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Polymorphisms of the *CYR61* gene in patients with acute myeloid leukemia in a Han Chinese population

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Abstract

It was demonstrated in previous studies that cysteine-rich angiogenic inducer 61 (Cyr61) plays vital roles in hematological disorders, and we have already reported that the Cyr61 protein is a tumor promoter in acute myeloid leukemia (AML). Here, we investigated the association between *CYR61* gene polymorphisms and susceptibility to AML.

We genotyped 2 single-nucleotide polymorphisms (rs2297141 and rs6576776) in the region of the *CYR61* gene by improved multiplex ligase detection reaction genotyping assays in a total of 275 samples, including samples from 137 AML patients and 138 healthy controls. Chi-squared tests and logistic regression analysis were performed to compare the different distributions of the genotypes and alleles between patients and healthy controls.

The rs2297141 A allele was associated with lower risk of AML compared with the G allele (odds ratio [OR] = 0.704, 95% confidence interval [CI] = 0.503–0.985, $P = .04$) in both the dominant (OR = 0.447, 95% CI = 0.22–0.909, $P = .025$, AA vs GG) and recessive inheritance models (OR = 0.419, 95% CI = 0.23–0.763, $P = .004$, AA vs GA + GG). Although the distribution of the rs6576776 alleles was not different between patients with AML and normal controls, the CC genotype significantly increased the risk of AML in the dominant inheritance model (OR = 6.064, 95% CI = 1.303–28.216, $P = .01$, CC vs GG) and the recessive inheritance model (OR = 5.937, 95% CI = 1.291–27.306, $P = .01$, CC vs GC + GG). Additionally, it was shown that the rs2297141 and rs6576776 genotypes were associated with AML-M5 and AML-M2, respectively.

Our findings indicated that genetic polymorphisms in the *CYR61* gene may be considered potential AML risk factors in the Han Chinese population.

Abbreviations: AML = acute myeloid leukemia, CI = confidence interval, Cyr61 = cysteine-rich angiogenic inducer 61, FAB = French–American–British, HWE = Hardy–Weinberg equilibrium, OR = odds ratio, SNP = single-nucleotide polymorphism, WHO = World Health Organization.

Keywords: AML, Cyr61, polymorphism

1. Introduction

Acute myeloid leukemia (AML) is a type of hematopoietic stem cell tumor. Genomic changes play vital roles in AML; mutations in AML inform the disease classification and prognostic stratification,[1,2] and gene analysis has significantly improved the diagnostic criteria and the classification of myeloid neoplasms (World Health Organization [WHO] 2016 edition).[3]

Cysteine-rich 61 (Cyr61), is a member of the connective tissue growth factor, cysteine-rich protein, which consists of 6 members. Cyr61 has diverse functions in several types of tumors, for example, cervical cancer,[4] breast cancer,[5] pancreatic cancer,[6] malignant melanoma,[7] esophageal squamous cell carcinoma,[8] and hepatocarcinogenesis.[9] Moreover, in recent years, there has been a focus on the association between polymorphisms of the *Cyr61* gene and diseases; the *Cyr61* rs12756618 variant increases the risk of Graves' ophthalmopathy,[10] the *Cyr61* p.R47W variant is related to atrial septal defect,[11] and the *Cyr61* rs3753793 variant might contribute to prostate cancer risk.[12] In our previous study, we reported that *Cyr61* is overexpressed in AML cell lines and patient samples and is a tumor promoter in AML through the MEK/ERK pathway.[13]

The results from ANLN clinical and RNA-seq data showed that there were diverse *CYR61* expression patterns in different AML subtypes, and the expression of *CYR61* was associated with FTL3 mutation, PML/RAR-fusion, or RAS activation (Fig. 1). In this study, to further understand the roles played by Cyr61 in AML, we analyzed the distributions of *Cyr61* polymorphism in patients with AML and healthy controls to explore the relationship between *Cyr61* polymorphisms and the risk of AML. It has been demonstrated that *CYR61* polymorphism rs3753793 was related to Graves' ophthalmopathy,[10] prostate cancer risk,[12] and HDL-cholesterol levels in obese individuals.[14] And, the data from population of CHB and CHS in 1000 genome database[15] show strong linkage relationship among single-nucleotide polymorphisms (SNPs) rs2297141, rs6576776, and rs3753793 in *CYR61* gene through Haploview

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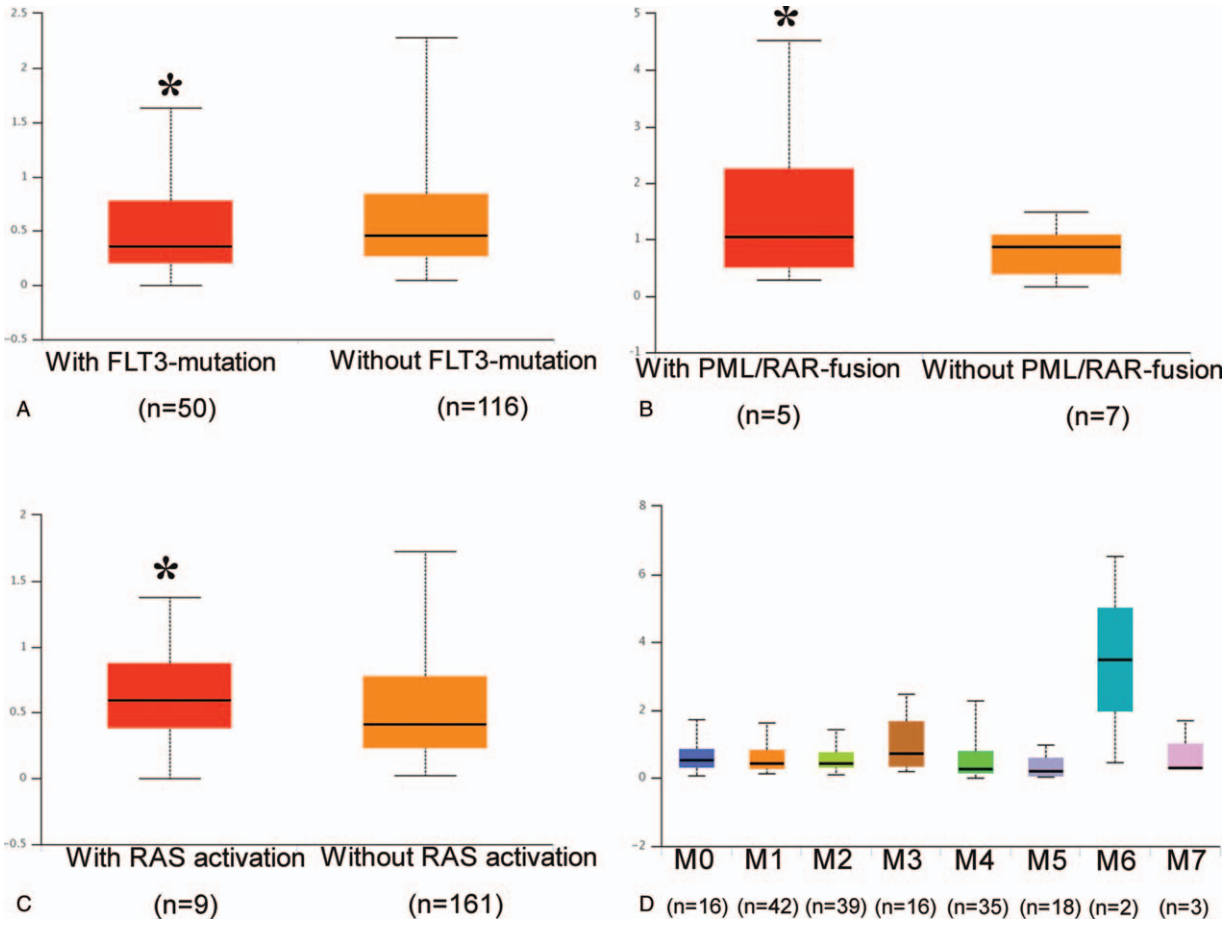

Figure 1. Comparison of *CYR61* RNA-seq expression level based on FLT3 mutation status (A), PML/RAR-fusion status (B), RAS activation status (C), and French–American–British classification (D) in the Cancer Genome Atlas samples. $*P < .05$.

analysis. Therefore, theses SNPs were chosen for current investigation.

2. Methods

2.1. Patient cohorts and healthy controls

Bone marrow samples from AML patients (n = 137) and peripheral blood samples of healthy controls (n = 138) were obtained from Chongqing General Hospital and Chongqing Xinqiao Hospital from November 2016 to September 2017. All participants were of Chinese Han ethnicity. The patients were diagnosed according to the French–American–British (FAB) and WHO classifications, and the patients who received hematopoietic stem cell transplantations were excluded. Healthy subjects who had no self-reported cancer history or known blood disorders were enrolled as the controls. The patients with AML and the healthy donors provided informed consent, and the study was approved by the Ethics Committee of Chongqing General Hospital. In addition, the authors did not have access to information that could identify individual participants during or after data collection.

2.2. Molecular genetic analysis

Genomic DNA was extracted with the Tiangen kit (Beijing, China) according to the manufacturer's instructions. Samples were stored at −80°C. The *CYR61* genotypes were determined by improved multiplex ligase detection reaction, which was developed by Genesky Biotechnologies Inc. (Shanghai, China) as previously reported.[16] The sequences of the probes are as follows:

rs2297141:

- 5′-TTCCGCGTTCGGACTGATATGCAGCCTTCCGAGGT GGACG-3′, 5′- TACGGTTATTCGGGCTCCTGTGCAGCC TTCCGAGGTGGACA-3′, 5′-GGCTGGACGAGATCAGA GGCTTTTTTT-3′;

rs6576776:

- 5′- TTCCGCGTTCGGACTGATATGCAAAGGAATGCAA GGAATTTCTAG -3′, 5′-TACGGTTATTCGGGCTCCTGT GCAAAGGAATGCAAGGAATTTCTAC-3′, 5′-TACTTTTC-CAATAGCGTGAGGGCTTTTTTTTTTTTTTTTTTTTT TTTTT-3′.

The mutations in AML genes (*c-kit*, *FLT3-TKD*, *CEBPA*, *NPM1*, *DNMT3A*, *U2AF1*, *SRSF2*, *ASXL1*), which play crucial roles in the diagnosis, stratification, and treatment of AML,[2,3,17,18] were analyzed with Sanger sequencing: DNA was amplified for the genes (c-kit, FLT3-TKD, CEBPA, NPM1, DNMT3A, U2AF1, SRSF2, ASXL1) with S1000 Thermal Cycler, then amplified products were analyzed with 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham).

2.3. Statistics

ANLN clinical and RNA-seq data were downloaded from the Cancer Genome Atlas (https://portal.gdc.cancer.gov/) and analyzed by using the online software UALCAN (http://ualcan.path.uab.edu/analysis.html) following their policies. Transcripts per million expression values employed for the generation of box plots were also used to estimate the significance of difference in gene expression levels between groups. The Student *t* test was performed using a PERL script with Comprehensive Perl Archive Network module.

SPSS 20.0 software was used for the statistical analysis. The quality control was assessed with Hardy–Weinberg equilibrium by the differences in the distributions of the patients and the healthy donors. Chi-squared tests or/and Fischer exact tests were used to analyze the genotype and allele frequency data. The association between *CYR61* gene polymorphisms and AML risk was evaluated by odds ratio (OR) with 95% confidence intervals (CIs). Logistic regression analysis was used to analyze the independent factors. $P < .05$ indicated a statistically significant result.

3. Results

3.1. The information of AML patients and healthy controls

There were no statistically significant differences between the groups with regard to gender (77/60 vs 76/62, $P = .850$) or age (mean 39.4 ± 15.6, 37.8 ± 17.2, $P = .445$) in the total 275 samples (137 AML patients and 138 healthy controls) (Table 1). The distribution of the AML subtypes based on the FAB classification was as follows: AML-M1, 9 (6.6%); AML-M2, 44 (32.1%); AML-M3, 10 (7.3%); AML-M4, 35 (25.6%); AML-M5, 29 (21.2%); AML-M6, 4 (2.9%); and AML-other type, 6 (4.4%). We analyzing the AML 8 gene mutations of AML samples (from 137 AML patients) with Sanger sequencing, and the results showed that the distribution of the AML 8 genes mutations were as follows: *c-Kit*, 6 (4.4%); *FLT3-TKD*, 16 (11.7%); *CEBPA*, 10 (7.3%); *NPM1*, 8 (5.8%); *DNMT3A*, 5 (3.7%); *U2AF1*, 1 (0.7%); *SRSF2*, 2 (1.5%); and *ASXL1*, 2 (1.5%).

3.2. The distribution of CYR61 gene polymorphisms in the patients with AML

As shown in Table 2, the rs2297141 A allele significantly decreased the risk of AML compared with the G allele (OR = 0.704, 95% CI = 0.503–0.985, $P = .04$) in both the dominant (OR = 0.447, 95% CI = 0.22–0.909, $P = .025$, AA vs GG) and recessive inheritance models (OR = 0.419, 95% CI = 0.23–0.763, $P = .004$, AA vs GA + GG). Although the G allele of rs6576776 was more frequent in AML cases (25.5%) than in controls (19.6%), it was not a statistically significant difference ($P = .093$). The genotype CC of rs6576776 significantly increased the risk of AML, whether in the dominant (OR = 6.064, 95% CI = 1.303–28.216, $P = .01$, CC vs GG) or the recessive inheritance model (OR = 5.937, 95% CI = 1.291–27.306, $P = .01$, CC vs GC + GG).

Furthermore, logistic regression analyses adjusted for gender and age were used to analyze the association of 2 *CYR61* polymorphisms with the risk of AML. As shown in Table 3, the CC genotype of rs6576776 in patients with AML was associated with a higher risk of AML, but the genotype AA of rs2297141 in patients with AML was associated with a lower risk of AML.

3.3. Patient characteristics in relation to CYR61 polymorphisms

Eight AML gene mutations were analyzed in the patients with AML, and the mutations were found in 39 patients. We also analyzed the relationship of rs2297141 and rs6576776 with the mutation of 8 AML genes, and the data showed that there were no associations (Table 4).

The association of *Cyr61* polymorphisms with the AML FAB subtypes was also analyzed. Chi-squared tests showed that the rs2297141 genotype was associated with AML-M5 ($P = .001$),

Table 1

Demographics and clinical characteristics of the patients with AML and the healthy controls.

Characteristics	Patients with AML (n = 137)	Control (n = 138)	P
Age, y	39.4 ± 15.6	37.8 ± 17.2	.445
Gender, male/female	77/60	76/62	.850

AML = acute myeloid leukemia.

Table 2

The allelic and genotypic frequencies of CYR61 polymorphisms in AML patients and controls.

Genotype/allele	AML (n = 137)	Control (n = 138)	P	OR (95% CI)
rs2297141				
GG (dominant)	38	34		
GA	79	64	.732	1.104 (0.626–1.949)
AA	20	40	**.025**	0.447 (0.22–0.909)
Multiple comparison			**.015**	
GG + GA (recessive)	117	98		
AA	20	40	**.004**	0.419 (0.23–0.763)
G (allele)	155	132		
A	119	144	**.04**	0.704 (0.503–0.985)
rs6576776				
GG (codominant)	78	86		
GC	48	50	.824	1.058 (0.641–1.746)
CC	11	2	**.01**	6.064 (1.303–28.216)
Multiple comparison			**.036**	
GG + GC (recessive)	126	136		
CC	11	2	**.01**	5.937 (1.291–27.306)
G (allele)	204	222		
C	70	54	.093	1.411 (0.943–2.110)

AML = acute myeloid leukemia, CI = confidence interval, OR = odds ratio.
The bold values are statistically significant.

Table 3

Logistic regression analysis for the association of rs2297141 and rs6576776 polymorphisms with AML.

Genotype	P	OR (95% CI)
rs2297141	.035	0.687 (0.485–0.974)
GA + GG vs AA	**.004**	0.416 (0.228–0.759)
AA + GG vs GA	.061	1.579 (0.980–2.546)
GG vs AA + GA	.551	1.179 (0.686–2.024)
rs6576776	.077	1.460 (0.960–2.22)
GC + GG vs CC	**.022**	6.022 (1.300–27.885)
CC + GG vs GC	.892	1.035 (0.628–1.707)
GG vs CC + GC	.321	1.283 (0.784–2.098)

AML = acute myeloid leukemia, CI = confidence interval, OR = odds ratio.
The bold values are statistically significant.

Table 4

The relationship of rs2297141 and rs6576776 with the mutations of 8 AML genes.

	rs2297141				rs6576776			
	AA	GA	GG	*P*	CC	GC	GG	*P*
c-Kit				.862				.406
Positive	1	3	2		1	1	4	
Negative	19	76	36		10	47	74	
FLT3-TKD				.756				1
Positive	1	10	5		1	6	9	
Negative	19	69	33		10	42	69	
CEBPA				.73				.784
Positive	2	5	3		0	3	7	
Negative	18	74	35		11	45	71	
NPM1				.531				1
Positive	0	5	3		0	3	5	
Negative	20	74	35		11	45	73	
DNMT3A				.839				1
Positive	0	4	1		0	2	3	
Negative	20	75	37		11	46	75	
U2AF1				1				.431
Positive	0	1	0		0	1	0	
Negative	20	78	38		11	47	78	
SRSF2				.673				1
Positive	0	1	1		0	1	1	
Negative	20	78	37		11	47	77	
ASXL1				.673				.673
Positive	0	1	1		0	0	2	
Negative	20	78	37		11	48	76	

AML = acute myeloid leukemia.

and the rs6576776 genotype was associated with AML-M2 ($P=.005$) (Table 5), compared with the healthy control. Furthermore, the dominant inheritance models, recessive inheritance models, and alleles were analyzed in the AML-M2 and AML-M5 subtypes. In addition, the results (Tables 6 and 7) showed that compared with the control, there was a lower risk of AML-M5 with the AA genotype (OR = 0.087, 95% CI = 0.012–0.665, $P=.002$, AA vs GG + GA) and a higher risk of AML-M2 with the CC genotype (OR = 12.286, 95% CI = 2.313–65.25, $P=.002$, CC vs GG; OR = 10.737, 95% CI = 2.082–55.36, $P=.003$, CC vs GG + GC; OR = 2.021, 95% CI = 1.184–3.4, $P=.013$, C vs G).

Table 5

Chi-squared tests for the association of *CYR61* polymorphisms with the AML FAB subtypes.

	rs2297141				rs6576776			
	AA	GA	GG	*P*	CC	GC	GG	*P*
AML-M1	3	4	2	1	0	1	8	.26
AML-M2	10	18	16	.316	6	17	21	**.005**
AML-M3	1	5	4	.37	1	1	8	.068
AML-M4	4	22	9	.071	3	13	19	.092
AML-M5	1	23	5	**.001**	1	12	16	.483
AML-M6	1	3	0	.81	0	1	3	1
AML-other	0	4	2	.367	0	3	3	.698
Control	40	64	34	.042*	2	50	86	.097*

AML = acute myeloid leukemia, FAB = French–American–British.
The bold values are statistically significant.
* Multiple comparison.

4. Discussion

Genetic heterogeneity explains the variations in cancer predisposition.[19] In this study, we analyzed the distribution of *CYR61* polymorphisms in patients with AML, and it was demonstrated that rs2297141 and rs6576776 genotypes were associated with the risk of AML in a Han Chinese population. Moreover, these 2 genotypes were associated with AML-M5 and AML-M2, respectively. Together with our previously finding,[13] this study confirmed the role of Cyr61 in the development of AML.

Cyr61 regulates hematological tumorigenesis by diverse mechanisms.[20] The *Cyr61* gene is located on chromosome 1p22.3. It was demonstrated in this study that the rs2297141 genotype and the rs6576776 genotype are associated with the risk of AML. Rs2297141 is in intron 1 of *CYR61*, and rs6576776 is on the 3′-flanking region of *Cyr61*. Although the SNPs cannot directly affect the sequence of the Cyr61 protein, they may regulate the transcription of *Cyr61* or other genes at many different levels.[21] That intron has been shown to regulate gene expression,[22,23] and it was reported that breast carcinogenesis was accompanied by a shift in intron 3 of the *CYR61* gene moving toward a phenotype wherein intron 3 is missing from the mRNA transcript.[24] It was also demonstrated that the 5′-flanking region of the *Cyr61* gene regulated its expression,[25,26] but little is known about the 3′-flanking region of the *Cyr61* gene. Although this study provides a clue regarding the roles of *CYR61* polymorphisms in AML, whether and how the roles of *Cyr61* polymorphisms are associated with hematological tumorigenesis remain unclear and need further study.

It has been demonstrated that *CYR61* polymorphisms (rs3753793, rs6682848, and rs12756618) were related to Graves' ophthalmopathy,[10] and that a *CYR61* polymorphism (rs3753793) was associated with prostate cancer risk[12]; however, these polymorphisms were not associated with AML in the Chinese population (data not shown). Based on these studies, we speculated that the distribution of *CYR61* polymorphisms in AML may be different from the distribution in other diseases, which may be the result of the roles played by Cyr61 in different diseases or/and ethnic groups.

Table 6

The dominant inheritance models, recessive inheritance models, and alleles were analyzed in the AML-M2 subtype.

Genotype/allele	AML-M2 (n = 44)	Control (n = 138)	*P*	OR (95% CI)
rs2297141				
GG (dominant)	16	34		
GA	18	64	.200	0.598 (0.271–1.319)
AA	10	40	.171	0.531 (0.213–1.324)
GG + GA (recessive)	34	98		
AA	10	40	.418	0.721 (0.325–1.596)
G (allele)	50	132		
A	38	144	.142	0.697 (0.43–1.13)
rs6576776				
GG (codominant)	21	86		
GC	17	50	.372	1.392 (0.672–2.88)
CC	6	2	**.002**	12.286 (2.313–65.25)
GG + GC (recessive)	38	136		
CC	6	2	**.003**	10.737 (2.082–55.36)
G (allele)	59	222		
C	29	54	**.013**	2.021 (1.184–3.4)

AML = acute myeloid leukemia, CI = confidence interval, OR = odds ratio.

Table 7

The dominant inheritance models, recessive inheritance models, and alleles were analyzed in the AML-M5 subtype.

Genotype/allele	AML-M5 (n = 29)	Control (n = 138)	*P*	OR (95% CI)
rs2297141				
GG (dominant)	5	34		
GA	23	64	.089	2.444 (0.853–7.003)
AA	1	40	.104	0.17 (0.019–1.527)
GG + GA (recessive)	28	98		
AA	1	40	**.002**	0.087 (0.012–0.665)
G (allele)	33	132		
A	25	144	.209	0.694 (0.392–1.229)
rs6576776				
GG (codominant)	16	86		
GC	12	50	.545	1.29 (0.565–2.946)
CC	1	2	.415	2.688 (0.23–31.4)
GG + GC (recessive)	28	136		
CC	1	2	.438	2.429 (0.213–27.7)
G (allele)	44	222		
C	14	54	.432	1.308 (0.669–2.559)

AML = acute myeloid leukemia, CI = confidence interval, OR = odds ratio.
The bold values are statistically significant.

CEBPA, *c-Kit*, *FLT3*, *DNMT3A*, *NPM*, *ASXL1*, *U2AF1*, and *SRSF2* mutations are used in clinical practice and affect the diagnosis, stratification, and treatment of AML.[2,18] Combined with the data from ANLN clinical tests and RNA-seq, we speculated that there were relationships between these mutations and *Cyr61* polymorphisms, but no correlations were found. Furthermore, we analyzed the distributions of rs2297141 and rs6576776 polymorphisms in the subtypes of AML. The genotype distributions of the polymorphisms with respect to the subtypes of AML revealed that the rs2297141 SNP was associated with AML-M5, and the rs6576776 polymorphism was associated with AML-M2; the results were also analyzed with the dominant inheritance models and recessive inheritance models. In recent years, gene analysis has played a vital role in the subclassification of AML, providing more information on which to base treatment and prognostic decisions,[3,27] and this study also showed the potential subclassification role of *Cyr61* in AML. The role of the rs2297141 polymorphism in AML-M5 and the role of the rs6576776 polymorphism in AML-M2 contributed the most to their roles in AML. The finding that there are roles played by different polymorphisms in different subtypes of AML is consistent with the diversity of Cyr61 functions.[28]

Overall, our findings revealed a significant association between *Cyr61* rs2297141 and rs6576776 polymorphisms and the risk of AML, especially the AML-M2 and AML-M5 subtypes. Whether and how the *Cyr61* polymorphisms can affect *CYR61* gene expression or the function of the *CYR61* gene product should be studied further. Our observations provided a clue to guide further exploration of the leukemogenic roles of Cyr61.

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