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LETTER

Hyperdiploid acute lymphoblastic leukemia in children with *LZTR1* germline variants

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First described in 1967, hyperdiploidy (HD) is the most frequent genetic abnormality in B-cell-precursor leukemia (BCP-ALL) in children, comprising about 25% of all cases. A not yet exactly defined proportion has predisposing pathogenic germline variants in DNA repair pathways, chromatin remodeling factors, transcription factors regulating B-cell development (particularly *ETV6*), or receptor tyrosine kinases pathways like RAS/RAF.[1] Among the latter, mutations have mostly been described in *PTPN11* and *SOS1* but not yet in other components of this central regulatory hub of cellular communication.[2] In the course of whole-exome-sequencing (WES) as part of the routine germline testing of children with cancer, we compiled data from several pediatric oncology centers in Germany and noticed an accumulation of *LZTR1* sequence variants in children with classical hyperdiploid BCP-ALL. Through further *in vivo* functional assays of the presumably pathogenic patient-derived *LZTR1* variants in a *Drosophila* model, we determined the impact of those on the alteration of RAS-pathway activity.

The *LZTR1* gene is located on chromosome 22q11.21, consists of 21 coding exons and encodes for the 840 aminoacid large LZTR1 protein, an adaptor for the E3 ubiquitin ligase Cullin3 (CUL3). *LZTR1* acts as a negative regulator of the RAS-MAPK signaling pathway, facilitating RAS ubiquitination and degradation.[3] Germline loss-of-function (LOF) *LZTR1* mutations are typically linked to hereditary nerve sheath tumors and mostly schwannomatosis.[4] However, it remains largely unknown if other tumor entities are associated with *LZTR1* LOF germline mutations, especially in the absence of a syndromic clinical phenotype, potentially broadening the spectrum of malignancies associated with RASopathies.

In a single-center study, we initially collected germline WES data from 353 pediatric patients with a broad variety of malignancies. Focusing on children with BCP-ALL (n = 105), we observed an enrichment of pathogenic/likely pathogenic (P/LP) *LTZR1* variants, as all putatively pathogenic variants were segregated with the diagnosis of a BCP-ALL. There was additionally a striking association between the identification of a P/LP *LTZR1* variant and the presence of an *in vivo* HD karyotype. Specifically, 5/105 (4.8%) BCP-ALL patients harbored a variant in the *LZTR1* gene and three (2.8%) of the identified variants were classified as P/LP through the application of *in silico* prediction tools. All the patients who carried a pathogenic variant were diagnosed with an HD BCP-ALL. Subsequently, we functionally tested two of the P/LP *LZTR1* variants, p.Arg283Trp and p.Tyr535Ter, as well as a presumably benign *LZTR1* variant, p.Lys761Arg, which was identified in a pediatric patient with anaplastic large cell lymphoma. We utilized a *Drosophila* model that is particularly suited for the functional evaluation of Ras pathway activity on proliferation.[5–7] Thus, we assessed the functionality of $hLZTR1^{p.Arg283Trp}$, $hLZTR1^{p.Tyr535Ter}$ and $hLZTR1^{p.Lys761Arg}$ *in vivo*. We employed the "ReDDM" (Repressible Dual Differential Marker, Supporting Information S1: Figure S1A) tracing method to determine the role of *LZTR1* variants in Ras signaling-dependent intestinal stem cell (ISC) lineage production. In addition, ReDDM allows the detection of

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morphological properties and programmed cell death.[6,8] In total, we assessed four different parameters indicative of a RAS pathway activation: ISC lineage production, apoptosis, ERK activity and mitotic recombination.

First, we confirmed that dLZTR1 knockdown increases Ras signaling activity and ISC lineage production by crossing established *dLztr1-RNAi* flies targeting *Drosophila Lztr1* (*dLztr1*) to esg^{ReDDM} (Figure 1C, Supporting Information S1: Figure S1A). Esg^{ReDDM} when crossed with oncogenic Ras^{G12V} served as positive control as they increased ISC lineage production by 2.5-fold (Supporting Information S1: Figure S1A–E).[8] Likewise, *dLztr1* depletion increased ISC lineage cell production by 1.5-fold (Figure 1C,H, Supporting Information S1: Figure S1E), which clearly suggests that dLztr1 mimics the effects of RAS ubiquitination in ISCs. Importantly, in dLztr1-depleted ISCs, the expression of wild-type *hLZTR1* (>$hLZTR1^{wt}$) restored ISC lineage production to control levels (Figure 1B,D,H), demonstrating the capability of $hLZTR1^{wt}$ to substitute its fly orthologue in ISC.

Next, we functionally characterized the patient-derived *LZTR1* variants in dLztr1-depleted ISCs. Much like wild-type $hLZTR1^{wt}$, $hLZTR1^{p.Arg283Trp}$ (Figure 1E) and $hLZTR1^{p.Lys761Arg}$ (Figure 1G) restored control-like ISC lineage production (Figure 1H), suggesting that $hLZTR1^{p.Arg283Trp}$ and $hLZTR1^{p.Lys761Arg}$ still regulate Ras ubiquitination. In contrast and confirming its functionality, $hLZTR1^{p.Tyr535Ter}$ resulted in even 1.8-fold higher ISC lineage production (Figure 1F,H)

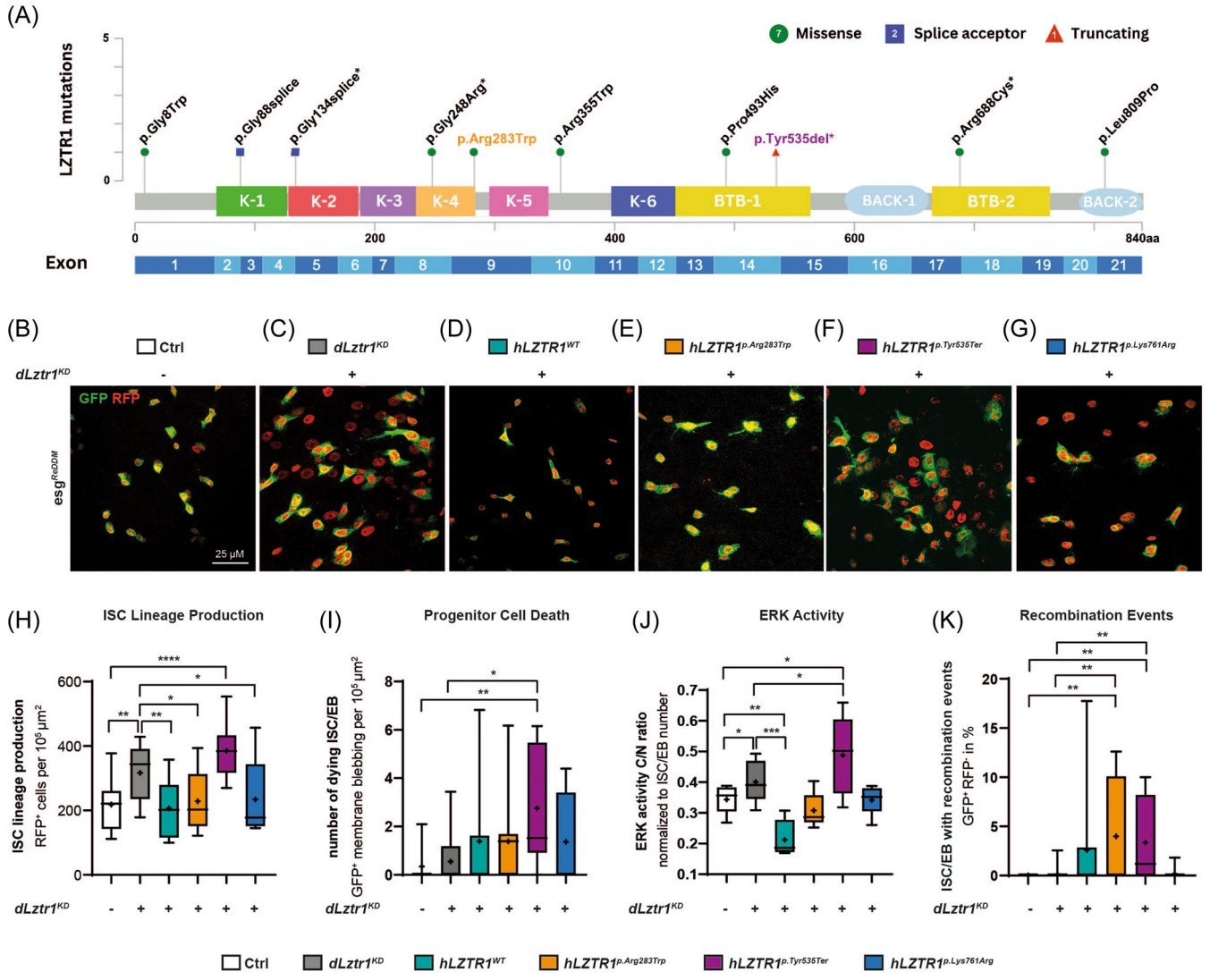

FIGURE 1 (A) Depiction of the ten *LZTR1* variants detected in the pediatric cohort of the 283 BCP-ALL patients. Germline variants that were tested by applying the *Drosophila* model are highlighted in colored fonts. (B–G) Confocal images showing (B) Controls, (C–G) specific inducible knockdown (KD) of *Drosophila Lztr1* (*dLztr1*), and simultaneous expression of (D) wild-type human LZTR1 ($hLZTR1^{wt}$), (E) variant $hLZTR1^{p.Arg283Trp}$, (F) $hLZTR1^{p.Tyr535Ter}$ and (G) $hLZTR1^{p.Lys761Arg}$ of adult *Drosophila* midguts after seven days of stem cell progeny tracing using esg^{ReDDM}. (H) Quantification of intestinal stem cell (ISC) lineage production encompassing ISC, EB, and newly differentiated EC (GFP^+ RFP^+ and RFP^+ cells) revealed an increase upon KD of *dLztr1* and expression of variant $hLZTR1^{p.Tyr353Ter}$. (I) Progenitor cell death assessed by the number of dying ISC/EB is increased upon expression of variant $hLZTR1^{p.Tyr353Ter}$, (J) ERK phosphorylation as an indication of Ras activation induced by $hLZTR1^{p.Tyr353Ter}$. (K) Of note, recombination frequency revealed by loss of double fluorescence is increased by $hLZTR1^{p.Arg283Trp}$ and $hLZTR1^{p.Tyr353Ter}$. Mean is indicated by (+) (n = 15,16,10,10,10,10 [H]; 11,12,7,10,10,10 [I]; 6,8,8,8,8,6 [J]; 19,20,10,10,10,10 [K]) and asterisks indicate significances from Student's *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). EB, enteroblasts; EC, enterocytes.

TABLE 1 Description of the 10 *LZTR1* germline variants detected in a cohort of 283 BCP-ALL pediatric patients.

ID	LZTR1 protein change	Clinical phenotypic presentation	Age at diagnosis	Hyperdiploidy	Suspicion of Noonan (like) syndrome before disease manifestation	Variant segregates with Noonan syndrome	Variant classification (Varsome)
P1	p.Arg283Trp	Mild developmental delay	9 years	Yes	No	No	Likely pathogenic
P2	p.Pro493His	Unremarkable	1.1 years	No	No	No	Likely benign
P3	p.Arg355Trp	Unremarkable	6 months	No	No	No	Likely benign
P4	p.Tyr535Ter	Unremarkable	17 years	Yes	No	Yes, AR inheritance	Pathogenic
P5	p.Leu809Pro	Unremarkable	3.4 years	Yes	No	No	Likely pathogenic
P6	c.263+7G>A	Unremarkable	10 years	Yes	No	No	Benign
P7	p.Gly8Trp	Unremarkable	6 months	No	No	No	Likely benign
P8	p.Gly248Arg	Mild developmental delay; facial dysmorphism with noticeably plump, broad facial skull, ear dimple on the left helix	2.1 years	Yes	No	Yes, AD inheritance	Pathogenic
P9	c.401-2_401-1delAG	Unremarkable	10.2 years	Likely HD	No	No	Pathogenic
P10	p.Arg688Cys	Unremarkable	9.4 years	No	No	Yes, AR inheritance	Likely pathogenic

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; HD, hyperdiploidy; VUS, variant of unknown significance.

than dLztr1-RNAi (Figure 1C). We also noticed that *hLZTR1*$^{p.Tyr535Ter}$ induced extensive membrane blebbing and nuclear fragmentation of GFP-positive cells (Figure 1F,I) likely indicating programmed cell death.[6] Quantification of apoptotic cells revealed an 11.1-fold increase compared to controls (Figure 1I), which was not observed for *hLZTR1*$^{p.Arg283Trp}$ and *hLZTR1*$^{p.Lys761Arg}$ (Figure 1E,G,I).

Beyond ISC production and progenitor cell death as a readout, we employed a third experimental paradigm directly addressing Ras signaling activity with a translocating modified ERK *in vivo* sensor (modERK, Supporting Information S1: Figure S2A–G).[9] Briefly, the modERK-sensor is a UAS-driven construct containing the docking site of mammalian ERK substrate Elk1 fused to a nuclear localization signal (NLS), a nuclear export signal (NES), and the mClover fluorescent protein. Upon phosphorylation of ERK the NLS activity is decreased while NES activity is increased leading to the shuttling of the mClover fluorophore to the cytoplasm (Supporting Information S1: Figure S2A). In line with Ras activity control of Lztr1, we measured that dLztr1-RNAi elicits a significant 1.1-fold increase of ERK activity (Figure 1J) in ISC when cytoplasmic versus nuclear fluorescence signal (Supporting Information S1: Figure S2C) was compared with controls (Supporting Information S1: Figure S2B). Confirming our previous results, *hLZTR1*^{wt} significantly reduces ERK activity (Figure 1J, Supporting Information S1: Figure S2D), while the putatively LOF variant *hLZTR1*$^{p.Tyr535Ter}$ variant considerably induces ERK phosphorylation by 1.4-fold over controls (Figure 1J, Supporting Information S1: Figure S2F), whereas both other variants did not elicit modERK activity changes (Figure 1J, Supporting Information S1: Figure S2E,G).

Finally and most notably, forced expression of *hLZTR1*$^{p.Arg283Trp}$ and *hLZTR1*$^{p.Tyr535Ter}$ induced mitotic recombination by 4-fold and 3.4-fold, respectively (Figure 1K, Supporting Information S1: Figure S3A,B). Importantly, mitotic recombination was neither observed upon increases of RAS activity such as *dLztr1-RNAi* and *Ras*^{G12V} (Supporting Information S1: Figure S3C) nor in the benign variant of *LZTR1* (hLZTR1$^{p.Lys761Arg}$, Figure 1k). Further studies are required to determine whether the ability to induce mitotic recombination and putatively chromosomal missegregation is tightly coupled to the development of HD BCP-ALL, either alone or in combination with concomitant enhanced RAS signaling. The development of aneuploidy in the context of RAS-pathway hyperregulation was recently demonstrated in a study where increased RAS/MAPK activity profoundly misregulated chromosome alignment and segregation in glioblastoma cells. Interestingly, this effect was reversible upon MEK inhibition.[10]

Taken together, using an *in vivo Drosophila* model, we demonstrated the functional impact of the *hLZTR1*$^{p.Tyr535Ter}$ variant on ERK activity, progenitor cell proliferation and death. We have also shown the effects of mitotic recombination for both the *hLZTR1*$^{p.Arg283Trp}$ and *hLZTR1*$^{p.Tyr535Ter}$ variants. Applying a phenotype-genotype correlation, despite the demonstrated impaired *in vivo* function of the *hLZTR1*$^{p.Tyr535Ter}$ variant, the patient expressed phenotypically no clinical signs of an underlying RASopathy. In contrast, the patient harboring the *hLZTR1*$^{p.Arg283Trp}$ variant with the milder functional effect presented with concomitant clinical signs of a syndromic condition, namely psychomotor delay and learning disabilities. Both patients developed HD BCP-ALL and the girl with *hLZTR1*$^{p.Tyr535Ter}$ variant was *in vivo* diagnosed at a rather atypical age (17 years old) for the occurrence of an HD karyotype, as the HD genetic subtype while predominant in childhood, is observed less frequently in adults and young adolescents.[11]

Moreover, sequencing of the respective HD BCP-ALL of both children harboring the two functional significant germline variants, *hLZTR1*$^{p.Tyr535Ter}$ and *hLZTR1*$^{p.Arg283Trp}$, unveiled secondary somatic mutations in RAS pathway genes: a stop gain *LZTR1*:p.Glu339Ter

mutation and a *NRAS*:c.38G>A mutation, respectively, which could be interpreted as an oncogenic second hit.[12]

After obtaining the above encouraging *in vivo* functional results, we mined germline WES data from other 178 children BCP-ALL who had been treated in several additional German pediatric oncology centers. Thus, overall our study comprised 283 children with BCP-ALL, of whom we collected comprehensive clinical and germline data after obtaining informed consent.[13,14] The median age at diagnosis was 4.8 years with an age range of 3 months to 17.4 years. We observed a slight male predominance in our cohort with 156 male (55%) and 127 female patients (45%). All in all, germline variants affecting an *LZTR1* variant were identified in 10/283 (3.5%) patients. Interestingly, in six out of 283 (2.1%) children, the *LZTR1* variants were classified through *in silico* prediction tools as P/LP (Table 1). Notably, as already observed in our initial in-house cohort, the majority of patients (5/6) harboring a P/LP germline *LZTR1* variant presented with a classical HD BCP-ALL. Of note, none of the patients carried an *ETV6* germline variant or other pathogenic variants apart from *LZTR1*. Remarkably, only two of the patients displayed concomitant phenotypic features indicative of an underlying syndromic condition, despite the presence of P/LP LZTR1 variants.

In summary, approximately 2% of all children with BCP-ALL in our cohort harbored a P/LP *LZTR1* germline variant, not necessarily linked with the clinical appearance of Noonan-syndrome-like features, but to the development of a BCP-ALL with a classical hyperdiploid karyotype. By applying a *Drosophila* model, we could demonstrate that the presumably pathogenic *LZTR1* germline variants affect RAS pathway activation, ERK accumulation, cell proliferation and apoptosis induction in various ways. The impact of the variants on mitotic recombination may especially comprise an interesting cellular link to the development of aneuploidy and HD leukemia initiation. Our study further emphasizes the necessity to apply different readouts to assess the full spectrum of functional consequences of the RAS pathway's mutated genes.

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AUTHOR CONTRIBUTIONS

Rabea Wagener, Anna Hoffmann, Lisa Zipper, Arndt Borkhardt, Triantafyllia Brozou, and Tobias Reiff organized the project, analyzed and interpreted the data, and performed validation and functional experiments. Danielle Brandes and Sanil Bhatia analyzed and interpreted the data. Lisa Zipper, Tobias Reiff, Arndt Borkhardt, and Triantafyllia Brozou wrote the paper. Triantafyllia Brozou and Stavrieta Soura recruited patients, provided clinical information, and obtained informed consent. Franziska Auer and Julia Hauer provided data on the patients of the Dresden pediatric cancer cohort. Stefanie V. Junk and Martin Stanulla provided the data of the ALL SMN cohort. Carolin Walter, Julian Varghese, Ammarah Anwar, Layal Yasin, and Martin Dugas performed the bioinformatic processing of the WES data. Ute Fischer, Triantafyllia Brozou, Tobias Reiff, and Arndt Borkhardt supervised the study. All authors read and approved the final paper.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the ethics committee of Heinrich Heine University, Düsseldorf, Germany (ethics vote number 4886R and study registration number 2014112933) and the ethical committee of the TU Dresden (ethics vote number EK 181042019).

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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