

# Relevance of ID3-TCF3-CCND3 pathway mutations in pediatric aggressive B-cell lymphoma treated according to the non-Hodgkin Lymphoma Berlin-Frankfurt-Münster protocols

Marius Rohde,<sup>1</sup> Bettina R. Bonn,<sup>1</sup> Martin Zimmermann,<sup>1</sup> Jonas Lange,<sup>2,3</sup> Anja Möricke,<sup>4</sup> Wolfram Klapper,<sup>5</sup> Ilse Oschlies,<sup>5</sup> Monika Szczepanowski,<sup>5</sup> Inga Nagel,<sup>6</sup> Martin Schrappe,<sup>4</sup> MMML-MYC-SYS Project,<sup>7</sup> ICGC MMML-Seq Project,<sup>6,8</sup> Markus Loeffler,<sup>7</sup> Reiner Siebert,<sup>6,8</sup> Alfred Reiter<sup>1</sup> and Birgit Burkhardt<sup>2</sup>

<sup>1</sup>Department of Pediatric Hematology and Oncology, Justus-Liebig-University Giessen; <sup>2</sup>Pediatric Hematology and Oncology, University Hospital Münster; <sup>3</sup>Translational Oncology, Department of Medicine A, University Hospital Münster; Cluster of Excellence EXC 1003, Cells in Motion, Münster; <sup>4</sup>Pediatric Hematology and Oncology, University Medical Center Schleswig-Holstein, Campus Kiel; <sup>5</sup>Department of Pathology, Hematopathology Section and Lymph Node Registry, University Hospital Schleswig-Holstein, Campus Kiel/Christian-Albrecht University, Kiel; <sup>6</sup>Institute of Human Genetics, Christian-Albrechts-University Kiel & University Hospital Schleswig-Holstein, Campus Kiel; <sup>7</sup>Institute for Medical Informatics Statistics and Epidemiology, University Leipzig and <sup>8</sup>Institute of Human Genetics, University of Ulm and University Medical Center Ulm, Germany

## ABSTRACT

**M**ature B-cell non-Hodgkin lymphoma is the most common subtype of non-Hodgkin lymphoma in childhood and adolescence. B-cell non-Hodgkin lymphomas are further classified into histological subtypes, with Burkitt lymphoma and Diffuse large B-cell lymphoma being the most common subgroups in pediatric patients. Translocations involving the *MYC* oncogene are known as relevant but not sufficient for Burkitt lymphoma pathogenesis. Recently published large-scale next-generation sequencing studies unveiled sets of additional recurrently mutated genes in samples of pediatric and adult B-cell non-Hodgkin lymphoma patients. *ID3*, *TCF3* and *CCND3* are potential drivers of Burkitt lymphomagenesis. In the study herein, frequency and clinical relevance of mutations in *ID3*, *TCF3* and *CCND3* were analyzed within a well-defined cohort of 84 uniformly diagnosed and treated pediatric B-cell non-Hodgkin lymphoma patients of the Berlin-Frankfurt-Münster group. Mutation frequency was 78% (*ID3*), 13% (*TCF3*) and 36% (*CCND3*) in Burkitt lymphoma (including Burkitt leukemia). *ID3* and *CCND3* mutations were associated with more advanced stages of the disease in *MYC* rearrangement positive Burkitt lymphoma. In conclusion, *ID3-TCF3-CCND3* pathway genes are mutated in more than 88% of *MYC*-rearranged pediatric B-cell non-Hodgkin lymphoma and the pathway may represent a highly relevant second hit of Burkitt lymphoma pathogenesis, especially in children and adolescents.

## Introduction

Non-Hodgkin lymphoma (NHL) belong to the most common hematological malignancies in childhood and adolescence.<sup>1</sup> About two thirds of all pediatric NHL belong to mature B-cell lymphomas, with Burkitt lymphoma (BL) and Diffuse large B-cell Lymphoma (DLBCL) representing the most prevalent entities in this subgroup.<sup>2,3</sup> During the past decades remarkable increases in patient survival were achieved by continuous advancement of treatment approaches worldwide.<sup>4</sup> Nowadays, risk-stratified polychemotherapy treatment reaches up to a total of 90% probability of event-free survival (pEFS) in pediatric NHL.<sup>5</sup> More than 95% of



EUROPEAN  
HEMATOLOGY  
ASSOCIATION



Ferrata Storti  
Foundation

Haematologica 2017  
Volume 102(6):1091-1098

## Correspondence:

birgit.burkhardt@ukmuenster.de

Received: September 27, 2016.

Accepted: February 7, 2017.

Pre-published: February 16, 2017.

doi:10.3324/haematol.2016.156885

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: [www.haematologica.org/content/102/6/1091](http://www.haematologica.org/content/102/6/1091)

©2017 Ferrata Storti Foundation

Material published in *Haematologica* is covered by copyright. All rights are reserved to the Ferrata Storti Foundation. Use of published material is allowed under the following terms and conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>.

Copies of published material are allowed for personal or internal use. Sharing published material for non-commercial purposes is subject to the following conditions:

<https://creativecommons.org/licenses/by-nc/4.0/legalcode>, sect. 3. Reproducing and sharing published material for commercial purposes is not allowed without permission in writing from the publisher.



all pediatric patients diagnosed with mature B-cell lymphoma in Germany are registered in the clinical trials of the non-Hodgkin Lymphoma - Berlin-Frankfurt-Münster (NHL-BFM) study group and are treated according to standardized treatment plans.

Classical genetic and molecular pathological studies on the pathogenesis of B-cell lymphoma provided distinct pathogenetic features, like translocation of the tumor oncogene *MYC* in BL<sup>6</sup> and specific molecular gene expression signatures in DLBCL.<sup>7</sup> *MYC* translocation was shown to be involved in cell cycle regulation, cellular growth, metabolism and apoptosis.<sup>6,8,9</sup> However, *MYC* translocation alone is not sufficient to initiate malignant transformation of B cells.<sup>10,11</sup> In DLBCL multiple co-acting molecular alterations have been described. Differentiation between activated B-cell-like (ABC) and germinal center B-cell-like (GCB) by gene expression is well established, profiling revealed differences in prognosis, especially in adult patients.<sup>12</sup>

Recent next-generation sequencing (NGS) studies provided valuable insight into the landscape of genomic alterations in B-cell non-Hodgkin lymphoma (B-NHL) and independently introduced Inhibitor of DNA binding 3 (*ID3*) to be recurrently mutated in BL.<sup>13-15</sup> *ID3* encodes for a helix-loop-helix (HLH) protein that typically lacks a basic DNA-binding domain and therefore inhibits other HLH proteins from binding to their transcriptional target sites by heterodimerization.<sup>16-18</sup> One such *ID3*-inhibited protein is Transcription Factor 3 (*TCF3*), which is consecutively expressed at high levels during B-cell development.<sup>19,20</sup> *TCF3* itself was also shown to be recurrently mutated in BL in the transcriptional study from Schmitz and colleagues, who additionally showed that both *TCF3* and *ID3* mutations resulted in increased expression of *TCF3* targets,<sup>14</sup> promoting growth and survival by activation of B-cell receptor signaling. A direct target of *TCF3* is the cell cycle regulating Cyclin D3 (*CCND3*),<sup>21,22</sup> which was also shown to harbor activating mutations in different subtypes of B-NHL cases.<sup>13,14</sup> The above mentioned studies described *ID3* mutations to accumulate in the HLH domain and functional analyses showed *ID3* mutant proteins to be less effective or completely ineffective in inhibiting *TCF3*, thus forcing increased cell proliferation and survival *via* phosphoinositide 3-kinase (*PI3K*) and Cyclin D3.<sup>13-15</sup> While *TCF3* mutations also affected the basic HLH (bHLH) domain of its isoform *E47*, *TCF3* mutant proteins did not lose their effect on downstream targets when compared to wild-type *TCF3*, but displayed *ID3/TCF3* interaction, turning them immune to the inhibitory effect of *ID3*.<sup>14</sup> *CCND3* mutant proteins showed an increase in cell cycle stimulation when compared to unaffected *CCND3*, thereby indicating a gain-of-function.<sup>14</sup> In summary, mutations in each of the candidate genes are thought to contribute to cellular growth, cell survival and proliferation.<sup>23,24</sup>

Within the index studies there was a large variation in the incidence of *ID3* mutations in BL. The frequency of *ID3* mutations varied between 34% (Love *et al.*), 58% (Schmitz *et al.*) and 68% (Richter *et al.*) (Online Supplementary Table S1). Schmitz *et al.* reported *TCF3* mutations in 27% and additional *CCND3* mutations in 38% of sporadic BL cases. *CCND3* mutations were also analyzed in the study by Richter *et al.*, who also reported 38% of the cases to display these aberrations.<sup>13-15</sup>

In this study we analyzed a well-defined cohort of 84

pediatric B-NHL patients, diagnosed and treated according to the NHL-BFM protocols for mutations in *ID3*, *TCF3* and *CCND3* to describe the incidence and relevance of such mutations in a uniformly diagnosed and treated representative pediatric cohort. Furthermore, we analyzed samples from 96 pediatric patients diagnosed with precursor B-cell acute lymphoblastic leukemia (pB-ALL), to examine whether disruption of this pathway also occurs in this precursor B-cell malignancy.

## Methods

### Patient samples

1117 Pediatric patients diagnosed with “Burkitt lymphoma”, “Burkitt leukemia”, “Diffuse large B-cell lymphoma” or “B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma” between January 2000 and December 2012 were eligible for the recruited population based study cohort. Pretreatment tumor samples from fresh frozen tissue, bone marrow or effusion samples were available for 84 patients (“study cohort”). For a more robust examination regarding the relevance of mutation status with respect to patient outcome, initial tumor samples from an additional 10 patients with a known history of relapse or progress were analyzed as the “extended cohort”.

All patients analyzed were registered in the NHL-BFM data center and treated according to the NHL-BFM protocols (NHL-BFM 95 and analogous protocol B-NHL BFM 04).<sup>3</sup>

Tumor DNA samples from 96 pediatric patients diagnosed with precursor B-ALL were kindly provided by the ALL-BFM study center, University of Kiel, Germany. All patients had previously been diagnosed between 2000 and 2006 and were treated according to the ALL-BFM 2000 protocol.<sup>25</sup> More detailed clinical characteristics of the analyzed patients can be found in the online supplement (Online Supplementary Methods).

This study was approved by the Ethical Advisory Board of the University of Giessen, Germany (A89/11 Amendment 2013).

### *ID3*, *TCF3* and *CCND3* mutation analysis

In the study cohort the full coding region of the *ID3* gene, exon 17 of the *TCF3* gene and the coding region of *CCND3* exon 5 were sequenced. pB-ALL samples were analyzed for *ID3* mutations only. Cases presenting with mutations were confirmed within a repetition experiment. More detailed descriptions of primer pairs, sequencing modalities, reference sequence annotation and exclusion of singular nuclear polymorphisms are given in the online supplement (Online Supplementary Methods).

### Statistics

Statistical analysis was performed in order to identify differences in typical patient characteristics, such as sex, age, stage of disease, bone marrow (BM) involvement, central nervous system (CNS) involvement, lactate dehydrogenase (LDH) levels, diagnosis, pEFS and probability of overall survival (pOS) according to the mutational status of the analyzed candidate genes. *MYC* rearrangement status was available from the study database. Clinical data for each calculation referred to patients with successful investigation of the respective criteria. Differences in the distribution of individual parameters among patient subsets were analyzed using Pearson's  $\chi^2$  test<sup>26</sup> or Fisher's exact test<sup>27</sup> where appropriate. pEFS was calculated according to Kaplan and Meier,<sup>28</sup> taking into consideration the time between the date of diagnosis and either the date of event or date of last follow up. pOS was calculated according to Kaplan and Meier<sup>28</sup> under consideration of the

time between date of diagnosis and death from any cause. Survival estimates were compared by the log-rank test.<sup>29</sup> Significant differences were assumed when the respective *P* value (*P*) was lower than 0.05. Calculations were conducted using the SAS statistical program (SAS-PC, Version 9.3, Cary, NC, USA: SAS Institute Inc.). Fisher's exact tests were calculated using the software Prism 6 for Mac OS X (GraphPad Software, Version 6.0c, San Diego, CA, USA). The two-tailed option was used.

## Results

### Patient characteristics of the study cohort

Characteristics of the 84 analyzed patients in the study cohort are shown in Table 1. Clinical characteristics of patients analyzed and not analyzed were similar regarding age, sex, BM involvement, CNS involvement, stage of disease and outcome. Histological subtype was BL in 64 cases (including 14 Burkitt leukemias, B-AL), DLBCL in 13 cases and B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma (B-NHL not further classifiable [nfc]) in 7 cases. Comparing patient characteristics of the analyzed cohort with the not analyzed patients revealed a trend towards

higher LDH levels in the study cohort and an overrepresentation of BL and B-NHL nfc over DLBCL cases. These mild differences, at least in part, might be related to the availability of tissue for molecular analysis. BM obtained in B-AL and tumor after ileocecal resection of BL was more likely to be sent to the NHL-BFM study center for research than samples of small biopsies of e.g., cervical lymph nodes in DLBCL. These circumstances may also explain the trend towards higher LDH levels in the study cohort, as a high tumor burden is associated with higher LDH levels, which in turn is key to a larger availability of sample material in the study center. To compensate for this slight imbalance in the representation of histological subtypes, the analyses were run for the study cohort and for the subgroup of *MYC* rearrangement positive BL/B-AL separately.

Incidence and relevance of *ID3*, *TCF3* and *CCND3* mutation status were analyzed in the study cohort. A detailed description of all genomic variants including the predicted change on protein level is presented in the *Online Supplementary Table S2*.

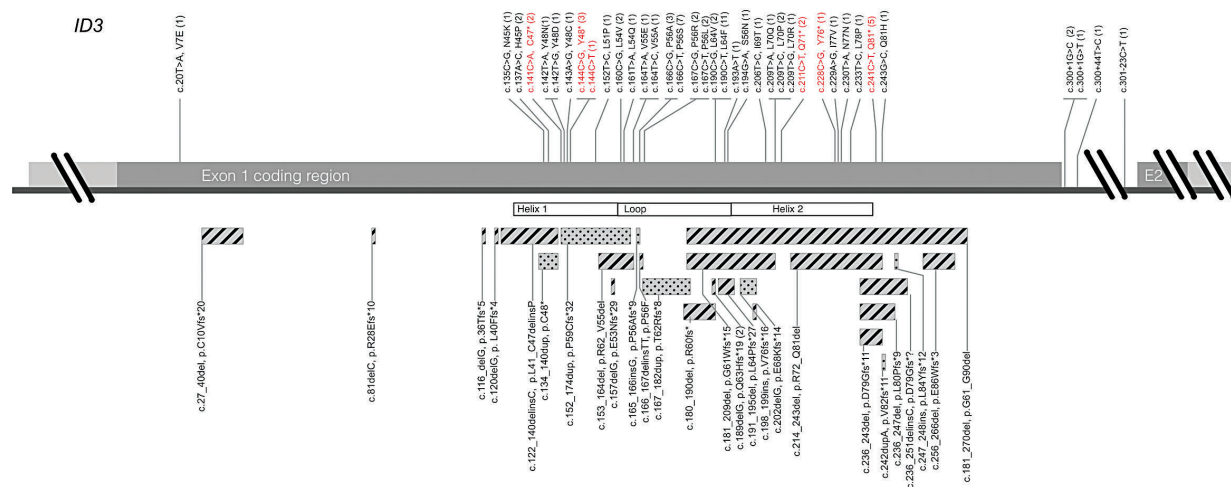
### *ID3*, *TCF3* and *CCND3* sequencing results

*ID3* mutations were found and verified in 56 out of 84 B-NHL samples (Figure 1). Thirty-one of 56 cases showed

**Table 1.** Patient characteristics of the study cohort.

Characteristics	Patients not analyzed (n=1033)		Patients analyzed (n=84)		<i>P</i>
Sex					
male	819	79%	69	82%	
female	214	21%	15	18%	.53
Age					
< 10 y	520	50%	43	51%	
10-14 y	346	34%	27	32%	
> 14 y	167	16%	14	17%	0.97
Stage of disease					
stage I	97	10%	5	6%	
stage II	247	25%	14	18%	
stage III	412	41%	43	55%	
stage IV	73	7%	3	4%	
B-AL	165	17%	14	18%	0.17
BM involvement	201	20%	14	17%	0.53
yes					
CNS involvement	102	10%	8	10%	0.92
yes	606	59%	39	46%	
LDH					
< 500 U/l					
500-1000 U/l	155	15%	16	19%	
> 1000 U/l	264	26%	29	35%	0.07
Diagnosis					
BL	576	56%	50	60%	
B-AL	165	16%	14	17%	
DLBCL	265	26%	13	15%	
B-NHL nfc	27	3%	7	8%	0.0008
Outcome					
pEFS (2y)	90 ± 1%		89 ± 3%		0.87 (LR)
pOS (2y)	93 ± 1%		92 ± 3%		0.83 (LR)

Y: years; BM: bone marrow; CNS: central nervous system; LDH: lactate dehydrogenase serum level; BL: Burkitt lymphoma; B-AL: Burkitt leukemia; DLBCL: Diffuse large B-cell lymphoma; B-NHL nfc: B-cell non-Hodgkin lymphoma with features intermediate between BL and DLBCL; pEFS: probability of event-free survival; pOS, probability of overall survival; LR: log-rank



**Figure 1.** *ID3* gene plot with annotated mutations of the study cohort. *ID3* coding region of exon 1 is illustrated with single base-pair substitutions on the upper and more complex alterations (insertions, deletions, InDels, duplications) on the lower site. Substitutions resulting in a nonsense mutation are depicted in red. Hatched bars delineate deletions and InDels, dotted bars characterize insertions and duplications. Each mutation is labeled with a correspondent description on the genomic and protein level, as well as the absolute number of occurrences in brackets. The functional helix-loop-helix domain is mapped according to UniProt (Q02535).

multiple *ID3* mutations, 26 cases with 2 mutations, 4 cases with 3 mutations and 1 case with 4 mutations. Ten of those cases were randomly selected for cloning, and biallelic involvement was shown in all cases. With respect to hotspots, single nucleotide substitutions affecting position C190 were the most frequent (13 cases), followed by C166 (10 cases) and C241 (5 cases). Four disambiguates were genomic variants that were not predicted to result in changes on amino acid levels: 144C>T, 193A>T, 300+44T>C, and 301-23C>T. Notably, each of the cases with one of those silent mutations also harbored at least a second *ID3* mutation. On the genomic level, 77 of 93 (83%) mutations directly affected the functional HLH coding region. The remaining 18 mutations were allocated either close to the splice-site of exon 1 (4 mutations), upstream or downstream of the HLH domain (13 mutations) or in the intronic region between exon 1 and 2 (1 mutation). Again, all cases with mutations not directly affecting the HLH domain or the splice-site were associated with at least a second mutation in the HLH domain. The frequency of *ID3* mutations according to diagnosis was 50/64 (78%) for BL/B-AL and 2/13 (15%) for DLBCL. In the subgroup of 7 analyzed B-NHL nfc, 4 showed mutations in *ID3*.

Mutations in *TCF3* (8/84, 10%) were considerably lower compared to the high frequency of cases with *ID3* and were only found in BL/B-AL cases (8/64, 13%). All 8 mutations occurred in the coding region of the bHLH binding domain of *TCF3* (Online Supplementary Figure S1). Mutation 1675G>A was present in 2 cases.

Twenty-six cases harbored *CCND3* mutations (Online Supplementary Figure S2). Mutations affected nucleotide C811 with a cytosine duplication in 9 cases, resulting in a protein elongating frameshift. Four cases presented with T869G substitution and 3 cases showed C580T mutations. Twenty-three out of 64 BL/B-AL cases (36%) presented with *CCND3* mutations. In DLBCL there were 2 out of 14 cases affected. One mutation was present in a case with a B-NHL nfc diagnosis.

### Mutational pattern of *ID3*, *TCF3* and *CCND3* and correlation with *MYC* rearrangement status

In total, 63 out of 84 cases (75%) had at least 1 mutation in 1 of the investigated genes. Exclusive *ID3* mutations were the most frequent (51%). This was followed by cases with concurrent *ID3* and *CCND3* mutation (31%). Cases 33 and 12 harbored mutations in all 3 genes. The pattern of mutations within the study cohort is depicted in Figure 2.

Results of fluorescence *in situ* hybridization (FISH) for detection of *MYC* rearrangements were available for 77 cases. Fifty-eight of 65 *MYC* rearrangement positive cases (89%) had at least one mutation in *ID3* and/or *TCF3* and/or *CCND3*. In contrast, within 12 *MYC* rearrangement negative patients only 1 case was affected by *ID3* mutations ( $P<0.0001$ ). In this patient (case 68) *ID3* mutations 20T>A and 164T>A were present.

### Clinical characteristics according to mutational status in *ID3*, *TCF3* and *CCND3*

Clinical characteristics and outcome regarding *ID3*, *TCF3* and *CCND3* mutational status were first analyzed in the study cohort (Table 2). *ID3* mutations were positively correlated with reference diagnosis of BL/B-AL ( $P=0.0003$ ), higher LDH serum levels ( $P=0.0038$ ) and higher stage of disease ( $P=0.03$ ) (Table 2). *ID3* mutations occurred at a higher frequency in BL/B-AL cases when compared to DLBCL ( $P=0.0001$ ). However, these results are strongly biased by diagnosis, as BL/B-AL patients comprised higher LDH serum levels, higher stage of disease and a frequent discovery of *ID3* mutations. To investigate the actual clinical relevance of mutations we further analyzed patient characteristics within BL/B-AL *MYC* rearrangement positive cases ( $n=61$ ) (Online Supplementary Table S3). *ID3* mutated cases were still associated with higher LDH serum levels ( $P=0.0431$ ). Furthermore, *CCND3* mutated cases were positively associated with advanced stage of disease ( $P=0.0482$ ). Regarding pEFS and pOS, there were no significant differences between wild-

Table 2. Clinical characteristics of study cohort regarding ID3, TCF3 and CCND3 mutation status.

	ID3 <sup>mutated</sup>		ID3 <sup>wt</sup>		P	TCF3 <sup>mutated</sup>		TCF3 <sup>wt</sup>		P	CCND3 <sup>mutated</sup>		CCND3 <sup>wt</sup>		P
All	n=56		n=28			n=8		n=76			n=26		n=58		
Sex															
male	47	84%	22	79%		6	75%	63	83%		21	81%	48	83%	
female	9	16%	6	6%	0.55	2	25%	13	17%	0.58	5	19%	17	17%	0.83
Age															
< 10 y	31	55%	12	43%		7	88%	36	47%		16	62%	27	47%	
10-14 y	19	29%	11	39%		1	12%	26	34%		7	27%	20	35%	
> 14 y	9	16%	5	18%	0.52	0	0%	14	18%	0.09	3	12%	11	19%	0.43
Stage of disease															
I	1	2%	4	14%		0	0%	5	7%		1	4%	2	8%	
II	8	17%	6	21%		4	50%	10	14%		5	19%	6	17%	
III	29	52%	14	50%		2	25%	41	58%		10	39%	24	62%	
IV	1	2%	2	7%		0	0%	3	4%		2	8%	0	2%	
B-AL	13	27%	1	7%	0.03	2	25%	12	17%	0.09	8	31%	6	11%	0.11
BM involvement	13	25%	1	7%	0.27	2	25%	12	16%	.51	8	31%	6	10%	0.03
yes															
CNS involvement	6	11%	2	7%	0.60	1	13%	7	9%	0.76	5	19%	3	5%	0.06
yes															
LDH															
< 500 U/l	19	34%	20	71%		4	50%	35	46%		9	35%	30	52%	
500-1000 U/l	12	21%	4	14%		2	25%	14	18%		5	19%	11	19%	
> 1000 U/l	25	45%	4	14%	<0.01	2	25%	27	36%	0.81	12	46%	17	29%	0.27
Diagnosis															
BL	37	66%	13	46%		6	75%	44	58%		15	58%	36	60%	
B-AL	13	23%	1	4%		2	25%	12	16%		8	31%	6	10%	
DLBCL	2	4%	11	40%		0	0%	13	17%		2	8%	11	19%	
B-NHL nfc	4	7%	3	11%	<0.01	0	0%	7	9%	0.42	1	4%	6	10%	0.08
Outcome															
pEFS (2y)	88 ± 4%		93 ± 5%.43 (LR)			88 ± 12%		89 ± 4%.82 (LR)			85 ± 7%		91 ± 4%		0.38 (LR)
pOS (2y)	90 ± 4%		93 ± 6%.26 (LR)			88 ± 12%		92 ± 3%.61 (LR)			89 ± 6%		93 ± 3%		0.52 (LR)

Wt: wild-type; y: years; BM: bone marrow; CNS: central nervous system; LDH: lactate dehydrogenase serum level; BL: Burkitt lymphoma; B-AL: Burkitt leukemia; DLBCL: Diffuse large B-cell lymphoma; B-NHL nfc: B-cell non-Hodgkin lymphoma with features intermediate between BL and DLBCL; pEFS: probability of event-free survival; pOS: probability of overall survival; LR: log-rank

type and mutated cases either in the study cohort or in subgroup analyses.

Next we analyzed for clinical relevance of certain mutational patterns. Of particular note was the combination of *ID3* and/or *TCF3* mutations, these cases were again associated with higher LDH levels ( $P=0.0023$ ), which is also reflected in an increased frequency of these cases in higher risk groups. Also, in patients with simultaneous and exclusive *ID3* and *CCND3* mutations, the frequency of BM involvement ( $P=0.014$ ) and as a consequence a diagnosis of B-AL ( $P=0.0175$ ) was increased.

Regarding *ID3* mutation hotspots it is of note that mutations affecting position 241C, resulting in Q81\* nonsense mutation on the protein level, accumulated in cases with B-AL ( $P=0.0073$ ) compared to BL. Further investigated *ID3* hotspots at 190C and 166C did not show association to any clinical criteria.

#### Outcome and event-free survival with respect to mutational status

In the study cohort 9 out of 84 patients suffered disease progression or relapse. Detailed analyses of *ID3*, *TCF3* and *CCND3* mutation frequencies and mutational patterns were not significantly associated with pOS and pEFS (Table 2). This observation was confirmed within the analysis of

an additional 10 initial BL samples from patients with a medical history of subsequent disease progression or relapse, adding up to a total of 19 cases with refractory or relapsed disease compared to 75 event-free cases.

#### ID3 sequencing results in pB-ALL

In the cohort of 96 pediatric pB-ALL patients DNA isolated from leukemic blasts was analyzed for *ID3* mutations. There were no pathogenic *ID3* mutations found.

#### Discussion

Burkitt Lymphoma is the most common subtype of NHL in children. With current polychemotherapy treatment regimen event-free survival rates of 90% can be achieved. However, the outcome of patients who suffer from relapse is often fatal. Most patients do not achieve second remission despite intensive salvage treatment. Therefore, new treatment concepts are urgently needed to salvage these patients. New drugs directly targeting pathogenic pathways of lymphoma cells might represent one possible strategy. However, despite MYC-activating translocations, detectable in the majority of cases,<sup>30</sup> little is known about Burkitt pathogenesis and molecular-based risk factors are lacking.

Specimen	case 33	case 12	case 86	case 32	case 23	case 57	case 1	case 71	case 44	case 5	case 55	case 50	case 76	case 59	case 64	case 9	case 48	case 24	case 64	case 14	case 7	case 43	case 40	case 16	case 49	case 15	case 53	case 61	case 13	case 67	case 25	case 8	case 11	case 10	case 84	case 79	case 37	case 81	case 83	case 75	case 35	case 36	case 26	case 45	case 69	case 39	case 73	case 78	case 47						
Diagnosis	BL	BL	B-AL*	BL	BL	BL	BL	BL*	B-AL*	BL	BL	B-AL*	B-AL*	BL	B-AL*	BL	B-AL*	BL	BL	B-AL	B-AL	BL	B-AL*	B-AL	BL	BL	BL*	BL*	BL	BL	BL	BL*	BL	BL	BL	B-AL	BL	B-AL*	BL	BL*	BL*	BL*	BL	BL	BL*	B-AL	BL	BL							
MYC rear.	+	+	+	+	+	o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
ID3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red			
TCF3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red		
CCND3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red

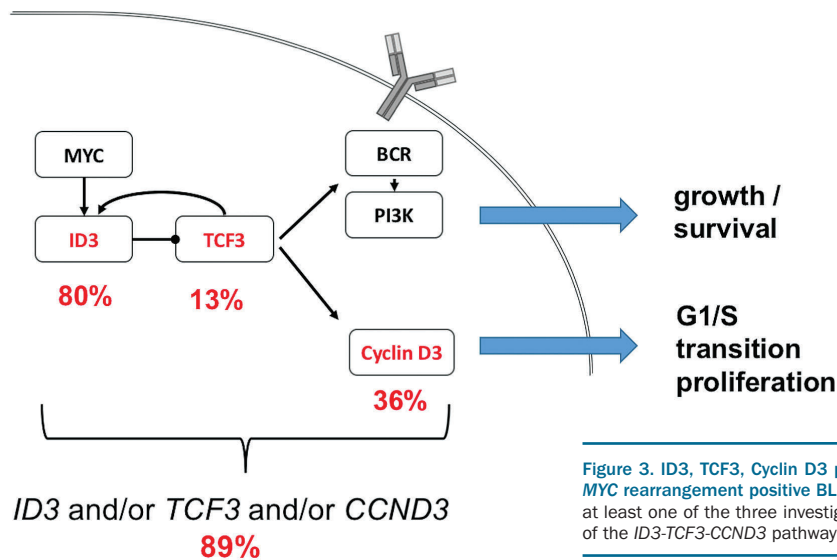
Specimen	case 2	case 52	case 63	case 74	case 38	case 22	case 82	case 29	case 3	case 77	case 20	case 65	case 54	case 4	case 80	case 68	case 27	case 56	case 19	case 42	case 30	case 31	case 70	case 60	case 6	case 28	case 18	case 58	case 51	case 46	case 62	case 17	case 21	case 41																		
Diagnosis	BL	B-AL*	BL*	BL*	BL	BL	BL	BL*	BL	BL	BL	BL	BL	BL	B-NHL Nfc	B-NHL Nfc	B-NHL Nfc	B-NHL Nfc	B-NHL Nfc	B-NHL Nfc	B-NHL Nfc	DLBCL <sup>†</sup>	DLBCL <sup>†</sup>	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL	DLBCL																		
MYC rear.	+	+	+	+	+	+	-	-	+	+	+	+	+	+	o	-	+	+	+	+	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o																	
ID3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red																	
TCF3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red																
CCND3	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red

**Figure 2. Overview of B-NHL patient sequencing results on ID3, TCF3 and CCND3.** Reference diagnosis according to NHL-BFM study. Red block indicates a case with mutation, black block indicates wild-type. MYC rear.: MYC status as reported in the study database; +: MYC rearrangement positive; -: MYC rearrangement negative; o: MYC status unknown. \*: No reference pathology review available. Diagnosis according to study center review. †: No reference diagnosis available. Diagnosis according to local pathology report. ‡MYC FISH analysis not available. However, MYC-Ig PCR report was positive for MYC rearrangement. BL: Burkitt lymphoma; B-AL: Burkitt leukemia; DLBCL: Diffuse large B-cell lymphoma; B-NHL nfc: B-NHL unclassifiable, with features intermediate between BL and DLBCL.

Therefore, the study herein aimed at identifying the frequency and clinical relevance of *ID3-TCF3-CCND3* pathway mutations and presents the largest analysis of such mutations in pediatric B-NHL thus far. While the 3 NGS studies cited previously were the first to describe genomic alterations of *ID3* and equally evaluated these finding as a new hallmark of BL, there were striking differences regarding the *ID3* mutation frequency in the studied cohorts, ranging between 34% and 68%.<sup>13-15</sup> Those variations point to relevant differences in inclusion criteria (i.e., histological-/morphological-/study-based vs. molecular-based definitions of BL) and clinical characteristics of the analyzed patients. This was also stressed by Havelange *et al.*, who recently published a series of 13 pediatric and 11 adult BL patients with respect to age-related genetic differences.<sup>31</sup> In their cohort they found 10 out of 10 evaluable *ID3* mutated cases in the adult group compared to only 5 out of 13 pediatric ones and discussed a potential higher prevalence of *ID3* mutations in adults. However, Richter *et al.* found an age-related correlation of *ID3* mutations towards younger patients and another study of only adult BL patients reported a rather low mutation rate of 47%.<sup>32</sup> With the finding of 78% *ID3* mutations in BL in the current study and comparison of the age structure of the recent studies (*Online Supplementary Table S1*), we conclude that *ID3* mutations occur at high frequency in pediatric BL patients. As patient age itself was not associated with mutation frequency within pediatric cases, these observations lead to the conclusion that *ID3* mutation frequency might in fact be associated with more homogeneously presenting pediatric BL and occur less often in the more heterogeneous group of Burkitt-like adult B-cell lymphoma. This is also supported by previously found differences in molecular presentation of BL between pediatric and adult patients, with the general mutational load being significantly higher in older BL patients.<sup>33</sup> Furthermore, BL

in general is known for homogeneous gene expression profiles, especially in comparison to the related group of DLBCL.<sup>34,35</sup> Results from whole genome sequencing of 13 pediatric BL cases of the NHL-BFM group supported these observations on the genomic level, showing a median of only 28 protein changing somatic mutations per tumor and a high frequency of recurrently affected genes, even in the small number of 13 cases.<sup>36</sup> The frequency of *TCF3* mutations in BL (13%) occurred less often in our cohort, while the incidence of *CCND3* mutations in BL (36%) cases was consistent with the findings of other groups.<sup>13,14,31</sup>

Within the subgroup of 61 *MYC* rearrangement positive BL, *ID3* mutations were significantly associated with a more disseminated presentation of disease and *CCND3* showed positive correlation to an advanced stage of disease, supporting their pro-proliferative and cell cycle driving role. These effects became even more evident when evaluating cases with *ID3* and/or *TCF3* mutations, which can be regarded as equal with respect to the resulting functional disruption of the pathway. The frequency of simultaneous *ID3* and *CCND3* mutations was significantly higher in patients with BM involvement, hinting at a potential relevance in terms of blast migration. Havelange *et al.* found a poorer outcome for patients affected by such simultaneous mutations in *ID3* and *CCND3*.<sup>31</sup> In our cohort we could not confirm this finding for pediatric patients, and it is, however, difficult to compare pediatric and adult patients with respect to clinical characteristics and prognosis as treatment regimens are generally different and outcome is inferior in adult patients.<sup>37</sup> In our study there was no association between clinical outcome and mutational status. These findings represent a contrast to previous data of Richter *et al.*, where superior outcome for patients with *ID3* mutations was reported. Again, one possible explanation



**Figure 3.** ID3, TCF3, Cyclin D3 pathway with frequencies of respective mutations in MYC rearrangement positive BL. BL with positive MYC translocation had mutations in at least one of the three investigated candidate genes in 89%, representing affection of the ID3-TCF3-CCND3 pathway in the vast majority of pediatric BL cases.

are the aforementioned general differences between BL in pediatric and adult patients. The high number of *ID3* mutations and recurrent involvement of its partners suggest a role of these alterations in Burkitt-lymphomagenesis, rather than a role for disease recurrence in a small subgroup of patients. In the current study, 89% of BL with positive *MYC* translocation had mutations in at least 1 of the 3 investigated candidate genes, representing affection of the *ID3-TCF3-CCND3* pathway in the vast majority of pediatric BL cases (Figure 3).

The study cohort included 7 cases of B-NHL nfc and 13 cases of DLBCL. Among them we detected 5 and 3 cases with *ID3-TCF3-CCND3* pathway mutations, respectively. Information on the *MYC* rearrangement status was limited in some of these patients, however, the overall strong association of *ID3-TCF3-CCND3* pathway mutations with *MYC* rearrangements in BL could similarly be observed in B-NHL nfc and DLBCL. Despite the defining histological diagnosis some of those cases show Burkitt-like features with respect to the genetic findings. In many malignancies certain discrepancies between histological and molecular diagnosis have been observed after the establishment of molecular profiling. In this context, proof of *ID3-TCF3-CCND3* pathway impairment might be helpful to better discriminate such borderline cases as BL in the future. This is supported by the recently updated World Health Organization (WHO) classification of lymphoid neoplasms, wherein *ID3* and *TCF3* mutations were added to the molecular characteristics of BL.<sup>38</sup>

The analysis of 96 samples of pB-ALL patients representing an immature B-cell malignancy did not show any pathogenic *ID3* mutations, supposing their exclusive occurrence in mature B-cell lymphoma. Regarding the process of malignant transformation in mature B cells, initial studies attributed the occurrence of the pathognomonic *MYC* translocations in BL to altered recombination-activating gene (RAG)-mediated recombination, however, more recently it has been widely accepted that aberrant somatic hypermutation processes involving activation-induced cytidine deaminase (AID) lead to these

changes.<sup>39,40</sup> Regarding *ID3*, it is of note that mutations were shown to recurrently occur in the RGYW-motif that is favorably affected by AID as well.<sup>13</sup> As *MYC* translocation alone seems not to be sufficient to induce lymphomagenesis,<sup>10</sup> one might speculate that subsequent impairment of the investigated pathway serves as a relevant second hit for BL development.

This hypothesis is furthermore supported by the detection of *ID3* mutations in mature B-cell malignancies exclusively. In addition, the lack of associations with clinical characteristics or prognosis may even imply an essential function of *ID3-TCF3-CCND3* pathway disruption. Cases presenting without mutations might still be affected by focal loss of *ID3* or mutations in other functional partners that are involved up- or downstream within the same pathway, likely in the B-cell receptor (BCR), PI3K and cyclin-dependent kinase (CDK)4/6 pathways and their regulators. However, additional candidates will less likely present at similar high frequencies, as NGS studies thus far should have covered most of the highly recurrent genomic events in BL.<sup>13-15,41</sup>

The overall high number of affected cases asks for therapeutic targeting of this pathway. There is initial promising evidence for successful application and efficacy of the orally available CDK4/6 inhibitor, palbociclib (PD) 0332991, as demonstrated by tumor mass reduction in a BL mouse model by Schmitz *et al.*<sup>14</sup> CDK4/6 inhibitors have also recently been shown to be effective in renal cell carcinoma cell lines and breast cancer cell lines<sup>42,43</sup> and are in preparation for clinical phase I and II studies in breast cancer patients (*clinicaltrials.gov* Identifier: 02297438). Further functional investigation of this pathway will shed more light on molecular processes in BL and hopefully reveal more specific therapeutic options.

In the context of relatively homogeneous genomic alterations in pediatric Burkitt lymphoma, the high number of *ID3* mutations found in this study of pediatric B-NHL patients suggests an essential role for this pathway with respect to lymphomagenesis and the phenotype of Burkitt lymphoma.

### Funding

The research on MYC-positive lymphomas is supported by the BMBF in the framework of E:bio Network Molecular Mechanisms in Malignant Lymphoma with MYC-Deregulation (MMML-MYC-SYS) Grant 0316166. The ICGC MMML-Seq Network is supported by the grants of the BMBF (01KU1002A-J and 01KU1505G). Support of research infrastructure by the Forschungshilfe-Peiper, Giessen, is acknowledged. WK, RS, MS

and IO are supported by the Kinderkrebsinitiative Buchholz, Holm-Seppensen (KKI). The research of JL is supported by the Clinician-Scientist Programme of the Cells-in-Motion Cluster of Excellence, Muenster, Germany.

### Acknowledgments

We thank the patients, their parents and participating clinics of the NHL-BFM studies.

### References

1. Swerdlow SH, Campo E, Harris NL, et al. (eds.). WHO classification of tumours of hematopoietic and lymphoid tissue. IARC press, Lyon 2008
2. Burkhardt B, Zimmermann M, Oshlies I, et al. The impact of age and gender on biology, clinical features and treatment outcome of non-Hodgkin lymphoma in childhood and adolescence. *Br J Haematol.* 2005; 131(1):39-49.
3. Woessmann W, Seidemann K, Mann G, et al. The impact of the methotrexate administration schedule and dose in the treatment of children and adolescents with B-cell neoplasms: a report of the BFM Group Study NHL-BFM95. *Blood.* 2005;105(3):948-958.
4. Rossig C, Juergens H, Schrappe M, et al. Effective childhood cancer treatment: The impact of large scale clinical trials in Germany and Austria. *Pediatr Blood Cancer.* 2013;60(10):1574-81.
5. Worch J, Rohde M, Burkhardt B. Mature B-Cell lymphoma and leukemia in children and adolescents-review of standard chemotherapy regimen and perspectives. *Pediatr Hematol Oncol.* 2013;30(6):465-83.
6. Boxer LM, Dang CV. Translocations involving c-myc and c-myc function. *Oncogene.* 2001;20(40):5595-5610.
7. Miles RR, Raphael M, McCarthy K, et al. Pediatric diffuse large B-cell lymphoma demonstrates a high proliferation index, frequent c-Myc protein expression, and a high incidence of germinal center subtype: Report of the French-American-British (FAB) international study group. *Pediatr Blood Cancer.* 2008;51(3):369-374.
8. Sanchez-Beato M, Sanchez-Aguilera A, Piris MA. Cell cycle deregulation in B-cell lymphomas. *Blood.* 2003;101(4):1220-1235.
9. Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol.* 1999;19(1):1-11.
10. Roschke V, Kopantzev E, Dertzbaugh M, Rudikoff S. Chromosomal translocations deregulating c-myc are associated with normal immune responses. *Oncogene.* 1997; 14(25):3011-3016.
11. Nepal RM, Zaheer A, Basit W, Li L, Berger SA, Martin A. AID and RAG1 do not contribute to lymphomagenesis in Emu c-myc transgenic mice. *Oncogene.* 2008;27(34):4752-4756.
12. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med.* 2002; 346(25):1937-1947.
13. Richter J, Schlesner M, Hoffmann S, et al. Recurrent mutation of the ID3 gene in Burkitt lymphoma identified by integrated genome, exome and transcriptome sequencing. *Nat Genet.* 2012;44(12):1316-1320.
14. Schmitz R, Young RM, Ceribelli M, et al. Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature.* 2012;490(7418):116-120.
15. Love C, Sun Z, Jima D, et al. The genetic landscape of mutations in Burkitt lymphoma. *Nat Genet.* 2012;44(12):1321-1325.
16. Benezra R, Davis RL, Lassar A, et al. Id: a negative regulator of helix-loop-helix DNA binding proteins. Control of terminal myogenic differentiation. *Ann N Y Acad Sci.* 1990;599:1-11.
17. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 1990;61(1):49-59.
18. Murre C. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol.* 2005;6(11):1079-1086.
19. Hsu LY, Laurant J, Liang HE, et al. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity.* 2003;19(1):105-117.
20. Beck K, Peak MM, Ota T, Nemazee D, Murre C. Distinct roles for E12 and E47 in B cell specification and the sequential rearrangement of immunoglobulin light chain loci. *J Exp Med.* 2009;206(10):2271-2284.
21. Song S, Cooperman J, Letting DL, Blobel GA, Choi JK. Identification of cyclin D3 as a direct target of E2A using DamID. *Mol Cell Biol.* 2004;24(19):8790-8802.
22. Cato MH, Chintalapati SK, Yau IW, Omori SA, Rickert RC. Cyclin D3 is selectively required for proliferative expansion of germinal center B cells. *Mol Cell Biol.* 2011;31(1):127-137.
23. Campo E. New pathogenic mechanisms in Burkitt lymphoma. *Nat Genet.* 2012;44(12):1288-1289.
24. Schmitz R, Ceribelli M, Pittaluga S, Wright G, Staudt LM. Oncogenic mechanisms in Burkitt Lymphoma. *Cold Spring Harb Perspect Med.* 2014;4(2).
25. Moricke A, Zimmermann M, Valsecchi MG, et al. Dexamethasone vs prednisone in induction treatment of pediatric ALL: results of the randomized trial AIEOP-BFM ALL 2000. *Blood.* 2016;127(17):2101-2112.
26. Pearson K. X. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. *Philos Mag.* 1900;50(302):157-175.
27. Fisher RA. On the Interpretation of 2 from Contingency Tables, and the Calculation of P. *J R Stat Soc.* 1922;85(1):87-94.
28. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc.* 1958;53(282):457-481.
29. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep.* 1966;50(3):163-170.
30. Salaverria I, Martin-Guerrero I, Wagener R, et al. A recurrent 11q aberration pattern characterizes a subset of MYC-negative high-grade B-cell lymphomas resembling Burkitt lymphoma. *Blood.* 2014; 123(8):1187-1198.
31. Havelange V, Pepermans X, Ameye G, et al. Genetic differences between paediatric and adult Burkitt lymphomas. *British journal of haematology.* *Br J Haematol.* 2016; 173(1):137-144.
32. Forero-Castro M, Robledo C, Lumbrales E, et al. The presence of genomic imbalances is associated with poor outcome in patients with burkitt lymphoma treated with dose-intensive chemotherapy including rituximab. *Br J Haematol.* 2016;172(3):428-438.
33. Trautmann H, Hadzidimitriou A, Darzentas N, et al. Evidence for antigen-driven development of molecularly classified Burkitt Lymphomas. *Blood (ASH Annual Meeting Abstracts).* 2009; 114(22):Abstract Number 317.
34. Lenze D, Leoncini L, Hummel M, et al. The different epidemiologic subtypes of Burkitt lymphoma share a homogenous micro RNA profile distinct from diffuse large B-cell lymphoma. *Leukemia.* 2011; 25(12):1869-1876.
35. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. *N Engl J Med.* 2006;354(23):2419-2430.
36. Rohde M, Schlesner M, Richter J, et al. Pediatric Burkitt Lymphoma of the NHL-BFM group analyzed within the ICGC-MMML-SEQ: Whole genome sequencing data from 12 cases. *Hematol Oncol.* 2013; 31 Suppl 1(Abstract number 081):96-150.
37. Perkins AS, Friedberg JW. Burkitt lymphoma in adults. *Hematology Am Soc Hematol Educ Program.* 2008;341-348.
38. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375-2390.
39. Goossens T, Klein U, Kuppers R. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. *Proc Natl Acad Sci USA.* 1998;95(5):2463-2468.
40. Casellas R, Yamane A, Kovalchuk AL, Potter M. Restricting activation-induced cytidine deaminase tumorigenic activity in B lymphocytes. *Immunology.* 2009; 126(3):316-328.
41. Kretzmer H, Bernhart SH, Wang W, et al. DNA methylome analysis in Burkitt and follicular lymphomas identifies differentially methylated regions linked to somatic mutation and transcriptional control. *Nat Genet.* 2015;47(11):1316-1325.
42. Logan JE, Mostofizadeh N, Desai AJ, et al. PD-0332991, a potent and selective inhibitor of cyclin-dependent kinase 4/6, demonstrates inhibition of proliferation in renal cell carcinoma at nanomolar concentrations and molecular markers predict for sensitivity. *Anticancer Res.* 2013; 33(8):2997-3004.
43. Finn RS, Dering J, Conklin D, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Res.* 2009;11(5):R77.