Modeling ETV6-JAK2-induced leukemia: insights from the zebrafish

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Cloning and functional characterization of a large number of chromosomal translocations revealed that aberrant activation of protein kinases is a key event for expansion of the malignant clone in chronic myeloproliferative disorders, as well as in acute leukemia. The best known example is t(9;22)(q34;q11), leading to the expression of the BCR-ABL1 (BCR-ABL) tyrosine kinase fusion associated with chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). In 1997, two studies reported that the *JAK2* gene on the short arm of chromosome 9p24 encoding for just another cytoplasmic protein tyrosine kinase is involved in rare chromosomal translocations resulting in fusions to the ETS-family member ETV6 (also known as TEL). Interestingly, particular breakpoints were found resulting in variant fusion proteins that were associated with different disease phenotypes. Peeters and colleagues cloned a t(9;12)(p24;p13) from a patient with early pre-B cell acute lymphoblastic leukemia (ALL) leading to a fusion of exon 4 of ETV6 to exon 17 of JAK2 (ETV6-JAK2, 4-17). In addition, they identified a t(9;15;12)(p24;q15;p13) in a patient with atypical CML in a transformation that fused exon 5 of ETV6 to exon 12 of JAK2 (ETV6-JAK2, 5-12).[1] In parallel, Lacronique and colleagues cloned a t(9;12)(p24;p13) from blasts from a childhood T-cell ALL patient leading to a fusion of ETV6 exon 5 to JAK2 exon 19 containing only the JH1 tyrosine kinase domain (ETV6-JAK2, 5-19).[2] Since their original description, very few additional cases of hematologic malignancies with ETV6-JAK2 have been reported.[3]

Later, additional translocations involving the *JAK2* gene were identified, such as t(9;22)(q34;q11.2) leading to a BCR-JAK2 fusion, t(8;9)(p22;p24) leading to a fusion to the pericentrilar material 1 (*PCM1*) gene, or t(4;9)(q21;p24) fusing JAK2 to the *SEC31A* gene.[4-6] Interestingly, in all of the PCM1-JAK2 positive cases reported so far, large parts of JAK2 (exon 9) were included and associated with a wide disease phenotype spectrum, including atypical CML, myelodysplasia/-proliferation with erythroid hyperplasia, or T-cell lymphoma.[7]

To address the biological activity of an ETV6-JAK2 fusion, several groups over-expressed the three fusion variants in IL-3 dependent murine Ba/F3 cells. Expression of all ETV6-JAK2 variants rendered Ba/F3 cells IL-3-independent with similar efficacy. Transformation of the cells by ETV6-JAK2 expression was associated with constitutive activation of the signal transducers and activators of transcription (STATs), predominantly of STAT5, but also STAT1 and STAT3, and expression of several putative STAT targets such as oncostatin M, CIS or PIM1.[8-10]

To model ETV6-JAK2 disease *in vivo*, we transplanted bone marrow cells with retroviral expression of the 5-19 fusion variant in lethally irradiated mice. All the mice developed an aggressive lethal mixed T-cell lympho- and myeloproliferative disease after a latency of 5-10 weeks.[8] Similarly, transgenic mice expressing the ETV6-JAK2 (5-19) variant from an SRα promoter/enhancer element developed a fatal $CD8^+$ T-cell acute leukemia.[11] Interestingly, breeding of the SRα-ETV6-JAK2 transgenics into a Cde-/- background with a block in T-cell development resulted in the development of B-cell lymphoma/leukemia.[12] As in Ba/F3 cells, transgenic expression of ETV6-JAK2 (5-19) was associated with activation of STAT5 and STAT1.[11]

During these early studies using retrovirally transduced bone marrow cells, we saw that it was difficult to obtain high titers of the ETV6-JAK2 (5-19). Retronectin-coated plates were used to improve the transduction efficacy. We speculated that the observed phenotype might have been influenced by the experimental system. However, our research did not answer the question of how the structural differences of the fusion variants would indeed be translated into the different disease phenotypes. The ETV6-JAK2 (5-12) fusion generates a product composed of the dimerization/ oligomerization pointed domain provided by ETV6 fused to the entire JH2 (also referred to as the 'pseudokinase domain') and the JH1 kinase domain of JAK2. In sharp contrast, the 5-19 fusion lacks JH2, and the 4-17 fusion contains parts of JH2 (Figure 1). These structural ETV6-JAK2 fusion variations might be translated into quantitative and/or qualitative differences in the transduced signals that might lead to a different disease phenotype. However, most probably due to the rarity of ETV6-JAK2 fusions, and also based on the similarity of their behavior in Ba/F3 cells, no special efforts were made to identify the cell of origin or the signaling differences between these variants.

Cloning of *JAK2* mutations as the molecular hallmark of classic myeloproliferative neoplasms (MPNs) stimulated new interest in the JAK2-STAT5 signaling axis.[13-14] Interestingly, the most prevalent *JAK2V617F* mutation found in more than 90% of polycythemia vera patients is located in the JAK2 JH2 domain. Many studies have shown that the *JAK2V617F* mutation results in increased kinase activity with transforming potential *in vitro* and *in vivo*. However, the molecular mechanism remained poorly understood. Ungureanu and colleagues recently proposed that the JH2 'pseudokinase' domain is indeed a dual-specificity protein kinase that phosphorylates two negative regulatory sites in JAK2. MPN-associated JAK2 mutations abrogated JH2 activity and increase the basal JAK2 activity and downstream signaling.[15] Resolution of the crystal structure revealed that the JH2 domain adopts the fold of a prototypical protein kinase and that the V617F mutation might stabilize an alpha helix in the JH2's N-lobe, thereby facilitating trans-phosphorylation of the JH1 kinase domain.[16]

These new studies clearly support an active role for JH2, initially called 'pseudokinase domain', for the regulation of JAK2 activity. Therefore, they also suggest significantly biological differences in the ETV6-JAK2 variants. These observations give rise to important questions on the pathogenesis of malignancies induced by these variants. During hematopoiesis, in which cell or cells do the translocations occur that lead to expression of different ETV6-JAK2

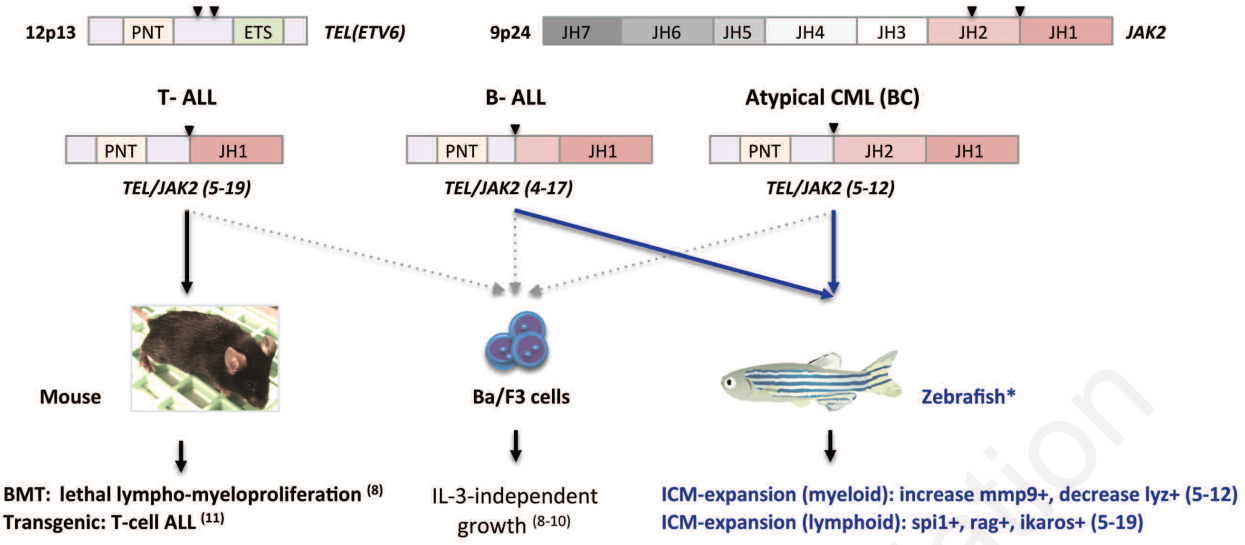

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Figure 1. Modeling TEL/JAK2 fusion gene associated leukemia.

fusion? Which cell or cells are the most sensitive to fusion expansion? How do ETV6-JAK2 fusion variants qualitatively and/or quantitatively affect different signaling pathways resulting in B-cell leukemia, T-cell leukemia or chronic myeloproliferation? In the past, it had been proposed that tyrosine kinase-driven leukemia is most probably based on genetic hits taking place in the hematopoietic stem cell compartment. Studies focusing on Philadelphia-chromosome positive hematologic malignancies suggested that both BCR-ABL1 fusion-induced chronic myeloid leukemia (CML) and B-cell acute lymphoid leukemia (B-ALL) might originate from cells resembling long-term hematopoietic stem cells (LT-HSCs). CML stem cells seem to function as cancer stem cells, maintaining the disease, whereas B-ALL originating cells seem to differentiate into cancer stem cells corresponding to pro-B cells.[17] In contrast, the cellular origin of acute leukemia is less clear and seems to be primarily dependent on the type of initial driver mutation. Elegant reconstitution experiments using separately transduced flow-sorted hematopoietic stem and progenitor fractions suggested that certain oncogenes, like the MLL-AF9, MLL-ENL or the MOZ-TIF2 fusions, have leukemogenic potential in stem cells, as well as in more committed progenitors cells.[18-21]

Many studies have demonstrated that zebrafish (*Danio rerio*) hematopoiesis follows a similar developmental program as that of humans with diversification into erythroid, myeloid and lymphoid lineage. In addition, the functional characterization of several zebrafish homologs of human transcriptional key regulators of hematopoiesis suggests functional interspecies conservation of molecular mechanisms.[22] Mutational screens revealed several genetic alterations that lead to hematologic diseases in the fish that mimicked many aspects of the human disease.[23] Several transgenic zebrafish leukemia models have been established, including T-cell ALL induced by overexpression of c-Myc or Notch1, and B-cell leukemia driven by the ETV6-RUNX1 (TEL-AML1) fusion.[24]

To validate zebrafish as a model for myeloid malignancies, Onnebo and colleagues have generated a transgenic fish line expressing a zebrafish etv6-jak2a fusion ortholog to the human ETV6-JAK2 (5-12) variant associated with atypical CML.[25] Injection of a spi1::etv6-jak2a transgenic construct severely compromised viability most probably due to severe perturbations of the hematopoietic system. Detailed analysis of the injected embryos revealed the accumulation of large white blood cells that were negative for erythroid and lymphoid markers. Although attempts to generate stable transgenic zebrafish lines expressing the tel-jak2a fusion failed, the phenotype observed in transiently injected embryos phenocopied, at least in part, the myeloid expansion seen in patients with ETV6-JAK2 (5-12) and atypical CML.

In their paper in this issue of *Haematologica*, Onnebo and colleagues have extended their studies by comparing the effects of ETV6-JAK2 (5-19) and ETV6-JAK2 (5-12) fusion type in zebrafish by expressing the respective zebrafish etv6-jak2a homologs as transgenes driven by the blood cell-specific *spi1* promoter or the ubiquitously expressed cytomegalovirus (CMV) promoter.[26] Zebrafish embryos were microinjected at the one-cell stage and followed for phenotypic pertubations from 24 hours post fertilization (hpf) to five days post fertilization (dpf). At 24 hpf, all etv6-jak2a constructs resulted in an expansion of the so-called posterior intermediate cell mass (pICM), a site of zebrafish hematopoiesis. At 48 hpf, an increased number of large non-hemoglobinized white blood cells appeared in the circulation. They used *in situ* hybridization to further characterize the observed hematopoietic changes. Interestingly, using lysozyme (lyz) and mmp9 as myeloid markers, only etv6-jak2a (5-12, CML) resulted in an increase of $mmp9^+$ and decrease in lyz^+ cells. In contrast, only the etv6-jak2a (5-19, ALL) fusion led to an expansion of $spi1^+$ cells in the pICM, whereas both constructs were associated with

ectopic expression of spi1 in anterior tissues. Importantly, comparing the expression of lymphoid markers rag1 and ikaros revealed an increase in embryos injected with the spi1::etv6-jak2a (5-19, ALL) compared to normal controls. No alterations of the rag1 or ikaros signals were observed upon injection of the other three constructs. Comparative biochemical analysis *in vitro* of HEK 293T cells revealed only minor differences between the etv6-jak2a fusion variants, with the 5-12 variant showing a slightly lower degree of autophosphorylation than the 5-19 variant. Expanded pICMs in embyros injected with both types of fusion showed a similar sensitivity to treatment with a JAK2 inhibitor (AG490). These findings strongly support a lineage-specific activity of the different etv6-jak2a fusion variants when expressed as transgenes in zebrafish.

This study of zebrafish is the first to provide evidence that there might indeed be intrinsic differences between the three ETV6-JAK2 variants that could contribute to the observed disease phenotype. However, a more rigorous functional analysis of biochemical structure might be needed to identify which of the different kinase activities is responsible for the observed differences. The currently available multi-color flow cytometric sorting of distinct hematopoietic cell populations would also allow comparative modeling in the mouse. Equal levels of the different ETV6-JAK2 fusion variants could be expressed in hematopoietic stem cells, and more myeloid or lymphoid committed progenitor cells, to compare the transforming potential and lineage specificity *in vivo*. Comparative profiling of phospho-proteins in derived leukemic cells could reveal different signaling pathways driving the disease. Domain-swapping experiments replacing the ETV6 part with other known JAK2 partners (PCM1, BCR, SEC31A) could provide an insight into potential regulatory circuits between the domains of the fusion. Comparative protein-protein interaction screens could help to identify different interactions to potential cell-specific regulators and/or downstream targets.

Taken together, the zebrafish modeling of ETV6-JAK2 leukemia by Onnebo and colleagues described in this issue of the Journal provides evidence for the *in vivo* potential of ETV6-JAK2 fusion variants initially cloned from patients with acute and chronic leukemia. This work also underlines the importance of generating *in vivo* models to better understand the molecular events underlying human leukemogenesis. Adequate models of ETV6-JAK2 variants in the fish or in the mouse are not only important tools to study oncogenic JAK2 signaling on a molecular level, but also for future testing of novel small molecule inhibitors and associated resistance mechanisms.

Professor Juerg Schwaller is a research group leader at the Department of Biomedicine of the University Hospital Basel, Switzerland. He is mainly interested in acute leukemia.

Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

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