Hedgehog pathway mutations in T-cell acute lymphoblastic leukemia

Hedgehog signaling plays an important role in both embryonic pattern formation and in proliferation, survival and differentiation of adult tissues, including T cells.[1,2] We report here the presence of rare acquired mutations in genes encoding components of the Hedgehog signaling pathway in T-cell acute lymphoblastic leukemia (T-ALL). In the 4 cases in which a detailed analysis was possible, we document the mutations in both neoplastic and normal hematopoietic cells, but not in epithelial cells. Functional analysis confirms the gain-of-function properties of two truncated smoothened (SMO) mutations identified in this study.

In mammalian cells, Hedgehog signaling involves three different ligands, Sonic Hedgehog (SHH), Indian Hedgehog (IHH) or Desert Hedgehog (DHH), each with different spatial and temporal distribution pattern. Hedgehog ligand binds to and inhibits its receptor patched 1 (PTCH1) or patched 2 (PTCH2), two multipass transmembrane proteins, and thereby results in release, accumulation and activation of SMO in the primary cilium. This in turn activates GLI transcription factors (GLI1, GLI2, and GLI3), which translocate into the nucleus and act as transcriptional activators or repressors. Previous studies have shown that mutations in *PTCH1* are responsible for the majority of nevoid basal cell carcinoma syndrome and basal cell carcinoma cases, and also mutations of *SMO* and *SUFU* have been identified.[3]

Several lines of evidence suggest that the Hedgehog pathway is important for normal T-cell development. *In vivo* studies in fetal[4] and adult[5] thymus have shown that Smo-induced signals are essential for homeostasis, differentiation and proliferation of early T thymocytes, especially at the DN1-DN2 stage. Likewise, Shh ligand is important for differentiation and proliferation of more immature T cells (at the DN stage) but is also important for DN to DP transition, as well as influencing the CD4/CD8 ratio.[2] Finally, Ihh promotes T-cell differentiation before pre-TCR signal transduction, but acts as negative T-cell regulator in later developmental stages.[4]

Despite the frequent mutation of the Hedgehog pathway in solid tumors, mutations in hematologic malignancies have not yet been described. We recently performed exome sequencing in T-ALL and identified several single nucleotide variants (SNVs) in genes encoding members of the Hedgehog pathway.[6] As these SNVs were also detected in DNA from remission, we initially believed that these SNVs were rare polymorphisms. However, upon more

Table 1. Mutations identified in different Hedgehog components in T-ALL patients.

Patient	Gene	Transcript number	Diagnosis DNA nucleotide change	Germline DNA nucleotide sequence	Amino acid change	Protein affecting score	Other characteristics
TLE41[p]	SMO	NM005631	C2176T	wild type	R726*	Damaging	Male, 16 years, relapsed, MLL fusion, NUP214-ABL1, NOTCH1 L2429fs, IL7R I244 delinsNDCS
TLE67	SMO	NM005631	C2287T	wild type	R763*	Damaging	Male, 14 years, complete remission, TAL1, NUP214-ABL1, NOTCH1 F1606LGEIP,
TLE40	GLI1	NM005269	C1613T	wild type	S538F	Damaging	Male, 38 years, complete remission
TLE44	GLI3	NM000168	G2179A	wild type	G727R	Damaging	Female, 29 years, relapsed, TLX3, NOTCH1 S2467fs, JAK3 M511I
TLE61[p]	PTCH1	NM000264	G1306A	G1306A	D436N	Benign	Male, 13 years, complete remission, RPL10 R98S
TLE59[p]	GLI3	NM000168	G3001A	G3001A	G1001S	Benign	Male, 7 years, allotransplant, TLX3, NOTCH1 L1678P, WT1 R369G
TLE70	PTCH1	NM000264	C2173T	NA	P725S	Benign	PTPN2 deletion
TLE37	PTCH2	NM003738	C2711T	NA	P904L	Damaging	Male, 37 years, relapsed, PTPRC D433N
TBR1	GLI3	NM000168	G1097T	NA	R366L	Damaging	Male, 35 years, IL7R F250V253 delinsPLGE

Sequence variations in Hedgehog components were identified in 9 of 50 T-ALL samples. All variations were heterozygous. Germline DNA was obtained from buccal swabs (n=4), skin (n=1), or liver (n=1) for 6 cases. The PTCH1-D436N variation detected in case TLE61 is known as a SNP (rs142274954). The protein score reflects the prediction by 3 different algorithms (Condel, SIFT, Polyphen): the score was considered to be damaging when at least 2 of the 3 predictions were damaging. Nucleotide counting started at the A from the start codon of the indicated transcript. NA: not available; fs: frameshift mutations; [p]pediatric patient.
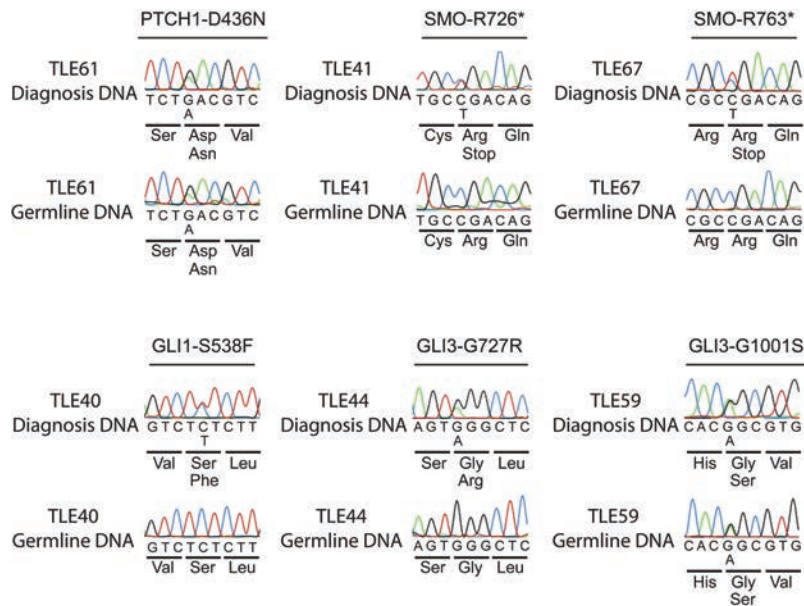
Figure 1. Sanger sequencing profiles of T-ALL cases with Hedgehog pathway mutations. Sanger sequencing in 6 T-ALL cases on DNA from diagnosis and germline DNA confirmed that 4 of the 6 Hedgehog pathway mutations were somatic mutations.

detailed analysis, we confirmed that these SNVs were not present in DNA extracted from non-hematopoietic tissues, indicating that these variants were only present in the leukemia cells and in normal hematopoietic cells.

We identified SNVs in different genes of the Hedgehog pathway in 9 T-ALL samples (Table 1). Four of these 9 sequence variations were confirmed to be somatic mutations, based on the analysis of available germline DNA of the same individuals (Figure 1). Remarkably, these four somatic mutations were still detected in DNA extracted from blood cells at time of remission, while other driver mutations (NOTCH1, etc.) detected in these samples were, as expected, absent at remission (Table 1). This finding suggests that the Hedgehog pathway mutations were acquired as *de novo* mutations in the hematopoietic compartment, potentially early in life. A similar finding was recently reported as one of the two possible explanations for TP53 mutations in hypodiploid ALL.[7]

Of the five other variations detected in Hedgehog components, two were shown to be present in germline DNA, indicating that these were likely rare polymorphisms, and these variations were also predicted not to alter protein structure. The other 3 sequence variations, identified in T-ALL samples for which no germline DNA was available, could still represent true somatic mutations, and predictions indicate a damaging effect for two of these variations (Table 1).

Two of the detected acquired mutations were truncating mutations of *SMO* (R726* and R763*), located in the C-terminus of the protein. This C-terminus contains several arginine clusters that are important in blocking the SMO cell surface expression and inhibit the conformational switch of SMO to its active form.[8] The two other acquired mutations are a *GLI1* point mutation (S538F) and a *GLI3* point mutation (G727R), which are both located downstream of the zinc finger domain and predicted to be damaging for the protein. Moreover, the *GLI3-G727R* mutation was previously identified in individuals with postaxial polydactyly (PAP),[9] a disease known to be caused by GLI3 defects, supporting the view that the *GLI3-G727R* mutation identified in T-ALL also affects GLI3 function.

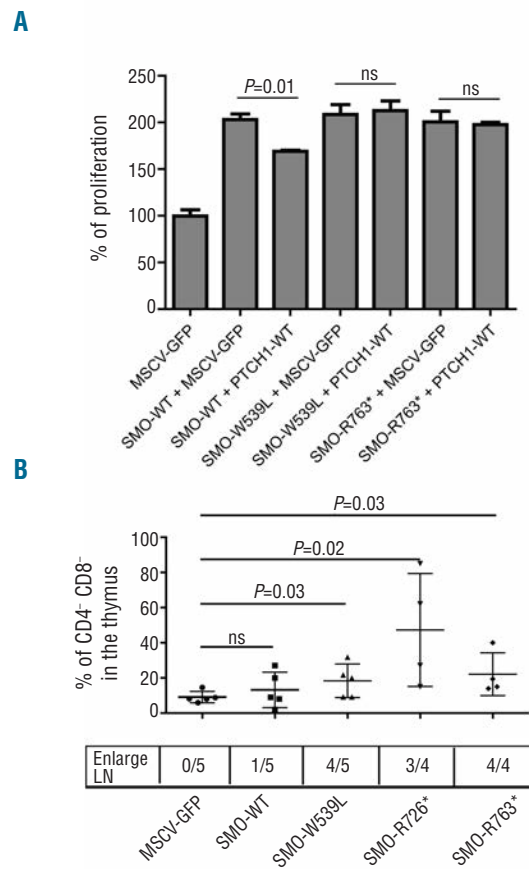To confirm the oncogenic properties of SMO-R763*, we

Figure 2. Identification of acquired mutations in the Hedgehog pathway. (A) Functional analysis of the *SMO-R763** mutant was performed in HEK293T cells. The proliferation of the cells was measured 4, 5 and 6 days after transfection (cell numbers at day 4 are shown). The experiment was repeated three times and each sample was analyzed in triplicate. The total amount of DNA used for the transfections was constant. WT: wild type. (B) Frequency of CD4CD8 double negative (DN) population in the thymus of Balb/C mice transplanted with hematopoietic progenitor cells expressing various SMO mutants.

used the HEK293T cell system previously reported by Barnes *et al.* in a study of PTCH1 mutants.[10] We reasoned that if the SMO-R763* mutant identified in our study was an activating mutant, we would be able to increase the proliferation of HEK 293T cells by expressing this mutant, and this effect on proliferation would be insensitive to PTCH1 inhibition. As control we used the SMO-wild type and a known activating mutant SMO-W539L.[11] To distinguish between the effects caused by the overexpression and by the overactivation of SMO, we co-transfected each SMO construct with a PTCH1-wild-type construct, with the aim of testing if PTCH1 could still inhibit the SMO mutants. HEK 293T cells transfected with wild-type SMO had a clear proliferation advantage that could be partially inhibited by co-expression of PTCH1 (Figure 2A). Similarly, expression of the SMO-W539L or SMO R763* resulted in increased proliferation of the 293T cells, but these SMO mutants could not be inhibited by PTCH1 co-expression (Figure 2A). These data demonstrate that the *SMO-R763** mutation is a typical SMO-activating mutation that is insensitive to PTCH1 inhibition and is capable of stimulating cell proliferation.

We next evaluated the capacity of these two SMO mutants to activate the Hedgehog pathway *in vivo*. Hematopoietic progenitors were transduced with the SMO-R763* or the SMO-R726* mutants, or with controls (empty MSCV-GFP vector, SMO-wild type vector, or the known activating mutant SMO-W539L). Transduced progenitor cells were injected into irradiated Balb/c mice. Flow cytometric analysis of these mice revealed an increase of CD4CD8 double negative cells in the thymus expressing the 3 SMO mutants (GFP positive cells) compared to GFP negative cells from the same mice or cells obtained from MSCV-GFP or SMO-wt mice (Figure 2B). Moreover, 11 of 13 mice expressing the SMO mutants showed enlarged lymph nodes with a major T-cell infiltration (Figure 2B). Nevertheless, none of these mice developed leukemia implying that activation of the hedgehog pathway is not sufficient to cause leukemia. Taken together, these data clearly demonstrate that SMO-R763* and SMO-R726* mutants can block the T-cell development *in vivo* in a similar way as the mutant SMO-W539L that activates the Hedgehog pathway.

T-ALL is a genetically complex leukemia where multiple genomic aberrations co-operate to transform normal T-cell precursors to fully malignant thymocytes. Here we provide evidence that also components of the Hedgehog pathway can be mutated in T-ALL. As a direct proof of activation of the Hedgehog pathway in T-ALL, we identified acquired mutations in 4 T-ALL samples (*SMO-R726**, *SMO-R763**, *GLI1-S538F* and *GLI3-G727R*). In 2 additional T-ALL cases we identified variations in PTCH2 and GLI3 that are predicted to affect these proteins, but for which we do not have evidence that these sequence variations are acquired, as no germline DNA was available for these patients.

Remarkably, and in contrast to other somatic mutations detected in the T-ALL samples, the somatic mutations in SMO, GLI1 and GLI3 were still present at high frequency at time of remission. This observation can potentially be explained by the *de novo* acquisition of these mutations early in life in the hematopoietic compartment. Similar findings had previously been reported for a set of TP53 mutations in ALL, as the same mutations were identified in non-tumor hematopoietic cells in remission.[7] It would be interesting to explore this further in future studies. It is also remarkable that this was observed for all 4 somatic mutations in SMO, GLI1 and GLI3, which seems to indicate that such mutations in the Hedgehog pathway are more deleterious early on than during adult hematopoiesis. This is in agreement with previous work illustrating an important role for Gli3 in the control of T-cell differentiation during fetal development and not during adult development.[12]

Taken together, our data indicate that the Hedgehog pathway can be activated in a subset of T-ALL through mutations in critical proteins of the pathway. These data contrast previous reports that showed that the Hedgehog pathway was dispensable for a NOTCH1-driven T-ALL mouse model.[13] These different conclusions may be explained by the differences between the mouse model and the human disease. NOTCH1 is a very strong oncogene in mouse models (especially when it is over-expressed), while the human leukemias have lower NOTCH1 activation and still require additional mutations for their further evolution towards an aggressive leukemia. In addition, we have to take into account that we only detected a low frequency of Hedgehog pathway mutations in T-ALL. Also, high expression of Hedgehog pathway components and sensitivity to Hedgehog antagonists in mouse and T-LBL patients as well as B-ALL cell lines further illustrate a role for the Hedgehog pathway in ALL.[14,15] Our data identify the Hedgehog pathway as an oncogenic pathway in a subset of T-ALL cases and as a potential target for therapy in T-ALL. Additional data are required to determine the exact origin of these mutations and to determine if treatment with Hedgehog pathway inhibitors could be beneficial in these cases.

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References

1. Petrova R, Joyner AL. Roles for Hedgehog signaling in adult organ homeostasis and repair. Development. 2014;141(18):3445-3457.
2. Crompton T, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. Nat Rev Immunol. 2007;7(9):726-735.
3. Teglund S, Toftgård R. Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim Biophys Acta. 2010;1805(2):181-208.
4. Outram SV, Hager-Theodorides AL, Shah DK, et al. Indian hedgehog (Ihh) both promotes and restricts thymocyte differentiation. Blood. 2009;113(10):2217-2228.
5. Andaloussi AE, Graves S, Meng F, et al. Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. Nat Immunol. 2006;7(4):418-426.
6. De Keersmaecker K, Atak ZK, Li N, et al. Exome sequencing identi-

fies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia. Nat Genet. 2012;1-6.

7. Holmfeldt L, Wei L, Diaz-Flores E, et al. The genomic landscape of hypodiploid acute lymphoblastic leukemia. Nat Genet. 2013;45(3):242-252.

8. Zhao Y, Tong C, Jiang J. Hedgehog regulates smoothened activity by inducing a conformational switch. Nature. 2007;450(7167):252-258.

9. Radhakrishna U, Bornholdt D, Scott HS, et al. The phenotypic spectrum of GLI3 morphopathies includes autosomal dominant preaxial polydactyly type-IV and postaxial polydactyly type-A/B; No phenotype prediction from the position of GLI3 mutations. Am J Hum Genet. 1999;65(3):645-655.

10. Barnes EA, Heidtman KJ, Donoghue DJ. Constitutive activation of the shh–ptc1 pathway by a patched1 mutation identified in BCC. Oncogene. 2004;24(5):902-915.

11. Taipale J, Chen JK, Cooper MK, et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature. 2000;406(6799):1005-1009.

12. Hager-Theodorides AL. The transcription factor Gli3 regulates differentiation of fetal CD4-CD8- double-negative thymocytes. Blood. 2005;106(4):1296-1304.

13. Gao J, Graves S, Koch U, et al. Hedgehog Signaling Is Dispensable for Adult Hematopoietic Stem Cell Function. Cell Stem Cell. 2009;4(6):548-558.

14. Gonzalez-Gugel E, Villa-Morales M, Santos J, et al. Down-regulation of specific miRNAs enhances the expression of the gene Smoothened and contributes to T-cell lymphoblastic lymphoma development. Carcinogenesis. 2013;34(4):902-908.

15. Lin TL, Wang QH, Brown P, et al. Self-Renewal of Acute Lymphocytic Leukemia Cells Is Limited by the Hedgehog Pathway Inhibitors Cyclopamine and IPI-926. PLoS ONE. 2010;5(12):e15262.