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

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Abstract

Acute lymphoblastic leukemia (ALL) is an aggressive hematological tumor resulting from the malignant transformation of lymphoid progenitors. Despite intensive chemotherapy, 20% of pediatric and over 50% of adult ALL patients fail to achieve a complete remission or relapse after intensified chemotherapy, making disease relapse and resistance to therapy the most significant

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AUTHOR CONTRIBUTIONS GT and APG performed validation and recurrence mutation analysis, enzymatic activity and cell drug resistance assays; ZC performed structure function analysis and analyzed Illumina sequence data; HK analyzed Illumina sequence data; VT analyzed genomic data from diagnostic and relapse T-ALLs; MA performed validation analysis of Illumina sequencing results; MP, GB, EP, JR, JH and RK-S contributed clinical samples and clinical correlative information; TP directed and supervised mutation analysis; RR directed and supervised the analysis of Illumina sequencing data; AF designed the study, directed and supervised research and wrote the manuscript.

Methods are included in the supplementary material

challenge in the treatment of this disease[1,2]. Using whole exome sequencing, here we identify mutations in the cytosolic 5'-nucleotidase II gene (*NT5C2*), which encodes a 5'-nucleotidase enzyme responsible for inactivation of nucleoside analog chemotherapy drugs, in 20/103 (19%) relapse T-ALLs and in 1/35 (3%) relapse B-precursor ALLs analyzed. 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 of ALL includes initial treatment with high dose combination chemotherapy, which obtains clinical and hematologic remission in over 90% of cases. This is typically followed by additional rounds of highly intensive therapy aimed to further reduce disease burden; and then by a 2 year long lower intensity maintenance therapy in which treatment with oral 6-mercaptopurine plays a particularly important role[3,4]. Patients with relapsed ALL generally receive a more intense treatment. However, despite these efforts, their outcome remains unsatisfactory with cure rates of less than 40%[5]. This is particularly the case in patients with relapsed T-ALL and in cases with primary resistance or early relapse, which is associated with higher risk of failure to achieve a second complete remission, shorter duration of chemotherapy response and poor survival[6,7]. Much effort has been spent on 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 question we performed whole exome sequencing of matched diagnosis, remission and relapse DNA samples from 5 pediatric T-ALL patients (Supplementary Table 1). This analysis identified a mean mutation load of 13 somatic mutations per sample (range 5 – 17) (Supplementary Table 2). Out of 60 somatic mutations identified in total, 17 mutations were present at diagnosis and at relapse, 24 genes were selectively mutated in relapsed T-ALL samples and 19 mutations were present only at diagnosis. Moreover, 4 of the 5 relapsed cases analyzed showed the presence of at least one somatic mutation present also at diagnosis, together with secondary mutations specifically acquired at the time of relapse. In addition, 4 out of the 5 cases showed absence of at least one mutation marker present at diagnosis during disease progression leading to relapse. Single nucleotide polymorphism analysis of exome sequencing results ruled out that loss of these markers was due to loss of heterozygosity at relapse (Supplementary Table 3). This result is consistent with previous studies based on copy number alteration analyses[8–10] and supports that relapsed ALLs can originate as derivates 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*[11], *WT1*[8] and *DNM2*[12] in addition to numerous new genes not implicated before 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 (TP53 R213Q), *BANP* (BANP H391Y)[13] and *RPL11* (RPL11 R18P)[14]. Notably, mutations in *TP53* have been reported in about 10% of relapsed ALL cases and are associated with a particularly poor prognosis[15]. Given the prominent role of TP53 pathway in DNA damage induced apoptosis[16], we performed

extended mutation analysis of the *TP53*, *BANP* and *RPL11* genes in 18 additional diagnostic and relapsed T-ALL samples (Supplementary Table 1). This analysis failed to identify additional *TP53* or *BANP* mutations, but showed the presence two additional somatic *RPL11* mutant alleles; one present both at diagnosis and relapse (RPL11 X178Q); and the other one (RPL11 G30fs) specifically mutated at relapse. Relapse-associated mutations also included a prototypical activating mutation in the *NRAS* oncogene (*NRAS* G13V). Notably, *NRAS* mutations in ALL have been associated with poor outcome[17] and are particularly prevalent in early T-cell precursor ALLs[12,18], a group of high risk leukemias with poor prognosis[19]. Extended mutation analysis of *NRAS* in relapsed T-ALL cases demonstrated the presence of 2 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 remarkable finding in our exome sequence analysis was the presence of a relapse-associated heterozygous mutation in the *NT5C2* gene (NT5C2 K359Q). NT5C2 is a ubiquitous enzyme responsible for the final dephosphorylation of 6-hydroxypurine nucleotide monophosphates such as IMP, dIMP, GMP, dGMP and XMP before they can be exported out of the cell[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-mercaptopurine (6-MP) and 6-thioguanine (6-TG)[22], two nucleoside analogs commonly used in the treatment of ALL. Mutation analysis of an extended panel of 98 relapse T-ALL (Supplementary Table 1) and 35 relapse B-precursor ALL samples (Supplementary Table 1) identified 22 additional mutations T-ALL and one additional *NT5C2* mutation in a B-precursor ALL patient in first relapse (Fig. 1 and Supplementary Table 4). Strikingly, 13 of these samples harbored the same NT5C2 R367Q mutation, 4 cases showed a recurrent NT5C2 R238W mutation and 2 samples harbored a L375F single amino acid substitution (Fig. 1 and Supplementary Table 4). In each of the 9 cases for which original diagnostic DNA was available for analysis, *NT5C2* mutations showed to be specifically acquired at the time of relapse. No *NT5C2* mutations were identified in 23 T-ALL and 27 B-precursor ALL additional diagnostic samples, further supporting the specific association of *NT5C2* mutations with relapsed disease. Analysis of clinical and molecular features associated with *NT5C2* mutant relapsed T-ALLs treated in Berlin Frankfurt Münster (BFM) group based clinical trials (ALL-BFM 95, ALL-BFM 2000, COALL 06-97, NHL-BFM 95 and Euro-LB 02) (Supplementary Table 1) showed an association of *NT5C2* mutations with early disease recurrence (very early or early relapse *vs.* late relapse, $P < 0.05$) and relapse under treatment ($P = 0.002$) independently of treatment protocol (Supplementary Tables 5–10).

Given the described role of NT5C2 in the metabolism and inactivation of nucleoside analog drugs[22–24]; the recurrent finding of the NT5C2 R367Q, NT5C2 R238W and NT5C2 L375F alleles; and the reported association of increased levels of nucleotidase activity with thiopurine resistance and worse clinical outcome[25], we hypothesized that relapse-associated *NT5C2* mutations may represent gain of function alleles with increased enzymatic activity. Detailed structure-function analysis of the NT5C2 K359Q mutation further supported this hypothesis. Thus, comparison of the wild type NT5C2 structure and models of the mutant

NT5C2 K359Q protein show that this mutation could result in increased NT5C2 activity by mimicking the effect of positive allosteric regulators (Fig. 2a). Allosteric activation of NT5C2 is mediated by binding of ATP, dATP, Ap_4A and 2,3-BPG to an allosteric pocket proximal to the NT5C2 active site (Fig. 2a). Occupancy of this regulatory site results in increased ordering of an alpha helix formed by residues G355-E364 (Helix A), which in turn displaces F354 from the catalytic center and moves D356 into the active site of the protein (Fig. 2b,c). Similarly, our model predicts that the NT5C2 K359Q mutation could increase the Helix A stability and reduce its solvent accessibility, resulting in an active configuration with displacement of F354 out of the NT5C2 active site and positioning D356 into the catalytic center of the enzyme (Fig. 2b–e). Consistent with this prediction, 5'-nucleotidase assays using NT5C2 K359Q recombinant protein demonstrated a 48-fold increase in enzymatic activity compared wild type NT5C2 (Fig. 3). An additional structurally interesting allele is the NT5C2 Q523* nonsense mutation, which removes an inhibitory region located in the C-terminal segment of the NT5C2 protein[26]. In addition, and despite the absence of clear structural cues suggesting a role of other mutations in NT5C2 activation, nucleotidase activity analysis of NT5C2 R367Q and NT5C2 D407A mutant proteins revealed an 18 fold and a 16 fold increase in their 5'-IMP nucleotidase activity compared with wild type NT5C2, respectively (Fig. 3).

Finally, and 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-mercaptopurine (6-MP) and 6-thyogunanine (6-TG) (Fig. 4). Cell viability analysis in the presence of increased drug concentrations demonstrated increased resistance to 6-MP and 6-TG therapy in cells expressing NT5C2 K359Q, NT5C2 R367Q and NT5C2 D407A compared with empty vector and wild type NT5C2 controls (Fig. 4, Supplementary Fig. 2 and Supplementary Table 11). Similar results were obtained in the CUTLL1 T-ALL cell line (Fig. 4, Supplementary Fig. 2 and Supplementary Table 11). Finally, we tested the effects of relapsed-associated NT5C2 mutations in the response to nelarabine – an AraG precursor highly active in relapsed T-ALL– and AraG [27–30]. Strikingly, both nelarabine and AraG showed to be equally active in cells expressing relapse-associated NT5C2 mutations compared to controls (Supplementary Fig. 3).

Prolonged maintenance treatment with 6-mercaptopurine is essential to obtain durable remissions in the treatment of ALL[3,4]. Indeed, low-adherence to 6-mercaptopurine treatment, defined as less than 95% compliance, results in increased relapsed rates and may account for as much as 59% of all ALL relapses[31]. 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, the lack of nelarabine cross resistance in 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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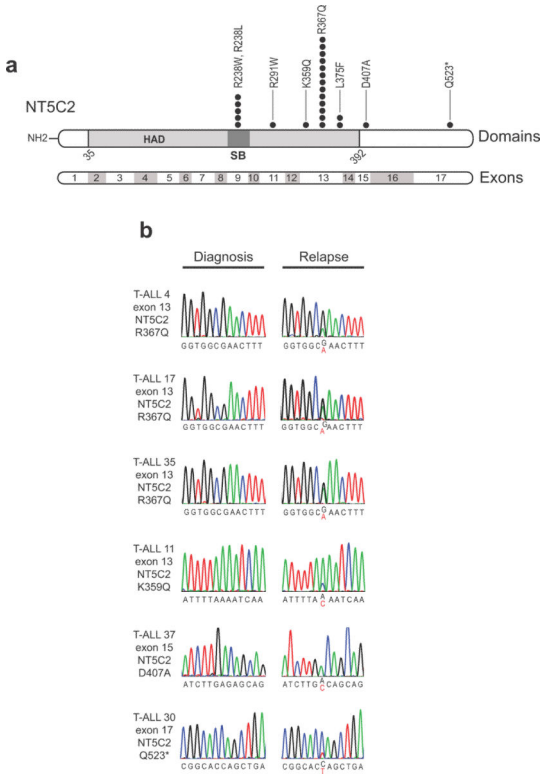

Figure 1. *NT5C2* mutations in relapsed pediatric T-ALL

(**a**) Schematic representation of the structure of the NT5C2 protein. The haloacid dehalogenase (HAD) and the substrate binding domains (SB) are indicated. *NT5C2* mutations identified in relapsed pediatric samples are shown. Filled circles represent heterozygous mutations. Multiple circles in the same amino acid position account for 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.

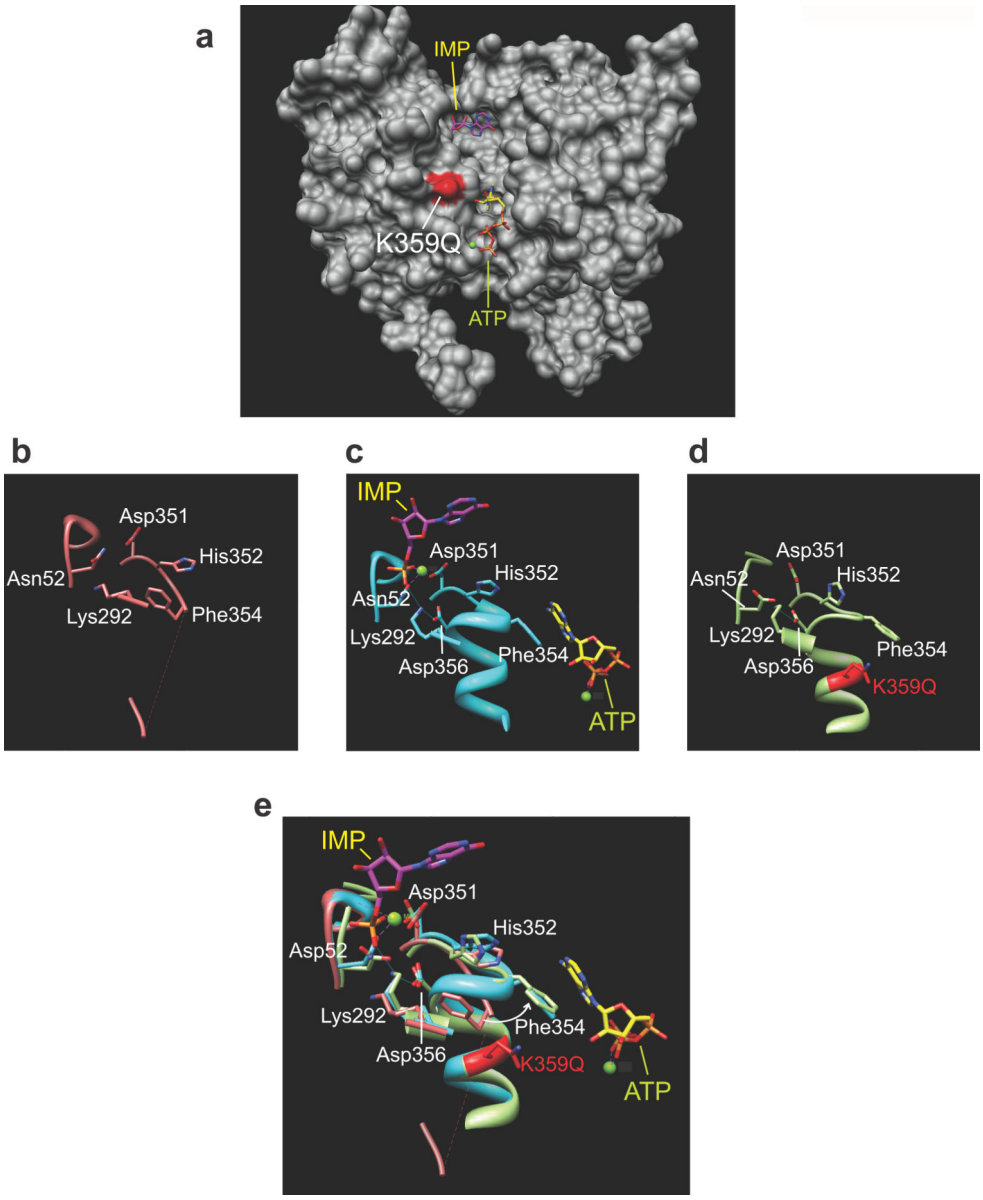

Figure 2. Structure-function analysis of the NT5C2 K359Q mutant protein

(**a**) Molecular surface representation of NT5C2 protein structure. The position of the NT5C2 K359Q mutation found is highlighted in red. The substrate inosine monophosphate (IMP) is depicted in purple; the ATP allosteric activator is shown in yellow. (**b**) Structure representation of the NT5C2 catalytic center and allosteric regulatory site devoid of substrate or ligands (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 shown in **b–d**. The white arrow indicates the repositioning of Phe354 from the inactive NT5C2 configuration to the active –ATP-bound NT5C2 and NT5C2 K359Q– structures. Mg^{2+} ions are depicted as green spheres.

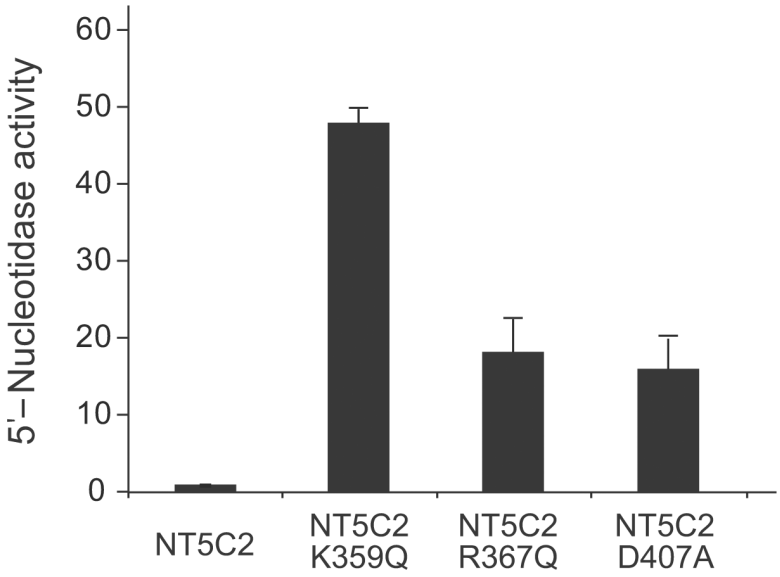

Figure 3. Increased 5'-IMP nucleotidase activity in NT5C2 mutant proteins

5'-Nucleotidase activity levels of recombinant mutant proteins relative to wild type NT5C2 control are shown. Data are means ± s.d.

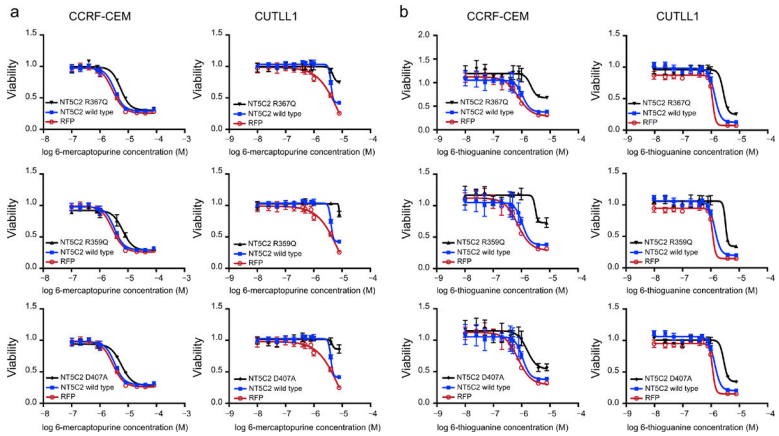

Figure 4. Expression of *NT5C2* mutations in ALL cells induces resistance to chemotherapy with 6-MP and 6-TG

(**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 control (RFP), treated with increased concentrations of 6-mercaptopurine (6-MP). (**b**) 6-Thioguanine (6-TG) dose response cell viability curves. Data is shown as means ± s.d.