



Acute lymphoblastic leukemia

Molecularly distinct models of zebrafish *Myc*-induced B cell leukemia

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Zebrafish are a valuable leukemia model due to highly conserved hematopoietic and oncogenic pathways, facile genetics, and ease of use in chemical genetic screens. However, until recently, robust zebrafish B-cell leukemia models had not been described [1, 2]. The first transgenic zebrafish leukemia model was created 15 years ago and targeted murine *c-Myc* (*mMyc*) to thymocytes of AB strain zebrafish, leading to the rapid development of T-cell acute lymphoblastic leukemia (T-ALL) [3]. Additional genetic models were subsequently developed that result in induction of T-ALL [4], but B-cell leukemia models lagged behind [5, 6].

In two recent reports published in *Leukemia*, our groups independently demonstrated the development of zebrafish B-ALL using transgenic expression of *mMyc* or human *c-MYC* (*hMYC*) controlled by the zebrafish recombination activating gene 2 (*rag2*) promoter [1, 2]. Both models shared genetic and phenotypic features, but there were also key differences including strain background and species

differences in the *MYC* transgene that was used to generate each model. Here, we compare and contrast these models, making the important finding that zebrafish develop at least four molecularly-distinct ALL types, including cortical thymocyte-arrested *cd4⁺cd8⁺* T-ALL, *ighm⁺* B-ALL, *ighz⁺* B-ALL, and biphenotypic T/B-ALL.

Thirteen ALLs were purified from *rag2:hMYC;lck:eGFP* double-transgenic fish [2]. As previously reported, these leukemias had heterogeneous GFP expression, with T-ALL being exclusively GFP^{hi}, B-ALL exclusively GFP^{lo}, and other fish harboring mixed-ALL with both GFP^{hi} and GFP^{lo} cells, representing simultaneous T- and B-ALL, respectively [2]. Notably, simultaneous B- and T-ALL in single *hMYC* fish were frequently observed by Borga et al. [2]. In contrast, *mMyc* ALL were propagated by single cell allo-transplantation and then assessed by single cell transcript expression, confirming the existence of a single biphenotypic ALL in the *mMyc* cohort [1]. *hMYC* ALL were subjected to RNA-seq transcriptomic profiling and compared to transplanted leukemias generated from single *mMyc* ALL clones described by Garcia et al. [1]. Principal Component Analysis clearly distinguished *mMyc*-induced T-ALL from B-ALL, with the single *mMyc*-induced biphenotypic B/T-ALL clustering between these samples (Fig. 1A). The eight *hMYC*-induced ALLs that were largely GFP^{hi} clustered with known T-ALLs (*hMYC* 2, 6, 8–11, 13, 14), while three primarily GFP^{lo} ALL clustered near the *mMyc* B-ALLs (*hMYC* 3–5). Two *hMYC*-induced ALLs (*hMYC*1, 12) with substantial populations of both GFP^{lo} and GFP^{hi} cells grouped near the *mMyc* biphenotypic B/T leukemia. Hierarchical clustering using the top 100 positively- and negatively-correlated genes from PC2 confirmed that these genes defined B and T lymphocytes, respectively (Fig. 1B), with B-ALLs expressing *cd79b*, *syk*, *pax5*, *blnk* and *ebf1*, while T-ALLs expressed *cd8b*, *lck*, *runx3* and *gata3*. As previously reported, the biphenotypic B/T-ALL and mixed *hMYC*-induced ALLs expressed both T- and B-cell lineage genes [1, 2]. PC2 up-regulated genes were enriched for B cell signaling pathways when independently assessed by GSEAsig (Supplemental Tables 1 and 2).

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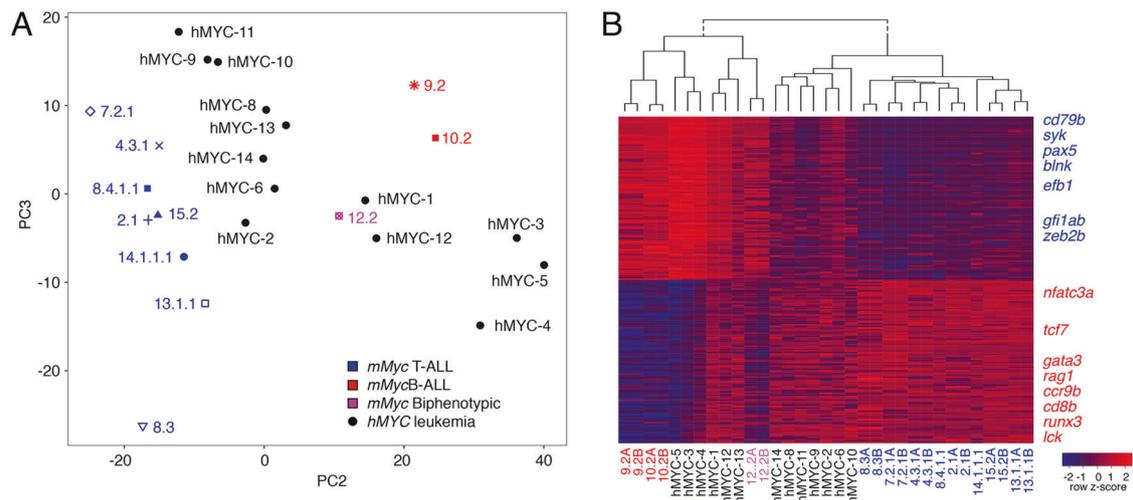


Fig. 1 Gene expression differences define T- vs. B-lineages in ALL of both the *rag2:mMyc* and *rag2:hMYC;lck:eGFP* models. **A**, Principal component analysis of RNAseq expression profiles of previously-classified *mMyc*-induced T- (blue, $n = 8$), B- (red, $n = 2$), or biphenotypic ALL (pink, $n = 1$) compared with 13 unknown *hMYC*-induced

ALL (black). **B**, Heatmap and hierarchical clustering using the top 100 positively- and negatively-correlated genes from PC2. B cell-specific genes are denoted in blue and T cell-specific genes in red at the right

To examine clonality and maturation in *mMyc*- and *hMYC*-induced ALL, we next analyzed the expression of constant and variable regions of the T cell receptor β (*tcrc*) and immunoglobulins μ and ζ (*ighm*, *ighz*; Fig. 2A). Every *mMyc* and *hMYC* T-ALL exhibited *tcrc* expression, with V(D)J recombination occurring in most samples as determined by expression of specific variable regions [1]. Conversely, *mMyc* and *hMYC* B-ALL did not recombine or express *tcrc*, but expressed constant regions of *ighm* or *ighz*. Ig variable regions were not detected, indicating V(D)J rearrangement likely had not occurred in these leukemias and suggesting B-ALLs arrest at the early pro-B cell stage. Ig constant regions without variable regions are termed 'sterile transcripts', and these non-coding mRNAs are transiently expressed during both early V(D)J recombination and during Ig class switching. Such sterile transcripts are detectable in mammalian pro-B cells before V(D)J rearrangement is complete, supporting our interpretation that *mMyc* and *hMYC* induce pro-B ALL [7–9]. As expected, *hMYC* mixed-ALL contained distinct T- and B-ALL clones expressing both *tcrc* and *ig* mRNAs, with their relative expression correlating well with the percentage of GFP^{hi}/T-ALL vs. GFP^{lo}/B-ALL cells found in each sample (Fig. 2A). Intriguingly, *mMyc* B-ALL expressed exclusively *ighm* while *hMYC* B-ALL favored *ighz* expression, indicating that *mMyc* and *hMYC* might be oncogenic in distinct B cell lineages.

To further explore differences between these models, we next identified genes uniquely-expressed by T-ALL, *mMyc/ighm*⁺ B-ALL, or *hMYC/ighz*⁺ B-ALL (Fig. 2B). As expected T-ALLs expressed known T cell lineage markers,

yet *mMyc/ighm*⁺ and *hMYC/ighz*⁺ B-ALLs were transcriptionally distinct. *mMyc/ighm*⁺ B-ALL expressed *gfi1ab*, *zfhx3*, *notch1a*, *nf1b*, and *gf3aa*. By contrast, *hMYC/ighz*⁺ B-ALL expressed higher *cd79a*, *cd83*, *mef2cb*, and *jak2a* levels. To further test for differences in these two molecular subtypes of B-ALL, we next performed GSEAsig using these same differentially-regulated genes. From this analysis, we uncovered that *mMyc/ighm*⁺ B-ALLs exhibited significant enrichment for pathways regulating ribosome biogenesis and RNA binding (Fig. 2C and Supplemental Tables 3 and 4). By contrast, *hMYC/ighz*⁺ B-ALLs were enriched for intracellular signaling, protein binding, and germinal center B cell maturation pathways. In support of our findings, Liu et al. recently reported the identification of molecularly and biologically distinct *ighz*⁺ and *ighm*⁺ B cell lineages using *rag2:mCherry*; *cd79b:GFP* transgenic zebrafish [10]. In the context of normal B cell development, *ighz*⁺ B cells were mCherry^{hi}/GFP^{lo} while *ighm*⁺ B cells were mCherry^{hi}/GFP^{hi}. Overall, these results demonstrate that *mMyc/ighm*⁺ and *hMYC/ighz*⁺ B-ALLs are not subtle B cell leukemia variants, but rather distinct malignancies that arise in different B cell types with vastly different molecular pathway signatures.

In summary, although zebrafish B cell leukemia models were lacking for many years, our analyses reveal two highly-divergent types of B-ALL. This is surprising, as both models utilize the same promoter (*rag2*) to regulate a near-identical oncoprotein, c-Myc/MYC, with the only differences being the MYC transgene species of origin and the genetic backgrounds upon which the models were developed. Yet, despite the high molecular similarity of both

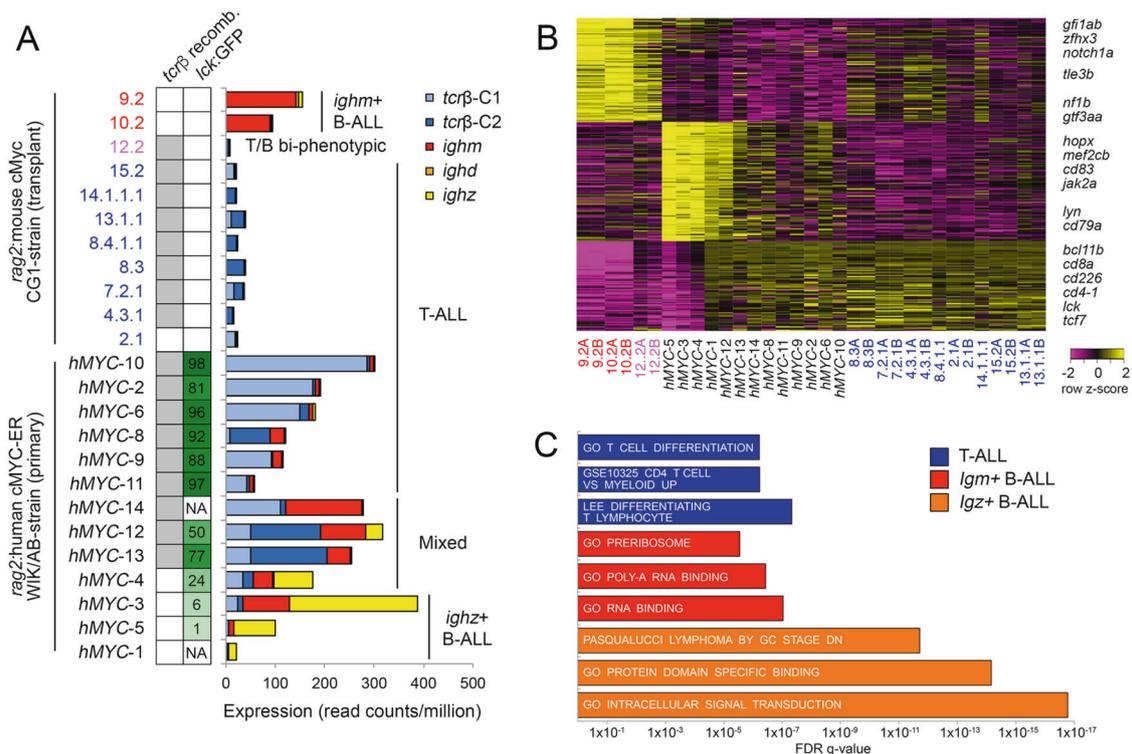


Fig. 2 Identification of two molecularly-distinct B-ALL types arising independently in either the *ighm*⁺ or *ighz*⁺ B cell lineages. **A**. T cell receptor beta and Ig heavy chain expression in individual ALLs. *tcrc* recombination is denoted by grey-shaded boxes (left column), with percentage of GFP^{hi} cells in each *rag2:hMYC;lck:eGFP* ALL noted in right column. Histograms depict expression of *tcrc* and *igh* constant regions by each ALL. Not available (NA). **B**. Heatmap showing

expression of genes differentially expressed in T-ALL, *mMyc/ighm*⁺ B-ALL, and *hMYC/ighz*⁺ B-ALL. **C**. Geneset enrichment analysis using genes positively correlated with each ALL molecular subtype. T-ALL (blue), *mMyc/ighm*⁺ B-ALL (red), and *hMYC/ighz*⁺ B-ALL (orange). Complete gene set and GSEAs results are provided in Supp. Tables 3 and 4

models, these B-ALL subtypes also show unique gene expression signatures when compared to one another, which likely reflects differences in both their lineage (*ighm* vs. *ighz*) and potential differences in MYC transcriptional targets expressed by the early developmental stages of these distinct pro-B cell populations. Our new analysis of these models reconciles the perceived differences in the manuscripts published by our groups, identifying four molecularly-distinct ALL subtypes in zebrafish: cortical *cd4*⁺/*cd8*⁺ T-ALL, biphenotypic B/T ALL, *ighm*⁺ B-ALL, and *ighz*⁺ B-ALL. Developing a wider array of leukemia models and refining mechanisms that drive their growth, aggression, and stem cell frequency will surely lead to new insights into human disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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