The multifaceted role of protein kinase CK2 in high-risk acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is an aggressive malignancy of developing lymphocytes. Despite our understanding overall cure rates, patients with the refractory or relapsed disease have a poor prognosis.1 In order to improve treatments for these high-risk (HR)-ALL patients, it is critical to gain an in-depth understanding of the disease pathogenesis. The enhanced expression of the protein kinase CK2α gene and proto-oncogene MYC are common in T cell ALL (T-ALL) and B cell ALL (B-ALL).2 CK2 is a constitutively active serine/threonine kinase composed of two catalytic (α or α′) and two regulatory (β) subunits that are overexpressed in a broad spectrum of human cancers. Despite the demonstrated anti-leukemic efficacy of CK2 inhibitors,3 how CK2 contributes to HR-ALL development remains incompletely understood. Here we utilized transgenic zebrafish models to elaborate the multifaceted role of CK2 in HR-ALL pathogenesis, providing therapeutic implications for this stubborn disease.

Overexpression of the CK2α subunit under the immunoglobulin gene promoter induces low penetrance of T-cell lymphomas in a murine model.4 In order to further understand the oncogenic potential of CK2 in T and B lineages, we generated transgenic zebrafish that overexpress the wild-type or the kinase-dead version (K68M) of the human CK2α gene in T and B cells through the zebrafish tyrosine kinase gene (lck) promoter.5 Western blotting analysis revealed elevated expression of CK2α in transgenic CK2 fish, compared to age-matched wild-type (wt) fish (Online Supplementary Figure S1A). Despite relatively normal thymus development and no observable difference in fish survival (Online Supplementary Figure S1B), lymphocytes in Tg(lck:CK2α wtrag2:mCherry) fish survived much longer than the control Tg(lck:EGFP) or Tg(rag2:EGFP) transgenic fish (Online Supplementary Figure S1C). By 8 months, CK2 transgenic fish still had clearly defined red-fluorescent thymi, while control transgenic fish began to lose their thymic fluorescence as early as 5 months of age (Online Supplementary Figure S1C and data not shown). Strikingly, CK2 transgenic fish can retain their thymic fluorescence till 18 months. In order to determine the effect of CK2α in inducing lymphoid malignancies in zebrafish, starting at 21 days post-fertilization (dpf), we monitored both wt and mutant CK2α expression at different stages of ALL developments. To this end, we bred our lck-promoter-driven wt or kinase-dead CK2 transgenic fish to conditional Tg(rag2:MYC-ER/lck:EGFP) fish, in which aberrant MYC activity is regulated by tamoxifen and induces leukemia in both T and B lineages.6,7 We raised their offspring in fish water containing 4-hydroxy-tamoxifen (4HT) beginning at 5 dpf when thymic fluorescence was first visible (Figure 1A), and monitored the fish for tumor onset using previously defined criteria.8,9 At 4 weeks of age, all groups showed normal-sized thymi. However, by 6 weeks, all three fish lines exhibited evidence of tumor initiation compared to Tg(lck:EGFP) and Tg(rag2:K68M) controls (Figure 1C; Online Supplementary Figure S1B). By 12 weeks of life, tumors developed in more than 90% of Tg(rag2:MYC-ER/lck:EGFP,lck:CK2α wtrag2:mCherry) fish, referred to as MYC-ER/CK2α wt (Figure 1B and C). However, tumors developed in less than 60% of Tg(rag2:MYC-ER/lck:EGFP) fish, referred to as MYC-ER (Figure 1B and C).

Interestingly, overexpressing the enzyme-dead version of CK2α in Tg(rag2:MYC-ER/lck:EGFP,lck:CK2αK68M;rag2:mCherry) fish, referred to as MYC-ER,CK2αK68M, failed to accelerate the disease, with approximately 50% of fish developing tumors at 12 weeks of life (Figure 1B and C). These results demonstrate that the HR-ALL development depends on the enzymatic activity of CK2 since wt, but not kinase-dead CK2α, significantly accelerated the onset of MYC-induced ALL.

Next, we questioned whether CK2α could hasten the progression of MYC-induced ALL by quantifying the tumor burden in the above three groups of fish. We found that ALL developed in MYC-ER,CK2α wt fish much more aggressively, as demonstrated by a significantly heavier tumor burden in these fish compared to those in MYC-ER sibling fish (Online Supplementary Figure S2A and B). However, overexpression of CK2αK68M failed to enhance disease aggression as the tumor burden in MYC-ER,CK2αK68M fish was similar to those in MYC-ER fish (data not shown). Since MYC-ER fish develop both T- and B-ALL,10 we then asked which types of leukemia MYC-ER,CK2α wt fish developed by performing semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using zebrafish T- and B-cell specific primers.11 Our results show that MYC-ER,CK2α wt fish also developed ALL of T and B lineages (Online Supplementary Figure S3).

In order to determine whether MYC-induced transformation is restricted to the particular stages of lymphocyte development, we treated MYC-ER fish with 4HT at 30 dpf instead of 5 dpf, and monitored fish for tumor development with weekly fluorescent imaging (Figure 2A). Surprisingly, none of these MYC-ER fish developed tumors after 8 weeks of 4HT treatment (Figure 2B and C). However, if these fish were treated with 4HT at 5 dpf, more than 90% of MYC-ER fish had already developed tumors at this time (Figure 1C). Next, we determined if the enhanced CK2α expression could overcome this temporal restriction of lymphocyte transformation. In order to do so, we bred CK2α wt transgenic fish to MYC-ER fish and treated the fish with 4HT at 30 dpf. Strikingly, tumors started to arise in the MYC-ER,CK2α wt fish within 1 week of 4HT treatment (Figure 2C). Within less than 2 weeks of 4HT treatment, approximately 80% of MYC-ER,CK2α wt fish developed aggressive ALL (Figure 2B and C). These results demonstrate that CK2α can overcome the temporal restriction of MYC-mediated lymphocyte transformation and induce ALL at a later developmental stage.

Since the aggressive nature of leukemia in MYC-ER,CK2α wt fish depends on the kinase activity of CK2 (Figure 1), we next performed phos-tag western blotting to determine whether enforced CK2 expression increases MYC phosphorylation in vivo. Compared to tumors in MYC-ER fish, we detected increased CK2α and relatively more phosphorylated MYC (upper bands) protein levels.
in tumors from MYC-ER;CK2αwt fish (Online Supplementary Figure S4A). In order to determine whether increased phosphorylation of MYC led to the stabilization of MYC protein in vivo, we analyzed the half-life of MYC-ER protein in the presence or absence of CK2α overexpression in zebrafish developing lymphocytes. We isolated premalignant thymocytes from 5-week-old MYC-ER and MYC-ER;CK2αwt fish, dissociated the thymocytes, and treated them with cycloheximide (CHX) to inhibit protein synthesis. Western blotting analysis was then performed to measure MYC-ER protein levels at different time points. We found that MYC-ER was stabilized in lymphocytes with CK2α overexpression, compared to those without CK2α overexpression (Online Supplementary Figure S4B). In order to understand whether CK2 can promote MYC-mediated leukemogenesis through other mechanisms, we performed quantitative RT-PCR analysis of zebrafish homologs of human anti-apoptotic genes, BCL2, BCL-XL, and MCL1. No significant difference was found in leukemic cells from MYC-ER versus MYC-ER;CK2αwt fish (Online Supplementary Figure S5). Together, these data indicate that CK2’s ability in phosphorylating and stabilizing MYC in vivo serves as one mechanism to promote leukemia initiation and aggressiveness.

In order to determine whether overexpression of CK2α alleviates the necessity of MYC in established tumors, we treated fish with 4HT starting at 5 dpf for 11 weeks to induce tumor development. We then removed 4HT from MYC-ER and MYC-ER;CK2αwt tumor fish to inactivate MYC and monitored disease regression for 8 weeks by fluorescent imaging (Figure 3A). We categorized tumor phenotypes based on the extent of change in tumor size as previously described: complete regression, partial

Figure 1. Overexpression of wild-type but not enzyme dead CK2α promotes the onset of MYC-induced acute lymphoblastic leukemia in zebrafish. (A) Diagram of the experimental design. (B) Thymic fluorescence in the Tg(rag2:MYC-ER;ick:EGFP) (left), Tg(rag2:MYC-ER;ick:EGFP;ick:CK2αwt;rag2:mCherry) (middle), and Tg(rag2:MYC-ER;ick:EGFP;ick:CK2αK68M;rag2:mCherry) (right) fish raised in 129 nM 4-hydroxytamoxifen (4HT) at 12 weeks of life. One representative fish is shown for each group. (C) Kaplan-Meier analysis of tumor-free fish revealed that overexpression of CK2αwt but not CK2αK68M significantly accelerated the onset of MYC-induced acute lymphoblastic leukemia (ALL) (P=0.0013 for Tg(rag2:MYC-ER;ick:EGFP) [MYC-ER; green line] vs. Tg(rag2:MYC-ER;ick:EGFP;ick:CK2αwt;rag2:mCherry) [MYC-ER; red line] n=19 and n=22, respectively; and P=0.0008 for MYC-ER;CK2αwt [red line] vs. Tg(rag2:MYC-ER;ick:EGFP;ick:CK2αK68M;rag2:mCherry) [MYC-ER;CK2αK68M; black line] n=22 and n=13, respectively). There was no statistical significance between MYC-ER and MYC-ER;CK2αK68M fish. Statistical analysis was performed using the log-rank test. The scale bar in the left and middle panel of Figure 1B =1 mm and in the right panel =200 μm.
regression, stable disease, and progression. By 4 weeks post-withdrawal of 4HT, approximately 35% of MYC-ER fish and approximately 50% of MYC-ER;CK2αwt fish had already exhibited complete tumor regression (Figure 3B). We found that there were no statistically significant differences between MYC-ER versus MYC-ER;CK2αwt fish for the changes of tumor status at both 4 and 8 weeks post 4HT removal (Figure 3C and data not shown). These results demonstrate that CK2 overexpression alone cannot substitute for aberrant MYC activity in maintaining the established disease.

In this study, we elaborated on the contribution of CK2 to different stages of HR-ALL development using the tamoxifen-regulated zebrafish model of MYC-induced ALL. Our data show that the kinase activity of CK2 promotes both the onset and progression of T- and B-ALL in the presence of aberrant MYC activation, but cannot maintain the disease upon MYC inactivation through the removal of 4HT. When MYC-ER fish are treated with 4HT to activate MYC at a later stage of development, these fish can no longer develop leukemia, indicating a temporal restriction of MYC-induced lymphocyte trans-

Figure 2. CK2α overexpression overcomes temporal restriction of MYC-induced lymphocyte transformation and induces leukemia at the later stage of development. (A) Diagram of the experimental design. (B) Thymic fluorescence in MYC-ER and MYC-ER;CK2αwt zebrafish that were raised in 129 nM 4-hydroxytamoxifen (4HT) beginning at 30 dpf. (C) Kaplan-Meier analysis of tumor-free fish based on genotype (P<0.0001; n=18 for MYC-ER and n=34 for MYC-ER;CK2αwt fish). Statistical analysis was performed using the log-rank test and scale bars = 1 mm.
formation. Strikingly, however, this temporal restriction can be overcome by enforced CK2 expression, leading to high penetrance of leukemia development. Although CK2α overexpression alone cannot induce leukemia, it promotes the survival of lymphocytes. Hence, it is likely that MYC activation at a later stage of development induces apoptosis in lymphocytes that is overcome by CK2 overexpression, enabling the rapid induction of leukemia in these fish.

As CK2 inhibition with the selective and potent inhibitor, CX-4945, exhibits anti-tumor activities,7 CX-4945 has been included in clinical testing to treat hematological malignancies (clinicaltrials.gov. Identifier: NCT01199718) and solid cancers (clinicaltrials.gov.

Figure 3. CK2α overexpression alone cannot maintain acute lymphoblastic leukemia in the absence of aberrant MYC activation. (A) Diagram of the experimental design. (B, top panel) Thymic fluorescence in MYC-ER (left) and MYC-ER;CK2αwt (right) zebrafish raised in 129 nM 4-hydroxytamoxifen (4HT) for 5 weeks showing tumor initiation in MYC-ER;CK2αwt fish. (B, middle panel) shows both MYC-ER (left) and MYC-ER;CK2αwt (right) with aggressive disease at 11 weeks although MYC-ER;CK2αwt fish developed more aggressive ALL than MYC-ER fish. (B, bottom panel) shows thymic fluorescence 4 weeks after 4HT withdrawal. One representative fish is shown for each group. (C) Zebrafish were classified by the indicated tumor phenotype at 8 weeks post 4HT removal (P=0.13; MYC-ER vs. MYC-ER;CK2αwt; n=11 per group). The difference in observed tumor phenotypes between each group as a whole was statistically insignificant, as calculated by a two-way ANOVA test. Scale bars =1 mm. ALL: acute lymphoblastic leukemia.
MYC and CK2. As such, it is highly effective and beneficial to patients with HR-ALL, and possibly other cancers with high expression of MYC and CK2.

Yun Zhou,1,2 Haiwei Lian,1,3, Ling Shen,1,3 Sovannarith Korn,1 Andrew Kwok Ping Lam,2 Olivia Layton,2 Leah N Huiting,2 Dun Li,2 Kelly Miao,2 Aozhuo Zeng,2 Esther Landesman-Bollag,4 David C. Seldin,4 Hui Fu,2 Li Hong1 and Hui Feng2,4

1Department of Gynecology, Wuhan University Renmin Hospital, Wuhan, Hubei, P. R. China; 2Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, USA; 3Department of Anatomy and Embryology, Wuhan University School of Basic Medical Sciences, Wuhan, Hubei, P. R. China and 4Department of Medicine, Section of Hematology and Medical Oncology, Boston University School of Medicine, Boston, MA, USA

*YZ and HL contributed equally as co-first authors.

Correspondence: HUI FENG - huifeng@bu.edu

HONG LI - drhongli1011@yeah.net


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