Clinical significance of high c-MYC and low MYCBP2 expression and their association with Ikaros dysfunction in adult acute lymphoblastic leukemia

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ABSTRACT

Increased expression of c-MYC is observed in both Acute Myeloid Leukemia (AML) and T- cell Acute Lymphoblastic Leukemia (T-ALL). MYC binding protein 2 (MYCBP2) is a probable E3 ubiquitin ligase and its function in leukemia is unknown. IKZF1 deletion is associated with the development and poor outcome of ALL. Here, we observed significant high c-MYC expression and low MYCBP2 expression in adult ALL patients. Patients with high c-MYC expression and/or low MYCBP2 expression had higher WBC counts and a higher percentage of CD34+ or CD33+ cells, as well as splenomegaly, liver infiltration, higher BM blasts, and lower CR rate. Ikaros bound to the regulatory regions of c-MYC and MYCBP2, suppressed c-MYC and increased MYCBP2 expression in ALL cells. Expression of c-MYC mRNA was significantly higher in patients with IKZF1 deletion; conversely MYCBP2 mRNA expression was significantly lower in those patients. A CK2 inhibitor, which acts as an Ikaros activator, also suppressed c-MYC and increased MYCBP2 expression in an Ikaros (IKZF1) dependent manner in the ALL cells. In summary, our data indicated the correlation of high c-MYC expression, low MYCBP2 expression and high c-MYC plus low MYCBP2 expression with high-risk factors and proliferation markers in adult ALL patients. Our data also revealed an oncogenic role for an Ikaros/MYCBP2/c-MYC axis in adult ALL, providing a mechanism of target therapies that activate Ikaros in adult ALL.

INTRODUCTION

The MYC families of proteins are transcription factors with essential roles in cell growth and proliferation through their ability to regulate gene expression [1, 2]. Activation or amplification of the MYC oncogene family is one of the most frequent events associated with cancer [2]. c-MYC is frequently activated in acute myeloid leukemia (AML), and plays an important role in the induction of leukemogenesis [3, 4]. High c-MYC expression, a result of activating mutations in the Flt3 receptor tyrosine kinase, correlates with poor prognosis in AML [5, 6]. c-MYC is also frequently reported to be upregulated in acute lymphoblastic leukemia (ALL), however, the correlation of c-MYC expression with clinical features of ALL has not been fully described. The cause of c-MYC overexpression in adult ALL is also unknown.

MYCBP2 is likely an E3 ubiquitin ligase that binds specifically to MYC [7]. The region in MYC, which is responsible for MYCBP2 interaction, is frequently mutated in Burkitt’s and AIDS-associated lymphomas, indicating MYCBP2 suppress MYC activity [7]. Moreover, recently it is found that MiR-1247-5p overexpression resulted in a decreased expression of MYCBP2 in prostate...
cancer [8]. Additionally, it is reported that MYCBP2 is a candidate for the transformation-associated gene that maps to the 13q22.3 locus in Angioimmunoblastic T-cell lymphoma (AITL) [9]. However both the expression of MYCBP2 and its correlation with clinical features are unknown in ALL.

IKZF1 (Ikaros) encodes a kruppel-like zinc finger protein that is essential for normal hematopoiesis and acts as a tumor suppressor in ALL. The impairment of Ikaros function, as a result of deletion and/or inactivating mutation of a single IKZF1 allele, is linked to the development of ALL that is characterized by a high rate of relapse and poor outcome. Therefore, Ikaros inactivation results in high-risk leukemia that is resistant to treatment. Ikaros exerts its anti-tumor effect by regulation of its target genes. Ikaros activates or represses expression of target genes by directly recruiting the NuRD/Mi2 or SWI/SNF chromatin remodeling complexes [10]. CK2 directly phosphorylate Ikaros, which results in suppression of its activity [11–14]. Recently we found that CK2 inhibition restores Ikaros function in ALL cells (15, 16). CK2 inhibitors can be used as Ikaros activator (15, 16). We also identified Ikaros binding profiling in ALL cells (15), and found that Ikaros exert its antitumor effect by regulating the expression of its gene targets (15); and CK2 inhibitors restore Ikaros function by increasing Ikaros binding to the gene targets and regulation of their expression in ALL cells [15, 16].

Here, we observed the expression of c-MYC and MYCBP2 and their correlation with clinical features in adult ALL. We found c-MYC expression is negatively correlated with MYCBP2 expression in ALL; and high c-MYC expression and/or low MYCBP2 expression is associated with high-risk leukemia. We also observed the obvious Ikaros binding peaks in promoter region of c-MYC and MYCBP2 in ALL cells by ChIP-seq, and found that Ikaros directly suppresses c-MYC and activates MYCBP2 expression. Our results suggest that Ikaros dysfunction is partially responsible for the changes of c-MYC and MYCBP2 in adult ALL.

RESULTS

Association of c-MYC expression with characteristics of adult ALL

We assessed c-MYC mRNA expression in 104 newly diagnosed adult B-ALL and T-ALL patients. We found that c-MYC expression is significantly higher in both B-ALL and T-ALL patients compared to normal control (Fig. 1A). Patients were divided into high (n = 66) and low (n = 85) c-MYC expression groups. Patients with high c-MYC expression showed higher median white blood cell counts (WBC) (64.9 × 10^9/L vs 30.9 × 10^9/L, P = 0.009), a higher percentage of CD33(+) cells (58.7% vs 35.4%, P = 0.006), and a lower complete remission (CR) rate (82.3% vs 95.8%, P = 0.010) than those with low c-MYC expression (Supplemental Table 1, Fig. 1C). The percentage of patients exhibiting splenomegaly, liver infiltration, and increased lactate dehydrogenase (LDH) was significantly higher in the high c-MYC expression group than in the low expression group (53.0% vs 36.1%, P = 0.039; 34.8% vs 15.5%, P = 0.006; 49.2% vs 31.2%, P = 0.033) (Supplemental Table 1, Fig. 1D). The high c-MYC expression group had a significantly lower median PLT and high median BM blast than the low c-MYC expression group (38.0 vs 46.0, P = 0.035; 90.4% vs 84.4%, P = 0.006) (supplemental Table 1, Fig. 1D and 1E). No significant differences in c-MYC expression were observed with age, sex, or peripheral blood blasts. These data indicate that high c-MYC expression is associated with high-risk ALL.

Association of MYCBP2 expression with characteristics of adult ALL

We also assessed MYCBP2 mRNA expression in 85 and 46 newly diagnosed adult B-ALL and T-ALL patients, respectively. We found that MYCBP2 expression is significantly lower in both B-ALL and T-ALL patients when compared to normal control (Fig. 1B). Patients were divided into high (n = 65) and low (n = 66) MYCBP2 expression groups. The high expression group showed lower median WBC counts (30.2 × 10^9/L vs 46.5 × 10^9/L, P = 0.025), a lower median percentage of CD33(+) cells (32.3% vs 57.1%, P = 0.004) and bone marrow blasts (87.2% vs 88.8%, P = 0.042) than the low expression group (Supplemental Table 2). The percentage of patients exhibiting splenomegaly and liver infiltration was significantly higher in the low MYCBP2 expression group than in the high expression group (54.3% vs 35.9%, P = 0.033; 35.7% vs 15.4%, P = 0.007) (Supplemental Table 2). No significant differences in MYCBP2 expression were observed with age, sex, or CR rate. These data indicated that low MYCBP2 expression is to some extent associated with high-risk ALL.

Association of c-MYC high MYCBP2 low expression with characteristics of adult ALL

We also observed that c-MYC mRNA expression is negatively correlated with MYCBP2 expression in the ALL patients (Supplemental Fig. 1A). We analyzed the correlation of c-MYC with MYCBP2 in other reported cohorts [16, 17] and found that c-MYC expression is negatively correlated with MYCBP2 expression (Supplemental Fig. 1B). We also observed the c-MYC is interacts with MYCBP2 in ALL cells (Supplemental Fig. 2). We further compared the clinical features of patients with both high c-MYC expression and low MYCBP2 expression (c-MYC high MYCBP2 low) with that of patients with both low c-MYC expression and high MYCBP2 expression.
expression (c-MYC<sup>high</sup>MYCBP2<sup>low</sup>). Our data showed that c-MYC<sup>high</sup>MYCBP2<sup>low</sup> patients showed higher median WBC counts (101.5 × 10<sup>9</sup>/L vs 29.4 × 10<sup>9</sup>/L, P = 0.007), a higher percentage of CD34(+) and CD33(+) cells (90.0% vs 61.3%, P = 0.025; 80.0% vs 25.8%, P = 0.000), and a lower CR rate (60.0% vs 92.0%, P = 0.027) than c-MYC<sup>low</sup>MYCBP2<sup>high</sup> patients (supplemental Table 3, Fig. 2A and 2B). The percentage of patients exhibiting splenomegaly, liver infiltration, increased LDH (>1000 u/L) and BM blasts was significantly higher in the c-MYC<sup>high</sup>MYCBP2<sup>low</sup> group than in the c-MYC<sup>low</sup>MYCBP2<sup>high</sup> group (75.0% vs 33.3%, P = 0.004; 75.0% vs 19.4%, P = 0.000; 70.0% vs 29.0%, P = 0.004, 93.6% vs 80.2%, P = 0.001) (supplemental Table 3, Fig. 2A and 2B). The percentage of patients exhibiting splenomegaly, liver infiltration, increased LDH (>1000 u/L) and BM blasts was significantly higher in the c-MYC<sup>high</sup>MYCBP2<sup>low</sup> group than in the c-MYC<sup>low</sup>MYCBP2<sup>high</sup> group (75.0% vs 33.3%, P = 0.004; 75.0% vs 19.4%, P = 0.000; 70.0% vs 29.0%, P = 0.004, 93.6% vs 80.2%, P = 0.001) (supplemental Table 3, Fig. 2A and 2B). The c-MYC<sup>high</sup>MYCBP2<sup>low</sup> group had a significantly lower median PLT than the c-MYC<sup>low</sup>MYCBP2<sup>high</sup> group (16.5% vs 39.0%, P = 0.013) (Supplemental Table 3, Fig. 2C). No significant differences between the two groups were observed with age, sex, or peripheral blood blasts. These data indicate that c-MYC high expression with MYCBP2 low expression is correlated with high-risk ALL.

**Ikaros binds to the promoter of c-MYC and MYCBP2 and regulate their expression**

In order to understand how c-MYC and MYCBP2 expression are regulated in ALL, we analyzed the transcription factor motifs in the promoter region of c-MYC and MYCBP2. We identified strong Ikaros binding motifs in the promoter region of each gene. c-MYC is reported to be a direct target of Ikaros. Our Ikaros ChIP-seq data also showed strong Ikaros binding peaks in the promoter region of both c-MYC and MYCBP2 (Fig. 3A and 3B). We explored the binding of Ikaros on the promoter regions of c-MYC and MYCBP2 by qChIP and found that Ikaros significantly binds to their promoter regions in Nalm6 B-ALL and Molt4 T-ALL cells (data not show). We further observed that Ikaros suppresses the promoter activity of c-MYC and activates that of MYCBP2 by luciferase reporter assay (Fig. 4A); and Ikaros knockdown block Ikaros-induced effect on the promoter activity of c-MYC and MYCBP2 (Supplemental Fig. 3). These data indicated that direct
Effect of Ikaros on transcription of \( c-MYC \) and \( MYCBP2 \).

Moreover, expression of \( Ikaros \) suppressed \( c-MYC \) mRNA level and increased \( MYCBP2 \) mRNA level in both Nalm6 and CEM cells (Fig. 4B and 4C), and this effect was observed in protein level (Supplemental Fig. 4A). Conversely, \( Ikaros \) knockdown induced an increase in \( c-MYC \) expression and a decrease in \( MYCBP2 \) expression in Nalm6 (Fig. 5A) and CEM cells (Fig. 5B). The efficient knockdown for Ikaros was evidenced in mRNA level (Right panel for Fig. 5A and 5B) and protein level (Supplemental Fig. 4B). Treatment of Nalm6 and CEM cells with Ikaros activator, TBB (CK2 inhibitor) could suppress \( c-MYC \) expression and increase \( MYCBP2 \) expression in a dose-dependent manner (Fig. 5C and 5D). Ikaros knockdown with shRNA could block the TBB-induced decrease in \( c-MYC \) expression and increase in \( MYCBP2 \) expression (Fig. 5E and 5F). These data indicate that both \( c-MYC \) and \( MYCBP2 \) are direct \( Ikaros \) targets in ALL and \( Ikaros \) regulates their expression.

\( c-MYC \) and \( MYCBP2 \) expression in patients with an \( Ikaros \) deletion

We explored \( Ikaros \) mRNA level by qPCR in our cohort study, and analyzed the correlation of Ikaros expression with clinical feature in the patients (Supplemental Table 4). We found that patients with low \( Ikaros \) expression showed higher median WBC counts (64.9 × 10^9/L vs 29.5 × 10^9/L, \( P = 0.016 \)), higher percentage of CD13(+) cells (57.1% vs 33.3%, \( P = 0.009 \)) and CD33 (+) cells (60.0% vs 40.5%, \( P = 0.033 \)), higher percentage of liver infiltration and splenomegaly.
and lower CR rate (79.2% vs 94.3%, \( P = 0.013 \)) than those with high Ikaros expression. These data indicated that low Ikaros expression, as that of high c-MYC and low MYCBP2, is correlated with high-risk ALL.

In order to further explore the relationship of Ikaros with c-MYC and MYCBP2 expression, we observed Ikaros binding to their promoters in primary B-ALL and T-ALL cells (Fig. 6A and 6B). We analyzed the correlation of Ikaros expression with that of c-MYC or MYCBP2 in the reported cohort of B-ALL or T-ALL patients [17–19], and found that Ikaros expression is negatively correlated with high c-MYC expression and low MYCBP2 expression in both B-ALL (Supplemental Fig. 5A and 5B) and T-ALL (Supplemental Fig. 6A and 6B). We also analyzed the correlation of Ikaros mRNA level with that of c-MYC and MYCBP2 in our cohort study, and found that Ikaros is negatively correlated with c-MYC expression and positively correlated with MYCBP2 expression (Supplemental Fig. 7A and 7B). Importantly, c-MYC expression was significantly increased and MYCBP2 expression significantly decreased in patients with Ikaros deletion compared to that of Ikaros wild type (Fig. 6C and 6D). These data further demonstrated a regulatory effect of Ikaros on both c-MYC and MYCBP2 in ALL patients and suggested that Ikaros deletion is one of the reasons for high c-MYC and low MYCBP2 expression in the patients. Additionally, TBB can suppress c-MYC expression and increase MYCBP2 expression in primary B-ALL (Fig. 6E) and T-ALL (Fig. 6F). This data not only indicated the effect of Ikaros activator on their expression, but also suggested that Ikaros-induced changes of c-MYC/MYCBP2 expression is at least partially responsible for the success of CK2 inhibitors in ALL therapy.

**DISCUSSION**

Our findings indicate the significance of high c-MYC, low MYCBP2 and low Ikaros expression in ALL patients, also demonstrate c-MYC and MYCBP2 is the direct target of Ikaros and reveal a model for the oncogenic effect of an Ikaros/MYCBP2/c-MYC axis in adult ALL.
Ikaros dysfunction results in the upregulation of c-MYC and downregulation of MYCBP2. This is the first report that shows Ikaros dysfunction results in the increase of c-MYC and decrease of MYCBP2, as well as a negative correlation between c-MYC and MYCBP2 in ALL patients.

MYC, as one of the key transcription factors in hematopoiesis, is frequently overexpressed in human acute lymphoblastic and myeloid leukemia [20–27]. We observed a significant increase in c-MYC mRNA in adult B-ALL and T-ALL compared to that in normal bone marrow. We also observed that high c-MYC expression is correlated with proliferation markers such as splenomegaly, liver infiltration, high LDH and BM blasts, and high-risk ALL factors: high WBC, CD33+ cells, and low CR rates. These findings indicate the oncogenic effect of high c-MYC expression.

The expression of MYCBP2 in ALL is not well determined. Here, we observed a significant decreased MYCBP2 expression in B-ALL and T-ALL patients. Low MYCBP2 expression is correlated with both proliferation markers and high-risk factors for ALL. We also observed a negative correlation between c-MYC and MYCBP2 expression. More importantly, c-MYC<sup>high</sup>MYCBP2<sup>low</sup> patients showed higher median WBC counts, higher percentage of CD34(+) and CD33(+) cells, higher rate of splenomegaly, liver infiltration, increased LDH, and lower CR rate compared with that of c-MYC<sup>low</sup>MYCBP2<sup>high</sup> patients. These data indicate that high c-MYC and low MYCBP2 expression have a synergistic oncogenic effect in ALL patients.

In human ALL, upregulation of c-MYC has been reported through chromosome translocations [28], aberrant
We also reported that high LEF1 expression increases c-MYC expression in ALL [31]. Ikaros is reported to inhibit pre-B cell proliferation by suppression of c-MYC expression [32]. Here, we found c-MYC expression is significantly increased in patients with an Ikaros deletion; and c-MYC expression is negatively correlated with Ikaros expression in ALL patients in the cohort studies. These data indicated that Ikaros plays an important role in the suppression of c-MYC expression in ALL, and that Ikaros deletion is one of the major reasons for high c-MYC expression in ALL patients. Moreover, Ikaros also induces MYCBP2 expression. Ikaros expression is positively correlated with MYCBP2 expression in ours and the other reported cohort studies. We also observed that MYCBP2 expression is significantly increased in patients with an Ikaros deletion. These data indicated that Ikaros dysfunction is an important mechanism underlying low MYCBP2 expression in the patients.

Our results also showed that Ikaros binds to c-MYC and MYCBP2 promoter, directly suppresses or increase their promoter activity, and further results in the changes

Figure 5: Ikaros knockdown induces upregulation of c-MYC and downregulation of MYCBP2. A–B. qRT-PCR of c-MYC, MYCBP2, and Ikaros expression in Nalm6 cells (A) and CEM cells (B) following Ikaros shRNA treatment as compared to scramble shRNA cells. Gene expression is determined by RT-qPCR using total RNA isolated from the cells transfected with scramble shRNA (siControl) or Ikaros shRNA (siIkaros) for 2 days; C. Effect of Ikaros activator (CK2 inhibitor, TBB) on expression of c-MYC and MYCBP2 in primary B-ALL (C) and primary T-ALL (D) cells with TBB treatment for 2 days; E–F. Ikaros knockdown rescues the TBB-induced change of c-MYC and MYCBP2 in Nalm6 (E) and CEM (F) cells.
of their expression in ALL cells. As a transcription factor, Ikaros regulates gene expression by recruiting the NuRD/ Mi-2 or SWI/SNF chromatin remodeling complexes [10, 15, 37]. Whether Ikaros regulating c-MYC and MYCBP2 expression through chromatin remodeling needs to be further clarified. CK2 inhibitor, TBB as an indirect Ikaros activator, inhibits c-MYC expression and promotes MYCBP2 expression in an Ikaros-dependent manner. CK2 inhibitors are reported to be potential therapies for leukemia [15, 13, 33]. Therefore, our data suggests that CK2 inhibitors exert their anti-leukemia effect through Ikaros-mediated suppression of c-MYC expression and activation of MYCBP2 expression.

Interestingly, we observed a negative correlation between c-MYC and MYCBP2 expression. We also found that c-MYC expression is negatively correlated with MYCBP2 expression in microarray data of ALL patients from other cohort studies. MYCBP2 is a large protein that binds specifically to MYC [7]. The mechanism underlying the negative correlation needs to be clarified further. However, MYCBP2 as an ubiquitin ligase may directly downregulate c-MYC protein. We did observe c-MYC is associated with MYCBP2 in ALL cells. Ikaros-induced

Figure 6: Ikaros binds to the c-MYC and MYCBP2 promoters and Ikaros deletion results in changes of their expression in primary ALL cells. A–B. Ikaros binding to the promoters of c-MYC and MYCBP2 in primary B-ALL (A) and T-ALL (B) cells; C–D, comparison of c-MYC (C) and MYCBP2 (D) in patients with or without Ikaros deletion; E–F, Effect of Ikaros activator (CK2 inhibitor, TBB) on expression of c-MYC and MYCBP2 in primary B-ALL(E) and T-ALL (F) cells with TBB treatment for 2 days.
low MYCBP2 expression might further increase the c-MYC protein level in the patients.

In conclusion, we observed that c-MYC expression is significantly increased and MYCBP2 expression significantly decreased in adult ALL patients. High c-MYC and/or low MYCBP2 or low Ikaros expression is correlated with high-risk leukemia. Ikaros dysfunction is one of the underlying reasons for high c-MYC and low MYCBP2 expression in ALL patients. Our data reveals the oncogenic effect of an Ikaros/MYCBP2/c-MYC axis in adult ALL, and suggests a mechanism by which CK2 inhibitors exert their anti-leukemia effect.

MATERIALS AND METHODS

Patients and samples

BM samples [90 male, 61 female; median age 32 (14–77) years old] with ALL (104 B-ALL, 47 T-ALL) were collected from 151 patients between June 2008 and June 2014 at the First Affiliated Hospital of Nanjing Medical University. The ALL diagnosis was made according to the cytogenetic, morphologic, immunophenotypic, and molecular criteria of WHO Diagnosis and Classification of ALL (2008). Written informed consent was provided before enrollment in the study by all patients in accordance with the Declaration of Helsinki. The cohort study was also approved by the Institutional Review Board of the Nanjing Medical University.

Cytogenetic and molecular analyses

Conventional cytogenetic analysis was performed at the time of diagnosis by using unstimulated short-term cultures according to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN). At least 20 bone marrow metaphase cells were analyzed for each sample. Flow cytometry was performed on fresh pretreatment BM samples for immunophenotypic analyses. A cell-surface antigen was defined as positive when fluorescence intensity of at least 20% of cells exceeded fluorescence of negative control as previously described [31].

Cell culture reagents, plasmid construction, and retroviral gene transfer

Nalm6 and MOLT4 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Hyclone). Cells were cultured with or without TBB and collected for total RNA isolation. Human HA-tagged Ikaros (IKZF1) retroviral construct and retroviral production was described previously [11, 12, 34].

Luciferase assay

The pGL3 luciferase reporter construct for the c-MYC promoter was previously reported [31] and the pGL3-MYCBP2 promoter construct was purchased from Addgene. Transient luciferase assays were performed in HEK293T cells using Promega luciferase assay reagents and measured with a luminometer following the manufacturer’s instructions. Luciferase activity was calculated as fold change relative to values obtained from pGL vector only control cells and expressed as a percentage of pcDNA 3.1-Ikaros transfection-induced luciferase activity versus that of pcDNA3.1 vector alone. All transfection and reporter assays were performed independently, in triplicate, at least three times.

For Ikaros knockdown, the same amount of Ikaros shRNA were transfected with the Ikaros and promoter constructs, and the resulting promoter activity was compared to that with scramble shRNA control.

Real time-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN). A 1 μg aliquot of RNA was reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR Kit (Invitrogen). qRT-PCR was performed with qSTAR SYBR Master Mix (OriGene) using a StepOne Plus real-time PCR system (Applied Biosystems). Each experiment was performed in triplicate.

c-MYC and MYCBP2 expression in patient samples was quantitated similarly by the formula achieved with serial dilutions of their plasmids as template standards as previously reported [31]. Gene expression values of c-MYC or MYCBP2 were achieved in each patient by a formula obtained with a scatter graph of the Ct values from the serial dilutions of template plasmid as previously reported [31, 35]. The expression level of c-MYC or MYCBP2 was subsequently normalized to 18s RNA and expressed as gene expression value of c-MYC or MYCBP2/18s RNA.

All the patients were divided into high and low c-MYC/MYCBP2 expression groups (Q3-4 vs Q1-2, respectively), which was determined by SPSS 17.0.

The qPCR for c-MYC and MYCBP2 expression was performed in Nalm6 and Molt4 cells transfected with Ikaros. The results were normalized to those obtained with 18sRNA and presented as fold induction over vector controls. Primers: 18s RNA, Sense: 5’-GTAACCCGGTTGACCCATT-3’, Antisense: 5’-CCATCCAAATCGTGTTAGCGG-3’; c-MYC Sense: 5’-AATGAAAGGCCCAAGGTATCC-3’,
Quantitative chromatin immunoprecipitation (qChIP)

qChIP assays were performed by incubation of the chromatin with antibodies against Ikaros and normal rabbit IgG (Abcam) as control [16]. Enrichment of the ChIP sample over input was evaluated by qPCR with three or more replicates, using specific primers in the promoter region of c-MYC (forward: 5′-AGGGGAAGGGAGGGGAGGAA-3′, reverse: 5′-CCCTTCTCCCTCCCTCCTCT-3′) and MYCBP2 (forward: 5′-GGTTTTCTCTGCTTAAACTCTGAA-3′, reverse: 5′-GCTGCCAACAGGAAGATTTACTG-3′). The relative concentration of the qPCR product was presented as the fold change of the level of DNA in Ikaros samples in comparison to controls.

Ikaros shRNA knockdown

Nalm6 cells were transiently transfected with human Ikaros shRNA constructs in the GFP vector (pGFP-v-RS) (Origene) using the Neon Transfection System (Invitrogen). The 29-mer scrambled shRNA cassette in the pGFP-VRS vector was also used as a control. After transfection for 1 day, Nalm6 and CEM cells with transfection efficiency around 80% (green cells) and greater than 95% cell viability were further treated with 20 μM TBB or vehicle control (0.01% DMSO) for 2 days to total RNA isolation. Knockdown of Ikaros was confirmed by measurement of Ikaros mRNA level using qPCR. Primers: IKZF1-F: 5′-gggcgcggtgctcctcct-3′, IKZF1-R: 5′-tccgacacgccctacgaca-3′

Co-immunoprecipitation and western blot

Nuclear extracts from Nalm6 and CEM cells were prepared as previously reported [36]. Nuclear extracts were precleared with protein A-Sepharose beads. The precleared extracts were incubated with 5 μg of antibody to c-Myc or MYCBP2 or normal rabbit IgG control for 2 hours, and then added the pre-washed protein A-Sepharose beads at 4°C overnight. Immunoprecipitated complexes were extensively washed with lysis buffer and then boiled in SDS sample buffer for 10 minutes. The immunoprecipitation products were run on SDS-PAGE and transferred to the membrane.

For western blot, membranes were blocked with 5% nonfat dry milk at room temperature for 1 hour and then incubated overnight at 4°C with primary antibody (anti-c-MYC; 1:500, Sigma; anti-MYCBP2; 1:1000, Abcam). After being washed, the membrane was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000) at room temperature for 2 hours. The blots were developed by the enhanced chemilluminescence technique (ECL Plus, Amersham, Arlington Heights, IL) according to the manufacturer’s instructions.

Statistical analysis

Patients were divided into high and low c-MYC or MYCBP2 expression groups (Q3-4 vs Q1-2, respectively). For quantitative parameters, overall differences between the cohorts were evaluated using a Mann – Whitney U-test. For qualitative parameters, overall group differences were analyzed using a χ² test. All statistical analyses were performed using the SPSS 17.0 and P < 0.05 was considered statistically significant.

The experimental data are shown as the mean value with bars representing the standard error of the mean (S.E.M.). Determinations of statistical significance were performed using a Student t-test for comparisons of two groups or using analysis of variance (ANOVA) for comparing multiple groups. The P < 0.05 was considered statistically significant.

CONFLICTS OF INTEREST

All the authors have no conflicts of interest to declare.

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