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Original Article

IKAROS6 is associated with *BCR-ABL1* and myeloid-associated antigens but indicates poor prognosis independently in Chinese adult B-cell acute lymphoblastic leukemia

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Abstract: *Ikaros6* was specifically associated with clinical and genetic features of acute lymphoblastic leukemia (ALL) and could be used for prediction of inferior survival. The present study aimed to further investigate the correlation between *Ikaros6* and other prognostic factors, and to explore the novel prognosis prediction function by combining *Ikaros6* and other factors in Chinese adult B-ALL. We examined the expression of *Ikaros6* in 108 patients by reverse transcription polymerase chain reaction and confirmed the results by sequencing, gene scanning and real-time PCR. *Ikaros6* was associated with *BCR-ABL1* (P=0.010) and myeloid-associated antigens (P=0.009), but had an independent negative impact on survival. In multivariable Cox analysis, *Ikaros6* was an independent prognostic marker for overall survival (P=0.013, HR=2.140), event-free survival (P=0.016, HR=1.972) and relapse-free survival (P=0.002, HR=3.636). This study indicated closed relation between *BCR-ABL1*, myeloid-associated antigens and *Ikaros6*.These three risk factors played an important role in evaluation of prognosis in Chinese adult B-ALL. Furthermore, *Ikaros6* is more beneficial for the disease recurrence prediction.

Keywords: *IKAROS6*, *BCR-ABL1*, myeloid-associated antigens, B-ALL, prognostic factor

Introduction

Ikaros protein encoded by the *IKZF1* gene, as a tumor suppressor, plays an important role in high-risk acute lymphoblastic leukemia (ALL) [1-7]. *Ikaros6*, as one of the dominant-negative (DN) *Ikaros* isoforms, was characterized by loss of exons 4 to 7 on chromosome 7p12 with breakpoints in introns 3 and 7 [8, 9]. Due to the absence of necessary zinc fingers, *Ikaros6* interferes with DNA binding activity to the longer isoforms, thereby reducing Ikaros activity [9-12]. Elevated expression of DN isoforms may disturb normal lymphocyte development and lead to leukemic transformation and progression [6, 12].

The frequency and prognostic relevance of *IKZF1* deletions, especially *Ikaros6* that lacks all of the N-terminal zinc fingers, have been reported previously in children ALL [4, 7, 13-15]. Mullighan *et al*. identified *IKZF1* deletions in 83.7% Ph+ALL patients, suggesting that DN isoforms were of importance to leukemic pathogenesis [3]. Moreover, significant correlation (P<0.001) was identified between *Ikaros6* and the *BCR-ABL1* transcript levels [6, 12, 16, 17]. Furthermore, several studies suggested that *IKZF1* deletions were involved in the pathogenesis of *BCR-ABL1*-negative ALL and were also indicative of poor outcomes of the condition [4, 13-15].

Immunophenotype has been often used as an essential approach for the diagnosis of ALL. Expression of myeloid-associated antigens (MY) in B-ALL patients has been studied systematically previously [18-22]. Some reports showed that most cases of *BCR-ABL1*-positive B-ALL exhibited myeloid antigens [18, 19], and others demonstrated significant correlation between *Ikaros6* and *BCR-ABL1* [6, 12, 16].

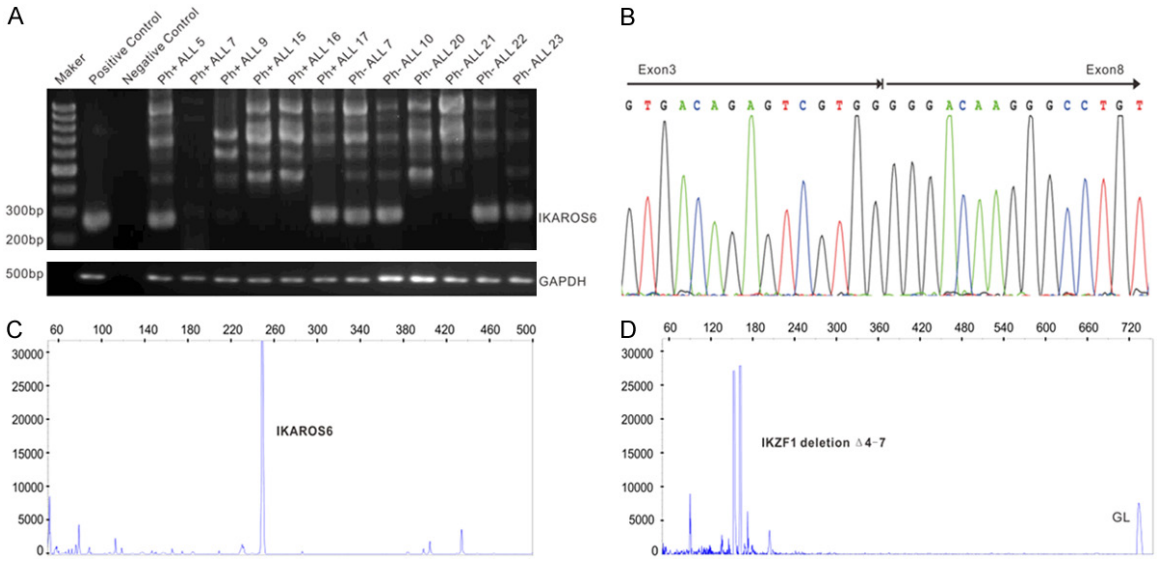

Figure 1. Molecular analysis on *IKZF1* in B-ALL. A. RT-PCR for *IKZF1* transcripts in representative cases, *Ikaros6* were detected in both *BCR-ABL1*+B-ALL and *BCR-ABL1*− B-ALL. B. Sequencing of RT-PCR products confirmed the expression of *Ikaros6*. C. Electrophotography of *IKZF1* PCR transcript product was performed by using a forward primer conjugated with the FAM at its 5' end. Different *Ikaros* isoforms are represented in the electropherogram by different peaks. The x-axis displays the computed length of the PCR products in base pairs, as determined automatically in terms of an internal lane standard. The y-axis represents the peak height in fluorescence units. D. Genomic gene scanning of *IKZF1* Δ 4-7 was performed.

Therefore, we supposed there may be a potential correlation between *Ikaros6* and myeloid-associated antigens (MY).

In this study, we analyzed the molecular features of *Ikaros6* and assessed its prognostic value in a cohort of 108 Chinese adult patients with B-ALL. Furthermore, we discussed the potential relationship between *Ikaros6*, *BCR-ABL1* and myeloid-associated antigens. Furthermore, explored deeply the prognostic value of three factors mentioned above.

Materials and methods

Subjects and the cell line

The study examined 108 de novo Chinese adult B-ALL who, from Jan 2007 to Dec 2013, were diagnosed and treated at the Hematological Centre of Tongji Hospital in accordance with the CALLG2008 Protocol [23]. The median follow-up was 10 months. The study was approved by a review committee of medical ethics of Tongji Hospital. Bone marrow samples were collected from these patients after obtaining their written consent in accordance with the Declaration of Helsinki. BV-173, as the positive control for PCR, was obtained from DMSZ (Braunschweig, Germany) and maintained in a culture according to DMSZ culture protocol. Cells were kept in an incubator at 37°C in 5% CO_2.

RT-PCR, sequencing and real-time PCR

Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Total RNA was extracted by employing the RNEASY total RNA isolation kit (QIAGEN, Germany). 1 μg total RNA sample was reversely transcribed into cDNA. *IKZF1* primers for PCR were: 5'-ATGGATGCTGATGAGGGTCAAGAC-3' (with fluorescently tagged FAM) and 5'-GATGGCTTGGTCCATCACGTGG-3'. RT-PCR products were purified by using GeneJET Gel Extraction kit (Thermo, USA) and the resultant segments were cloned into pGEM-T-Easy vector (Promega, USA). The cloned PCR products were sequenced by utilizing 3500 Genetic Analyzer (Applied Biosystems, USA). *Ikaros6* transcript was quantitatively detected as previously described [3] by employing a 7900 Real-Time PCR system (Applied Biosystems, USA).

Gene scanning

Ikaros6 isoform was detected and quantified by gene scanning as described previously [24]. *IKZF1* transcripts were detected by using gene

Table 1. Clinical characteristics, molecular features, genomics markers and Myeloid-associated antigens in B-ALL patients

Characteristic	IKAROS6 Positive (n=37)		IKAROS6 Negative (n=71)		
	No.	%	No.	%	p
Age, years					*0.781*
Median	30		36		
Range	16-68		16-72		
Sex					*0.315*
Male	22	59.5	35	49.3	
Female	15	40.5	36	50.7	
WBC count, $\times 10^9$/L					*0.049*
Median	13.7		12.6		
Range	1.17-522.0		0.87-363.0		
Hemoglobin , g/dL					*0.132*
Median	72.0		76.2		
Range	32.8-122.0		27.0-140.0		
Platelet count, $\times 10^9$/L					*0.557*
Median	33.0		38.0		
Range	3.0-252.0		2.2-279.0		
Bone marrow blasts, %					*0.238*
Median	93.2		89.6		
Range	32.49-99.5		31.0-99.2		
Major ALL subtypes					*0.879*
Early precursor B-ALL	5	13.5	12	16.9	
Common B-ALL	24	64.9	43	60.6	
Precursor B-LL	8	21.6	16	22.5	
Missing					
Genomics Markers of Prognosis					
Hyperdiploidy	0	0.0	1	1.4	*0.739*
Hypodiploidy	1	2.7	2	2.8	*0.560*
t(9;22)(q34;q11.2)	16	43.2	14	19.7	*0.010*
t(v;11q23)	3	8.1	4	5.6	*0.933*
t(1;19)(q23;P13.3)	0	0.0	3	4.2	*0.350*
Complex karyotype	4	10.8	7	9.9	*0.857*
Normal karyotype	8	21.6	30	42.3	*0.033*
Other	5	13.6	10	14.1	*0.935*
BCR-ABL1					*0.010*
Positive	16	43.2	14	19.7	
Negative	21	56.8	57	80.3	
MLL					*0.933*
Positive	3	8.1	4	5.6	
Negative	34	91.9	67	94.4	
E2A-PBX1					*0.350*
Positive	0	0	4	5.6	
Negative	37	100.0	67	94.4	
Myeloid-associated Antigens					*0.009*
Positive	27	73.0	33	46.5	
Negative	10	27.0	38	53.5	

ALL, acute lymphoblastic leukemia; Myeloid-associated antigens: CD13, CD33, CD15, MPO; WBC, white blood cells.

scanning with the primers used aforementioned. Genomic DNA was isolated by using QIAamp DNA Blood Mini Kit (QIAGEN, Germany). Genomic gene scanning of *IKZF1* Δ 4-7 was performed as described by Caye *et al.* [25].

Genetic detection and phenotype

Bone marrow samples were examined for common translocations *E2A-PBX1*, *TEL-AML1*, *BCR-ABL1*, and *MLL-AF4* by employing RT-PCR and Real-time PCR. Other *MLL* gene rearrangements were detected by using fluorescence in situ hybridization (FISH). Immunophenotype was identified by utilizing 4-color flow cytometry.

Statistical analysis

The Kaplan-Meier (log-rank test) and Cox proportional hazards regression models were used to calculate the risk factors affecting overall survival (OS), event-free survival (EFS) and relapse-free survival (RFS). The distributions of prognostic factors in subgroups were analyzed by using χ^2 or Fisher's exact test. All tests were two-sided and difference was considered to be statistically significant when a $P<0.05$. All statistical analyses were performed using SPSS 18.0 software (Chicago, USA).

Results

Clinical features of patients

Ikaros6 was identified in 37 of 108 B-ALL patients (34.3%) by RT-PCR (**Figure 1A**), then we confirmed the results by sequencing (**Figure 1B**) and gene scanning (**Figure 1C**, **1D**). *Ikaros6* was predomi-

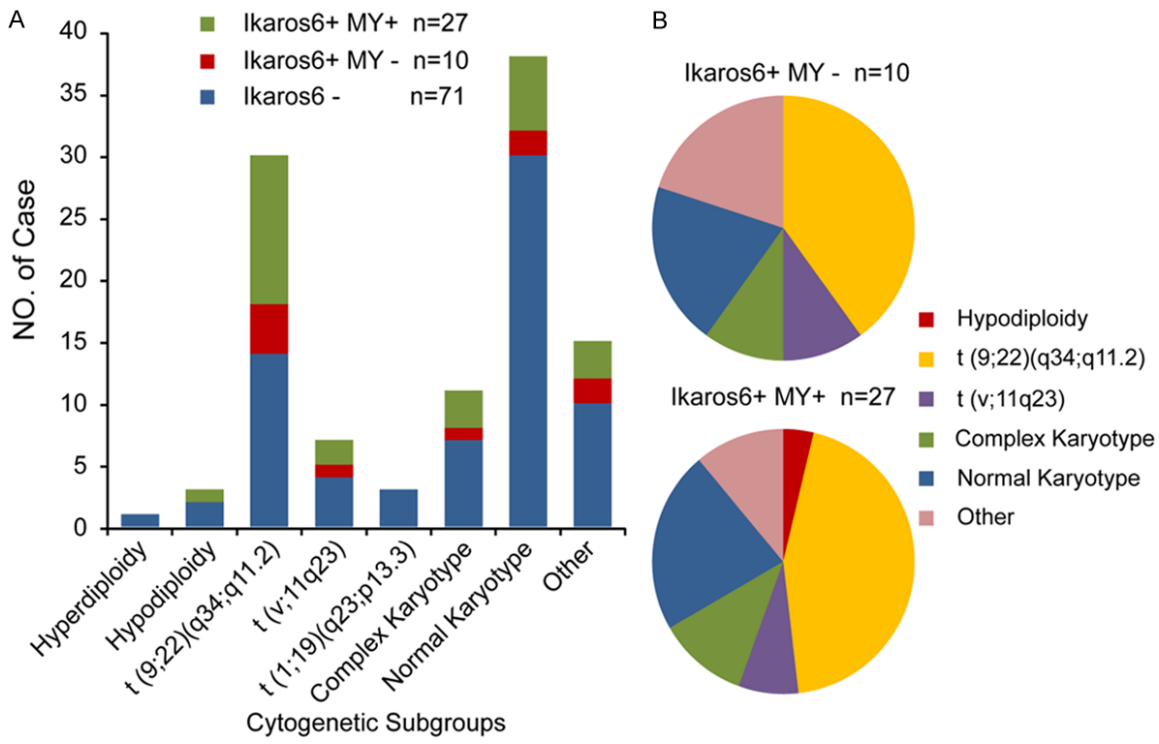

Figure 2. Hierarchical cluster analysis on the basis of *Ikaros6* associated signature in B-ALL. A. The distribution of cytogenetic subgroups. The hierarchical cluster analysis was conducted on the principal chromosomal abnormalities observed in adult B-ALL according to NCCN Guidelines (2013): t(9;22)(q34;q11.2)/*BCR-ABL1*; t(v;11q23)/*MLL* rearranged; t(1;19)(q23;p13.3)/*E2A-AML1*; hyperdiploidy (51 to 65 chromosomes), hypodiploidy (<46 chromosomes), complex karyotype (5 or more chromosomal abnormalities). *Ikaros6* was intimately associated with the presence of t(9;22)(q34;q11.2). Because one patient with *Ikaros6+BCR/ABL1+* and the other with *Ikaros6-BCR-ABL1+* harbored other karyotypes, they were attributed to complex karyotype group. B. The pie charts showed, among B-ALL patients positive for *Ikaros6*, differences in the distribution of principal chromosomal abnormalities were found between patients expressing myeloid-associated antigens and those not expressing myeloid-associated antigens.

nantly found in the common B-ALL subtype (24 of 37, 64.9%), whereas in the early precursor B-ALL subtype and precursor B-ALL, only 5 (5 of 37, 13.5%) and 8 (8 of 37, 21.6%) were found respectively according to NCCN guideline 2013 (**Table 1**). In all cases positive for *Ikaros6*, 22 were males and 15 females, with a median age of 30 years. *Ikaros6* had no significant relationship with age, gender, hemoglobin, PLT count, bone marrow blasts and extra medullary invasion (P>0.05), while it was correlated with WBC count (13.7×10^9/L *versus* 12.6×10^9/L, P= 0.049) (**Table 1**).

Association of Ikaros6 with cytogenetic, molecular markers and myeloid-associated antigens

Diagnostic material from a total of 108 adult B-ALL patients was available for cytogenetic analysis. *Ikaros6* was principally found in high-risk group (23 of 37, 62.2%) according to NCCN guideline (2013), especially in cases of Ph+ALL (16 of 37, 43.2%). The frequency of *Ikaros6* in subjects with the normal karyotype was 21.6% (8 of 37) and those with other unclassified chromosomal abnormalities was 13.5% (5 of 37) (**Figure 2**). *Ikaros6* was intimately associated with the presence of *BCR-ABL1* (16 of 37, P=0.010). No significant correlation was revealed between *Ikaros6* and *MLL* (P=0.933) or *E2A-PBX1* (P=0.350) (**Table 1**).

Four myeloid-associated antigens, CD13, CD33, CD15, MPO, were detected by flow cytometry. In all subjects, significant associations were observed between *Ikaros6* and myeloid-associated antigens (27 of 37, P=0.009) (**Table 1**). Of note, 12 of 16 (75.0%) ALL patients positive for both *Ikaros6* and *BCR-ABL1* expressed myeloid-associated antigens (Figure S1).

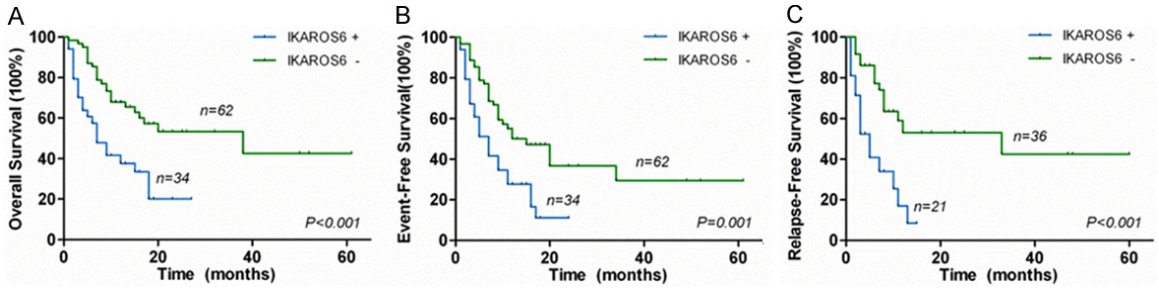

Figure 3. Kaplan-Meier survival estimates for patients with B-ALL according to *Ikaros6* status. A-C. Represented overall survival (OS), event-free survival (EFS) and relapse-free survival (RFS) for this cohort of patients.

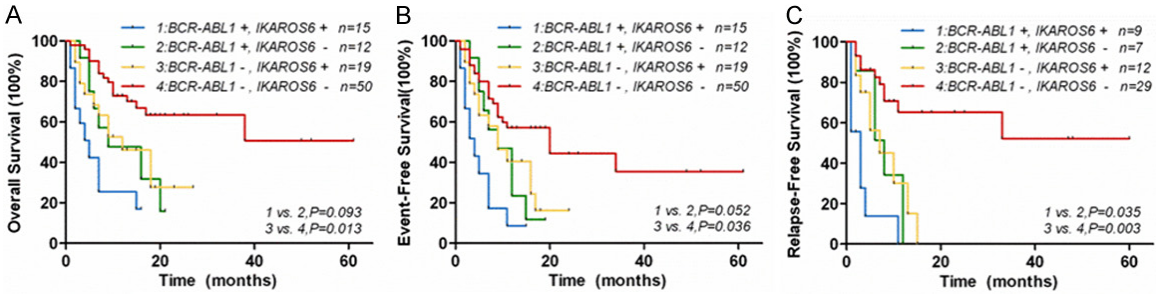

Figure 4. Kaplan-Meier survival estimates for patients with B-ALL according to *Ikaros6* and *BCR-ABL1*. A-C. Represented overall survival (OS), event-free survival (EFS) and relapse-free survival (RFS) for *BCR-ABL1*+ patients and *BCR-ABL1*- patients.

Minimal residual disease (MRD) detected by gene scanning and real-time PCR

In this study, MRD was detected by using gene scanning and real-time PCR in bone marrow samples harvested from adults with Ph+ALL. The MRD was monitored at four time points: initial diagnosis (point 1), partial remission (point 2), complete remission (point 3) and relapse (point 4). Notably, *Ikaros6* and *BCR-ABL1* were highly expressed at the first time point. When the patient achieved CR, both levels decreased to undetectable. Furthermore, the two genes presented similar expression patterns at these chosen time points (Figure S2).

Survival analysis

Among identified 108 patients with B-ALL, survival analysis was mainly performed in 96 cases due to 12 people receiving hematopoietic stem cell transplantation. Note that 3 of these 12 patients were detected Ikaros6, and, more remarkable, all of them had satisfied overall survival and relapse-free survival. Kaplan-Meier survival analysis revealed that *Ikaros6* was associated with inferior survival, and that estimated survival rate for *Ikaros6*-positive versus *Ikaros6*-negative patients were: OS, 32.4% *versus* 59.7% (P<0.001) (**Figure 3A**); EFS, 23.5% *versus* 48.4% (P=0.001) (**Figure 3B**), and RFS, 19.0% *versus* 58.3% (P<0.001) (**Figure 3C**). However, Ikaros6, *BCR-ABL1* and myeloid-associated antigens (MY) were strongly correlated; therefore comparison of survival by *Ikaros6* expression was easily confounded by *BCR-ABL1* or MY. In order to solve this question, the impact of *Ikaros6* on survival would be further explored in different subgroups.

Survival estimates for patients with Ikaros6 and/or BCR-ABL1

Patients were divided into four subgroups due to the co-occurrence of *Ikaros6 and BCR-ABL1*. The first subgroup included patients co-occurring *Ikaros6* and *BCR-ABL1*, which seemed to have the worst survival; cases in the second and the third subgroup, which with Ikaros6+ or *BCR-ABL1*+ seemed to have worse survival than those in the fourth subgroup without neither of these two factors. Significant statistical difference was found in RFS between *Ikaros6+BCR-ABL1+* patients and *Ikaros6*-

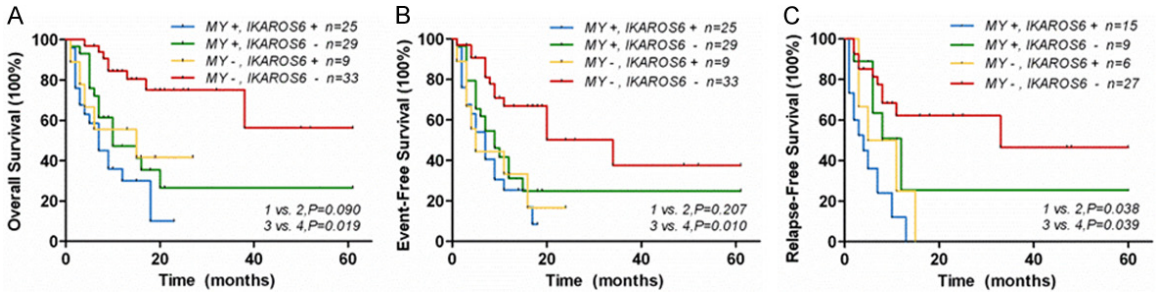

Figure 5. Kaplan-Meier survival estimates for B-ALL patients according to *Ikaros6* and myeloid-associated antigens (MY). A-C. Represented overall survival (OS), event-free survival (EFS) and relapse-free survival (RFS) for MY+ patients and MY-patients.

Table 2. Multivariable analyses for patients with B-ALL (COX regression model)

	OS			EFS			RFS		
Risk Factor	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Ikaros6	2.140	1.178-3.887	0.013	1.972	1.136-3.424	0.016	3.636	1.598-8.273	0.002
Log_{10}WBC	1.921	0.848-2.151	0.205	1.877	1.262-2.791	0.002	4.116	2.213-7.654	0.000
HB	0.993	0.980-1.006	0.293	0.995	0.984-1.007	0.440	0.986	0.968-1.004	0.130
BCR-ABL1	2.028	1.095-3.757	0.025	1.827	1.024-3.260	0.045	2.197	1.017-4.746	0.047
Myeloid-associated antigens	1.980	0.980-4.000	0.057	1.333	0.721-2.467	0.359	1.920	0.909-4.055	0.087

ALL, acute lymphoblastic leukemia; OS, overall survival; EFS, event-free survival; RFS, relapse-free survival; HR, hazard ratio; 95% CI, 95% Confidence intervals; Myeloid-associated antigens: CD13, CD33, CD15, MPO.

BCR-ABL1+ counterparts (P=0.035) (**Figure 4C**), while no difference in OS (P=0.093) and EFS (P=0.052) were noted between the two groups (**Figure 4A**, **4B**). Moreover, *Ikaros6* has a predictive value for inferior OS (P=0.013), EFS (P=0.036) (**Figure 4A**, **4B**) and RFS (P=0.003) (**Figure 4C**) in *BCR-ABL1*− ALL.

Survival estimates for patients with Ikaros6 and/or myeloid-associated antigens

Then patients were divided into four subgroups due to the co-occurrence of *Ikaros6 and* myeloid-associated antigens (MY). The first subgroup included patients co-occurring *Ikaros6* and MY, which seemed to have the worst survival; cases with one of these two risk factors had worse survival than those with Ikaros6- and MY-. Patients co-expressed *Ikaros6* and myeloid-associated antigens (MY) had higher risk of relapse than those expressed MY alone (P=0.038) (**Figure 5C**), but OS and EFS showed no statistically significant difference between the two groups (**Figure 5A**, **5B**). Interestingly, among the patients without MY, there was a significant difference in OS (P=0.019), EFS (P=0.010) (**Figure 5A**, **5B**) and RFS (P=0.039) (**Figure 5C**) between Ikaros6+ patients and Ikaros6-ones.

Cox proportional hazards model

Multivariate analysis showed that *Ikaros6* was a significant marker for inferior OS, EFS and RFS. Among a number of factors, including age, sex, leukocyte count, hemoglobin, platelet count, bone marrow blasts, *Ikaros6, BCR-ABL1*, *MLL* rearrangements, *E2A-PBX1*, extra medullary invasion and myeloid-associated antigens, *Ikaros6* was found to be significantly associated with OS (hazard ratio [HR], 2.140; 95% CI, 1.178-3.887; P=0.013) , EFS (HR,1.972; 95% CI, 1.136-3.424; P=0.016) and RFS (HR, 3.636; 95% CI, 1.598-8.273; P=0.002) (**Table 2**).

Discussion

Evaluation of relapse risk after the initial treatment is critical for ALL patients. For many years, risk stratification has been based on clinical features, such as age, WBC count, and genetic backgrounds [2]. In general, patients with *BCR-ABL1* and *MLL* gene rearrangements have poor outcomes, but obviously these genetic changes cannot be used for relapse prediction in all patients [15]. It has been well known that *Ikaros6*, as a novel molecular marker, play an important role of in prognosis estimates of adult ALL. Furthermore, we have shown that

combinatory detection of Ikaros6 and myeloid associated antigens, as well as *BCL-ABL1*, play a novel role in the recurrence prediction of the B-ALL patients.

Ikaros6, *BCR-ABL1* and myeloid-associated antigens were useful parameters to evaluate the prognosis. On the basis of study, it indicated closed relation between these three factors. Patients with B-ALL expressing myeloid-associated antigens were more likely to be positive for *Ikaros6* or to have the *BCR-ABL1* translocation. Among them, 13 of 57 cases co-expressed *Ikaros6*, *BCR-ABL1* and myeloid-associated antigens. Theocharides AP *et al*. used a humanized experimental leukemia model to gain insight into the important roles of *BCR-ABL1* and *Ik6* in AML that progress from myeloproliferative neoplasm (MPN) [26]. In this study, it showed that the more additional adverse factors patients harbored, the poorer prognosis patients had. These interrelated factors would play an important role in the transition or development of diseases.

Consistent with previous literature [3], this study showed that *Ikaros6* was identified in 34.3% of B-cell ALL and frequently present in *BCR-ABL1*-positive ALL patients (53.3%). By employing a number of detection methods together, we dynamically examined the expression level of *Ikaros6* and *BCR-ABL1*. It showed that the expression pattern of *Ikaros6* was synchronized with that of *BCR-ABL1*, suggesting that *Ikaros6* may serve as a valuable factor reflecting tumor burden accurately and a reliable marker for MRD detection, which was confirmed by recent research [17, 25].

Several studies have shown that alterations of *IKZF1* were involved in the pathogenesis of both *BCR-ABL1*-positive and *BCR-ABL1*-negative ALL with poor outcome [4, 13-15]. Mullighan *et al*. suggested that *IKZF1* deletions were correlated to an increased frequency of relapse and drug resistance, furthermore it was independent of *BCR-ABL1* and other risk factors in children B-cell progenitor ALL [4]. Our study demonstrated that in Chinese adult B-ALL patients, the presence of *Ikaros6* was associated with an inferior outcome. We further discussed the impact of Ikaros6 on survival in different subgroups, in order to exclude the interference of other factors, such as *BCR-ABL1* and myeloid-associated antigens. Significant increased relapse was observed in patients co-expressing *Ikaros6* and *BCR-ABL1*, as compared with cases positive for *BCR-ABL1* alone, which was supported by previous result [3]. The notion that *Ikaros6* can serve as a good predictor for inferior RFS in *BCR-ABL1*-negative ALL was consistent with other opinions [4, 14, 27]. We concluded that *Ikaros6*, as an independent adverse factor, could predict recurrence of B-ALL.

The prognostic relevance of myeloid-associated antigens is controversial, but most studies suggested that myeloid-associated antigens expression was associated with poor prognosis [20, 21]. In this study, patients co-expressed *Ikaros6* and myeloid-associated antigens had high risk of relapse. The same result was obtained from patients who positive for *Ikaros6* but not expressing myeloid-associated antigens. Thus *Ikaros6* was an adverse factor for RFS, which was independent of myeloid-associated antigens. Therefore *Ikaros6* could serve as an appropriate prognostic marker for RFS. Furthermore, multivariate analysis confirmed that *Ikaros6* had a negative impact on survival independent of other prognostic factors. It also indicated that WBC and BCR-AB*L1* were independent factors with a strong adverse effect. However, myeloid-associated antigens were not showed to be an independent predictor of survival.

In conclusion, we demonstrated that *Ikaros6* was highly expressed in *BCR-ABL1*-positive cases and patients who exhibited myeloid-associated antigens. There was a significant correlation between *BCR-ABL1*, myeloid-associated antigens and *Ikaros6*. *Ikaros6* was associated with increased risk of relapse, which was independent of *BCR-ABL1* and myeloid-associated antigens. However, the more additional adverse factors patients harbored the poorer prognosis patients would have. Furthermore, *Ikaros6* was an independent prognostic marker negatively impacted on survival and a valuable factor to evaluate the risk of relapse in Chinese adult B-ALL.

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Disclosure of conflict of interest

None.

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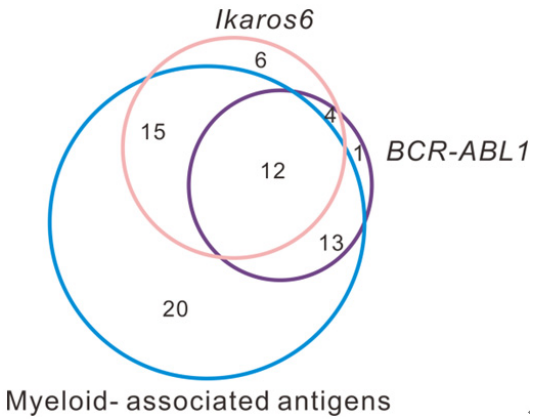

Figure S1. Venn diagrams show the overlap of patients who expressed three risk factors. The region of overlap between all circles indicates the number of patients co-expressing all three risk factors. Regions of overlap between two circles indicate cases co-expressing any two of three risk factors. Regions that do not overlap between circles indicate patients expressing only one risk factor. There was a close correlation between *Ikaros6*, *BCR-ABL1* and myeloid-associated antigens.

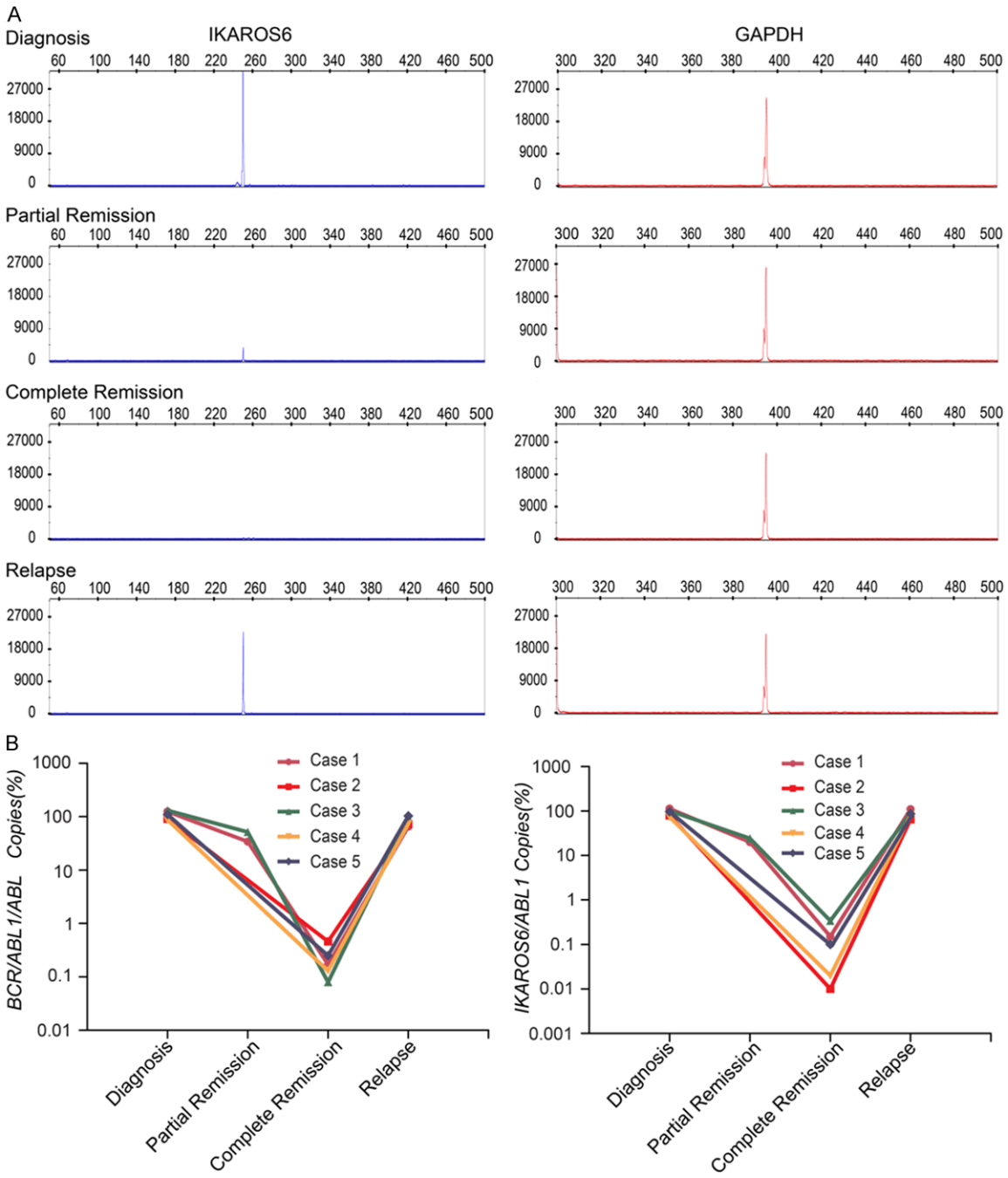

Figure S2. The MRD was monitored by gene scanning and real-time PCR at four time points. The MRD was monitored at four time points in 5 patients co-expressing *BCR-ABL1* and *Ikaros6*: initial diagnosis (point 1), partial remission (point 2), complete remission (point 3) and relapse (point 4). A. With *GAPDH* gene serving as an internal control, *Ikaros6* was discordantly expressed at four points, as was detected by gene scanning. B. Real-time PCR showed the expressions of *Ikaros6* and *BCR-ABL1* when a segment of *ABL* gene was used as an internal control (bottom panels).