

# Recurrent *RHOA* Mutations in Pediatric Burkitt Lymphoma Treated According to the NHL-BFM Protocols

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Burkitt lymphoma (BL) is the most frequent B-cell lymphoma in childhood. Genetically, it is characterized by the presence of an *IG-MYC* translocation which is supposed to be an initiating but not sufficient event in Burkitt lymphomagenesis. In a recent whole-genome sequencing study of four cases, we showed that the gene encoding the ras homolog family member A (*RHOA*) is recurrently mutated in pediatric BL. Here, we analyzed *RHOA* by Sanger sequencing in a cohort of 101 pediatric B-cell lymphoma patients treated according to Non-Hodgkin's Lymphoma Berlin–Frankfurt–Münster (NHL-BFM) study protocols. Among the 78 BLs in this series, an additional five had *RHOA* mutations resulting in a total incidence of 7/82 (8.5%) with c.14G>A (p.R5Q) being present in three cases. Modeling the mutational effect suggests that most of them inactivate the *RHOA* protein. Thus, deregulation of *RHOA* by mutation is a recurrent event in Burkitt lymphomagenesis in children.

## INTRODUCTION

Burkitt lymphoma (BL) is the most common subtype of B-cell non-Hodgkin's lymphoma (NHL) in pediatric and adolescent patients (Burkhardt et al., 2005). On the molecular level, BL is characterized by translocation of the *MYC* oncogene in 8q24 to one of the three immunoglobulin gene (*IG*) loci, *IGH* in 14q32, *IGK* in 2p12, or *IGL* in 22q11 (Klapproth and Wirth, 2010). These *IG-MYC* translocations result in deregulation of *MYC* and, therefore, in reinforced cell survival and proliferation (Klapproth and Wirth, 2010). Although studies showing that nearly all molecular BLs carry an *IG-MYC* translocation, this alone does not seem to be sufficient for full malignant transformation (Roschke et al., 1997; Dave et al., 2006; Hummel et al., 2006). Therefore, it is assumed that additional genetic changes cooperate with *MYC* deregulation in Burkitt lymphomagenesis.

Recently, we performed whole-genome sequencing of four *IG-MYC* positive pediatric BL cases (Richter et al., 2012). Seven genes were

affected by recurrent mutations, namely *DDX3X*, *FBXO11*, *ID3*, *MYC*, *RHOA*, *SMARCA4*, and *TP53* (Richter et al., 2012). Alterations affecting *TP53*

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and *MYC* have been previously described in BL (Johnston and Carroll, 1992; Wilda et al., 2004) and changes in *FBXO11* (Duan et al., 2012) and *DDX3X* (Wang et al., 2011) have been recently characterized in other B-cell malignancies, like diffuse large B-cell lymphomas (DLBCL), and chronic lymphocytic leukemia, respectively. Moreover, recent studies showed *ID3* mutations to activate the PI3-kinase pathway in BLs (Love et al., 2012; Richter et al., 2012; Schmitz et al., 2012). Nevertheless, the role of mutations in *RHOA* encoding a Rho GTPase family protein that is involved in a variety of cellular processes, such as cell migration, cell adhesion, and cell cycle progression, has not yet been explored in Burkitt lymphomagenesis or other B cell malignancies (Ridley, 2013). Thus, we here performed mutation screening of *RHOA* in an extended cohort of pediatric B-cell lymphomas and modeled the effect of the detected mutations on protein function.

## MATERIALS AND METHODS

### Patient Samples

Initial pretreatment tumor samples from 101 pediatric patients, diagnosed with mature aggressive B-cell lymphoma comprising 78 BL (including 16 cases of Burkitt leukemia), 16 DLBCL and seven B-cell lymphomas, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and BL (B-UCL), were analyzed. All patients analyzed were registered in the NHL-BFM data center and treated according to the NHL-BFM protocols (Woessmann et al., 2005). The BL cases did not overlap with those four studied by Richter et al. (2012). Tumor cell content of tumor samples was estimated to be at least 60%. DNA of the different tumor specimens was extracted using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. This study was approved by the Ethical Advisory Board of the University of Giessen (A89/11 Amendment 2013).

### *RHOA* Mutation Analysis

Exons 2 and 3 of the *RHOA* gene (NM\_001664) were PCR amplified using OneTaq Polymerase 2× MM with Standard Buffer (New England BioLabs, Frankfurt am Main, Germany) and the following primers: exon 2: forward: 5'-caggtggatcggcgtacta-3'; reverse: 5'-ttggactaagatgcaggatg-3'; and exon 3: forward: 5'-tttctaaaagtgggtggg-3'; reverse: 5'-actgatgccagaaaccag-3'. PCR products

were subjected to Sanger sequencing using the same primers and the ABI BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit. Sequence analysis was performed using an ABI 3130XL sequencer.

### *RHOA* Protein Modeling

Protein structures of mutated *RHOA* and interacting partner complexes were modeled using the structures of DIAPH1 (Rose et al., 2005) (Protein Data Base code 1z2c), ARHGDI1A (Tnimov et al., 2012) (4f38), ARHGDI1B (Tnimov et al., 2012) (4f38), ARHGEF12 (Kristelly et al., 2004) (1×86), MCF2L (Snyder et al., 2002) (1b1), and ARHGEF25 (Lutz et al., 2007) (2rgn).

## RESULTS AND DISCUSSION

Using whole-genome sequencing, we previously detected somatic missense mutations in *RHOA* in two out of four analyzed pediatric BL (BL2, BL4; Table 1; Richter et al., 2012). Remarkably, both cases carried the same mutation c.14G>A (p.R5Q) and, moreover, in one biallelic involvement was detected (c.14G>A, p.R5Q; c.68T>G, p.I23R; Fig. 1A). Transcriptome analysis showed expression of the mutated alleles [relative expression of the mutated allele, BL2: 0.55 (p.R5Q); BL4: 0.26 (p.R5Q), 0.29 (p.I32R)] (Richter et al., 2012). The recurrence of the mutations as well as the biallelic involvement prompted us to investigate further the *RHOA* gene in pediatric Burkitt lymphomagenesis.

In a first step, we mined published mutational analyses of BL and other aggressive B-cell lymphomas. Schmitz et al. (2012) identified *RHOA* mutations in two BL patients and Zhang et al. (2012) in three DLBCL patients, among whom two carried a p.R5Q mutation. Remarkably, the hitherto identified mutations in aggressive B-cell lymphomas seem to affect conserved positions particularly in exons 2 and 3 of the *RHOA* gene. Indeed, 6/7 previously described mutations in aggressive B-cell lymphomas locate to these two exons (Richter et al., 2012; Schmitz et al., 2012; Zhang et al., 2013).

Next, we performed Sanger sequencing of *RHOA* in 101 tumor samples from pediatric B-cell lymphomas of the NHL-BFM group. The series included 78 BL, 16 DLBCL, and 7 B-UCL. Patients' age at diagnosis ranged between 1.5 and 18.3 years (mean  $9.7 \pm 4.3$ ) with 82% being males and 18% being females. The 2-year event-free survival of the whole group was  $0.86 \pm 0.03$ , and the 2-year overall survival was  $0.90 \pm 0.03$ .

Because of the distribution of the mutations and limited material, we focused the mutation

TABLE 1. Summary on *RHOA* Mutations and Clinical Characteristics

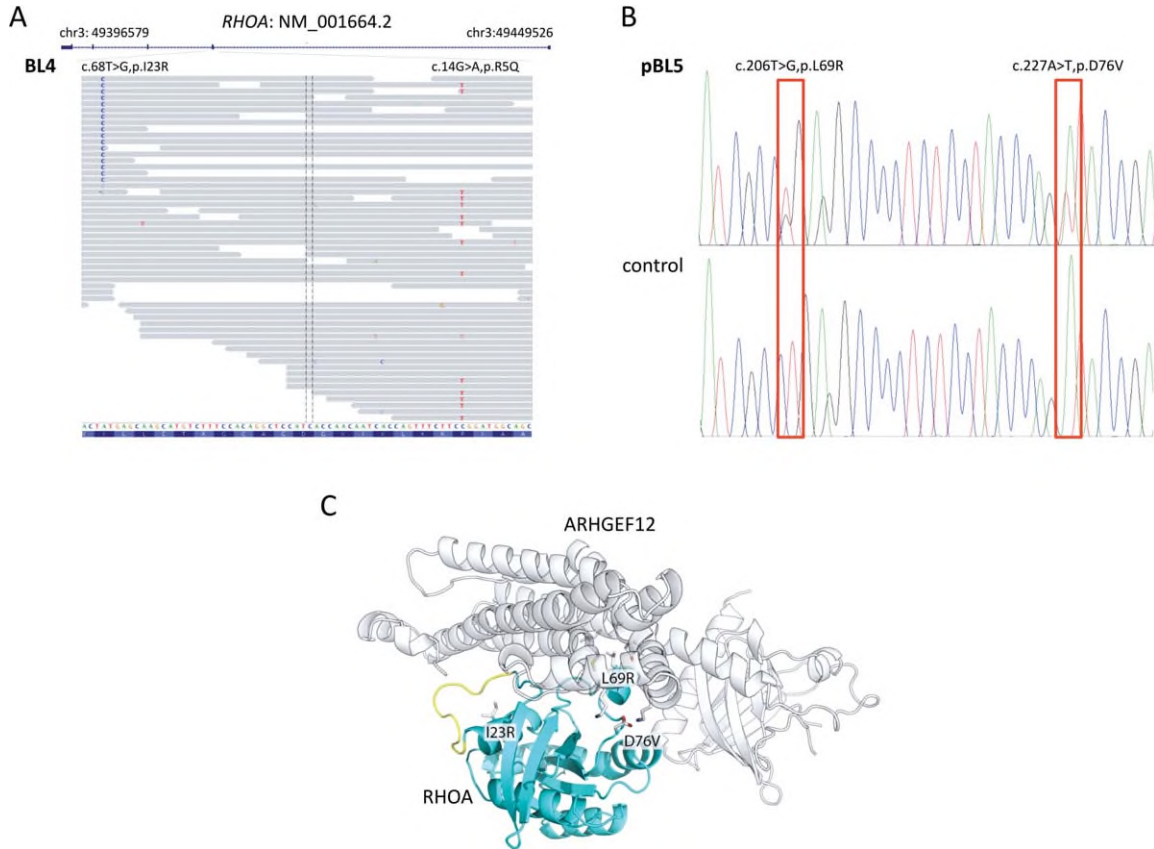
Sample ID	Chr	Position (hg19)	Ref	Alt	<i>RHOA</i> mutation	Germline	Protein modeling (confidence)
Pediatric Burkitt lymphoma							
BL2 <sup>a</sup>	3	49413009	C	T	exon2:c.14G>A,p.R5Q	wt	Deactivating (high)
BL4 <sup>a</sup>	3	49413009	C	T	exon2:c.14G>A,p.R5Q	wt	Deactivating (high)
	3	49412955	A	C	exon2:c.68T>G,p.I23R	wt	Deactivating (high)
pBL1	3	49413009	C	T	exon2:c.14G>A,p.R5Q	wt	Deactivating (high)
pBL2	3	49412958	T	A	exon2:c.65T>A,p.L22H	NA	Deactivating (low)
pBL3	3	49412898	T	A	exon2:c.125A>T,p.Y42F	wt	Deactivating (low)
pBL4	3	49412898	A	C	exon2:c.125A>C,p.Y42S	wt	Deactivating (low)
pBL5	3	49405932	A	C	exon3:c.206T>G,p.L69R	wt	Deactivating (high)
	3	49405911	T	A	exon3:c.227A>T,p.D76V	wt	Deactivating (high)
Sample ID	Chr	Age at diagnosis	Gender	Diagnosis	<i>MYC</i> translocation	Treatment/clinical trial	Outcome
Pediatric Burkitt lymphoma							
BL2 <sup>a</sup>	3	12	Male	BL, sporadic	<i>IGH-MYC</i>	B-NHL BFM 04	NED
BL4 <sup>a</sup>	3	15	Male	BL, sporadic	<i>IGL-MYC</i>	B-NHL BFM 04	R
	3						
pBL1	3	13	Male	BL, sporadic	<i>IGH-MYC</i>	B-NHL BFM 04	NED
pBL2	3	6	Female	BL, sporadic	<i>IGH-MYC</i>	NHL-BFM 90	R
pBL3	3	18	Female	BL, sporadic	<i>IGH-MYC</i>	B-NHL BFM 04	NED
pBL4	3	14	Male	BL, sporadic	<i>MYC</i>	B-NHL BFM 04	NED
					rearrangement positive		
pBL5	3	5	Male	BL, sporadic	<i>IGH-MYC</i>	B-NHL BFM 04	NED
	3						

<sup>a</sup>Published by Richter et al. (2012); BL, Burkitt lymphoma; Chr, chromosome; Ref, reference allele; alt, alternative allele; wt, wildtype at the site of mutation; NA, not available; BFM, Berlin–Frankfurt–Münster NHL protocols; NED, no evidence of disease; and R, relapse.

screening to exons 2 and 3. We identified nonsynonymous *RHOA* mutations in five of the 78 analyzed BL (Table 1). Thus, including the four previously published cases (Richter et al., 2012) from the same trial, we detected protein-changing *RHOA* mutations in a total of seven of 82 pediatric BL (8.5%) in the NHL-BFM trial. In all six cases with germline material available for comparison the mutations could be shown to be of somatic origin. As more than 95% of children in Germany are registered in the NHL-BFM data center, the frequency of mutations determined herein can be regarded representative for the German population. Regarding the clinical characteristics, none of the patients with *RHOA* mutation showed bone marrow or central nervous system involvement. Two of the seven patients suffered from relapse. However, the number of patients was too small to draw valid conclusions on clinical characteristics typically associated with *RHOA* mutations. No *RHOA* mutation was identified in the 16 DLBCL and 7 B-UCL.

A total of six different amino acids were affected by *RHOA* missense mutations in seven

pediatric BL, with two mutations being recurrent: Arg5 (p.R5) was affected in three and Tyr42 (p.Y42) in two cases. Modeling of the mutations suggests that they have deactivating effects on the protein. *RHOA* encodes a GTPase which is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Those either encourage dissociation of guanosine diphosphate (GDP) or hydrolysis of guanosine triphosphate (GTP) to GDP and thereby activate or deactivate *RHOA*, respectively (Dvorsky and Ahmadian, 2004; Rossman et al., 2005). Furthermore, a phosphate binding and two switch loops are involved in GTP/GDP binding (Vetter and Wittinghofer, 2001). The mutations p.R5Q, p.L69R, and p.D76V lie at the interface between *RHOA* with GEFs within crystal structures involving *RHOA*. The mutations p.L69R and p.D76V disrupt hydrophobic and electrostatic contacts, respectively, and are predicted to diminish most *RHOA*-GEF interactions; p.R5Q has a less obvious effect, although it might favor binding to certain GEFs by removing a disfavorable interaction with a GEF arginine residue. In line with



**Figure 1.** *RHOA* mutation and its effect on the protein interaction. (A) Biallelic *RHOA* mutation in sample BL4 is shown using IGV visualization of whole genome sequencing results. Sanger sequencing confirmed both mutations. (B) Two mutations (c.206T>G, p.L69R, and c.227A>T, p.D76V) in the tumor sample pBL5 detected by Sanger sequencing (reverse sequence is shown). (C) The structure of *RHOA* (cyan) in complex with *ARHGEF12* (gray) showing the likely deactivating effects of p.I23R, p.L69R, and p.D76V mutations. p.I23R interacts

with the switch I loop (yellow) of *ARHGEF12*, probably favoring GDP over GTP binding. p.L69R lies in a largely hydrophobic pocket; thus the interaction is likely destabilized by mutation from hydrophobic leucine to positively charged arginine. p.D76V interacts with two lysines in *ARHGEF12*. Mutation to valine would remove these salt bridges and weaken the interaction. Mutated residues and those with which they interact are shown as sticks colored by atom types.

this, Arg5 (p.R5) was described to be important for the selective binding of *RHOA* to distinct GEFs of the Dbl family (Snyder et al., 2002). Additionally, the mutations p.Y42F and p.Y42S are likely at interfaces with GEFs as modeled by homology using other GTPases (e.g., *CDC42*) although Y42 makes limited contacts with the interacting GEFs implying a possible deactivating mutation with lower confidence. Mutation p.I23R is not predicted with confidence to interact with any other protein, but very likely lies at the interface with  $Mg^{++}$  or GTP (as modeled also on *CDC42*), suggesting a disruptive effect on catalysis. p.L22H is not obviously close to any protein or small molecule, but lies buried in a hydrophobic pocket in *RhoA* and would thus be predicted to affect the overall structure (by a mutation to a polar residue).

Interestingly, p.L69R might also promote interactions with *ARHGAP20* relative to other GEF,

*GDI*, or *GAPs*, as the wildtype *RHOA* has a hydrophobic residue (leucine) in contact with negatively charged or polar residues in *ARHGAP20* that would likely prefer to interact with an arginine (PDB code 3msx; manuscript not yet published). Generally, these three residues seem to adjust subtle preferences for *RHOA* regulatory proteins in a fashion reminiscent of the edgetic perturbations proposed for disease mutations at interaction interfaces (Zhong et al., 2009). The p.I23R mutation observed in BL4 affects the conformation of the switch-I-loop which may directly favor GDP binding over GTP (Snyder et al., 2002; Fig. 1C). No clear prediction could be made for the effects on Tyr42 and on Leu22.

Remarkably, during the preparation of this manuscript, *RHOA* mutations were also reported to be recurrently found in peripheral T-cell lymphoma (PTCL; Palomero et al., 2014; Sakata-

Yanagimoto et al., 2014). In particular, the p.G17 has been shown to be common in angioimmunoblastic T-cell lymphoma (Palomero et al., 2014; Sakata-Yanagimoto et al., 2014; Yoo et al., 2014). However, the sites affected by mutations in BL as described herein were not found to be recurrently mutated in PTCL. Mutations of RHOA have also been detected in solid tumors, particularly head and neck tumors (Lawrence et al., 2014). Also in those tumors the spectrum of mutations was different from that in pediatric BL. These findings might indicate that deregulation of RHOA is common in tumorigenesis but that there might exist some specificity with regard to the residues and, consequently, probably interaction partners affected.

In summary, we show that *RHOA* mutations occur recurrently in pediatric BL, are biallelic in a subset of cases, target important sites for protein function and seem to predominantly inactivate RHOA. However, the highly nonrandom distribution of mutations indicates that not a complete loss of function but more specific regulation of RHOA activity confers a selective advantage. RHOA plays a role in early and late stages of normal B-cell development, is activated by B-cell receptor signaling and PI3K activation and regulates IP<sub>3</sub> production via direct activation of the PLCγ2 and thereby calcium mobilization (Saci and Carpenter, 2005; Zhang et al., 2012). Downstream effectors of RHOA mediating cellular processes include ROCK (Sahai et al., 1998). RHOA alterations could result in impairment of B cell signaling by suppression of PI3K via insufficient ROCK and PTEN activation (Papakonstanti et al., 2007; Carracedo and Pandolfi, 2008). Thus, considering that defects in the normal regulation of B cell receptor signaling and the PI3-kinase pathway due to mutations in *ID3*, *TCF3*, and *CCND3* are well known to play a role in pathogenesis of BL, it is intriguing to speculate that *RHOA* mutation might yet add another mechanism deregulating this pathway in BL lymphomagenesis (Papakonstanti et al., 2007; Love et al., 2012; Richter et al., 2012; Sander et al., 2012; Schmitz et al., 2012).

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