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

Marius Rohde,1 Julia Richter,2† Matthias Schlesner,3 Matthew J. Betts,4 Alexander Claviez,5 Bettina R. Bonn,6 Martin Zimmermann,1 Christine Damm-Welk,1 Robert B. Russell,6 Arndt Borkhardt,7 Roland Eils,7,8 Jessica I. Hoell,7 Monika Szczepanowski,7 Ilse Oschlies,9 Wolfram Klapper,5 Birgit Burkhardt,1,6,9* and Reiner Siebert,2,9† on behalf of the German ICGC MMML-Seq-Project and the NHL-BFM Study Group1

INTRODUCTION

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.
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’-cagttggatatgcgtacta-3’; reverse: 5’-ttggactaatagttgagg-3’; and exon 3: forward: 5’-atttctaaagttggttggtg-3’; reverse: 5’-actttgatcaggcattering-3’. PCR products were subjected to Sanger sequencing using the same primers and the ABI BigDye® 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), ARHGDI (Tnimov et al., 2012) (4f38), ARHGDIB (Tnimov et al., 2012) (4f38), ARHGEF12 (Kristelly et al., 2004) (1x86), MCF2L (Snyder et al., 2002) (lb1), 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 hereby 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 ± 4.3) with 82% being males and 18% being females. The 2-year event-free survival of the whole group was 0.86 ± 0.03, and the 2-year overall survival was 0.90 ± 0.03.

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

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Chr</th>
<th>Position (hg19)</th>
<th>Ref</th>
<th>Alt</th>
<th>RHOA mutation</th>
<th>Germline</th>
<th>Protein modeling (confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>49413009</td>
<td>C</td>
<td>T</td>
<td>exon2:c.14G&gt;A, p.R5Q</td>
<td>wt</td>
<td>Deactivating (high)</td>
</tr>
<tr>
<td>BL4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>49413009</td>
<td>C</td>
<td>T</td>
<td>exon2:c.14G&gt;A, p.R5Q</td>
<td>wt</td>
<td>Deactivating (high)</td>
</tr>
<tr>
<td>pBL1</td>
<td>3</td>
<td>49412955</td>
<td>A</td>
<td>C</td>
<td>exon2:c.68T&gt;G, p.I23R</td>
<td>wt</td>
<td>Deactivating (high)</td>
</tr>
<tr>
<td>pBL2</td>
<td>3</td>
<td>49412958</td>
<td>T</td>
<td>A</td>
<td>exon2:c.65T&gt;A, p.L22H</td>
<td>NA</td>
<td>Deactivating (low)</td>
</tr>
<tr>
<td>pBL3</td>
<td>3</td>
<td>49412898</td>
<td>T</td>
<td>A</td>
<td>exon2:c.125A&gt;C, p.Y42F</td>
<td>wt</td>
<td>Deactivating (low)</td>
</tr>
<tr>
<td>pBL4</td>
<td>3</td>
<td>49412898</td>
<td>A</td>
<td>C</td>
<td>exon2:c.125A&gt;C, p.Y42S</td>
<td>wt</td>
<td>Deactivating (low)</td>
</tr>
<tr>
<td>pBL5</td>
<td>3</td>
<td>49405932</td>
<td>A</td>
<td>C</td>
<td>exon3:c.206T&gt;G, p.L69R</td>
<td>wt</td>
<td>Deactivating (high)</td>
</tr>
<tr>
<td>pBL11</td>
<td>3</td>
<td>49405911</td>
<td>T</td>
<td>A</td>
<td>exon3:c.227A&gt;T, p.D76V</td>
<td>wt</td>
<td>Deactivating (high)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Chr</th>
<th>Age at diagnosis</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>MYC translocation</th>
<th>Treatment/clinical trial</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>12</td>
<td>Male</td>
<td>BL, sporadic</td>
<td>IGH-MYC</td>
<td>B-NHL BFM 04</td>
<td>NED</td>
</tr>
<tr>
<td>BL4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>15</td>
<td>Male</td>
<td>BL, sporadic</td>
<td>IGL-MYC</td>
<td>B-NHL BFM 04</td>
<td>R</td>
</tr>
<tr>
<td>pBL1</td>
<td>3</td>
<td>13</td>
<td>Male</td>
<td>BL, sporadic</td>
<td>IGH-MYC</td>
<td>B-NHL BFM 04</td>
<td>NED</td>
</tr>
<tr>
<td>pBL2</td>
<td>3</td>
<td>6</td>
<td>Female</td>
<td>BL, sporadic</td>
<td>IGH-MYC</td>
<td>NHL-BFM 90</td>
<td>R</td>
</tr>
<tr>
<td>pBL3</td>
<td>3</td>
<td>18</td>
<td>Female</td>
<td>BL, sporadic</td>
<td>MYC</td>
<td>B-NHL BFM 04</td>
<td>NED</td>
</tr>
<tr>
<td>pBL4</td>
<td>3</td>
<td>14</td>
<td>Male</td>
<td>BL, sporadic</td>
<td>MYC</td>
<td>B-NHL BFM 04</td>
<td>NED</td>
</tr>
<tr>
<td>pBL5</td>
<td>3</td>
<td>5</td>
<td>Male</td>
<td>BL, sporadic</td>
<td>IGH-MYC</td>
<td>B-NHL BFM 04</td>
<td>NED</td>
</tr>
</tbody>
</table>

<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.

TABLE 1. Summary on RHOA Mutations and Clinical Characteristics

- **RHOA** mutations in five of the 78 analyzed BL (Table 1).
- **NHL-BFM** trial: detected protein-changing in seven pediatric BL, two mutations being recurrent.
- RHOA encodes a GTPase.
- Deactivating effects on the protein.
- Modeling suggests changes in interactions with GEFs.
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 pY42S 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 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-...
In particular, the p.G17 mutations have 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 IP3 production via direct activation of the PLCγ2 and thereby calcium mobilization (Saci 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).

ACKNOWLEDGMENTS

The authors thank C. Keller, J. Schieferstein, and G. Buck for their excellent technical support. Support of infrastructure by the KinderKrebsInitiativ Buchholz/Holm-Seppensen is gratefully acknowledged. The authors declare no competing financial interests.

REFERENCES


