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DISSERTATION

Clinical and biological relevance of mutations in the *cytosolic 5´-nucleotidase II* (*NT5C2*) gene in children with relapsed acute lymphoblastic leukemia

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Abstract

Relapse of acute lymphoblastic leukemia is a major cause of death in childhood cancer. Activating mutations in the *cytosolic 5´-nucleotidase II* (*NT5C2*) are considered to drive relapse formation in acute lymphoblastic leukemia (ALL) by conferring purine analogue resistance. *NT5C2* mutations are found in 20% of relapses of pediatric T-ALL and in 3-45% of relapses of the more frequent B-cell precursor ALL. To examine the clinical effects of *NT5C2* mutations in relapsed ALL, we analyzed *NT5C2* in 455 relapsed B-cell precursor ALL patients treated within the ALL-REZ BFM 2002 relapse trial using sequencing and sensitive allele-specific real-time PCR. We detected 105 *NT5C2* mutations in 74 (16%) of 455 patients with B-cell precursor ALL relapse. Mutation hot spots were p.R39Q and p.R367Q. Two-thirds of relapses harboured subclonal and only one third clonal mutations. *NT5C2* mutations rarely occurred in relapses with *ETV6/RUNX1* fusion gene (p=0.001), but were frequent in relapses of so-called B-other-ALL (p<0.001). Surprisingly, both subclonal and cloncal *NT5C2* mutations were associated with inferior event-free survival compared to wild-type (19% and 25% *vs* 53%, p<0.001). Clonal *NT5C2* mutations were highly associated with an increased cumulative incidence of second relapse (clonal 56%, subclonal 41%, wild-type 28.8%, p=0.002). Relapses with subclonal *NT5C2* mutations showed an increased rate of nonresponse (subclonal 33%, clonal 9%, wild type 9%, p<0.001) and 80% of those patients who responded to relapse treatment presented with high minimal residual disease levels at the end of relapse induction treatment. However, subclonal, but not clonal *NT5C2* mutations were associated with reduced event-free survival in multivariable analysis (HR 1.89 [95% CI 1.28-2.69], p=0.001). To investigate whether outgrowth of *NT5C2* mutant subclones during relapse treatment contributes to the poor patient response and outcome, we analyzed follow-up samples and found that subclonal *NT5C2* mutations never grew out to clonal mutations at the time of nonresponse or second relapse. In fact, 82% of subclonal *NT5C2* mutations became undetectable at the time of nonresponse or second relapse, and in 71% patients subclonal *NT5C2* mutations were undetectable already after relapse induction treatment. These results show that subclonal *NT5C2* mutations define relapses associated with high risk of treatment failure and at the same time emphasize that their role in outcome is complex and goes beyond mutant NT5C2 acting as a targetable driver during relapse progression. Sensitive, prospective identification of *NT5C2* mutations is warranted to improve the understanding and treatment of this aggressive ALL relapse subtype.

Zusammenfassung

Kinder mit Rezidiv einer akuten lymphoblastischen Leukämie (ALL) werden mit einer intensiven Zweitlinien-Chemotherapie behandelt, dennoch zeigen rezidivierte Leukämien häufig Resistenzen. Aktivierende Mutationen im *NT5C2* (*cytosolic 5´-nucleotidase II*) Gen wurden beschrieben eine Resistenz gegen Purin Analoga zu verursachen und somit das Auswachsen des ALL-Rezidives zu unterstützen. Mutationen in *NT5C2* wurden in 20% bei T-ALL-Rezidiven und 3-45% bei B-Zell Vorläufer ALL-Rezidiven identifiziert. Um die klinischen Auswirkungen von *NT5C2* Mutationen zu untersuchen, analysierten wir *NT5C2* in 455 B-Zell Vorläufer ALL-Rezidiven von pädiatrischen Patienten, welche nach der ALL-REZ BFM 2002 Rezidivtherapie behandelt worden waren, mittels Sequenzierung und sensitiver allelspezifischer Echtzeit-PCR. Insgesamt identifizierten wir 110 Mutationen in 75 (16,5%) von 455 ALL-Rezidiv Patienten. Die häufigsten Mutationen waren p.R39Q und p.R367Q. Zwei Drittel aller Mutationen waren subklonal in unter 15% der Leukämiezellen vorhanden und nur ein Drittel hauptklonal. Mutationen in *NT5C2* traten selten zusammen mit dem Fusionsgen *ETV6/RUNX1* (p=0.001), jedoch häufig in der Gruppe ohne etablierte zytogenetische Veränderungen, der sogenannten B-other ALL, auf (p=0.001). Patienten mit einem B-Zell ALL-Rezidiv mit subklonalen sowie hauptklonalen *NT5C2* Mutationen hatten ein signifikant schlechteres ereignisfreies Überleben als Patienten mit wildtypischem *NT5C2* (19% und 25% versus 53%, p<0.001). Allerdings waren nur subklonale, aber nicht hauptklonale *NT5C2* Mutationen in der multivariablen Analyse unabhängig mit verringertem ereignisfreiem Überleben assoziiert (HR 1.89 [95% CI 1.28-2.69], p=0.001). Zudem hatte die Patientengruppe mit ALL-Rezidiv mit subklonalen *NT5C2* Mutationen eine erhöhte Rate an Fällen, die nicht auf die Rezidivtherapie ansprachen (*engl*. nonresponse) (subklonal 33%, hauptklonal 9%, Wildtyp 9%, p<0.001). Patienten mit subklonalen *NT5C2* Mutationen, welche auf die Rezidivtherapie ansprachen, hatten in 80% der Fälle erhöhte Level von minimaler Resterkrankung nach der Zweitlinien-Induktionsbehandlung. Um zu untersuchen, ob subklonale *NT5C2* Mutationen während der Rezidivtherapie auswachsen und das schlechte Ansprechen auf die Rezidivtherapie verursachen, untersuchten wir Proben, die im Verlauf sowie nach der Rezidivtherapie entnommen wurden. Tatsächlich wuchsen subklonale *NT5C2* Mutationen nie zu einer hauptklonalen Mutation aus, und 27 von 33 (82%) subklonalen Mutationen waren zum Zeitpunkt der Nonresponse oder des zweiten Rezidivs nicht mehr zu detektieren. In 10 von 14 Patienten konnte die subklonale *NT5C2* Mutation bereits nach der Zweitlinien-Induktionsbehandlung nicht mehr detektiert werden. Diese Ergebnisse zeigen, dass subklonale *NT5C2* Mutationen Rezidive definieren, welche mit einem hohen Risiko des Therapieversagens nach dem Rezidiv assoziiert sind. Zudem weisen sie darauf hin, dass die Rolle der subklonalen Mutationen bei der Progression des ALL-Rezidivs komplex ist und über *NT5C2* als einzelnen Verursacher hinausgeht.

Extended Introduction

(*German* "Manteltext")

In the following, an in-depth review of the current stage of research, a detailed description of the methods and of the most important findings including a discussion of their clinical and scientific importance are given. The extended introduction serves a deeper understanding of the publication Barz et al. Subclonal *NT5C2* mutations are associated with poor outcomes after relapse of pediatric acute lymphoblastic leukemia, *Blood*, 2020.

All figures and tables referenced with "Publication Table (Pub. Tab.)" or "Publication Figure (Pub. Fig.)" are presented in the publication (Barz et al. 2020, *Blood*[1]) and are not included in this extended introduction. All other figures and tables are referenced with "Table/Figure" as usual.

1. Introduction

1.1 Leukemia

Leukemia (Greek: *leukós* "white", *haima* "blood") is a blood and bone marrow disease caused by uncontrolled growth of malignant hematopoietic progenitor cells located in the bone marrow, which has a strong impact on normal hematopoiesis. The consequence is a reduction in erythrocytes, granulocytes and platelets in the blood leading to clinical symptoms such as anemia, increased sensitivity to infections and an increased bleeding tendency. The leukemic cells can spread from the bone marrow throughout the body, infecting more tissues and the organ system.[2] Leukemia can be classified by disease course (acute versus chronic) and affected hematopoietic cell type (lymphocytic/lymphoblastic versus myeloid).[3] Whereas acute leukemia develops and progresses within a few months, chronic leukemia persists over years.[3] Acute myeloid leukemia (AML) is caused by aberrant precursors of granulocytes, monocytes and platelets, which are formed from myeloid stem cells.[2–4] This form of leukemia occurs more frequently in adulthood.[2,4] Acute lymphoblastic leukemia (ALL) is caused by malignant transformation of B- or T-cell progenitors developing from lymphoid stem cells. ALL mainly appears in infancy between the ages of 3 to 5 and is the most commonly diagnosed malignant disease in childhood.[4–6]

1.2 Acute lymphoblastic leukemia

ALL accounts for approximately 30% of all childhood cancers and is the most common cause of cancer-related death before the age of 20.[5–7] Approximately 500 children and adolescents up to the age of 14 are diagnosed with ALL in Germany per year. Due to the small annual number of new cases (3.8 cases per 100,000 people) it is considered a rare disease (≤50 cases per 100,000 people).[5,8] ALL is usually diagnosed by cytological examination of the bone marrow, the blood and cerebrospinal fluid. The diagnosis is considered certain if more than 25% of bone marrow cells with a nucleus are lymphoblasts.[2] Furthermore, immune phenotyping is determined by flow cytometry to describe the hematopoietic cell type and the degree of hematopoietic differentiation. Within lymphoblastic leukemia, two major subtypes can be distinguished, specifically the B- and T-lineage subtype.[4,6] B-cell precursor (BCP) ALL is the predominant subtype accounting for 85% of pediatric cases, whereas T-lineage ALL (T-ALL) occurs less frequently.[7] B-lineage ALL is divided into the following subtypes or differentiation grades: pro-B-ALL, common-ALL, pre-B-ALL and mature B-ALL. The subtypes pro-B-ALL, common-ALL and pre-B-ALL are summarized as BCP-ALL. The mature B-ALL is equivalent to Burkitt's lymphoma, which has different cytomorphologic and genetic characteristics and is treated differently from BCP-ALL. T-ALL is defined by the expression of T-cell associated antigens. It is subclassified as early pro/pre-T-ALL,

cortical/thymic T-ALL and mature T-ALL. The cortical/thymic T-ALL is the most common T-ALL subtype.[4,9]

Environmental conditions as well as a genetic predisposition may influence the development of ALL.[2,4,6] Genome-wide studies have identified polymorphisms in for example the *ARID5B*, *ETV6* and *IKZF1* genes associated with high-risk of developing certain genetic ALL subtypes such as hyperdiploid ALL and T-ALL.[6,10,11] The association of inherited genetic variants with specific ALL subtypes indicates that inherited and early acquired genetic lesions (initiating events) may interact during development of leukemia (Fig. 1).[10–12] Additional acquired genetic alterations then further contribute to the formation of fully malignant ALL cells (Fig. 1), however, their interaction during this process is not yet fully understood in detail and subject of current research. Genetically, ALL is a heterogenous disease with a number of genetic subtypes.[13] Since the focus of this thesis lies on BCP-ALL, genetic alterations of this ALL subtype are described below. Early cytogenetic studies identified alterations such as aneuploidy (copy number alterations of whole chromosomes) and chromosomal translocations causing deregulation of tumor suppressors, oncogenes or hematopoietic regulators. The most common cytogenetic alterations in BCP-ALL are high hyperdiploidy (>50 chromosomes) found in 25% of cases and the t(12;21)(p13;q22) translocation (generating the *ETV6/RUNX1* fusion gene) found in another 25% of cases.[14,15] Other translocations are t(9;22)(q34;q11) encoding the *BCR/ABL1* fusion gene in 3% of pediatric cases, t(1;19)(q23;p13) encoding the *TCF3/PBX1* fusion gene in 5% of cases and *MLL*-rearrangements in 5% of cases (mostly in infants).[6,16–18] Hypodiploidy (<44 chromosomes) occurs in approximately 5% of cases.[19] These chromosomal alterations define the classical cytogenetic subtypes of childhood ALL.[13] More recently, genomic studies identified additional genetic alterations (e.g. mutations/focal deletions) in genes encoding transcriptional regulators of B-cell differentiation such as *PAX5*, *EBF1* and *IKZF1*, as well as components of signaling pathways, cell cycle and apoptosis regulators and tumor suppressors that may cooperate with large structural lesions to drive leukemogenesis in ALL (Fig. 1).[20,21,22]

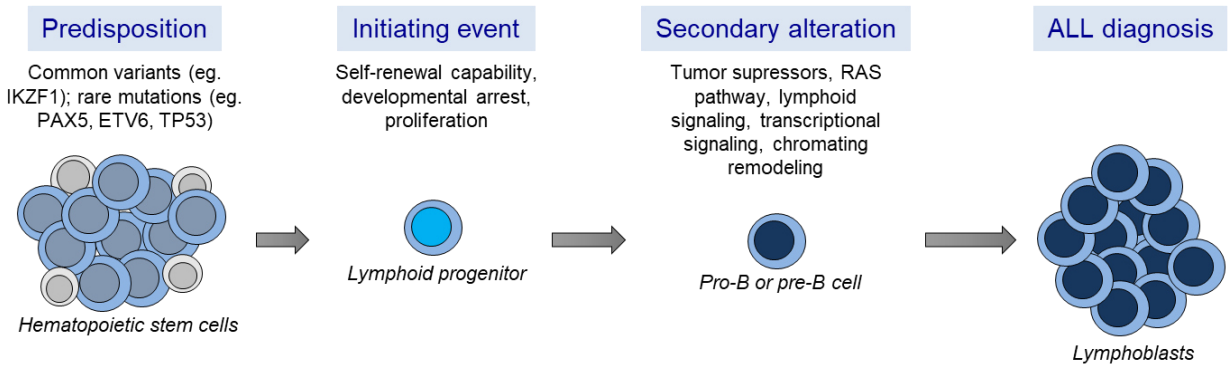

Figure 1. Model of successive acquisition of genetic alterations in the development of B-cell precursor ALL. Inherited genetic variants confer predisposition to ALL. Initiating alterations (e.g. translocations) are required to transform a lymphoid progenitor. Secondary alterations (e.g. RAS pathway mutations) contribute for example to developmental arrest resulting in clinical manifestation of ALL. Adapted from Hunger and Mullighan, 2015.

Treatment of childhood ALL is based on risk-adapted multi-agent chemotherapy courses. Typically, curative ALL treatment protocols include an induction phase to induce remission, a consolidation phase with intensive treatment and a maintenance phase.[23] Allogeneic stem cell transplantation (allo-HSCT) is only used for very high-risk cases in primary ALL.[24] The probability of children with ALL for long-term survival after treatment has greatly improved in the past 40 years due to extensive clinical research. Several study groups from developed countries performed clinical trials aiming at treatment optimization (e.g. clinical trials of the German ALL-BFM [Berlin Frankfurt Münster] group, of the American Children's Cancer Group and Children's Oncology Group and of the British UK Medical Research Council Working Party on Leukemia in Children).[23,25–29] Among other things, these trials showed that the chance of long-term remission of ALL was improved, when patients received several months of post-remission anti-metabolite-based maintenance therapy.[13,30] The backbone of maintenance therapy consists of daily oral administration of 6-mercaptopurine and weekly methotrexate for a duration of up to three years.[30] Some trials tested 6-thioguanine instead of 6-mercaptopurine and others monthly impulses of vincristine and glucocorticoids, but there was not much evidence for added benefit in overall survival.[13,31] Furthermore, 6-MP is the drug of choice due to less liver toxicity in comparison to 6-TG.[31] The clinical trials improved the survival rate of children with ALL from below 30% in 1960 to up to 90% in 2015.[6,13,17] The survival rate describes the percentage of treated patients, who achieved a continuous complete remission of ALL. The remaining 10% to 20% of children, who still succumb to the disease, had mostly suffered a relapse, which remains a major challenge in the treatment of ALL.[32,33]

1.3 Relapsed acute lymphoblastic leukemia in childhood

Statistically, 0.7 of 100,000 children in Germany suffer an ALL relapse per year.[5] Relapse of ALL implies that leukemic cells that had not been eradicated by ALL front-line therapy re-proliferate and expand. Several mechanisms were described to cause relapse of ALL, including amongst others the positive selection of residual, aggressive malignant cells, the presence of rare quiescent stem cells escaping chemotherapy or protection of malignant cells from chemotherapy by microenvironment niches.[34–43] Relapses frequently affect extramedullary tissues next to the bone marrow, such as the central nervous system (CNS) or the testes, which makes treatment of relapsed ALL more difficult than primary ALL.[4] In addition, the development of resistances to chemotherapy significantly impacts the induction of a second remission in relapsed patients.[36,37,44,45]

1.3.1 Development of ALL relapse

Several studies showed that ALL is a clonally heterogeneous disease that undergoes genetic and/or epigenetic changes from primary diagnosis to relapse.[38–40,42,43,46,47] Overall, the mutational burden of leukemic cells is higher at relapse than at primary diagnosis of ALL.[48,49] Relapse-acquired secondary alterations are for example deletions of *CDKN2A/B*[40], *IKZF1*[50] and *TP53*[36] as well as somatic mutations of *TP53*[36], *CREBBP*[40], *PRPS1*[38,51], *NT5C2*[38,39,52,53] and mismatch repair genes[38,39,42,54]. A crucial factor for the acquisition of mutations for example is the long systematic exposure to 6-mercaptopurine and methotrexate during maintenance therapy. The nucleotide analogue 6-mercaptopurine is a prodrug and intracellularly activated through the purine salvage pathway. PRPS1 (phosphoribosyl pyrophosphate synthetase 1) and NT5C2 (cytosolic 5´-nucleotidase II) participate in the metabolism of purine nucleotides and in the regulation of the intracellular purine nucleotide pool.[55] Mutations in these two genes were found specifically in relapsed ALL and were associated with early disease recurrence.[51–53] Leukemic cells harboring mutant PRPS1 or NT5C2 showed resistance against 6-mercaptopurine and 6-thioguanine *in vitro*, supporting a role in the formation of resistant leukemic cells during ALL maintenance treatment (Fig. 2).[38,39,51–53]

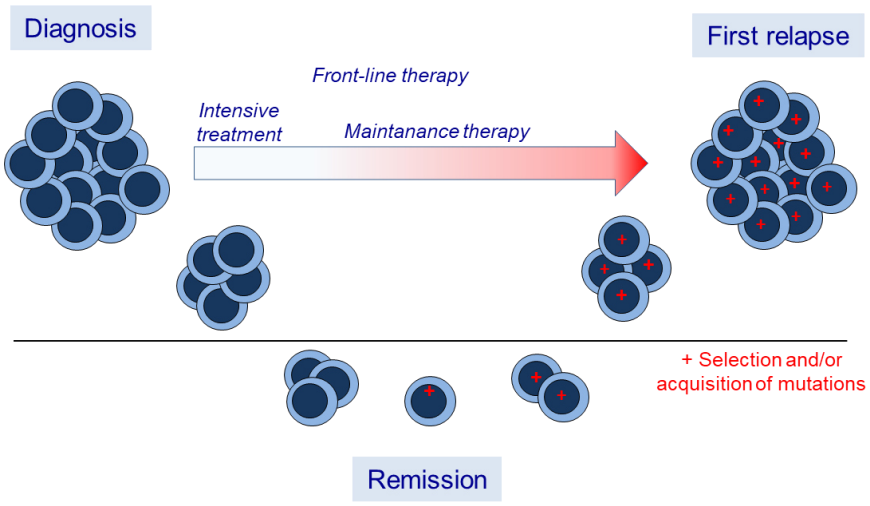

Figure 2. Acquisition of genetic alterations contributes to the development of ALL relapse. Front-line treatment eliminates leukemic cells, but leave subclones that acquire additional mutations (red crosses), which confer resistance to maintenance therapy and enable leukemic clones to expand. Model adapted from Mullighan et al. 2008 and Bhatla et al. 2014.

1.3.2 Treatment of relapsed ALL

In the early 1970s, first attempts were made to treat children with relapsed ALL. Even though second remissions could be achieved by intensive chemotherapy, long-term remission and survival rates after relapse were still poor.[56,57] Since the 1980s clinical trials for relapsed ALL from different study groups as the BFM (Berlin-Frankfurt-Münster, Germany), UKALL (United Kingdom ALL) and the POG (Pediatric Oncology, U.S.A.) optimized intensive chemotherapy regimen for relapsed ALL and defined risk factors that affect the patients' chance of survival.[56–59] The most

important factors are the time to relapse (duration of first remission from diagnosis to relapse), the location of the relapse (with or without bone marrow involvement) and the immunophenotype (BCP-ALL or T-ALL).[56–62] Patients with very early relapses of ALL (18 to 24 months from diagnosis) have the worst prognosis while patients with late relapses (≥6 months after completion of primary treatment or >3 years from diagnosis) have the best prognosis.[56–62] Patients with isolated bone marrow relapses have an inferior prognosis compared to patients with involvement of an extramedullary compartment and to patients with isolated extramedullary relapses.[63] Patients with relapsed T-ALL have a dismal prognosis compared to patients with relapsed BCP-ALL.[56–58] Accordingly, risk groups were defined using the time to relapse, location of relapse and immunophenotype,[57,60,63] supporting the implementation of risk-adapted treatment protocols in the mid-1990s including among other things allocation of high-risk patients to HSCT as most intensive treatment option[56,57]. Several studies showed that patients with high-risk relapsed ALL benefit from HSCT.[56–59] More recently, minimal residual disease (MRD) measurement and MRD-based allocation of intermediate-risk patients to HSCT was included in relapse treatment.[58,59,64,65] The MRD levels during treatment reflect the patients' responses to chemotherapy.[66] Similar to primary ALL, the early response of relapsed patients to second-line induction treatment is a strong prognostic factor.[66,67] The treatment optimization efforts raised the survival rates of relapsed patients to approximately 50%.[60,61,63,68–70] However, in subgroups event-free survival after relapse is highly heterogeneous and ranges between 10% and 75%.[60,61,63,68–70] For instance, survival rates of patients with T-lineage ALL relapse or with very early BCP-ALL relapse do not exceed 15%, whereas patients with late isolated extramedullary BCP-ALL relapse reach an event-free survival rate of 75%.[60,61,63,68–70]

1.3.3 The ALL-REZ BFM 2002 protocol

The ALL-REZ BFM 2002 protocol was developed for the Berlin-headed clinical trial on relapsed ALL of the same name that was open from 2002 till 2012. Similar to primary ALL, the protocol consisted of three phases: intensified induction, consolidation and maintenance therapy or allo-HSCT (Fig. 3). Based on the risk factors described above, relapsed patients were allocated to three different treatment arms corresponding to four risk (stratification) groups: S1 low-risk, S2 intermediate-risk, S3/S4 high-risk (Fig. 3, Tab. 1). For instance, a patient with very early bone marrow relapse occurring during initial therapy was stratified into S4, whereas a patient with late relapse after cessation of primary treatment and no bone marrow involvement was stratified into S1 (Tab. 1).[71] The response to relapse treatment was evaluated in serial bone marrow samples by microscopic, cytomorphological analysis and by quantification of minimal residual leukemic cells using real-time quantitative PCR of immunoglobulin and T-cell receptor (Ig/TCR) rearrangements (MRD measurement).[66,72–74]

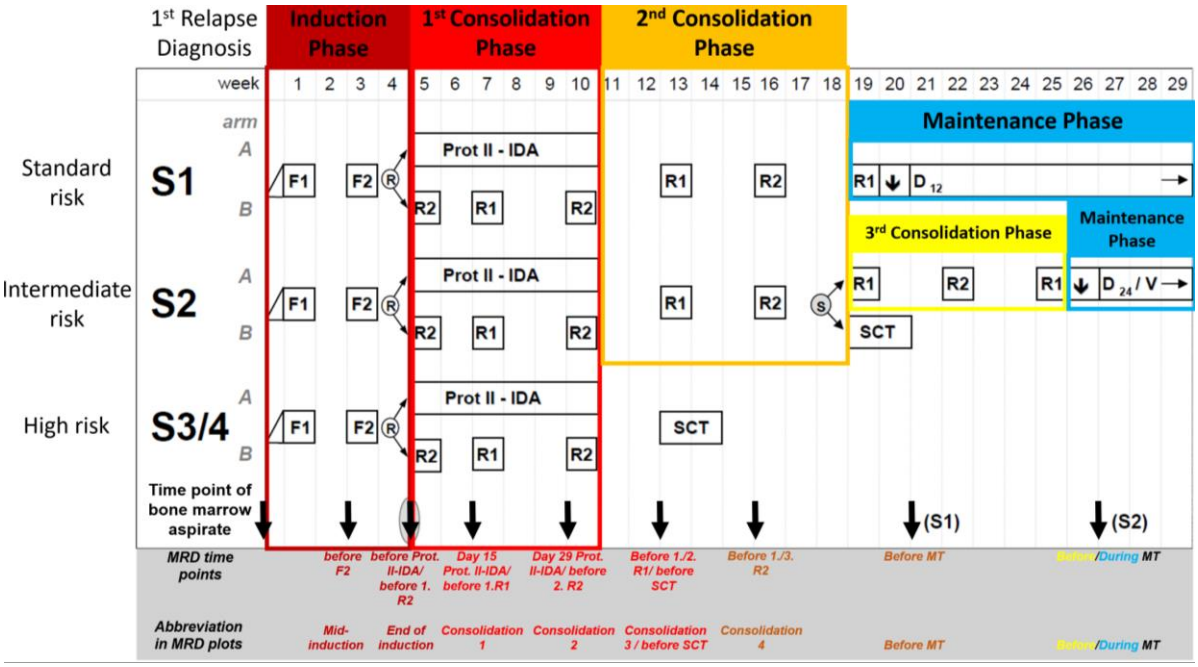

Figure 3 Treatment schedule of the ALL-REZ BFM 2002 protocol. Black arrows indicate time points of bone marrow aspiration for MRD. The encircled black arrow indicates the time point for post-induction stratification based on MRD. F1, F2, R1, R2 and Protocol II-IDA represent blocks of chemotherapy. Abbreviations: ↓ local radiation therapy, D12/D24, 12 or 24 months of maintenance therapy; MRD, minimal residual disease; R, randomization; S, stratification; SCT, allogeneic stem cell transplantation; V, VP-16-reinduction pulse. Reproduced from Eckert et al., JCO 2012.

Table 1. Definition of risk (stratification) groups S1 to S4 in the ALL-REZ BFM 2002 clinical trial for relapsed ALL.

	Immunophenotype BCP-ALL			**Immunophenotype T-ALL**		
Localization	Extra-medullary	Bone marrow	Bone marrow	Extra-medullary	Bone marrow	Bone marrow
*Time of relapse**	isolated	combined	isolated	isolated	combined	isolated
very early	S2	S4	S4	S2	S4	S4
early	S2	S2	S3	S2	S4	S4
late	S1	S2	S2	S1	S4	S4

Induction treatment lasted four weeks and aimed to induce complete remission that was defined as a regenerating bone marrow with less than 5% leukemic blasts, peripheral blood without blasts and with evidence of regeneration, and absence of extramedullary leukemic involvement.[50,64,68] Chemotherapy agents used in this phase were dexamethasone (glucocorticoid), vincristine (mitotic spindle inhibitor), methotrexate (antifolate), cytarabine (antimetabolite, analog of deoxycytidine) and asparaginase (breaks down asparagine, that ALL cells cannot synthesize). Consolidation lasted 6 months and aimed to minimize drug resistance and to sustain remission. It consisted either of different R-blocks or of a continuous therapy (protocol II-IDA), depending on randomization. In both randomization arms, dexamethasone, vincristine, cytarabine, asparaginase as well as the purine analogues 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP) were used. Protocol II-IDA additionally included idarubicine (topoisomerase II inhibitor) and cyclophosphamide (alkylating agent), whereas the R-blocks used daunorubicine (topoisomerase

II inhibitor) and ifosfamide (alkylating agent). All low-risk (S1) patients received one year of maintenance therapy with daily oral 6-MP and weekly methotrexate. Patients of the intermediate-risk group (S2) with low levels of MRD (leukemic burden less than 1 in 1,000 normal cells, ≤1E-03) at the end of induction therapy (week 5) received two years of maintenance therapy with additional etoposite re-induction pulses (VP-16, topoisomerase II inhibitor). Intensification of treatment by allo-HSCT was recommended for high-risk (S3/S4) patients and for intermediate-risk (S2) patients with high levels of MRD (≥1E-03) at the end of induction therapy.[50,64,68] Relapsed patients with relapsed ALL who did not respond to treatment were diagnosed with nonresponse, which was defined as failure to achieve complete remission after induction and the first two courses of consolidation therapy (week 9).[50,64,68]

1.3.4 Novel risk factors and new therapeutic approaches

Further improvement of treatment outcomes of relapsed patients, in particular of those at high-risk of standard treatment failure, relies on the identification of novel biomarkers and drug targets and the development of new therapeutic approaches. Along these lines, an early and accurate identification of patients with high-risk relapsed ALL is essential to support their allocation to alternative treatment protocols, such as phase I/II trials including targeted drugs, as early as possible. Genetic studies in relapsed ALL have revealed genetic alterations that are associated with high risk of treatment failure and/or poor event-free survival after relapse.[36,50,75] *TP53* mutations and/or deletions, that were frequently acquired at relapse, were identified as poor prognosis marker in patients with relapsed ALL.[36] Accordingly, the drug APR-246, which restores the wild-type function of mutant TP53 by confirmation changes, is currently considered for a phase I/II clinical trial in relapsed ALL patients with mutant TP53.[76] *TP53* alterations were associated with hypodiploidy (<44 chromosomes), which represents a poor prognosis markers in relapsed ALL, too.[36,45,77] Furthermore, deletions of *IKZF1* in relapses of BCP-ALL were described to be associated with inferior survival rates.[50] Another pathway that is often deregulated in ALL is the RAS/Raf/MEK/ERK pathway (short RAS pathway).[78] RAS pathway deregulation occurs for example by mutations in the *KRAS* and *NRAS* genes and is often acquired at relapse.[39,75] Mutations in *NRAS* and *KRAS* were identified in 14.6% and 14.1% of patients with relapsed BCP-ALL, respectively.[75] In contrast to primary ALL, where *NRAS* and *KRAS* mutations are associated with inferior event-free survival rates,[79] only *KRAS* mutations seemed to have an effect on patient survival in the relapse setting.[75] Nevertheless, relapsed patients harboring *RAS* pathway activating mutations may individually benefit from targeted treatment with, for instance, selumetinib, an inhibitor of the downstream MAPK/ERK pathway that was effective in *RAS*-mutated ALL both *in vitro* and *in vivo*.[75] For patients with relapses harboring alterations affecting purine nucleotide metabolism such as *PRPS1* or *NT5C2* mutations, response to treatment and outcome after first relapse is currently unknown.

1.4 Cytosolic 5´-nucleotidase II (NT5C2)

Cytosolic 5´-nucleotidase II (NT5C2) is a hydrolase that plays an important role in cellular purine metabolism.[80] The enzyme serves the purine salvage and the *de novo* purine biosynthesis pathway by its role in cellular purine homeostasis.[80] NT5C2 catalyzes the 5´-dephosporylation of 6-hydroxy purine nucleoside monophosphates and thereby facilitates the export of the resulting nucleosides out of the cell. Thus, the enzyme is involved in maintenance of a constant intracellular purine/pyrimidine nucleotide pool.[80–82] NT5C2 preferentially degrades inosine 5´-monosposphate (IMP), guanosine monophosphate (GMP) and xanthosine monophosphate (XMP) as well as the deoxyribose forms of IMP and GMP (dIMP and dGMP, respectively). Less efficiently, the enzyme can also degrade adenosine monophosphate (AMP) and uridine monophosphate (UMP, Fig. 4).[80–83] The preferred substrate of NT5C2, IMP, is a central metabolite of purine metabolism. IMP is required for the synthesis of either GMP or AMP that are central precursors of GTP and ATP (Fig. 4).[84] Thio-nucleoside monophosphates resulting from thiopurine drugs are metabolized by the cell in the same way as normal nucleoside monophospates.[84] Different allosteric activators regulate the activity of NT5C2 as for example ATP, dATP and ADP.[80] The human *NT5C2* gene is located on chromosome 10q24.32. Germline mutations in *NT5C2* were associated with hereditary spastic paraplegia, a neurodegenerative disorder.[85] The *NT5C2* transcript encodes 561 amino acids with a molecular weight of 65 kDa and is ubiquitously expressed.[81,86] The *NT5C2* protein is highly conserved across species with the protein sequence sharing 99% identity with that from other mammals and 51% with that from *Caenorhabditis elegans*, underscoring its key role in the maintenance of the intracellular nucleotide pool.[80]

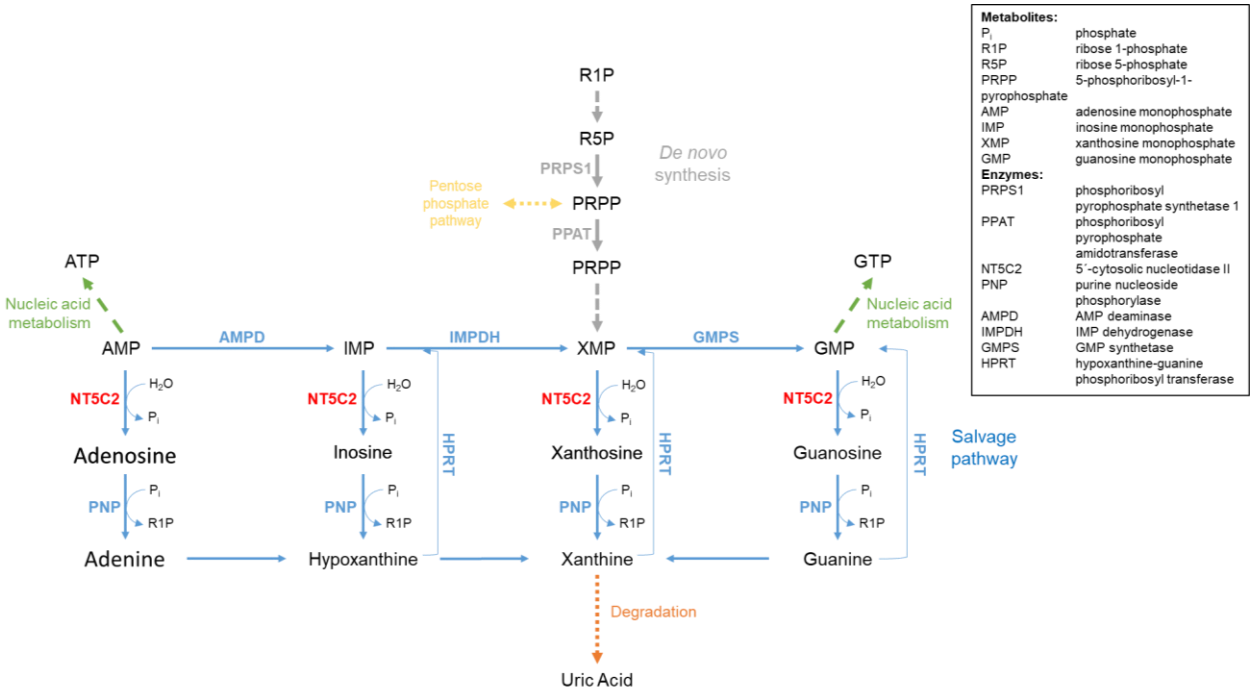

Figure 4. Role of NT5C2 in nucleotide metabolism and 6-mercaptopurine (6-MP) resistance. Schematic representation of the nucleotide metabolism. Adapted from Tzoneva et al. 2018.

NT5C2 mutations in ALL relapses were first described in March 2013 by Tzoneva et al. and Meyer et al. who investigated relapsed patients treated in various trials from the European ALL-REZ BFM and AIOP-BFM as well as from the American COG study group. Beyond reporting mutational frequencies of 19% (20/103) in T-ALL relapses and 3% (1/35) or 10% (7/71) in BCP-ALL relapses, the molecular and cellular functions of mutant NT5C2 were the primary focus of both papers, relating that NT5C2 mutant proteins have increased nucleotidase activities *in vitro* and confer resistance to purine analogue drugs 6-MP and 6-TG in ALL cell lines.[52,53] In a T-ALL mouse model, Tzoneva et al. demonstrated a selective growth advantage of *NT5C2* mutant cells under purine analogue treatment, suggesting that mutant *NT5C2* may help drive relapse formation.[87] At the same time, Tzoneva et al. showed that the growth advantage under thiopurine treatment is accompanied by high fitness costs of *NT5C2*-mutant cells under normal growth conditions, such as decreased proliferation rate and reduced leukemia-initiating cell activity.[87] The impact of *NT5C2* mutations found in relapsed ALL on protein structure and enzyme function was studied by Dieck and colleagues, who divided *NT5C2* mutations into three functional classes: i) Class I mutations alter the helix A region and induce an active conformational state in the absence of allosteric modulator, ii) Class II mutations cause an increased basal enzymatic activity and are located in the positively charged pocket at the interface of the tightly associated dimer and in the tip region of the arm segment, and iii) Class III mutations cause a loss of the C-terminal acid tail segment. A similar structural analysis was performed by Hnizda et al. 2018 who included a few additional *NT5C2* variants such as p.R29Q.[88]

2. Aims and objectives of the thesis

Up to 20% of children with ALL suffer a relapse. Event-free survival rates after second-line treatment of relapse are highly heterogeneous, and further improvement of outcomes of relapsed patients at high risk of second-line treatment failure relies on their early identification and allocation to alternative treatment approaches. Mutations in the *NT5C2* gene were found specifically in relapsed ALL and are considered to drive relapse development by conferring resistance to 6-mercaptopurine, which is administered in primary ALL maintenance therapy. However, all patient-directed studies were hampered by small cohorts and produced widely varying *NT5C2* mutation frequencies (3-45% in relapsed BCP-ALL and 19-38% in relapsed T-ALL).[39,52,53,89] Only one report investigated the impact of *NT5C2* mutations on survival of patients after relapse, concluding that the *NT5C2* mutation had no prognostic impact in the 67 T-ALL patients analysed.[89] In contrast to the clear idea of the role of *NT5C2* mutations in ALL relapse formation, detailed knowledge on their relevance for response to relapse treatment and outcome of patients with relapsed BCP-ALL after relapse therapy is still lacking. The aim of this study was to elucidate the potential of *NT5C2* mutations as a novel marker and therapeutic target in relapsed BCP-ALL.

To achieve this aim, the objectives of my thesis were

i) to analyze the frequency of *NT5C2* mutations in a large, representative cohort of patients with relapsed BCP-ALL by comprehensive next generation sequencing (NGS) in collaboration with A. Ferrando (Columbia University, New York, USA) and H. Khiabanian (Rutgers University, New York, USA) in an identification cohort and cost-efficient Sanger sequencing in a validation cohort.

ii) to investigate the co-occurrence of *NT5C2* mutations with other recurrent genetic alterations in relapsed BCP-ALL,

iii) to investigate the effects of *NT5C2* mutations on clinical presentation and outcomes after relapse of patients and

iv) to analyze the development of *NT5C2* mutant clones during and after treatment of first relapse.

3. Patients, Materials and Methods

The selection of patients, sample processing and statistical analyses are described in detail in the section “Material and Methods” in the main manuscript of the publication Barz et al. 2020. Briefly, the 455 patients with relapsed ALL treated accordingly to the ALL-REZ BFM clinical trial protocol included in this study were selected based on the successful DNA analyses. Patient samples were withdrawn from the in-house biobank. Samples from primary diagnosis and from time-points during or after relapse ALL treatment were used to track *NT5C2* mutations. In the following section, *NT5C2* mutation analysis including NGS, Sanger Sequencing and allele-specific quantitative real-time PCR (ASQ-PCR) as well as the identification of key genetic alterations are described since they were not specified in detail in the publication or were included in the supplemental information only. Additional details regarding NGS are given in the section “Material and Methods” in the main manuscript of the publication Barz et al. 2020 and the including supplemental material in the section “Identification of small mutations by targeted deep next generation sequencing (NGS)”. *NT5C2* mutation analysis using Sanger sequencing and ASQ-PCR, the identification of key genetic alterations and statistical analyses with the SPSS software (all except Cox regression with HSCT as time-dependent variable that was done with the STATA software by my supervisor Dr. Cornelia Eckert, Charité) were performed by myself. NGS and capillary gel electrophoresis for Sanger sequencing were performed in collaboration with other research groups as specified below.

3.1 *NT5C2* sequence analysis

3.1.1 Next generation sequencing

NGS allows high-throughput parallel sequencing of different genes with high sensitivity.[47] The analysis of all *NT5C2* exons in 228 relapses of BCP-ALL was done by the laboratory of A. Ferrando (Columbia University, New York, USA) using a targeted multiplex amplicon-based NGS approach. The NGS methodology is described in detail in Oshima et al. 2016[47] and in the dissertation of G. Tzoneva[90]. The NGS data analysis pipeline used by Oshima et al. included a variant allele frequency (VAF) cut-off of >10%.[47] However, this cut-off was insufficient to identify low-frequency *NT5C2* mutations. Therefore, the NGS data was reanalyzed in collaboration with H. Khiabanian (Rutgers University, New York, USA). Using a Bayesian approach to detect true mutations against background error.[91] The method of NGS data reanalysis is described in detail in the supplemental information accompanying the publication Barz et al. 2020 by H. Khiabanian.[1] To validate the NGS-identified mutations, we compared the *NT5C2* p.R367Q mutations identified by NGS to those detected by Sanger sequencing (n=6) and/or ASQ-PCR (n=15). Of 21 p.R367Q *NT5C2* mutations 17 (81%) were identified by NGS (Tab. 2). The four mutations with a very low clonal frequency of ≤1E-03 by ASQ-PCR were not reliably detected by NGS (Tab. 2). Taken

together, NGS reliably identified the *NT5C2* p.R367Q mutation up to a VAF of 2% (Tab. 2). Therefore, the threshold of 2% VAF was chosen for the detection of *NT5C2* mutations by NGS. Besides p.R367Q, all low-frequency *NT5C2* variants with a NGS VAF ≥2% and ≤20% were validated using individual ASQ-PCR assays (see chapter 2.3.3; Pub. Supplemental (Suppl.) Tabs. 5 and 6). *NT5C2* mutations with NGS VAFs ≥20% were validated by Sanger sequencing (exemplary shown for p.R367Q in Tab. 2, Pub. Suppl. Tab. 5).

Table 2. Comparison of results from NGS data reanalysis, ASQ-PCR and Sanger Sequencing data for the *NT5C2* mutation p.R367Q.

Patient ID	*Variant Depth (NGS)*	*Total Depth (NGS)*	*FDR (NGS)*	*Exon*	*Amino Acid Change by NGS*	*Variant allele frequency (NGS)*	*Clone frequency by ASQ-PCR without reference gene*	*MS simplified allele ratio*
223	*no data*	1423	*no data*	13	**WT**	0	7E-04	*no data*
240	*no data*	*no data*	*no data*	13	**WT**	0	1E-03	*no data*
414	*no data*	2951	*no data*	13	**WT**	0	1E-03	*no data*
419	*no data*	*no data*	*no data*	13	**WT**	0	3E-04	*no data*
369	37	2994	8,7E-08	13	**R367Q**	1	4E-04	*no data*
215	27	1207	1,9E-11	13	**R367Q**	2	7E-03	*no data*
248	27	1130	4,3E-12	13	**R367Q**	2	3E-03	*no data*
336	34	1366	2,0E-15	13	**R367Q**	2	9E-03	*no data*
423	111	4190	1,4E-50	13	**R367Q**	3	4E-03	*no data*
281	32	1162	9,0E-16	13	**R367Q**	3	5E-03	*no data*
290	29	960	1,7E-15	13	**R367Q**	3	2E-03	*no data*
228	57	1340	4,9E-37	13	**R367Q**	4	2E-02	*no data*
310	51	1152	3,9E-34	13	**R367Q**	4	8E-03	*no data*
396	363	4512	0,0E+00	13	**R367Q**	8	1E-02	*no data*
363	230	2584	1,2E-220	13	**R367Q**	9	2E-02	*no data*
326	295	1498	0,0E+00	13	**R367Q**	20	8E-02	21%
244	219	960	8,6E-294	13	**R367Q**	23	9E-02	50%
403	1263	3890	0,0E+00	13	**R367Q**	32	5E-01	34%
377	798	2439	0,0E+00	13	**R367Q**	33	4E-02	39%
234	567	1381	0,0E+00	13	**R367Q**	41	2E-01	46%
371	1017	2367	0,0E+00	13	**R367Q**	43	5E-01	50%

Abbreviations: ASQ-PCR, allele-specific sensitive real-time PCR, FDR, false discovery rate; ID, patient identifier, MS, Mutations Surveyor Software; NGS, next generation sequencing.

3.1.2 Sanger Sequencing

The *NT5C2* hot spot exons (2, 9, 13, 15, and 16) were sequenced by the method of Sanger (for primers see Pub. Suppl. Tab. 2). The workflow for Sanger Sequencing including PCR amplification, sequencing reaction, capillary gel electrophoresis and data analysis is described below. PCR to amplify *NT5C2* exons was performed as shown in Tab. 3. The PCR reaction buffer contained 1.5 mM $MgCl_2$ to provide Mg^{2+}-Ions as co-factor for the polymerase. After the PCR reaction, samples were stored at 4°C or, for long-term storage, at -20°C. Quality control of PCR products was done by agarose gel electrophoresis.

Table 3. PCR reaction setup and PCR thermocycler protocol

PCR reaction for one sample (25 µL)		**PCR thermocycler protocol**		
Amount in µL	*Agent*		*Temperature in °C*	*Time duration*
2.5	10X PCR buffer	Initial denaturation	95	10 min
2.5	dNTPs (2 nM)		Cycle 35x	
0.5	Primer forward (10 nM)	Denaturation	95	30 sec
0.5	Primer reverse (10 nM)	Annealing	60	30 sec
0.15	AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Foster City, USA); 5 U/µL)	Elongation	72	45 sec
17.85	H_2O	Final elongation	72	10 min
1	DNA (25 – 100 ng)	Storage	4/ -20	∞

Abbreviations: min, minutes; ng, nanogram; sec, seconds; U, units.

Prior to the sequencing reaction, 2.5 µl of the PCR products were treated with 4 units Exonuclease I (Exo I, New England Biolabs, Ipswich, Massachusetts, USA) and 2 units Antarctic phosphatase (AP, New England Biolabs) to inactivate surplus primers and dNTPs in a final volume of 10 µl with addition of enzyme-specific buffers as recommended. The reaction was performed in a thermo cycler for 15 minutes at 37°C, followed by 20 minutes at 80°C. The product was stored at -20°C until it was used in the sequencing reaction steps. For the sequencing reaction the BigDye Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used as specified in Tab. 4. The kit contained dNTPs and fluorescence marked ddNTPs. The product of the sequencing reaction was stored at -20°C until further processing.

Table 4. Sequencing reaction and cycling protocol.

Sequencing reaction (10 µL)		**Cycling protocol**		
Amount in µL	*Agent*		*Temperature in °C*	*Time duration*
1.8	BigDye buffer	Initial denaturation	96	1 minute
0.2	Big Dye v3.1	Cycle 35x		
0.4	Primer forward or reverse (10nM)	Denaturation	96	10 seconds
6.6	H_2O	Annealing	50	5 seconds
1	Treated PCR product	Elongation	60	4 minutes
		Hold	4	∞

Prior to capillary gel electrophoresis, the sequencing reaction products were purified with the magnetic Dynabeads® Sequencing Clean-Up Kit (ThermoFisher Scientific). First, one µL sequencing product was added to 20 µL Dynabeads® Sequencing Clean-Up solution and incubated for 15 minutes at room temperature in a dark place. Next, the sample was placed on a magnetic to pull down the DNA-Dynabeads® complexes and to discard the supernatant. The sample was washed with 70% ethanol and dried for 30 minutes at 37°C. Finally, the DNA was eluted from the Dynabeads® with highly purified water. This purification process was performed by a specific pipetting robot at the Molecular Diagnostic Laboratory of the Institute for Medical Genetics at the Charité – Universitätsmedizin Berlin. Capillary gel electrophoresis of the sequencing reaction products was then performed at the same laboratory using the ABI 3730 sequencer from Applied Biosystems (Foster City, California, USA).

Sanger sequence chromatograms were visualized and evaluated with the Mutation Surveyor software version 5.0.0 (Softgenetics, State College, USA) and compared to the *NT5C2* GenBank sequence NG_042272.1 hg18/38 as reference. The mutations surveyor indicates the level of confidence of a called mutation with a mutation score. Clonal mutations were always called by the mutation surveyor with mutation scores ranging between 13 and 185 (example given in Fig. 5A). Subclonal mutations were not always recognized by the software, but were seen as minimal peaks in forward and reverse sequence and were validated using ASQ-PCR. Comparing the NGS VAF with the mutation score, the mutation surveyor software called mutations up to a VAF of approximately 15%. Mutations below 15% VAF were seen in the chromatogram traces in forward and reverse sequence, but not called as mutations (example given in Fig. 5B). For the

quantification of mutant alleles, the allelic peak ratio was calculated from the sequencing chromatograms using the Mutation Surveyor's algorithm for 'simplified allele ratio'. Resequencing of all NGS-identified mutations by the method of Sanger revealed a correlation between the VAF from NGS and the allelic peak ratio (r^2=0.871, p<0.001; Pub. Suppl. Fig. 1A). For the definition of low-frequency mutations (so called subclonal mutations), the VAF and allelic peak ratios from NGS- and Sanger-detected mutations, respectively, were compiled into one data set. Based on the distribution of the VAF/allelic peak ratios (Pub. Suppl. Fig. 1B), a common cut-off point of 15% was set to separate subclonal from clonal mutations.

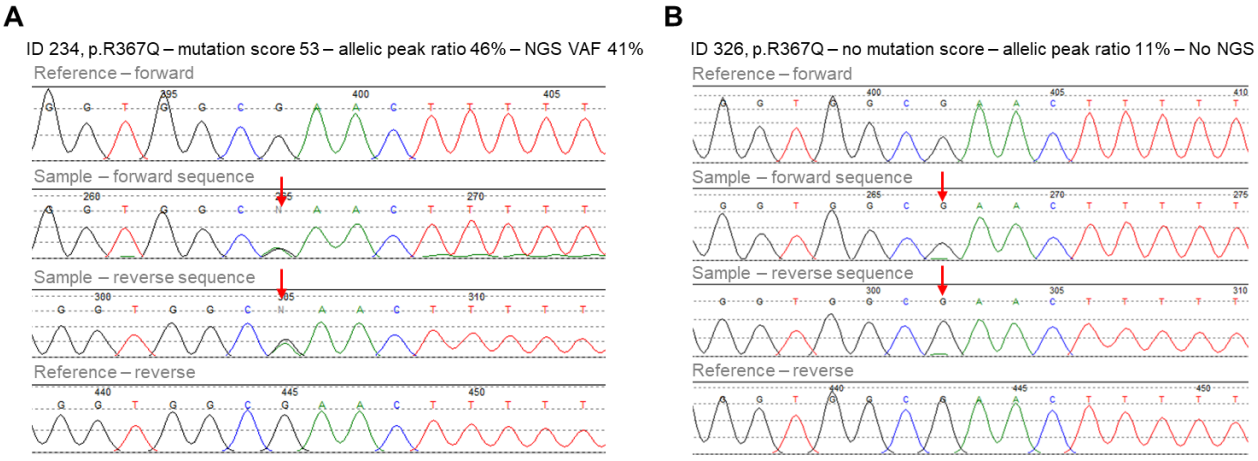

Figure 5. Illustrative chromatogram traces from Sanger sequencing of NT5C2 in BCP-ALL relapses (**A**) clonal *NT5C2* mutation detected by the Mutation Surveyor software (**B**) subclonal *NT5C2* mutation not detected by the Mutation Surveyor software, but recognized as minimal peaks in forward and reverse sequence.

3.2 Allele-specific quantitative real-time PCR

For sensitive detection and validation of mutations, allele-specific quantitative real-time PCR (ASQ-PCR) assays targeting 14 different *NT5C2* mutations (p.R39Q, p.R238W, p.R238L, p.R367Q, p.K404N, p.P414S, p.M192L, p.V301A, p.R303H, p.R363L, p.N411D, p.D415G, p.S445F and p.V454M) were developed. In addition, five assays detecting *NRAS* p.G12D and p.G13D and *KRAS* p.G12D, p.G12V and p.G12R mutations were designed. Analysis of assay performance and evaluation of results was performed according to the MRD guidelines.[73] The design, evaluation and quantification of the ASQ-PCR assays is described below.

3.2.1 Assay design

Primers and probes for ASQ-PCR assays were designed using the Primer3 program available from http://primer3.ut.ee/ (last access October 2019).[92,93] The allele-specific primer was built manually in forward and reverse design with a length of 18 nucleotides and the 3' terminal nucleotide matching the mutation. Corresponding reverse/forward primers and hydrolysis probes were picked by Primer3 using the default settings and the human mispriming library. If Primer3 was unable to find an acceptable reverse/forward primer, the allele-specific primer was extended

in length by one nucleotide at the 5' terminus in an iterative process up to a maximum of 27 nucleotides. From the Primer3 results, the ASQ-PCR assays with the best possible conditions was chosen for further use. An additional mismatch was inserted on the second base or the third base closest to the 3' end using all three possible nucleotides to increase the specificity of the allele-specific primer.[94] By this method, six different allele-specific primers for the *NT5C2* p.R39Q (c.116G>A) mutation together with a universal hydrolysis probe and a universal reverse primer were obtained (p.R39Q ASQ-PCR assays V2-V7, Pub. Suppl. Tab. 3). The third base mismatch approach showed a superior performance (described in detail in the next section). Therefore, all other allele-specific primers for the detection of *NT5C2* and *NRAS*/*KRAS* mutations were designed according to the third base mismatch approach (Pub. Suppl. Tab. 3). The hydrolysis probes were dual-labelled with 6-Carboxyfluorescein (6-FAM) and Black Hole Quencher 1 (BHQ-1).

3.2.2 Assay evaluation and selection

The performance of the ASQ-PCR assays was evaluated by measuring serial 10-fold dilutions of a mutation-positive patient DNA sample in a mutation-negative control DNA ranging from 1:10 (1E-01) to 1:10,000 (1E-04). Pooled DNA from blood samples of at least 10 healthy individuals was used as mutation-negative control. For each ASQ-PCR assay, the dilution series and the mutation-negative DNA control was measured in at least two independent runs at 65°C annealing temperature in a StepOnePlus real-time PCR System (ThermoFisher, Waltham, MA, U.S.A.). As template for the standard dilution series 500 ng DNA were used which corresponds to 75,000 cells based on the amount of a diploid human cell of 6.7 pg.[95] The standard conditions used for the ASQ-PCR runs are listed in Tab. 5. For the ASQ-PCR assays targeting *NT5C2* mutations that were found as subclonal mutations only (VAF <15%) by NGS (p.M192L, p.V301A, p.R303H, p.R363L, p.N411D, p.D415G, p.S445F and p.V454M), the evaluation of ASQ-PCR assay performance by serial dilutions was not possible due to the lack of a positive control.

Table 5. ASQ-PCR reaction setup and ASQ-PCR thermocycler protocol

ASQ-PCR reaction for one sample (20 µL)		**ASQ-PCR protocol for StepOnePlus**		
Amount in µL	*Agent*		*Temperature in °C*	*Time duration*
2	10X PCR buffer	Initial denaturation	94	5 min
2	$MgCl_2$ (50mM)			
2	dNTPs (2 mM)	**Cycle 50x**		
1	Primer forward (10 µM)	Denaturation	94	8 sec
1	Primer reverse (10 µM)	Annealing & Elongation	65	23 sec
0.5	BSA (250 ng/µl)			
0.2	Probe (10 µM)			
0.2	Platinum™ Taq DNA polymerase (ThermoFisher, 5 U/µL)			
6.1	H_2O			
5	DNA template	Storage	4/ -20	∞

Abbreviations: ASQ-PCR, allele-specific real-time PCR; BSA, bovine serum albumin; min, minutes; rxn, reaction; sec, seconds; u, units.

For each ASQ-PCR assay, a semi-logarithmic regression line plot of C_T (cycle threshold) values versus decimal logarithm (log10) of mutation-positive cell input was generated using at least three

independent measurements of each dilution point (depicted for p.R39Q and p.R367Q in Pub. Suppl. Figs. 2 and 3). Evaluation of the standard curves following the MRD analysis guidelines for quantitative real-time PCR of immunoglobulin and T-cell receptor gene rearrangements[72] (Tab. 6) lead to exclusion of assays that did not fulfil the following three criteria: i) slope of the standard curve < -2·6, ii) correlation coefficient $R^2 \geq 0{\cdot}98$ and iii) mean C_T of the lowest dilution (1E-01) < 29. For the p.R39Q mutation, this concerned all assays that were designed to the second base mismatch approach (V2-V4) and one assay designed according to the third base mismatch approach (V5; Pub. Suppl. Fig. 2). Based on this result, all assays targeting other *NT5C2* variants than p.R39Q were designed solely according to the third base mismatch approach. Applying the three criteria listed above, the best-performing allele-specific primer for each mutation was chosen (Pub. Suppl. Tab. 4). Regarding the *NT5C2* ASQ-PCR assays (p.R39Q and p.R367Q) used for screening of the study cohort, p.R39Q assays V6 and V7 showed a highly similar performance (Pub. Suppl. Fig. 2). Therefore, V6 was arbitrarily chosen for further experiments. For *NT5C2* p.R367Q, allele-specific primers F8 and F10 were discarded and F9 chosen (Pub. Suppl. Fig. 3).

In a next step, the best-performing ASQ-PCR assay for each mutation was assessed for the degree of non-specific amplification by calculating the mean C_T distance between the 1E-03 dilution of the mutation-positive sample and the mutation-negative DNA control (Pub. Suppl. Tab. 4). In agreement with the MRD guidelines[72], the distance was ≥ 2 C_T values for each assay (Pub. Suppl. Tab. 4). Finally, the annealing temperature was optimized using slope and R^2 of the standard curve as well as C_T of the lowest dilution as quality criteria for each of the selected ASQ-PCR assays (Pub. Suppl. Fig. 4). The annealing temperature was set to 65°C for p.R39Q primer V6, p.R238L primer F2, and p.P414S primer F3, to 64°C for p.R238W primer F2 and p.R367Q primer F9 and to 66°C for p.K404N primer F1.

3.2.3 Sensitive detection of *NT5C2* mutations in patient samples

For sensitive detection of mutations by ASQ-PCR, the standard reaction conditions were used as defined above. Of each sample 100 ng DNA corresponding to 15,000 cells was used as template.[95] This amount was chosen due to limited availability of DNA from patient samples. Five to seven replicates of the mutation-negative DNA control and a series of dilutions (1E-01, 1E-02, 5 x 1E-03, 1E-03, 5 x 1E-04 and 1E-04) of a mutation-positive reference sample (see above) were used in each ASQ-PCR run, next to duplicates of the samples to be tested. The 1E-01 and 1E-04 dilution were performed in duplicate and all other dilutions in triplicate. As recommended[72], all replicates of the dilution points and of the patient samples differed by less than 1.5 C_T values in each run. According to the MRD guidelines[72], a background cut-off C_T was defined in each run by subtracting one C_T from the lowest C_T value of the mutation-negative DNA control replicates. A sample was considered “positive” for a given mutation if the mean C_T of the sample duplicates

was lower than the background cut-off C_T. Each positive result was confirmed by at least one independent ASQ-PCR run. If multiple confirmation runs were performed, the sample was considered positive if the majority of all measurements were positive. For *NT5C2* mutations that were found as subclonal mutations only (lacking positive control), the sample was considered "positive" if it's mean C_T achieved a distance of 1.5 C_T values to the lowest mutation-negative DNA control C_T value in runs of two of the three third base mismatch ASQ-PCR assays designed for each mutation (Pub. Suppl. Tab. 6).

3.2.4 Quantification of *NT5C2* mutations in patient samples

For quantification of *NT5C2* mutations p.R39Q, p.R238W, p.R238L, p.R367Q, p.K404N and p.P414S in patient samples, the limit of quantification (LoQ) was defined using standards adopted from the analysis of MRD for each ASQ-PCR run.[72] The LoQ was defined by the lowest dilution of the dilution series that met the criteria listed in Tab. 8 in column "*NT5C2* ASQ-PCR adapted guidelines". The 1:1000 (1E-03) dilution of the mutation-positive reference DNA control reliably defined the LoQ in all runs and for all ASQ-PCR assay. Subsequently, the ratio of mutation-positive cells to wild-type cells was calculated for each tested sample from the mean C_T using the equation of the LoQ standard curve and a PCR input of 15,000 cells, as described above. For the final quantitative value of a given mutation in a sample (as listed in Pub. Suppl. Tab. 5), the mean of the results from all independent ASQ-PCR runs was taken. Mutation-positive samples with values below the LoQ were considered "NT5C2 mutation-positive, but not quantifiable" (Pub. Suppl. Tab. 5). The limit of detection (LoD) was individually calculated for each run of a given ASQ-PCR assay as described in Tab. 6.

Table 6. Allele-specific real-time PCR guidelines adapted from the minimal residual disease measurement guidelines by Van der Velden et al. 2007.

Parameter	***MRD guidelines (TCR/Ig-rearr. quantification)***	***NT5C2 ASQ-PCR adapted guidelines***
Standard dilution curve		
Slope	≥-3.1 to -3.9	≥-2.7
Y-axis intercept (representing 10^{-0})	not specified	not specified
Regression	≥0.98	≥0.98
C_T of 10^{-1} Dilution	not specified	<29
distance between each 10-fold dilution	2.6 – 4.0 C_T	2.6 – 4.0 C_T
distance between each two-fold dilution	0.5 – 1.5 C_T	0.5 – 1.5 C_T
used DNA amount for standard dilution	500 ng	500 ng
serial dilution range	1E-01, 1E-02, 5E-03, 1E-03, 5E-04, 1E-04, 5E-5, 1E-5	1E-01, 1E-02, 5E-03, 1E-03, 5E-04, 1E-04
other factors		
ΔC_T of replicates	≤1.5	≤1.5
quantitative range/ limit of quantification (LoQ)	C_T values ≥3.0 lower than the lowest C_T value of the background (mutation-negative DNA control)	C_T values ≥2.0 lower than the lowest C_T value of the background (mutation-negative DNA control)
sensitivity/ limit of detection (LoD)	C_T values ≥1.0 lower than the lowest C_T value of the background (mutation-negative DNA control)	C_T values ≥1.0 lower than the lowest C_T value of the background (mutation-negative DNA control)
Calculation		
PCR input/template	500 ng DNA (equivalent to 75,000 cells)	100 ng DNA (equivalent to 15 000 cells)
normalization (reference gene)	beta-globin	beta-globin or wild-type *NT5C2*

Abbreviations: ASQ-PCR, allele-specific quantitative real-time PCR; CT, cycle threshold; MRD, minimal residual disease guidelines; rearr. rearrangement.

3.2.5 Screening for low-frequency *NT5C2* p.R39Q and p.R367Q mutations

Screening for *NT5C2* p.R39Q and p.R367Q mutations was performed using the ASQ-PCR assays and adapted guidelines described above. In total, 455 first relapse samples of patients with BCP-ALL were screened for low-frequency mutations of *NT5C2* p.R39Q and p.R367Q. To minimize the chance of false positive results, an overall background cut-off C_T was calculated by subtracting twice the standard deviation from the mean C_T of the mutation-negative controls across all ASQ-PCR runs of each mutation assay. This calculation included 214 measurements of the mutation-negative control in 32 runs for the p.R39Q assay and 201 measurements in 29 runs for the p.R367Q assay. The overall background cut-off C_T was 32.67 for p.R39Q and 32.35 for p.R367Q. All samples that were defined positive for a *NT5C2* mutation by individual ASQ-PCR runs, but of which the majority of measured C_T values still fell below the overall background cut-off C_T, were subsequently set to "negative". This concerned three samples (ID 303, 492 and 524). The p.R39Q ASQ-PCR assay had a median LoD of 3 mutation-positive cells in 100,000 wild-type cells (3E-05, range 2E-03 - 8E-06) across all runs and the p.R367Q assay of 4 mutation-positive cells in 100,000 wild-type cells (4E-05, range 3E-04 - 1E-05).

3.3 Identification of recurrent genetic alterations in relapsed ALL

The presence of *NT5C2* mutations was correlated with recurrent genetic alterations in ALL and relapsed ALL including gene fusions, aneuploidy, copy number alterations and mutations (listed in Pub. Fig. 2). Data on recurrent genetic alterations in relapses of the study cohort was mostly generated in previous work as summarized in Tab. 7. In the context of this thesis, the existing data on genetic alterations was extend as listed in Tab. 7 in the column "Added data (n=patient number)". For Sanger sequencing of *JAK1*, *JAK2* and *JAK3,* primers were designed and optimized (Tab. 8). Furthermore, low-frequency *NRAS/KRAS* mutations were analyzed in 136 patients with relapsed BCP-ALL using ASQ-PCR. The detection of subclonal *NRAS/KRAS* mutations was restricted to two *NRAS* mutations (p.G12D and p.G13D) and three *KRAS* mutations (p.G12D, p.G12V and p.G12R). The allele-specific primer for the *KRAS* and *NRAS* variants were designed according to the third base mismatch approach (for details see chapter 2.3.3; Pub. Suppl. Tab. 3). The assays for these five mutations were designed, tested, performed and analyzed according to the same procedure as described in detail in chapter 2.3.3. The allele-specific primers, universal primers and universal probes, as well as the non-specific amplification of the chosen ASQ-PCR assays are listed in Pub. Suppl. Tabs. 3 and 4. The optimized annealing temperature for the final assay performance was set to 65°C for *KRAS* G12D primer F1, *KRAS* G12V primer F2 and *KRAS* G12R primer F2, to 64°C for *NRAS* G12D primer F3 and to 66°C for *NRAS* G13D primer F2. The analysis of ASQ-PCR data for *NRAS/KRAS* mutations was performed according to the *NT5C2* ASQ-PCR guidelines (Tab. 6). For all established *NRAS* and

KRAS ASQ-PCR assays, the 1:1000 (1E-03) dilution of the mutation-positive control reliably defined the LoQ in all runs.

Table 7. Detection of key genetic alterations in patients with relapsed ALL in previous studies.

Gene(s)/ Alterations	***Alteration***	***Method***	***Previously existing data (n=patient number)***	***Reference***	***Added data (n=patient number)***
TP53	mutations* in key exons	Sanger sequencing, amplicon-based NGS	480	Hof et al 2011[36], Oshima et al.[47] 2016	6
NRAS	mutations* in key exons	Sanger sequencing, amplicon-based NGS	436	Irving et al.[75] 2014, Oshima et al.[47] 2016	47
KRAS	mutations* in key exons	Sanger sequencing, amplicon-based NGS	436	Irving et al.[75] 2014, Oshima et al.[47] 2016	47
FLT3	mutations* in key exons	Sanger sequencing, amplicon-based NGS	323	Irving et al.[75] 2014, Oshima et al.[47] 2016	88
PTPN11	mutations* in key exons	Sanger sequencing, amplicon-based NGS	206	Irving et al.[75] 2014	116
JAK1	mutations* in key exons	Sanger sequencing	none	none	110
JAK2	mutations* in key exons	Sanger sequencing, amplicon-based NGS	230	Oshima et al.[47] 2016	68
JAK3	mutations* in key exons	Sanger sequencing, amplicon-based NGS	230	Oshima et al.[47] 2016	68
BCR/ABL1, TCF3/PBX1, ETV6/RUNX1, KMT2A-rearr.	Fusion genes	reverse transcriptase PCR, fluorescence *in situ* hybridization	475	Krentz et al. 2013[50], Groeneveld-Krentz et al. 2019[45]	0
P2RY8/CRLF2	Fusion gene	reverse transcriptase PCR; Sanger sequencing	45	Krentz et al. 2013[50]	178
EBF1, IKZF1, CDKN2A/B, PAX5, ETV6, BTG1, RB1, PAR1	deletion	MLPA kit P335 (MRC Holland, Amsterdam, Netherlands)	450	Krentz et al. 2013[50], Groeneveld-Krentz et al. 2019[45]	23
TP53	deletion	MLPA kit P056 (MRC Holland)	461	Hof et al. 2011[36]; Groeneveld-Krentz et al. 2019[45]	0
Aneuploidy		MLPA P181 (MRC Holland), DNA Index and SNP array	467	Groeneveld-Krentz et al. 2019[45]	0

Abbreviations: ALL, acute lymphoblastic leukemia; MLPA, multiplex ligation-dependent probe amplification; NGS, next generation sequencing; rearr., rearrangement; SNP, single nucleotide polymorphism.

Table 8. Primer for Sanger sequencing of *JAK1*, *JAK2*, *JAK3, NRAS* and *KRAS.*

Gene	***Exon***	***Forward primer (5'-3')***	***Reverse primer (5'-3')***
JAK1	15	CACTTCAGGGCACAGAGAGG	AAACCACTGGGCCACAAGAA
	16	AAACCTCACATCCCTGCCAG	CCACCCACCCCTTTGAAAGA
	17	AGGGGATGAAGGAGAGGACC	CTTGGCTAGCACCTCCTTCC
JAK2	18	TCTCAATGCATGCCTCCAAA	CCCTTTACACCACTGCCCAA
	23	AGACAGTCTGCTAATTCCAGCT	CCCTCTGGGCATTGGCATAA
JAK3	4	CCCCACCATAATGTCACTCC	ATGCCAGTCCTCATGTTGC
	14	CTGTGTCTGGCCCCCTTAG	GGAATGAAAGTGGGATCAGG

4. Results

4.1 Frequency of *NT5C2* mutations in relapsed B-cell precursor ALL

Overall, 51 mutations in 46 (10%) of 455 patients with relapsed BCP-ALL were identified by NGS and Sanger sequencing (Pub. Fig. 1A). The most prevalent *NT5C2* mutations were p.R39Q and p.R367Q accounting for nearly two-thirds of all identified mutations (Pub. Fig. 1A). Surprisingly, 23 (45%) of 51 mutations were low-frequency mutations, so called subclonal mutations, with a variant frequency of below 15% (Pub. Fig. 1A; Pub. Suppl. Fig. 1). To investigate subclonal *NT5C2* mutations in detail, ASQ-PCR assays for p.R39Q and p.R367Q were developed and used to screen the total cohort of 455 relapses for low-frequency mutations (Pub. Fig. 1B). By ASQ-PCR, additional 39 low-frequency *NT5C2* mutations in 29 relapses were detected (Pub. Fig. 1C). Compiling all three detection methods (NGS, Sanger sequencing and ASQ-PCR), a total of 110 mutations in 75 (16.5%) of 455 patients with relapsed BCP-ALL were identified. Sixty-three% of *NT5C2*-mutated relapses harbored subclonal mutations only (Pub. Fig. 1D). All clonal mutations (28 of 110) were confirmed as somatic mutations by matched samples from diagnosis, remission or second event being wild-type for *NT5C2*.

4.2 Co-occurrence of *NT5C2* mutations with other recurrent genetic alterations

NT5C2 mutations were not detected in hypodiploid relapses, but in relapses with *BCR/ABL1*, *ETV6/RUNX1*, *TCF3/PBX1* and *KMT2A*-rearrangements as well as with high hyperdiploidy. However, *NT5C2* mutations were rare in relapses with the prognostically favorable *ETV6/RUNX1* rearrangement (p=0.001) or high hyperdiploidy (p=0.031) compared with relapses of other cytogenetic groups (Pub. Fig. 2A; Pub. Suppl. Tab. 7). Differentiating between relapses with clonal and subclonal *NT5C2* mutations, enrichments of *NRAS* mutations and *CDKN2B* deletions were strongly pronounced in the subgroup with clonal *NT5C2* mutations (p=0.009 and p<0.001, respectively; Pub. Fig. 2B; Pub. Suppl. Tab. 7).

4.3 Effects of *NT5C2* mutations on clinical parameters, response and outcome in relapsed BCP-ALL

Patients with relapsed ALL and either clonal or subclonal *NT5C2* mutations were associated with very early and early relapse in comparison to patients with wild-type *NT5C2* (p<0.001; Pub. Tab. 1), confirming the findings from earlier studies.[52,53,89] Accordingly, a higher proportion of patients with relapsed ALL and either clonal or subclonal *NT5C2* mutations relapsed while they were still being treated for primary ALL compared to patients with wild-type *NT5C2* (89% and 75% *vs* 19%,

p<0.001; Pub. Tab. 1). Patients with either clonal or subclonal *NT5C2* mutations had a significantly reduced event-free survival compared to patients with wild-type *NT5C2* (25% [95% confidence interval (CI) 11.06-41.78 and 19.2% [9.46-31.39], respectively, *vs* 52.6% [47.43-57.46], p<0.001; Pub. Fig. 3A). The cumulative incidence of second relapse was significantly higher in patients with relapsed ALL and either clonal or subclonal *NT5C2* mutations compared to patients with wild-type *NT5C2* (56% and 41% *vs* 29%, p=0.002; Pub. Fig. 3C). This result was consistent with a second relapse being the most frequent event in patients with relapsed ALL and clonal *NT5C2* mutations (Pub. Tab. 2). In patients with relapsed ALL and subclonal *NT5C2* mutations the proportion of patients that showed a nonresponse to relapse treatment was significantly increased compared to patients with clonal *NT5C2* mutations or with wild-type *NT5C2* (p=0.001; Pub. Tab. 2). Even if patients with relapsed ALL harboring subclonal *NT5C2* mutations responded to relapse treatment, they had high MRD levels at the end of induction treatment (p=0.030; Pub. Tab. 2). In multivariable Cox regression analysis, subclonal but not clonal *NT5C2* mutations were an independent marker for reduced event-free survival next to site of relapse, time to relapse, allo-HSCT and *TP53* mutation and/or deletion (hazard ratio 1.89 [95% CI 1.28-2.69], p=0.001; Pub. Tab. 3).

4.4 Development of *NT5C2* mutant clones after first relapse

To analyze the development of *NT5C2* mutant clones after first relapse treatment, 13 clonal *NT5C2* mutations and 33 subclonal *NT5C2* mutations in 34 patients with relapsed ALL were sensitively tracked by ASQ-PCR in matched samples from nonresponse to treatment or from second relapse. Seven (54%) of 13 clonal *NT5C2* mutations persisted as clonal *NT5C2* mutations, two (15%) of 13 were detected as subclonal mutations and four (31%) of 13 became undetectable in nonresponse or second relapse samples (Pub. Fig. 4A; Pub. Suppl. Fig. 7). Subclonal *NT5C2* mutations became undetectable in 27 (82%) of 33 cases and only six (18%) of 33 persisted as subclonal *NT5C2* mutations to nonresponse or second relapse (Pub. Fig. 4A; Pub. Suppl. Fig. 7). Surprisingly, none of the subclonal *NT5C2* mutations grew out to a clonal mutation (Pub. Fig. 4A; Pub. Suppl. Fig. 7). In-depth analysis of the clonal development of *NT5C2* mutations was performed by quantitative tracking of mutations by ASQ-PCR and comparison to MRD levels in follow-up samples taken during relapse treatment in 30 of 34 patients with relapsed BCP-ALL (n=16 patients with clonal and n=14 patients with subclonal mutations). Quantification of p.R39Q, p.R238W, p.R238L, p.R367Q, p.K404N and p.P414S in samples taken at the end of relapse induction therapy of 30 patients revealed that levels of clonal *NT5C2* mutations corresponded to MRD levels, whereas subclonal *NT5C2* mutations were reduced below the detection limit, but MRD levels remained high (Pub. Fig. 4B).

5. Discussion

ALL is an aggressive hematological malignancy and despite advances in ALL treatment, which have made ALL a curable disease in most cases, still up to 20% of children suffer a relapse.[7,29,31] Relapsed ALL remains a clinical challenge with heterogeneous survival rates depending on clinical and genetic factors.[58,60,61,63,66,67] Relapse-specific mutations in the *NT5C2* gene were described to drive relapse formation. However, the evolutionary trajectories of *NT5C2* mutations after first relapse of ALL and the importance of *NT5C2* mutations for relapse treatment remains unclear. All previously performed patient-directed *NT5C2* sequencing studies were hampered by small cohorts and the clinical significance after relapse of ALL is unknown.

In this study, the frequency and clinical relevance of *NT5C2* mutations was studied in a large unbiased cohort of 455 patients with relapsed BCP-ALL that were all treated according to the ALL-REZ BFM 2002 clinical trial protocol. In this context, our results are statistically meaningful. Moreover, a comprehensive analysis of genetic and clinical factors was performed to assess the clinical importance of *NT5C2* mutations after relapse of ALL. This study confirms the *NT5C2* p.R367Q mutation as the most frequent mutation in relapsed BCP-ALL. However, it reveals a slightly different mutation spectrum compared with previous studies with p.R39 being the second most frequent mutation site instead of R238.[21,22] All clonal *NT5C2* mutations identified in our study had a disrupted regulatory switch-off mechanism (Class II mutations).[88,96] Furthermore, we describe previously unidentified *NT5C2* mutations, including M192L, V301A and R303H, where no functional annotation existed. Hnízda et al. characterized a mutation in a nearby codon to p.M192, p.R195Q, which maps to the oligomeric interface B-substrate channel.[39,88] The p.R363L mutation, which was very recently identified by Li et al. in 2019, was detected as a subclonal *NT5C2* mutation in our study. Li et al. showed that p.R363L confers resistance to purine analogues in an ALL cell line model in the same way as the well-characterized *NT5C2* mutations do.[38] By using ASQ-PCR with 3-prime allele-specific primers with an additional third base mismatch as a cost-efficient, highly sensitive and robust tool that is comparable with NGS, two-thirds of *NT5C2* mutations in our study were detected subclonal and only one-third clonal. This finding is in line with previous findings from two earlier, but smaller studies by Ma et al. 2015[39], who described subclonal *NT5C2* mutations in 64% (9 of 14) of patients with relapsed BCP-ALL, and by Richter-Pechanska et al. 2017[89], who identified subclonal *NT5C2* mutations in 71% (15 of 21) of patients with relapsed T-ALL. This study confirms these earlier findings in a large and representative patient cohort and emphasizes that *NT5C2* mutations most commonly appear in subclones in relapses of BCP-ALL.

Recently, *NT5C2*-mutant leukemic cells were described to show a loss-of-fitness phenotype in a T-ALL mouse model.[87] In this model, *NT5C2* mutant leukemic cells were positively selected under thiopurine treatment, but mutant *NT5C2* caused imbalances in the intracellular nucleotide pool

and led to a reduced proliferation rate and leukemia initiating cell activity.[87,97] The increased export of purine metabolites out of the *NT5C2* mutant cells may result in potential satellite effects modulating the response of *NT5C2* wild type cells to purine analogue drugs.[87] Since *NT5C2* mutations are mostly identified in subclones only, *NT5C2* mutant cells may have such a supportive effect on nearby wild-type cells and, therefore, may rarely grow out to major clones.[34,38,39,52,87,97] Another possible reason for the predominantly subclonal occurrence of NT5C2 mutations may be that mutant *NT5C2* is insufficient for ALL cells to escape the selective pressure of 6-MP and methotrexate during maintenance therapy for primary ALL and that cooperative genetic alterations as for example *NRAS* mutations and/or *CDKN2A/B* deletions, that are both enriched in relapses with clonal *NT5C2* mutations, are needed to overcome the negative selection during relapse development.

Clinically, subclonal but not clonal *NT5C2* mutations were independently associated with inferior event-free survival after first relapse of ALL and with an increased rate of nonresponse to relapse treatment. These results suggest that patients with relapsed BCP-ALL harboring subclonal *NT5C2* mutations form a previously unidentified subgroup with high risk of treatment failure. Even though subclonal *NT5C2* mutations are rapidly eradicated by relapse treatment and *NT5C2* wild-type leukemic cells persist to nonresponse or second relapse, the results raise the question if *NT5C2* mutations should be implemented in relapse diagnostics and in treatment decisions. The rapid eradication of subclonal *NT5C2* mutations by relapse polychemotherapy excludes the incorporation of targeted treatment of mutant *NT5C2* into the therapy of patients with relapses harboring subclonal *NT5C2* mutations. However, these patients may still benefit form alternative therapies, as for example immunotherapies like antibody or CAR-T cell treatment, if standard relapse treatment fails. Nevertheless, the patients with relapsed BCP-ALL and clonal *NT5C2* mutations persisting to second relapse, could benefit from a third-line personalized treatment targeting *NT5C2* with mizoribine[87], a immunosuppressive drug,[47] which could be combined with RAS/Raf/MEK/ERK pathway inhibitors, as for example Selumetinib, in patients with a co-occurring *NRAS* mutation.[75] All in all, *NT5C2* mutations are a marker for poor outcome, which raises the question if *NT5C2* mutation analysis should be implemented in diagnostics of relapsed ALL and could be used similar to MRD as a prognostic marker. The relevance of *NT5C2* mutations for risk stratification and possible allocation of intermediate-risk patients to HSCT could not be assessed due to the very small number of intermediate-risk patients with low MRD levels after induction therapy in the present study. The integration of *NT5C2* mutation analysis into relapse diagnostics and a possible third-line personalized approach for patients, in whom standard relapse therapy fails, need to be further evaluated in future studies.

If detection of *NT5C2* mutations during maintenance therapy of primary ALL can serve an early identification of *NT5C2*-mutated relapses and in that way help to prevent relapse outgrowth at an early stage needs to be elucidated. However, the rapid eradication of *NT5C2*-mutant clones by

standard relapse therapy emphasizes that their role in resistance and patient outcome is complex and goes beyond mutant *NT5C2* acting as a single targetable oncogenetic driver during relapse progression. Systematic and prospective identification of *NT5C2* mutations in relapsed patients should be integrated into trials currently in planning to support an improved understanding of the biology of the aggressive relapses harboring *NT5C2* mutations. Understanding this biological basis will be essential for the development of novel treatment approaches, whether intensified treatment or novel targeted agents, that will help to improve outcome of patients after first relapse of BCP-ALL in the long-term.

6. References

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Statutory Declaration

"I, Malwine Jeanette Barz (born Pogodzinski), by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "*Clinical and biological relevance of mutations in the cytosolic 5´-nucleotidase II (NT5C2) gene in children with relapsed acute lymphoblastic leukemia*" (English)/ "Klinsche und biologische Bedeutung von Mutationen in der zytosolischen 5´-Nukleotidase (*NT5C2*) bei Rezidiven der akuten lymphoblastischen Leukämie im Kindesalter" (German), independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date 19.02.2020 Signature

Declaration of my contribution to the top-journal publication for a PhD degree

I, Malwine Jeanette Barz contributed the following to the below listed publication:

Publication:

Malwine J. Barz, Jana Hof, Stefanie Groeneveld-Krentz, Jui Wan Loh, Annabell Szymansky, Kathy Astrahantseff, Arend von Stackelberg, Hossein Khiabanian, Adolfo A. Ferrando, Cornelia Eckert, Renate Kirschner-Schwabe, **Subclonal *NT5C2* mutations are associated with poor outcomes after relapse of pediatric acute lymphoblastic leukemia**, *Blood*, 2020;epub ahead of print 22th January 2020, doi:10.1182/blood.2019002499

IMPACT FACTOR: 16.562

Contribution (in detail):

The mutation spectrum of the *NT5C2* gene in relapsed ALL (Publication Figure 1A, Publication Supplemental Table 5) was based on my data from sequencing of the *NT5C2* key exons 2, 9, 13, 15 and 16 by the method of Sanger in a cohort of n=442 patients with relapsed BCP-ALL from the ALL-REZ BFM 2002 clinical trial. My work included withdrawal of patient samples from the in-house biobank of relapsed ALL, sample quality control, PCR and cycle-sequencing, capillary gel electrophoresis and data analysis using the Mutation Surveyor software. My work was built on a pilot study from Jana Hof, a former PhD student of the department, who had sequenced 259 of 442 patients previously. I re-analyzed part of the sequencing data of this pilot study and performed additional sequence analysis for validation in cases with insufficient data quality. I compiled all sequencing data (NGS and Sanger) into the final cohort of 455 patient with relapsed ALL and classified the mutations by their allele frequency into clonal and subclonal mutations (Publication Figure 1A, Publication Supplemental Figure 1). I designed, tested and optimized ASQ-PCR assays for the validation of all subclonal (low-frequency) *NT5C2* mutations (14 different assays, summarized in Publication Supplemental Tables 3, 4 and 6 and Publication Supplemental Figures 2 to 4) and used the two best-performing ASQ-PCR assays (p.R39Q and p.R367Q) for systematic screening of all 455 patients of the study cohort subclonal p.R39Q and p.R367Q *NT5C2* mutations (Publication Figure 1B and 1C, Publication Supplemental Table 5). My work included the analysis and interpretation of the ASQ-PCR data as well as correlation of ASQ-PCR data with NGS and Sanger sequencing data (Publication Supplemental Figure 5, Publication Supplemental Table 5) and compilation of all *NT5C2* mutational data in to one data set (Publication Figure 1D). The

correlations of the *NT5C2* mutation status with recurrent genetic alterations (Publication Figure 2) was based on a previously established in-house data base of genetic alterations of patients with relapsed ALL treated within the ALL-REZ BFM 2002 clinical trial that I extended by approximately 17% to increase patient numbers. I designed, established and optimized ASQ-PCR assays for two *NRAS* and three *KRAS* mutations to analyze subclonal mutations in these genes and correlate their presence with the presence of *NT5C2* mutations (Publication Supplemental Figure 6). My work included all statistical analyses underlying Figure 2 and Tables 1 and 2 (cross tabulation analyses). Furthermore, I performed survival analyses using Kaplan-Meier analysis and Cox regression modelling (Publication Figure 3A and 3B, Publication Table 3). For Cox regression modelling, I performed a sensitivity analysis including and excluding different factors in the model to test the robustness of my model (Supplemental Tables 8 and 9). Additionally, my work included calculation of the cumulative incidence of competing events (Publication Figure 3C-D). By Sanger sequencing and ASQ-PCR, I tracked *NT5C2* mutations in samples from initial diagnosis, in follow-up samples taken during relapse treatment and in samples from the time of nonresponse or second relapse (Publication Figure 4A, Supplementary Figure 7). My work included comparison of *NT5C2* ASQ-PCR data and MRD data in follow-up samples taken during relapse treatment, nonresponse samples and/or second relapse samples (Figure 4B). I created all charts, graphs and tables for the manuscript and for the supplemental material and participated in structuring and writing of the manuscript as well as in manuscript revision (each at least 50%).

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Journal Summary List (ISI Web of Knowledge)

BLOOD: rank #1/73 category "Hematology"

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"HEMATOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 73 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	BLOOD	161,827	16.562	0.240720
2	CIRCULATION RESEARCH	52,988	15.862	0.072290
3	Lancet Haematology	1,934	11.990	0.010520
4	LEUKEMIA	24,555	9.944	0.054750
5	Journal of Hematology & Oncology	5,366	8.731	0.013620
6	Blood Cancer Journal	2,247	7.895	0.009060
7	HAEMATOLOGICA	16,255	7.570	0.037660
8	ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY	33,223	6.618	0.036000
9	AMERICAN JOURNAL OF HEMATOLOGY	10,375	6.137	0.022930
10	BLOOD REVIEWS	2,889	6.125	0.005980
11	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	19,766	6.040	0.028050
12	STEM CELLS	21,467	5.614	0.030220
13	BRITISH JOURNAL OF HAEMATOLOGY	23,963	5.206	0.037720
14	CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY	7,401	5.012	0.012890
15	THROMBOSIS AND HAEMOSTASIS	16,590	4.733	0.022810
16	BONE MARROW TRANSPLANTATION	12,031	4.674	0.020710
17	JOURNAL OF THROMBOSIS AND HAEMOSTASIS	18,886	4.662	0.028230
18	CYTOTHERAPY	5,969	4.297	0.009690
19	JOURNAL OF LEUKOCYTE BIOLOGY	16,921	4.012	0.019570
20	SEMINARS IN HEMATOLOGY	2,157	3.738	0.003950
21	TRANSFUSION MEDICINE REVIEWS	1,434	3.610	0.002890

Selected Publication (incl. Supplementary information)

Malwine J. Barz, Jana Hof, Stefanie Groeneveld-Krentz, Jui Wan Loh, Annabell Szymansky, Kathy Astrahantseff, Arend von Stackelberg, Hossein Khiabanian, Adolfo A. Ferrando, Cornelia Eckert, Renate Kirschner-Schwabe, **Subclonal *NT5C2* mutations are associated with poor outcomes after relapse of pediatric acute lymphoblastic leukemia**, *Blood*, 2020, epub ahead of print 22.01.2020, doi:10.1182/blood.2019002499

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Abstract of Bachelor thesis entitled “Point Mutations and Expression of the Mismatch Repair Gen MLH1 by Relapsed Acute Lymphoblastic Leukemia in Childhood”.

Acute lymphoblastic leukaemia (ALL) is the most common cancer in childhood. While the treatment of primary ALL approaches survival rates of up to 90%, patients with a relapse often show resistance to chemotherapeutic drugs. Studies on primary ALL and ALL cell lines have shown that defects in the mismatch repair (MMR) system are associated with resistance to cytotoxic drugs including 6-Mercaptopurin and 6-Thioguanin. Those chemically modified nucleotides (thiopurines) are part of the ALL relapse therapy and cause a mismatch in the DNA during the replication of a cell. An intact MMR system recognizes the mismatch and in case of excessive DNA damage cell death is induced. In case of a defective MMR system this pathway is disrupted and this can lead to resistances to DNA damaging drugs. The MMR repair protein MLH1 is essential for the generation of the MMR component MutL and can be inactivated by mutations or hypermethylation. The mutation c.790+1G>A was detected in the ALL cell lines REH and CCRF-CEM at the MLH1 5´-donor splice site of exon 9 leading to a very low MLH1 expression level. In this thesis 216 patients with first ALL relapse were examined for mutations in MLH1 exon 9 and flanking intronic regions by Sanger sequencing. In those 216 patients 5 (3%) sequence variations were found. One patient showed the c.790+1G>A mutation. In three patients the single nucleotide polymorphism c.790+10A>G was detected and in another patient the nucleotide variation c.790+87A>G was found. The variation c.790+10 was also found in available remission samples. In eight patients was the splicing variance analyzed. For the patient with the 5’-donor splice site mutation skipping of exon 9 was shown. Interestingly all eight relapse patients also showed additional MLH1 splicing variants lacking exon 10 or exon 9 and 10. These splice variations could result in an MLH1 protein with aberrant MutS homolog interaction domain, which can lead to a dominant negative effect on the MMR system. This requires further examinations. As a last step relative MLH1 gene expression was examined by real-time PCR in 68 relapse patients. The patient and the REH showed a low expression. Within the B-cell precursor immunophenotype, patients with a late ALL relapse had a significantly lower MLH1 expression than patients with a very early or early relapse ($p=0.031$). In summary, this work shows the mutation rate of the MLH1 Exon 9 and flanking introns is very low, which indicates that the mutation c.790+1G>A only has a minor influence on the MLH1 inactivation in patients with relapsed ALL. Nevertheless, interesting insights from the alternative splicing- and expression analysis were attained that need further examinations.

Abstract of Master thesis entitled "Molecular Genetic Basis of Resistance to Polychemotherapy in Children with Relapsed Acute Lymphoblastic Leukemia".

Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood. While treatment of primary ALL approaches survival rates of up to 90%, patients suffering from a relapse often show resistances to chemotherapeutic drugs. A study on relapse ALL have shown that mutations in the nucleotide pathway and in the tumor suppressor gene *p53* are associated with resistance to cytotoxic drugs including 6-Mercaptopurine (6-MP), 6-Thioguanine (6-TG), Idarubicin (Ida) and Daunorubicin (DNR). 6-MP and 6-TG (nucleotide analoga) are chemically modified nucleotides inducing DNA mismatches and Ida and DNR are alkylating agents inducing DNA damage. Those drugs are part of the ALL relapse therapy. The nucleotide analoga are conversed from prodrugs into its active form. This conversion can be overcome by mutations of *PRPS1* causing an overactivtiy of this protein, which is the first, rate limiting factor in the nucleotide pathway. In relapsed patients' mutations in the N-terminal domain were at amino acid position p.S103, p.N114 and p.N144 and in the pyrophosphokinase domain at the position p.G174, p.V178, p.T179 and p.A190. A cohort of n=517 patients from the ALL-REZ BFM has been sequenced for *PRPS1* in exon3, 4 and 5. In this cohort n=458 were B-ALL patients and n=59 T-ALL patients. 20 patients (3.87%) carry a nucleotide variation in the *PRPS1* gene. Four of these 20 variations have been a single nucleotide polymorphism p.I159I (n=4/ 0.77% of total patients). 16 patients (3.1% of total cohort) harbored a mutation which leads to an amino acid exchange influencing the possibility of the response to 6-TG and 6-MP during the therapy. The *PRPS1* mutations are relapse specific mutations because 11 of 12 sequenced patients (91.67%) were wild type for *PRPS1* at the initial diagnosis. Those patients carrying a *PRPS1* mutation showed a poor response to the relapse treatment and suffered an early relapse. Additionally, more males (n=14) than females (n=2) have been affected. Moreover, it has been shown that mutation of *PRPS1* in relapsed ALL patients are correlated with mutation in the *RAS* pathway genes. 12 of 16 (75%) *PRPS1* mutated patients harbored a *RAS* mutation as well. 63.64% of studied patients had the *RAS* pathway mutation at primary diagnosis and gained the PRPS1 mutation at the relapse diagnosis. Furthermore, a functional study of p53 was performed in ALL cell lines to assess whether mutated p53 is causing a resistance against alkylating drugs. The cell lines MHH and Loucy harbor following mutations of *p53*: p.R248P and p.V272M. The mutation p.R248P of *p53* was detected in a patient who is a nonresponder. Other used cell lines had been wild type (wt) for p53. The drug sensitivity profile and a correlation between DNA damage inducing drugs and p53 mutated cell lines were determined. Loucy and MHH had been less sensitive to the treatment in comparison to wt *p53* cell lines. Knock out of mutant p53 and introduction of wt *p53* in Loucy made the cells highly sensitive to the drug treatment with Ida. The introduction of wt *p53* in MHH and the drug sensitivity

result of these cells showed that p.R248P has a dominant negative effect on the wt p53 and is suppressing the function of the wt protein. All in all, this study showed that resistances or possible nonresponse to the treatment are caused by mutations in *PRPS1* and *p53*. But these genes alone cannot drive relapse and resistances and are associated with other aberrations in relapsed ALL. Nevertheless, interesting insights from the mutational analysis and functional study were attained that need further examinations.

List of publications

Barz MJ, Hof J, Groeneveld-Krentz S, Loh JW, Szymansky A, Astrahantseff K, von Stackelberg A, Khiabanian H, Ferrando AA, Eckert C, Kirschner-Schwabe R, Subclonal *NT5C2* mutations are associated with poor outcomes after relapse of pediatric acute lymphoblastic leukemia, *Blood*, 2020, epub ahead of print 22.01.2020, doi:10.1182/blood.2019002499

Groeneveld-Krentz S, Schroeder MP, Reiter M, **Pogodzinski MJ**, Pimentel-Gutiérrez HJ, Vagkopoulou R, et al. Aneuploidy in children with relapsed B-cell precursor acute lymphoblastic leukaemia: clinical importance of detecting a hypodiploid origin of relapse. *Br J Haematol* 2019. doi:10.1111/bjh.15770.

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