Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL

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Acute lymphoblastic leukemia (ALL) is an aggressive hematological tumor resulting from the malignant transformation of lymphoid progenitors. Despite intensive chemotherapy, 20% of pediatric patients and over 50% of adult patients with ALL do not achieve a complete remission or relapse after intensified chemotherapy, making disease relapse and resistance to therapy the most substantial challenge in the treatment of this disease1,2. Using whole-exome sequencing, we identify mutations in the cytosolic 5′-nucleotidase II gene (NT5C2), which encodes a 5′-nucleotidase enzyme that is responsible for the inactivation of nucleoside-analog chemotherapy drugs, in 20/103 (19%) relapse T cell ALLs and 1/35 (3%) relapse B-precursor ALLs. NT5C2 mutant proteins show increased nucleotidase activity in vitro and conferred resistance to chemotherapy with 6-mercaptopurine and 6-thioguanine when expressed in ALL lymphoblasts. These results support a prominent role for activating mutations in NT5C2 and increased nucleoside-analog metabolism in disease progression and chemotherapy resistance in ALL.

Therapy for ALL includes an initial treatment with high-dose combination chemotherapy, which results in clinical and hematologic remission in over 90% of cases. This is typically followed by additional rounds of highly intensive therapy aimed at further reducing disease burden and then a 2-year-long lower-intensity maintenance therapy in which treatment with oral 6-mercaptopurine (6-MP) has a particularly important role3,4. Patients with relapsed ALL generally receive more intense treatment. However, despite these efforts, the outcome of these patients remains unsatisfactory, with cure rates of less than 40%5. This is the case particularly in patients with relapsed T cell ALL (T-ALL) and in individuals with primary resistance or early relapse, which is associated with a higher risk of failure to achieve a second complete remission, shorter duration of chemotherapy response and poor survival6,7. Much effort has been directed toward the study of the molecular basis of relapse and chemotherapy resistance in ALL. However, the specific mechanisms mediating escape from therapy, disease progression and leukemia relapse remain largely unknown. To address this issue, we performed whole-exome sequencing of matched diagnosis, remission and relapse DNA samples from five pediatric patients with T-ALL (Supplementary Table 1). This analysis identified a mean mutation load of 13 (range, 5–17) somatic mutations per sample (Supplementary Table 2). Out of 60 somatic mutations identified in total, 17 were present at diagnosis and relapse, 24 genes were selectively mutated in relapsed T-ALL samples and 19 mutations were present only at diagnosis. Moreover, four of the five relapsed leukemias analyzed showed the presence of at least one somatic mutation at diagnosis together with secondary mutations specifically acquired at the time of relapse. In addition, four out of these five individuals showed absence of at least one mutation marker present at diagnosis during disease progression leading to relapse. Single-nucleotide polymorphism (SNP) analysis of the exome sequencing results ruled out that loss of these markers was caused by loss of heterozygosity at relapse (Supplementary Table 3). This result is consistent with previous studies based on copy number alteration analyses8–10 and supports that relapsed ALLs can originate as derivatives of ancestral subclones related to, but distinct from, the main leukemic population present at diagnosis.

Somatically mutated genes at diagnosis included known T-ALL tumor suppressor genes, such as FBXW7 (ref. 11), WT1 (ref. 8) and DNM2 (ref. 12), in addition to numerous new genes not previously implicated in the pathogenesis of this disease. Analysis of mutant alleles found at the time of relapse identified mutations in three genes encoding proteins involved in positive regulation of TP53 signaling, including TP53 itself (R213Q), BANP (H391Y)13 and RPL11.

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**Figure 1** NT5C2 mutations in relapsed pediatric ALL. (a) Schematic representation of the structure of the NT5C2 protein. The haloacid dehalogenase (HAD) and substrate-binding (SB) domains are indicated. NT5C2 mutations identified in relapsed pediatric samples are shown. Filled circles represent heterozygous mutations. Multiple circles in the same amino acid position indicate multiple patients with the same variant. (b) DNA sequencing chromatograms of paired diagnosis and relapse genomic T-ALL DNA samples showing representative examples of relapse-specific heterozygous NT5C2 mutations, with the mutant allele sequence highlighted in red.

(R18P)\(^\text{14}\) (Supplementary Fig. 1). Notably, mutations in TP53 have been reported in ~10% of relapsed patients with ALL and are associated with a particularly poor prognosis\(^\text{15}\). Given the prominent role of the TP53 pathway in DNA damage–induced apoptosis\(^\text{16}\), we performed extended mutation analysis of TP53, BANP, and RPL11 in 18 additional diagnostic and relapsed T-ALL samples (Supplementary Table 1). This analysis did not identify additional TP53 or BANP mutations but did show the presence of two additional somatic RPL11 mutant alleles: one (X178Q) was present both at diagnosis and relapse, and the other (G305S) was specifically mutated at relapse. Relapse-associated mutations also included a prototypical activating mutation in the NRAS oncogene (G13V). Notably, NRAS mutations in ALL have been associated with poor outcome\(^\text{17}\) and are particularly prevalent in early T cell–precursor ALLs\(^\text{12,18}\), a group of high-risk leukemias with poor prognosis\(^\text{19}\). Extended mutation analysis of NRAS in relapsed patients with T-ALL showed the presence of two diagnostic and relapse sample pairs harboring a prototypical NRAS G12S activating allele and a third patient with a heterozygous activating NRAS G12R mutation, which was present at diagnosis and showed loss of heterozygosity at the time of relapse.

However, the most notable finding in our exome sequence analysis was the presence of a relapse-associated heterozygous mutation in NT5C2 (K359Q). NT5C2 is a ubiquitous enzyme that is responsible for the final dephosphorylation of 6-hydroxypurine nucleotide monophosphates such as inosine monophosphate (IMP), deoxynucleosine monophosphate (dIMP), guanosine monophosphate (GMP), dGMP and xanthosine monophosphate (XMP) before they can be exported out of the cell\(^\text{20,21}\). In addition, and most notably, NT5C2 can also dephosphorylate and inactivate 6-thioinositol monophosphate and 6-thioguanosine monophosphate, which mediate the cytotoxic effects of 6-MP and 6-thioguanine (6-TG)\(^\text{22}\), two nucleoside analogs commonly used in the treatment of ALL. Mutation analysis of an extended panel of 98 relapse T-ALL and 35 relapse B-precursor ALL samples (Supplementary Table 1) identified 22 additional mutations in T-ALL and one additional NT5C2

**Figure 2** Structure-function analysis of the NT5C2 K359Q mutant protein. (a) Molecular surface representation of the NT5C2 protein structure. The position of the NT5C2 K359Q mutation is highlighted in red. The substrate IMP is in purple; the ATP allosteric activator is in yellow. (b) Structure representation of the NT5C2 catalytic center and allosteric regulatory site devoid of substrate or ligands (Protein Data Bank (PDB) 2XCX). (c) Structure representation of the NT5C2 catalytic center and allosteric regulatory site bound to IMP and ATP, respectively (PDB 2XCW). (d) Structure representation of the NT5C2 K359Q mutant model corresponding to the catalytic center and allosteric regulatory sites. (e) Overlay of the structures in b-d. The white arrow indicates the repositioning of Phe354 from the inactive NT5C2 configuration to the active (ATP-bound NT5C2 and K359Q NT5C2) structures. Mg\(^{2+}\) ions are depicted as green spheres.
The role of NT5C2 in the metabolism and inactivation of nucleoside-analog drugs, as well as its potential role in relapse, is supported by the findings in this study. NT5C2 mutations were identified in samples from patients with ALL, and the recurrence of these mutations in relapsed disease suggests a role in relapse resistance. The observed association of NT5C2 mutations with increased nucleotidase activity and resistance to chemotherapy, particularly 6-MP and 6-TG, indicates that NT5C2 mutations play a significant role in drug resistance in ALL.

In summary, the findings in this study highlight the importance of NT5C2 mutations in the recurrence and resistance of ALL, providing new insights into the molecular mechanisms of relapse and therapeutic implications for the treatment of ALL.

**Figure 3** Increased 5′-IMP nucleotidase activity in NT5C2 mutant proteins. 5′-NT activity levels of recombinant mutant proteins relative to wild-type NT5C2 control are shown. Data are shown as the means ± s.d.

**Figure 4** Expression of NT5C2 mutations in ALL cells induces resistance to chemotherapy with 6-MP and 6-TG. (a) Viability assays in CCRF-CEM and CUTLL1 T-ALL cells expressing wild-type NT5C2, relapse-associated mutant NT5C2 alleles or a red fluorescent protein (RFP) control and treated with increasing concentrations of 6-MP. (b) 6-TG dose-response cell-viability curves. Data are shown as the means ± s.d.
18-fold and a 16-fold increase in their 5′-IMP nucleotidase activity compared with wild-type NT5C2, respectively (Fig. 3).

To formally test the role of NT5C2 mutations in chemotherapy resistance, we analyzed the effects of wild-type and relapse-associated mutant NT5C2 expression in the response of CCRF-CEM T-ALL cells to 6-MP and 6-TG (Fig. 4). Cell viability analysis in the presence of increasing drug concentrations demonstrated increased resistance to 6-MP and 6-TG therapy in cells expressing K359Q, R367Q and D407A NT5C2 compared with empty vector and wild-type NT5C2 controls (Fig. 4, Supplementary Fig. 2 and Supplementary Table 11). We obtained similar results in the CUTL11 T-ALL cell line (Fig. 4, Supplementary Fig. 2 and Supplementary Table 11). We then tested the effects of relapse-associated NT5C2 mutations in the response to nelarabine, an arabinosylguanine (AraG) prodrug that is highly active in relapsed T-ALL, and AraG7–30. Notably, both nelarabine and AraG were equally active in cells expressing relapse-associated NT5C2 mutations compared to controls (Supplementary Fig. 3).

Prolonged maintenance treatment with 6-MP is essential to obtain durable remissions in the treatment of ALL2–4. Indeed, low adherence to 6-MP treatment, defined as less than 95% compliance, results in increased relapsed rates and may account for as many as 59% of all ALL relapses5. In this context, our results highlight the prominent role of relapse-specific mutations in NT5C2 as a mechanism of resistance to 6-MP and a genetic driver of relapse in ALL. In addition, and most notably, a lack of nelarabine crossresistance in the cells expressing activating NT5C2 alleles analyzed here suggests that these mutations may not impair the effectiveness of nelarabine-based salvage therapies in relapsed T-ALL.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
G.T. and A.P.-G. performed validation and recurrence mutation analysis, enzymatic activity and cell drug-resistance assays. Z.C. performed structure function analysis and analyzed Illumina sequence data. H.K. analyzed Illumina sequence data. V.T. analyzed genomic data from diagnostic and relapse T-ALLs. M.A. performed validation analysis of Illumina sequencing results. M.P., G.B., E.P., J.R., J.H., I.M.R., M.S.T. and R.K.-S. contributed clinical samples and clinical correlative information. T.P. directed and supervised mutation analysis. R.R. directed and supervised the analysis of Illumina sequencing data. A.F. designed the study, directed and supervised research and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Patient samples. DNAs from leukemic T-ALL blasts at diagnosis and relapse and matched remission lymphocytes were provided by the Hemato-Oncology Laboratory at University of Padua, Italy; the Eastern Cooperative Oncology Tumor Bank Laboratory in New York, New York, USA; and the Department of Pediatric Oncology/Hematology at the Charité-Universitätsmedizin Berlin in Berlin, Germany. Informed consent was obtained at study entry. We collected and analyzed samples under the supervision of the local Columbia University Medical Center Institutional Review Board. We selected samples for whole-exome sequencing on the basis of the availability of sufficient DNA from diagnosis, remission and relapse samples, and we evaluated high tumor content at relapse on the basis of copy number analysis of T cell receptor–associated deletions.

Whole-exome capture and next generation sequence analysis. We used matched diagnostic remission and relapsed DNA samples from five patients with T-ALL from the University of Padua treated under Associazione Italiana Ematologia Oncopedia Pediatrica (AIEOP) protocols (Supplementary Table 1) for exome capture with the SureSelect 50 Mb All Exon kit (Agilent Technologies) following standard protocols. We performed paired-end sequencing (2 × 100 bp) by using HiSeq2000 sequencing instruments at Centrillion Biosciences. Illumina HiSeq analysis produced between 60 million and 120 million paired-end reads per sample. We mapped reads to the reference genome hg19 using the Burrows-Wheeler Aligner (BWA) alignment tool version 0.5.9. The mean depth (defined as the mean number of reads covering the captured coding sequence of a haploid reference) was 50×, with 80% of the genome covered more than 10× and 57% covered more than 30×. We identified sites that differed from the reference (called here variants) in each sample independently. We constructed empirical priors for the distribution of variant frequencies for each sample. We obtained high-credibility intervals (posterior probability ≥10−5) for the corresponding change in frequency between tumor and normal samples using the SAVI (Statistical Algorithm for Variant Identification) algorithm developed at Columbia University32,33. The number of germline SNPs in the coding region was 18,000, which is comparable with previous reports2,5. Most of the candidate germline SNPs (16,000, or ~90% of germline variants) were reported in the dbSNP database. We identified candidate somatic variants using the following criteria: variant total depth in tumor and normal >10× and <300×, variant frequency >15% in tumor and <3% in normal and ≥2× change in frequency from the normal with high posterior probability (2 × 10−5). Also, to remove systematic errors, we excluded all variants that were found in unaffected individuals. In addition, to eliminate ambiguous mapping from captured pseudogenes and regions of low complexity, each variant with a flanking 20-base context sequence around its genomic position was mapped to the hg19 reference using the BLAST algorithm. We kept in the list only those with unique mappability; that is, we required the 41-base sequence to uniquely map to the reference genome with only one mismatch.

To discern the regions of loss of heterozygosity (LOH), we used the SAVI-calculated high-credibility intervals for the variants in dbSNP, which correspond to the change in their frequency between tumor and normal samples. In an LOH event, depending on whether the reference or the dbSNP allele was lost, at least a 1% or at most a −1% change in frequency from the normal is expected. Therefore, by segmenting the regions covering more than ten dbSNP variants with significantly changed frequencies, we were able to identify the LOH regions.

Mutation validation and analysis of recurrence. We designed primers flanking exons containing candidate somatic variants using Primer3 (http://frodo.wi.mit.edu/primer3/) and used them for PCR amplification from whole genome–amplified (WGA) tumor, relapse and matched normal (remission) DNAs. We analyzed the resulting amplicons by direct bidirectional dideoxynucleotide sequencing with a validation rate of 97%. After exome sequence analysis of 5 diagnostic relapse and remission T-ALL AIEOP samples from the University of Padua (Supplementary Table 1), we used 18 additional patient samples from the same institution for the analysis of recurrence of TP53, BANP, RPL11, NRAS and NT5C2 (Supplementary Table 1).

We subsequently extended this series to additional relapse T-ALL samples from the University of Padua (n = 13) and the Charité-Universitätsmedizin Berlin (n = 67) (Supplementary Table 1) and to relapsed patients with B-precuror ALL from the University of Padua (n = 35) for extended mutation analysis of NT5C2 (Supplementary Table 1). We used two cohorts of diagnostic patients with T-ALL from ECOG (n = 23) and diagnostic patients with B-precuror ALL from the University of Padua (n = 27) to verify the absence of NT5C2 mutations in diagnostic ALL specimens (Supplementary Table 1).

Structural depiction and analysis. We identified structural coverage of the NT5C2 protein through use of the PSI-BLAST and SKAN algorithms; we subsequently mapped viable structures to all NT5C2 isoforms and analyzed them using Chimera Suite34,35. We aligned structurally the PDB structures 2XCW, 2XCX, 2XCB, 2XCV, 2XJR, 2XJC, 2XJD, 2XJE, 2JC2 and 2JC9 and subsequently analyzed the composite structure to assess conformational flexibilities34. We structurally modeled NT5C2 mutations using the I-TASSER software suite and subsequently refined and analyzed them by minimization and rotamer library analysis in Chimera34,36. We predicted protein stability changes resulting from mutation through use of the SDM potential energy statistical algorithm and associated software37. We created all structural images using UCSF Chimera34.

Site-directed mutagenesis. We generated the NT5C2 mutations K359Q, R367Q and D407A by site-directed mutagenesis on the mammalian expression pLOCT5C2 vector (Open Biosystems) using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions.

Cell lines. We cultured CCRF-CEM and CUPLL1 cells in RPMI-1640 medium supplemented with 10% FBS, 100 U ml−1 penicillin G and 100 μg ml−1 streptomycin at 37 °C in a humidified atmosphere under 5% CO2. We maintained HEK293T cells under similar conditions in DMEM media.

Lentiviral production and infection. We transfected the lentiviral constructs pLOC-NT5C2, pLOC-NT5C2-359, pLOC-NT5C2-367 and pLOC-NT5C2-407 and the pLOC-RFP control plasmid with Gag-Pol– and V-SVG–expressing vectors into HEK293T cells using JetPEI transfection reagent (Polyplus). We collected viral supernatants after 48 h and used them for infection of CCRF-CEM and CUPLL1 cells by spinoculation. After infection, we selected cells for 5 μg ml−1 blasticidin and ficolled them the day before experiments.

Western blot. Western blot analysis was performed using a rabbit polyclonal antibody to NT5C2 (1:1,000, Abcam, ab96084) and a goat polyclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000, Santa Cruz Biotechnology, sc-20357) using standard procedures.

Cell viability and chemotherapy drug response. We determined cell viability by measurement of the metabolic reduction of the tetrazolium salt MTT using the Cell Proliferation Kit I (Roche) following the manufacturer’s instructions. We performed experiments in triplicate. We analyzed viability at 48 h or 72 h after initiation of treatment with 6-MP, 6-TG, nelarabine and AraG.

Recombinant protein production and purification. We cloned full-length complementary DNA constructs encoding wild-type, K359Q, R367Q and D407A NT5C2 with a N-terminal hexahistidine (His6) tag in the pET28a-LIC expression vector using the In-Fusion HD PCR cloning system (Clontech) as per the manufacturer’s instructions. We expressed recombinant proteins from Rosetta 2(DE3) Escherichia coli cells by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C. We harvested cells and lysed them in lysis buffer (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.5 mg ml−1 lysozyme and 20 mM imidazole) supplemented with Complete EDTA-free protease inhibitor (Roche). We purified His6-tagged NT5C2 proteins by binding them to nickel-Sepharose beads and eluting them with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and 300 mM imidazole. We removed imidazole by buffer
exchange using PD-10 desalting columns (GE Healthcare). We assessed protein expression and purity by SDS-PAGE and Coomassie staining.

**5′-NT assay.** We assessed 5′-NT activity of purified recombinant wild-type and mutant NT5C2 proteins using the 5′-NT Enzymatic Test Kit (Diazyme) according to the manufacturer’s instructions. The assay measures the enzymatic hydrolysis of inosine 5′-monophosphate to inosine, which is reacted further to hypoxanthine by purine nucleoside phosphorylase and then to uric acid and hydrogen peroxide by xanthine oxidase. H₂O₂ is quantified using a Trinder reaction. We calculated 5′-NT activity levels using a calibrator of known 5′-NT activity as standard. We performed assays in triplicate in an Infinite M200 Tecan plate reader.

**Statistical analyses.** We evaluated differences in the percentages of wild-type and mutant NT5C2 in patients with ALL in different relapsed categories using Fisher’s exact test. We analyzed the equality of categorical and continuous variables by Fisher’s exact test and Mann-Whitney U test, respectively.