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Research Paper

RAG1 high expression associated with *IKZF1* dysfunction in adult B-cell acute lymphoblastic leukemia

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Abstract

The recombination mediated by recombination activating gene (RAG) is not only the dominant mutational process but also the predominant driver of oncogenic genomic rearrangement in acute lymphoblastic leukemia (ALL). It is further responsible for leukemic clonal evolution. In this study, significant *RAG1* increase is observed in the subsets of B-ALL patients, and high expression of *RAG1* is observed to be correlated with high proliferation markers. *IKZF1*-encoded protein, IKAROS, directly binds to the *RAG1* promoter and regulates *RAG1* expression in leukemic cells. CK2 inhibitor by increasing IKAROS activity significantly suppresses *RAG1* expression in ALL in an IKAROS-dependent manner. Patients with *IKZF1* deletion have significantly higher expression of *RAG1* compared to that without *IKZF1* deletion. CK2 inhibitor treatment also results in an increase in *IKZF1* binding to the *RAG1* promoter and suppression of *RAG1* expression in primary ALL cells. Taken together, these results demonstrate that *RAG1* high expression is associated with high proliferation markers in B-ALL. Our data for the first time proved that *RAG1* expression is directly suppressed by IKAROS. Our results also reveal drive oncogenesis of B-ALL is driven by high expression of *RAG1* with IKAROS dysfunction together, which have significance in an integrated prognostic model for adult ALL.

Key words: *RAG1*; *IKZF1*; adult; acute lymphoblastic leukemia

Introduction

The recombination-activating gene 1 (RAG1) is the member of a recombination-activating gene family which play an important role in the rearrangement and recombination of the genes of immunoglobulin and T cell receptor molecules and is critical for the generation of T and B cells. However, the abnormalities in RAG family members are observed quite commonly in lymphoid malignancies [1-7]. The RAG-mediated gene deletions and translocations have been reported in acute lymphoblastic leukemia (ALL) [8-13]. RAG and activation-induced cytidine deaminase (AID) together drive leukemic clonal evolution due to chronic exposure to inflammatory stimuli, parallel to infections noted in children during their childhood [13]. It is also reported that a higher *RAG1* mRNA level predicts shorter relapse free survival and overall survival in patients with ALL [13]. These reports suggest *RAG1* is involved in the oncogenesis and relapse of ALL.

IKAROS, a kruppel-like zinc finger protein is encoded by the *IKZF1* gene, and it plays an essential role for normal hematopoiesis and acts as a tumor suppressor in ALL. The genetic defects of *IKZF1* in a single allele are associated with the development of

human acute lymphoblastic leukemia, which is characterized by an increased risk of relapses and poorer prognosis. Recently, we reported the global binding profiling of IKAROS in ALL cells and found that IKAROS regulates the expression of gene targets through chromatin remodeling in ALL [14-20]. Our data from chromatin immunoprecipitation followed by sequencing (ChIP–seq) showed IKAROS binding peaks in the promoter region of the genes in multioncogenic pathways. We also observed that CK2 inhibitors increased the tumor suppressor activity of IKAROS and act as a functional activator of IKAROS [14-20]. However, it still remains unclear whether IKAROS regulates *RAG1* expression.

Here we examined *RAG1* expression in an adult patient with B-ALL and analyzed the correlation of *RAG1* expression with clinical features. We observed that high expression of *RAG1* correlates with high proliferation markers in adult ALL. We also identified *RAG1* as a direct target of *IKZF1*-encoded protein, IKAROS. Deletion of *IKZF1* was significantly associated with increased expression of *RAG1* in adult B-ALL. Our findings reveal that *IKZF1* deletion may be responsible for the high expression of *RAG1* in B-ALL. High expression of *RAG1* may work in conjunction with *IKZF1* deletion to drive oncogenesis and relapse in B-ALL.

Materials and Methods

Patients and samples

Subjects with newly-diagnosed B-ALL (age12-77 years old) were recruited between June 2008 and June 2016. There were 131 patients who were recruited at the First Affiliated Hospital of Nanjing Medical University and Zhongda Hospital Southeast University. The patients' diagnosis was based on the WHO Diagnosis and Classification of ALL (2008). The approval for this study was received from the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University and Zhongda Hospital Southeast University, Nanjing, China.

Cytogenetic and molecular analyses

Cytogenetics and detection of most common *IKZF1* deletion, Ik6 [27], CRLF2 rearrangements [28-30], BCR-ABL fusion gene/Ph chromosome [25, 31], PAX5 mutations [26] and SH2B3 mutations [17] etc. were performed as previously described. The StepOne Plus Real-time PCR system (Applied Biosystem-Thermofisher, Foster, CA, USA) was used for qPCR. Expression values of genes of interest (GOI) were calculated in each sample by a formula derived from a scatter graph of Ct values from serial dilutions of a template standard as described [16, 18, 20, 25]. Expression levels of GOIs were expressed as gene expression value of GOI/18s rRNA by normalized to the housekeeping genes. Subjects were allocated into high or low *RAG1*expression cohorts (3rd-4th quartiles vs. 1st-2nd quartiles) with a cut-off value determined by SPSS 20[16, 20].

The qPCR for *RAG1* expression was similarly performed as above in Nalm6 cells. The results were n presented as fold induction over vector controls after normalized to those obtained with 18sRNA. Primers: 18s RNA, Sense: 5'-GTAACCCGTTGAACCCCATT-3', Antisense: 5'-CCATCCAATCGGTAGTAGCG-3'; *RAG1* Sense: 5'-CGAGGAACGTGACCATGGA-3', Anti-sense: 5'-GGCAGTGTTGCAGATGTCACA-3'.

Plasmid construction, retroviral gene transfer,and cell culture reagents

The Nalm6 [32] has been previously described. RPMI 1640 medium (Cellgro, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and DMEM (Cellgro) supplemented with 10% fetal calf serum and 1% L-glutamine (Cellgro) were used for culture of Nalm6 and HEK293 cell, respectively, incubated at 37°C in a humidified atmosphere of 5% CO2. Primary human B-ALL were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Hyclone). CX4945 was obtained from Sigma (St. Louis, MO, USA). Cells were cultured with or without CX4945 and collected for total RNA isolation. Human *IKZF1* retroviral construct and retroviral production were described previously [14, 15, 19, 20].

Quantitative Chromatin Immunoprecipitation (qChIP)

For the qChIP assays, the chromatin was incubated with antibodies against IKAROS [14, 15, 19] or normal rabbit IgG (Abcam) as a control [14, 15, 19, 20]. The enrichment of the ChIP sample was evaluated over input with the qPCR data obtained in three or more replicates, using specific primers in the promoter region of *RAG1* (forward: 5'-GCAACCAACCAACTCTGTTCAC-3', reverse: 5'-ACTGGTTTGTTTTGGACTTTCCTT-3'). The relative concentration of the qPCR product was presented as the fold change of the level of DNA-IKAROS samples in comparison to controls.

Luciferase reproter Assay

The promoter of *RAG1* was cloned into the pGL4.15 vector (Promega, WI, USA), and transiently expressed in HEK293 cells for luciferase reporter assay. The promega luciferase assay reagents were used to measure the result on the luminometer following the manufacture's instruction [14, 16]. The

firefly luciferase activities were expressed as a percentage of pcDNA3.1-*IKZF1* transfection-induced luciferase activity versus that of the pcDNA3.1 vector. All transfection for reporter assays were performed independently, in triplicate, at least three times.

IKZF1 shRNA knockdown

The human*IKZF1* shRNA constructs in the GFP vector (pGFP-v-RS) (Origene) was transiently transfected into Nalm6 cells using the Neon Transfection System (Invitrogen, USA). The scrambled 29-mer shRNA cassette in the pGFP-v-RS vector was used as a control. *IKZF1* Knockdown efficiency was confirmed by measurement of *IKZF1* mRNA level with qPCR [14, 15, 19, 20]. Primers: *IKZF1*-F: 5'-GGCGCGGTGCTCCTCCT-3', *IKZF1*-R: 5'-TCCGACACGCCCTACGACA-3'.

Statistical analysis

The Mann–Whitney U-test was used for evaluation of median differences between the cohorts. Univariate and multivariate Cox models were performed for frequency differences with SPSS version 20.0. Mean values with bars representing the standard error of the mean (SEM) was used for data presentation. The shown data was the representative of 3 independent experiments. Statistical significance was determined by Student t-test for comparison of two groups or analysis of variance (ANOVA) for comparing more than two groups.

Results

RAG1 is highly expressed and associated with high proliferation in adult B-ALL

Firstly, we detected the *RAG1* mRNA expression in 131 newly diagnosed adult B-ALL patients. We found that the *RAG1* mRNA levels were significantly higher in patients with B- ALL compared to that in the normal controls (Fig 1). We also examined the *RAG1* expression in microarray cohorts and found that *RAG1* is highly expressed in the cohort studies of B-ALL patients (Supplemental Fig 1).

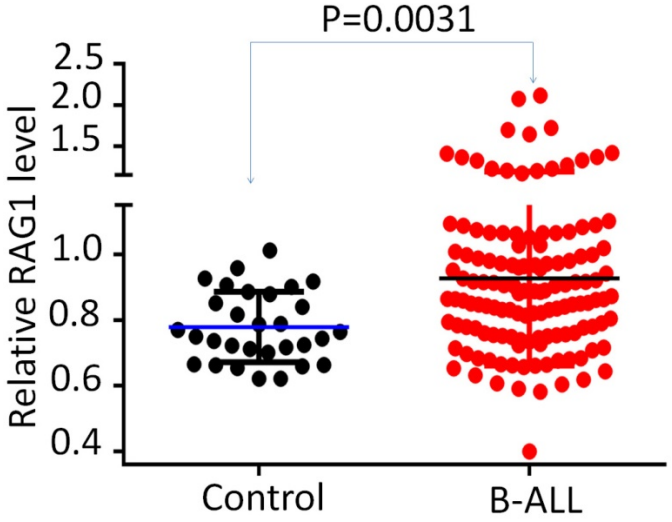

Fig 1. *RAG1* expression in adult B-ALL. Comparison of *RAG1* mRNA level in B-ALL with normal BM control by qPCR.

We further divided the patients into two subgroups based on the high (Quartile 1-2) or low (Quartile 3-4) *RAG1* mRNA levels. The relationships between the *RAG1* expression and clinical characteristics in this cohort of B-ALL were analyzed (Fig 2 and Supplemental Table 1). *RAG1* high expression group demonstrated a significantly higher percentage of white blood cell (WBC) ≥30x10^9/L (67.7% vs. 38.5%, P<0.001) and higher median WBC (47.9×10^9/L vs. 17.0×10^9/L, P=0.025), which are markers of poorer prognosis in B-ALL (Fig 2A and Supplemental Table 1). In addition, patients with high *RAG1* expression had a higher rate of blasts in peripheral blood (PB) (75.0% vs. 61.0%, P=0.026) (Fig 2B and Supplemental Table 1), indicating the higher proliferation of leukemic cells in patients with high *RAG1* expression.

We did not observe the significant association of mutations in PAX5 and SH2B3 with *RAG1* expression (Fig 2C, Supplemental Table 1); however, the percentage of CRLF2 mutations, which includes R186S in exon5, F232F in exon6 and A11A in exon 1 is significantly higher in the patients with *RAG1* high expression compared to that of low expression (Fig 2C). Also, the rate of *IK*6, the most common deletion form of *IKZF1* is significantly higher in patients with *RAG1* high expression (Fig 2C). These data suggest *RAG1* high expression associates with CRLF2 mutations and *IKZF1* deletion.

IKAROS binds to the promoter of *RAG1* and regulates its expression in B-ALL

To further address the potential link between the high *RAG1* expression and high proliferation of B-ALL cells, we analyzed the motifs of transcription factors in the promoter region of *RAG1*. As we expected, certain core IKAROS binding motifs (GGGA or GGAA) were identified in the promoter region of *RAG1* (Fig 3A). Notably, our chromatin immunoprecipitation followed by sequencing (ChIP–seq) data showed the strong binding peaks for *IKZF1* in the *RAG1* promoter region in B-ALL cell lines and primary cells from the B-ALL patients (Fig 3B and Supplemental Fig 2)[14, 15]. To further confirm, quantitative ChIP (qChIP) assays were performed and showed significant binding of IKAROS at the *RAG1* promoter region in both B-ALL cell line Nalm6 (Fig 3C) and primary cells (Fig 3D).

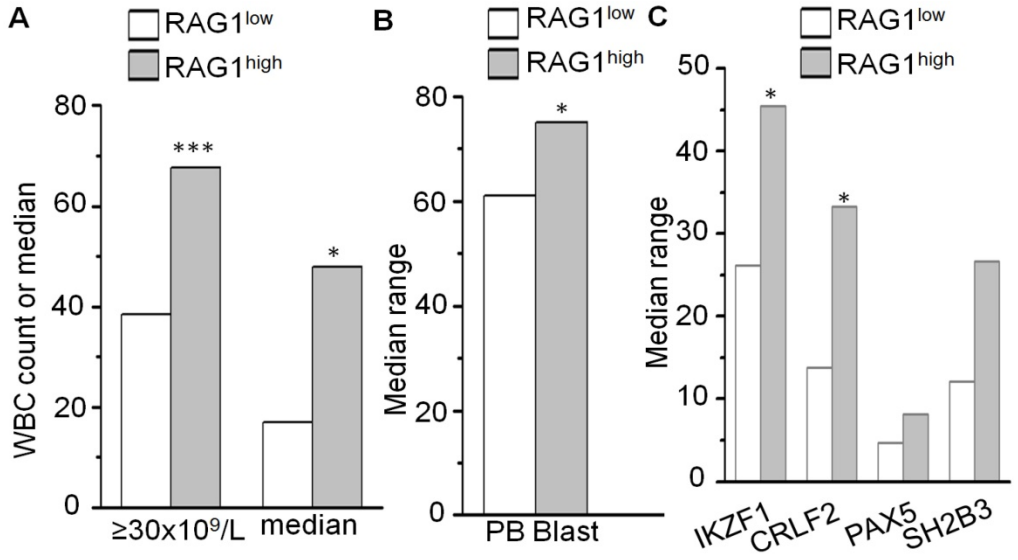

Fig 2. Correlation of *RAG1* expression with clinical features in adult B-ALL. (A-B) Correlation of *RAG1* mRNA level with WBC count and blast in peripheral blood in B-ALL. (C) *RAG1* expression with deletion and mutations in adult B-ALL.*P<0.05, ***P<0.001.

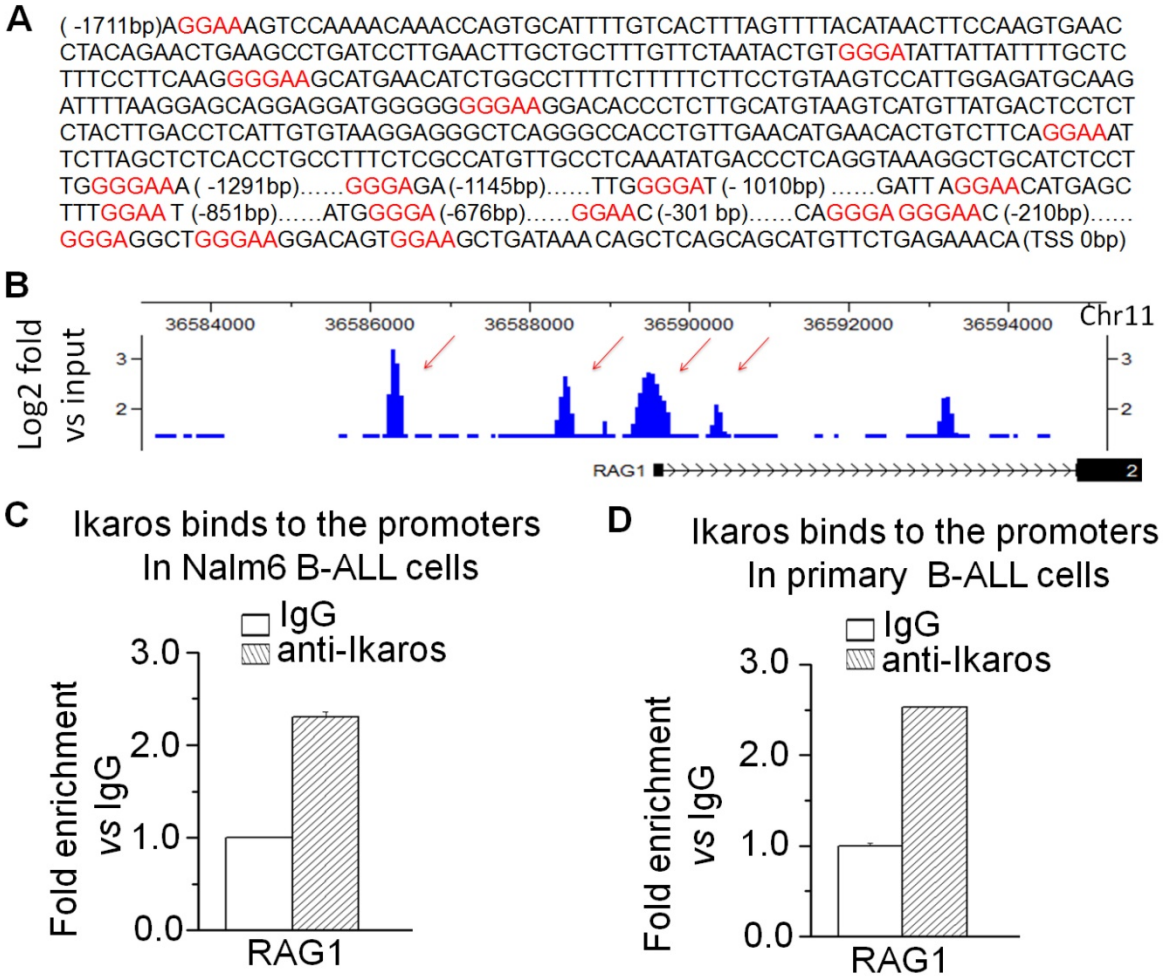

Fig 3. IKAROS binds the promoters of *RAG1*. (A) *RAG1* promoter region with conserved *IKZF1* core binding motifs (GGGA or GGAA, red). (B) IKAROS binding peaks in the *RAG1*promoter region identified by ChIP-seq. (B) qChIP data for IKAROS binding on *RAG1* promoter in Nalm6 B-ALL (B) and primary B-ALL cells (C).

Moreover, we addressed whether *RAG1* is the direct target of IKAROS and how IKAROS regulates *RAG1* expression in B-ALL cells. We observed that IKAROS suppresses the promoter activity of *RAG1* by luciferase reporter assay (Fig 4A). By over-expression of IKAROS in Nalm6 B-ALL cells, we observed that

IKAROS could suppress *RAG1* mRNA levels (Fig 4B). Conversely, efficient *IKZF1* knockdown increased *RAG1* expression in Nalm6 cells (Fig 4C).To further demonstrate that IKAROS negatively regulates *RAG1* expression, Nalm6 B-ALL cells were treated with CK2 inhibitor (CX4945) which functioned as IKAROS activator. Significantly, CX4945 could suppress *RAG1* expression in a dose-dependent manner (Fig 5A). Similarly, CK2 knockdown with shRNA could reduce *RAG1* expression (Fig 5B). It worth noting that the CX4945-induced decrease of *RAG1* expression could be blocked by *IKZF1* knockdown with shRNA in Nalm6 cells (Fig 5C). These data indicate that *RAG1* is the direct target of IKAROS and IKAROS suppresses *RAG1* expression in B-ALL cells.

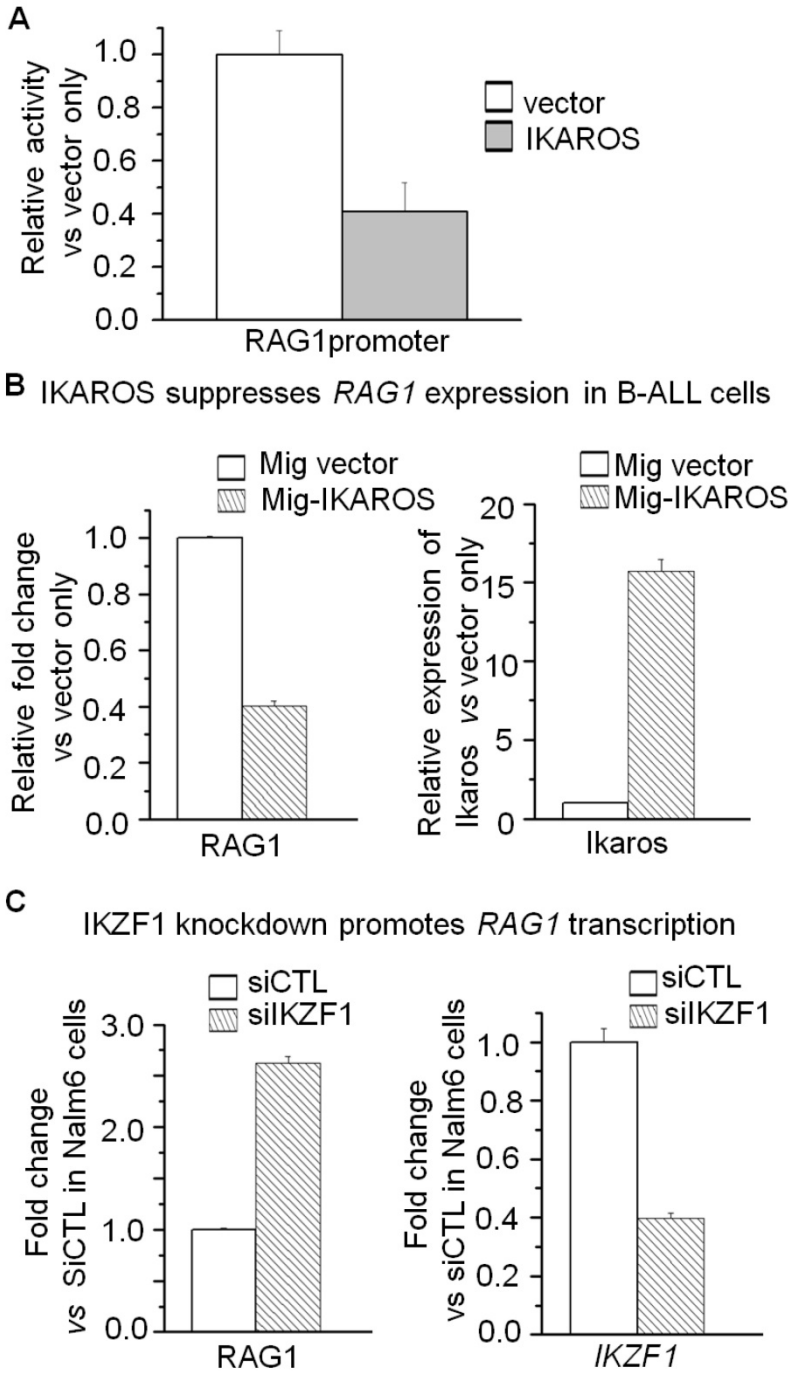

Fig 4. IKAROS suppresses *RAG1* expression. (A) The promoter activity of *RAG1* promoters measured by luciferase reporter assay following transfection with IKAROS or control vector in HEK293 cells. (B) Effect of expression of IKAROS in Nalm6 cells. The cells were transduced with a vector containing IKAROS as compared to the control vector. (C) qPCR of *RAG1* and *IKZF1* expression in the Nalm6 cells following *IKZF1* shRNA treatment as compared to scramble shRNA cells. Gene expression is evaluated by RT-qPCR using total RNA isolated from the cells transfected with scramble shRNA (siControl) or *IKZF1* shRNA (si*IKZF1*) for 2 days.

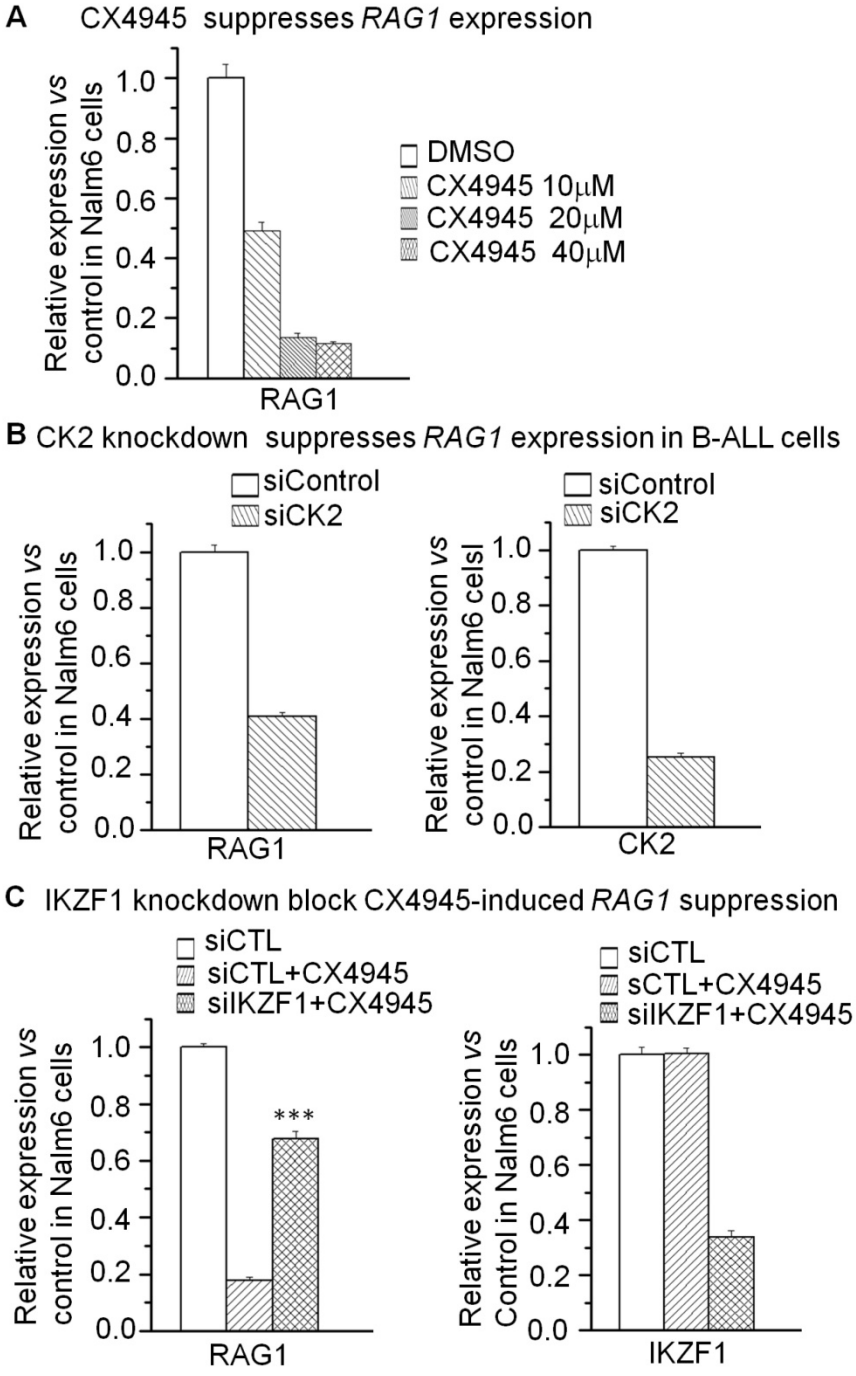

Fig 5. CK2 inhibitor CX4945 suppresses the expression of *RAG1* in an IKAROS-dependent manner. (A) Effect of CK2 inhibitor CX4945, which functions as IKAROS activator, on the expression of *RAG1* mRNA level in Nalm6 cells with CX4945 treatment for 2 days. (B) Effect of CK2 knockdown on the expression of *RAG1* in Nalm6 cells. (C) *IKZF1* knockdown rescues the CX4945-induced change of *RAG1* in Nalm6 B-ALL cells. Cells were treated with 10mM CX4945 for 2 days.***P<0.001.

Correlation of *IKZF1* deletion with high *RAG1* expression in patients with B-ALL

To explore the clinical association of IKAROS and *RAG1* expression, we observed the significantly negative correlation of *RAG1* mRNA level with *IKZF1* expression in the cohort of B-ALL and B lymphoma patients (Supplemental Fig 3). More importantly, we analyzed the correlation of *IKZF1* deletion with *RAG1* expression in patients with B-ALL. A higher incidence of *IK6*, the most common protein produced by *IKZF1* deletion was observed in B-ALL patients with the high *RAG1* expression group (45.5% vs 26.2%, P=0.021) (Supplemental Table 1); and *RAG1* mRNA level was significantly higher in patients with *IKZF1* deletion than those without *IKZF1* deletion (Fig 6A). These data indicate that *IKZF1* deletion is associated with upregulation of *RAG1* expression in primary B-ALL cells.

We have shown that CK2 inhibitor (CX4945) suppressed *RAG1* expression in Nalm6 cells. To further demonstrate the potential clinical significance, we treated the primary cells from the patients with B-ALL. CX4945 could increase the binding of IKAROS on the *RAG1* promoter region in primary B-ALL as compared to untreated controls (Fig 6B). In addition, CX4945 treatment inhibited expression of *RAG1* mRNA in a dose-dependent manner in primary B-ALL cells (Fig 6C). These results indicate that *IKZF1* binds to the promoter of *RAG1* and that treatment with CK2 inhibitors, which can restore *IKZF1* tumor suppressor activity, results in suppression of *RAG1* expression in primary cells from B-*ALL patients.*

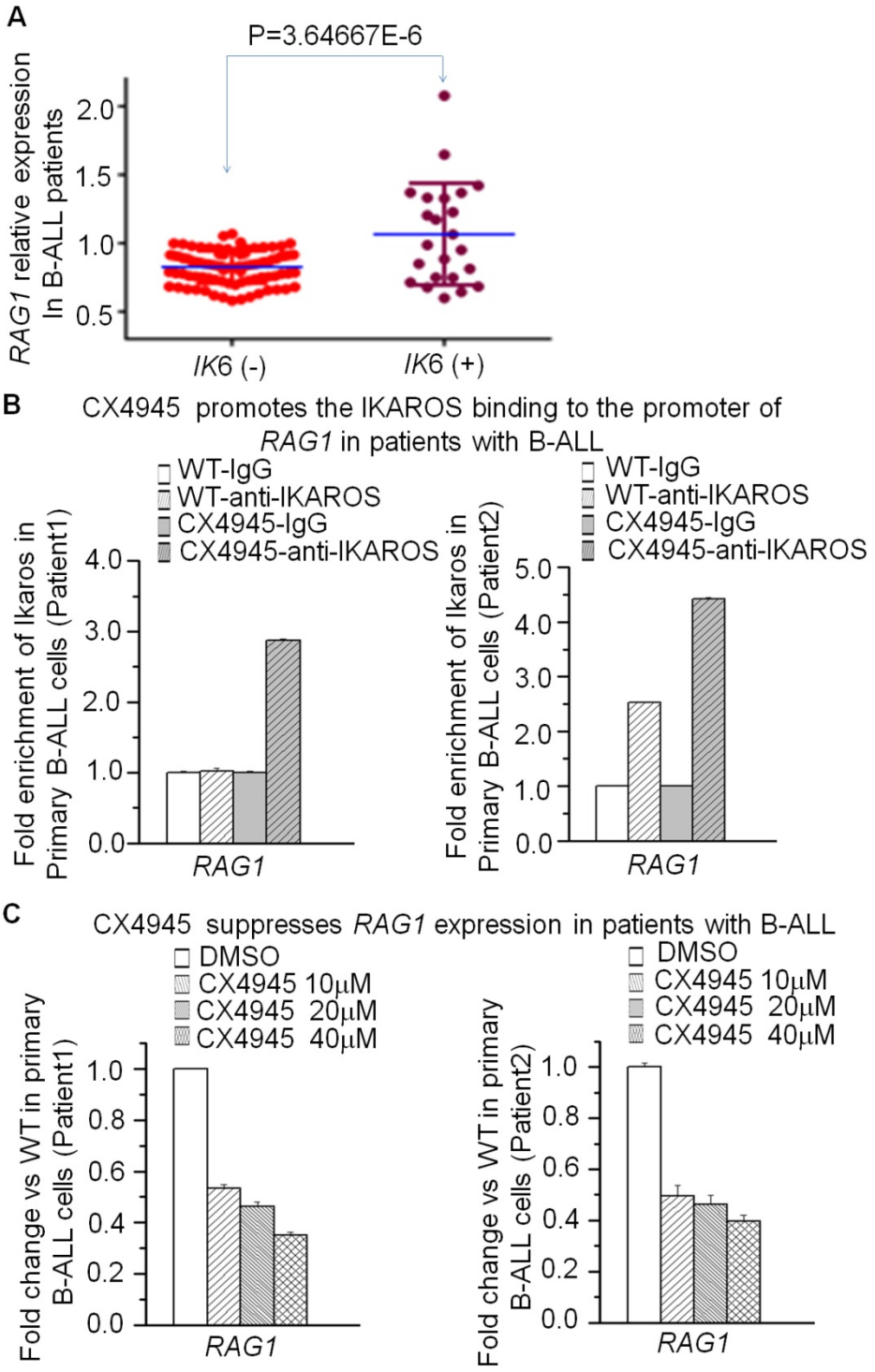

Fig 6. Correlation of *RAG1* high expression with *IKZF1* deletion in primary ALL cells. (A)Comparison of *RAG1* expression in patients with or without *IKZF1* deletion presented as *RAG1*/18s rRNA. The detection method for Ik6 (the most common IKAROS deletion) was done as our previously reported [23]. (B) CK2 inhibitor-CX4945 increased the *IKZF1* binding to the promoters of *RAG1* in primary B-ALL cells. (C) Effect of CK2 inhibitor-CX4945 functioning as *IKZF1* activator on the expression of *RAG1* in primary B-ALL cells with CX4945 treatment for 2 days.

Discussion

We observed that *RAG1* is significantly higher in patients with B-ALL. Higher expression of *RAG1* is associated with proliferation markers in B-ALL. IKAROS is crucial for the development of the hematopoietic system and its genetic deletion/mutations have been linked to the development of lymphoid leukemia. In particular, IKAROS functions as a major tumor suppressor involved in human B-cell acute lymphoblastic leukemia. In addition to a genetic defect, we also found that CK2 mediated-phosphorylation of IKAROS is another key reason leading to IKAROS dysfunction in ALL. Here, we found IKAROS directly suppresses *RAG1* expression in B-ALL cells; CK2 inhibitor-CX4945 could suppress *RAG1* expression by restoring IKAROS function in B-ALL cells. *IKZF1* deletion is significantly associated with *RAG1* high expression in B-ALL patients. These data reveal that *RAG1* high expression together with IKAROS dysfunction may be responsible for the oncogenesis in B-ALL.

RAG1 is the key component of RAG complex which is the predominant driver of oncogenic genomic deletion and translocation, and also the cause of the clonal expansion in ALL.RAG complexes are constitutively expressed in pro- and pre-B cells and target recombination signal sequences (RSSs) in both Ig and non-Ig genes. Motif analyses show that deletion breakpoints in the tumor suppressor genes such as *IKZF1*, etc. have RSSs motif and RAG complex target to the RSS motifs [12, 13, 23]. The patients with *IKZF1* deletion and mutations have increased risk of relapses and poor prognosis. Here we observed that *RAG1* high expression is associated with *IKZF1* deletion and patients with *RAG1* high expression have a significantly higher detective rate of *Ik6*, the most common isoform of *IKZF1* deletion. These data suggest *RAG1* high expression may also affect *IKZF1* deletion in adult B-ALL. Therefore, both *RAG1* high expression and *IKZF1* deletion may work together to amplify the oncogenic signaling responsible for oncogenesis in B-ALL (Supplemental Fig 4).

It is also reported that RAG-mediated recombination is the predominant driver of oncogenic rearrangement ETV6-RUNX1 in ALL [12] and plays critical roles underlying clonal expansion in pediatric ALL [12, 13]. Particularly RAG complex drives leukemic clonal evolution with repeated exposure to inflammatory stimuli, paralleling chronic infections in childhood under the same conditions [13]. We did observe the *RAG1* high expression is associated with a high frequency of CRLF2 mutations in addition to *IKZF1* deletion (Supplemental Table 1, Fig 2C). It is reported that both *IKZF1* deletion and CRLF2 rearrangement are the drivers of high-risk ALL particularly the Ph-like ALL [17, 23-26]. Thus, these data suggested that high *RAG1* expression may be also through oncogenic CRLF2 rearrangement to drive oncogenesis in B-ALL, although the association and underlying mechanisms of RAG activity with CRLF2 rearrangement need to be further determined.

Constitutive Rag expression is observed in a large portion of B-ALL cases [33]. Transcription factors such as FOXO1, FOXP1, NF-κB, PAX5β, E2A and Ikaros are reported to regulate Rag1 expression [34,35]. Here our data showed the Rag1 high expression in subsets of B-ALL and *RAG1* high expression is associated with the cell proliferation markers in B-ALL, and also Rag1 expression is directly regulated by Ikaros in B-ALL. The *RAG1* high expression may result in the Ikaros deletion and CRLF2 rearrangements, and then Ikaros dysfunction and may result in the Ikaros suppression on *RAG1* expression and further induce the *RAG1* high expression. This might be a positive feedback loop to amplify the oncogenic signaling in B-ALL (Supplemental Fig 4). Restoring IKAROS function by CK2 inhibitor can suppress the *RAG1* expression and block the oncogenic amplification loop.

In summary, we for the first time reported *RAG1* is a direct target of tumor suppressor gene *IKZF1*. We found that *IKZF1* deletion (characterized by *IK6* expression) is significantly correlated with high *RAG1* expression. *RAG1* is highly expressed and significantly correlated with high proliferation markers in a cohort of adult B-ALL patients. Our data suggested *RAG1* high expression work together with *IKZF1* deletion may drive the oncogenesis in B-ALL.

Supplementary Material

Supplementary figures.
http://www.jcancer.org/v10p3842s1.pdf

Acknowledgments

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Ethics approval and consent to participate

The written informed consent from all the patients was obaitned in accordance with the Declaration of Helsinki before enrollment in the study. The Institutional Review Board of the Nanjing Medical University and Zhongda Hospital Southeast University, Nanjing, China approved this study.

Competing Interests

The authors have declared that no competing interest exists.

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