , high *CD97* ( $Z \geq 1$ ) expression was associated with shorter OS compared with low *CD97* ( $Z < 1$ ) (median OS: 3.3 months vs. 11 months,  $p = 0.0019$ ; **Figure 7A**) and a decrease in DFS though not significant (median DFS: 5.9 months vs. 17 months,  $p = 0.092$ ; **Supplementary Figure E6A**, online only, available at [www.exphem.org](http://www.exphem.org)). A similar trend but not statistically significant association was observed in younger patients (median OS: 27.75 months vs. 53.9 months;  $p = 0.2$ ; **Figure 7B**) and (median DFS: 11.7 months vs. 20.8 months,  $p = 0.1$ ; **Supplementary Figure E6B**).

### Pathways involved in the leukemia stem cell interaction with the bone marrow niche are activated in patients with high *CD97* expression

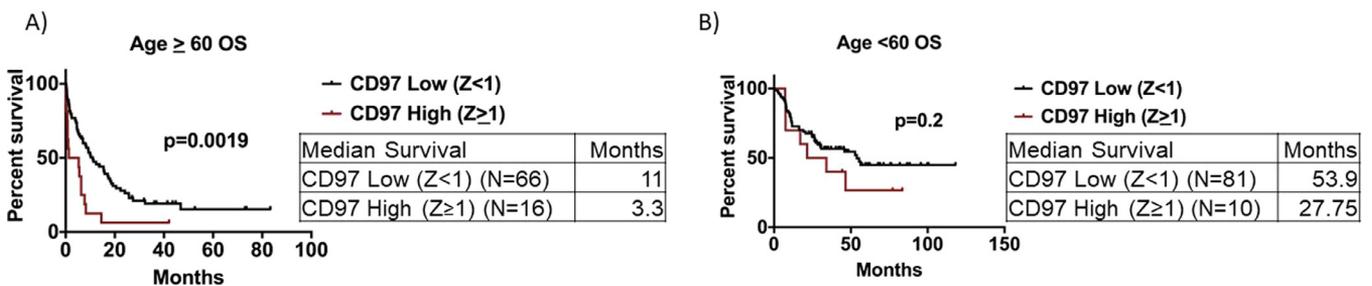
We further evaluated the *CD97*-associated cell signaling pathways through ingenuity pathway analysis (**Supplementary Figure E7**, online only, available at [www.exphem.org](http://www.exphem.org)). Patients were divided into *CD97* high ( $Z > 2$ ,  $N = 11$ ) and *CD97* low ( $Z < -1$ ,  $N = 10$ ). Pearson

correlation scores of *CD97* versus all the available gene expression data in TCGA were calculated and analyzed in IPA. Our analysis revealed that the integrin signaling, IL-8 signaling, CXCR4 signaling, and ILK signaling pathways were all activated in patients with high *CD97* expression ( $Z > 2$ ). Importantly, these signaling pathways were also downregulated in patients with low *CD97* expression ( $Z < -1$ ). This further validates the association between *CD97* levels and activation of pathways involved in the LSC–BM niche interaction.

### Discussion

*CD97* has been reported to play a role in disease progression of a variety of malignancies by regulating cell invasion and metastasis. In glioma, high *CD97* expression is associated with worse survival [23]. In colorectal cancer, high *CD97* expression correlated with the cells' dedifferentiation ability [14]. With use of several cancer models including AML cells, *CD97* knockdown was found to decrease cell migration and adhesion [16,23,24].

Here we report that high *CD97* mRNA expression is significantly associated with poorer overall survival in AML patients. This is particularly relevant in older patients: median overall survival of patients  $> 60$  years with high *CD97* was less than 4 months. This is



**Figure 7.** Survival analysis of AML patients with respect to *CD97* expression after stratification based on age. (A) Overall survival of patients  $\geq 60$  years of age with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ). (B) Overall survival of patients  $< 60$  years of age with *CD97* high ( $Z$  score  $\geq 1$ ) versus *CD97* low ( $Z$  score  $< 1$ ).

significantly lower than the already dismal survival of these patients. In AML, the median age at diagnosis is 67 years, and more than 60% of newly diagnosed patients are older than 60 years [25]. Because of several factors, including comorbidities and disease complications, older patients are faced with challenging and not optimal therapeutic approaches compared with younger patients. Therefore, the identification of outcome predictors and possible viable targets in this subset of patients will greatly affect disease understanding and treatment outcome.

Interestingly, we found that patients with *FLT3* mutations had high *CD97* expression. Previous *in vitro* studies found that AML cell lines harboring a *FLT3*-ITD mutation had higher *CD97* expression than cell lines with *FLT3*-WT [26]. Similarly, it was also reported that inhibiting *FLT3*-ITD in AML cell lines with PKC412, a *FLT3*-ITD inhibitor, resulted in reduced *CD97* expression [26]. The mechanism by which *FLT3*-ITD and *CD97* pathways interact remains unclear. Additionally, our analysis also reveals a positive association between *CD97* and *NPM1* mutations. *NPM1* mutations are associated with a favorable outcome in AML [27]. Contrastingly, there is a trend for patients with *NPM1* mutation and high *CD97* expression ( $Z \geq 1$ ) to have a worse survival outcome compared with patients with *NPM1* mutation and low *CD97* expression. Additionally, patients with *NPM1* wild type and high *CD97* expression ( $Z \geq 1$ ) have significantly worse survival outcome compared with patients with *NPM1* wild type and low *CD97* expression. This suggests a benefit to including *CD97* expression in prediction of survival in patients with *NPM1* mutation. The mechanistic interplay between *CD97* and *NPM1* is yet to be determined.

The identification of integrin and ILK signaling pathway to be activated in *CD97* high groups is of particular interest, as *CD97* was previously reported to act as a chemoattractant for migration and invasion of human umbilical vein endothelial cells (HUVECs) in an integrin-dependent manner [11]. Importantly, integrin and ILK signaling pathways have been shown to contribute to leukemia stem cell survival [10]. Additionally, IL-8 and CXCR4 signaling pathways are among the most significant pathways in the high *CD97* group. IL-8 was previously reported to be regulated by hypoxia, as well as to influence the niche formation in the bone marrow by inducing the migration of mesenchymal stromal cells (MSC) [28]. Furthermore, high IL-8 expression also correlated with a worse clinical outcome in non-APL AML [28]. Interestingly, mAbCD97 inhibits IL-8-induced HSC/HPC mobilization [29], suggesting that *CD97* may potentially regulate the IL-8 signaling pathway. Similarly, *CXCR4* expression is associated with poor prognosis of patients with AML [30]. *CXCR4* plays an important role in the crosstalk

between leukemic cells and the bone marrow niche [31,32]. The association between *CXCR4* and *CD97* is yet to be determined. Yet, knockdown of *CD97* in MV4-11 AML cells impaired cell adhesion on a MSC monolayer [26]. Additionally, the extracellular domain of *CD97* binds to integrins  $\alpha 5 \beta 1$  and  $\alpha v \beta 3$ , stimulating endothelial cell migration and invasion [11]. Furthermore, the interaction of *CD97* with Thy-1 plays a role in the regulation of leukocyte trafficking to inflammatory sites [33]. Because *CD97* binding partners (integrins and Thy-1) are also present in the MSCs, it is plausible that *CD97* upregulation may contribute to the interaction between LSCs and the bone marrow niche.

## Conclusions

Patients with high *CD97* expression had shorter overall and disease-free survival. *CD97* was higher in cytogenetically normal patients compared with cytogenetically abnormal patients. Additionally, high *CD97* expression was also associated with *NPM1* mutation. Our findings demonstrate that *CD97* expression contributes to the clinical outcome of patients with AML, particularly older patients. This study provides a rationale for further functional and mechanistic studies aiming to understand the role of *CD97* in AML.

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## Conflict of interest disclosure

The authors declare no competing interests.

## Authorship

VPV performed the analysis and generated the figures. SW performed the multivariate statistical analysis. JW performed the IPA analysis. VPV and HA wrote the article. HA conceived the project, designed the research, and supervised the project.

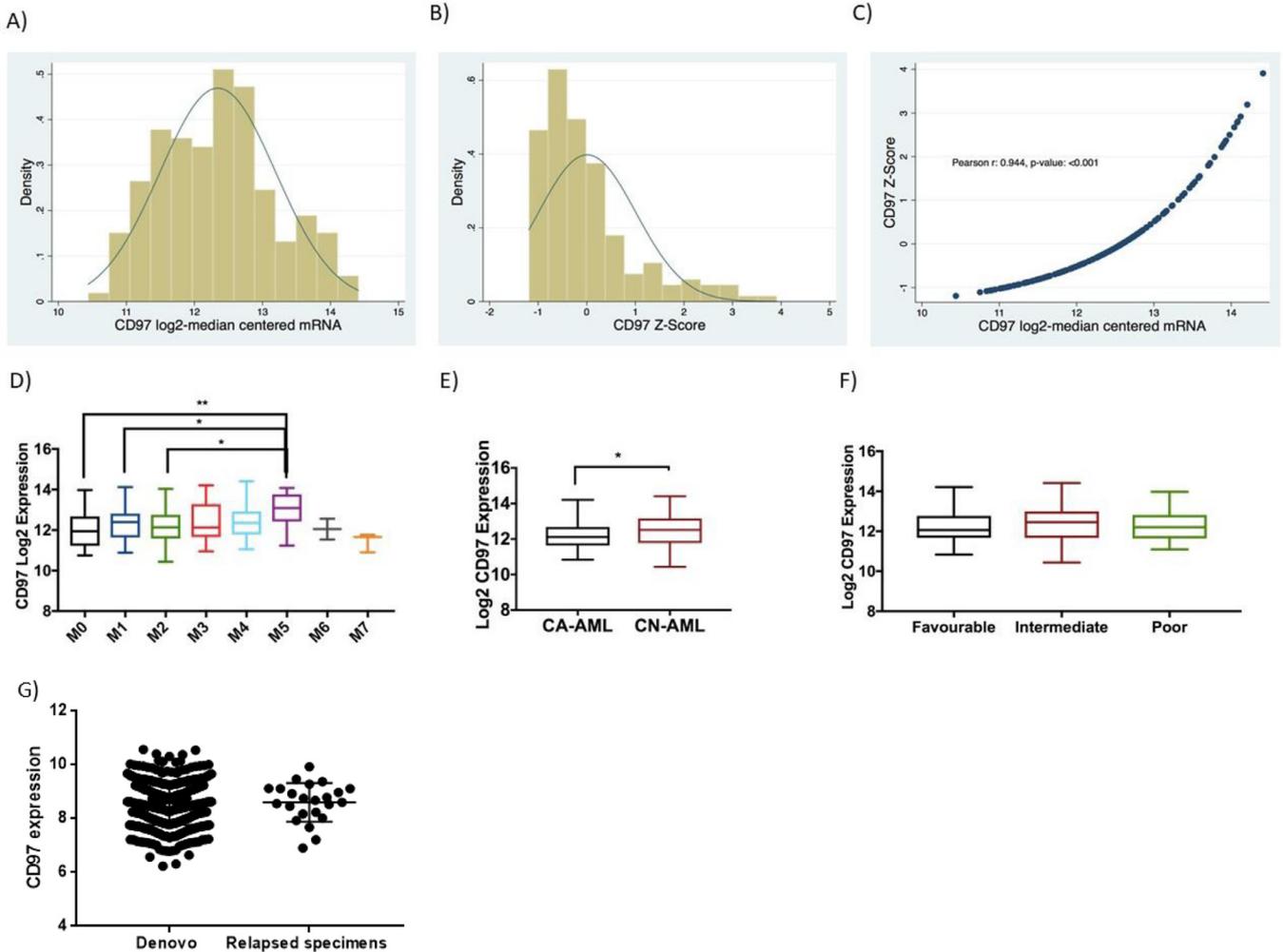
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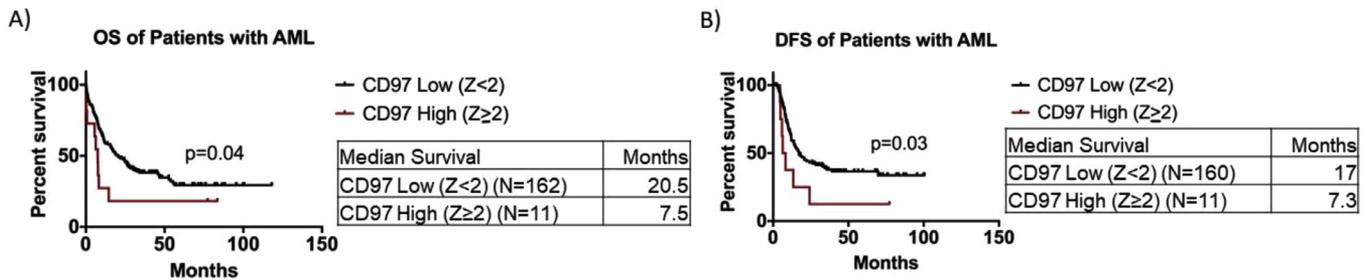
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### Supplementary materials

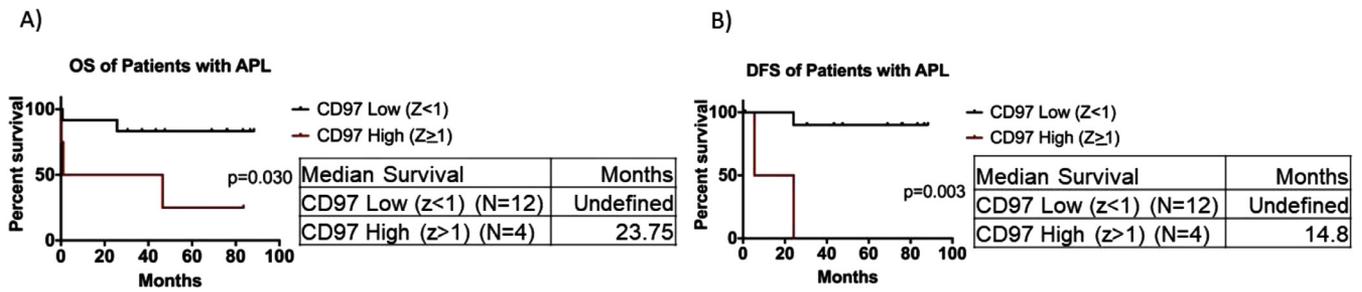
The supplemental materials includes supplemental figures.



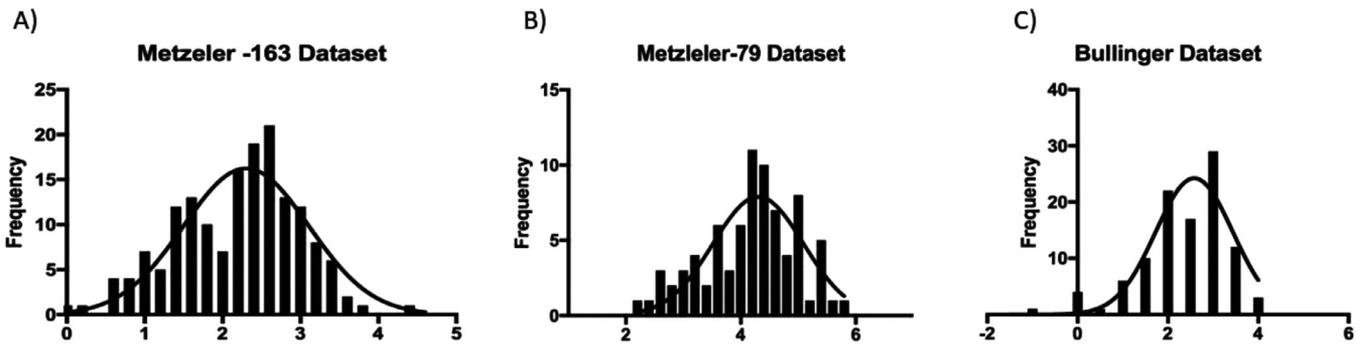
**Figure S1. *CD97* Z-score and mRNA expression distribution.** (A) Histogram of *CD97* Log2 transformed mRNA expression; (B) *CD97* mRNA expression Z score; (C) Scatterplot of *CD97* Z-score vs *CD97* log2 mRNA expression. Relative *CD97* log2 mRNA expression categorized by (D) FAB classification; (E) cytogenetic status and (F) risk status. (G) *CD97* expression in relapsed vs. de novo AML samples from the BEAT AML cohort analyzed using Vizome tool. \* $P < 0.05$  \*\* $P < 0.0$



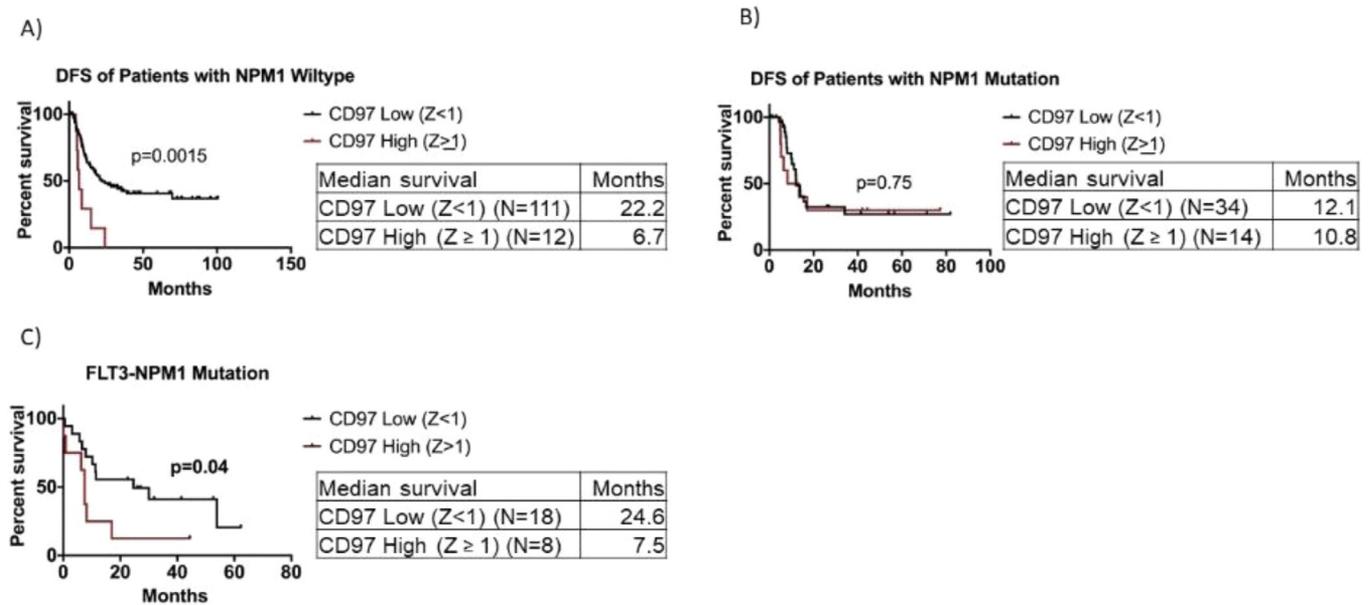
**Figure S2. Survival analysis of AML patients according to *CD97* expression.** (A) Overall Survival of 173 AML Patients with *CD97* Z-score  $> 2$  and *CD97* Z-score  $< 2$ ; (B) Disease-free Survival of 171 AML Patients with *CD97* Z-score  $> 2$  and *CD97* Z-score  $< 2$ .



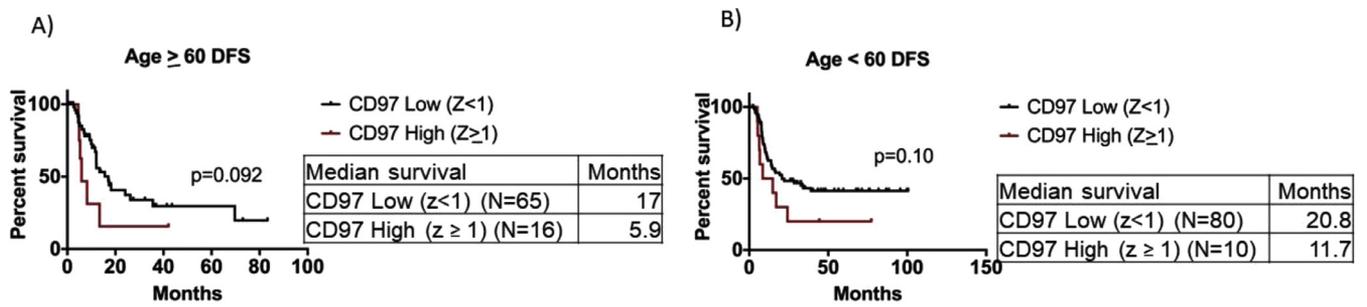
**Figure S3. Survival analysis of AML patients with APL according to CD97 expression.** (A) Overall Survival of AML patients with APL with CD97 Z-score $\geq 1$  and CD97 Z-score $< 1$ ; (B) Disease-free Survival of AML patients with APL with CD97 Z-score $\geq 1$  and CD97 Z-score $< 1$ .



**Figure S4. CD97 mRNA expression distribution.** Histogram of CD97 mRNA expression of (A) Metzeler-163 dataset; (B) Metzeler-79 dataset; (C) Bullinger dataset.

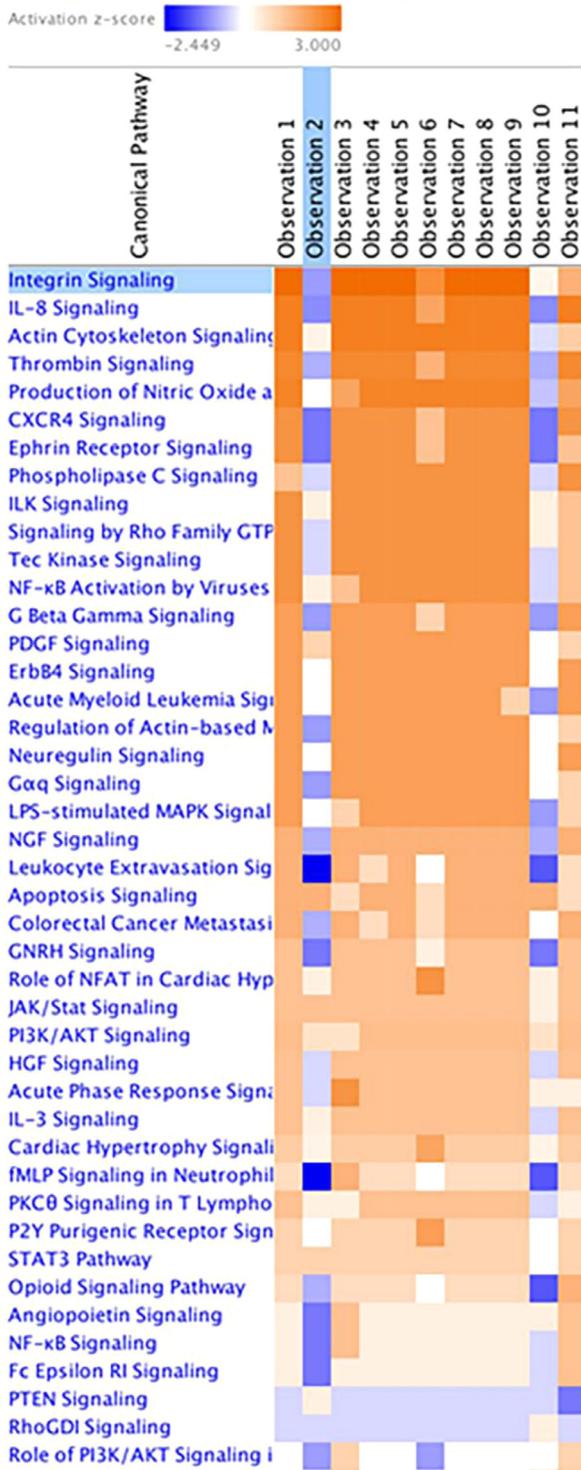


**Figure S5. Survival analysis of AML patients according to CD97 expression after stratification based on NPM1 mutation status** (A) Disease free Survival of AML patients with CD97 high (Z-score $\geq 1$ ) vs CD97 low (Z-score $< 1$ ) in patients with NPM1 wild type gene. (B) Disease free Survival of AML patients with CD97 high (Z-score $\geq 1$ ) vs CD97 low (Z-score $< 1$ ) in patients with NPM1 mutation. (C) OS AML patients with CD97 high (Z-score $\geq 1$ ) vs CD97 low (Z-score $< 1$ ) in patients with FLT3 and NPM1 mutation

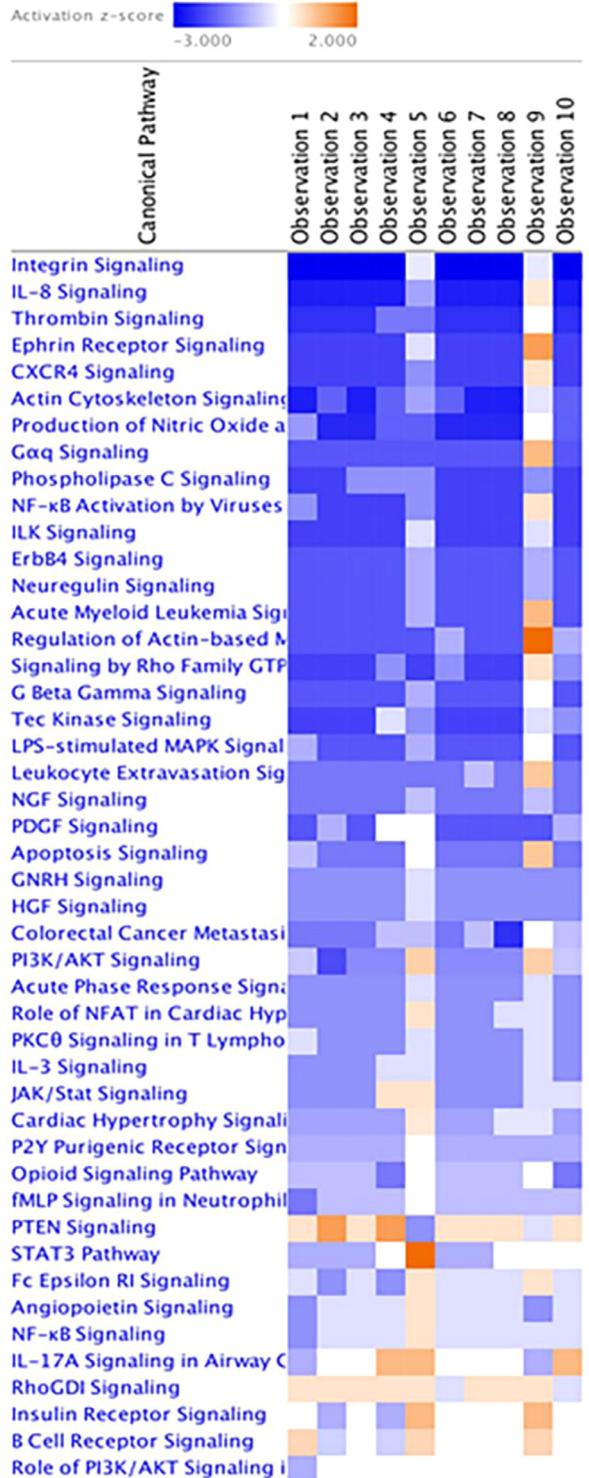


**Figure S6. Survival analysis of AML patients according to *CD97* expression after stratification based on age.** (A) Disease free Survival of AML patients with *CD97* high ( $Z$ -score $\geq 1$ ) vs *CD97* low ( $Z$ -score $< 1$ ) in patients  $\geq 60$  years of age. (B) Disease free Survival of AML patients with *CD97* high ( $Z$ -score $\geq 1$ ) vs *CD97* low ( $Z$ -score $< 1$ ) in patients  $< 60$  years of age

### Z-SCORE>2 COMPARISON ANALYSIS



### CD97 Z-SCORE<-1



**Figure S7. Cell signaling pathway analysis associated with CD97** Patients were grouped in CD97 high ( $Z > 2$ ) and CD97 low ( $Z < -1$ ). Pearson correlation scores of CD97 vs all the available gene expression data in TCGA were calculated and analyzed in ingenuity pathway analysis