Cryptic MYC insertions in Burkitt lymphoma: New data and a review of the literature

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Abstract

The occurrence of MYC-negative Burkitt lymphoma (BL) has been discussed for many years. The real frequency of the MYC insertion in MYC-negative BL is still unknown. Fine-needle aspiration biopsies of 108 consecutive patients with clinicopathologically suspected BL (suspBL) were evaluated by flow cytometry, classical cytogenetics, and fluorescence in situ hybridization (FISH). We found 12 cases (11%) without the MYC rearrangement by FISH with a MYC breakapart probe: two patients (1.9%) with cryptic MYC/IGH fusion (finally diagnosed as BL) and 10 patients (9.3%) with 11q gain/loss (finally diagnosed as Burkitt-like lymphoma with 11q aberration). The exact breakpoints of the cryptic MYC/IGH were investigated by next-generation sequencing. The MYC insertions’ breakpoints were identified in PVT1 in the first case, and 42 kb upstream of 5’MYC in the second case. To date, a molecular characterization of the MYC insertion in BL has only been reported in one case. Detailed descriptions of our MYC insertions in a routinely and consecutively diagnosed suspBL cohort will contribute to resolving the issue of MYC negativity in BL. In our opinion, the presence of the MYC insertions in BL and other lymphomas might be underestimated, because routine genetic diagnostics are usually based on FISH only, without karyotyping.

Introduction

Burkitt lymphoma (BL) is a highly aggressive B-cell lymphoma and the fastest-growing human tumor type. The genetic hallmark of BL is MYC rearrangement (MYCR). This aberration is present in nearly all BL cases, mainly as a result of the chromosomal translocation t(8;14)(q24;q32), and less often due to the variant translocation t(2;8)(p11;q24) or t(8;22)(q24;q11) [1–3]. The molecular consequence of these translocations is the deregulated expression of the MYC oncogene. The overexpression arises as a result of the juxtaposition of MYC to the enhancer
elements of one of the immunoglobulin (IG) genes: IGH (14q32), IGK (2p11), or IGL (22q11) [4]. Recent studies have described lymphomas, which morphologically and phenotypically resemble BL but have unique chromosome 11q aberrations (11q gain/loss) instead of MYCR. For these lymphomas, the term Burkitt-like lymphoma with 11q aberration (BLL,11q) was proposed as a new provisional entity in the revised 4th edition of the World Health Organization’s WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues [5]. Some rare cases with 11q gain/loss also have MYCR (BL,MYCR/11q) and are diagnosed as BL or high-grade B-cell lymphoma, not otherwise specified (HGBL,NOS), or even double-hit lymphoma (DHL) [6–8]. MYCR is also observed in other aggressive mature B-cell lymphomas (BCLs), such as HGBLs and diffuse large B-cell lymphomas (DLBCLs).

The breakpoints of the MYC are widely dispersed across the large >1 Mb region, and depend on the lymphoma subtype and translocation partner. In sporadic BL (sBL) with MYC/IGH fusion, breakpoints of the MYC are mapped within MYC or in close proximity to 5’MYC [9, 10]. On the other hand, the breakpoints of MYC involved in the variant translocations are located 16–350 kb from 3’MYC [9–13]. Most breakpoints within the IGH region in the MYC/IGH of sBL are located within switch regions (S), and only a minority, within the joining locus [14].

In almost all BLs, and other BCLs, the MYC fusions are the results of karyotypically visible translocations. However, there are few data in the literature describing the MYC fusions arising from cryptic MYC insertion in different types of lymphomas [10, 15–19]. According to these data, the occurrence of the cryptic MYC insertion in BL is very rare, and only occasional cases of such insertions have been described. Detailed molecular characterizations of insertion breakpoints in BL are even more scarce—only one such case has been recorded [19].

Because the detection of every type of the MYCR is crucial for determining the final BL diagnosis, detailed knowledge regarding the molecular features and frequency of the MYC insertions in BL is very important. Herein, we present two cases of BL without typical, chromosomal MYC translocations and without 11q gain/loss out of 108 consecutive, mainly adult patients with BL/BLL,11q diagnosis. In these cases, with clinicopathological characteristics of classical BL, the karyotypically invisible insertion of MYC into the IGH locus and that of IGH into the MYC region were detected. We present a detailed characterization of these fusions on a molecular level obtained by next-generation sequencing (NGS). Thus, we confirm the rare occurrence of cryptic MYC fusions in BL patients with a frequency of 1.9% in patients with clinicopathologically suspected BL diagnosis (susBL). We also discuss the significant role of the flow cytometry (FCM) evaluation of CD38 expression in establishing the final diagnosis of BL/BLL,11q and the value of karyotyping in distinguishing MYC insertions during routine BL diagnosis.

Materials and methods

Patients

The classical cytogenetics (CC) and/or fluorescence in situ hybridization (FISH) status of MYC was routinely analyzed in 108 consecutive patients with suspicion of BL, diagnosed at Maria Skłodowska-Curie National Research Institute of Oncology (Warsaw, Poland) from 2003 to 2020. In all patients with clinical or histopathology/immunohistochemistry (HP/IHC)-suspected BL diagnosis, fine needle aspiration biopsy (FNAB) for FCM/CC/FISH was performed. A diagnosis was established according to the 2016 revision of the WHO Classification of Lymphomas [5] and our practical FCM and IHC-based approach to the diagnosis of BL and BLL,11q [7]. All the BLL,11q cases diagnosed before the latest revision of the WHO classification were primarily diagnosed as MYC-negative BL and treated as MYC-negative BL at our
institute. Finally, MYC-positive BL was confirmed in 93 cases. MYC-negative BLL,11q and BL, MYC/11q were established in 10 and 5 cases, respectively. Patients with HGBL,NOS with MYC and DLBCL with MYC were excluded because of different diagnoses after reviews of the HP slides, combined with FCM/IHC data and a more complex karyotype or clinical data obtained at follow-up.

Classical cytogenetics

Cells prepared from the FNAB sample were fixed directly and cultured for 24 h without mitogen or for 48 or 72 h with DSP-30 (2 μM; TIBMolBiol, Berlin, Germany) together with IL-2 (200 U/mL; R&D Systems, Minneapolis, MN, USA). Chromosomes were stained with Wright for G,C-banding and analyzed using the MetaSystems Ikaros Imaging system (MetaSystems, Altlussheim, Germany), and the karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2016) [20].

FISH

FISH analysis was performed on cultured cells in 104/108 patients. In 4/108 patients, a formalin-fixed paraffin-embedded (FFPE) tumors were used. In six patients both type of samples were used. FFPE specimens were prepared with a Pretreatment Reagent Kit (Vysis Abbott Molecular, Downers Grove, IL, USA) according to the manufacturer’s protocol. For the routine diagnostics of patients with suspBL diagnoses, the following probes were used: BCL2 breakapart (BAP), BCL6 BAP, and MYC BAP (Vysis Abbott Molecular). For the precise evaluation of the MYC aberrations, the following probes were used: IGH BAP, IGH/MYC;CEP8 (Vysis Abbott Molecular), IGK/MYC, and IGL/MYC (CytoTest, Rockville, MD, USA). For the assessment of 11q gain/loss, the following panel of probes (11q gain/loss panel) was used: ATM SO, CCND1 SO, MLL BAP, TelVision 11q (D11S1037), and CEP11 (Vysis Abbott Molecular). The FISH results were analyzed using a fluorescence microscope, Axioskop2 (Carl Zeiss, Jena, Germany), documented by the ISIS Imaging System (MetaSystems, Altlussheim, Germany).

Histopathology and immunohistochemistry

FFPE tissues were examined by standard HP/IHC, as described previously [7, 21] and characterized in S1 File. The IHC was performed using monoclonal antibodies specific for CD(3/5/10/20/38/43/44/56), BCL2, BCL6, LMO2, MYC, MUM1, Tdt, and Ki-67. Depending on the date of diagnosis, the panel of IHC varied but always included CD(3/10/20), BCL2, BCL6, and Ki-67. In the following years, the panel was expanded to include CD(5/38/43/44/56), MYC, LMO2, MUM1, and Tdt. Latent membrane protein 1 (LMP1) expression by IHC and Epstein–Barr virus (EBV) small nuclear RNA transcripts (EBER) by in situ hybridization (ISH) method was performed in some patients as described previously [7].

Flow cytometry with cytological smears evaluation

The immunophenotype (CD38 PE-conjugated HB7 clone and other monoclonal antibodies) for the BL/BLL,11q diagnosis of cells obtained by the FNAB or ultrasound-guided FNAB of the lymph nodes/tumors was determined as previously described (see S1 File) [7, 21]. Antigen expression was quantified using FACSCalibur and FACSCanto II cytometers (Becton Dickinson Biosciences, San Jose, CA, USA) and was categorized according to the percentages of positive cells into three groups: ‘(−)’—no expression (<20% of neoplastic cells), ‘(+/−)’—expression in ≥20% but <100% of cells, and ‘(+’)—expression in 100% of lymphoma cells. Quantitative
expression was defined as (+)\textsuperscript{higher} or (+)\textsuperscript{comparable} than on control lymphocytes (i.e., CD38 (+)\textsuperscript{higher} or CD38(+) in BL and BL,MYC/11q or BLL,11q cells, respectively, compared to normal B- and T-lymphocytes). Simultaneously, cytological smears were stained with a hematoxylin–eosin (HE) and May–Grünwald–Giemsa stain for morphological evaluation, as described previously [7, 21].

Next-generation sequencing

DNA quality assessment. Before the creation of the NGS libraries, the quality of the DNA was evaluated by real-time quantitative PCR using our personally developed method (see the detailed description in S1 File).

Libraries and sequencing. The NGS libraries used in this study were created, pooled, and enriched according to the SeqCap EZ HyperCap Workflow (Roche, Basel, Switzerland), using the NimbleDesign software to design the set of SeqCap EZ Choice hybridization probes (Roche) covering the following two regions in the human GRCh38 genome assembly:

chr8:127,351,112–128,172,319 (MYC) and chr14:105,199,125–106,860,200 (IGH). The estimated coverage of the design equaled 92.8%. The obtained enriched multilibrary was then sequenced on a MiSeq next-generation sequencer (Illumina, San Diego, CA, USA) in paired-end mode (2 × 76 bp).

Bioinformatic analysis of the NGS results. The quality of the FASTQ files was checked with the Fastqc app. Afterwards, the reads were mapped to the reference human genome (the GRCh38 assembly) with the HISAT2 aligner. The obtained BAM files were first analyzed with Qualimap [22] and Samtools [23] apps to determine the mapping quality and then subjected to deduplication with the MarkDuplicates program, a part of the Genome Analysis Toolkit (GATK) [24]. Finally, the deduplicated BAM files were used for the detection of intra- and interchromosomal translocations (CTX) with the CTX-explorer application (version:1.0), a personally developed piece of open-source software, available for download at https://github.com/lukszafirn/CTX-explorer. In order to verify the sensitivity and specificity of the CTX-explorer-based breakpoint predictions, our results obtained with this piece of software were compared with the output of Breakdancer [25] and Delly [26], open-source programs developed by other research teams.

In vitro verification of the CTX found in silico. To verify the existence of interchromosomal translocations in the genomes, a unique pair of PCR primers for each patient was designed: Case 1 (forward primer: 5′-AGGAGCAACATAATGGGGGC-3′; reverse primer: 5′-CCCTTTTCAGTTTCGGTCAGCC-3′); Case 2 (forward primer: 5′-GACGGTCAGCCACTTC TCTC-3′; reverse primer: 5′-GACTTGGACCTTGCCTGTCC-3′). The PCR and Sanger sequencing reactions were performed under conditions described in S1 File.

Results

Patients

Clinicopathological features and the results of HP/IHC revealed 108 patients with suspBL diagnosis from a total cohort of approximately 11,000 FCM/CC/FISH diagnoses of lymphomas obtained by FNAB material. This group of patients consisted of 102 adults with median age of 35 years (range, 19–79 years) and 6 children with median age of 8 years (range, 3–12 years). Among adult patients, 81 were male and 21 were female (ratio, 3.86:1). Among pediatric patients, 5 were male and 1 was female (ratio, 5:1). For the precise establishing of final diagnosis, FCM, CC and FISH of FNABs were performed (Table 1 and S1 Table). Both the CC and FISH were conducted in 86/108 patients. In the remaining 22/108 patients, FISH (20/108) or CC (2/108) were carried out. Some of the HP, FCM, molecular, and clinical data of these
patients have been published previously [6, 7, 21, 27]. Routine FISH analysis with MYC BAP, BCL2 BAP, and BCL6 BAP probes, performed in 106/108 patients, demonstrated a lack of BCL2 and BCL6 rearrangements in all cases and confirmed MYC R in 94/108 patients. In 2/108 patients (lack of FISH examination), MYC R was confirmed by a karyotype demonstrating the t(8;14)(q24;q32) translocation, for a total of 96/108 MYC R cases.

Among all the patients with MYC R (96/108), five had 11q gain/loss (5/108; 4.7%) (final BL, MYC R/11q diagnosis). In these patients, 11q gain was observed in the karyotype and further FISH examination, with an 11q gain/loss probe panel confirming the 11q aberration. The remaining 12 patients demonstrated a lack of MYC R (12/108; 11%). All these patients were examined with the use of IGH/MYC, IGL/MYC, and IGK/MYC probes. In two patients (2/108), cryptic MYC/IGH fusions were confirmed (final BL diagnosis). In a further 10 patients, the karyotype and FISH with the 11q gain/loss probe panel revealed an 11q aberration (final BLL,11q diagnosis) (10/108; 9.3%). None of the patients without MYC R had cryptic MYC/IGL or MYC/IGK fusions.

The presence of two cases with MYC insertion among the patients with suspBL resulted in an MYC insertion frequency of 1.9% (2/108). Considering patients with final BL diagnosis, this frequency was 2% (2/93).

All the BL cases with just MYC R or the translocation of the 8q24 locus (91/108) were characterized by CD38(+) higher expression by the FNAB/FCM method. The BL,MYCR/11q cases (5/108) also demonstrated CD38(+) higher expression, while the expression of CD38 in BLL,11q cases (10/108) was significantly weaker—CD38(+). The BL cases without MYC R but with MYC/IGH fusion (2/108) (the MYC insertions described below) had CD38(+) higher expression. In both cases, despite the initial failure to confirm the MYC R, the FCM and HP/IHC results pointed to a BL diagnosis. Some epidemiological data of patients with suspBL, including BL with MYC insertions as well as BLL,11q and BL,MYCR/11q are presented in Table 1.

Table 1. The MYC and CD38 status with epidemiological data in 108 patients with suspected Burkitt lymphoma.

| FISH + karyotype | Number of cases | FCM: CD38 | Final diagnosis | Age (years median, range) | Sex (male: female) |
|------------------|----------------|-----------|----------------|--------------------------|--------------------|
| MYCR and/or t(8;V) | 91/108 (84%) | (+)higher | BL | 48 (3–68) | 3:47:1 |
| MYCR and t(8;V) and 11q gain/loss | 5/108 (4.7%) | (+)higher | BL,MYCR/11q | 31 (20–65) | 5:0 |
| MYCnoR: | 12/108 (11%) | | | |
| MYC/IGH | 2 (1.9%) | (+)higher | BL | 29 (22–36) | 1:1 |
| MYC/IGL | 0 | | BL | |
| MYC/IGK | 0 | | BL | |
| 11q gain/loss | 10 (9.3%) | (+)weaker | BLL,11q | 29 (20–79) | 10:0 |

Abbreviations: FCM, flow cytometry; MYCR, the MYC rearrangement detected by MYC BAP probe; t(8;V), translocation of 8q24 (MYC locus) and one of the loci: 14q32 (IGH), 22q11 (IGL), and 2p11 (IGK); BL, Burkitt lymphoma; 11q gain/loss, duplication and deletion of 11q observed in karyotype and confirmed by FISH; BL, MYCR/11q, Burkitt lymphoma with both the MYC rearrangement and 11q gain/loss; MYCnoR, lack of the MYC rearrangement detected by MYC BAP probe; BLL,11q, Burkitt-like lymphoma with 11q gain/loss.

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Case 1

Clinical presentation, and pathomorphological and flow cytometry features. A 22-year-old, HIV-negative Caucasian male presented with a bulky abdominal, extranodal tumor. His serum lactate dehydrogenase (LDH) (940 IU/L, \( n < 240 \)), \( \beta \)-2-microglobulin (4.44 ng/mL, \( n = 0.7–1.8 \)), d-dimer (1247 ng/mL, \( n < 500 \)), C-reactive protein (CRP) (36.2 mg/L, \( n < 5 \) mg/L) and fibrinogen (3.59 g/L, \( n = 1.7–3.5 \)) levels were elevated, with the biochemical features of renal failure, an ECOG performance status of 0, and Ann Arbor stage of IVA without B symptoms. Positron emission tomography (PET-CT) showed numerous extranodal lesions in the abdominal space and kidney. The patient has undergone a hemicolectomy and specimen from the tumor of the cecum revealed BL with a reduced number of apoptotic bodies and starry sky appearance in HP. IHC showed EBV-positive classic MYC-positive BL immunostaining for CD20+/CD10+/BCL6+/ BCL2−/MYC+ 

\[ \text{strong}^{100\%} / \text{LMO2−/MUM1−/CD38} \]

\[ \text{strong} / \text{EBER+/EBV-LMP1−/CD43−/CD44−/CD56−/Ki-67 index > 98%/ CD3−/CD5−/TdT−} \] (Fig 1). The FCM immunophenotype was determined three weeks after hemicolectomy on the recurrent abdominal tumor. BL cells were positive for CD45\[\text{weaker}/\text{CD20}^{\text{bright}}/\text{CD19}^{\text{bright}}/\text{CD22} (\text{with the order according to median fluorescence intensity (MFI) being CD20 > CD19 > CD22}))/\text{CD10}/\text{CD38}^{\text{higher}} (\text{with a MFI of 698 for CD38, compared to a MFI of 37 on T lymphocytes—Fig 2})/\text{MYC}/\text{CD81}^{\text{higher}}/\text{BCL6}^{\text{higher}}/\text{CD79}^{\beta}/\text{HLA-DR}/\text{FMC7}/\text{CD43}^{\text{weaker}}/\text{CD49d}^{\text{weaker}}/\text{CD52}^{\text{higher}} \text{and surface immunoglobulin (IgM/κ), and negative for CD5/CD8/CD11c/CD23/CD25/CD44/CD16&CD56/CD56/CD138/CD200/CD305/BCL2/IgG/IgD/λ} \) (Fig 3). The intracellular expression of MYC/BCL6 and a lack of BCL2 were detected after the permeabilization procedure. In addition, CD71 (+++) expression was detected in 100% of the cells. CD62L± and CD54± expression was weak in slightly over 20% of the tumor cells. The cytological smear stained with HE showed monomorphic medium-sized lymphoid cells with a small number of apoptotic bodies. A bone marrow (BM) HP/IHC was negative. The FCM confirmed the minimal cerebrospinal fluid (CSF) involvement of BL cells. The patient was treated with three courses of the R-CODOX-M/IVAC regimen (fractionated cyclophosphamide, doxorubicin, vincristine, and high-dose methotrexate alternating with fractionated ifosfamide, etoposide, and high-dose cytarabine, and triple-dose intrathecal therapy), leading to a complete response. Fifty-four months after diagnosis, the patient was still alive.

Cytogenetics, FISH, and NGS. CC and FISH with the MYC BAP probe of a recurrent abdominal biopsy demonstrated a normal karyotype 46,XY [20] and a lack of MYCR. FISH with the 11q gain/loss probe panel revealed normal results. However, subsequent FISH with IGH BAP and IGH/MYC dual fusion probes revealed the rearrangement of IGH and IGH/MYC fusion via the insertion of the IGH into the MYC locus on a normal chromosome 8 (Fig 4). At the same time, FISH was performed on a FFPE abdominal tumor before recurrence, and we confirmed the IGH/MYC fusion.

The interchromosomal translocation analysis with our original CTX-explorer software (see Material and methods for details), followed by PCR and Sanger sequencing, showed that the breakpoint on chromosome 8 was located 158 kb downstream of 30 MYC, in the PVT1 region (chr8:127,901,209) (Figs 4 and 5). Regarding the breakpoint on chromosome 14, it was between joining and constant IGH regions, 1.6 kb upstream of the Sμ switch region (ch14:105,862,125), according to the recently mapped IGH switch regions (Sμ: 105,856,501–105,860,500) (S1A Fig) [28].

FISH with the IGH BAP probe revealed an atypical signal pattern (2Y1R), suggesting a breakpoint in the IGH region complementary to the 3′IGH BAP probe or duplication of this complementary region (Fig 4B). Considering the data regarding the exact location of the IGH BAP probe (according to the manufacturer’s information), the IGH breakpoint was in the gap.
between the 3′IGH BAP probe and the 5′IGH BAP probe, which pointed to a duplication in the area complementary to the 3′IGH probe (S1C Fig).

Case 2
Clinical presentation, and pathomorphological and flow cytometry features. A 35-year-old, HIV-negative Caucasian female presented with disseminated, mainly extranodal abdominal tumors, showing thickening of the stomach wall, an enlarged ovary, numerous lesions in the liver, ascites, and spinal canal infiltration with neurological symptoms and pain. Her serum LDH (2318 IU/L), β2-microglobulin (2.43 ng/L), d-dimer (1666 ng/mL), and fibrinogen (6.58 g/L) levels were elevated; ECOG performance status, 1; Ann Arbor stage, IVB with B symptoms. PET-CT showed numerous extranodal lesions in the abdominal space and nodal, massive BM involvement. BL cells from the peritoneal fluid and liver tumor were positive for CD45<sup>weaker</sup>/CD20<sup>bright</sup>/CD19<sup>bright</sup>/CD22 (with an order according to MFI of CD20 > CD19 > CD22)/CD10/CD38<sup>higher</sup> (with an MFI of 873 for CD38, compared to an MFI of 36 on T lymphocytes)/CD81<sup>higher</sup>/CD79α/HLA-DR/CD43<sup>weaker</sup>/CD52<sup>higher</sup>/CD54<sup>higher</sup>/CD305/ MYC and surface immunoglobulin (IgD/IgM), while they were negative for CD5/CD8/CD11c/CD23/CD25/CD44/CD16&CD56/CD56/CD62L/CD200/ IgG/λ/κ and BCL6/. CD71 (++) expression was detected in 100% of cells. FMC7(±) and BCL2(±) expression was weak in slightly over 20% of the tumor cells (Fig 6). A monoclonal population of neoplastic lymphoid cells with a small number of apoptotic bodies in the background was visible in the cytological smear obtained from the peritoneal fluid and
FNAB of the liver tumor (Fig 7A). A trephine biopsy revealed a diffuse proliferation of intermediate-sized atypical lymphoid cells with prominent central nucleoli, morphologically raising concern for BL, but also with a reduced number of apoptotic bodies and reduced starry sky appearance in HP (Fig 7B). The HP/IHC studies revealed that 90% of the BM involved BL cells. IHC showed classic MYC-positive BL immunostaining but still with BCL2 (±) weaker. A surgical biopsy from the stomach infiltrate revealed BL with the reduced number of apoptotic bodies and starry sky appearance in HP (Fig 7C). The IHC showed EBV-negative classic MYC-positive BL (but partial BCL2 stronger positive) immunostaining for CD20+/CD10+/BCL6−/MYC+/MYC+strong,100%/LMO2−/CD38+/EBER−/EBV-LMP1−/MUM1−/CD43−/CD44−/CD55−/Ki-67 index > 98%/CD3−/CD5−/TdT− (Fig 7D). No CSF involvement by BL cells was confirmed by FCM. The patient was treated with four CODOX-M and IVAC alternating courses for patients with elevated risk. Thirty-nine months after diagnosis, the patient was still alive.

Cytogenetics, FISH, and NGS. The CC and FISH with a MYC BAP probe of peritoneum fluid cells demonstrated karyotype 46,XX,dup(1)(q21q42) [7]/46,idem,del(11)(q23) [6] and a lack of MYCR. However, subsequent FISH with an IGH/MYC dual fusion probe showed MYC/IGH fusion as an insertion of the MYC into the IGH locus on normal chromosome 14 (Fig 8).

The usage of the CTX-explorer app for identifying chromosomal breakpoints, followed by PCR and Sanger sequencing, revealed that the breakpoint on chromosome 8 was located 43 kb upstream of the 5′MYC (chr8:127,692,550), and the breakpoint on chromosome 14 was in a
diversity IGH region, 2 kb downstream of 3′IGHD2-2 (chr14:105,914,873) (Figs 5 and 8 and S1B Fig).

**CTX-explorer software for intra- and interchromosomal translocation detection**

The greatest methodical problem in the accurate identification of IG translocations in NGS data is caused by the vast sequence diversity within the IGH due to somatic hypermutations and the genomic instability of malignant cells. These aspects significantly hamper the bioinformatic analysis of the NGS data. In order to increase the chance of inter-chromosomal translocation detection, we developed the CTX-explorer app, capable of identifying such genetic
alterations even in short NGS paired-end reads (75 bp or longer) with single-nucleotide precision. Notably, the mate pair reads are not required for this app to work. This feature is particularly advantageous if only poor-quality DNA (e.g., that extracted from FFPE samples) is available. The CTX-explorer program proved its usefulness, showing 100% specificity combined with an outstanding precision of detection—noticeably higher than that offered by other open-source apps (see Discussion for details). In order to reduce the risk of CTX misidentification, either the gene set enrichment (as in this study) or exclusion of repetitive and low-complexity genome regions should be performed (with the windowmasker and dustmasker apps from the BLAST+ package [30]) before running the CTX-explorer software.

**Lymphoma MYC insertions reported in the literature**

Table 2 presents 19 cases of MYC insertions in lymphomas previously reported in the literature [10, 15–19]. In all these cases, the MYC status was examined by FISH; in two cases, the
Fig 5. Schematic view of the MYC insertion breakpoints in our data and previous literature. In Case 1, the breakpoint on chromosome 8 was 158 kb downstream of 3' MYC, in the PVT1 region. In Case 2, the breakpoint was 42 kb upstream of the 5' MYC. Green arrows indicate the MYC insertion breakpoints in lymphomas reported in the literature [17–19]. Visualization based on Ensembl 101: Aug 2020 [29]. BL control, BL without insertion, but with typical translocation t(8;14)(q24;q32) and with MYC/IGH fusion.

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Fig 6. Flow cytometry immunophenotyping including analysis of CD38 expression of Case 2. FCM analysis of BL cells from the peritoneal fluid. A: Forward scatter/side scatter dot plots present both small normal T lymphocytes (red cells) and larger lymphoma cells (green cells) with a reduced number of apoptotic bodies (marked by circles). B-D: BL expresses CD20/CD19/CD22 (with median fluorescence intensity (MFI) of CD20> CD19> CD22), as shown by monoclonal antibodies conjugated with the same fluorochrome, APC-A as well as CD45+weaker/HILA+. E-I: BL expresses a homogeneous phenotype of germinal center origin (CD81+higher/CD10±/CD38+higher/CD44– but BCL6 negative. H (enlarged dot plot): FCM-based analysis of MFI of CD38 expression in BL. MFI of CD38 expression on BL (873) in R1 was higher (CD38(+)higher) compared to normal T-lymphocytes (36) in R2 and apoptotic bodies (599) (in a circle). J-P: BL expresses CD54+higher/CD305+higher/ BCL2±weaker (very low expression on a small subpopulation of cells) but is negative for CD62L/kappa/lambda with a restricted expression of IgM±IgD heavy immunoglobulin chain. In addition, CD71++ expression is detected in 100% of BL cells. Antigen expression of few macrophages was marked with a pink asterisk (CD10/CD38/CD44/CD54/CD71/ CD305). Antigen expression of BL cells is compared to the expression on a subpopulation of normal T-lymphocytes (most antigens) (i.e. CD38/CD43/ CD44/CD45/CD54/CD81/BCL2) and on macrophages (i.e. CD54/ CD305) of the tumor and described as + higher for an antigen with a higher expression in BL cells compared to normal lymphocytes/macrophages in 100% of cells; +, positive in 100% of BL cells; + weaker, for an antigen with a weaker expression than in lymphocytes/macrophages in 50% of cells; ± weaker, for an antigen with a weaker expression in BL cells compared to normal lymphocytes/macrophages in 20% to <100% of BL cells; –, no expression (i.e. expression in <20% BL cells). Dot-plots.

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karyotype was also available. The type of insertion was defined in seven cases, revealing MYC inserted into IGH in four cases and IGH inserted into MYC in three cases. The diagnoses of all cases with MYC insertion were various and included BL, DHL, HGBL, DLBCL, primary cutaneous large B-cell lymphoma, leg type, mantle cell lymphoma, and plasma cell neoplasm. Detailed molecular analyses with the use of NGS were performed in four cases (Fig 5). In one HGBL reported by Peterson et al., a 200-kb fragment of IGH was inserted into MYC, upstream of and close to 5′PVT1 [18]. King et al. described two cases of MYC insertion in BCL without precise diagnosis [17]. In the first case, MYC was inserted into IGH and the breakpoint was located in PVT1, 217 kb downstream of 3′MYC. In the second case, IGH was inserted into MYC, down-stream of and close to 5′PVT1. Wagener et al. presented a case of BL with the insertion of exons 2 and 3 of MYC into the IGH locus [19].

The IGH breakpoints were specified by Wagener et al. and Peterson et al. and were located within Sα1 and within both Sα2 and 3′ to Sµ, respectively. King et al. did not specify the break location in IGH, reporting that variable and diversity regions were affected.

Discussion

The genetic hallmark of BL is a translocation of MYC and one of the IG genes. The real occurrence of BL without MYCR has been a subject of discussion for many years. Recently, some
MYC-negative BL cases have been described as having characteristic 11q gain/loss [31, 32]. The revised 4th edition of the WHO Classification of Lymphomas describes this entity as “Burkitt-like lymphoma with 11q aberration” [5]. On the other hand, some of the MYC-negative BL cases may still have MYC-Rs, which appear as cryptic during testing by standard genetic methods. Since the presence of MYC-R is crucial for establishing BL diagnosis, in rare MYC-negative BL cases, detailed examination is needed to determine the exact status of the MYC [8].

In our cohort of 108 cases with the clinicopathological features of BL, which accounted for approximately 1% (108/11,000) of all FNAB/FCM diagnosed lymphomas, we found 12 cases without MYC-R as confirmed by CC and FISH with the MYC BAP probe. The one percent incidence of BL in our FNAB/FCM diagnosed cohort is in line with the incidence of sBL in Poland, in western Europe, and in the USA, where BL constitutes only 1–2% of all lymphomas.

Fig 8. Genetic findings in Case 2. The thick black arrow indicates chromosome 14 with insertion of the MYC and with the MYC/IGH fusion. A: Karyotype 46,XX,dup(1)(q21q42) [7]/46, idem,del(11)(q23) [6]. B: Metaphase, FISH with IGH/MYC/CEP8 dual fusion probe: two centromere 8 (blue) signals on chromosomes 8; two MYC (red) signals on chromosomes 8, one IGH (green) signal on chromosome 14, and one MYC/IGH (yellow) signal on chromosome 14, indicating MYC/IGH fusion. C: Detailed breakpoints identified by PCR and Sanger sequencing: the break on chromosome 8 maps 43 kb upstream of the 5'MYC; the break on chromosome 14 is 2 kb downstream of 3'IGHD2-2.

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Among our MYC-negative cases, 10 cases demonstrated 11q gain/loss, leading to a final diagnosis of BLL,11q. In the remaining two cases with final BL diagnosis, karyotypically cryptic MYC/IGH fusions were detected. In our report, BLL,11q were the majority of MYC-negative suspBL (83%) and accounted for approximately 9% of adult aggressive CD10(+) BCLs suspected of BL. For comparison, the studies in the literature reported the BLL,11q' incidence of 3 or 13% in suspBLs [32, 34]. On the other hand, we demonstrated that nearly 17% of the MYC-negative suspBL had cryptic MYC insertions. As we have previously emphasized, MYC negativity defined by using break apart probes and karyotyping does not exclude cryptic rearrangements, because both these methods cannot detect insertions of small chromosomal segments, which did not change the morphology of chromosomes. Considering these limitations, we have applied dual fusion probes to assess MYC status in our two MYC-negative cases, which did not have 11q gain/loss. Moreover, in all the cases with 11q aberrations and without MYCR, the status of MYC/IGH, MYC/IGK, and MYC/IGL fusions was also verified. None of these BLL,11q patients had a cryptic MYC insertion. This verification was necessary because, as we and others have described before [6–8], the occurrence of 11q gain/loss does not rule out MYCR. In this study, the concurrent presence of MYCR and 11q gain/loss was observed in approximately 5% of patients with suspBL. This information is worth underlining, because the data on the frequency of BL,MYC/11q has not been published.

The literature data regarding the cryptic MYC insertions in lymphomas are scarce; only a few incidences of this aberration in BL have been reported (Table 2) [10, 15–19]. Moreover, a detailed molecular description of the cryptic MYC/IGH fusion breakpoints was given only in one case of BL, by Wagener et al. [19], and both breakpoints of MYC and IGH were typical of MYC/IGH in sBL. In our study, the MYC breakpoint in Case 2 was also typical of IGH/MYC fusions in sBL, which are mapped most often within MYC (in exon 1 and intron 1) or close to the 5'MYC [9, 13, 14]. However, in Case 1, the breakpoint resembled MYC breakpoints of variant MYC/IGL or MYC/IGK fusions in sBL, in which the MYC breakpoints are most often located more than 100 kb from the 3'MYC, in the PVT1 [9, 11, 13, 14]. With respect to the IGH breakpoints, they were also only partially typical of the MYC/IGH fusions in sBLs. As described in the literature, most breakpoints within the IGH in sBLs with MYC/IGH fusions map to the switch and joining regions, and result from failed class switching (CSR) and VDJ recombinations, respectively [14, 35, 36]. In Case 1, the IGH break occurred outside but near the switch Sμ region, and errors in CSR may be the cause of this break. However, in Case 2, the

### Table 2. Review of the literature data regarding MYC insertion in lymphomas.

| Authors                  | Number of cases | Type of insertion | Genetic methods       | Diagnosis               |
|--------------------------|-----------------|-------------------|-----------------------|-------------------------|
| Haralambieva et al., 2004 [10] | 1               | MYC into IGH      | FISH                  | sBL (Caucasian)         |
| May et al., 2010 [15]    | 3               | IGH into MYC      | FISH, classical cytogenetics | Suspected BL          |
|                         |                 | MYC into IGH      | FISH, classical cytogenetics | PCLBCL                |
|                         |                 | one case—no data | FISH                  | HGBL                   |
| Sun et al., 2012 [16]    | 11              | no data           | FISH                  | Various diagnoses (BL, DLBCL, MCL, DHL) |
| Peterson et al., 2019 [18] | 2               | IGH into MYC      | FISH, NGS (MPseq)    | DHL                    |
| King et al., 2019 [17]   |                 | IGH into MYC      | FISH, NGS (MPseq)    | Various diagnoses (HGBL, DLBCL, PCN) |
| Wagener et al., 2020 [19] | 1               | MYC into IGH      | FISH                  | BL                    |

Abbreviations: sBL, sporadic Burkitt lymphoma; BL, Burkitt lymphoma; PCLBCL, primary cutaneous large B-cell lymphoma, leg type; HGBL, high-grade B-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; DHL, double hit lymphoma; NGS, next-generation sequencing; MPseq, mate-pair sequencing; PCN, plasma cell neoplasm.

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IGH break was 2 kb downstream of the IGHD2-2. The distance from the recombination signal sequences (RSS) region and the lack of N nucleotides at the breakpoint suggest that this insertion was not attributed to VDJ recombination [37].

It is worth mentioning that all the cryptic MYC insertions in lymphomas reported in the literature and in the present study result in the fusion of MYC with IGH. There are no data regarding cryptic fusions of MYC with IGK or IGL. The reason is that variant MYC fusions are less common than MYC/IGH fusions. The other cause is that IGK/MYC and IGL/MYC testing in MYC-negative lymphomas is performed sporadically. The additional emerging question is whether variant MYC fusions might occur in BLL,11q cases. In our report, we excluded variant fusions in BLL,11q cases, but further studies are needed.

Our results obtained with the CTX-explorer app were compared with the output of Breakdancer [25] and Delly [26], open-source programs developed by other research teams, the functionality of which included CTX detection. This comparison, performed for the two BL cases described herein and on two peripheral blood samples from healthy donors, revealed that CTX-explorer outperformed both competing apps in terms of the precision and specificity of the analysis. In Case 1, the breakpoint on chromosome 14 was identified perfectly by CTX-explorer (the results were identical to those obtained with the Sanger sequencing). On the contrary, Breakdancer missed the correct breakpoint by −284 bp, and Delly, by +1 bp. The breakpoint on chromosome 8 was misidentified by each application used (CTX-explorer: −23 bp, Breakdancer: −63 bp, and Delly: +5 bp). The detection of this breakpoint was tricky due to the low proportion of DNA molecules with this translocation, as assessed by examining the relevant BAM file with the Integrative Genomics Viewer (IGV). In Case 2, the breakpoint on chromosome 8 was identified by CTX-explorer; Delly made no mistake, while Breakdancer erred by +287 bp. On chromosome 14, each program made a mistake of −1 bp when trying to find the exact breakpoint. In fact, this error was caused by the HISAT2 aligner being unable to determine whether the last matching nucleotide was a part of the translocation or not (the same nucleotide was present on both chromosomes at the junction site). Finally, it is worth noting that the breakpoint detection with both CTX-explorer and Breakdancer was 100% specific, whereas the Delly app reported a t(8;14) translocation in one of two healthy blood donors.

The vast majority of reports regarding MYC insertions in lymphomas are based on retrospective analyses or isolated cases. In the present study, we describe two BL cases with MYC insertion, which were found during routine diagnostics for 108 patients with suspBL. At our institution, in all cases of clinically suspected BL/BLL,11q or HP/IHC-confirmed BL/BLL,11q, attempts are made to perform FNAB for further diagnostic tests [7,27]. The high diagnostic accuracy and effectiveness of FNAB/FCM in BL/BLL,11q have been presented before [7]. According to these data, a lack of CD56 with CD38 higher expression and CD56 expression without CD38 higher proves to be a reliable, fast, easy, and cost-effective method for the estimation of MYCRs and the 11q aberration in CD10(+) BCL, respectively. Moreover, FNAB samples enable us to culture cells for karyotyping, regardless of FISH. In the present report, all cases with MYCR as detected using the MYC BAP probe and without MYCR as detected using the MYC BAP probe, but with MYC insertions (BL and BL,MYC/11q), were characterized by CD38(+) higher expression. On the other hand, the expression of CD38 in cases without MYCR (BLL,11q) was significantly weaker and comparable to CD38 expression in T cells. These data show that the overexpression of CD38 and MYCR detected using the MYC BAP probe allowed us to confirm MYCR in the vast majority of BL and BL,MYC/11q cases, as well as to select cases for further examination of the MYC aberration type. In addition to FCM results, BL cases with the MYC insertion were characterized by the reduced number of apoptotic bodies and starry sky appearance in the histopathological examination, by strong MYC(+) expression.
and a lack of LMO2(−) by IHC. Recently, such IHC:MYC(+)/LMO2(−) staining was found to be significantly associated with MYCR in CD10(+)BCL, including BL [7, 38], and consistent with low levels of LMO2 expression in MYC-positive BL [39]. In both our MYC-negative cases with insertion, the false negative rate for the MYC BAP probe, comprehensively described by King et al. [17], with concomitant FCM/IHC results were enough for the use of MYC/IG probes. The MYC/IGH probe enabled us to detect the fusion in these cases; however, confirmation of the insertion was possible after CC and FISH on metaphases. Considering the significance of chromosome analysis in the detection of insertion, it is possible that the presence of the MYC insertions in lymphomas is undervalued because routine genetic diagnosis of suspBL in most laboratories is based on FISH only; karyotyping is rarely performed.

In summary, to the best of our knowledge, this is the largest study devoted to cryptic MYC insertions in consecutive mainly adult suspBL patients, routinely diagnosed by HP/IHC and FNAB/FCM/CC/FISH examinations at a single institution. We confirmed that cryptic MYC insertions in BL are extremely rare but not incidental. In our large group of patients clinicopathologically suspected of BL, the frequency of this aberration was 1.9% and constituted 17% of MYC-negative suspBL. The remaining cases of MYC-negative suspBL were represented by BLL,11q. We detected the insertions through chromosome analyses and performed NGS examination of these alterations, which will extend our knowledge of the molecular features of very rare BL MYC insertions. The insertions we described were observed in sBL patients and resulted in cryptic MYC/IGH fusions. In one case, the breakpoint of the MYC was typical of IGH/MYC fusions in sBL, contrary to the other case in which the MYC break was as in variant IG/MYC fusions of sBL. Despite the rarity of MYC insertions, we believe that our study will substantially add to the understanding of MYC-negative BL and BLL,11q.

Conclusions

The phenomenon of MYC insertions in lymphoma is known; however, data regarding the occurrence of this abnormality in BL are limited. Knowledge of the cryptic MYC insertion is important, particularly with respect to MYC-negative suspBL. We showed the molecular characteristics of insertion breakpoints in two sBL cases found in 108 consecutive patients with suspBL. MYC insertions constituted 17% of the MYC-negative group and 1.9% of the whole cohort. We expect that the appearance of the MYC insertions in lymphoma might be underestimated and that more studies on the frequency of this alteration in BL and BLL,11q are needed.

Supporting information

S1 Fig. Schematic view of IGH breakpoints in Cases 1 and 2 with MYC insertions.
(PPTX)

S1 Table. Summarized data of classical cytogenetic analyses in patients with suspected Burkitt lymphoma.
(DOCX)

S1 File. Methods.
(DOCX)

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